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LARGE-LIGAND ADSORPTION TO MEMBRANES

I. LINEAR LIGANDS AS A LIMITING CASE

STEFAN STANKOWSKI

Biozentrum der Universität, Klingelbergstrasse 70, CH-4056 Basel (Switzerland)

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It is shown that the usual evaluation of binding data in terms of linear Scatchard plots or using simple mass-action equations is not applicable to the binding of ligands covering more than one receptor on a membrane. As a first step in a general treatment of large-ligand adsorption to membranes, a simple formula is derived which applies to linear chain molecules binding to a membrane without cooperative interactions. The formula may be considered as an extension to surface problems of the well-known one-dimensional treatment of Mc Ghee and Von Hippel (Mc Ghee, J.D. and Von Hippel, P.H. (1974) J. Mol. Biol. 86, 469–489). Being applied to non-linear ligand molecules, our treatment yields a lower limit estimate of the stoichiometric number.

Introduction

Adsorption of molecules to surfaces is involved in a wide spectrum of biological phenomena, e.g. ligand-receptor interactions on cells or the binding of ions, peptides or proteins to lipid membranes.

In this article we shall be concerned with one particular aspect of adsorption which is frequently encountered in biological systems: the effect of large size of the adsorbate molecules. By 'large size' we mean that one ligand binds more than one subunit on the surface, i.e. several receptors, several lipid molecules etc. Despite the widespread attention received in the case of large-ligand binding to linear biopolymers [1–3], this problem seems to have largely escaped notice in the field of biomembrane research.

To state it more quantitatively we assume that the adsorbing surface (membrane) is made up of N regularly arranged subunits S, e.g. lipid molecules or receptors. They are supposed to be all equivalent with respect to their ligand binding properties. Let one adsorbate molecule A cover n subunits upon binding. By 'covering' we mean that the subunits are excluded from further ligand adsorption. Usually, in the literature, such an adsorption process is described by introducing a binding constant K and writing a 'mass-action law'

$$K = nc_a/(c_A c_S) \tag{1}$$

c_a, c_A and c_S represent the concentrations of bound and free ligand A and of free subunits S, respectively. Eqn. 1 may readily be converted to the well-known Scatchard representation [4]:

$$\frac{r}{c_A} = \frac{K}{n}(1 - nr) \tag{2}$$

where $r = c_a/c_S^0$ is the amount of bound ligand per subunit (c_S^0 = total subunit concentration). The important point to note is that such a description is only correct (i.e. K is constant, independent of concentrations) if the binding sites are isolated from each other and do not overlap, as had been

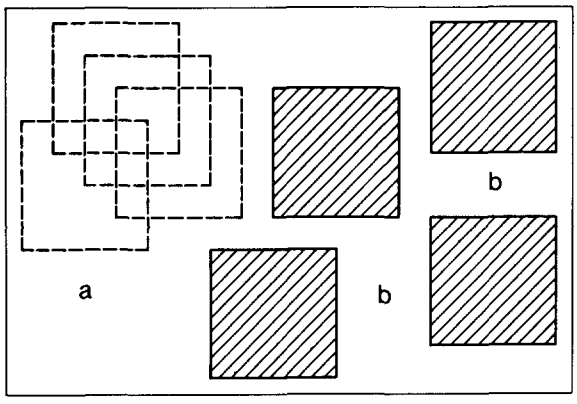

Fig. 1. Large-ligand adsorption to an array of receptors showing overlapping of potential binding sites (a) and the formation of gaps which can only be filled by reshuffling of bound ligand (b).

the situation originally treated by Scatchard. Non-overlapping implies that the number of available binding sites is equal, at any degree of saturation, to N/n minus the number of already occupied sites. Thus, as the number of occupied sites at full saturation is N/n, there should also be N/n possible ways for the first ligand to bind to the free membrane.

However, in practical cases such an assumption is usually not valid. If the subunits are all equivalent, the first adsorbing ligand may bind anywhere on the N subunits so that it actually 'sees' N potential sites and not N/n (cf. Fig. 1). The number of sites available to the second ligand is N minus the number blocked by the first one and so on. Since the potentially available sites overlap (see Fig. 1) the adsorbed molecules will not necessarily form a regular array, and gaps too small to accommodate further ligand may occur between them. Full saturation of the membrane is only reached after extensive reshuffling of already bound ligands (by dissociation-reassociation processes or by diffusion on the surface; but this concerns the kinetic pathway, whereas we shall limit our discussion to equilibrium properties). Therefore binding curves (r versus c_A or c_A^0) become very flat already at r values far below saturation. The corresponding Scatchard plots are bent downwards, as discussed by Schwarz [5] (note that this article gives a general account of Scatchard plots and is not restricted to linear systems). We should emphasize that these considerations have already been fully discussed, e.g. by McGhee and Von Hippel [1], in the context of large-ligand binding to linear polymers. However, in order to make these concepts fruitful for the discussion of surface adsorption phenomena, a suitable formalism is needed for the actual evaluation of experimental data. There are two main obstacles to such a formalism. First, mathematics of the two-dimensional problem are so complex that a general and exact closed-form solution is at present not available. Second, a general two-dimensional treatment is rendered difficult by the enormous variability of ligand shapes. In principle, separate theories must be considered for rod-like, disk-like and each other type of ligand. What to do then if the shape of the ligand is not exactly known as it often happens to be the case in biophysical or biochemical experiments?

In a series of articles we have attacked the problem from different sides in order to find a way out of this dilemma. In this first part, we consider surface adsorption of linear chain molecules. This is not only an important class of ligands but may in addition be shown to lead to a maximal large-size effect, at a given stoichiometry. In cases where the exact ligand shape is not known, application of the linear chain formalism thus yields a well-defined limit of the stoichiometric number n (subunits covered per ligand).

The method used here is the one of Miller [6] and Guggenheim [7] which we present in a particularly simple form. It may be considered as a direct extension of the one-dimensional McGhee and Von Hippel treatment [1] to surface problems. In fact the results for linear polymer binding obtained by these authors come out automatically as a special case. However, whereas the formalism can be shown to be exact for linear systems [8], it is only approximate for two and higher dimensions.

In the subsequent article, part II [16], we derive an exact formula for the low saturation regime, applicable to arbitrary ligand shapes. With the help of this result, the influence of ligand shape on the adsorption isotherms can be estimated.

In addition, a treatment is given for compact, symmetric ligands valid at arbitrary saturation.

These two articles will constitute the basis for a more general treatment, valid for a large class of different ligand shapes and including cooperativity, which we are working out presently.

In the present article cooperativity is neglected, i.e. we assume that there are no attractive or repulsive interactions between bound ligand molecules.

In our presentation we shall make extensive use of Scatchard plots [4], though we do not ignore the problems related to this plotting procedure (experimental errors are weighted differently in different parts of the plot, and exact knowledge of the saturation level is needed for correct evaluation: this leads to serious problems especially in the case of large ligands). Nevertheless, Scatchard plots are the best way to visualize large-size effects, namely as a curve bending strongly away from a straight line. In addition, Scatchard plots still are among the most familiar methods of evaluation of binding data. It is one of the aims of this paper to show that much caution is needed when using them: in particular, straight-line extrapolations should always be avoided when dealing with large ligands which cover more than one subunit.

Lattice structure and ligand shape

There exist two extreme ways of how to visualize adsorption of ligands to a surface. The surface may either be considered as a continuum, to which the adsorbate molecules stick in any possible orientation such as leaves falling to the ground (but not covering one another). Or else the surface may be viewed as a regular array of binding contacts to which the ligands bind in a well-defined way. We think that the latter model is much closer to most systems of practical interest. We shall therefore give a treatment of adsorption to a two-dimensional lattice. Clearly, in the limit of very large ligands ($n > 1000$, say) the lattice model converges to the continuum model.

We thus imagine the surface to be formed of N binding contacts (subunits) which are all equivalent with respect to their ligand binding properties and are regularly arranged forming a two-dimensional lattice. The lattice structure may be characterized by its coordination number z, defined as the number of nearest neighbors around each subunit. The maximal value of z in two dimensions is six, corresponding to a close-packed hexagonal lattice. z will be smaller for more 'loosely packed' lattices, e.g. $z = 4$ (square lattice), $z = 3$ (honeycomb lattice) and finally $z = 2$ for a linear arrangement of subunits.

The lattice picture does not necessarily involve the assumption of subunits fixed at given positions. Diffusion is allowed, in the model, by exchange between lattice sites. The lattice representation can be supposed to yield reasonable results even for systems with less regular subunit distribution or liquid-like subunit diffusion, provided that the distributional irregularities do not produce some special effects in the adsorption process. Since we are mainly interested in average properties, such as the global degree of saturation of the membrane, the effects of random irregularities will normally cancel out.

The number N of lattice points is assumed to be so large that end effects are negligible. This remains true for finite N on closed surfaces, which is the situation generally encountered with vesicles, liposomes and cells. N should in any case be much larger than the size of the adsorbing molecules.

A ligand molecule, upon adsorption, is assumed to cover n subunits. Thereby, the word 'covering' should not be understood physically but in the sense that n subunits are prevented from binding further ligand. In the following, we shall often use the short-hand expression 'ligand shape' to denote the shape of the surface region covered by a ligand molecule. The ligand is further thought to bind in a well-determined orientation with respect to the lattice axies. To give an example, rod-like ligands adsorbing to a square lattice may bind parallel to the lattice axes, i.e. horizontally or vertically, whereas diagonal binding would be a different adsorption mode. While the model may in principle be extended to include several adsorption modes at a time, we shall in the following limit the discussion to one well-defined mode.

Up to here, our considerations are analogous to those applying to one-dimensional systems, e.g. unspecific protein-DNA interactions [1]. The only difference is given by the variability in two-dimensional lattice structures, with z ranging from 2 to 6, whereas for linear systems z always equals 2. A more serious complication arises from the enor-

mous variability of ligand shapes which may range from slim rods to compact disks, including irregular structures. This is in sharp contrast to the one-dimensional situation where the ligand-covered region cannot be anything else than linear.

The influence of ligand shape on the adsorption behavior will be discussed in detail in part II [16]. One main result, however, may already be anticipated on the basis of a simple heuristic argument: Comparing the adsorption behavior of ligands of different shape but all covering the same number of subunits, n, the most pronounced large-size effect (most strongly bent down Scatchard graph) is produced by what we call a 'stretched linear ligand'. That is the type of ligand which covers a linear sequence of subunits not bending back on itself. On the contrary, the smallest large-size effect is produced by ligands having the shape of a regular polygon (hexagon on a hexagonal lattice, square on a square lattice etc.). The latter is the most strongly connected structure, the 'linear ligand' being the most loosely connected one. High connectedness means a high probability of finding other bound subunits in the neighborhood of a given one. This 'clustering' may be interpreted as a sort of (pseudo-)cooperativity (though it is not mediated by finite interaction energies but by the steric form of the ligand, hence the attribute 'pseudo'). Cooperativity, in turn, is known to counterbalance the downward bending of Scatchard plots [5]. This is an intuitive argument to explain the finding (discussed in the subsequent part) that the large-size effect is less pronounced the more compact the ligand shape.

The Bragg-Williams approximation

We now come to the heart of the problem, namely to establish a relation between the degree of binding, r, and the free ligand concentration, c_A, at arbitrary membrane saturation. In what follows, indices 1 and 2 will be used to denote free and bound subunits, respectively. Their mole fractions are given by

$$x_2 = c_2/c_s^0$$

$$x_1 = 1 - x_2 = c_1/c_s^0 \tag{3}$$

The degree of saturation of the membrane, θ, is defined as the concentration of bound subunits, divided by the total concentration of subunits; thus

$$\theta = x_2 = nr \tag{4}$$

We neglect cooperative interactions between bound ligand molecules and assume that they distribute randomly on the membrane. We then have to find a useful expression for the concentration of free sites $c_{(n)}$, or the corresponding mole fraction

$$x_{(n)} = c_{(n)}/c_s^0 \tag{5}$$

$x_{(n)}$ may be interpreted as the probability of finding a set of n free subunits suitably arranged to fit the form of a ligand molecule. The simplest thing to do is just not to care about the ligand shape and set $x_{(n)}$ proportional to the nth power of x_1 (the probability of finding one free subunit). This may be called a Bragg-Williams approximation to the large-size problem (in the sense that all probabilities are reduced to single subunit probabilities x_1 and x_2). Introducing a proportionality coefficient ρ we write

$$x_{(n)} = \rho x_1^n \tag{6}$$

The statistical factor ρ indicates the number of distinct ways a ligand can be arranged on the lattice if one of its components is held fixed. (We refer to a component as to that part of a ligand covering one subunit). For a rigid ligand, ρ is simply given by [7]

$$\rho = z/\text{symmetry number} \tag{7}$$

For flexible chains composed of m residues which may orientate freely with respect to each other [7,9]:

$$\rho = z(z-1)^{m-1}/\text{symmetry number} \tag{7a}$$

(In the case of a hexagonal subunit lattice, $(z-1)$ in Eqn. 7a should be replaced by $(z-3)$ if the chain is so stiff to exclude acute angles between neighboring residues; this should be assumed for 'stretched linear chains' to be considered in the following).

The symmetry number is 2 for rods, 6 for hexagons etc. Clearly, in the lattice picture, the highest possible symmetry number equals z. (This is due to our definition of well-defined orientation with respect to the lattice axes, cf. previous section). Thus, $\rho = 1$ for a ligand symmetric in all lattice directions (square in a square lattice, hexagon in a hexagonal lattice, disk in any lattice). ρ is always unity in a linear lattice, because then the coordination number and symmetry number both equal 2. The relevance of this statistical factor is easily visualized by considering, for instance, binding of a 'dimeric' ligand (covering two neighboring subunits) to a completely free square lattice. Such a ligand has N possibilities (ignoring end effects) to bind horizontally and further N possibilities to bind vertically. The total number of available sites is thus $2N$. The factor of 2 is calculated as the coordination number $z = 4$, divided by the symmetry number of 2.

We may now write the mass action-law in its correct form

$$Kc_A = c_a/c_{(n)} = r/x_{(n)} \tag{8}$$

and insert from Eqn. 6 to obtain

$$Kc_A = r/\rho x_1^n \tag{9}$$

It is advantageous to include the constant factor ρ in the binding constant by defining an effective binding constant K_{eff}:

$$K_{eff} = K\rho \tag{10}$$

With the help of Eqns. 3 and 4 we then get

$$K_{eff}c_A = \frac{r}{(1-nr)^n} \tag{11}$$

or, in the Scatchard representation:

$$r/c_A = K_{eff}(1-nr)^n \tag{12}$$

The nth power form of Eqn. 12 had been derived by Schwarz [5] for the high saturation regime. Despite the rather crude approximation used in calculating $x_{(n)}$, Eqn. 12 displays the main qualitative features outlined in the introduction: r/c_A is no longer proportional to $(1 - nr)$, as in Eqn. 2 which would correspond to a linear Scatchard plot, but to the nth power of that expression, yielding a parabola with ordinate intercept K_{eff} and abscissa intercept $1/n$. The resulting Scatchard curve is thus strongly bent downward.

The main disadvantage of the Bragg-Williams method is that it contains no information neither on the structure of the lattice nor on the shape of the ligand (apart from the factor ρ which, however, is independent of θ and acts only as a normalization of the binding constant). From the heuristic argument mentioned above we may anticipate that the Bragg-Williams approximation generally overestimates the large-size effect: The n free subunits forming a binding site are treated as independent neglecting that they must in reality be connected to have the correct shape. Thus the clustering or pseudo-cooperative effect is ignored which would compensate for some of the downward bending of the Scatchard curve.

The doublet closure method

A considerable improvement over Eqn. 15 is obtained by using the doublet closure method. In addition to probabilities x_1 and x_2 describing fractions of free and occupied lattice points, respectively, we now take into account pair probabilities x_{11}, x_{12}, x_{21} and x_{22}. These give the probabilities that pairs of neighboring lattice points, picked up at random, are made up of two free (x_{11}), one free and one occupied (x_{12} or x_{21}) or two occupied (x_{22}) subunits. Clearly, $x_{12} = x_{21}$ by symmetry. The important step which allows to deal with large ligands is to separate x_{22} into two different parts:

$$x_{22} = x_{22}^i + x_{22}^e \tag{13}$$

x_{22}^i describes all pairs lying at the interior of an adsorbed molecule, whereas x_{22}^e refers to the pairs formed between adjacent ligand molecules. (Again we use the short-hand notation 'ligand molecule' to designate a surface region covered by an adsorbed ligand molecule). The total fraction of pairs connecting ligand molecules with their environment, either with free or with other adsorbed subunits, equals $x_{12} + x_{22}^e$. We may therefore construct the ratio of pairs at the interior to that at

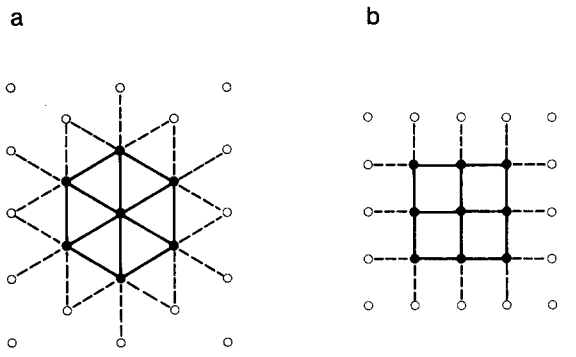

Fig. 2. Determination of the geometrical parameters β and λ: $n = 7$ hexagon on a hexagonal lattice (a) and $n = 9$ square on a square lattice (b) as examples. β is the ratio of interior bond lines (straight line connections: 12 in (a), 12 in (b)) to half the exterior bond lines (dashed connections: 18 in (a), 12 in (b)). Thus, $\beta = 12/9 = 4/3$ in (a), $\beta = 12/6 = 2$ in (b). λ is $(\beta+1)^{-1}$ or, alternatively, the number of exterior bond lines divided by nz. Thus, $\lambda = 18/(7\times 6) = 3/7$ in (a) and $\lambda = 12/(9\times 4) = 1/3$ in (b).

the edges of bound ligands

$$\beta = \frac{x_{22}^{i}}{x_{12} + x_{22}^{e}} \tag{14}$$

β is a purely geometrical factor and depends only on the ligand shape, but not on the degree of saturation. This ratio is easily determined by drawing the shape of a ligand molecule on the lattice, connecting all points by 'bond lines' and counting the bonds at the interior and the edges. The procedure is demonstrated in Fig. 2. Note that in order to obtain the correct pair statistics each bond line at the edges counts only one half. (The general algorithm considers each lattice point individually counting all the bond lines emanating from it; the final result is then divided by two to correct for the fact that each pair has thus been counted twice).

To proceed further we note that the fraction of free subunits, x_1, is related to the pair probabilities by the condition that a free lattice point may be found in either a (11) or a (12) pair. The same holds for x_2 as related to (12) or (22) pairs. Thus the following logical constraints must hold:

$$x_1 = x_{11} + x_{12} \tag{15a}$$

$$x_2 = x_{22}^{i} + x_{22}^{e} + x_{12} = (\beta+1)(x_{12} + x_{22}^{e}) \tag{15b}$$

The last equation has been simplified by introducing the concentration independent geometrical factor β. A more convenient notation is given by Eqn. 16:

$$\lambda x_2 = x_{12} + x_{22}^{e}, \quad \lambda = 1/(\beta+1) \tag{16}$$

The physical meaning of the parameter λ is easily understood noting that each bound ligand covers n subunits and that bond lines can be drawn in z directions from each of these lattice points. Thus

$$zn\lambda = \text{number of pairs at the edges of a bound ligand} \tag{17a}$$

$$zn(1-\lambda)/2 = \text{number of pairs in the interior of a bound ligand} \tag{17b}$$

Again, the factor one half in the second expression prevents from counting pairs twice.

The parameter λ is the essential new element brought in by the doublet closure method. It depends, in general, both on the size n and shape of the ligand, as well as on the lattice structure. (On the other hand, knowledge of λ, n and z does not define the shape in a unique way). For a given lattice and a given size n, λ is the smaller the more compact the ligand. Values of λ for various types of ligands will be given in part III. For the moment we shall only be interested in the value referring to linear ligands. In view of Eqn. 17a we need only count the bonds starting at points covered by the ligand and pointing to the outside. λ is obtained by dividing this number by nz. For a linear ligand, there are $(z-2)$ bonds pointing to the outside for each of the n covered subunits, plus two bonds, one at each end. (We assume that the ligand does not form closed loops). Thus [7,9]:

$$\lambda = (z-2)/z + (2/nz) \text{ for linear ligands} \tag{18}$$

For a more detailed description of shape it would be necessary to introduce additional parameters relating to triplet, quadruplet etc. probabilities. The essential approximation made by the doublet closure technique is to reduce all these quantities to functions of only single state (x_1, x_2) and pair (x_{11}, x_{12}, x_{22}) probabilities. In the case of linear systems of subunits (and only then) this assumption can be shown to be exact [8].

At any given degree of saturation $\theta = x_2 = 1 - x_1$, there is thus only one independent variable for which we choose x_{12}. The remaining pair probabilities follow from the constraints, Eqn. 15. The probability of finding a free subunit adjacent to an occupied one clearly depends on the distribution of bound ligands on the surface: if they all tend to cluster together (strong attraction between ligands, high cooperativity), x_{12} will become very small; if the ligands repell each other (anticooperativity), x_{12} will tend to be maximal (or, equivalently, x_{22}^e very small) [10]. We shall only consider the case of random distribution, in a system where the ligands neither attract nor repell each other. Mathematically, the condition of randomness may be formulated by the following 'quasi-chemical equation' [7]:

$$x_{11}x_{22}^e = (x_{12})^2 \tag{19}$$

This equation states that it makes no difference whether two adsorbed ligands are separated from each other, giving rise to two (12) terms (right hand side of Eqn. 19) or whether they come into contact with each other forming a (22^e) pair and 'releasing' a (11) pair of free subunits (left hand side of Eqn. 19; for a more rigorous derivation of Eqn. 19 see [7]). Substituting for x_{11} and x_{22}^e with the help of Eqns. 15a and 16 we solve for x_{12} and find the following expressions for the pair probabilities:

$$x_{12} = \lambda x_1 x_2/(x_1 + \lambda x_2) \tag{20a}$$

$$x_{11} = x_1^2/(x_1 + \lambda x_2) \tag{20b}$$

$$x_{22}^e = (\lambda x_2)^2/(x_1 + \lambda x_2) \tag{20c}$$

$$x_{22}^i = (1-\lambda)x_2 \tag{20d}$$

We have retained the symbol x_1 to make the symmetry of these equations more evident. In particular, for $n = 1$, λ equals unity and the trivial equations for random distribution, $x_{11} = x_1^2$, $x_{22} = x_2^2$ etc. are obtained. For practical calculations, x_1 should be replaced everywhere by $(1 - x_2)$, so that the degree of saturation, $\theta = x_2$ remains as the only variable. Note that $x_1 + \lambda x_2 = 1 - x_{22}^i$ represents the total fraction of pairs which are not at the interior of adsorbed ligands. Clearly only these pairs can be meaningful in a combinatorial analysis [7]. Therefore, at random distribution, we find the conditional probability of having a (11) pair if we already know that one subunit is free:

$$x_{11}/x_1 = x_1/(x_1 + \lambda x_2)$$

which corresponds exactly to Eqn. 20a. With the same argument, Eqns. 20b,c can be rederived.

'Linear ligands': the Miller-Guggenheim approach

With these notations in hand, Guggenheim [7] has shown how to calculate $x_{(n)}$ in the case of ligands covering linear sequences of subunits without forming closed loops. Considering this particular ligand shape has the advantage that the assumptions inherent in the doublet closure approximation remain a priori internally consistent.

The probability $x_{(n)}$ for finding a linear sequence of n free subunits is obtained by multiplying the following probabilities: First, the probability x_1 of finding the initial free subunit at one end of the sequence. Second, a conditional probability x_{11}/x_1 for the first pair, knowing already that the first subunit is free. Similarly, conditional probabilities x_{11}/x_1 for all the remaining pairs, proceeding along the sequence up to its end. Eventually, the constant factor ρ must be included, as in the Bragg-Williams approximation:

$$\begin{aligned} x_{(n)} &= \rho x_1 (x_{11}/x_1)^{n-1} \\ &= \rho x_1 (x_1/(x_1 + \lambda x_2))^{n-1} \\ &= \rho(1-x_2)[(1-x_2)/(1-(1-\lambda)x_2)]^{n-1} \end{aligned} \tag{21}$$

In the second equation we have introduced the condition of randomness (zero cooperativity). With $K_{\text{eff}} = K\rho$, as before, and with the help of Eqn. 8, the large-ligand isotherm reads

$$K_{\text{eff}} c_A = \frac{x_2}{n(1-x_2)} \left(\frac{1-(1-\lambda)x_2}{1-x_2} \right)^{n-1} \tag{22}$$

with $x_2 = nr$. Eqn. 22 may be converted to the form of a Scatchard plot, yielding

$$\frac{r}{c_A} = K_{\text{eff}}(1-nr) \left(\frac{1-nr}{1-(1-\lambda)nr} \right)^{n-1} \tag{23}$$

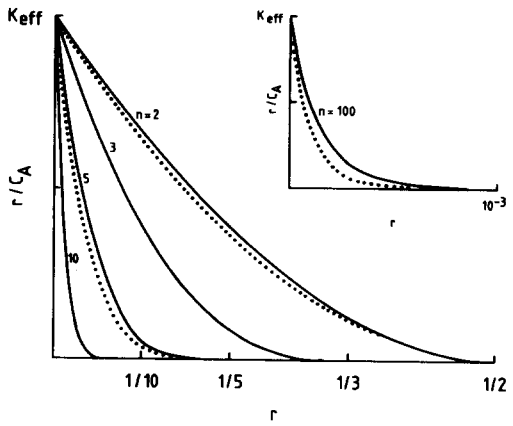

Fig. 3. Scatchard plots for binding of linear ligands of varying size n (as indicated in the figure) to a hexagonal lattice of binding contacts (receptors). Curves are calculated from Eqn. 23 (straight lines). Results of the Bragg-Williams approximation, Eqn. 12, are indicated as dotted lines. (Note that in the insert, the abscissa ends at one tenth of the saturation limit $r = 1/100$).

Inserting λ from Eqn. 18, Eqns. 22 or 23 allow to calculate the degree of saturation of a membrane as a function of the free ligand concentration c_A. The only remaining parameters are the effective binding constant K_{eff} and the stoichiometric number n.

Binding curves in the form of Scatchard graphs are shown in Figs. 3–5 for linear ligands of various sizes. In Fig. 4, the abscissa is scaled so that all curves have the same saturation limit and the degree of bending may be compared easily (cf. Ref. 1). Even for moderate n, bending is strong and saturation is achieved very slowly. It therefore appears to be virtually impossible to determine the correct stoichiometric number for the abscissa intercept of an experimentally obtained Scatchard curve. Since the high-saturation branch is often hardly accessible under normal experimental conditions, most data points must be expected to fall on the initial, rather steep branch of the curve. Extrapolating to the abscissa from near-to-linear parts of this branch, as is often done in the literature, leads to an apparent intercept I_x^{app} which bears very little relation to the true value of $1/n$. Stoichiometric numbers evaluated in such a way will normally be much too large.

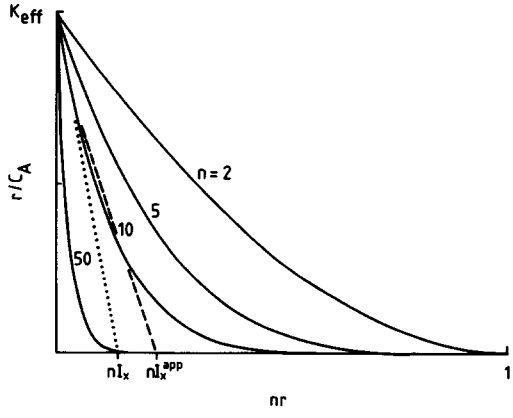

Fig. 4. Same as Fig. 3, with the abscissa scaled so that the saturation limit $nr = 1$ is the same for all curves. Also indicated are the intercept of the initial slope line, nI_x, and an estimated apparent intercept, nI_x^{app}, which would result from extrapolation of apparently near-to-linear parts of the plot.

By inspection of Fig. 3 we estimate that I_x^{app} will usually be somewhat larger, by a factor of 1.5 to 2, say, than the abscissa intercept of the initial slope, I_x. The latter is readily calculated from Eqn. 23, as a function of n. Differentiation with respect to r first yields the initial slope of the Scatchard curve:

$$S_0 = -nK_{eff}[1+\lambda(n-1)] \tag{24}$$

and extrapolation to the r-axis gives

$$I_x^{-1} = n[1+\lambda(n-1)] \tag{25}$$

As an example, for the close-packed lattice:

$$I_x(z=6) = \frac{3}{2n(n+1)-1} \tag{26}$$

and for the square lattice:

$$I_x(z=4) = \frac{2}{n(n+2)-1} \tag{27}$$

For large n, these intercepts are rather related to $1/n^2$ than to $1/n$. This makes clear how large the error may be if $1/I_x^{app}$ is illegally regarded as representing the true stoichiometric number.

Discussion

Starting from the well-known concept that large ligands produce special effects upon adsorbing to

a lattice of binding contacts just by their mere size [1,2,5], we have attempted to device a suitable formalism for the description of large-ligand binding to membranes. Unfortunately, a complete and exact treatment is rendered impossible by the high mathematical complexity of two-dimensional systems. In the present article we have therefore concentrated on an approach using simple approximation procedures: the Bragg-Williams and the Miller-Guggenheim approximations. Both approximations lead to the characteristic concave curvature of the corresponding Scatchard plots.

1. Comparison of Bragg-Williams and Miller-Guggenheim approximations

Comparing the Bragg-Williams and the Miller-Guggenheim results, Eqns. 12 and 23, respectively, the only difference is seen to be the $(1-(1-\lambda)nr)^{n-1}$ denominator in Eqn. 23. Noting that λ as well as nr always remain smaller than or equal to unity, this denominator must always be smaller than unity. Thus Scatchard graphs defined by Eqn. 23 lie higher than those of the Bragg-Williams approximation, i.e. are less strongly bent downwards. As anticipated above, the Bragg-Williams approximation thus overestimates the large-size effect (cf. Fig. 3). Both approximations approach each other as λ approaches unity. From Eqn. 18 this is seen to occur only in the trivial case of $n=1$, or for z tending to infinity (a purely theoretical limit).

Whereas the Bragg-Williams result is independent of ligand shape, the Miller-Guggenheim formulae apply only to linear ligands. However, we have given an argument telling that non-linear ligands give rise to flatter Scatchard plots. Consequently, the result of Eqn. 23 must still be an improvement with respect to the Bragg-Williams formula, even for more compact ligands.

From Figs. 3 and 5, it is evident that the Bragg-Williams approximation is the better the smaller n and the larger the coordination number of the subunit lattice. Especially for the hexagonal lattice, the Bragg-Williams method may well be used for n up to the order of 10 (cf. Fig. 3), but it is less useful for the other lattices.

2. Linear polymer binding as a special case

Since the lattice character enters the theory only via the coordination number z, one-dimensional arrays of subunits like those defined by long chain molecules, can be treated as a special case of the formalism. Setting $z=2$, λ is found from Eqn. 18 to equal $1/n$. The ligand-covered region on a linear polymer cannot be anything else than linear so that the quasichemical approach applies. Furthermore, $\rho=1$ and thus $K_{\mathrm{eff}}=K$ as discussed previously in connection with Eqn. 7. Eqn. 23 may then be written

$$\frac{r}{c_A}=K\frac{(1-nr)^n}{(1-(n-1)r)^{n-1}} \tag{28}$$

which is the well-known result for large-ligand binding to linear polymers derived by McGhee and Von Hippel [1]. Since the reduction to pair probabilities or 'doublet closure' is exact in one-dimensional systems [8], Eqn. 28 is also exact. Its extension to higher dimensions, Eqn. 23, is only approximate.

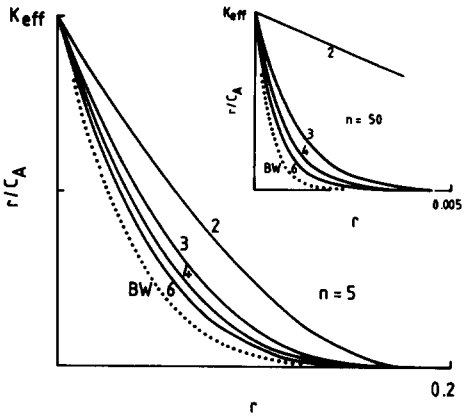

Fig. 5. Scatchard plots for binding of linear ligands of size $n=5$ and $n=50$ (insert) to different lattices: $z=2$ (linear), 3 (honeycomb), 4 (square) and 6 (hexagonal). Curves are calculated from Eqn. 23. Also shown is the Bragg-Williams approximation (dotted lines) which is lattice independent.

3. Scatchard plots

Scatchard plots calculated from Eqn. 23 have the following characteristics (cf. Figs. 3–5):

They are bent downwards. Bending becomes more and more pronounced as the stoichiometric number n increases. Comparing the effect of one and the same ligand on different lattices of bind-

ing contacts (Fig. 5), Scatchard curves are seen to be the flatter the smaller the coordination number z. However, the differences are not very large among true two-dimensional systems (i.e. $z > 2$), whereas linear arrangements of subunits ($z = 2$) show a very different behavior at large n (see insert of Fig. 5).

The ordinate intercept is not the intrinsic binding constant K but an effective binding constant K_{eff}. The latter involves a factor ρ indicating the number of distinguishable orientations of the ligand on the lattice. ($\rho = 1$ for completely symmetric ligands, and $\rho = z/2$ for rigid linear ligands).

At high saturation Scatchard curves become so flat that it is practically impossible to evaluate the stoichiometric number n from the endpoints. Instead, it is advisable to determine n from the initial slope, according to Eqn. 24. We are aware that such a procedure might present some difficulties due to the usually large experimental uncertainties at very low saturation, especially in a Scatchard representation. Therefore we have calculated the slope of the Scatchard plot at half-height, $S_{1/2}$, i.e. at $r/c_A = K_{\text{eff}}/2$. We have found that for stoichiometric numbers ranging from $n = 5$ to about $n = 1000$, we have to about 6% precision:

$$S_0/S_{1/2} \cong 1.6 \tag{29}$$

Even if the half-height is not determined exactly, taking $S_{1/2}$ from an experimental plot, multiplying by a factor of 1.6 to obtain S_0, and estimating n by means of Eqn. 24 will obviously lead to much better results than evaluating n from any straight-line extrapolation to the abscissa.

Note that even strongly bent Scatchard plots may look quite straight if data are available only from a limited range of saturations and erroneous assumptions have been made about the level of full saturation. Parameters evaluated from such plots may be very far from reality. In principle, it would be preferable to avoid Scatchard plots but to determine binding parameters by direct fitting of Eqn. 23 to the binding curves, θ versus c_A or c_A^0. In the latter case, when using c_A^0 as the abscissa, mass conservation has to be applied to relate c_A and c_A^0:

$$c_A^0 = c_A + nrc_S^0 \tag{30}$$

(c_A^0, c_S^0 = total concentrations of ligand and subunits, respectively).

4. Linear ligands as a limiting case

In the Introduction, a heuristic argument has been given (see part II [16] for a more rigorous analysis) to demonstrate that the large-size effect is most pronounced for stretched linear ligands. Among differently shaped ligands, all covering the same number of binding subunits, n, the linear ligands yield the steepest, the compact ligands yield the flattest Scatchard curves. As a consequence, if a given Scatchard plot (or other binding curve) is fitted with the assumption of a linear ligand, the resulting value of n must be a lower limit: all other ligand shapes would need larger n values in order to achieve the same degree of downward bending. In cases where the shape of the ligand is not known, Eqn. 23 may thus be applied in order to obtain a lower limit of the stoichiometric number.

5. Example: Stoichiometry of melittin binding to lipid bilayers

In order to demonstrate the practical importance of our considerations we shall apply our formalism to published data on the adsorption of melittin to phosphatidylcholine vesicles [11]. Melittin, a 26 amino acid peptide, is the main component of bee venom. It is known to adsorb to lipid bilayers, its hydrophobic parts penetrating into the hydrocarbon core without crossing the bilayer [12]. Clearly, the lipid matrix may be distorted upon embedding of the peptide, and this complication is not accounted for by our formalism. Nevertheless we shall use it as a first approximation assuming that distortions become relevant only in the high saturation regime which is of minor experimental importance.

We take the lipid molecules as the binding subunits and assume that n of them are so tightly bound to each adsorbed melittin molecule that they are prevented from taking part in further melittin binding (cf. definition of n in the Introduction). The shape of a binding site (n lipid molecules ready to accept a melittin molecule) is not known. In the following we assume it to be a linear sequence of lipid just to get the corresponding limiting values of the binding parameters (a

minimum stoichiometric number will thus be obtained).

Binding curves of melittin to dimyristoylphosphatidylcholine (DMPC) have been published by Vogel [11], using the observed changes in circular dichroism. The author interpreted his curves by means of the mass-action equation (Eqn. 1). The fit is excellent and yields stoichiometric numbers $n_1 = 59$ at 26°C (above the lipid phase transition) and $n_2 = 96$ at 15°C (below the phase transition). These results are quite unsatisfactory for two reasons: First, it is difficult to see why the lipid molecules should not be all equivalent with respect to their melittin-binding properties; if they are, however, Eqn. 1 does not apply (cf. Introduction). Secondly, the evaluated stoichiometric numbers seem to be very large. Even if one accounts for the fact that melittin should only bind to the outside of the vesicles, dividing n by a factor of about 2, the resulting numbers drastically exceed those found by independent techniques: From the decrease of the transition enthalpy, $n_1 = 10$ is evaluated [13,14], and a similar figure has been estimated from an analysis of electrical properties [15].

Using Eqn. 23 together with mass conservation, Eqn. 30, we have fitted the linear-ligand model directly to the binding curves of Ref. 11. z was set equal to 6 and K_{eff} and n varied as free parameters. The quality of the fit is essentially the same as that obtained by Vogel by (illegally) using the mass-action law. (On the scale of Fig. 1b of Ref. 11 the differences would not be visible). However, the corresponding parameter values are much more plausible: at 26°C we get $n_1 = 21$, at 15°C $n_2 = 29$. Dividing by a factor of two by the above argument would lead to a real stoichiometry of $n = 10$–11 on the outside of the vesicles, above the phase transition. This is in perfect agreement with the results from other techniques. For both 15°C and 26°C, our K_{eff} values also come out by a factor of 2.5 lower than those evaluated by Vogel. Having applied the linear shape assumption we know that $n = 10$–11 constitutes a lower limit of the stoichiometric number. This value may nevertheless be realistic if anticooperative interactions, e.g. electrostatic repulsion between the positively charged melittin molecules, play an important role.

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