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# LARGE-LIGAND ADSORPTION TO MEMBRANES

## II. DISK-LIKE LIGANDS AND SHAPE-DEPENDENCE AT LOW SATURATION

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**Large-ligand adsorption to membranes or cells is considered in the absence of cooperative interactions. For the low-saturation regime, a general and exact treatment is given by means of the concept of excluded areas. With the help of this formalism, shape dependence of the adsorption behavior can be discussed quantitatively. In addition, a formalism is presented which allows to calculate binding curves at arbitrary saturation for ligands having a symmetric shape (disks, regular polygons). The underlying model is a modified version of the hard core fluid theory of Andrews (Andrews, F.C. (1976) J. Chem. Phys. 64, 1941–1947). Apart from applications to symmetric ligands, the results can be used to derive limiting conditions for ligands of any shape.**

## Introduction

Large ligands adsorbing to an array of binding contacts or receptors are known to show some pecularities in their binding behavior [1–4]: Typical binding curves start quite steep, because the adsorbing ligand molecules cover several contacts or receptors at a time. On the other hand, binding curves become very flat already at moderate saturation, due to steric hindrance between adsorbing and already bound molecules. These features are easily visualized if the data are represented according to Scatchard [5]: large-ligand Scatchard plots strongly deviate from straight lines and are bent downwards [1,2], at least if the large-size effect is not compensated by strong cooperative interactions [2]. In what follows, cooperativity will be neglected. The topic will be taken up, however, in a later publication.

Large-size effects have been studied extensively in the case of ligand binding to linear polymers [3,4,6], such as proteins to DNA, especially since the appearance of the work of McGhee and Von Hippel [3]. In the present series of publications we try to make these concepts available also for practical work concerned with the adsorption of large ligands to surfaces (cells, membranes) in the fields of cell biology, biochemistry and biophysics. The aim is to give a comparatively simple treatment readily applicable to the evaluation of binding data.

Apart from the substantial increase in mathematical complexity, there appears to be one fundamental aspect which distinguishes two-dimensional (surface) adsorption from linear polymer binding: The binding depends crucially not only on the size, but also on the shape of the ligand molecule. Here we use the word 'size' to designate the number of binding contacts covered by each ligand molecule, i.e. the stoichiometric number $n$. 'Shape' then refers to the geometrical arrangement of the $n$ binding contacts forming a binding site. A heuristic argument has been given previously to make a first classification: Linear chain molecules can be

expected to produce the strongest large-size effects, the weakest effects being made by bulky, compact structures, e.g. disk-shaped molecules [1]. For the former of these extreme types of shape, a simple treatment has been given [1], based on the Miller-Guggenheim theory of polymer mixtures [7].

The present article is divided into two parts. Firstly, shape dependence is analyzed in an exact and general form at low membrane saturation. The basic concept is then further used in order to build up a simple treatment of the surface adsorption of compact ligands having the shape of disks or regular polygons. This complements the linear ligand model given in Ref. 1, and offers the possibility to derive limiting conditions for large ligands of any shape. The method used is adapted from the hard core fluid model of Andrews [8,9]. Assumptions and approximations involved in this model are analyzed, as well as possible extensions to other ligand shapes.

## Large-ligand adsorption at low saturation

As in the preceding article, we consider an array of subunits (binding contacts, receptors) arranged on a surface which is either closed (as on a cell or vesicle) or large enough that end effects may be neglected. In any case, the number of subunits, $N$, is presumed to be much larger than the size of one ligand molecule. By 'ligand size' we mean the stoichiometric number $n$, i.e. the number of binding contacts covered by an individual ligand molecule upon binding. The subunit lattice is characterized by its coordination number, $z$, i.e. the number of nearest neighbors surrounding each subunit. All $N$ subunits are supposed to be equivalent with respect to their ligand binding properties.

As a consequence, the first ligand molecule adsorbing to the free lattice may bind with its center anywhere on the $N$ binding contacts: it 'sees' $N$ potential binding sites (a more general account will be given below). Once the first ligand molecule is bound, $n$ subunits are covered and thus prevented from further ligand binding. In addition, a certain number of potential binding sites in the neighborhood of the bound molecule is blocked by excluded-area effects. This is where the ligand shape enters.

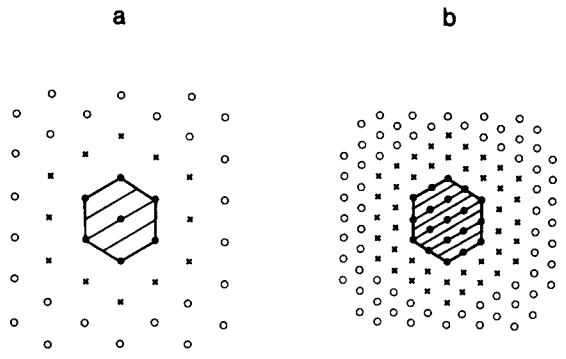


Fig. 1. Excluded areas of hexagons on a hexagonal lattice. (a) $n = 7$ hexagon with an excluded area of 12 lattice points; (b) $n = 19$ hexagon with an excluded area of 42 lattice points.

The phenomenon is particularly easy to visualize in the case of disk-shaped ligands which are so large that the lattice of binding contacts may be approximated by a continuum. One bound disk then covers $n$ subunits corresponding to an area $\pi R^2$, with $R$ the disk radius. Since bound disks cannot penetrate or overlap each other, they can only bind with their centers at least a distance $2R$ away from each other. Thus the first bound disk effectively blocks an area of radius $2R$ for the center of the second one. Of this area of $\pi(2R)^2$, $\pi R^2$ or $n$ subunits are actually covered by the ligand molecule. The rest, $3\pi R^2$ or $3n$ subunits, is the 'excluded area', which is blocked for the center

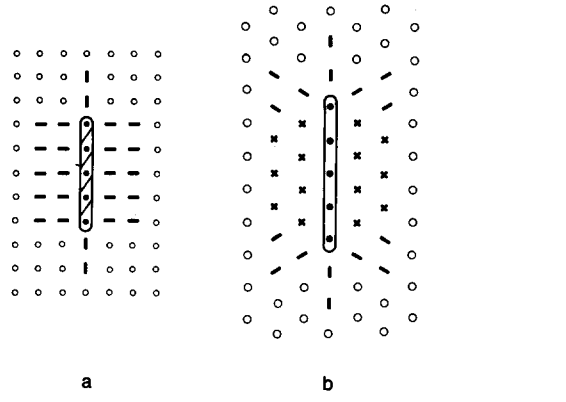


Fig. 2. Excluded areas of linear rods: (a) on a square lattice; points with horizontal (vertical) bars are excluded for the centers of horizontal (vertical) rods; (b) on a hexagonal lattice; points with bars are excluded for centers of rods in one orientation (weight 1/3), points with crosses are excluded for centers of rods in two orientations (weight 2/3).

TABLE I

EXCLUDED-AREA PARAMETERS $\alpha$ FOR LIGANDS OF DIFFERENT SHAPES ON THE FOLLOWING LATTICES: HEXAGONAL ($z = 6$), SQUARE ($z = 4$) AND HONEYCOMB LATTICE ($z = 3$)

Non-linear structures are well defined only for integer values of $a$, $b$ and $k$.

| $z$ | Shape | Excl.-area parameter | Remarks |
|---|---|---|---|
| 6 | linear rod, length $n$ | $(2n^2 - n - 1)/3n$ | – |
| | hexagon, $k$ concentric shells around center | $3k(3k+1)/n$ | $k = [-1+(1+4(n-1)/3]^{1/2}/2$<br>$n = 1+3k(k+1)$ |
| 4 | linear rod, length $n$ | $(n^2-1)/2n$ | – |
| | rectangle, side lengths $a$ and $b$, $a/b = p$ | $[(a+b)(a+b-4)+2(n+1)]/2n$<br>$(p^2+4p+1)/2p - 2(p+1)/(np)^{1/2} + 1/n$ | $n = pb^2 = a^2/p = ab$ |
| | square, side length $a$ | $(3a-1)(a-1)/n$ | $n = a^2$ |
| 3 | linear rod, length $n$ | $(n^2+n-2)/3n$ | – |
| | hexagon, $k$ concentric shells | $2(3k-1)^2/n$ | $n = 6k^2$ |

(but not for the periphery) of other bound ligand molecules [10].

Let us now turn from large disks to small $n = 7$ hexagons on a hexagonal lattice, consisting of one central point surrounded by a shell of six lattice points. Obviously, the excluded area of such a hexagon is given by its nearest neighbor shell of 12 lattice points (cf. Fig. 1). In the same way, a larger $n = 19$ hexagon (one central point plus two concentric shells) has an excluded area of two neighboring shells corresponding to a total of 42 lattice points (cf. Fig. 1), and so forth.

The situation is slightly more complicated in the case of ligands having a non-symmetric shape. Consider for instance a linear rod adsorbed in a vertical position on a square lattice. Points in the neighborhood of such a rod are excluded for the binding of the center of horizontally oriented rods, but not of vertically oriented ones (Fig. 2). This may be accounted for by giving a weight one half to each of these semi-excluded points. As a general rule, the excluded area can thus be determined by the following simple recipe:

Draw the structure of one ligand molecule on the lattice. Fix one given point on that structure (possibly the center or a point near to it, though such a choice is not obligatory). Count all the lattice points in the neighborhood which would cause overlapping if a second ligand molecule were placed there with its fixed point (e.g. its central point). In cases where overlapping occurs only for some fraction of the possible ligand orientations, weight the lattice point by that fraction.

With this prescription in mind, an excluded-area parameter $\alpha$ may be defined [10]:

$$\alpha = \text{excluded area}/n \tag{1}$$

Thus, $\alpha = 3$ for disks, whereas $\alpha = 12/7$ for $n = 7$ hexagons etc., according to the above determined excluded areas. Values of $\alpha$ for some ligand shapes on the most important lattices are collected in Table I.

At high saturation of the membrane, bound ligand molecules must clearly come close together so that they share their exclusion areas. At very low saturation, on the other hand, it is legitimate to assume that the bound molecules are far apart from each other so that their exclusion areas do not overlap. The concentration of free sites of size $n$, $c_{(n)}$, can then be calculated readily. For symmetric ligands (disks, hexagons etc.) it is given as the difference between the total subunit concentration $c_s^0$ (i.e. the total number of sites on the free membrane) and the concentration of sites blocked by the bound ligand, namely

$$c_{(n)} = c_s^0 - (1+\alpha) n c_a \tag{2}$$

($c_a$ = concentration of bound ligand). $(1+\alpha)n$ represents the covered plus the excluded area, i.e. the number of subunits effectively blocked for the

center (or other fixed point) of further ligand. In the case of non-symmetric ligands a statistical factor $\rho$ has to be included which accounts for the number of distinct orientations of the ligand on the lattice [1,7]. For instance, linear rods may adsorb to a square lattice in two different orientations (horizontal or vertical). In general, $\rho$ is given, for rigid ligands, by [7]

$$\rho = z/\text{symmetry number} \tag{3}$$

(For a more detailed discussion cf. Ref. 1). $\rho$ equals unity for the completely symmetric structures, e.g. squares on a square lattice or regular hexagons on a hexagonal lattice.

The concentration of available sites on the free membrane is thus $\rho c_s^0$, and by the same argument the blocked subunits have to be counted $\rho$ times. Thus Eqn. 2 transforms to the more general form

$$c_{(n)} = \rho c_s^0 - (1+\alpha) n \rho c_a \tag{4}$$

We now introduce the degree of binding $r$,

$$r = c_a/c_s^0 \tag{5}$$

and an intrinsic binding constant $K$:

$$K = c_a/(c_A c_{(n)}) \tag{6}$$

$c_A$ being the concentration of free ligand. The following adsorption isotherm is then obtained in the low-saturation limit:

$$K\rho c_A = \frac{r}{1-(1+\alpha)nr}, \quad r \to 0 \tag{7}$$

In this equation, $K$ and $\rho$ may be combined in an effective binding constant $K_{eff}$:

$$K_{eff} = K\rho \tag{8}$$

In the Scatchard representation, Eqn. 7 reads, using $K_{eff}$:

$$r/c_A = K_{eff}(1-(1+\alpha)nr) \tag{9}$$

Theoretically speaking, this equation corresponds to a virial expansion up to the second virial coefficient. Inserting $\alpha$ from Table I (or computing it according to the above recipe) one may get from Eqn. 9 the initial part of binding curves for ligands of different shapes. Since the expansion, Eqn. 9, is exact up to linear terms in the degree of binding $r$, the initial slope of Scatchard plots, $S_0$, is readily calculated:

$$S_0 = -nK_{eff}(1+\alpha) \tag{10}$$

Eqn. 10 allows to calculate the stoichiometric number $n$ from low-saturation data in those cases where the ligand shape is known so that $\alpha$ may be determined.

**Shape dependence of binding curves**

Scatchard plots of large ligand binding in the absence of cooperative interactions have a typical near-to-hyperbolic shape [1,2]: a steeply falling branch at small degree of binding, a sharp bending and a flat branch at high $r$. Quite obviously, the degree of downward bending of such a graph is strongly related to the steepness of the initial branch. Initial slopes, as given by Eqn. 10, should therefore be valuable tools for discussing large-size effects (i.e., the degree of bending of the corresponding Scatchard plots) for ligands of different shapes.

Comparing ligands of equal size $n$, the initial slope appears to be determined by the excluded area parameter $\alpha$: In view of Eqn. 10, $\alpha$ may be interpreted as a direct measure of the large-size effect.

Considering in a first instance the case of linear rods, as the least compact structures, we note that

$$\alpha = n\frac{z-2}{z} + \frac{4-z}{z} - \frac{2}{nz} \tag{11}$$

For large $n$, the second and third terms may be neglected. Thus, for very large linear rods, $\alpha$ increases indefinitely with the rod length $n$. This is in sharp contrast to large disks, for which we already know that $\alpha = 3$, independent of $n$. As a consequence, extremely different Scatchard plots are to be expected for very large ligands of strongly different shapes. Taking $n = 100$, for instance, $\alpha$ equals 66.33 in the case of linear rods on a hexagonal lattice, to be compared to $\alpha = 3$ for disk-shaped ligands.

With the help of Table I, the decrease of $\alpha$ with

increasing compactness of structures of a given size can be followed. Going over from a linear rod covering a single sequence of subunits, to a more compact rod covering two or more neighboring rows of subunits, we have (with $n = 100$, on a square lattice):

| | |
|---|---|
| $\alpha \cong 50$ | one row ($a = 100, b = 1$ in Table I) |
| $\alpha \cong 13.5$ | two rows ($a = 50, b = 2$) |
| $\alpha \cong 4.64$ | four rows ($a = 25, b = 4$) |
| $\alpha = 2.61$ | ten rows × ten columns square ($a = b = 10$) |

The last value refers to a square which represents the most compact structure to be realized on a square lattice. It yields the lowest $\alpha$ for a given $n$. The same is true for regular hexagons on a hexagonal lattice etc. At small stoichiometric numbers $n$, the $\alpha$ values corresponding to these compact symmetric polygons are definitely smaller than the large-disk value of 3. The latter is approached as $n$ becomes large. E.g. for squares on a square lattice:

| | | |
|---|---|---|
| $\alpha = 1.25$, | $n = 4$ | (cf. $\alpha \cong 1.88$ for the corresponding linear rod) |
| $\alpha \cong 1.78$, | $n = 9$ | |
| $\alpha = 2.24$, | $n = 25$ | |
| $\alpha = 2.61$, | $n = 100$ | |
| $\alpha \cong 2.87$, | $n = 900$ | |

and for regular hexagons on a hexagonal lattice

| | | |
|---|---|---|
| $\alpha \cong 1.71$, | $n = 7$ | (cf. $\alpha \cong 4.29$ for the corresponding linear rod) |
| $\alpha \cong 2.21$, | $n = 19$ | |
| $\alpha \cong 2.64$, | $n = 91$ | |
| $\alpha \cong 2.89$, | $n = 919$ | |

Since these compact, fully symmetric shapes are only defined for some particular values of $n$, rather large variations in $\alpha$ occur as $n$ is slightly increased beyond these numbers, leading to squares or hexagons with unsymmetric protrusions.

In summary, consideration of initial slopes or, equivalently, excluded-area parameters $\alpha$, shows that adsorption isotherms depend quite strongly on the ligand shape, especially for ligands of large size $n$. Scatchard plots are most strongly bent downwards for slim linear rods, whereas bending is least pronounced for the most compact shapes, namely the regular polygons on the respective lattices.

This confirms, on a quantitative basis, a statement made previously on more heuristic grounds [1]: compact shapes lead to a clustering of bound subunits, mediated by the steric properties of the ligand, which may be considered as a sort of (pseudo-)cooperativity compensating for some of the large-size effect.

## Compact ligands: Simplified Andrews model

A semi-empirical approach has been worked out by Andrews [8,9] allowing to extend the excluded-area concept to arbitrary saturation, in the case of disks. The theory, originally conceived as a hard core fluid model, is easily adopted to the adsorption picture.

Consider a disk-shaped ligand of radius $R$, covering so many subunits that the lattice of binding contacts may be approximated by a continuum (i.e. $n \gg 1$). Let $M$ disks be already bound so that the degree of binding is $r = M/N$ ($N$ = total number of subunits). The fraction of free sites remaining, $x_{(n)} = c_{(n)}/c_s^0$, is then calculated as the product of two probabilities, $P_1$ and $P_2$, the latter being conditional on the former. $P_1$ simply represents the probability of finding a free subunit:

$$P_1 = 1 - (nM/N) = 1 - nr \tag{12}$$

(note that the $M$ disks bound cover $nM$ subunits).

Having chosen a free subunit, it is already certain that the center of any bound disk is at least a distance $R$ away, since some part of it would otherwise cover the subunit. In order to place the $(M+1)$th disk center on the free subunit, an area of $\pi R^2$ around it must also be unoccupied or, equivalently, there cannot be other disk centers between distances $R$ and $2R$. $P_2$ therefore is the probability to have the excluded area (the area between distances $R$ and $2R$) free of disk centers. Andrews calculates $P_2$ as the $M$th power of the probability that any one of the $M$ bound molecules, taken individually, is not centered in that area. The latter probability is set equal to unity minus the ratio of the excluded area, $\alpha n = 3n$, to the free area available for an individual bound molecule, $N - Mw$ [8,9]. $w$ is defined [8,9] as the average area (expressed in numbers of subunits, in our notation) 'taken up by each molecule when

they are crowded into one side' of the surface, packed close enough to avoid molecular-sized holes, but without reshuffling (see Discussion for further comments). With $w$ considered as a parameter, $P_2$ may be written

$$P_2 = \left(1 - \frac{\alpha n}{N - Mw}\right)^M = \exp\left(-\frac{\alpha n M}{N - Mw}\right) \tag{13}$$

The last expression arises in the limit of $N$ and, consequently, $M$ tending to infinity.

The fraction of free sites of size $n$, $x_{(n)}$, is then given by

$$x_{(n)} = P_1 P_2 = (1 - nr) \exp\left(-\frac{\alpha n r}{1 - wr}\right) \tag{14}$$

where we have used $r = M/N$. Binding curves are finally obtained using the following simple equation:

$$Kc_A = \frac{r}{(1 - nr)} \exp\left(\frac{\alpha n r}{1 - wr}\right) \tag{15}$$

and, in the Scatchard representation,

$$\frac{r}{c_A} = K(1 - nr) \exp\left(-\frac{\alpha n r}{1 - wr}\right) \tag{16}$$

with $\alpha = 3$, for disks. (Remember that $K_{eff} = K$, for fully symmetric ligands).

The value of $w$ remains to be determined. It is exactly known only in the limit of maximum saturation where each disk 'takes up' an area of $2\sqrt{3}\,R^2$ or, expressed in numbers of subunits with $\pi R^2 = n$, $w_{sat} = 2\sqrt{3}\,n/\pi$ (corresponding to a maximum value of $r = 1/w_{sat}$). For lower saturation, $w$ is expected to be larger than $w_{sat}$. Andrews actually uses a linear interpolation between the low-saturation value of $w$, taken to fit the third (and eventually fourth) virial coefficient, and $w_{sat}$. It turns out, however, that the numerical improvement reached by such a procedure is only small with respect to the result obtained by simply keeping $w$ constant and equal to $w_{sat}$ over the whole range of saturations. The reason is that the denominator in the exponential of Eqn. 14 has little influence on the total expression as long as $r$ remains small, and becomes important only at comparatively high saturation with $wr$ approaching its limiting value of unity: $wr \to w_{sat} r \to 1$. For our purposes, it is fully sufficient to set $w = w_{sat}$ throughout, as shown in Fig. 3: the resulting discrepancy is much smaller than typical experimental uncertainties in protein binding studies and may be safely neglected for the evaluation of such data.

We see no reason why this simple formalism should not be applied to lattices instead of continuous surfaces (in fact, we have already used the corresponding terminology), and to symmetric structures others than disks. The best candidates would be the regular polygons listed in Table I (hexagons, squares etc.). Since these structures are capable of covering the surface completely at maximum saturation, the corresponding value of $w_{sat}$ is simply equal to $n$, the number of subunits covered by each ligand molecule. Binding curves are then readily calculated from Eqns. 15 or 16, setting $w = n$ and taking the appropriate value of $\alpha$ from Table I.

Corresponding Scatchard plots are shown in Fig. 3a for a small ($n = 7$) and a large ($n = 331$) hexagon on a hexagonal lattice and an $n = 25$ square on a square lattice. The abscissa is scaled so that the abscissa intercepts are the same for all $n$: $x = nr$. Disk isotherms are shown in the same representation in Fig. 3b (note that the abscissa intercepts for the disks are at $nr = \pi/2\sqrt{3} = 0.907$

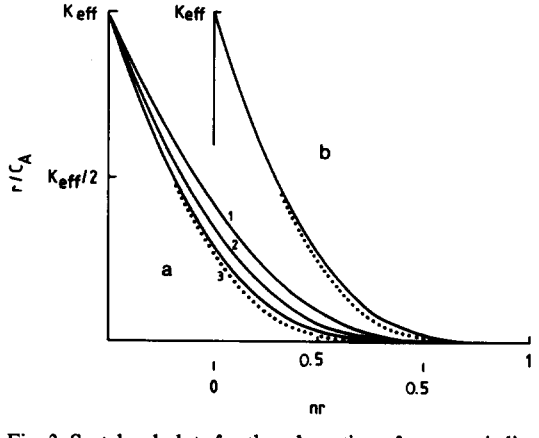


Fig. 3. Scatchard plots for the adsorption of symmetric ligands; the abscissa is scaled by a factor $n$. (a) $n = 7$ hexagon (1), $n = 25$ square (2) and $n = 331$ hexagon (3). Dotted line: same as (3), with $w = n(1.329 - 0.329nr)$ in Eqn. 16; (b) disks with $w = w_{sat}$ (full line) and with $w = n(1.397 - 0.1713nr - 0.1694n^2r^2)$, (dotted line), according to Eqn. 8 of Ref. 9, involving the third and fourth virial coefficients.

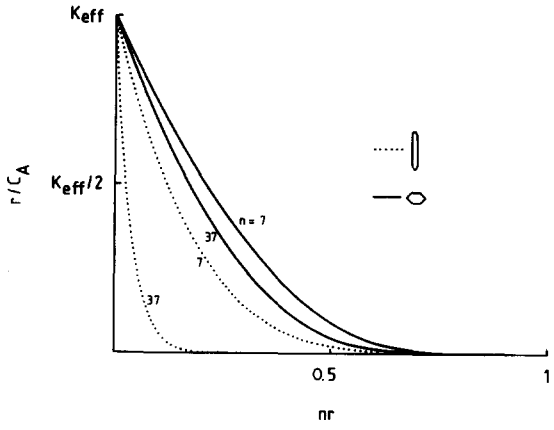


Fig. 4. Scatchard plots for the adsorption of regular hexagons (full lines) of size $n = 7$ and $n = 37$, respectively, and linear rods (dotted lines) with the same values of $n$, on a hexagonal lattice.

instead of $nr = 1$, cf. above). With the abscissa proportional to $n$, the disk isotherms fall on top of each other, reflecting the constant value of $\alpha = 3$. The bending of the hexagon isotherms increases with increasing $n$ until it reaches a limit as $\alpha$ reaches its limiting value of 3. In Fig. 3, this limit is practically reached for the $n = 331$ hexagon.

For the hexagons, we have also calculated the more sophisticated expressions according to Andrews' theory involving third virial coefficients. For the small hexagon, this happens to coincide with the simple form given above. For the $n = 331$ hexagon, $w_{sat}$ has to be replaced by $w = n(1.329 - 0.329nr)$ (dotted line in Fig. 3a). Again, the simplified model involving only the second virial coefficient (the excluded-area parameter $\alpha$) is seen to be fully sufficient for the practical needs of data evaluation in protein binding studies.

The Andrews model has been shown to be in excellent agreement with numerical calculations in the case of disks [9]. In view of the general relevance of the excluded-area concept and the small numerical importance of minor modifications in the parameter $w$, it should be safe to expect Eqns. 15 and 16 to remain fully reliable also for the regular polygons. Complications arise only when non-symmetric ligands are considered (see Discussion).

## Discussion

Adsorption of large ligands to a two-dimensional array of binding contacts has been considered: (1) at low saturation, in an exact and general form; (2) at arbitrary saturation, in an approximate treatment of ligands having a symmetric shape.

From the low-saturation formula, Eqn. 9, initial slopes of Scatchard plots can be obtained (Eqn. 10) and compared for ligands of different shape. A very strong shape dependence is found, especially for ligands of large size. In agreement with a result obtained previously [1], the large-size effect is shown to be maximal (steepest Scatchard plots) with thin, rod-like ligands and minimal (flattest Scatchard plots) with bulky ligands having the shape of regular polygons.

A treatment for the former of these extremes, the 'linear ligands', had already been given in part I [1]. A simple formula has now been presented for the adsorption of regular polygons and disks, Eqns. 15 and 16, using a slight modification and extension of an approach due to Andrews [8,9]. Binding curves for the two limiting cases of rods and regular polygons can thus be compared not only at low degree of binding, but over the whole range of saturations. Model curves are shown in Fig. 4 comparing the adsorption behavior of linear rods (dotted lines) and regular hexagons (full lines) having the same stoichiometric numbers. The discrepancy between the two types of ligands is seen to be large for the $n = 37$ molecules. It would even increase with larger $n$. For $n = 7$, on the other hand, the discrepancy is still there, but much less drastic. In a Scatchard plot with $r$, and not $nr$, as the abscissa, a difference of this kind would correspond to about a 30–40% change in the stoichiometric number. We have found about the same difference between $n = 9$ rods and $n = 9$ squares on a square lattice (not shown).

These results allow to draw an interesting conclusion for the evaluation of binding data in those cases where the exact shape of the ligand is actually not known: If the ligand is small enough, i.e. if it covers not more than about ten binding contacts, any shape assumption will still lead to rough, but reasonable estimates of the binding parameters. If the ligand is much larger ($n > 10$), it

should be tried to get some information about its shape from electron microscopy or some other technique, otherwise the evaluation of the binding data might prove difficult.

Nevertheless, the following procedure can be used quite generally to obtain upper and lower limits for the stoichiometric number $n$:

(1) The obtained binding curve may first be fitted to the 'linear ligand' adsorption, Eqns. 22 and 23 of part I: the value of $n$ obtained will be a lower limit for the stoichiometric number. (In terms of Scatchard graphs, higher $n$ is needed to yield the same amount of bending with more compact shapes).

(2) In the same way, the experimental data may be compared to the formalism for regular polygons (squares on a square lattice, hexagons on a hexagonal lattice etc.), Eqns. 15 and 16, with the corresponding parameters taken from Table I. Strictly speaking, these symmetric shapes exist only for some particular values of $n$. However, the general expressions in the table may be used for arbitrary $n$, as an interpolation. Fitting to the experimental curve will yield an upper limit of $n$.

In this way it is always possible to establish the allowed range of values of the stoichiometric number.

It has been pointed out in part I that the abscissa intercept of the initial slope line of Scatchard plots is proportional to $1/n^2$ rather than to $1/n$ in the case of long linear ligands. The same is true, to a good approximation, for the intercept of the slope line at half height (i.e. at $r/c_A = K_{eff}/2$) [1]. We think that the latter is a good measure of apparent intercepts as they may be obtained from straight-line extrapolations through data points in a Scatchard representation, and which may differ very much from the true abscissa intercept, $r = 1/n$. In the case of bulky, compact ligands, the risk of erroneous data evaluation is much smaller: In fact, the excluded-area parameter being nearly constant for large enough ligands ($\alpha = 3$), the initial slope $S_0$ is simply proportional to $n$, and the corresponding intercept therefore proportional to $1/n$. We have also verified numerically that the slope at half height, $S_{1/2}$, can be written to an accuracy of 7.5% or better:

$$S_{1/2} = S_0/1.45$$

for values of $n$ ranging from 7 to infinity. Thus, the abscissa intercept of the half-height slope line is also approximately proportional to $1/n$. Straight-line extrapolations through data points in a Scatchard representation will therefore overestimate the stoichiometric number by a factor of the order of 2 (cf. Fig. 3), but not by an order of magnitude as it may occur in the case of very large linear ligands.

As to the ordinate intercept of Scatchard plots, we note that it equals the intrinsic binding constant $K$ in the case of symmetric ligands, in contrast to the linear ligands. It should be clear from Fig. 3 that straight-line extrapolations through data points must generally underestimate the true value of $K$.

*On the nature of the approximation used in the model*

Having discussed the possible applications of the formalism (to give its raison d'être, so to speak), we would like to add some comments on the nature of the approximation used in the Andrews model [8,9], and especially in the definition of the parameter $w$. The latter was introduced in order to calculate the probability that the center of an individual bound molecule did not occupy a given area $A_x = \alpha n$ (needed as the excluded area for the $(M+1)$th molecule to be placed on the lattice). This probability was taken as unity minus the ratio of $A_x$ and $A_f$, the remaining free area. However, strictly speaking, it is not the areas which have to be compared (or, equivalently, the number of subunits in them), but the number of free sites they contain. The latter is clearly smaller than the former, due to the excluded areas of the other bound molecules. At low saturation, it is not very probable that a small given area $A_x$ be influenced by excluded-area effects of bound molecules in the immediate neighborhood. The remaining free area $A_f$, on the other hand, always includes the excluded-area effects of all bound molecules, per definition. This is a rigorous explanation for the fact that the ratio to be calculated is larger than $A_x/A_f$, if $A_f$ is taken to represent the real unoccupied area, i.e. $A_f = N - nM$. Replacing $n$ by a concentration-dependent parameter $w$, may then be considered as a phenomenological correction to account for the different influence of excluded

areas on $A_x$ and $A_f$. Of course, $w > n$ must hold in order that the ratio considered become larger than that of the elementary areas. In the limit of high saturation only, there is a high probability of finding bound ligands near $A_x$, and the excluded-area effects on $A_x$ and $A_f$ tend to become comparable. Thus the ratio of elementary areas can be used in this limit, i.e. $w$ tends to $n$ (or, more generally, to $w_{sat}$).

The problem of establishing the exact value of $w$ at moderate saturations has been solved by Andrews in a phenomenological way [8,9] which has the practical disadvantage that it requires calculation of higher order virial coefficients. Extension of the formalism to other than disk-like shapes is therefore very much simplified by realizing that the approximation remains fully satisfactory even in the simplified version with $w$ set equal to $n$ (or to $w_{sat}$) throughout the whole range of saturations.

The situation gets, however, much more complicated if one tries to adapt the method to non-symmetric ligands. The distinct orientations of the molecules on the lattice have then to be taken into account. Without going into details we state only that this produces a new problem in the calculation of the ratio $A_x/A_f$, as we shall demonstrate for the case of a linear rod on a square lattice: for such a system, there are two potential binding sites centered on each lattice point, a horizontal and a vertical one. Binding of a linear rod on a given subunit thus destroys two potential sites on that subunit. At high saturation, however, there is a high probability of finding other bound rods in the neighborhood of a free subunit, which already preclude one of the two possible orientations. Binding of a linear rod then only destroys one potential site at its central point. Such effects would lead to modifications of the parameter $w$ at moderately high saturation, that is to say, in a regime where the numerical contribution of the denominator of the exponential in Eqn. 14 is in general not negligible.

In view of these difficulties, the present treatment has been restricted to the consideration of symmetric ligands. Work is in progress, however, to establish a formalism (which will combine ideas of this and of the preceding article) valid for a very large class of ligand shapes ranging from linear ligands to polygons.

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