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STEROIDS AND THE PRACTICAL ASPECTS OF PERFORMING BINDING STUDIES

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SUMMARY

Many cellular regulatory processes are described by ligand interactions with macromolecules. Steroid complexing with specific cytoplasmic receptor proteins and the subsequent binding of these complexes to nuclear constituents are two such processes which are fundamental to the understanding of steroid hormone action. These reactions are characterized by the binding parameters k, the equilibrium association constant, and M_0, the binding site molarity. The current methods used to determine these two parameters are discussed in relation to the general theory of binding parameter measurement. A number of practical aspects of binding parameter measurement are considered. These include problems encountered by working at dilute non-uniform protein concentrations, non-specific binding, and the limitations of all such experimental systems. An exact solution to the non-linear Scatchard plot is presented for the case when only two non-interacting classes of binding sites need be considered. Finally, a set of specific conclusions is offered which should enable investigators to obtain the most accurate information possible whenever carrying out steroid hormone or receptor binding studies.

INTRODUCTION

The interaction of macromolecules with small ligand molecules is a ubiquitous phenomenon of importance to many aspects of biochemistry. These interactions can be extremely strong, such as antigen-antibody interactions or the binding of the vitamin biotin to the egg white protein avidin. Such strong interactions are characterized by equilibrium association constants of 10^{15} M^{-1}. Weaker interactions have also been described. These include substrate binding to enzymes, oxygen binding to hemoglobin, cyclic AMP binding to the regulatory subunit of protein kinases, and finally, steroid hormone binding to serum globulins or to specific cytoplasmic receptor proteins. On a more complex plane, the interaction of regulatory proteins such as the lac repressor, CAP protein, and steroid–hormone receptor complexes with specific regions of the prokaryotic or eukaryotic genome may also be considered as ligand-macromolecule interactions.

All such interactions can be characterized by studying the following parameters: (1) k, the equilibrium association constant; (2) M_0, the binding site molarity; and (3) independence or co-operativity of the binding sites. The classical method employed to evaluate these parameters is the method of Scatchard[1]. Since Scatchard's initial treatment of ligand interactions with macromolecules, a number of other approaches have been developed. These approaches are well founded, but unfortunately often deeply mired in mathematics. The purposes of this paper are to: (1) review some of the theory necessary to accurately determine the binding parameters k and M_0; (2) indicate several methods of graphically determining these parameters; (3) indicate the strengths or weaknesses of these approaches; (4) discuss some practical aspects of measurement; and (5) present an exact solution for the case of two independent sets of binding sites which will appear in the form of a nonlinear Scatchard plot. Application of this knowledge to evaluate steroid hormone receptor interactions with nuclear constituents is crucial to the understanding of emerging molecular mechanisms of gene control.

RESULTS AND DISCUSSION

Theory of binding parameter measurement

Generally the macromolecule under study is maintained at a dilute fixed total concentration M_0 and titrated with the ligand up to a total concentration L_0. Equilibrium conditions are completely defined when three parameters are known under fixed conditions of solvent composition and temperature. These three parameters are: (1) free binding site concentration, M; (2) free ligand concentration, L; and (3) the concentration of the complex, ML. Under these conditions the equilibrium association constant, k, is given by:

$$k = \frac{ML}{M \cdot L}. \quad (1)$$

The binding site molarity is nothing more than the sum of the concentrations of free and filled sites on the macromolecules:

$$M_0 = M + ML. \quad (2)$$

In most systems, particularly in steroid hormone work, the parameters ML and L are most easily

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measured. Following a number of determinations of these parameters, one is able to calculate M_0 and k graphically.

Benesi and Hildebrand[2], Scott[3], Bjerrum[4], and others have developed methods useful in the analysis of binding data. Unfortunately, most of these methods require measurement of the saturation fraction, ϕ:

$$\phi = ML/M_0 \; (0 \leq \phi \leq 1). \tag{3}$$

But, since M_0 is unknown, these methods are not useful in the study of steroid hormone interactions with receptor, or in the study of interactions of steroid-receptor complexes with nuclei. Equations (1) and (2) may be combined to yield:

$$ML = \frac{M_0 kL}{1 + kL}. \tag{4}$$

After the appropriate linear transformation of equation (4), k and M_0 are determined graphically as shown in Fig. 1. Of these various methods for obtaining estimates of k and M_0, the Scatchard plot (Fig. 1a) is far superior to the others. This is because the Scatchard plot does not have open limits at either end of the plot. Thus, all the theoretically obtainable data can be plotted. By way of contrast, the double reciprocal and Eadie–Hofstee plots (Figs. 1b and 1d) have open limits on the abscissa and hence any range of values of ϕ can be represented as a "complete" plot. Moreover, least-squares fitting of a line to such data in the absence of statistical weighting factors[8,9] biases the estimates of k and M_0. The Michaelis–Menten plot (Fig. 1c) requires accurate determination of the asymptote. The logarithmic plots (Figs. 1e and 1f) are best handled by a computer.

Weber[10] has applied a propagation of errors treatment to the parameters k and M_0. With appro-

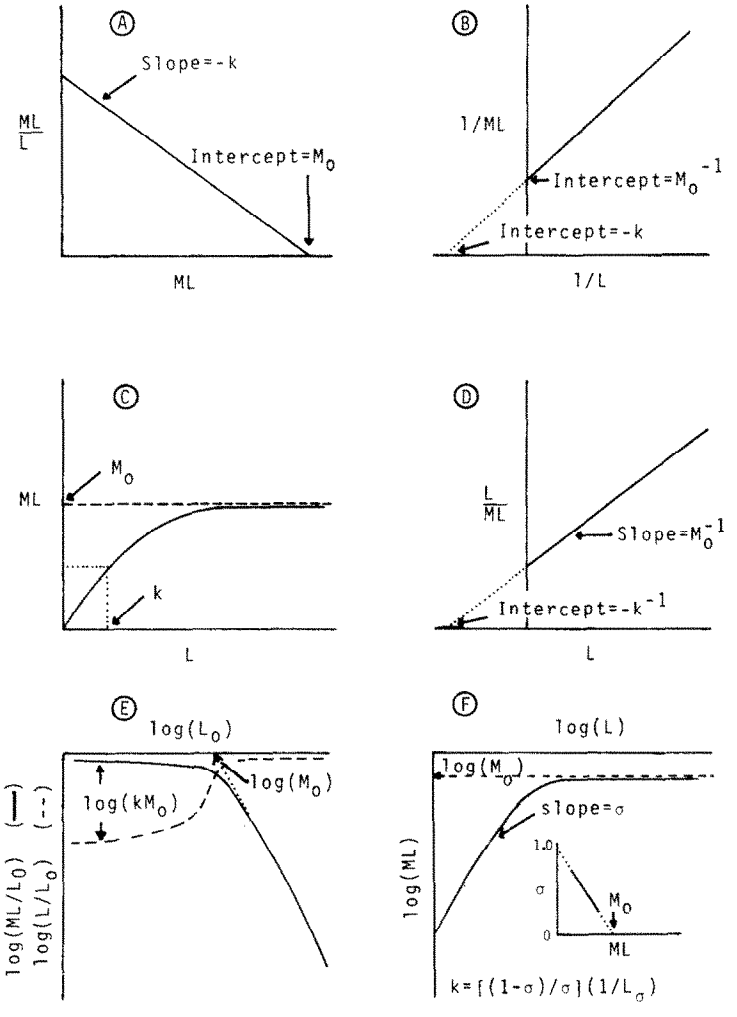

Fig. 1. Graphical methods for binding parameter determination. The appropriate slopes, intercepts, and asymptotes of different linear transformations of equation (4) can be used to determine binding constants: A, Scatchard Plot [1]; B, Lineweaver–Burk or Double Reciprocal Plot; C, Michaelis Menten, or bound vs free plot; D, Eadie–Hofstee Plot [5]; E, Proportion Plot [6]; F, Log Bound versus log Free Plot [7].

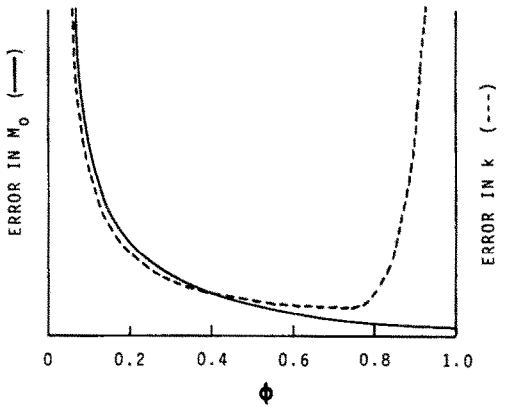

Fig. 2. Relationships of ϕ, k, and M_0. Errors inherent in the measurement of k and M_0 plotted as a function of the saturation fraction ϕ.

priate boundary conditions, the errors in measurement of these two parameters simultaneously are related as shown in Fig. 2. When the saturation fraction ϕ approaches 1, error in measurement of M_0 reaches a minimum. However, at the same time the error in measurement of k increases asymptotically without bound. Conversely, over the initial range of saturation ($\phi \ll 1$), both k and M_0 are measured imprecisely. The point to be made is that binding should be measured over a wide range of values in order to most accurately determine both k and M_0. When $0.2 \leq \phi \leq 0.8$, the most accurate measurements of k and M_0 are made[9]. This is particularly true in the case of multiple equilibria. The left hand panel of Fig. 3 shows a linear Scatchard plot analysis of chick oviduct progesterone receptor protein binding to nuclei over a small range in ϕ. However, when the range of ϕ was increased as in the right hand panel of Fig. 3, an obvious departure from linearity occurred. Such non-linearity is usually interpreted as a reflection of the presence of a second set of non-interaction binding sites. However, as Rodbard[8] and McGhee and von Hippel[12] have indicated, such an interpretation may be erroneous. Other possible meanings of non-linearity include: (1) positive ligand-ligand co-operatively (concave down); (2) ligands occupying more than one binding site (concave up); (3) differences in affinity between unlabeled and labeled ligand (concave up); (4) negative ligand-ligand co-operatively (concave up); (5) error in separation of bound and free (concave up); (6) errors in estimation of total or non-specific counts (concave down); and (7) lack of true equilibrium (concave down). Of these six, certainly the most frequently encountered situations are (5), (6), and (7).

There is another possible cause of Scatchard plot non-linearity which has not been considered elsewhere. The non-linearity caused in this case is artifactual and may be of significance in studies of receptor interactions with nuclei or chromatin at very dilute non-uniform protein concentrations. Under these conditions, the amount of protein in the nuclei or chromatin under study is very large when compared with the protein added in the receptor fraction. The nuclear or chromatin proteins in the incubation medium may serve as a sink for hormone dissociated from the receptors. Moreover, at such dilute receptor concentrations, the rate of hormone (S) dissociation

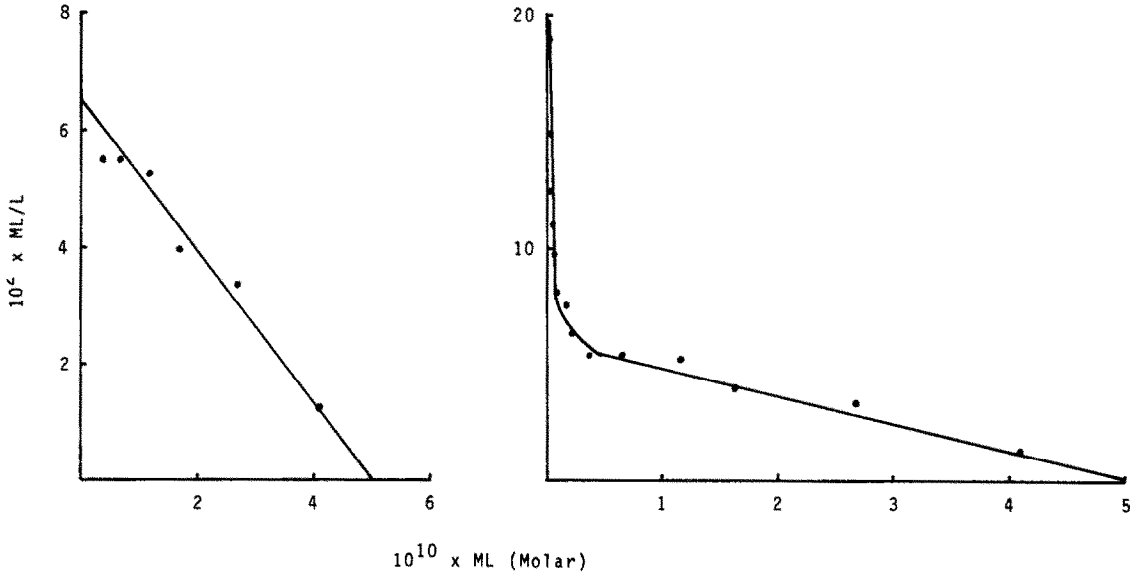

Fig. 3. Importance of using a wide range of total ligand concentration. Scatchard plots of chick oviduct progesterone receptor binding to oviduct nuclei. Left, only a small range in L_0. Right, use of a wider range in L_0. Experimental details can be found elsewhere [11].

from receptor (R) may easily exceed the rate of hormone receptor complex association with nuclei (N). Therefore, the reactions:

$$SR \rightarrow S + R \tag{5}$$

$$S + N \rightarrow SN \tag{6}$$

can become as favorable as:

$$SR + N \rightarrow SRN \tag{7}$$

the reaction that is actually of interest. Stated another way the total amount of steroid–receptor complex in the reaction system (SR + SRN) cannot be assumed to remain constant. Mathematically, for a total steroid concentration S_0, when $S_0 \rightarrow 0$:

$$\frac{\partial S_0}{\partial (SRN)} \rightarrow 0 \tag{8}$$

but at a rate much slower than:

$$\frac{\partial S_0}{\partial SR} \rightarrow 0. \tag{9}$$

The net result is that SRN/SR increases without bound producing a Scatchard plot which is concave up. To rule out such artifactual non-linearity in any studies of very high affinity nuclear binding, the receptor fraction should be added at constant protein concentration. Then, if an interfering reaction was initially observed, repeating the experiment at constant protein concentration will reverse the rate at which equations (8) and (9) approach zero so that SRN/SR will rapidly approach zero causing a downward concavity.

It should be evident from the preceding discussions that it is imperative to vary ϕ over as wide a range as possible. When this is done, the experimental problem becomes one of determining how to distribute the data points over this interval in order to obtain the maximum possible information with the fewest measurements. Through the use of conditional probabilities, which are related to the saturation fraction ϕ, it is possible to interrelate probability, information theory, and binding studies. Complete detailed mathematical descriptions of this approach appear in Weber[10] and Deranleau[9]. Results of such an approach indicate that only 83% of the total possible information can be obtained in any binding study. This is because of the errors inherent in making significantly different measurements. Only if different measurements are made can new information be obtained. If $\delta\phi$ represents one standard deviation on an independent measurement of ϕ, then two measurements of bound ligand which fall within $2\delta\phi$ of each other convey no more information than a single measurement. Thus, if one is limited in the number of determinations which can be made in a given assay, it is more informative to have five single determinations spread over the entire saturation range than to have three duplicate determinations (6 points) spread over the same region. To re-emphasize this from another standpoint it is erroneous to choose a narrow range of values for ϕ, fit a few points to a straight line and then to claim the existence of a single class of binding sites. Nearly any function, no matter how wild, can be made to approximate a straight line over a small interval. Such a fit cannot be invoked as proof of any model.

Methods of measuring binding parameters

Several articles have appeared in the steroid hormone literature which have detailed numerous useful methods for measurement of binding parameters[13,14]. In using any of these methods, one should consider possible sources of systematic errors.

The dextran charcoal assay of Korenman[15] is an extremely useful and prevalent technique for measuring bound steroid. (The charcoal adsorbs free steroid while leaving receptor steroid complexes in the supernatant fraction.) Specific receptor binding of steroid is determined as the difference in non-adsorbable ligand in the presence and absence of a 100-fold excess of unlabelled ligand. There is nothing magical about the 100-fold excess of unlabeled ligand, or any other fixed-multiple excess, unless the excess is sufficient to saturate all true binding sites. In some instances, this method of determining specific binding must be used. The presence of non-receptor proteins such as ovalbumin or BSA, which interact strongly with estradiol, makes measurement of estrogen receptor titer in chick oviduct cytosol meaningless without eliminating non-specific binding. However, whenever one uses the charcoal assay, it is essential to remember that denaturation of the proteins will occur at the charcoal surface in dilute solutions. Thus, if protein concentrations are not maintained constant, serious systematic errors can be introduced by this assay. This point is emphasized by Table 1. Table

Table 1. Dextran-charcoal binding assay (D-C) compared to sephadex (G-75) method

Step*	Fold Purified	mg Protein Assayed	% Bound c.p.m. Charcoal Method	% Bound c.p.m. G-75 Method	Charcoal/ G-75
Cytosol	1	2.22	63	82	77
$(NH_4)_2SO_4$ Pellet	20	.15	65	99	65
DE-52 A	80	.003	6	32	19
DE-52 B	300	.001	3	55	6

* The steps refer to those used by Schrader and O'Malley[16] for purification of the chick oviduct progesterone receptor A and B proteins.

1 compares receptor bound [^{3}H]-progesterone as determined by both the Dextran charcoal assay and by Sephadex G-75 gel filtration. In every case, the dextran charcoal assay gives a lower determination than does gel filtration. Moreover, the relative difference in bound counts is accentuated as the receptor fraction is successively purified. Finally, even such a gentle procedure as gel filtration does not give accurate values for bound steroid in purified receptor solutions. Since DE-52 A subunit and B subunit elute from a DEAE cellulose column as "bound" counts, one would expect to find nearly 100% of the [^{3}H]-progesterone in these fractions as bound hormone. This was clearly not the case when they were assayed by either the Dextran-charcoal assay or gel filtration.

Measurement of bound steroid receptor complexes in nuclear or chromatin bonding studies introduces a new problem. What is non-saturable (non-specific) binding in this system and how does one correct for it? It is virtually impossible to agree on criteria for completely clean nuclei or chromatin. Thus, one must be concerned with binding of free steroid in the receptor fraction to contaminating unlabeled receptors and with an artifactual binding of the complexes themselves. One way to circumvent this problem is to prepare the nuclei of chromatin in the presence of unlabeled steroid. However, such a procedure could artifactually lower M_0 since unlabelled complexes could then occupy specific nuclear acceptor sites. Another approach is to use a receptor fraction which has been labeled in the presence and absence of excess unlabeled steroid. However, such a measurement hides true non-saturable binding of labeled receptors to non-acceptor sites which may be functionally significant. The best measurements are done using a receptor fraction which contains no free hormone[11]. A second problem emerges in the form of hormone dissociation from the hormone–receptor complex should the half life for this process be comparable to the time course of the binding reaction. Therefore, to accurately measure non-specific binding in nuclear systems requires the presence of excess steroid–receptor complexes in the absence of free hormone. Such studies can most advantageously be carried out using purified receptors. Finally, it should be noted that non-specific nuclear or chromatin binding may be analogous to non-operator DNA binding of the lac repressor protein[17,18], or to non-promoter binding of RNA polymerase[18]. In both cases, such non-specific binding may be highly significant from a functional standpoint.

System limitations

There are numerous Scatchard plots in the literature which show ML/L-axis intercepts on the order of 0.001. With such tiny ratios, it should be clear that small errors in separation of bound from free ligand could produce large errors in ML/L. What is not common knowledge, is the fact that simply by changing the binding site molarity M_0, it is possible to overcome this problem. This is because the experimental system limits the possible observable values of ML/L. To see this, consider the Scatchard equation when the ligand concentration L_0 is much less than the binding site molarity M_0 and equilibrium has been reached:

$$k(M_0 - ML) = \frac{ML}{L} \approx kM_0 \qquad (10)$$

Table 2. Interrelationships of k and M_0

Relation of k to M_0	(ML/L) Max.
$k = 0.001\ M_0$	0.001
$k = M_0$	1.0
$k = 1000\ M_0$	1000.0

Table 2 shows maximum values for ML/L corresponding to three of the possible intrinsic relationships between k and M_0. Clearly $kM \approx 1$ gives ratios of ML/L which are most accurately and precisely measured.

Multiple classes of binding sites: a special case

Klotz and Hunston[19] have treated the general case of multiple classes of noninteracting binding sites quite rigorously using three different linear transformations. From a practical standpoint, methodology is probably no better than to allow the assignment of the parameters M_{0_1}, M_{0_2}, k_1 and k_2 for two independent limiting classes of binding sites. Therefore, an exact solution of the general equations of Klotz and Hunston[19] was carried out for this case. Figure 4 is a sketch of a typical nonlinear Scatchard plot. These are four useful parameters obtained from it: α, β, γ, and δ. The slopes α and β of the two asymptotes to the curve can be determined by construction[20]. The midpoint of the shortest posssible line from the origin to the curve is also the point of intersection of the two asymptotes. For a class of binding

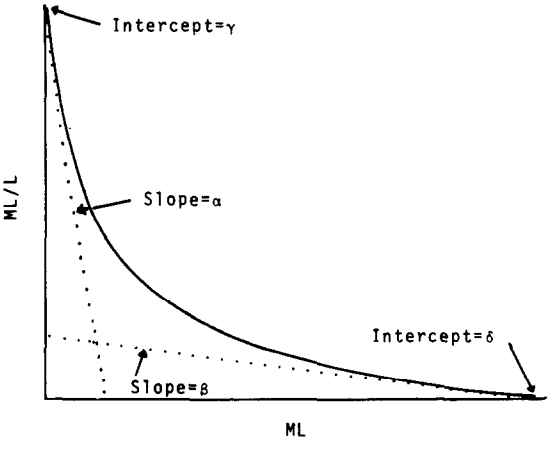

Fig. 4. Non-linear Scatchard plots. Asymptotes are drawn to the binding curve in order to obtain the values α, β, γ, and δ.

sites present at a concentration M_{0_1}, and characterized by k_1, which do not interact with a second class of binding sites present at a concentration M_{0_2}, and characterized by k_2, it can be shown that since $\delta = M_{0_1} + M_{0_2}$:

$$k_1 = \frac{-T \pm (T^2 - 4SU)^{1/2}}{2S} \tag{11}$$

where:

$$S = (\delta/\gamma) = \beta^{-1}$$

$$T = (\alpha/\beta) - 1$$

$$U = (\gamma/\delta) - \alpha^{-1}$$

$$k_2 = (k_1 + \alpha)/|(\delta/\gamma)k_1 - 1| \tag{12}$$

$$M_{0_2} = (\gamma - \delta k_1)/(k_2 - k_1) \tag{13}$$

$$M_{0_1} = \delta - M_{0_2}. \tag{14}$$

Moreover, in the limiting case where $|\alpha| \gg |\beta|$, equations (11) and (12) reduce to:

$$k_1 \approx \alpha \tag{15}$$

$$k_2 \approx \beta. \tag{16}$$

The error in these approximations can be as small as 1%. The x-intercept of the asymptote to the curve described α and γ is approximately n_1. Thus, when a non-linear Scatchard plot is obtained, exact values for the limiting binding parameters k_1, M_{0_1}, k_2, M_{0_2} can be obtained without the use of a computer. It must be remembered, however, that the asymptotes can only be drawn accurately if the ligand concentration L_0 is varied over a wide range ($\sim$100 fold). Finally one must realize that these parameters may only describe 2 limiting classes of binding sites present in a group of 3 or more such classes. Even the use of non-linear computer-fit least-squares regression techniques will only permit determination of parameters for 3 such classes. Accuracy of experimental measurements make it doubtful whether the presence of more than 2 or 3 classes of binding sites can accurately be detected[20].

CONCLUSION

Ligand interaction with macromolecules is fundamental to regulatory processes such as steroid hormone binding to receptor proteins or nuclear binding of these complexes. These interactions should be studied over a wide range of ligand concentrations ($\sim$100 fold). This will permit determination of the presence or absence of multiple classes of binding sites. By using a closed end transformation such as the Scatchard plot it is possible to obtain the best estimate of the binding parameters k (association constant) and M_0 (binding site molarity). It may be necessary to adjust M_0 so that $kM_0 \simeq 1$ in order to make the most accurate measurements of bound and free ligand. Collection of binding data spanning the range $0.2 \leq \phi \leq 0.8$ in increments of $2\delta\phi$ where ϕ is the saturation fraction and $\delta\phi$ is the error in measuring ϕ, will enable one to use the most information to estimate k and M_0.

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