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Interpretation of Double Reciprocal Plots Used to Determine the Spectroscopic Parameters of Bound Ligand for Binding Assays

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Abstract

The spectroscopic determination of binding data usually requires measurement of the spectral characteristics of the chromophore when all sites on the acceptor are occupied by the ligand. The double reciprocal plot employed for this purpose has been interpreted in diverse ways. This paper examines the theoretical and practical limitations which must be considered in making such interpretations, and shows that the linear extrapolation of the double reciprocal plot which is normally practised cannot be justified.

Introduction

The binding of a ligand to a macromolecular acceptor or to an assembly of molecules is frequently studied by observing the change in some spectroscopic property of the system (e.g. absorbance, fluorescence, optical rotation or n.m.r. line shift). In order to determine a binding function (r = moles of ligand bound per mole of acceptor) it is necessary to determine the characteristics of the ligand when it is completely free (x_f) and when it is completely bound (x_b). Whilst the former is determined in the absence of acceptor, the latter is usually found by varying the acceptor concentration $[\bar{A}]$ at constant ligand concentration $[\bar{S}]$ and constructing a double reciprocal plot to extrapolate the measured parameter (x_{obs}) to infinite acceptor concentration. The value of x_b at all other ligand concentrations is found by direct proportion, assuming that each additional ligand bound causes an equivalent change in the spectroscopic property observed, an assumption which is open to experimental test (Daniel and Weber 1966). The fraction of ligand bound is then $(x_{obs} - x_f)/(x_b - x_f)$ and the binding function is given by

$$\begin{aligned} r &= ([\bar{S}] - [S])/[\bar{A}] \\ &= [\bar{S}]\{(x_{obs} - x_f)/(x_b - x_f)\}/[\bar{A}], \end{aligned} \quad (1)$$

where $[S]$ is the concentration of free ligand. A Scatchard or double reciprocal plot ($1/r$ versus $1/[S]$) can then be made to determine the binding constant (K_a) and the number of binding sites (n) involved in the equilibrium.

The double reciprocal plot used to determine x_b (i.e. $1/x_{obs}$ versus $1/[\bar{A}]$) has received various interpretations. Harris (1971) concluded that this plot could be linear or non-linear depending on the magnitude of the equilibrium constant and the number of binding sites involved. Azzi (1975) considers that plots are linear only if a single set of equivalent and independent binding sites are involved. In studying the binding of the fluorescent probe 1-anilino-8-naphthalene sulphonate to mitochrondria,

both Harris (1971) and Layton *et al.* (1974) found non-linear plots and the latter authors argue strongly that linear extrapolations lead to incorrect estimations of the fluorescent yield of the bound probe. Recently, Gains and Dawson (1975) justified the use of linear extrapolation in situations where the concentration of probe is small compared to the concentration of binding sites. Finally, Radda (1971) has used the linearity of the plot to justify the assumption that each molecule of bound probe has the same fluorescent enhancement.

In view of these many and conflicting interpretations we have re-examined the theory and carried out numerical simulations of a simple binding model to determine those factors which influence the linearity of the plot. Although we specifically consider the use of fluorescence enhancement for determining the binding of a fluorescent ligand to a macromolecule or membrane, the theory applies equally to any spectroscopic binding study.

Materials and Methods

12-(9-Anthroyloxy)-stearic acid was synthesized from 12-hydroxystearic acid and anthracene-9-carboxylic acid by mixed anhydride synthesis (Barratt *et al.* 1974). The product was purified by preparative thin-layer chromatography and crystallized from hexane. The magnesium salt of 1-anilino-8-naphthalene sulphonate was purchased from Sigma and was recrystallized from water after passage of the hot solution through activated charcoal. Liposomes of egg phosphatidylcholine (Lipid Products, U.K.) were prepared by sonication of lipid in 0·1 M KCl–0·05 M tris (pH 7·5) for 3 min. Fluorescence measurements were made with a Perkin Elmer–Hitachi MPF 3 spectrofluorometer.

Theory and Results

Consider an acceptor A possessing n equivalent and independent sites for a ligand S:

$$A + nS \rightleftharpoons AS_n, \tag{2}$$

$$[AS_n] = K_a [A] [S]^n, \tag{3}$$

where K_a is the intrinsic association binding constant. Expressions for the total concentration of acceptor ($[\bar{A}]$) and the concentration of free acceptor ($[A]$) may be derived from first principles (Klotz 1946):

$$[\bar{A}] = [A](1 + K_a [S])^n, \tag{4}$$

$$[A] = \frac{[\bar{S}] - [S]}{nK_a[S](1 + K_a[S])^{n-1}}. \tag{5}$$

Substitution of equation (5) into (4) gives

$$[\bar{A}] = \frac{([\bar{S}] - [S])(1 + K_a [S])}{nK_a [S]}. \tag{6}$$

When the acceptor possesses two independent but non-equivalent binding sites with association constants $K_{a,1}$ and $K_{a,2}$, it may be shown (Klotz 1946) that

$$[\bar{A}] = \frac{([\bar{S}] - [S])\{1 + K_{a,1} [S] + K_{a,2} [S] + K_{a,1} K_{a,2} [S]^2\}}{K_{a,1} [S] + K_{a,2} [S] + 2K_{a,1} K_{a,2} [S]^2}. \tag{7}$$

Equations (6) and (7) describe the relationship between $[\bar{A}]$ and $[S]$ for given values of $[\bar{S}]$, n and K_a. These equations are not amenable to analytical solution but may

be solved numerically. The binding function r ($=\{[\bar{S}] - [S]\}/[\bar{A}]$) can be calculated for any value of $[S]$, and values of the observed fluorescence (x_{obs}) can be obtained from equation (1) for given values of x_b and x_f.

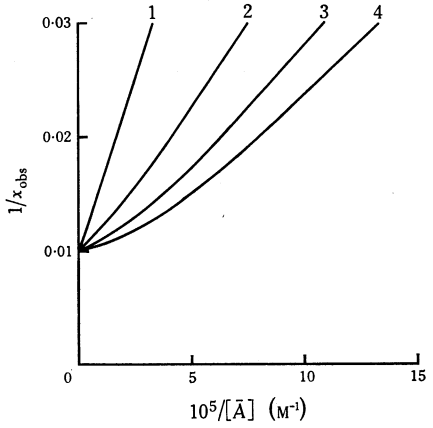

Fig. 1. The reciprocal of the observed physical parameter (x_{obs}) plotted against the reciprocal of the acceptor concentration ($[\bar{A}]$). The simulated examples are computed according to equation (6) for a fluorescence assay where $x_b = 100$, $x_f = 0$, $[\bar{S}] = 3 \times 10^{-6}$ M; there are two equivalent and independent binding sites with binding constants $0{\cdot}1 \times 10^6$ M, $0{\cdot}3 \times 10^6$ M, $0{\cdot}8 \times 10^6$ M and $1{\cdot}0 \times 10^6$ M for curves 1–4 respectively.

A simulated plot of $1/x_{obs}$ versus $1/[\bar{A}]$ is shown in Fig. 1 for several values of K_a. At high values of K_a (Fig. 1, curves 2, 3, 4) the graphs are non-linear but the degree of non-linearity increases as K_a increases since more probe becomes bound at lower acceptor concentrations. In all examples depicted in Fig. 1, x_b was held constant and thus the curves intercept the ordinate axis at a single value. A similar family of curves can be computed for varying values of n and, as expected, an increase in the number of binding sites also causes more probe to be bound at lower acceptor concentrations: the plots again have pronounced non-linearity.

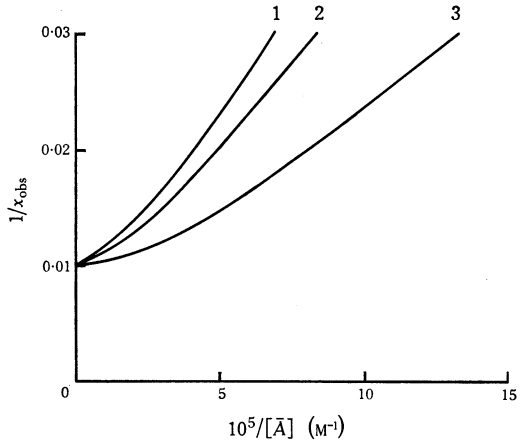

Fig. 2. The reciprocal of the observed physical parameter (x_{obs}) plotted against the reciprocal of the acceptor concentration ($[\bar{A}]$). The simulation [equation (7)] is for a fluorescence assay as in Fig. 1 except that the two binding sites are now non-equivalent. The equilibrium constants are as follows: curve 1, $K_{a,1} = 10^4$, $K_{a,2} = 10^6$; curve 2, $K_{a,1} = 10^5$, $K_{a,2} = 10^6$; curve 3, $K_{a,1} = K_{a,2} = 10^6$.

Fig. 2 presents the case of non-equivalent sites described in equation (7). The plots are again non-linear, the deviation from linearity being determined by the magnitude and ratio of the equilibrium constants involved.

Fig. 3 (curve 2) depicts an experimental situation, namely the binding of 12-(9-anthroyloxy)-stearic acid to egg lecithin liposomes, and it is clear that the

non-linearity is of a form predicted by theory. Extrapolation of the linear part of the graph would lead to erroneous estimates of x_b (broken line, Fig. 3). The results for 1-anilino-8-naphthalene sulphonate binding to egg lecithin liposomes are also non-linear (Fig. 3, curve 1). Our recent experiments indicate that the binding of this probe is approximately 10 times weaker than the binding of the fluorescent fatty acid.

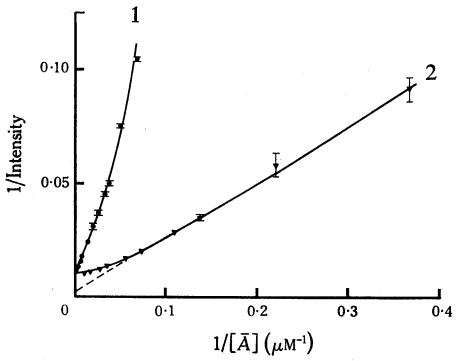

Fig. 3. The reciprocal of the fluorescence intensity versus the reciprocal of the lipid concentration for the binding of 1-anilino-8-naphthalene sulphonate (curve 1) and 12-(9-anthroyloxy)-stearic acid (curve 2) to egg phosphatidylcholine liposomes. The buffer systems were: 1·6 M KCl–0·05 M tris, pH 7·5, for the sulphonate; and 0·1 M KCl–0·05 M tris, pH 7·5, for the fluorescent fatty acid. Experimental points are averages of duplicates, and the curves have been normalized to give $x_b = 100$. The probe concentrations were 3·3 μM for the sulphonate and 3·1 μM for the fluorescent fatty acid.

Discussion and Conclusions

Use of a linear extrapolation to obtain x_b cannot be justified theoretically or in practice and may lead to erroneous results. Incorrect values of x_b can lead to non-linear Scatchard plots which might be interpreted as indicating either binding to non-equivalent sites or a form of positive or negative cooperativity. In designing experiments it is essential to examine a wide range of acceptor concentrations since under some circumstances graphs may appear linear over restricted ranges. It is particularly important to examine an acceptor concentration as high as possible to facilitate extrapolation to the ordinate axis. Non-linearity does not indicate the existence of binding sites of different affinity nor can the plot be used to determine if the binding of each ligand causes the same fluorescence enhancement. The important parameter which is derived from this plot is x_b, the fluorescence intensity of bound ligand.

Plots of the type depicted in Fig. 3 contain all the information necessary for the calculation of binding data suitable for a Scatchard plot or a double reciprocal plot ($1/r$ versus $1/[S]$): the use of x_{obs}, x_f and x_b permits calculation of the concentration of bound ligand ($[\bar{S}] - [S]$) and of r according to equation (1). If the resulting binding curve is non-linear, the data may be interpreted in terms of non-equivalent binding sites. Where it is shown by an alternative technique such as equilibrium dialysis that the binding sites are equivalent then the non-linearity of the binding plot must be interpreted in terms of non-identical enhancement associated with each successive binding site occupied by ligand.

The above analysis applies only to cases in which there is no self-association of the acceptor. Such self-association introduces dependence of r on $[\bar{A}]$ as well as on $[S]$. Unless one has prior knowledge that self-interactions are absent, it is wise

to obtain binding data from separate experiments in which the ligand concentration is varied while the acceptor concentration is held constant.

Acknowledgments

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