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## Reconciliation of classical and reacted-site probability approaches to allowance for ligand multivalence in binding studies

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### Abstract

The objective of this investigation is to engender greater confidence in the validity of binding equations derived for multivalent ligands on the basis of reacted-site probability theory. To that end a demonstration of the theoretical interconnection between expressions derived by the classical stepwise equilibria and reacted-site probability approaches for univalent ligands is followed by use of the traditional stepwise procedure to derive binding equations for bivalent and trivalent ligands. As well as demonstrating the unwieldy nature of the classical binding equation for multivalent ligand systems, that exercise has allowed numerical simulation to be used to illustrate the equivalence of binding curves generated by the two approaches. The advantages of employing a redefined binding function for multivalent ligands is also confirmed by subjecting the simulated results to a published analytical procedure that has long been overlooked.

### Keywords

Antigen–antibody affinity; Binding equations; Ligand multivalence; Reacted-site probability theory

### INTRODUCTION

The analysis of the binding of a univalent ligand to equivalent and independent sites on a multivalent acceptor was developed originally (Klotz, 1946) by consideration of the stepwise equilibria that constitute the overall process. Several other methods have been used to reduce the polynomial equation resulting from the stepwise approach to the familiar hyperbolic binding isotherm (e.g., Tanford, 1961; Cantor & Schimmel, 1980; Klotz, 1986). However, ligand multivalence has long been recognized as a phenomenon of particular relevance to the quantitative description of antigen–antibody interactions where neither reactant is univalent (Goldberg, 1952, 1953; Singer, 1965). Nevertheless, most quantitative

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immunochemical studies have employed the classical binding equation for univalent ligands derived from the stepwise, stoichiometric equilibria involved in the assembly of the multiligated acceptor (Klotz, 1946; Scatchard, 1949), a practice that has continued despite specific demonstrations of its invalidity (Calvert et al., 1979) as well as means of making adequate allowance for the consequences of ligand multivalence (Calvert et al., 1979; Hogg and Winzor, 1984, 1985; Harris et al., 1995). Such reluctance to take advantage of valid procedures for characterizing the binding of multivalent ligands presumably reflects distrust of these developments because of their reliance upon reacted-site probability theory (Flory, 1941, 1953; Stockmayer, 1943) rather than the classical stepwise binding approach.

The aim of the present communication is to bolster confidence in the equations derived from reacted-site considerations by using the traditional approach to derive an expression which predicts the same binding curves as those based on reacted-site probability theory for the simplest multivalentmultivalent system – that in which acceptor and ligand are both bivalent. Thereby demonstrated is the impracticality of adopting the traditional stepwise approach as a general procedure for treating multivalence because of its generation of binding equations involving the ratio of two indefinite multinomial series in free ligand concentration. That undesirable situation can be avoided completely by resorting to reacted-site probability theory, which provides binding equations with closed solutions for all combinations of acceptor and ligand valences.

## THEORETICAL CONSIDERATIONS

The derivation of binding equations by either the traditional (Klotz, 1946) or reacted-site probability (Calvert et al., 1979) approach is based on equivalence and independence of acceptor sites in their interaction with ligand – a combined set of circumstances that allows description of all interactions in terms of a single equilibrium constant, variously called the intrinsic equilibrium constant (Klotz, 1986), site-binding constant (Calvert et al., 1979), or microscopic equilibrium constant (Cantor & Schimmel, 1980). For systems involving the interaction of a univalent ligand ( $B$ ) with a  $p$ -valent acceptor ( $A$ ) the binding equation has traditionally been derived (Klotz, 1946) from the concentrations of all species generated by the stepwise addition of ligand molecules to form the complex with maximum stoichiometry,  $AB_p$ . However, multivalence of the ligand introduces virtually insurmountable complexity into this stepwise approach because of the need to establish the concentrations of an infinite number of species  $A_iB_j$  – the reason for the switch to reacted-site probability theory (Flory, 1941; Stockmayer, 1943; Calvert et al., 1979) and thereby avoidance of that unenviable task. This theoretical section begins with presentation of the reacted-site probability approach to derivation of the binding equation for the interaction between a univalent ligand and a  $p$ -valent acceptor to demonstrate that it yields the expression obtained by the classical stepwise procedure (Klotz, 1946). Such action serves to introduce in current terminology the approach used by Goldberg (1952) in the original application of reacted-site probability theory to antigen–antibody interactions.

## Binding equation for a univalent ligand: the reacted-site probability approach

The central parameters in reacted-site probability theory are  $P_A$ , the probability that any given site on an acceptor molecule ( $A$ ) has reacted with a site on ligand ( $B$ ); and  $P_B$ , the corresponding probability that a ligand site has reacted with an acceptor site. For a univalent ligand the single site is either occupied by acceptor or free, whereupon the free and total ligand concentrations ( $C_B$  and  $\bar{C}_B$  respectively) are related by the expression  $\bar{C}_B = C_B + P_B C_B$ ; or, on rearrangement,

$$C_B = \bar{C}_B (1 - P_B) \quad (1)$$

The corresponding relationship between total ( $\bar{C}_A$ ) and free ( $C_A$ ) concentrations of a  $p$ -valent acceptor is obtained by noting that  $\bar{C}_A$  is the sum of concentrations of free  $A$  and the complexes  $AB$ ,  $AB_2$ , ...,  $AB_p$ . On the grounds that the probability of  $i$  acceptor sites being occupied is given by the binomial density function  $\phi_i$ , the concentration of acceptor–ligand complex  $AB_i$  is given by the product  $\phi_i \bar{C}_A$ , where

$$\phi_i = \frac{p!}{(p-i)!i!} P_A^i (1 - P_A)^{p-i} \quad (2)$$

The relationship between free and total acceptor concentrations then assumes the form

$$C_A = \bar{C}_A \left( 1 - p P_A (1 - P_A)^{p-1} - [p(p-1)/2!] P_A^2 (1 - P_A)^{p-2} - [p(p-1)(p-2)/3!] P_A^3 (1 - P_A)^{p-3} - \dots - P_A^p \right)$$

which has the closed solution (Singer, 1965)

$$C_A = \bar{C}_A (1 - P_A)^p \quad (3)$$

In situations where all interactions involve identical and independent sites on the acceptor, the intrinsic equilibrium constant  $k$ , is defined as the ratio of the concentration of bound (reacted) ligand sites to the product of the concentrations of unreacted acceptor sites and unreacted ligand sites (Klotz, 1946). For a univalent ligand, reacted ligand sites equal bound ligand molecules. In terms of reacted-site probability, the concentration of bound ligand can be expressed as either the concentration of reacted ligand sites,  $P_B \bar{C}_B$ , or the concentration of reacted acceptor sites ( $p P_A \bar{C}_A$ ). The concentrations of unreacted acceptor sites and unreacted ligand sites are  $p(1 - P_A) \bar{C}_A$  and  $(1 - P_B) \bar{C}_B$ , respectively. Thus,

$$k = \frac{P_B \bar{C}_B}{p(1 - P_A) \bar{C}_A (1 - P_B) \bar{C}_B} = \frac{P_B}{p(1 - P_A) (1 - P_B) \bar{C}_A} \quad (4)$$

Defined in this way,  $k$  is an association constant with units of  $M^{-1}$ . The term in  $(1 - P_A)$  is eliminated by noting the necessity for the concentration of reacted  $A$  sites ( $p P_A \bar{C}_A$ ) to equal that of reacted  $B$  sites ( $P_B \bar{C}_B$ ), a requirement that leads to the expression

$$(1 - P_A) = \frac{(p\bar{C}_A - P_B\bar{C}_B)}{p\bar{C}_A} \quad (5)$$

Substitution of eqn (5) into eqn (4) then gives

$$\frac{P_B}{(1 - P_B)} = k(p\bar{C}_A - P_B\bar{C}_B) \quad (6)$$

or, on noting from eqn (1) that  $(1 - P_B) = C_B/\bar{C}_B$ ,

$$P_B\bar{C}_B(1 + k\bar{C}_B) = k p \bar{C}_A C_B \quad (7)$$

Incorporation of the definition of the experimental binding function,  $r$ , as the concentration of bound ligand divided by the total acceptor concentration ( $r = P_B C_B / \bar{C}_A$ ) then leads to the rectangular hyperbolic relationship

$$r = \frac{p k C_B}{(1 + k C_B)} \quad (8)$$

that was derived by Klotz (1946) in the classical stepwise treatment of ligand binding to equivalent and independent acceptor sites.

The stepwise equilibria define  $p$  constants, variously called macroscopic, stoichiometric or stepwise equilibrium constants. The relationship between the stoichiometric equilibrium constant for the  $i^{\text{th}}$  step ( $K_i$ ) and the intrinsic equilibrium constant,  $k$ ,

$$K_i = \frac{p - i + 1}{i} k \quad (9)$$

was derived by Klotz (1946) algebraically without using statistical or probability factors. This expression can also be derived by a statistical approach to the number of ways a total of  $i$  ligands can bind to a  $p$ -valent acceptor (e.g., Tanford, 1961; Cantor & Schimmel, 1980).

For interactions involving a univalent ligand there is no particular advantage in switching to the reacted-site probability approach. However, it provides a much simpler means of deriving a binding equation for systems in which the ligand also exhibits multivalence.

## Binding equation for a bivalent ligand: the reacted-site probability approach

Interactions between a  $p$ -valent acceptor  $A$  and a bivalent ligand  $B$  lead to networks of alternating  $A$  and  $B$  molecules with stoichiometric composition  $A_i B_j$ . The counterpart of eqn (1) for ligand now becomes

$$C_B = \bar{C}_B (1 - P_B)^2 \quad (10)$$

where the exponent accommodates the bivalence of ligand. The binding function may thus be written as

$$r = \frac{\bar{C}_B - C_B}{\bar{C}_A} = \frac{C_B \left( [1/(1 - P_B)]^2 - 1 \right)}{\bar{C}_A} \quad (11)$$

where  $r$  is the number of moles (not sites) of  $B$  bound per mole of acceptor. The definition of the intrinsic equilibrium constant,  $k$ , as the concentration of reacted ligand sites to the product of the concentrations of unreacted acceptor sites and free bivalent ligand sites is retained, which again produces eqn (4). The stoichiometry factor for bivalent ligand does not appear in eqn (4) because it cancels in the numerator and denominator. Again we take advantage of the necessity for equal concentrations of reacted acceptor and ligand sites, i.e.,

$$pP_A \bar{C}_A = 2P_B \bar{C}_B \quad (12)$$

to eliminate  $(1 - P_A)$  from eqn (4) – an exercise from which it follows that

$$P_B = k(1 - P_B) \left( p\bar{C}_A - 2P_B \bar{C}_B \right) \quad (13)$$

The free ligand concentration is now introduced into eqn (13) by means of eqn (10) to give a quadratic with solution (Calvert et al., 1979)

$$P_B = 1 + \frac{(2kC_B - 1) - \sqrt{\Delta}}{2(1 + pk\bar{C}_A)} = 1 + \frac{(\alpha - 1) - \sqrt{\Delta}}{2(1 + pk\bar{C}_A)} \quad (14a)$$

$$\Delta = (1 + 2kC_B)^2 + 8k^2 p\bar{C}_A C_B = (1 + \alpha)^2 + 4kp\bar{C}_A \alpha \quad (14b)$$

where  $\alpha = 2kC_B$ . As established by Calvert et al. (1979), the substitution of this expression for  $P_B$  into eqn (11) eventually leads to the following binding equation,

$$r = \frac{p\alpha}{1 + \alpha} + \Omega \quad (15a)$$

$$\Omega = \frac{(1 - \alpha)^2}{4k\bar{C}_A \alpha} \left[ 1 + \frac{2pk\bar{C}_A \alpha}{(1 + \alpha)^2} - \left\{ 1 + \frac{4pk\bar{C}_A \alpha}{(1 - \alpha)^2} \right\}^{1/2} \right] \quad (15b)$$

The first term on the right-hand side of eqn (15a) describes a rectangular hyperbolic dependence of binding function upon  $C_B$  with  $p$  and  $2k$  the characteristic parameters. However, the overall dependence deviates from the form of a rectangular hyperbola because of the contribution from the second term ( $\Omega$ ), which only assumes a value of zero when  $\alpha = 1$  [i.e.,  $C_B = 1/(2k)$ ] for all  $\bar{C}_A$ . Binding curves obtained at a series of fixed acceptor concentrations are thus predicted to intersect at the point corresponding to  $C_B = 1/(2k)$ ,  $r = p/2$ . This behavior has been illustrated (Calvert et al., 1979) by the numerical simulation of such binding curves. As noted by Calvert et al. (1979) deviations from rectangular

hyperbolic behavior that are consistent with eqn (15a) usually tend to be regarded as signifying either heterogeneity or negative cooperativity of acceptor sites, when in fact they may simply reflect ligand bivalence and a single intrinsic equilibrium constant.

Although over thirty years have elapsed since the publication of eqns (15a) and (15b), their use seems to have been limited to that illustrative application in the original investigation (Calvert et al., 1979) and a review article shortly thereafter (Nichol and Winzor, 1981). Such disregard of the only valid quantitative description of the traditional binding curve for the interaction between a bivalent ligand and a multivalent acceptor must reflect to some extent a reluctance to accept a finding based on reacted-site probability theory, an unfamiliar concept to immunochemists. To engender greater confidence in the validity of the analysis, a comparable quantitative description is derived by considering the stepwise equilibria involved – an approach with which experimenters are more familiar. However, a prerequisite for such an endeavour is the availability of expressions for the equilibrium concentrations of all complexes present in a mixture of multivalent acceptor and bivalent ligand.

### Quantitative description of the solution composition

The interaction between a  $p$ -valent acceptor and a bivalent ligand results in the equilibrium coexistence of unbound reactants and an array of  $A_iB_j$  complexes that can be formulated in stoichiometric terms as (Calvert et al., 1979)

	$A_iB_j$				
$i = 1:$	$A,$	$AB,$	$AB_2,$	....,	$AB_p$
$i = 2$	$A_2B,$	$A_2B_2,$	$A_2B_3,$	....,	$A_2B_{2p-1}$
$i = 3$	$A_3B_2,$	$A_3B_3,$	$A_3B_4,$	....,	$A_3B_{3p-2}$
$i = 4$	$A_4B_3,$	$A_4B_4,$	$A_4B_5,$	....,	$A_4B_{4p-3}$
.	.	.	.	.	.
.	.	.	.	.	.

The simplest approach to quantifying concentrations for these complexes is to obtain expressions for those in the first column of the array,  $A_iB_{i-1}$ , and then to consider the completion of each line of the array. Here we envisage formation of those  $A_iB_{i-1}$  complexes via the addition of  $AB$  to the previous member, noting that for  $i = 1$  this corresponds to complex formation between  $A$  and  $AB$ .

For the interaction of bivalent ligand with  $p$ -valent acceptor the relationship between stoichiometric ( $K_i$ ) and intrinsic ( $k$ ) binding constants [eqn (9) for the univalent case] becomes

$$K_i = \frac{p - i + 1}{i} (2k) \quad (16)$$

to incorporate the fact that there are two (rather than one) ways of ligand attachment to each of the  $(p - i + 1)/i$  possible arrangements of unoccupied acceptor sites. The concentration of the building block  $AB$  is thus given by

$$C_{AB} = K_1 C_A C_B = p(2kC_B) C_A \quad (17)$$

An expression for the concentration of  $A_2B$  can be generated from those of  $A$  and  $AB$  by regarding its formation as the first-step interaction of the univalent  $AB$  species with the  $p$  sites on  $A$ . In those terms it follows from eqn (9) that

$$C_{A_2B} = pkC_A (C_{AB}/2) = p^2 k^2 C_A^2 C_B \quad (18)$$

where, as a thermodynamic necessity, the halving of the  $AB$  concentration reflects the formation of  $A_2B$  from the interaction of  $A$  with either of the two forms of  $AB$ , which are present in equal proportions. In similar vein, the complex  $A_3B_2$  may be considered to result from the interaction of univalent  $AB$  with  $A_2B$ , which has  $(2p - 2i + 2)$  available sites. Indeed, on the grounds that the concentrations of all  $A_iB_{i-1}$  species can likewise be determined because all  $A_iB_{i-1}$  species (including  $A$ ) possess  $(pi - 2i + 2)$  sites for interaction with  $AB$ , the general form of eqn (18) is

$$C_{A_{i+1}B_i} = (pi - 2i + 2) k C_{A_iB_{i-1}} (C_{AB}/2) = [\Pi (pi - 2i + 2)] (2k)^{2i} C_A^{i+1} C_B^i \quad (19)$$

where the product of statistical factors,  $\pi(pi - 2i + 2)$ , covers the range 1 to  $i$ .

Because each  $A_iB_{i-1}$  complex also possesses  $(pi - 2i + 2)$  equivalent and independent sites available for the binding of  $(j - i + 1)$  molecules of bivalent  $B$  to form the  $A_iB_j$  complexes in each line of the array, the Klotz (1946) approach can be adopted by employing eqn (16) to obtain their concentrations as (Calvert et al., 1979)

$$C_{A_iB_j} = \left[ C_{j-i+1}^{pi-2i+2} \right] (2k)^{j-i+1} C_{A_iB_{i-1}} C_B^{j-i+1} \quad (20)$$

where  $\left[ C_{j-i+1}^{pi-2i+2} \right]$  is the combination of  $(pi - 2i + 2)$  sites taken  $(j - i + 1)$  at a time.

### Traditional binding equation for a bivalent ligand

Consider initially a system in which the acceptor is also bivalent ( $p = 2$ ), this being the simplest situation inasmuch as the consequent bivalence of all  $A_iB_{i-1}$  species ensures that complex formation is restricted to linear polymers of alternating  $A$  and  $B$  molecules: no branched polymers can form. An expression for total acceptor concentration is first derived in terms of the dimensionless parameter  $a = 2kC_B$  used in the earlier binding expressions, eqns (15a) and (15b). On the grounds that

$$\bar{C}_A = \left( C_A + C_{AB} + C_{AB_2} \right) + 2 \left( C_{A_2B} + C_{A_2B_2} + C_{A_2B_3} \right) + 3 \left( C_{A_3B_2} + C_{A_3B_3} + C_{A_3B_4} \right) + \dots \quad (21)$$

we clearly need to sum the concentrations of the three species,  $A_iB_{i-1}$ ,  $A_iB_i$  and  $A_iB_{i+1}$ , for each value of  $i$ . For  $i = 1$  and  $p = 2$  these species are  $A$ ,  $AB$  and  $AB_2$ , where  $C_{AB} = 2C_A\alpha$  [see eqn (17)] and  $C_{AB_2} = C_A\alpha^2$  [from eqn (20)]: the sum of these three concentrations may thus

be written as  $C_A(1 + \alpha)^2$ . In similar vein,  $C_{A_2B} = 2kC_A^2\alpha$  [eqn (18)], whereas

$C_{A_2B_2} = 4kC_A^2\alpha^2$  and  $C_{A_2B_3} = 2kC_A^2\alpha^3$  [from eqn (20)]: their sum can be written as

$2kC_A^2\alpha(1 + \alpha)^2$ . Continuation of this process leads to the conclusion that

$$\bar{C}_A = C_A(1 + \alpha)^2 \left( 1 + 2(2kC_A\alpha) + 3(2kC_A\alpha)^2 + 4(2kC_A\alpha)^3 + 4(2kC_A\alpha)^4 + 5(2kC_A\alpha)^5 + \dots \right) \quad (22)$$

To obtain the corresponding expression for the concentration of bound ligand,  $(\bar{C}_B - C_B)$ , terms in order of increasing acceptor content are again collected to give

$$(\bar{C}_B - C_B) = (C_{AB} + 2C_{AB_2}) + (C_{A_2B} + 2C_{A_2B_2} + 3C_{A_2B_3}) + (2C_{A_3B_2} + 3C_{A_3B_3} + 4C_{A_3B_4}) + \dots \quad (23)$$

where, from the expressions already presented to derive the general form of eqn (22),  $C_{AB} + 2C_{AB_2} = 2C_A\alpha + 2C_A\alpha^2$ , and  $C_{A_2B} + 2C_{A_2B_2} + 3C_{A_2B_3} = 2kC_A^2\alpha + 8kC_A^2\alpha^2 + 6kC_A^2\alpha^3$ . In these terms the overall expression for the concentration of bound ligand can be written in the form

$$\bar{C}_B - C_B = 2C_A\alpha(1 + \alpha) \left( 1 + (2kC_A)(1 + 3\alpha)/2 + (2kC_A)^2\alpha(1 + 2\alpha) + (2kC_A)^3(3 + 5\alpha)/2 + \dots \right) \quad (24)$$

whereupon the binding equation becomes

$$r = \frac{2\alpha}{(1 + \alpha)} \left[ \frac{1 + (2kC_A)(1 + 3\alpha)/2 + (2kC_A)^2\alpha(1 + 2\alpha) + (2kC_A)^3\alpha^2(3 + 5\alpha)/2 + (2kC_A)^4\alpha^3(2 + 3\alpha) + \dots}{1 + 2(2kC_A\alpha) + 3(2kC_A\alpha)^2 + 4(2kC_A\alpha)^3 + 5(2kC_A\alpha)^4 + \dots} \right] \quad (25)$$

An expression analogous in form to that in eqns (15a) and (15b) is then obtained by subtracting the quantity  $2\alpha/(1 + \alpha)$  from each side of eqn (25), the result being

$$r = \frac{2\alpha}{(1 + \alpha)} + \Psi \quad (26a)$$

$$\Psi = \frac{(1 - \alpha) \left( (2kC_A\alpha) + 2(2kC_A\alpha)^2 + 3(2kC_A\alpha)^3 + 4(2kC_A\alpha)^4 + \dots \right)}{(1 + \alpha) \left( 1 + 2(2kC_A\alpha) + 3(2kC_A\alpha)^2 + 4(2kC_A\alpha)^3 + 5(2kC_A\alpha)^4 + \dots \right)} \quad (26b)$$

The binding equation derived by conventional means is clearly not as convenient to use as that deduced from reacted-site probability considerations because the second term on the right-hand side ( $\Psi$ ) is in the form of a ratio of polynomial series rather than a closed solution. However, it is noted that eqns (26a) and (26b) also predict a value of unity (i.e.,  $p/2$ ) for  $r$  when  $\alpha = 1$ . The extent of agreement with eqns (15a) and (15b) for  $p = 2$  can only be deduced by numerical calculation, a task undertaken later.



Although the same approach can obviously be used for systems with a larger acceptor valence, the derivation becomes increasingly tedious because of the greater number of complexes to which an expression for the concentration has to be assigned. For example, an increase in acceptor valence from  $p = 2$  to  $p = 3$  raises the number of complexes requiring quantitative description from 11 to 29 in order to maintain the above truncation of polynomial series at the  $A_4B_j$  species. For a trivalent acceptor the counterpart of eqn (21) becomes

$$\bar{C}_A = (C_A + C_{AB} + C_{AB_2} + C_{AB_3}) + 2(C_{A_2B} + C_{A_2B_2} + C_{A_2B_3} + C_{A_2B_4} + C_{A_2B_5}) + \dots \quad (27)$$

where  $C_{AB} = 3C_A\alpha$  [eqn (7)],  $C_{AB_2} = 3C_A\alpha^2$ , and  $C_{AB_3} = C_A\alpha^3$  [both from eqn (20)]: the sum of  $AB_i$  concentrations is therefore  $C_A(1 + \alpha)^3$ . For the corresponding  $A_2B_i$  series  $C_{A_2B} = (9/2)kC_A^2\alpha$  [eqn (18)],  $C_{A_2B_2} = 18kC_A^2\alpha^2$ ,  $C_{A_2B_3} = 27kC_A^2\alpha^3$ ,  $C_{A_2B_4} = 18kC_A^2\alpha^4$  and  $C_{A_2B_5} = (9/2)kC_A^2\alpha^5$  [all from eqn (20)]; and these concentrations all need to be doubled in the summation to obtain  $\bar{C}_A$  [see eqn (27)]. The contribution of this series can be arranged to the form  $9kC_A^2\alpha(1+\alpha)^4$ . Upon extension of this approach the expression for total acceptor concentration becomes

$$\bar{C}_A = C_A(1+\alpha)^3 \left( 1 + 3(3kC_A\alpha(1+\alpha)) + 9[(3kC_A\alpha)(1+\alpha)]^2 + 30[(3kC_A\alpha)(1+\alpha)]^3 + 112.5[(3kC_A\alpha)(1+\alpha)]^4 + \dots \right) \quad (28)$$

whereas that for bound ligand concentration is

$$\bar{C}_B - C_B = 3C_A\alpha(1+\alpha)^2 \left[ 1 + 0.5(3kC_A)(1+6\alpha+5\alpha^2) + 2(3kC_A)^2\alpha(1+5.5\alpha+8\alpha^2+3.5\alpha^3) + 7.5(3kC_A)^3\alpha^2(1+6\alpha+12\alpha^2+10\alpha^3) + \dots \right]$$

Subtraction of  $3\alpha/(1 + \alpha)$  from the resulting binding function then gives

$$r = \frac{3\alpha}{(1+\alpha)} + \Psi \quad (30a)$$

$$\Psi = \frac{3\alpha(1-\alpha^2) \left[ 0.5(3kC_A) + 2(3kC_A)^2\alpha(1+\alpha) + 7.5(3kC_A)^3\alpha^2(1+\alpha)^2 + 30(3kC_A)^4\alpha^3(1+\alpha)^3 + \dots \right]}{(1+\alpha) \left[ 1 + 3\{3kC_A\alpha(1+\alpha)\} + 9\{3kC_A\alpha(1+\alpha)\}^2 + 30\{3kC_A\alpha(1+\alpha)\}^3 + 112.5\{3kC_A\alpha(1+\alpha)\}^4 + \dots \right]} \quad (30b)$$

As for the previous system with  $p = 2$ , the analogy with eqns (15a) and (15b) prevails in that a value of  $p/2$  is again predicted for the binding function when  $\alpha = 1$  [i.e.,  $C_B = 1/(2k)$ ].

Although these traditionally derived expressions for the classical binding function ( $r$ ) may be used to simulate numerically its dependence upon the reduced (dimensionless) variable  $\alpha$ , they are not particularly useful in an experimental context because  $k$  (the magnitude of which is being sought in the investigation) is encapsulated in the independent variable. Indeed, a similar situation applies to the practical utility of eqns (15a) and (15b), the corresponding expressions emanating from reacted-site probability theory. As shown previously (Hogg and Winzor, 1985; Harris et al., 1995), the solution to this problem entails redefinition of the binding function for a multivalent ligand.

## A revised definition of the binding function for a multivalent ligand

The requirement for a revised definition of the binding function for a multivalent ligand surfaced during the development of quantitative affinity chromatography as a means of characterizing the interaction of tetrameric and hence tetravalent glycolytic enzymes with the affinity matrix (Nichol et al., 1981; Hogg and Winzor, 1984); and was realized a year later (Hogg and Winzor, 1985) with the report of a generalized Scatchard (1949) analysis that takes into account the ligand valence. That expression emanated from the following consideration of the problem in terms of reacted-site probability theory.

For a system involving the interaction of a  $q$ -valent ligand with a  $p$ -valent acceptor eqn (3) continues to describe the conservation of acceptor, but the corresponding counterpart for ligand [eqn (1)] needs changing to

$$C_B = \bar{C}_B (1 - P_B)^q \quad (31)$$

Furthermore, the necessity for identical concentrations of reacted acceptor and ligand sites becomes

$$pP_A \bar{C}_A = qP_B \bar{C}_B \quad (32)$$

whereupon elimination of the  $(1 - P_A)$  term from eqn (4) gives

$$k = \frac{P_B}{(1 - P_B) (p\bar{C}_A - qP_B \bar{C}_B)} \quad (33)$$

as the expression for the intrinsic binding constant. Combination of a rearranged form of eqn (31), namely

$$P_B = \frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{\bar{C}_B^{1/q}} \quad (34)$$

with eqn (33) then leads to the conclusion that

$$\frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{\bar{C}_A C_B^{1/q}} = pk - qk \bar{C}_B^{(q-1)/q} \frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{\bar{C}_A} \quad (35)$$

Upon definition of the binding function for a  $q$ -valent ligand,  $r_q$ , as

$$r_q = \frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{\bar{C}_A} \quad (36)$$

eqn (35) becomes

$$\frac{r_q}{C_B^{1/q}} = pk - qk \bar{C}_B^{(q-1)/q} r_q \quad (37)$$

which allows evaluation of the intrinsic binding constant from a linear dependence of  $r_q/C_B^{1/q}$  upon  $\bar{C}_B^{(q-1)/q} r_q$ . On substituting a value of unity for  $q$ , eqn (37) simplifies to

$$r_1/C_B = pk - kr_1 \quad (38)$$

which is the linear transform of eqn (8) that was recommended by Scatchard (1949) for the characterization of interactions involving univalent ligands. Examples of the use of this generalized Scatchard analysis are to be found in studies of the interactions between glycolytic enzymes and muscle myofibrils (Harris and Winzor, 1989a,b).

In the normal course of events a linear transform is proposed to simplify the characterization of interactions by graphical analysis. However, in this instance the derivation of eqn (37) was achieved (Hogg and Winzor, 1985) without recourse to the rectangular hyperbolic relationship of which it was the linear transform. Indeed, a decade elapsed before the discovery that eqn (37) is a linear transform of the expression (Harris et al., 1995)

$$qr_q \bar{C}_B^{(q-1)/q} = \frac{pq \bar{C}_B^{(q-1)/q} k C_B^{1/q}}{1 + q \bar{C}_B^{(q-1)/q} k C_B^{1/q}} \quad (39)$$

which, as required, simplifies to eqn (8) for a univalent ligand ( $q = 1$ ). Whereas analysis according to eqn (8) only allows assessment of the equivalence and independence of acceptor sites in binding studies involving a univalent ligand, eqn (39) provides the general rectangular hyperbolic relationship that permits the same criteria to be used for multivalent ligands.

## An alternative derivation of the generalized Scatchard equation

An alternative approach to derivation of the general counterpart of the Scatchard equation [eqn (37)] has also been developed in the vain hope that it might gain greater acceptance because of closer adherence to the standard textbook approach. A slightly adapted version of that alternative procedure (Winzor, 2002) now follows.

Advantage is taken of the fact that description of the concentration of bound ligand only requires knowledge of the total and free ligand concentrations, whereupon the concentrations of the array of complexes  $A_i B_j$  can be regarded as merely contributing in some unspecified manner to the difference between  $C_B$  and  $\bar{C}_B$ . In writing an expression for the total concentration of ligand it suffices to note that there are  $(q + 1)$  possible states of a ligand molecule to consider: that in which all of its sites are unoccupied, and those in which 1, 2, ...,  $q$  of its sites are occupied by acceptor. The total concentration of ligand can thus be written as

$$\bar{C}_B = C_B + K_1 (C_A^*) C_B + K_1 K_2 (C_A^*)^2 C_B + \dots + K_1 K_2 \dots K_q (C_A^*)^q C_B \quad (40)$$

where  $K_i$  are stoichiometric binding constants and  $C_A^*$  is the concentration of free acceptor sites on the whole array of complexes as well as those on free A; and where assumed

equivalence and independence of ligand sites allows replacement of the stoichiometric binding constants by their intrinsic counterpart ( $k$ ) via the expression (Klotz, 1946)

$$K_i = [(q - i + 1) / i] k \quad (41)$$

which is eqn (9) with the roles of  $A$  and  $B$  reversed. Here we are essentially considering the interaction of univalent  $A$  sites (present at free concentration  $C_A^*$ ) with multivalent  $B$ . With those substitutions eqn (40) can be written as

$$\bar{C}_B = C_B + q (k C_A^*) C_B + q (q - 1) (k C_A^*)^2 C_B + \dots + (k C_A^*)^q C_B \quad (42)$$

which, on application of the binomial theorem, becomes

$$\bar{C}_B = C_B (1 + k C_A^*)^q \quad (43)$$

or, on rearrangement

$$\frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{C_B^{1/q}} = k C_A^* \quad (44)$$

The term in free acceptor-site concentration ( $C_A^*$ ) is now eliminated by writing the counterparts of eqns (40) and (42) for  $p\bar{C}_A = \bar{C}_A^*$ , the total concentration of acceptor sites. Specifically,

$$\begin{aligned} \bar{C}_A^* &= C_A^* + K_1 (C_A^*) C_B \\ &\quad + 2K_1 K_2 (C_A^*)^2 C_B \\ &\quad + \dots \\ &\quad + q K_1 K_2 \dots K_q (C_A^*)^q C_B \\ &= C_A^* + q (k C_A^*) C_B \\ &\quad + 2 [q (q - 1) / 2!] (k C_A^*)^2 C_B \\ &\quad + \dots \\ &\quad + (k C_A^*)^q C_B \end{aligned} \quad (45)$$

where advantage can again be taken of the binomial theorem to write eqn (45) as

$$C_A^* = p\bar{C}_A - q (k C_A^*) C_B (1 + k C_A^*)^{q-1} \quad (46)$$

This substitution for  $C_A^*$  in eqn (44) then gives

$$\frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{C_B^{1/q}} = pk\bar{C}_A - kq(kC_A^*) C_B (1+kC_A^*)^{q-1} \quad (47)$$

or, on replacing the first  $kC_A^*$  term by the left-hand side of eqn (44) and noting from eqn (43)

$$\text{that } (1+kC_A^*)^{q-1} = (\bar{C}_B/C_B)^{(q-1)/q},$$

$$\frac{(\bar{C}_B^{1/q} - C_B^{1/q})}{C_B^{1/q}} = pk\bar{C}_A - qk\bar{C}_B^{(q-1)/q} (\bar{C}_B^{1/q} - C_B^{1/q}) \quad (48)$$

Division of eqn (48) by the total acceptor concentration ( $\bar{C}_A$ ) reveals its identity with eqn (35), the corresponding expression obtained from reacted-site probability considerations.

## RESULTS AND DISCUSSION

New developments arising from the above theoretical considerations have been (i) the generation by the classical stepwise approach of an expression describing the solution composition for an equilibrium mixture of multivalent acceptor and bivalent ligand, and (ii) the consequent derivation of a binding equation for such systems without recourse to reacted-site probability theory. An obvious point to be established is demonstrated agreement between predictions based on the current expressions [eqns (26a) and (26b)] and their predecessors from reacted-site probability theory [eqns (15a) and (15b)] about the forms of binding curves for systems with bivalent ligands.

### Comparison of predicted binding curves

The simulation of normalized binding curves ( $r$  versus  $\alpha$ ) from the equations deduced by reacted-site probability considerations [eqns (15a) and (15b)] is relatively straightforward because of their expression in terms of  $kC_A^-$ , which is constant for a given binding curve with fixed total acceptor concentration. In their classically derived counterparts [eqns (26a) and (26b)] the corresponding product is  $kC_A^*$ , which depends upon  $\alpha$  for the system with fixed  $kC_A^-$ . Calculation of the magnitude of  $kC_A^*$  for assigned values of  $k\bar{C}_A^*$  and  $\alpha$  entails solution of the expression

$$k\bar{C}_A^* = kC_A^* (1+\alpha)^2 \left[ 1 + 2(2kC_A^* \alpha) + 3(2kC_A^* \alpha)^2 + 4(2kC_A^* \alpha)^3 + 5(2kC_A^* \alpha)^4 + \dots \right] \quad (49)$$

which is the specific polynomial in  $kC_A^*$  obtained by multiplying eqn (45), or indeed eqn (22) for the particular situation in which  $q = 2$ , by the intrinsic binding constant. This equation can be solved numerically by iterative adjustment of an input value of  $kC_A^*$  to achieve the assigned magnitude of  $k\bar{C}_A^*$  for any given  $\alpha$ .

The results of simulations for the interaction between a bivalent ligand and a bivalent acceptor are summarized in Figure 1, where the small symbols (●) depict the rectangular hyperbolic relationship stemming from the first term on the right-hand side of eqns (15a)

and (26a). The determination of  $\Omega$  from eqn (15b) for a system with  $kC_A^- = 1$  leads to a binding curve ( $\blacklozenge$ ) that deviates considerably from a rectangular hyperbolic dependence. The other set of solid symbols ( $\blacktriangle$ ) illustrates the exacerbation of this deviation by a 10-fold increase in acceptor concentration ( $kC_A^- = 10$ ). However, the purpose of presenting these findings, which merely confirm those of Calvert et al. (1979), is to demonstrate the essential coincidence of binding curves with their counterparts ( $\diamond$ ,  $\triangle$ ) predicted from eqns (26a) and (26b), the expressions deduced from classical considerations of ligand binding. In that regard particular care needed to be exercised to ensure the adequacy of the truncation of eqn (49) as well as the polynomials in eqn (26b). For the calculations with  $kC_A^- = 1$  it was necessary to extend the polynomials by a further three terms to achieve a final term contribution less than 0.1% of  $kC_A^-$ . At the higher total acceptor concentration ( $kC_A^- = 10$ ) those polynomials required extension to the term in  $(kC_A^-)^{12}$ . Fortunately, this undesirable and tedious aspect of the classical analysis is countered by the demonstrated equivalence between its predictions and those based on eqns (15a) and (15b), their counterparts arising from reacted-site probability considerations. The results shown in Figure 1 should thus serve to substantiate the validity of binding expressions based on reacted-site probability theory.

### A better binding function for multivalent ligands

Despite obvious advantages over its classically derived counterpart, the binding equation emanating from reacted-site probability theory [eqns (15a) and (15b)] still has shortcomings because of its expression in terms of a binding function ( $r$ ) that depends upon total acceptor concentration. In that regard the division of  $(C_B - C_B^-)$  by  $C_A^-$  for a univalent ligand did generate a binding function that was independent of total acceptor concentration, and hence a unique binding equation for the description of a binding curve. We now employ the simulated data sets from Figure 1 to demonstrate that this desirable feature is retained by invoking the more general definition of the binding function that takes into account the ligand valence (Hogg and Winzor, 1985).

Manipulation of the data from Figure 1 into a form compatible with presentation according to eqn (37) is accomplished by first noting that multiplication of the binding function  $r = (C_B - C_B^-)/C_A^-$  that was used in Figure 1 by  $kC_A^-$  yields a value of  $k(C_B - C_B^-)$  for each value of  $\alpha$ . On the grounds that  $kC_B^- = \alpha/2$ , the corresponding value of  $kC_B$  may be obtained as

$$k\bar{C}_B = r k\bar{C}_A - (\alpha/2) \quad (50)$$

Knowledge of  $k\bar{C}_B$  then allows calculation of the values for  $(k\bar{C}_B)^{1/2}$  and  $(k\bar{C}_B)^{1/2} = (\alpha/2)^{1/2}$  required for a plot of the results according to the expression

$$\frac{(k\bar{C}_B)^{1/2} - (kC_B^-)^{1/2}}{(k\bar{C}_A)^{1/2} (kC_B^-)^{1/2}} = \frac{r_2}{kC_B^{1/2}} = 2 - \frac{2\bar{C}_B^{1/2} r_2}{k} \quad (51)$$

which is eqn (37) for  $p = q = 2$  divided by the intrinsic binding constant. Such treatment of the simulated data for  $kC_A^- = 1$  ( $\blacklozenge$ ) and  $kC_A^- = 10$  ( $\diamond$ ) is summarized in Figure 2, which establishes their conformity with the predicted linear dependence characterized by values of

2 for the slope and ordinate intercept. It is hoped that a program for performing this multivalent Scatchard analysis will be available on the web shortly.

Redefinition of the binding function according to eqn (36) has thus had the desired consequence of yielding a unique description of results from measurements made with different total acceptor concentrations as well as an analysis in terms of a rectangular hyperbolic dependence of binding upon free ligand concentration raised to the appropriate power [eqn (39)]. That analytical description (Hogg and Winzor, 1985) has particular relevance to the characterization of antigen–antibody interactions by radioimmunoassays conducted with a fixed antibody concentration and a range of concentrations of labelled antigen (Hurrell et al., 1976) because of its seeming superiority over an earlier recommended practice involving characterization by simulation of a nonlinear dependence of the percentage of antigen bound upon the logarithm of total antigen concentration (Calvert et al., 1979).

### Apparent antibody univalence in ELISA studies of immunochemical interactions

Despite antibody bivalence, the results from ELISA studies of immunochemical interactions involving multivalent antigens ( $A$ ) often conform with a simple rectangular hyperbolic dependence of  $(\bar{C}_B - C_B)$  upon  $C_B$  (Hogg and Winzor, 1987; Winzor, 2011). This seemingly anomalous behavior of a multivalent ligand, first noted in a quantitative affinity chromatography study of lactate dehydrogenase on Sepharose-oxamate (Brinkworth et al., 1975), reflects a vast disparity between acceptor and ligand concentrations ( $C_A \ll C_B$ ) that justifies the simplification (Kalinin et al., 1995)

$$\bar{C}_B^{1/2} = C_B^{1/2}(1+\delta)^{1/2} = C_B^{1/2} [1 + (\delta/2) + \dots] \quad (52)$$

on the grounds that  $\delta = (C_B - C_B)/C_B \ll 1$ . On this basis

$(\bar{C}_B^{1/2} - C_B^{1/2})/C_B^{1/2} \approx (\bar{C}_B - C_B)/(2C_B)$ , whereupon eqn (35) for a bivalent ligand and can be approximated as

$$(\bar{C}_B - C_B) / (C_B \bar{C}_A) = 2pk - 2k (\bar{C}_B - C_B) / \bar{C}_A \quad (53)$$

which is a linear transform of the rectangular hyperbolic dependence

$$r = \frac{(\bar{C}_B - C_B)}{\bar{C}_A} = \frac{2pkC_B}{1+2kC_B} \quad (54)$$

Although ELISA systems do seemingly exhibit univalent antibody behavior, the equilibrium constant deduced from the analysis is not the intrinsic binding constant but rather the product  $2k$  for an IgG antibody. The physical explanation of this situation is the essential confinement of the antigen–antibody interaction to 1:1 complex formation ( $-A-B$  and  $B-A-$ ) because spatial constraints preclude the additional interaction to form the crosslinked species ( $A-B-A$ ) with antibody attached to two antigen sites (Nichol et al., 1974). A notable example of such restriction in the types of complexes that can form is provided by the FERRIZYME bead assay, for which it has been calculated that the cross-sectional area of

the antigen (ferritin,  $q = 24$ ) is some 10,000-fold smaller than the average surface area within which an immobilized anti-ferritin antibody molecule would be located (Hogg and Winzor, 1987): complex formation beyond the 1:1 species is thus precluded by the large distances between antibody molecules complexed 1:1 with antigen (ferritin).

## CONCLUDING REMARKS

The purpose of this investigation has been to engender greater confidence in the validity of binding equations derived for multivalent ligands on the basis of reacted-site probability theory rather than the classical stepwise equilibrium method. In addition to demonstration of the theoretical interconnection between the two approaches for a univalent ligand, the classical approach has been employed to derive binding equations for bivalent and trivalent ligands. This action has served not only to demonstrate the unwieldy nature of the classical binding equation for such systems but also to establish by numerical simulation the equivalence of binding curves generated by the two approaches. The advantages of switching to a redefined binding function for multivalent ligands have also been illustrated. It is hoped that these endeavors may lead to experimental adoption of the binding equations derived many years ago for multivalent ligands, and hence to the validity of reported quantitative analyses for antigen–antibody interactions.

## Acknowledgments

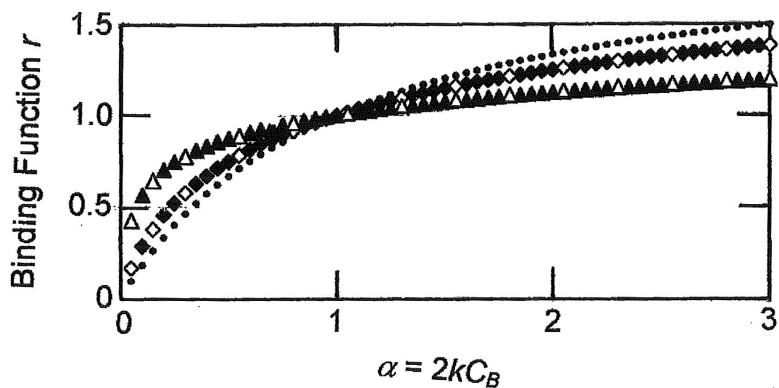
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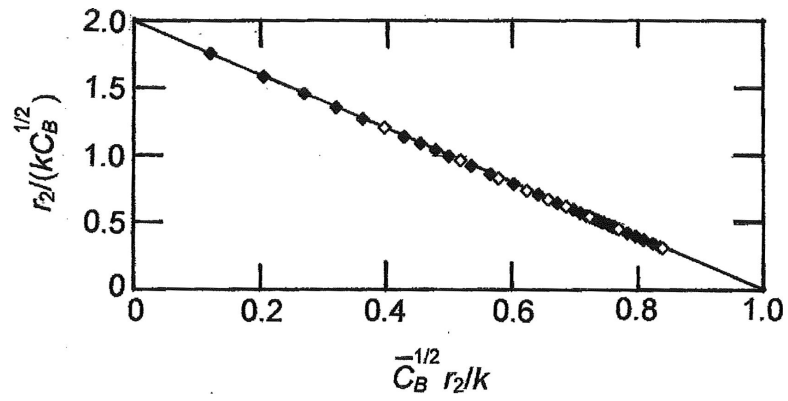
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**Figure 1.** Comparison of simulated binding curves calculated by means of expressions [eqns (15a,b) and (26a,b)] derived from reacted-site probability theory (solid symbols) and classical stepwise equilibria considerations (open symbols) respectively for the interaction between a bivalent ligand ( $B$ ) and a bivalent acceptor ( $A$ ). Diamonds refer to calculations with  $kC_A = 1$ ; and triangles to calculations with  $kC_A = 10$ ; ●, the rectangular hyperbolic dependence corresponding to the first term on the right-hand side of eqns (15a) and (26a).



**Figure 2.** Amalgamation of the two simulated sets of binding data from Figure 1 into a single set by the incorporation of a redefined binding function [eqn (36)] and analysis in terms of eqn (37), the counterpart of the Scatchard equation for a bivalent ligand.