CHAPTER 7
Theory of Binding Data Analysis

Clark's Theory

The quantitation of physical constants defining classical ligand-receptor interaction is dependent on the following assumptions:

1. The interaction is reversible; the association reaction is bimolecular while the dissociation is unimolecular.
2. All the receptor molecules are equivalent and independent
3. The biological response is proportional to the number of occupied receptor sites.
4. The interaction and response are measured after the reaction has reached equilibrium.
5. The active chemical agent does not undergo degradation or participate in other reactions, and only exists in either a free (L_F; unbound) form or bound to the receptor (B).

Under these assumptions, at equilibrium:

$$\textbf{Equation 7.1:}\quad \mathbf{Receptor_{free} + Ligand_{free} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Receptor{:}Ligand \xrightarrow{k_e} effect}$$

where k_1 and k_{-1} are the kinetic association and dissociation constants and k_e is the proportionality constant between response and occupancy. Because the determination of physical binding constants does not normally require the measurement of k_e, we can focus our discussion on the reversible reaction.

At equilibrium, mass action says that:

$$\textbf{Equation 7.2:}\quad \frac{[Ligand_{free}][Receptor_{free}]}{[Receptor{:}Ligand]} = \frac{[L_F][R_F]}{[B]} = \frac{k_{-1}}{k_1} = K_d$$

We can rewrite **Equation 7.2** as:

$$\textbf{Equation 7.3:}\quad \frac{[L_F][R_T - B]}{[B]} = K_d$$

where L_F is the free ligand concentration, R_T is the total receptor concentration, and (R_T - B) is the free receptor concentration, and B is the bound ligand:receptor complex concentration. **Equation 7.3** can be rearranged to:

$$\textbf{Equation 7.4:}\quad \frac{B}{R_T} = \frac{L_F}{(K_d + L_F)}$$

which is the equation for a rectangular hyperbola with horizontal asymptote corresponding to 100% saturation of R_T, such that [bound] = [receptor], as shown in **Figure 7.1**. The ratio $\mathbf{B/R_T}$ is also referred to as **f**, the *fractional occupancy*. **Equation 7.4** shows that the K_d is defined as the concentration of free ligand at which 50% of the receptor sites are occupied (*i.e.*, fractional occupancy = 0.5).

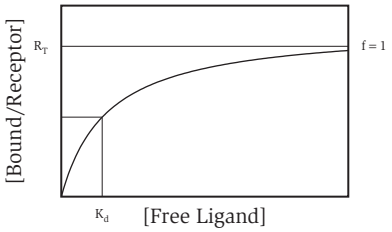


Figure 7-1. The classic hyperbolic binding curve, expressed at the fractional occupancy, f, of the receptor.

Non-specific Binding

The most common problem to deal with in receptor-ligand interactions is non-specific binding (NSB). NSB has been commonly, but incorrectly, defined as "**binding that is not saturating**" due to the presence of unlimited low affinity binding sites (*e.g.*, proteins sticking to the phospholipids of the cell membrane). Binding of the ligand would therefore be directly proportional to the concentration of **free** ligand alone. This definition has also been incorrectly stated as "**binding that was not displaceable by excess concentrations of unlabeled ligand.**" Specific binding to the receptor is routinely calculated by subtracting the measured NSB (after addition of excess unlabeled ligand to the system) from the total binding.

Since the latter definition for NSB is incorrect, results obtained using the definition above are often misleading. Binding thought to be non-displaceable and non-saturable can actually be both, if enough unlabeled or labeled ligand are added, respectively. It is better to treat NSB as binding to a set of identical and independent sites that have an affinity and capacity for the ligand in question. These sites are distinct from those of the receptor under study.

The binding of a ligand to two classes of binding sites (*e.g.*, a receptor and non-specific site) can be described by **Equation 7.5**:

$$\textbf{Equation 7.5:} \qquad B = \frac{R_1 \times L_F}{k_{d1} + L_F} + \frac{R_2 \times L_F}{k_{d2} + L_F}$$

where R_1 and R_2 are the concentrations of receptors for each site and K_{d1} and K_{d2} are the respective dissociation constants. When $K_{d2} >>> F$, **Equation 7.5** reduces to **Equation 7.6**:

$$\textbf{Equation 7.6:} \qquad B = \left(\frac{R_1 \times L_F}{R_{d1} + L_F} \right) + (K_{nsb} \times L_F)$$

The binding to the second site therefore appears linear (unsaturable). When the second site K_{d2} is very large, NSB can often be adequately described by a linear function.

When an excess of unlabeled ligand is used to estimate NSB, L_F approaches K_d and NSB will appear saturable. NSB correction of this type, with excess unlabeled ligand, results in overestimation of the number of receptor sites and underestimation of the receptor affinity. Because this overestimation increases with L_F, further artifacts can appear, incorrectly suggesting negative cooperativity or multiple binding sites.

Therefore, the best way to measure NSB would be to not to measure it at all. Rather, measure total binding and fit the data to **Equations 7.5** and **7.6** with one of the many nonlinear curve-fitting programs now available. This method requires no assumption and can account for complicated systems. This approach is especially true if NSB is in the range of $< 30\%$ of total binding. If NSB is $> 30\%$ of total binding, one should first attempt to experimentally reduce the percent of NSB in order to facilitate, with any confidence, the discovery of a solution. In this case, NSB is estimated as the binding that remains in the presence of 100-200 fold excess unlabeled ligand. NSB at each concentration is subtracted from total binding to arrive at specific binding. For an excellent review of NSB, see Mendel and Mendel (1985).

Determination of Binding Constants

It is not our aim to describe the details of performing a binding experiment, but a few issues should be addressed in the experimental design.

Equilibrium time: Analysis of equilibrium binding experiments assumes you measure binding at equilibrium. Before any equilibrium experiments are done, you should determine the incubation time that will allow the system to approach equilibrium. This is usually accomplished by incubating a low concentration of ligand (well below the presumed K_d) with receptor and following the amount of ligand bound over time. The incubation time for most assays is the time required for 90% of the ligand to bind (**Figure 7-2**).

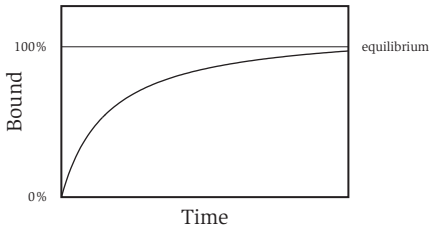


Figure 7-2. Representation of the time course for a binding event to reach equilibrium. Note that there is very little increase in binding after very long incubations.

Equilibrium binding experiment: The concentration of one of the binding agents (usually the Receptor) is kept constant and below the K_d ($R/K_d < 0.1$ or lower). Higher receptor concentrations tend to bind significant amounts of ligand leading to ligand depletion (discussed further in the section on **Free Ligand Concentration**).

The concentration of the other binding agent (usually the ligand) is varied from at least 100-fold below the K_d to 100-fold above the K_d. In this case, 99% of the fractional occupancy is covered by four orders of magnitude of free ligand concentration (two orders above and two orders below the K_d). In practical terms, binding experiments normally need to cover free ligand concentrations over three to four orders of magnitude (we usually aim for 20-fold below to 50-fold above the K_d). The number of points needed for analysis of the binding isotherms is dependent on the number of binding constants that need to be estimated. In general, simple models will require 15-20 single data points. Models with second binding sites and nonspecific binding will require more in order to statistically analyze the data properly.

• Mendel, C.M. and Mendel, D.B. (1985) *Biochem. J.* **228:**269-72.

Free Ligand Concentration: Equation 7.4 describes equilibrium in terms of the free ligand concentration, not the total ligand concentration. In many experimental systems, the amount of bound ligand is a very small percentage of the total ligand concentration, and the total ligand concentration can be used as an approximation of the free ligand concentration. Generally, if the bound ligand concentration is 5% or 10% of the total ligand concentration, the approximation holds.

If the total concentration cannot be used as an approximation of the free concentration (a situation usually referred to as ligand depletion), there are a few possible alternatives. These include:

1. Accounting for the discrepancy in the a more complex binding model (See Swillens, 1995, and Kenakin, 1993)
2. Changing the experiment so that less ligand is bound (in traditional experiments, this usually means reducing the receptor concentration which results in a concomitant decrease in the bound ligand signal)
3. Measuring the concentration of free ligand in each sample by either measuring the free ligand concentration directly or subtracting the bound ligand concentration from the total ligand concentration.

Swillens (1995) and Motulsky (1995) argue that the last method, while traditionally the most popular (*i.e.*, determining the free concentration by subtracting bound from total) has problems. Specifically, the bound and new free terms will be related (as will their errors), and certain calculations of non-specific binding are impossible. Instead, the explicit solution of the binding curve, with the bound and total values in place, is recommended instead. Rearranging **Equation 7.3** and substituting (L_T - B) for L_F leads to:

$$\textbf{Equation 7.7:} \qquad \frac{R_T \times (L_T - B)}{K_d + (L_T - B)} = B$$

solving for B, one real solution for the quadratic is:

$$\textbf{Equation 7.8:} \quad B = \frac{(L_T + K_d + R_T) - \sqrt{(-L_T - K_d - R_T)^2 - 4L_TR_T}}{2}$$

Several software packages can automatically solve this equation for K_d and R_T.

Data analysis: Traditionally, binding data were analyzed using linear transformations of the simple binding equations. These transformations provide considerable information, but they lack the ability to analyze binding isotherms (*i.e.*, binding curves) that deviate from Clark's Theory (*e.g.* one ligand, one receptor, no NSB). In addition, a plethora of statistical errors creep into the linear transformations, many times due to weighting effects. The most sophisticated analysis method involves the use of non-linear, least-square, curve-fitting computer programs that are capable of fitting the binding isotherms according to several different models and then compare the statistical quality of the resulting 'goodness of fit'. Many excellent Windows-based programs are commercially available.

The linear transformations are important tools in understanding binding isotherms. Here we describe one of the many step-by-step approaches to analyzing binding experiments.

- Swillens, S. (1995) *Molec. Pharm.* **47:**1197-1203.
- Kenakin, T. Pharmacologic Analysis of Drug/Receptor Interaction, 2nd Edition, **397**.
- Motulsky, H. (1996). The GraphPad Guide to Analyzing Radioligand Binding Data. GraphPad Software, Inc., San Diego, CA.

Saturation function

It is often a good idea to look at simple graphical representations of your data in order to understand what complications might be present. The simplest curve to consider is the **bound ligand vs. total ligand** curve. Note that in strict terms, the saturation function refers to the **bound receptor vs. free ligand** curve, but plotting this curve requires some calculation. The **bound vs. total** curve should be inspected for smoothness and should have no inflection points, maxima, or minima. If the curve does not level off, but continues upward linearly, it may contain an NSB component.

Klotz Plot

The most useful curve is the Klotz Plot, or semi-log plot (**Figure 7-3**). Many computer analysis programs use the Klotz plot as their primary graphical representation. The binding data are plotted as **bound ligand vs. log free ligand** and yield a sigmoidal curve. The graphical representation of the Klotz plot is used in two ways: determining the suitability of chosen ligand concentrations and analyzing the data for cooperativity.

The Klotz Plot should be symmetrical around the inflection point, which corresponds to the K_d along the free axis. The upper asymptote is R_T. The curve is nearly linear between $0.1 \times K_d$ and $10 \times K_d$. In addition, it is easy to see that 99% of occupancy is represented by four orders

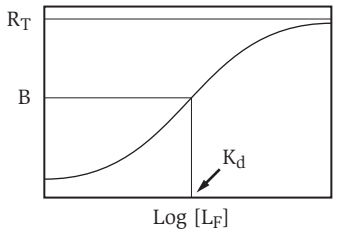


Figure 7-3. The classic semi-log Klotz plot. Note, that as with most binding curves, the free ligand, not the total ligand concentration, is plotted.

of magnitude of free concentration (two on either side of the K_d). Lack of sufficient points on either side of the inflection point (K_d) will be obvious, especially at the plateaus. Most experiment fail because they lack data points at high ligand concentrations, due to poor design, or more often because ligand is expensive or rare. Unfortunately, fewer points in this region sometimes mean additional binding sites or non-specific binding will be missed, or the total receptor concentration (R_T) will be imprecise.

The Klotz Plot can also indicate cooperativity. In the simplest model, the curve will rise from approximately 10% to 90% occupancy through $0.1X < K_d < 10X$ of free ligand concentration. If the curve completes this rise over a smaller range of concentration, this is indicative of positive cooperativity. If this rise requires a wider range of free ligand concentration, it is indicative of negative cooperativity. For a further discussion, see the section on the **Hill Plot and Cooperativity** on page 7-9.

Scatchard Analysis

Over the last thirty years, the so-called Scatchard plot has been the traditional method for analysis of binding data until the introduction of sophisticated non-linear curve-fitting software. Rearrangement of **Equation 7.3** yields:

Equation 7.9:

$$\frac{B}{L_F} = \frac{-B}{K_d} + \frac{R_T}{K_d}$$

which fits the equation of a line ($y = mx + b$; B/L_F vs. B) where R_T/K_d is the Y-intercept, R_T is the X-intercept, and $-1/K_d$ is the slope (See **Figure 7.4**). While many authors have written extensively on the errors inherent to analysis of binding curves by the Scatchard plot, it is a very powerful tool for identifying deviations from Clark's simple model, which without deviations, is represented by a straight line on the Scatchard Plot.

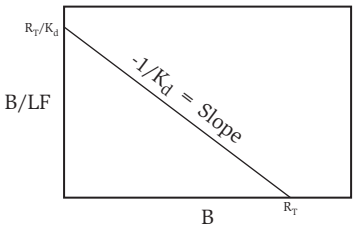


Figure 7-4. The Scatchard Curve. This representation is very sensitive to deviations from a simple binding model.

Non-specific Binding

A concave-up curve with an x-asymptote may indicate the presence of NSB (**Figure 7-5**). At first glance, it appears easy to estimate the NSB asymptote, but the mathematical subtraction of NSB from the total binding data is tedious (*i.e.,* NSB must be subtracted *radially* from the Scatchard curve because B/L_F and B are correlated, pursuant with Rosenthal's Construction). NSB in this case illustrates why computer analysis is preferred over the daunting task of **properly** deconvoluting the Scatchard plot (*i.e.,* mathematically divide it into its constituent curves).

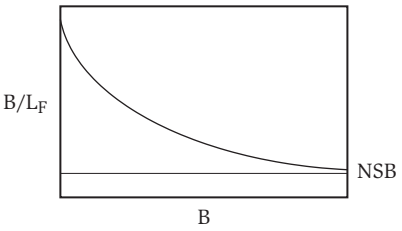
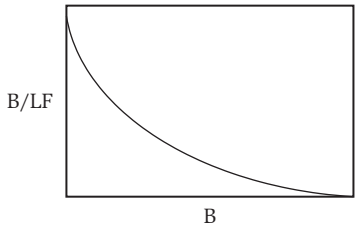


Figure 7-5. The Scatchard Curve indicating the presence of non-specific binding.

Negative Cooperativity

A concave-up curve that intersects the y-axis and the x-axis may indicate the presence of negative cooperativity between receptors (**Figure 7-6**). It is often difficult to differentiate between negative cooperativity and NSB on the Scatchard plot. One suspects negative cooperativity only when other data support the possibility.

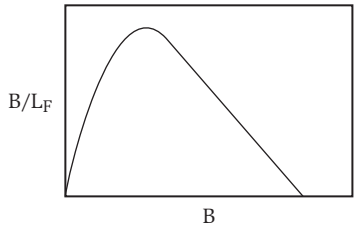
Figure 7-6. A Scatchard Curve indicating the presence of negative cooperativity.



Positive Cooperativity

A concave-down curve that intersects the origin is indicative of positive cooperativity (**Figure 7-7**). The maxima occurs at: f = (n-1)/n, where n is the slope of the Hill Plot (explained on page 7-10) and f = fractional occupancy.

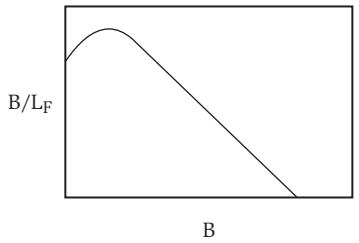
Figure 7-7. The Scatchard Plot indicating positive cooperativity.



Chemical Instability at Low Concentrations

A concave-down curve that intersects the y-axis is usually due to breakdown of the ligand at low concentrations and not positive cooperativity (**Figure 7-8**). It is difficult to differentiate between these two possibilities.

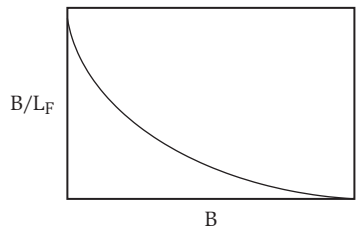
Figure 7-8. A Scatchard Plot depicting that the ligand is breaking down during the experiment.



Multiple Classes of Binding Sites

A concave-up curve may also indicate the presence of multiple classes of binding sites with differing K_d values, instead of negative cooperativity or NSB (**Figure 7-9**).

Figure 7-9. This Scatchard analysis indicates the presence of multiple binding sites on the receptor.



Hill Plot and Cooperativity

Many times we observe, especially with multimeric proteins, that the occupancy of some of the sites affects the affinity of the ligand for unfilled sites. The classic example is O_2-hemoglobin. The Hill Slope analysis allows for the differentiation of cooperativity (*i.e.,* when the Klotz plot (**Figure 7-3**) is steeper or shallower than predicted by Clark's Model) and multiple binding sites. The Hill Equation accounts for the possibility that not all receptor sites are independent and says that:

Equation 7.10:
$$f_{\text{(the fractional occupancy)}} = \frac{L_F^{\,n}}{K_d + L_F^{\,n}}$$

where n is the slope of the Hill Plot and is also the average number of interacting sites.

The linear transformation that is commonly used, the Hill Plot, is made by rearranging **Equation 7.8** and taking the log:

Equation 7.11:
$$\log\left[\frac{B}{R_T - B}\right] = n[\log(L_F)] - \log(K_d)$$

This equation is plotted as log $B/(R_T\text{-}B)$ vs. log L_F where the y-intercept is -log K_d and the slope n = the Hill Coefficient (see **Figure 7-10**).

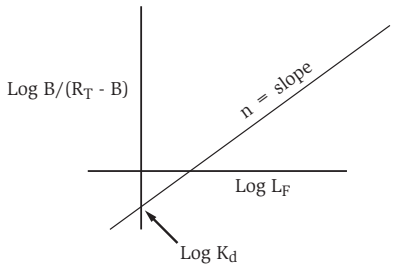


Figure 7-10. The classic Hill Plot.

Please note that if the Hill Plot is not linear, the model used is not applicable to the data set and needs reevaluation

Deviations from a slope of 1 tell us about deviation from the ideal model. **Table 7-1** shows the Hill Slope result and the effect on other transformations.

Table 7-1. Hill Slope Effects.

Hill Slope	B vs. L_F plot	Scatchard	Klotz
If slope = 1, there is a single class of binding sites	hyperbola	straight line	sigmoidal
If slope ≠ 1 and is an integer, multiple non-interacting binding sites	hyperbola	concave-down	sigmoidal
If slope ≠ 1 and is fractional, you have cooperativity:			
If slope > 1, Positive cooperativity	sigmoidal	concave-down	narrow sigmoidal
If slope < 1, Negative cooperativity	no effect	concave-up	wide sigmoidal

One of the most elegant methods to confirm negative cooperativity was developed by De Meyts (1976). It is based on the observation that with negative cooperativity, the decrease in affinity as percent saturation increases is due to a decrease in the rate of association of the complex (net drop in K_a, the equilibrium association constant). To put it more simply, K_d varies with the percent saturation.

Experimentally, De Meyts (1976) allowed the labeled ligand to bind to its receptor until equilibrium was reached. The remaining free ligand is removed, replaced with buffer, and the amount of ligand which remains bound to the receptor is measured over time. In an identical experiment, free ligand is replaced with buffer containing a large excess of unlabeled ligand. Both data sets are plotted as B_t (*i.e.,* bound at time t)/B_o (*i.e.,* bound at time zero) vs. time. If the addition of the unlabeled ligand leads to an increase in the dissociation rate (compared to the dissociation in the absence of excess unlabeled ligand), negative cooperativity is indicated.

The issue of describing the K_d for negative cooperativity among receptor sites is addressed below. Traditionally, three parameters are reviewed:

$K_{average}$ = average affinity value (it varies with R and L_F)

K_e = maximum value of K_d when virtually all the sites are empty

K_f = minimum value of K_d when virtually all the sites are full

K_e and K_f can be estimated from the concave-up Scatchard plot. K_e is calculated as the slope of the line running from the y-intercept to the x-intercept. K_f is the slope of the line tangent to the curve at the x-intercept. $K_{average}$ varies all along the curve depending on the ligand concentration chosen.

Non-linear, Least-Squares, Curve Fitting

As with most computer programs, the axiom 'garbage in, garbage out.' rings true during the analysis of binding curve data. Too often, this type of analysis is done without careful thought to what the data are telling you about deviations in your system from the ideal. Careful inspection of the data, using the methods described above, will allow you to most suitably fit your data to a model that accounts for the physical reality. If you see NSB, the computer programs will allow you to subtract it out. If you might have one receptor site or two, the programs will allow you to reanalyze your data with several models and compare them to each other with statistical robustness. People regularly report the K_d from a computer analysis and never take the time to see if the fitted curve actually fits the data well.

In general, the model that is chosen to explain a particular system should be the simplest and most statistically significant one. For example, if the data fit a model with two receptor sites no better than a model with one receptor site, the one receptor model should be chosen, unless other independent data suggest using the two-site model.

• De Meyts, P., Bianco, A.R. and Roth, J. (1976) *J. Biol. Chem.* **251:**1877-88.

Multiple Classes of Binding Sites

A common situation occurs with the presence of multiple classes of binding sites in a receptor preparation. The second site may be another high-affinity receptor or may be a low-affinity site more akin to NSB. It is important to properly identify multiple sites (see **Table 7-2**).

Table 7-2. Characteristics of Multiple Binding Sites.

Plots	Identifying Feature
B vs. LF plot	A polynomial of n + 1 degrees being the sum of two hyperbolas
Scatchard plot	Concave-up curve (**Figure 7-7**)
Hill plot	Slope not equal to 1
Klotz plot	Multiple inflection points if the K_d values differ enough

The changes in the Klotz plot may be very small. Usually, the ratio of the K_d values must be at least 100X before you will see a second inflection point. If there is a <14–fold difference in the K_d values, you will not be able to mathematically deconvolute the sites. On the traditional B vs. L_F plot, the composite curve will appear to be a normal hyperbola, but the apparent K_d is actually equal to $[(K_{d1})(K_{d2})]^{1/2}$. For this reason, the Scatchard Plot, which normally shows a straight line, is the easiest way to see multiple classes of binding sites because it will be concave-up when multiple sites are present. Note that the multi-site Scatchard curve is the sum of two (or more) linear lines with different intercepts and slopes. The K_d of a line forced through the total curve does not correspond directly to the K_d of either site. This relationship is presented mathematically in **Equation 7-12**:

$$\textbf{Equation 7.12:} \qquad B = \frac{R_{T1} \times L_F}{K_{d1} + L_F} + \frac{R_{T2} \times L_F}{K_{d2} + L_F}$$

This model can be complicated more by the addition of a linear NSB term (resulting in a total of five parameters: R_{T1}, K_{d1}, R_{T2}, K_{d2}, NSB). Models of more than five parameters are difficult to analyze. At least 80 data points are required to tell the difference between a five and seven parameter model. Generally, multiple sites are confirmed by fitting the data to a one-site (± NSB) model and two-site (± NSB) model, and then determining which model gives a statistically better fit.

Many times, it is not clear whether to call a low affinity second site a true binding site or nonspecific binding. This issue was addressed earlier in the chapter. As discussed there, if the experiment covers a concentration range such that $K_{d2} >>> L_F$, then the second site will collapse into a NSB component.

Competition in Binding

The term "competition," as used in this section, refers to the special case of antagonism in which two ligands are capable of binding to the same sites. The simplest case is the binding of a labeled ligand in the presence of various concentrations of an unlabeled ligand (also called the inhibitor or competitor) to a receptor with one class of binding sites. Competition experiments are useful for a few reasons:

Determining whether the labeling process has affected the ligand's affinity for the receptor. The labeled ligand's K_d determined by the direct binding experiment is compared to the K_i values of the unlabeled ligand and mock-labeled ligand that were determined from the competition assay. If the K_i and K_d value are not the same (within the error of the assay - say four-fold) then the labeling process may have affected the affinity.

Comparing the affinities of several ligands for the same receptor. It is more reliable to compare the affinities of several ligands when none are labeled. A single control labeled ligand is used for comparison to each unlabeled ligand. The control ligand may be of absolutely no interest itself. Each unlabeled ligand will generate its own binding isotherm, and as long as the conditions of the experiments are identical, the affinities of each can be directly compared. The advantage of this approach is that since only a single ligand is labeled, adverse effects on affinity caused by the labeling process will not affect the comparison of the unlabeled ligands.

The IC_{50} is the concentration of inhibitor necessary to displace 50% of the labeled ligand. It is a useful binding constant to characterize the inhibitory ligand (See **Figure 7-11**). If your aim is to directly compare the relative affinities of several inhibitors, comparing IC_{50} values obtained under identical conditions is sufficient.

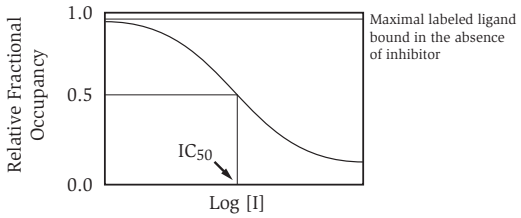


Figure 7-11. The standard competition curve. Please not that the IC_{50} is not necessarily equal to the K_i.

Historically, the affinity of the inhibitor for the receptor has been derived using the Cheng and Prussoff (1973) Corrections:

$$\textbf{Equation 7.13:} \qquad f_i = \frac{I}{I + K_i(1 + L_T/K_d)}$$

and

$$\textbf{Equation 7.14:} \qquad f = \frac{L_T}{K_d(1 + I/K_i) + L_T}$$

where: f_i = the fractional inhibition
f = fractional occupancy or saturation
L_T = total concentration of the labeled ligand
I = Added concentration of the unlabeled ligand (competitor/inhibitor)
K_d = dissociation constant for labeled ligand
K_i = dissociation constant for the unlabeled ligand

• Cheng, Y.C. and Prusoff, W.H. (1973) *Biochem. Pharm.* **22:**3099-108.

When the value of f_i or f is equal to 0.5, **Equations 7-9** and **7-10** can be simplified to:

Equation 7.15:
$$K_i = \frac{IC_{50}}{1 + L_T/K_d}$$

and

Equation 7.16:
$$K_d = \frac{EC_{50}}{1 + I/K_i}$$

where EC_{50} is the concentration of ligand that yields 50% binding in the presence of a given concentration of inhibitor.

Total vs. Free for the labeled ligand and unlabeled inhibitor. Equations 7.15 and **7.16** were originally derived in the context of competitive enzyme inhibition. In the original equation, the concentrations of L would be the **free** concentration (not initial or total) of labeled ligand and the IC_{50} would be the **free** concentration (not added or total) of inhibitor that reduces the binding of the labeled ligand by 50%. However, enzyme kinetic studies are usually done under conditions such that the free and total concentrations of substrate and the free and total concentrations inhibitor are nearly identical (*i.e.,* [Total] approximates [Free]). This may not be true of equilibrium binding studies, and therefore the total concentrations cannot be substituted for free concentrations without the possibility of introducing significant errors into the calculation of K_i (Hollenberg and Cuatrecasas, 1979). Several authors have approached this issue (see Rodbard, 1973; Jacobs *et al.*,1975; Kenakin, 1993; Munson and Rodbard, 1988). These authors show quite dramatically how high receptor and labeled ligand concentrations lead to an error in the IC_{50} and their papers are essential reading for anyone performing competitive displacement experiments.

Munson and Rodbard (1988) offered an exact solution (**Equation 7-17**) of the Cheng and Prusoff Correction that takes these possible problems into account.

Equation 7.17:
$$K_i = \frac{IC_{50}}{1 + \dfrac{L_T(y_0 + 2)}{2 \times K_d(y_0 + 1)} + y_0} - K_d\left(\frac{y_0}{y_0 + 2}\right)$$

where y_0 = the initial Bound to Free ratio for the labeled species before perturbation of equilibrium by the added inhibitor and IC_{50} = the concentration of inhibitor that reduces binding of the labeled ligand by 50%.

When y_0 is very small, **Equation 7-17** reduces to **Equation 7-15**. Generally, if y_0 is < 0.1, the problem can be ignored.

An alternative approach is to calculate the exact solution of **Equations 7.13** and **7.14** in terms of free ligand and inhibitor concentrations (*i.e.,* Free = Total - Bound). Kenakin (1993; also see Swillens, 1995) offers the following solution (**Equation 7-18**) and shows that if receptor concentration is 10-times the K_d, the IC_{50} will overestimate the K_i by about 10-fold. It should be noted that all errors caused by high receptor and ligand concentrations lead to overestimations of the K_i by the IC_{50}.

Equation 7.18:
$$B = \frac{(L_T \times R_T) - (B \times R_T)}{L_T - B + K_d + (I \times K_d/K_i)}$$

- Hollenberg, M.D. and Cuatrecasas, P. (1979) The receptors: a comprehensive treatise. New York:Plenum Press.
- Rodbard, D. (1973) Receptors for reproductive hormones. New York:Plenum Press.
- Jacobs, S. *et al.* (1975) *Biochem. Biophys. Res. Commun.* **66:**687.
- Kenakin, T. P. (1993) Pharmacologic analysis of drug/receptor interaction, 2nd ed. New York:Raven. 483 p.
- Munson, P.J. and Rodbard, D. (1988) *J. Receptor Res.* **8:**533-46.
- Swillens, S. (1995) *Molec. Pharm.* **47:**1197-1203.

Rearranging to solve for K_i:

Equation 7.19:

$$K_i = \frac{B \times I \times K_d}{(L_T \times R_T) + B \times (-R_T - L_T + B - K_d)}$$

In order to convert the observed IC_{50} to K_i, solve this equation for conditions when the added inhibitor concentration, I, equals the IC_{50}. If your model for competitive binding includes a non-specific binding term, Swillens suggests how this second method can be modified to account for deviations to NSB by high receptor and ligand concentrations.

Equilibrium and Non-equilibrium Conditions

Equilibrium Conditions

One of the central assumptions to the analysis of binding data is that the data are gathered after equilibrium between the receptor and ligand is reached. This may take minutes or several hours. **Before meaningful binding experiments can be done, the incubation time necessary to reach equilibrium must be determined.** Usually this is accomplished by following the time course of binding at a single ligand and receptor concentration (see **Figure 7-12**). Because equilibrium is reached more slowly when concentrations are low, choose the lowest concentration of ligand possible for the time course experiment. Binding experiments are then usually done at a time when > 90% of equilibrium is reached.

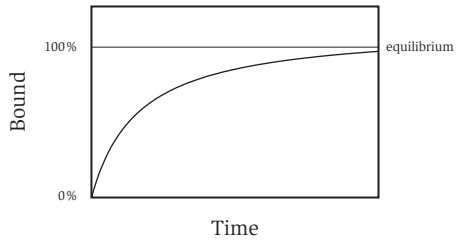


Figure 7-12. A graphical representation of an experiment used to determine the time required for equilibrium binding to occur.

Non-equilibrium Conditions

There are varieties of reasons why researchers may be interested in obtaining measurements of bound ligand versus time, under non-equilibrium conditions. For instance, the receptor/ligand K_d may be so low that concentrations of labeled ligand required in an equilibrium binding experiment might be lost in the background. The only way to calculate binding constants may be through kinetic experiments.

Determination of the Association Rate Constant, k_1

Introduction. Clarke's model can be written as:

Equation 7.20:

$$(R_T - B) + (L_T - B) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$$

Where R_T is the total receptor concentration, $(R_T - B)$ is the free receptor concentration, L_T is equal to the total ligand concentration and $(L_T - B)$ is equal to L_F, the free ligand concentration. During a very small time interval, δt, the concentration of bound ligand (*i.e.,* receptor:ligand complex) will change by a small amount, δB_t, and therefore the following equation applies:

Equation 7.21:

$$\frac{\delta B_t}{\delta t} = k_1(R_T - B_t)(L_T - B_t) - (k_{-1} \times B_t)$$

At equilibrium, $\delta B_t/\delta t = 0$ and the equation can be rewritten as a quadratic, with one meaningless solution, B', and a real solution, B_e. Bound, at any time t can then be written as **Equation 7.22**:

Equation 7.22:

$$B_t = B_e + \frac{(B' - B_e)}{1 = e^{(mt + C)}}$$

where $m = (k_1)(B' - B_e)$, C (the integration constant) $= \ln(B'/B_e)$, and B_e is the amount bound at equilibrium. When $t = 0$, $B_t = 0$, and at infinite time, B_t approaches B_e, the real solution (see **Figure 7-12**).

Solution of k_1. Solving **Equation 7.22** by non-linear regression analysis, using the time-dependent binding data at a single ligand concentration, will yield the four parameters of this equation: B_e, B', m, and C. If the experiment is repeated n times, at n different ligand concentrations, n sets of parameters will be obtained. From these parameters, obtained at many ligand concentrations, k_1 is determined by plotting the n values of m obtained with n different ligand concentrations versus the respective values of $(B' - B_e)$. This plot will generate a straight line passing through the origin, with a slope of k_1.

Calculation of the dissociation constant.

Equation 7.23:

$$B_e + B' = R_T + L_F + \left(\frac{k_{-1}}{k_1}\right)$$

Plotting the n values of $(B_e + B' - R_T)$ versus the n values of L_F should yield a straight line with a slope of 1 and a y-intercept of K_d. Knowing the k_1 and K_d, k_{-1} can be calculated as $k_{-1} = (K_d)(k_1)$.

Independent Determination of the Dissociation Rate Constant, k_{-1}

The determination of the dissociation rate constant, k_{-1}, of a labeled ligand from its receptor is commonly accomplished by allowing the labeled ligand to bind to the receptor until equilibrium is reached and then measuring the rate of dissociation of ligand from receptor. In solid-phase binding experiments, labeled ligand is allowed to bind to immobilized receptor and then the incubation medium is quickly removed from the dish or filter, and replaced by buffer. This step instantaneously removes any free ligand from the system (hence the term "instantaneous dilution"). As the bound ligand reaches a new equilibrium with the ligand in the buffer (zero ligand in solution at time = 0), the amount of ligand bound to receptor will decrease over time, as shown by the **Equation 7-24**:

Equation 7.24:

$$B_t = B_0 \times [e^{(-k_{-1} \times t)}]$$

where B_t is the amount of ligand bound at any time t, and B_o is the total ligand bound at time zero. When $B_t = 0.5\ B_o$, then $k_{-1} = 0.693/T_{1/2}$ where $T_{1/2}$ is the half-time of dissociation (see **Figure 7-13**).

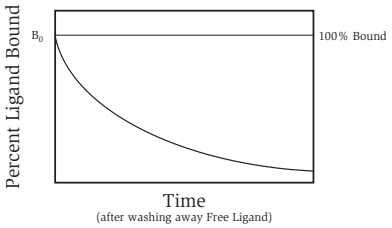


Figure 7-13. The dissociation binding curve.

$T_{1/2}$ is measured from a plot of ln (B_t/B_o) vs. t, and is the time when ln $(B_t/B_o) = 0.5$ (**Figure 7-14**).

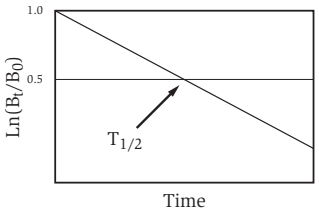


Figure 7-14. The graphical method of determining $T_{1/2}$.

This method for determining the dissociation constant will yield reliable results unless the system exhibits positive or negative cooperativity in binding.

Approximate Solutions for k_1 and k_{-1}

The complication of non-linear regression analysis of the association curves in order to calculate k_1 and k_{-1} can be avoided if one assumes that the amount bound is much less than the amount free ($B << L_F$), as is the case in many binding experiments. If $B << L_F$, then **Equation 7.21** can be simplified, and its integral yields **Equation 7.25**:

$$\textbf{Equation 7.25:} \qquad B_t = B_e \times \{1 - e^{[-(k_1 R_T + k_{-1})t]}\}$$

When $B_t = 0.5B_e$, then **Equation 7.25** can be transformed to **Equation 7.26**:

$$\textbf{Equation 7.26:} \qquad \frac{\ln(2)}{T_{1/2}} = k_1 L_T + k_{-1}$$

where $T_{1/2}$ is the half-time of association (between ligand and receptor). The rate constants are therefore calculated in the following way:

1. Repeat an association binding experiment at several different ligand concentrations, collecting data on bound vs. time.
2. Analyze each curve for B_e, the amount bound when equilibrium is reached.
3. Plot the association data from each experiment as log $(B_e - B_t)/B_e$ vs. time.
4. Determine the $T_{1/2}$ for each curve (point at which log $(B_e - B_t)/B_e = 0.5$).
5. Plot $\ln(2)/T_{1/2}$ vs. L_T. According to **Equation 7.26**, this plot will yield a straight line with slope $= k_1$ and y-intercept $= k_{-1}$.

Of course, you could always use the kinetic subroutines of the commercially available curve fitting programs to help in many of these calculations.

Ways to Improve Experimental Results

Interchangeability of Terms

While it is common to describe the analysis of binding with the terms "receptor" and "ligand," it is important to note that these terms are actually interchangeable. There is no reason why the "receptor" can't vary in concentration while the ligand concentration remains constant. These terms can also apply to a variety of systems. For instance, replace these terms with "antibody" and "antigen" and all of the same rules apply. In reviewing the issues discussed throughout **Chapter 7**, it is important to keep in mind how the guidelines on the analysis of binding data can be applied to specific, individual circumstances. In **Chapter 8**, specific examples using fluorescence polarization are discussed.

Important Points

- Upward concavity in the Scatchard Plot may result not only from multiple classes of sites but from negative cooperativity or from experimental artifacts.
- Although experimental points might appear to correspond to a curvilinear Scatchard plot, it is important to determine whether or not a straight line is an equally adequate fit.
- The most common experimental artifact results from incorrect estimation of free ligand concentration due to inadequate separation of the bound ligand. This may also result in neglecting NSB.
- If the receptor is more stable with bound ligand than without, the effective concentration of receptor, R, decreases as bound receptor:ligand, B, diminishes; this may result in apparent positive cooperativity.
- Binding curves will have meaning only if measurements are done under equilibrium conditions.
- Do not average replicate values. It decreases your degrees of freedom. In addition, it is better to run more individual concentrations, than fewer ones with replicates.

Common Binding Experiment User Errors

- Neglecting a proper correction for NSB.
- Determination of NSB by competition with excess unlabeled ligand. If the unlabeled ligand is present in excessive amounts, correction by simple subtraction may be misleading.
- Pooling data from separate experiments with different protein concentrations. This cannot be done unless the bound and bound/free values are normalized (dividing by the protein concentration).
- Presence of a non-binding contaminant in the labeled ligand. Since the contaminant is not bound, its signal will be computed as part of free ligand.
- Not taking into account different physical and/or chemical properties of the labeled and the unlabeled ligand with respect to the interaction with binding sites.
- Inadequate number or range of ligand concentrations (*i.e.,* only a 100-fold range of concentrations).
- Not taking into account internalization of the ligand/receptor complex or its degradation in experiments involving cells.

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