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EFFECTS OF THERMODYNAMIC NONIDEALITY IN LIGAND BINDING STUDIES

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Effects of thermodynamic nonideality are considered in relation to the quantitative characterization of the interaction between a small ligand, S, and a macromolecular acceptor, A, by two types of experimental procedure. The first involves determination of the concentration of ligand in dialysis equilibrium with the acceptor/ligand mixture, and the second, measurement of the concentration of unbound ligand in the reaction mixture by ultrafiltration or the rate of dialysis method. For each situation explicit expressions are formulated for the appropriate binding function with allowance for composition-dependent nonideality effects expressed in terms of molar volume, charge-charge interaction and covolume contributions. The magnitudes of these effects are explored with the aid of experimental studies on the binding of tryptophan and of methyl orange to bovine serum albumin. It is concluded for experiments conducted utilizing either equilibrium dialysis or frontal gel chromatography that, provided a correction is made for any Donnan redistribution of ligand, theoretically predicted acceptor-concentration dependence is likely to be negligible and that use of the conventional binding equation written for an ideal system is appropriate to the analysis of the results. Use of ultrafiltration or the rate of dialysis method requires examination of the assumption that the activity coefficient ratio $y_A y_S / y_{AS}$ for the reaction mixture approximates unity; but again reassurance is provided that nonideality manifested as a dependence of the binding function on acceptor concentration is unlikely to be significant.

1. Introduction

The binding of low molecular weight ligands to macromolecular acceptors is an event of considerable biological importance, and one which has been studied extensively. For this purpose there are several experimental methods available for obtaining binding results, the theoretical interpretation of which has also received considerable attention [1]. In general, the theoretical expressions have been formulated on the basis of thermodynamic ideality, an assumption that has received only occasional consideration [2,3]. It is recognized at the outset that thermodynamic nonideality effects are unlikely to influence significantly the interpretation of results obtained with relatively low acceptor concentrations, but there are systems for which physiological considerations indicate that studies of ligand binding at high acceptor concentration would be more relevant. The purpose of this paper is to explore the contribution of nonideality effects to the thermodynamic characterization of the multiple binding of ligand over a wide range of acceptor concentrations, the appropriate theory being written in terms of composition-dependent activity coefficients, which may be assessed on the statistical mechanical basis of excluded volumes [3,4].

2. Theory

Consider first an experiment in which the binding of a small uncharged ligand, S, to the single

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site on an acceptor, A, is to be studied by equilibrium dialysis or by frontal gel chromatography [5,6]. In either case the quantities available from the experiment are $\bar{m}_A^\alpha$ and $\bar{m}_S^\alpha$, the total acceptor and ligand concentrations in the protein-containing phase; and m_S^β, the concentration of ligand in the protein-free phase. Since the chemical potential of S is the same in both phases, it follows that

$$y_S^\alpha m_S^\alpha = y_S^\beta m_S^\beta \tag{1}$$

where y_S^α and y_S^β denote the activity coefficients of S in the appropriate phases. Moreover, the total ligand and acceptor concentrations are clearly

$$\bar{m}_S^\alpha = m_S^\alpha + m_{AS}^\alpha \tag{2a}$$

$$\bar{m}_A^\alpha = m_A^\alpha + m_{AS}^\alpha \tag{2b}$$

From the definition of the thermodynamic binding constant, K, the concentration of complex may be written as

$$m_{AS}^\alpha = K m_A^\alpha m_S^\beta (y_A^\alpha y_S^\beta / y_{AS}^\alpha) \tag{3}$$

The experimental binding function, r, is then defined as

$$r = (\bar{m}_S^\alpha - m_S^\beta)/\bar{m}_A^\alpha \tag{4a}$$

which, on combination with eqs. 1–3, may be expressed as

$$r = \frac{K m_S^\beta (y_A^\alpha y_S^\beta / y_{AS}^\alpha)}{1 + K m_S^\beta (y_A^\alpha y_S^\beta / y_{AS}^\alpha)} - \frac{m_S^\beta [1 - (y_S^\beta / y_S^\alpha)]}{\bar{m}_A^\alpha} \tag{4b}$$

In theory, therefore, the relationship between r and m_S^β is not rectangular hyperbolic; nor is it independent of acceptor concentration. It also follows from eq. 4b that the extent of deviation of the activity coefficient ratios from unity governs the magnitude of the departure from hyperbolic form. With the aid of eqs. 3, A2, and A9 of ref. 4, written in terms only of the second virial coefficient, these activity coefficient ratios may be expressed as

$$y_A^\alpha y_S^\beta / y_{AS}^\alpha = \exp\Big\{ U_{S,S} m_S^\beta + (U_{A,S} - U_{AS,S}) m_S^\alpha + (U_{A,A} - U_{AS,A}) m_A^\alpha + (U_{AS,A} - U_{AS,AS}) m_{AS}^\alpha + \frac{z_A^2 \kappa^2 (r_{AS} - r_A)}{2I(1 + \kappa r_A)(1 + \kappa r_{AS})} \times \left[\frac{r_A m_A^\alpha}{(1 + \kappa r_A)} - \frac{r_{AS} m_{AS}^\alpha}{(1 + \kappa r_{AS})} \right] \Big\} \tag{5a}$$

$$(y_S^\beta / y_S^\alpha) = \exp\{ U_{S,S}(m_S^\beta - m_S^\alpha) - (U_{S,A} - M_A \bar{v}) m_A^\alpha - (U_{S,AS} - M_{AS} \bar{v}) m_{AS}^\alpha \} \tag{5b}$$

where $U_{i,j}$ are covolumes, equal to $4\pi N(r_i + r_j)^3/3$ on the basis of spherical geometry; z_A the net charge borne by the acceptor, which is assumed equal to that on AS since S is uncharged; and κ is the Debye-Hückel parameter, which may be calculated from the ionic strength I. In relation to the molar volume terms, only those involving the species A and AS, considered to have the same partial specific volume, $\bar{v}$, have been included, since the molecular weight of S is very much smaller than M_A, which approximates M_{AS}. Indeed, by virtue of the smallness of S, it is entirely reasonable to assume that $r_{AS} \simeq r_A$, whereupon $U_{A,S} \simeq U_{AS,S}$, $U_{A,A} \simeq U_{AS,A} \simeq U_{AS,AS}$, and the above expressions simplify to

$$(y_A^\alpha y_S^\beta / y_{AS}^\alpha) = \exp\{ U_{S,S} m_S^\beta \} \tag{6a}$$

$$(y_S^\beta / y_S^\alpha) = \exp\{ U_{S,S}(m_S^\beta - m_S^\alpha) - (U_{S,A} - M_A \bar{v}) \bar{m}_A^\alpha \} \tag{6b}$$

It is now apparent why $(y_A^\alpha y_S^\beta / y_{AS}^\alpha)$ approximates unity even at high acceptor concentrations: it is simply that the self-covolume of ligand is likely to be extremely small (only 2.5 l/mol for a ligand with a radius of 0.5 nm). For the same reason eq. 6b may be approximated as $(y_S^\beta / y_S^\alpha) = 1 + (M_A \bar{v} - U_{S,A}) \bar{m}_A^\alpha$, which on combination with eq. 4b permits us to write

$$r = \frac{K m_S^\beta}{(1 + K m_S^\beta)} - (U_{S,A} - M_A \bar{v}) m_S^\beta \tag{7}$$

It is noted that the explicit acceptor-concentration dependence has been eliminated in this formulation, which nevertheless shows that at any m_S^β the value of r is the difference between the normally accepted hyperbolic contribution and a linear contribution given by the second term. With a protein such as serum albumin, for which r_A is in the vicinity of 3.5 nm (the Stokes radius), the quantity $(U_{S,A} - M_A \bar{v}) \simeq 100$ l/mol, and hence significant error would only arise from neglect of the second term in eq. 7 if the interaction were sufficiently

weak to require experiments with m_S^β in the 10 mM range.

In summary, for the simple system under discussion we have gained the reassurance that use of the conventional rectangular hyperbolic formulation of the binding function is entirely reasonable even when high total concentrations of acceptor are being studied. There are, of course, more complicated systems to consider, and comment is now made on cases where the ligand and acceptor both bear a net charge. As eq. A9 of ref. 4 shows, this offers no difficulty in the estimation of excluded volumes, but the Donnan redistribution of charged ligand is clearly a complication in the present experimental design. We therefore examine the merits of adopting ligand-binding procedures such as ultrafiltration [7] and sedimentation velocity [8,9], which yield $m\,m_S^\alpha$.

In the event that m_S^α may be determined, the appropriate expression for the concentration of complex, m_{AS}^α, for the interaction $A + S \rightleftarrows AS$ is then

$$m_{AS}^\alpha = k m_A^\alpha m_S^\alpha (y_A^\alpha y_S^\alpha / y_{AS}^\alpha) \tag{8}$$

and the expression for the experimental binding function, ν, then becomes

$$\nu = \frac{(\bar{m}_S^\alpha - m_S^\alpha)}{\bar{m}_A^\alpha} = \frac{K(y_A^\alpha y_S^\alpha / y_{AS}^\alpha) m_S^\alpha}{1 + K(y_A^\alpha y_S^\alpha / y_{AS}^\alpha) m_S^\alpha} \tag{9}$$

By reasoning entirely analogous to that adopted above it may be shown that the activity coefficient ratio for a system with no change of radius upon complex formation ($r_A \simeq r_{AS}$) is given by the relationship, after neglecting the $U_{S,S}$ term as before,

$$\begin{aligned} y_A^\alpha y_S^\alpha / y_{AS}^\alpha &= \exp\left\{ \frac{z_S^2}{2I(1+\kappa r_S)} \left[\frac{(1+2\kappa r_S)}{(1+\kappa r_S)} - \frac{(1+\kappa r_A + \kappa r_S)}{(1+\kappa r_A)} \right] m_S^\alpha \right. \\ &+ \sum_{i=0}^{i=1} \left[U_{S,AS_i} - M_A \bar{v} \right. \\ &\left. \left. + \frac{z_S z_{AS_i}}{2I(1+\kappa r_A)} \left\{ \frac{(1+\kappa r_A + \kappa r_S)}{(1+\kappa r_S)} - \frac{(1+2\kappa r_A)}{(1+\kappa r_A)} \right\} \right] m_{AS_i}^\alpha \right\} \end{aligned} \tag{10}$$

for a ligand and acceptor with valences z_S and z_A, respectively.

Two points merit immediate comment. First, if S is uncharged, combination of eqs. 9 and 10 yields

$$\nu = \frac{K\{\exp[(U_{S,A} - M_A \bar{v}) \bar{m}_A^\alpha]\} m_S^\alpha}{1 + K\{\exp[(U_{S,A} - M_A \bar{v}) \bar{m}_A^\alpha]\} m_S^\alpha} \tag{11}$$

Thus, in the situation where m_S^α (rather than m_S^β) is determined, a family of hyperbolic plots of ν vs. m_S^α would be obtained in a series of experiments conducted with fixed but different acceptor concentrations, provided the range of acceptor concentrations were sufficiently large for the exponential term to deviate significantly from unity. Nevertheless, a single value of K would emerge from the analysis of every curve provided suitable assessment of $(U_{S,A} - M_A \bar{v})$ were made. Secondly, it is possible to generalize eq. 10 for the case of ligand binding to p equivalent and independent sites [10] on an acceptor by extending the summation to cover all values of i ($0 \leqslant i \leqslant p$). Thus, it may readily be shown that the activity coefficient ratio $(y_{AS_i}^\alpha y_S^\alpha / y_{AS_{i+1}}^\alpha)$ is independent of i provided the reasonable assumptions are made that charge is conserved on successive ligand addition, and that all $r_{AS_i} \simeq r_A$. In these terms eq. 9 may be written

$$\nu = \frac{p k_A (y_A^\alpha y_S^\alpha / y_{AS}^\alpha) m_S^\alpha}{1 + k_A (y_A^\alpha y_S^\alpha / y_{AS}^\alpha) m_S^\alpha} \tag{12}$$

where k_A is the intrinsic association constant [10] for the interaction of ligand with acceptor. As with eq. 11, acceptor-concentration dependence of binding curves is predicted for the multiple binding situation, even when the ligand is uncharged; but this is likely to be observed only if a very wide range of acceptor concentrations is examined. Perhaps of even greater interest for many studies is the further inspection of eq. 10 in its generalized form in relation to the contribution of charge-charge interaction terms to results obtained with even moderate concentrations of acceptor. This question is best examined by considering a particular experimental system for which realistic values may be assigned to the various parameters in eq. 10.

3. Experimental

3.1. Materials

A salt-free crystalline sample of bovine serum albumin was obtained from Sigma Chemical Co., who also supplied the L-tryptophan used in this investigation. Methyl orange was obtained from B.D.H. Chemicals Ltd. Unless specified otherwises, concentrations of albumin solutions were based on spectrophotometric measurements and an absorption coefficient ($A^{1\%}_{1\,cm}$) of 6.6 at 280 nm [11]. Ligand concentrations were also estimated spectrophotometrically using molar absorptivities of 5650 at 279 nm for tryptophan [12] and 23 000 at 440 nm for methyl orange [13].

3.2. Studies of tryptophan binding to bovine serum albumin

Solutions of bovine serum albumin were prepared by direct dissolution of the crystalline protein in 0.10 M sodium phosphate buffer, pH 7.4, $I = 0.26$ M, the same buffer being used to prepare a stock solution of L-tryptophan (1.01×10^{-4} M). Mixtures (≈ 20 ml) were then prepared by mixing 10 ml of the tryptophan with 10 ml of albumin solution, these mixtures being prepared by weight in order to define more accurately the composition in terms of protein and ligand constituents. The mixtures were allowed to equilibrate at 37°C for 10 min and then subjected to frontal gel chromatography [5] at that temperature on a Sephadex G-25 column (1.2×9 cm) preequilibrated with 0.10 M sodium phosphate buffer, pH 7.4, a peristaltic pump being used to maintain a flow rate of 0.8 ml/min. The concentration of tryptophan in the protein-free plateau region of the trailing elution profile was determined from a continuous recording of the effluent absorbance at 279 nm. As shown previously [6], this concentration corresponds to m^{β}_{S}. The binding function, r, was then calculated in accordance with eq. 4a, a value of 66 000 for the molecular weight of bovine serum albumin [14] being used in the conversion of weight concentrations to their molar counterparts, $\bar{m}^{\alpha}_{A}$.

Differential gel chromatography [15] has been used to test for any volume change associated with complex formation. A solution of bovine serum albumin (25 ml, 2.2 mg/ml) in 0.10 M sodium phosphate buffer, pH 7.4, was applied to a column of Sephadex G-100 (1.4×9.6 cm) preequilibrated with the same buffer made 2 mM with respect to tryptophan, a concentration sufficient to saturate effectively the albumin-binding site. Throughout application of the sample and the subsequent elution with the phosphate buffer, the column was maintained at 37°C and a flow rate of 0.16 ml/min, the effluent being monitored continuously by the biuret procedure [16].

3.3. Studies of methyl orange binding to bovine serum albumin

Because of the limited solubility of methyl orange, mixtures of bovine serum albumin and this ligand were prepared by dialyzing protein in Tris-chloride buffer, pH 7.4, for 24 h at 25°C against buffer (four 500-ml portions) containing methyl orange, the concentration of which ranged between 50 μM and 1 mM. Such preparation of mixtures by equilibrium dialysis has necessitated the use of Tris-chloride buffers with ionic strength dependent on the albumin concentration used, in order to achieve the same ionic strength in the reaction mixtures. For experiments with an albumin concentration of 2.4 mg/ml the ionic strength of the Tris-chloride buffer (pH 7.4) used was 0.047 M, the estimated ionic strength of mixtures prepared by dialysis of more concentrated albumin solutions (13.1 mg/ml) against methyl orange in buffer with $I = 0.050$ M. In experiments with even higher protein concentration (35.0 mg/ml), the ionic strength of the Tris-chloride buffer was raised to 0.054 M. In these allowances for the Donnan redistribution of ions the average net protein charge was taken as -28, which was based on a valence of -22 for bovine serum albumin, the value obtained by combining ultrafiltration with dialysis [17], and the presence of an additional six negative charges due to bound methyl orange, corresponding to the mean of the range of ν values obtained. The concentration of albumin ($\bar{m}^{\alpha}_{A}$) in these dialyzed mixtures was determined colorimetrically [18] after suitable dilution by weight with Tris-chloride buffer. An iden-

tical concentration of methyl orange was included in the albumin solutions used as standards, which were also made up in the same Tris-chloride buffer in order to take into account the effect of Tris on color production in the method of Lowry et al. [19].

Values of ν for the interaction of methyl orange with bovine serum albumin were obtained by measuring the absorbances at 440 nm of protein/ligand mixtures and the ultrafiltrates obtained therefrom by means of an Amicon 8MC ultrafiltration assembly fitted with a YM10 membrane. Since 440 nm represents an isobestic point for the serum albumin/methyl orange system [13], the absorbance of each mixture yielded the total ligand concentration, $\overline{m}_S^a$, while that of the ultrafiltrate yielded m_S^a, the concentration of free ligand in the mixture [7,17]. Eq. 9 was then used to calculate ν.

3.4. Measurement of ligand binding by rate of dialysis

The concentrations of free methyl orange in the bovine serum albumin/methyl orange mixtures were also determined by a modified version of the procedure of Colowick and Womack [20], in which the liquid in the lower compartment of the dialysis assembly formed part of a closed system, a flow cell being placed in the return line to allow spectrophotometric analysis of the lower phase. A peristaltic pump was used to maintain a flow rate of 1.6 ml/min in this phase (4.0 ml), which was monitored at 460 nm, the wavelength at which methyl orange absorbs maximally [13]. Reaction mixture (1.0 ml) with defined constituent concentrations of albumin ($\overline{m}_A^a$) and methyl orange ($\overline{m}_S^a$) was placed in the upper part of the assembly, which was separated from buffer in the lower compartment by a cellulose dialysis membrane (Technilab Instruments). In experiments of this design attainment of the required steady state [20] is signified by linear time dependence of the measured absorbance at 460 nm, the slope being interpreted in terms of the free ligand concentration (m_S^a) in the reaction mixture by means of a calibration plot obtained with different concentrations of pure ligand in the upper compartment (fig. 1). As an empirical means of determining

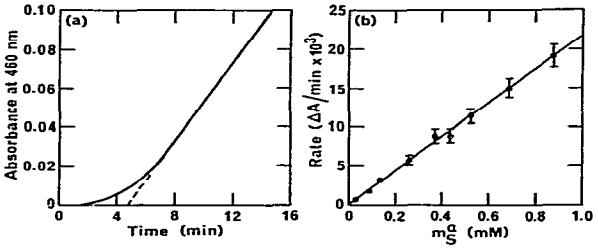

Fig. 1. Evaluation of methyl orange concentrations by the modified rate of dialysis method. (a) Time dependence of A_{460} in the lower compartment of the dialysis assembly in an experiment with an upper concentration, $(m_S^0)_U$, of 0.52 mM: (b) dependence of the steady-state slope upon $(m_S^0)_U = m_S^a$.

ligand concentrations this method has no obvious advantages over that of Colowick and Womack [20]; but it does provide a means of obtaining better insight into the nature of the empiricism.

The present rate of dialysis assembly bears a striking resemblance to the later version [21] of the diaphragm cell devised originally by Northrop and Anson [22] for the measurement of diffusion coefficients. In an experiment with an initial ligand concentration $(m_S^0)_U$ in the upper compartment, the concentrations of ligand in the two phases at time t are related to its diffusion coefficient, D, by [22,23]

$$D = \frac{1}{\beta t} \ln \frac{(m_S^0)_U}{(m_S)_U - (m_S)_L}; \beta = \frac{(A/\delta x)(V_U + V_L)}{V_U V_L} \qquad (13)$$

where β is a cell constant that depends not only on the thickness (δx) and effective pore area (A) of the membrane, but also on the volumes (V_U, V_L) of liquid in the two compartments [24]. Since $(m_S)_U = (m_S^0)_U - [(m_S)_L V_L / V_U]$, eq. 13 may be rewritten as

$$\{(m_S^0)_U - (m_S)_L[1 + (V_L/V_U)]\}/(m_S^0)_U = \exp(-\beta D t) = 1 - \beta D t \qquad (14)$$

the truncation of the exponential term being an acceptable approximation for small values of βDt. Sample calculations indicate that this situation should prevail for the first 20 min of the present experiments, during which time the concentration of ligand in the lower compartment is therefore

given by

$$(m_S)_L = \beta D t (m_S^o)_U / [1 + (V_L/V_U)] \quad (15)$$

In accord with fig. 1, the concentration of ligand in the lower compartment increases linearly with time, the slope, $d(m_S)_L/dt$, being directly proportional to $(m_S^o)_U$ under conditions where D may be regarded as a constant. Binding results obtained from rate of dialysis measurements are thus based on the reasonable approximation that the free diffusion coefficient of the ligand is unaffected by the presence of protein in the reaction mixture.

4. Results and discussion

4.1. Interaction of tryptophan with bovine serum albumin

A frontal gel chromatographic investigation of the binding of L-tryptophan to bovine serum albumin at pH 7.4 provides a system appropriate to the exploration of eqs. 4b and 7 written for the formation of a 1 : 1 complex. Thus, estimates of the stoichiometry of the complex range between 0.85 and 0.98 mol ligand bound per mol albumin [12,25]. Moreover, at neutral pH tryptophan is essentially uncharged [12], and the experimentally determined value of m_S^β [6] therefore requires no correction for the Donnan effect. In table 1, which summarizes results from eight such experiments, column 4 gives values of the binding function r calculated using eq. 4a, and column 5 the values of the second term of eq. 7, which are indeed seen to contribute negligibly to r. Accordingly, the first term of eq. 7 suffices in the calculation of the values of K, which are reported in column 6 of table 1. Clearly, the same value of K (9000 ± 700 M^{-1}) emerges from the results obtained over a 10-fold range of albumin concentration, which supports the reasoning that the activity coefficient ratios in eq. 4b closely approximate unity in such studies.

It should be noted that the results presented in table 1 are at variance with those reported by Bowmer and Lindup [26], who observed an inverse dependence of the binding constant upon bovine serum albumin concentration from equilibrium dialysis studies performed under similar conditions. The latter results could only find explanation in terms of thermodynamic nonideality if an approximation inherent in eq. 7 were invalid. In that regard, inspection of eq. 5a reveals that our approximation that $r_{AS} - r_A \simeq 0$ deserves further scrutiny. Numerical calculations based on eq. 5a show that the required volume change would need to be markedly greater than that associated with the acid expansion of bovine serum albumin, a phenomenon readily detected by differential gel chromatography [15]. In the event, the differential gel chromatography experiment performed to

Table 1

Binding of L-tryptophan to bovine serum albumin at pH 7.4, $I = 0.26$ M, and 37°C

$\bar{m}_A^o$ (M) ($\times 10^4$)	$\bar{m}_S^o$ (M) ($\times 10^5$)	m_S^β (M) ($\times 10^5$)	r	$(U_{S,A} - M_A\bar{v})m_S^\beta$ ($\times 10^3$) [a]	K (M^{-1})
3.67	5.04	1.24	0.104	1.24	9400
2.42	4.88	1.59	0.136	1.59	9900
1.86	5.04	2.12	0.157	2.12	8800
1.28	5.04	2.57	0.193	2.57	9300
0.65	5.04	3.45	0.245	3.45	9400
0.65	5.06	3.63	0.220	3.63	7800
0.57	5.04	3.54	0.263	3.54	10100
0.36	4.98	4.16	0.228	4.16	7100
				Mean($\pm 2s_m$)	9000 ± 700

[a] Based on a value of 100 l/mol for $(U_{S,A} - M_A\bar{v})$, as mentioned in the discussion of eq. 7.

compare the weight-average elution volumes of albumin in the presence and absence of tryptophan revealed no change in plateau concentration in the region where the ligand boundary emerged. This negative result leads us to the conclusion that $r_{AS} - r_A$ does approximate zero; and that, therefore, eq. 7 is appropriate to the description of the system, as confirmed by the results shown in table 1.

4.2. Interaction of methyl orange with bovine serum albumin

The binding of methyl orange to bovine serum albumin presents a system involving multiple equilibria between a charged acceptor species ($z_A = -22$) and a charged ligand ($z_S = -1$) at pH 7.4. Results of equilibrium dialysis experiments used in the preparation of the protein/ligand mixtures are presented in fig. 2, where r was calculated using eq. 4a on the basis of measured values of $\overline{m}_S^{\alpha}$ and m_S^{β} at fixed acceptor concentrations of 2.4 mg/ml (■) and 35 mg/ml (●). At first sight the apparent acceptor concentration dependence of the Scatchard [27] plots seems to be at variance with eq. 7, which was, however, derived on the basis of the ligand being uncharged. Accordingly, it is necessary to correct the results for the Donnan effect before any interpretation is made in terms of eq. 7

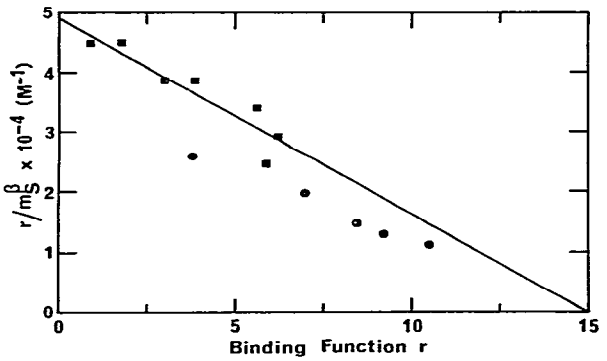

Fig. 2. Scatchard plot of binding results obtained by equilibrium dialysis at 25°C for the interaction of methyl orange with bovine serum albumin in experiments with protein concentrations of 2.4 mg/ml (■) and 35.0 mg/ml (●) in Tris-chloride buffer, pH 7.4, $I = 0.047$ M.

and its implicit approximation that no volume changes accompany complex formation. After such correction, all results are adequately described by the straight line shown in fig. 2, which is the relationship for a system with $p = 15$ and $k_A = 3300\ m^{-1}$. Again, the form of eq. 7 appropriate to multiple binding has been shown to apply, with no real acceptor concentration effects being operative, and with a negligible value of the second term.

With the growing interest in employing analysis techniques which determine m_S^{α} rather than m_S^{β}, it is timely to explore the same system in relation to eq. 12 and the generalized form of eq. 10. Fig. 3 presents the results of a series of binding experiments in which the values of m_S^{α} were determined either by ultrafiltration [7] or by a modified form

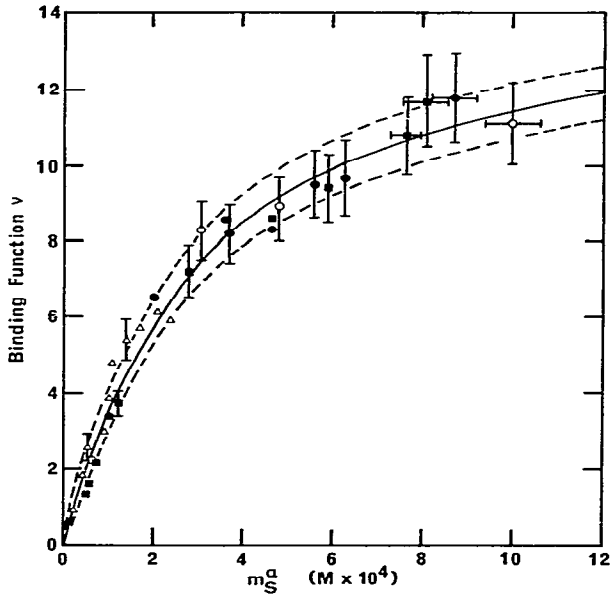

Fig. 3. Binding results (with error bars indicated) obtained by ultrafiltration (open symbols) and rate of dialysis (closed symbols) for the interaction at 25°C between methyl orange and bovine serum albumin in Tris-chloride buffer, pH 7.4, $I = 0.047$ M. The solid curve denotes the rectangular hyperbola that best describes the results from experiments with albumin concentrations of 2.4 mg/ml (△), 13.1 mg/ml (○, ●) and 35.0 mg/ml (■); and the broken curves the extent of uncertainty ($\pm 2s_m$) associated with the best-fit description ($p = 14.9$, $k_A = 3300\ M^{-1}$).

of the rate of dialysis method [20]. Open symbols are used to denote the former, and closed symbols the latter results, in experiments with constituent albumin concentrations ($\bar{m}_A^\alpha$) of 2.4 mg/ml (triangles), 13.1 mg/ml (circles) and 35.0 mg/ml (squares). Several points in relation to fig. 3 are noted. First, in view of the empirical nature of the procedure of Colowick and Womack, it is reassuring to observe agreement between results obtained by rate of dialysis measurements and those obtained by ultrafiltration, where the measured quantity is certainly m_S^α [7,17]. Secondly, there is no discernible dependence of the binding function ν upon the concentration of albumin used for its determination, all of the results being described adequately by the rectangular hyperbola shown as the solid line in fig. 3. This relationship, which was obtained by nonlinear regression analysis of the untransformed (r, m_S^α) results [28], indicates a value of 14.9 for p, the number of binding sites, and an intrinsic association constant of 3300 (± 600) M^{-1}: the broken lines define the region encompassed by the reported uncertainty ($\pm 2s_m$) in k_A. Thirdly, the value of 14.9 (± 1.2) for p, the number of sites for methyl orange, agrees with, but is more definitive than, the most recent estimate of 15 (± 6) for this parameter from studies of the system in 0.1 M phosphate, pH 5.68 [29]. Finally, the insensitivity of the present binding parameters to the concentration of albumin used for their determination is encouraging inasmuch as it signifies that the consequences of thermodynamic nonideality manifested in the activity coefficient ratio of eq. 12 have little or no effect on the experimental evaluation of k_A by methods in which m_S^α is the free ligand concentration that is measured. This implies that the ratio ($y_A^\alpha y_S^\alpha / y_{AS}^\alpha$) must closely approximate unity for the system under consideration.

A theoretical evaluation of the activity coefficient ratio was made using the generalized form of eq. 10 with $\kappa = 3.27 \times 10^7 \sqrt{I}$, $M_A = 66000$ [14], $\bar{v} = 0.734$ ml/g [30,31], $r_A = 3.5$ nm [32] and $r_S = 0.5$ nm [24], this being the Stokes radius of sucrose, which has a molecular weight comparable with that of methyl orange. The calculation was simplified by noting that the coefficient of $m_{AS_i}^\alpha$ in the summed term of eq. 10 taken from $i = 0$ to $i = 15$ varied between 188 and 240 l/mol as a consequence of the increasing value of z_{AS_i}: the reasonable approximation was therefore made that this coefficient was effectively a constant, which permitted eq. 10 to be written as

$$(y_A^\alpha y_S^\alpha / y_{AS}^\alpha) = \exp\{1.3 m_S^\alpha + 214 \bar{m}_A^\alpha\} \quad (16)$$

On this basis the activity coefficient ratio is calculated to be 1.12 in the experiments with the highest albumin concentration (5.30×10^{-4} M), 1.04 in those with $\bar{m}_A^\alpha = 1.98 \times 10^{-4}$ M, and 1.01 in those with lowest albumin concentration (3.6×10^{-5} M), the values being insensitive to the range of m_S^α encountered in each set of experiments. In strict terms these calculations show that the product $k_A(y_A^\alpha y_S^\alpha / y_{AS}^\alpha)$ appearing in eq. 12 should vary slightly in the series of experiments conducted with different acceptor concentrations by methods which determine m_S^α. However, with the present system the effect is negligible, being well within the limits of uncertainty ($\pm 18\%$) associated with the measurement of k_A.

5. Concluding remarks

As exemplified by eq. 7, table 1 and fig. 2, all applicable to situations in which m_S^β (corrected for Donnan effects) has been determined by equilibrium dialysis or frontal gel chromatography, little if any error is likely to be introduced by the neglect of thermodynamic nonideality effects even when high total concentrations of acceptor are employed. The one reservation that applies to this statement might arise if binding of ligand is accompanied by large volume changes of reaction: in that instance acceptor-concentration dependence might be observed and would necessitate reconsideration of the ratio ($y_A^\alpha y_S^\beta / y_{AS}^\alpha$) in terms of eq. 5a. In contrast, binding results determined by the evaluation of m_S^α merit closer scrutiny. It is true that such results need no correction for Donnan redistribution of ligand, and that, as fig. 3 exemplifies, system parameters may be such as to render conventional interpretation appropriate. Nevertheless, eq. 12 emphasizes that such interpretation neglecting thermodynamic nonideality is only appropriate when the ratio ($y_A^\alpha y_S^\alpha / y_{AS}^\alpha$) ap-

proximates unity for the acceptor concentration employed. The discussion of eq. 10 has indicated how this condition may be explored for other systems, where the origin of acceptor-concentration dependence is uncertain.

In summary, the present theory and experimental results have provided the reassurance that the consequences of thermodynamic nonideality in the multiple binding of small ligands to a single macromolecular state of a protein are likely at most to be a second-order effect. This reassurance has considerable simplifying implications in the formulation of binding theory for multiple interactions of ligand with several states of a protein acceptor coexisting in equilibrium. For such systems, a full treatment of all thermodynamic nonideality effects is prohibitively difficult, particularly if a space-filling macromolecule is also to be included [33–35]; but in view of the present work it now appears possible to focus attention primarily on the thermodynamic nonideality factors which influence the protein self-association rather than those affecting in a minor way the binding of ligand to the individual acceptor species.

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