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ON THE ANALYSIS OF LINEAR BINDING EFFECTS ASSOCIATED WITH CURVED SCATCHARD PLOTS

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First the question is examined as to which binding data, especially if given as Scatchard plots, can be described in terms of a basic model mechanism. This refers to a linear lattice of equivalent binding sites (as for example located on a linear biopolymer) which can exert cooperative interaction between nearest neighbors. It is shown that the effect of overlapping of potential sites (so-called "multiple-contact" binding), as may occur with larger ligands, will largely be compensated by a higher degree of cooperativity. Therefore, in practice such properties can scarcely be separated by means of ordinary binding experiments.

A pronounced inflection point in the Scatchard plot turns out to be clearly indicating a more complex mechanism involving at least two rather antagonistic cooperative interactions which may, however, occur even between equivalent binding sites.

Finally some consequences of different classes of binding sites are considered. In particular, a simple approach is introduced by which the binding to mutually exclusive classes of sites may be described. Such a model is of interest for multiple-mode binding of ionic ligands to oppositely charged polymers.

1. Introduction

The binding of many kinds of ligands to macromolecular structures plays an important role in biological processes. Pertinent theoretical analysis of experimental binding data is therefore of great interest in biophysical chemistry.

From the thermodynamic point of view we note fundamentally that the free and the bound ligand can be considered as existing in different phases. Hence the respective chemical potentials must be equal at equilibrium, so that we may write

$$\mu_A^0 + RT \ln f_A c_A = \mu_a^0 + RT \ln \varphi(\nu), \qquad (1)$$

where "A" denotes the free ligand state (μ_A^0 = standard potential, f_A = activity coefficient, c_A = concentration in moles per unit volume) while "a" refers to the bound state. The standard state of the latter may be taken arbitrarily according to convenience. The difference $\mu_a - \mu_a^0$ will be a more or less complicated function of the fraction of occupied binding sites depending on the binding mechanism. This is to be expressed as a function of ν, $\varphi(\nu)$, with $\nu = c_a/c_p$ being the binding ratio, i.e., the amount of bound ligand per macromolecule (c_p = concentration of the polymer).

Eq. (1) is easily seen to be equivalent to

$$Kc_A = \varphi(\nu), \qquad (2)$$

where $K = f_A \exp\{(\mu_A^0 - \mu_a^0)/RT\}$ represents a basic binding constant. By inversion of (2) we obtain a theoretical relation for ν, viz.

$$\nu = \varphi^{-1}(Kc_A).$$

In other words, any binding data can be expressed as a function of a dimensionless parameter

$$s = Kc_A,$$

which is the product of some suitable thermodynamic constant and the free ligand concentration. This function as well as the value of K depend of course on the special mechanism of binding. Therefore a natural way to represent experimental results would be a plot of ν versus c_A as is indeed frequently employed. On the basis of such a direct binding curve it should in principle be possible to determine the underlying

mechanism and the characteristic binding constants. In practice, however, other plots may be more convenient.

Very popular is the plot introduced by Scatchard [1], namely ν/c_A versus ν. One of its advantages stems from the fact that all data fall in a limited range of the coordinate system. This is rather suitable for extrapolations to the extremes of very low as well as very high saturation of binding sites. Moreover, the simplest mechanism is characterized by a linear plot with a negative slope. Deviations from such linearity are quite sensitive indications of cooperative behavior, different classes of binding sites, and/or multiple-contact binding (as to be pointed out below). Some pertinent aspects concerning the interpretation of Scatchard plots have been generally discussed elsewhere [2]. The present article is to deal with binding to linear structures. This is encountered quite often in systems of biological significance. Simple cases which imply only one class of binding sites as well as nearest neighbor cooperativity have already been treated in the literature (e.g., [3,4]). In practice the question arises of whether or not experimental data can actually be interpreted in the frame of such a model and as to how its parameters may be evaluated. Naturally the actual mechanism could be more complicated. Definite indications of this as reflected in the binding curve, especially if represented as a Scatchard plot, will be of considerable interest. These aspects are therefore to be examined quantitatively.

First it will be necessary to give a brief comparative survey of the theoretical methods to calculate equilibrium properties of linear binding. Then we shall investigate which features of binding curves are associated with the various possible modes of binding in the case when there is only one class of equivalent reaction sites. Finally we direct our attention to the consequences of more than one class of binding sites, considering especially the blocking of one type of sites due to binding to the other. The latter apparently applies in particular to some systems of actual interest where the binding is due to electrostatic interactions.

Together with other pertinent information the results of our analysis can be utilized to interpret experimental binding data with regard to the nature of the underlying mechanism and a quantitative evaluation of its characteristic parameters.

2. General theoretical principles

2.1. Matrix method and doublet closure

It is assumed that the system under consideration consists of linear lattices of N equivalent binding sites which are numbered as $i = 1, 2, ..., N$. A site may exist in a number of different elementary binding states r denoting for instance various forms of free, blocked and occupied sites. In case of cooperative interaction between neighboring sites a general equilibrium theory can be developed rather elegantly on the basis of the matrix method as first applied to linear biopolymers by Zimm and Bragg [5]. It can be shown to allow quite general calculations including end effects [6] and linear binding [3].

Provided cooperative interaction is only exerted between nearest neighbors the probability of a certain lattice state can be written as a product of weight factors $w_{\rho r}$ which are associated with an individual state r and its interaction with the respective left neighbor state ρ. End effects may be described by appropirate weight factors u_r and v_r. All these quantities could be expressed in terms of equilibrium constants for the various transformation steps of elementary binding (see section 2.3). Introducing the matrix

$$\mathbf{W} = (w_{\rho r})$$

and the vectors $\mathbf{u} = (u_r)$, $\mathbf{v} = (v_r)$ the partition function of the system is readily formulated as

$$Q = \sum_{r_1} \cdots \sum_{r_N} u_{r_1} w_{r_1 r_2} \cdots w_{r_{N-1} r_N} v_{r_N} = \mathbf{u}\mathbf{W}^{N-1}\mathbf{v}.$$

Furthermore, the probability to find a certain sequence of n elementary states $r_1 r_2 \ldots r_n$ starting at site i becomes

$$\{r_1 r_2 \ldots r_n\}^{(i)} = \frac{1}{Q}(\mathbf{u}\mathbf{W}^{i-1})_{r_1} \times w_{r_1 r_2} \cdots w_{r_{n-1} r_n} (\mathbf{W}^{N-i-n+1}\mathbf{v})_{r_n} \qquad (3)$$

(the expressions in parentheses being the r_1 th and r_n th components of the respective vectors).

For actual calculations $\mathbf{W}$ is suitably converted to its diagonal form

$$\mathbf{\Lambda} = \mathbf{M}\mathbf{W}\mathbf{M}^{-1} = (\lambda_r \delta_{\rho r})$$

(with the eigenvalues λ_r and transformation matrix $\mathbf{M}$ evaluated from $\mathbf{W}$ in the usual way). Then we can for

instance obtain

$$Q = a_0\lambda_0^N + a_1\lambda_1^N + \dots ,$$

where the coefficients $a_0, a_1 \dots$ do not depend on the lattice length N. In principle any equilibrium property of the system may be calculated along these lines including its dependence on N which is determined by the special end effects. The latter can, however, be neglected above a certain critical length where the system virtually behaves like an infinite lattice ($N \to \infty$). This case which often applies in practice involves comparatively simple theoretical relations, e.g.,

$$\ln Q = N \ln \lambda_0 , \tag{4}$$

with λ_0 being the largest eigenvalue of $\mathbf{W}$.

Pertinent calculations can be performed in a rather straight-forward way by means of the so-called doublet closure [7] which directly follows from (3). At neglect of end effects it reads

$$\{r_1 \dots r_n\} = \{r_1\}(r_1 r_2)(r_2 r_3) \dots (r_{n-1} r_n), \tag{5}$$

where generally

$$(\rho r) = \{\rho r\}/\{\rho\}$$

stands for the conditional probability of a state r with its left neighbor known to be in state ρ. Evidently relation (5) means that doublets are statistically independent elements in linear lattices with cooperative interaction between nearest neighbors. The apparent usefulness of this principle has already been proved [8]. Further applications will follow below.

2.2. The sequence generating functions method

The matrix method can in principle also be applied to cooperative interactions beyond nearest neighbors [6]. However, since this requires larger matrices the practical procedure may become extremely tedious. The problem can be very much simplified by the use of so-called sequence generating functions [9]. This SGF-method has only the disadvantage that it is restricted to practically infinite lattices. In that case it offers a rather straight-forward approach to the quantity λ_0 from which Q results according to (4).

It has been shown that λ_0 is obtained as the largest root of an equation for x resulting by evaluation of the determinant

$$|\mathbf{I} - \mathbf{U}(x)| = 0,$$

with $\mathbf{I}$ representing the identity matrix whereas

$$\mathbf{U} = (U_{\rho r})$$

is characterized by zero diagonal elements and the other $U_{\rho r}$ given by so-called sequence generating functions which characterize sequences of like states of type r following a sequence of type ρ. In terms of a dummy variable x they are defined as

$$U_{\rho r} = u_{\rho r}^{(1)} x^{-1} + u_{\rho r}^{(2)} x^{-2} + \dots ,$$

with $u_{\rho r}^{(j)}$ being the statistical weight of a sequence of j states of kind r following a state of kind ρ.

2.3. Treating a definite binding mechanism

The elementary steps of any linear binding mechanism can be generally formulated as

$$\mathrm{A} + \mathrm{xuy} \rightleftharpoons \mathrm{xay}, \tag{6}$$

with the symbols u, a representing the unoccupied and occupied site, respectively, whereas x and y are to characterize the instantaneous overall states on its left and right sides. Introducing a corresponding equilibrium (binding) constant K_{xy} the law of mass action for (6) reads

$$\{\mathrm{xay}\}/\{\mathrm{xuy}\} = K_{\mathrm{xy}} c_{\mathrm{A}} . \tag{7a}$$

Choosing arbitrarily a certain elementary binding constant as K, the probability of any occupied binding site may be expressed as

$$\{\mathrm{xay}\} = \sigma_{\mathrm{xy}} s \{\mathrm{xuy}\} \tag{7b}$$

involving the basic parameters $\sigma_{\mathrm{xy}} = K_{\mathrm{xy}}/K$ and $s = Kc_{\mathrm{A}}$. The probability of any sequence of elementary states can so be obtained in terms of the σ's and s by successive application of the respective versions of (7) starting at some reference lattice state, preferably the state of completely free sites. In this way the probability of a lattice with n occupied sites turns out to be proportional to the nth power of s so that the partition function must be

$$Q = \sum_{n=0}^{N} A_n s^n ,$$

where the A_n only depend on the various σ-quantities. Accordingly the fraction of occupied sites, θ, the so-

called degree of binding is generally evaluated as

$$\theta = \frac{1}{N}\frac{\Sigma_n n A_n s^n}{\Sigma_n A_n s^n} = \frac{1}{N}\frac{\partial \ln Q}{\partial \ln s} \xrightarrow[N\to\infty]{} \frac{\partial \ln \lambda_0}{\partial \ln s}. \quad (8)$$

Such an approach can be analogously extended to the treatment of more than one class of binding sites if respective σ- and s-parameters are defined for each of them. Then for a class r the degree of binding is simply

$$\theta_r = \frac{1}{N}\frac{\partial \ln Q}{\partial \ln s_r}. \quad (9)$$

We note that the total binding ratio will be

$$\nu = \sum_r g_r \theta_r, \quad (10)$$

where g_r represents the maximum number of bound ligands per macromolecule in class r. With $s_r = K_r c_A$ we then have

$$\nu/c_A = \sum_r g_r K_r \theta_r / s_r. \quad (11)$$

Thus by combining (10) and (11) the Scatchard plot of any theoretical binding model may be readily determined once the θ_r have been calculated as a function of s_r. Especially the intercepts I_0, I_∞ and slopes S_0, S_∞ as obtained upon extrapolating $c_A \to 0$ (i.e., $\nu \to 0$) and $c_A \to \infty$ (i.e., $\nu/c_A \to 0$), respectively, can be computed. These characteristic quantities can frequently be obtained from experimental data and may be utilized to analyze the binding mechanism [2]. In principle four parameters describing the latter should be quantitatively accessible in this way.

3. One class of binding sites

3.1. Simple case of nearest neighbor cooperativity

The most uncomplicated situation of cooperative binding will be encountered if a linear and infinitely long lattice of equivalent sites can be assumed where each site may be either completely occupied or unoccupied and these states can only interact with nearest neighbors. The equilibrium behavior of such a system has been theoretically analyzed in some detail using the matrix method [3]. The essential points needed later in this article may be briefly reviewed employing a new comparatively simple approach which takes advantage of the doublet closure.

A cooperative binding constant K is defined as the equilibrium constant of the growth step of binding

$$A + uua \text{ (or } auu) \rightleftharpoons uaa \text{ (or } aau, \text{ respectively}), \quad (12a)$$

whereas the nucleation step

$$A + uuu \rightleftharpoons uau \quad (12b)$$

has a binding constant $K^{(0)}$ which is by a factor of q smaller, i.e., $K^{(0)} = \sigma K$ with $\sigma = 1/q$. This cooperative parameter q is thus determined by the free enthalpy of cooperative interaction between two neighboring occupied sites.

Furthermore we define a quantity λ_0 according to

$$(uu) = 1/\lambda_0.$$

This λ_0 can indeed easily be shown to be identical with the quantity λ_0 introduced before in (4). Applying the doublet closure to the mass action relations (12) yields for the conditional probabilities of the other doublets

$$(aa) = s/\lambda_0, \qquad (ua) = (\sqrt{\sigma s}/\lambda_0)\sqrt{\theta/(1-\theta)},$$

$$(au) = (\sqrt{\sigma s}/\lambda_0)\sqrt{(1-\theta)/\theta}.$$

Because of $(uu) + (ua) = 1$ it then follows that

$$\lambda_0 = 1 + \sqrt{\sigma s}\sqrt{\theta/(1-\theta)}.$$

On the basis of these fundamental relations the concentration of any sequence of elementary binding states can be calculated, e.g., that of a_n (i.e., exactly n bound ligands in a row):

$$c_n = c_u(au)(aa)^{n-1}(au) = (\sigma/\lambda_0)(s/\lambda_0)^n c_u. \quad (13)$$

The average length of such sequences, n_0, is evidently equal to $\{a\}/\{au\} = 1/(au)$. A condition that practically infinite lattices can be assumed, could therefore be formulated as $n_0 \ll N$ or $(au) \gg 1/N$, respectively. Then we find owing to

$$c_a = \sum_n n c_n = [\sigma s/(\lambda_0 - s)^2]c_u,$$

$$\frac{c_a}{c_u} = \frac{\theta}{1-\theta} = \phi = (\xi + \sqrt{1+\xi^2})^2, \quad (14a)$$

with

$$\xi = (s - 1)/(2\sqrt{\sigma s}). \qquad (14b)$$

The function $\phi(\xi)$ may be interpreted as a quasi equilibrium constant of the overall transition of binding sites, i.e., u ⇌ a. As is easily seen we finally have

$$\theta = \frac{\phi}{1+\phi} = \tfrac{1}{2}(1 + \xi/\sqrt{1+\xi^2}),$$

which describes the degree of binding as a function of the quantity $\xi(\sigma, s)$.

The approach given here could be successfully applied to cooperative binding of positively charged acridine dyes to polyanions (e.g., [10]) although no specific binding sites exist there. It is rather a formation of dye stacks induced by electrostatic ligand binding and charge neutralization which takes place. Nevertheless the model mechanism is applicable because of the fact that the minimum distance between two stacks (corresponding to a "free binding site") is of about the same size as the distance covered by a bound ligand (corresponding to an "occupied binding site"). A situation involving ligands which may cover more space on the polymer is to be discussed in the next section.

3.2. Multiple-contact binding

A larger ligand may be bound to a macromolecular structure by means of more than one equivalent binding contact. Then potential binding sites will overlap each other. With binding contacts instead of binding sites the general linear problem can well be solved using any one of the theoretical methods discussed before. Employing an approach which actually implies the doublet closure it has recently been treated in some detail [4]. We must emphasize at this point that presuming the doublet closure requires that only cooperative interaction between nearest neighbor contacts is allowed.

The binding behavior in such multiple-contact systems is more complicated than under the simpler conditions of the preceding section. Because of its great fundamental interest we shall give here a brief and slightly simplified version of the basic theory for ligands which require n (> 1) binding contacts. The various possible elementary states of a single contact are to be denoted $f, b_1, ..., b_n$ if it is free or occupied by the first, second, ..., nth counter contact on a ligand, respectively. As is easily realized we have regarding the probabilities of these states

$$\{f\} = 1 - \theta, \qquad \{b_1\} = ... = \{b_n\} = \theta/n,$$

where θ again stands for the degree of binding. These individual state probabilities together with the conditional probabilities of doublet contact states may then be used to express the probability of any sequence of contact states, for instance that describing an isolated bound ligand, namely

$$\{fb_1 ... b_n f\} = (1 - \theta)(fb_1)(b_n f).$$

We note that only $(b b_1)$, $(b_n f)$, (ff) and $(b_n b_1)$ are variable whereas the other conditional probabilities must be either zero or unity. Furthermore we have to take into account the rather obvious relations

$$(ff) + (fb_1) = 1, \qquad (b_n f) + (b_n b_1) = 1, \qquad (15a,b)$$

as well as

$$(1 - \theta)(ff) + (\theta/n)(b_n f) = 1 - \theta. \qquad (16)$$

Thus the binding can be described in terms of only two independent variables which are to be taken here as θ and (ff). These can be related to appropriate thermodynamic parameters defined again on the basis of the elementary binding reactions of growth and nucleation, respectively. The former would read in the present model

$$A + f ... fb_1 \rightleftharpoons fb_1 ... b_n b_1 \quad \text{(involving } n + 2 \text{ contacts)},$$

so that application of mass action and doublet closure leads to

$$(b_n b_1)/(ff)^n = K c_A = s. \qquad (17)$$

On the other hand, the corresponding nucleation step yields analogously

$$(fb_1)(b_n f)/(ff)^{n+1} = \sigma s. \qquad (18)$$

It should be mentioned that this includes non-cooperative competitive binding of some ligand B to single contacts so that these are blocked for binding to A. With a binding constant K_b for the process $f_0 + B \rightleftharpoons f_b$ we have $\{f\} = \{f_0\}(1 + K_b c_B)$ where f_0, f_b, and f mean a contact binding neither A nor B, binding B, and not binding A, respectively. The relations (17) and (18) then still apply with

$$K = K_0/(1 + K_b c_B)^n,$$

with K_0 referring to the cooperative binding constant of A in the absence of B.

On dividing (17) by (18) the cooperative parameter is given by

$$q = 1/\sigma = (ff)(b_n b_1)/(fb_1)(b_n f). \tag{19}$$

Introducing now an appropriate λ_0 according to

$$(ff) = 1/\lambda_0 \tag{20}$$

it follows on the basis of (15) and (19)

$$(fb_1) = (\lambda_0 - 1)/\lambda_0, \tag{21a}$$

$$(b_n f) = 1/[1 + (\lambda_0 - 1)q], \tag{21b}$$

$$(b_n b_1) = (\lambda_0 - 1)q/[1 + (\lambda_0 - 1)q]. \tag{21c}$$

If we insert (20) and (21c) in (17) an algebraic equation of the $(n + 1)$th order for λ_0 is obtained which may be written as

$$(\lambda_0 - 1)(\lambda_0^n - s) = \sigma s.$$

From this λ_0 can be evaluated as a function of s and q (note there is one and only one appropriate root greater than one). Once λ_0 is known the degree of binding may be calculated by means of the proper version of (8) which reads in this case

$$\theta = n\,\partial \ln \lambda_0/\partial \ln s$$

(owing to the fact that n contacts are required for a binding site) or by taking advantage of (16). This results in

$$\frac{\theta}{1-\theta} = (nq/s)(\lambda_0 - 1)^2 \lambda_0^{n-1} = \phi(\sigma, n, s). \tag{22}$$

On the other hand, λ_0 may be expressed in terms of θ, n, and q as

$$\lambda_0 = 1 + \frac{1}{2q}[\zeta - 1 + \sqrt{(\zeta - 1)^2 + 4q\zeta}], \tag{23a}$$

where

$$\zeta = \frac{1}{n}\frac{\theta}{1-\theta}. \tag{23b}$$

This allows us to determine λ_0 and subsequently any other equilibrium property of the system (including the parameter s) for any given degree of binding (and known n, q). It should be emphasized that the model binding mechanism treated here is a more general version of the basic one discussed in the preceding section. The latter model is included in the present theoretical approach for $n = 1$. For instance, (22) is a general formulation of the overall quasi equilibrium constant introduced in (14). Thus the general behavior of binding of ligands to an infinite linear lattice of only one class of sites with nearest neighbor cooperative interaction and one or more binding contacts per ligand is completely described by the above relations. In order to get some fundamental ideas about the pattern and quantitative nature of experimental data corresponding to such a mechanism we consider the structure of respective Scatchard plots and their characteristic intercepts and slopes.

The concentration of the macromolecular lattices is conveniently measured with reference to the number of subunits. Thus

$$\nu = g\theta, \qquad \nu/c_A = gK\theta/s, \tag{24a,b}$$

with the parameter g being the maximum number of ligands which can be bound to a subunit. We note that according to (22) and (23)

$$s = (q/\zeta)(\lambda_0 - 1)^2 \lambda_0^{n-1} \tag{25}$$

is a definite function of q and ζ. Thus at given g, K, n and q the Scatchard plot can be calculated in a straight-forward way.

Typical Scatchard plots for $n = 1$ and various q have been presented in the literature [4,11]. For positive cooperativity ($q > 1$) they exhibit negative (i.e., convex) curvature everywhere while for negative cooperativity ($q < 1$) positive (i.e., concave) curvature is encountered. This is in accordance with a quite general principle [2]. The rule that a positively cooperative system can be recognized by a maximum in its Scatchard plot [12] is apparently less general since in this case a maximum occurs only for $q > 3/2$.

Let us now turn to the case of $n > 1$. For $q = 1$ the Scatchard plots have been found to appear rather similar to those for $n = 1$ at negative cooperativity [4]. It can indeed generally be shown that multiple contact binding leads to a reduced apparent degree of cooperative interaction between nearest neighbors. For a quantitative analysis we first consider the limit of small θ. There we derive from (25) that

$$\theta/s = (n/q)[1 + (2q - 2n - 1)(\theta/n)]$$

implying for the initial intercept and slope of the Scatchard plot

$$I_0 = ngK/q, \qquad S_0 = (2q - 2n - 1)K/q.$$

As far as these characteristic parameters are concerned it is easily seen that an increase of n can be largely compensated by an increased in q (especially at greater q-values).

For an extrapolation to the other end of the Scatchard plot we consider (25) at sufficiently small values of $1 - \theta$ so that $\theta \approx 1$ and $\lambda_0 = \zeta/q \gg 1$. This leads to

$$\theta/s = [nq(1 - \theta)]^n$$

and accordingly yields final intercepts and slopes of

$$I_\infty = g, \qquad S_\infty = -qK, \qquad n = 1,$$
$$= 0, \qquad n > 1.$$

Thus ordinary one-contact binding is associated with a finite slope at the high saturation intercept of the Scatchard plot. This slope even tends to become very steep as the cooperativity increases since $S_\infty/S_0 \to -q/2$. For multiple-contact binding, on the other hand, the theory predicts a rather flat behavior at $\nu \to I_\infty$ with an inflection point in that region. In practice, however, the situation is less definite as can be recognized from the typical plots shown in fig. 1. The solid curves refer to $n = 1$ and various degrees of cooperativity. If in each case q and n are changed under the condition of constant intercepts and initial slope, this makes obviously very little difference even when going to $n = 100$ as far as the overall course of the plot is concerned. The fact that we arrive at virtually the same curves with extremely different sets of q and n means of course that it will practically not be possible to determine all the four parameters of the model mechanism from pertinent experimental data even if these are rather accurate.

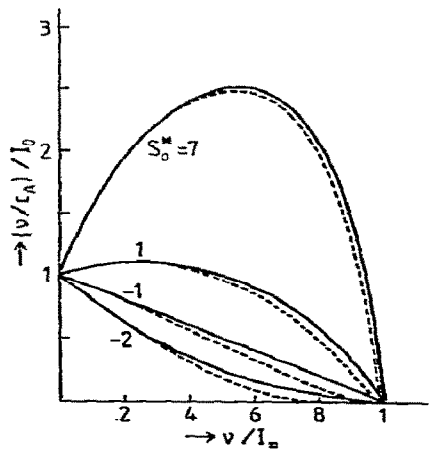

Fig. 1. Scatchard plots for various values of normalized initial slopes $S_0^* = (I_\infty/I_0)S_0 = [2(q-n) - 1]/n$ and $n = 1$ (solid) or 100 (dashed), respectively.

Let us consider a plot with a fairly steep initial slope and a pronounced maximum (corresponding to about $S_0 \gtrsim 5\ I_0/I_\infty$ or $q/n \gtrsim 3$, respectively). Under these circumstances the theoretically existing inflection point will hardly be detectable in actual experiments. In practice we shall find a rather large negative S_∞ (with the ratio $S_0 I_\infty/S_\infty I_0$ being virtually independent of n) even in cases of an extremely high degree of multiple contact binding. Nevertheless the apparent final intercept will still agree fairly well with the exact value. Accordingly we have in addition to

$$I_0 = g(n/q)K, \qquad S_0 \approx 2(1 - n/q)K,$$

apparent values of

$$I_\infty = g, \qquad S_\infty = -(q/n)K.$$

From this only g, K, and q/n can be evaluated. The same applies to an overall curve fit. Actually we could choose n arbitrarily as $n = \tilde{n}$ and would still be able to obtain a good fit of the data with correct values of g and K but a possibly quite false parameter $q = \tilde{q}$. We note, however, that a good value of the correct q follows as $q = (n/\tilde{n})\tilde{q}$ once the actual n is known.

In plots which exhibit weakly positive or even negative initial slopes the final part of the curve may be difficult to be extrapolated in practice. But as long as n is known a curve fit still offers good chances to evaluate g, K and q. Otherwise even g and K will become uncertain.

3.3. Cooperative interaction beyond nearest neighbors

Nearest neighbor cooperativity has been shown to imply Scatchard plots which do not exhibit a clearly pronounced inflection point. Nevertheless such behavior has been observed with certain experimental systems [13,14]. This may be due to the existence of (at least) two different classes of binding sites. However, it should be made clear that even with only one type of sites it is cooperative interaction beyond nearest

neighbors which could result in a phenomenon of this kind.

For the sake of simplicity we shall consider only cooperativity up to second neighbors and assume one contact per site. It should be quite obvious from our general theoretical approaches how more complex systems can be quantitatively treated if necessary.

The cooperative binding constant for the growth of sequences of at least two bound ligands is again denoted K. The cooperative parameters are to be defined as q_0 and q_1 in such a way that q_0 multiplies the nucleation binding constant in order to obtain the binding constant for a ligand occupying a site next to a nucleus (so that a sequence of just two bound ligands is formed) and in turn this binding constant is to be multiplied by q_1 to yield K. (q_1 thus describes the additional cooperative interaction with the second nearest neighbor.) We neglect end effects and suitably use the SGF-method for the calculations. Taking the statistical weight factors for a site unoccupied by a ligand as unity the sequence generating function of the unoccupied parts of the lattice becomes as usual

$$U(x) = \sum_{j=1}^{\infty} x^{-j} = 1/(x-1).$$

With $s = Kc_A$ the statistical weights of sequences of n occupied sites are found to be

$$v_1 = (1/q_0 q_1)s, \qquad v_j = (1/q_0 q_1^2)s^j \qquad (j > 1),$$

resulting in a corresponding sequence generating function of

$$V(x) = \sum_{j=1}^{\infty} (v_j/x^j)$$

$$= s[q_1 x - (q_1 - 1)s]/q_0 q_1^2 x(x-s).$$

The condition $U(x)\,V(x) = 1$ accordingly leads to an algebraic equation of the third order in x, namely

$$f(x) \equiv x^3 - (1+s)x^2 + (1-\sigma)sx + \sigma(1-\sigma_1)s^2 = 0. \quad (26)$$

We have to evaluate its largest root λ_0. It can be easily realized that this λ_0 is greater than both s and 1 and there is only one such root. The calculation is readily performed by means of a desk computer. We may then calculate θ using the expression

$$\frac{\theta}{s} = \frac{\lambda_0^2 - (1-\sigma)\lambda_0 - 2\sigma(1-\sigma_1)s}{(1+s)\lambda_0^2 - 2(1-\sigma)s\lambda_0 - 3\sigma(1-\sigma_1)s^2},$$

which can be derived on the basis of the relations (4) and (26). Scatchard plots are now numerically obtainable according to (24).

Let us first consider the two extreme cases of binding. At $s \to 0$ we may put

$$\lambda_0 = 1 + \sigma s$$

and find

$$\theta/s = \sigma[1 + (2q_0 - 3)\sigma s],$$

which leads to

$$I_0 = gK/q, \qquad S_0 = (2q_0 - 3)K/q. \quad (27a,b)$$

Note that the initial slope when extrapolated to the ν-axis intercepts at $I'_\infty = -g/(2q_0 - 3)$. Turning now to $s \to \infty$ it follows at sufficiently small values of $1/s$ that

$$s/\lambda_0 = 1 - q_0\sigma^2/s,$$
$$\theta/s = (1/s)(1 - \sigma_1\sigma/s),$$

so that

$$I_\infty = g, \qquad I_\infty/I'_\infty = -(2q_0 - 3), \qquad S_\infty = -q_1 qK. \quad (28a,b,c)$$

Thus by comparing I_∞ and I'_∞ the cooperativity of the dimerization step in binding may be determined. The quantity $K^{(\infty)} = q_1 qK = -S_\infty$ has been generally pointed out [2] to be the final binding constant, i.e., in this

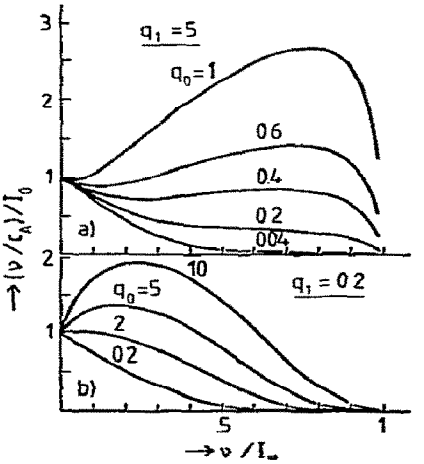

Fig. 2. The effect of changing q_0 on Scatchard plots with a constant value of q_1.

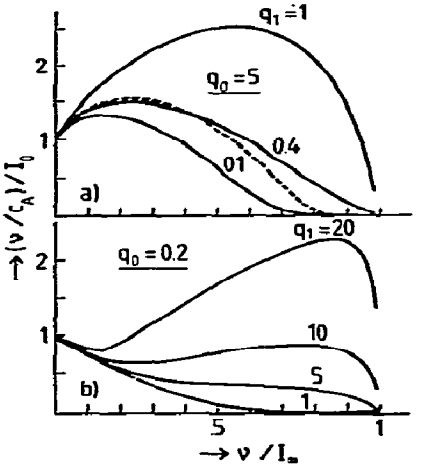

Fig. 3. The effect of changing q_1 on Scatchard plots with a constant value of q_0. The dashed curve in (a) refers to the special long range cooperativity discussed in the next.

case the binding constant with respect to an unoccupied site which has two occupied ones on each side.

Actual Scatchard plots of various typical shapes are represented in the fig. 2 and 3. Quite obviously the q_0- and q_1-values are (at least qualitatively) reflected in the curve's behavior at lower and higher binding, respectively, provided both parameters have fairly opposing effects. Steep slopes (rising at $\nu \to 0$ and falling at $\nu/c_A \to 0$) and positive curvature (or even a maximum) indicate (depending on the part of the plot where they occur) pronounced positive cooperativity of the first or second interactions, respectively. On the other hand, negative or flat slopes and positive curvature are characteristic for negative cooperativity under such circumstances. In principle, all the four parameters of the underlying model, viz. g, K, q_0, and q_1, can be determined quantitatively from experimentally extrapolated initial and final intercepts and slopes as described by (27) and (28).

Cooperative interactions beyond more than two neighbors have effects on Scatchard plots which can at least qualitatively be estimated from the properties of the curves discussed so far in this section. Quantitative treatment along the above lines would also be possible but usually require more computational effort. An example of long-range cooperative interaction which is nevertheless comparatively easy to handle mathematically may be given.

We consider a case described by a cooperative parameter q for the first step of binding after nucleation. The respective binding constant is to be denoted K. Further successive binding steps at this nucleus are assumed to become weaker and weaker (due to some unfavorable interaction, for instance electrostatic repulsion) as expressed by binding constants of $K/2$, $K/3$, and so on. With $s = Kc_A$ the sequence generating function of the bound ligands will then be

$$V(x) = \frac{s}{qx}\left\{1 + \frac{s}{x} + \frac{1}{2!}\left(\frac{s}{x}\right)^2 + \ldots\right\} = \frac{s}{qx} e^{s/x},$$

leading to an equation for $s/x = y$, namely

$$y^2 e^y = q(s - y).$$

As can be easily seen it has one real root $y_0 > 0$ which is associated with $y_0 = s/\lambda_0$ so that according to (8) the degree of binding can be written

$$\theta = \frac{(1 + y_0)(s - y_0)}{(1 + y_0)(s - y_0) + s}.$$

A Scatchard plot calculated on this basis with $q = 5$ is shown in fig. 3a. Apparently there is no essential difference compared with the plots corresponding to the basic model with the two parameters q_0, q_1 and only cooperative interaction up to two neighbors. In practice it would be especially difficult to distinguish it from the one where $q_0 \approx 5$ and $q_1 \approx 0.3$.

3.4. Interpretation of experimental data

We are now in a position to analyze measured Scatchard plots of linear binding which we assume or know to involve only one class of equivalent sites.

In the simple case that either negative or positive curvature is observed throughout (implying no detectable inflection point), nearest neighbor cooperativity in binding may be sufficient to interpret the data. Negative curvature and especially a maximum in any case reflect positive cooperativity. Positive curvature could be due to negative cooperativity or multiple-contact binding. Unfortunately the four parameters of the general model in section 3.2, including the number of binding contacts per ligand, can in practice not all be determined from a measured plot as we have pointed out above. Multiple-contact binding can therefore not be recognized by virtue of ordinary

binding experiments. The value of n should be known (for instance from structural information) in order to arrive at correct values of the other parameters. Otherwise only more or less approximate values of g, K and q/n can be evaluated. In any case we can describe the binding data with any n and appropriately adjusted g, K and q. If such curve fit fails, cooperative interaction beyond the immediate neighbors must be taken into account. One more parameter introduced in this way should yield a reasonably curve fit then. More parameters would hardly be of any use in practice.

Scatchard plots which exhibit one or more pronounced points of inflection can in principle occur. They indicate quite antagonistic cooperative interactions between one bound ligand and at least two neighbors. Which kind of cooperativity comes first (last) can apparently be recognized from the shape of the initial (final) part of the Scatchard plot. A basic model theory as presented in section 3.3 should usually be sufficient for a quantitative description. It can be extended to multiple-contact binding at the expense of introducing another parameter which should be known since it would otherwise not be accessible from the data.

Finally it must be emphasized that all the plots so far discussed – with the sole exception of purely negatively curved ones – can be also due to a superposition of different classes of binding sites which may have a less complex nature.

4. Different classes of binding sites

4.1. Independent classes

Naturally there may be more than one kind of equivalent binding sites for a ligand on a linear structure. The properties of such a system can be described by simply superimposing the binding behavior of the individual classes, provided these do not interact with each other. Binding curves and Scatchard plots then follow by means of (10) and (11) with

$$\theta_r = \phi_r/(1 + \phi_r)$$

where ϕ_r denotes the pertinent quasi-equilibrium constant of the overall saturation process $u_r \rightleftharpoons a_r$ regarding class r, i.e., $\phi_r = [c_a/c_u]_r$ which is of course a function of c_A. Neglecting multiple contacts as well as cooperative interaction beyond nearest neighbors we apparently have

$$\phi_r(c_A) = \phi\left(\frac{s_r - 1}{2\sqrt{\sigma_r s_r}}\right) \tag{29}$$

involving the ϕ-function of (14) with $s_r = K_r c_A$ and $\sigma_r = 1/q_r$.

Generally the shape of binding curves and Scatchard plots to be expected under the circumstances has already been discussed elsewhere [2]. We note that two non-cooperative classes imply a positively curved Scatchard plot which could be confused with one due to only one class of sites but exhibiting negative cooperativity or multiple contacts. One non-cooperative together with a positively cooperative class (of comparable K-values) will be associated with a Scatchard plot which starts linearly at a negative slope and then turns to negative curvature displaying a more or less pronounced hump similar to some of the plots in figs. 2a and 3b. In case of several classes of sites which all exert strongly positive cooperativity, a corresponding number of humps will usually appear in the plot.

4.2. Mutual blocking of sites

The situation will be more complex if sites of different classes interact with each other. Many possibilities of such interaction are conceivable [15]. We shall discuss here only a special case which is of significance for certain experimental systems.

Let us assume a linear structure to which a ligand can cooperatively bind in various different modes but so that occupied binding sites of one mode block sites for other modes on that part of the structure. This should for instance be a reasonable model for the binding of charged ligands to oppositely charged polymers, e.g., ATP to polylysine [14]. Since in such cases the nucleation binding constant is determined by electrostatic attraction, not more than one mode of binding can be effective at the same place on the polymer.

A rigorous theory for a mechanism of this kind encounters some difficulties so that a simplified approach is to be introduced as an apparently useful approximation. We start from the point that the different classes of bound ligands do not interact directly in any way but only reduce the number of free sites accessible to others. For each class we therefore

simply take the equilibrium proportion of bound ligands and those sites which can actually be occupied to be independent of the presence of other modes. Accordingly we set

$$\theta_r = \phi_r \beta,$$

where ϕ_r refers to the appropriate function applicable also to a corresponding single-mode binding mechanism and β equals the completely unoccupied fraction of polymer. Because of

$$\beta = 1 - \sum_j \theta_j = 1 - \beta \sum_j \phi_j$$

it follows that

$$\theta_r = \frac{\phi_r}{1 + \Sigma_j \phi_j}.$$

We note that the degree of saturation of any individual mode will always be smaller than in the case of independent classes. The formulation of ν and ν/c_A remains formally the same as before.

We shall investigate here only nearest neighbor cooperativity and single-contact binding. The respective functions ϕ_r are the same as given in (29). As may be derived from (14) they obey the limiting laws

$$\phi_r/s_r \to 1/q_r, \qquad \text{for } s_r \to 0,$$
$$\to q_r, \qquad s_r \to \infty.$$

Thus while still $\theta_r \to 0$ at $c_A \to 0$ we find

$$\theta_r \to \theta_{r,\infty} \approx \frac{q_r K_r}{\Sigma_j q_j K_j} < 1, \qquad \text{at } c_A \to \infty \tag{30}$$

implying $I_\infty < \Sigma_r g_r$. This phenomenon is of course due to the blocking of different types of sites for the ligands. The final distribution of ligands among types of binding is evidently proportional to $q_r K_r$, its initial distribution, however, proportional to K_r/q_r. This could result in a switch of bound ligands from one mode of binding to another as the level of free ligand increases. Typical binding curves and respective Scatchard plots are demonstrated in the figs. 4 and 5. First a non-cooperative binding mode ($q_1 = 1$) interacting with one of strongly positive cooperativity ($q_2 = 100$) is investigated (fig. 4). Up to $c_A = 1/K_2$ practically only a hyperbolic binding curve due to the first mode is observed. Then it becomes almost completely suppressed by the second mode (because of

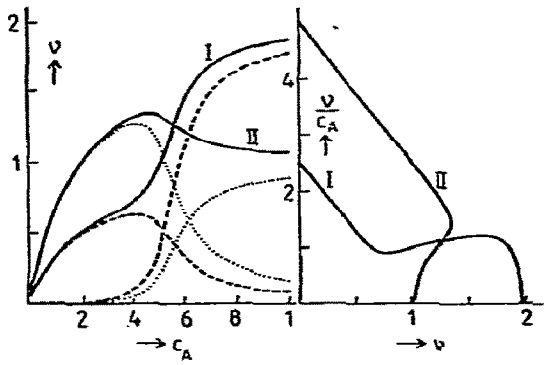

Fig. 4. Binding curves and Scatchard plots for two cases of a non-cocperative and a positively cooperative binding mode which exclude each other. Case I refers to $K_1 = 5, q_1 = 1, g_1 = 1; K_2 = 2, q_2 = 100, g_2 = 2$ (the individual binding ratios ν_1 and ν_2 are indicated by the dashed curves). In case II only the g-values are exchanged: $g_1 = 2; g_2 = 1$ (with individual binding curves dotted).

$q_2 K_2 \gg q_1 K_1$). Note that owing to the appreciable contribution of ϕ_1 to θ_2 the course of ν_2 versus c_A will be clearly flatter and also displaced to higher c_A-values as compared with an independent binding curve associated with the same parameters. On the other hand, an unusual effect may occur after the onset of second binding mode, namely a decrease of the overall binding curve. This would apparently be the case if $g_2 < g_1 K_1/(K_1 + K_2)$. The Scatchard plot must bend backwards then. Such an event can obviously never

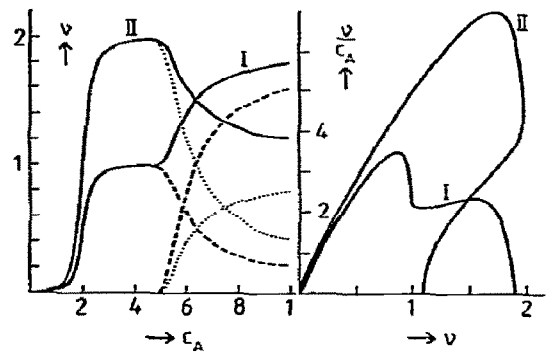

Fig. 5. Binding curves and Scatchard plots for two cases of strongly cooperative and mutually exclusive binding modes. Case I refers to $K_1 = 5, q_1 = 100, g_1 = 1; K_2 = 2, q_2 = 2500, g_2 = 2$ (individual binding behavior indicated by dashed curves). In case II again only the g-values have been exchanged: $g_1 = 2$, $g_2 = 1$ (with individual binding curves dotted).

take place with independent classes of sites.

Analogous behavior is encountered when both modes exert strongly positive cooperativity (fig. 5). Here no binding occurs below $c_A < 1/K_1 < 1/K_2$ (where $\phi_1 \approx \phi_2 \approx 0$). Once c_A is greater than $1/K_2$ both modes distribute themselves on the entire polymer according to the limits of θ_r as given in (30). Thus

$$\nu \to g_1\theta_{1,\infty} + g_2\theta_{2,\infty} = g_1 + (g_2 - g_1)\theta_{2,\infty}.$$

Again we find suppression of the first and flattening of the second mode as well as a decay in the overall binding implying a backward bending in the Scatchard plot if $g_2 < g_1$.

In a separate article this simplified approach to multiple mode cooperative binding will be used to interpret and evaluate the apparently dual-mode cooperative binding of nucleotides to basic polypeptides [14].

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