Analyzing Binding Data

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ABSTRACT

Measuring the rate and extent of radioligand binding provides information on the number of binding sites, and their affinity and accessibility of these binding sites for various drugs. This unit explains how to design and analyze such experiments. *Curr. Protoc. Neurosci.* 52:7.5.1-7.5.65. © 2010 by John Wiley & Sons, Inc.

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INTRODUCTION

A radioligand is a radioactively labeled drug that can associate with a receptor, transporter, enzyme, or any protein of interest. The term *ligand* derives from the Latin word *ligo*, which means to bind or tie. Measuring the rate and extent of binding provides information on the number, affinity, and accessibility of these binding sites for various drugs. While physiological or biochemical measurements of tissue responses to drugs can prove the existence of receptors, only ligand binding studies (or possibly quantitative immunochemical studies) can determine the actual receptor concentration. Radioligand binding experiments are easy to perform, and provide useful data in many fields. For example, radioligand binding studies are used to:

1. Study receptor regulation, for example during development, in diseases, or in response to a drug treatment.
2. Discover new drugs by screening for compounds that compete with high affinity for radioligand binding to a particular receptor.
3. Investigate receptor localization in different organs or regions using autoradiography (*UNITS 1.2 & 1.3*).
4. Categorize receptor subtypes.
5. Probe mechanisms of receptor signaling, via measurements of agonist binding and its regulation by ions, nucleotides, and other allosteric modulators.

This unit reviews the theory of receptor binding and explains how to analyze experimental data. Since binding data are usually best analyzed using nonlinear regression, this unit also explains the principles of curve fitting with nonlinear regression. For more general information on receptor theory and analyses of receptor data, see books by Limbird (2004) and Kenakin (2006).

BINDING THEORY

The Law of Mass Action

Binding of a ligand to a receptor is a complex process involving conformational changes and multiple noncovalent bonds. The details are not known in most cases. Despite this

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complexity, most analyses of radioligand binding experiments successfully use a simple model called the law of mass action:

$$\text{ligand} + \text{receptor} \rightleftarrows \text{ligand} \cdot \text{receptor}$$

Equation 7.5.1

The model is based on these simple ideas:

1. Binding occurs when ligand and receptor collide (due to diffusion) with the correct orientation and sufficient energy. The rate of association (number of binding events per unit of time) equals [ligand] × [receptor] × k_{on}, where k_{on} is the association rate constant in units of M^{-1} min^{-1}.
2. Once binding has occurred, the ligand and receptor remain bound together for a random amount of time. The rate of dissociation (number of dissociation events per unit time) equals [ligand · receptor] × k_{off}, where k_{off} is the dissociation rate constant expressed in units of min^{-1}.
3. After dissociation, the ligand and receptor are the same as they were before binding.

The equilibrium dissociation constant K_d

Equilibrium is reached when the rate at which new ligand·receptor complexes form equals the rate at which they dissociate:

$$[\text{ligand}] \times [\text{receptor}] \times k_{on} = [\text{ligand} \cdot \text{receptor}] \times k_{off}$$

Equation 7.5.2

Rearrange to define the equilibrium dissociation constant K_d.

$$\frac{[\text{ligand}] \times [\text{receptor}]}{[\text{ligand} \cdot \text{receptor}]} = \frac{k_{off}}{k_{on}} = K_d$$

Equation 7.5.3

The K_d, expressed in units of mol/liter or molar (M), is the concentration of ligand that occupies half of the receptors at equilibrium. To see this, set [ligand] equal to K_d in the equation above. In this case, [receptor] must equal [ligand·receptor], which means that half the receptors are occupied by ligand.

Affinity

The term affinity is often used loosely. If the K_d is low (e.g., pM or nM), that means that only a low concentration of ligand is required to occupy the receptors, so the affinity is high. If the K_d is larger (e.g., μM or mM), a high concentration of ligand is required to occupy receptors, so the affinity is low. The term equilibrium association constant (K_a) is less commonly used, but is directly related to the affinity of a compound. The K_a is defined to be the reciprocal of the K_d, so it is expressed in units of liters/mol. A high K_a (e.g., $>10^8$ M^{-1}) would represent high affinity.

Because the names sound familiar, it is easy to confuse the equilibrium dissociation constant (K_d, in molar units) with the dissociation rate constant (k_{off}, in min^{-1} units), and to confuse the equilibrium association constant (K_a, in liter/mol units) with the association

rate constant (k_{on}, in M^{-1} min^{-1} units). To help avoid such confusion, equilibrium constants are written with a capital "*K*" and the rate constants with a lowercase "*k*."

A wide range of K_d values are seen with different ligands. Since the K_d equals the ratio k_{off}/k_{on}, compounds can have different K_d values for a receptor either because the association rate constants are different, the dissociation rate constants are different, or both. In fact, association rate constants are all pretty similar (usually 10^7 to 10^9 M^{-1} min^{-1}, which is about two orders of magnitude slower than diffusion), while dissociation rate constants are quite variable (with half-times ranging from seconds to days).

Fractional occupancy at equilibrium

Fractional occupancy is defined as the fraction of all receptors that are bound to ligand. The law of mass action predicts the fractional receptor occupancy at equilibrium as a function of ligand concentration.

$$\text{fractional occupancy} = \frac{[\text{ligand} \cdot \text{receptor}]}{[\text{receptor}]_{\text{total}}} = \frac{[\text{ligand} \cdot \text{receptor}]}{[\text{receptor}] + [\text{ligand} \cdot \text{receptor}]}$$

Equation 7.5.4

A bit of algebra creates a useful equation. Multiply both numerator and denominator by [ligand] and divide both by [ligand receptor]. Then substitute the definition of K_d.

$$\text{fractional occupancy} = \frac{[\text{ligand}]}{[\text{ligand}] + K_d}$$

Equation 7.5.5

The approach to saturation as [ligand] increases is slower than one might imagine (see Fig. 7.5.1). Even using radioligand at a concentration equal to nine times its K_d will only lead to its binding to 90% of the receptors.

Assumptions of the law of mass action

Although termed a "law," the law of mass action is simply a model. It is based on these assumptions:

1. All receptors are equally accessible to ligands.

[ligand]/K_d	Fractional occupancy at equilibrium
0	0%
1	50%
4	80%
9	90%
99	99%

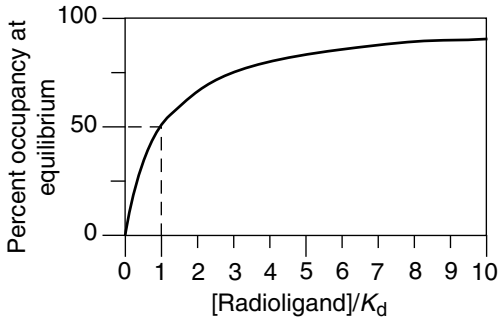

Figure 7.5.1 Occupancy at equilibrium. The fraction of receptors occupied by a ligand at equilibrium depends on the concentration of the ligand compared to its K_d.

2. All receptors are either free or bound to ligand. The model ignores any states of partial binding.
3. Binding alters neither ligand nor receptor.
4. Binding is reversible.

Nonspecific Binding

In addition to binding to the receptors of physiological interest, radioligands also bind to other (nonreceptor) sites. Binding to the receptor of interest is termed *specific binding*. Binding to other sites is called *nonspecific binding*. Because of this operational definition, nonspecific binding can represent several phenomena:

1. The bulk of nonspecific binding represents some sort of interaction of the ligand with membranes. The molecular details are unclear, but nonspecific binding depends on the charge and hydrophobicity of a ligand—but not its exact structure.
2. Nonspecific binding can also result from binding to receptor transporters, or to enzymes not of interest to the investigator (e.g., binding of epinephrine to serotonin receptors).
3. In addition, nonspecific binding can represent binding to the filters, tubes, or other materials used to separate bound from free ligand.

In many systems, nonspecific binding is linear with radioligand concentration. This means that it is possible to account for nonspecific binding mathematically, without ever measuring nonspecific binding directly. To do this, measure only total binding experimentally, and fit the data to models that include both specific and nonspecific components (see More Complicated Situations, below).

Most investigators, however, prefer to measure nonspecific binding experimentally. To measure nonspecific binding, first block almost all specific binding sites with an unlabeled drug. Under these conditions, the radioligand only binds nonspecifically. This raises two questions: which unlabeled drug should be used and at what concentration?

The most obvious choice of drug to use is the same compound as the radioligand, but unlabeled. This is necessary in many cases, as no other drug is known to bind to the receptors. Most investigators, however, avoid using the same compound as the hot and cold ligand for routine work because both the labeled and unlabeled forms of the drug will bind to the same specific and nonspecific sites. This means that the unlabeled drug will reduce binding purely by isotopic dilution. When possible, it is better to define nonspecific binding with a drug chemically distinct from the radioligand.

The concentration of unlabeled drug should be high enough to block virtually all the specific radioligand binding, but not so much that it will cause more general physical changes to the membrane that might alter specific binding. If studying a well characterized receptor, a useful rule of thumb is to use the unlabeled compound at a concentration at least 100 times its K_d for the receptors.

The same results should be obtained from defining nonspecific binding with a range of concentrations of several drugs. Ideally, nonspecific binding is only 10% to 20% of the total radioligand binding. If the nonspecific binding makes up more than half of the total binding, it will be hard to get quality data. If the system exhibits a great deal of nonspecific binding, use a different kind of filter, wash with a larger volume of buffer or a different temperature buffer, or use a different radioligand.

Ligand Depletion

The equations that describe the law of mass action include the variable [ligand], which is the free concentration of ligand. Unless specifically stated, all of the analyses presented later in this unit assume that a very small fraction of the ligand binds to receptors (or to nonspecific sites), so that the free concentration of ligand is approximately equal to the concentration added.

In some experimental situations, the receptors are present in high concentration and have a high affinity for the ligand. A large fraction of the radioligand binds to receptors (or nonspecific sites), depleting the amount of ligand remaining free in solution. The discrepancy is not the same in all tubes or at all times. Many investigators use this rule of thumb: if $<10\%$ of the ligand binds, don't worry about ligand depletion.

If possible, design the experimental protocol to avoid situations where $>10\%$ of the ligand binds. This can be done by using less tissue in the assays; however, this will also decrease the number of counts. An alternative is to increase the volume of the assay without changing the amount of tissue. In this case, more radioligand will be needed.

If radioligand depletion cannot be avoided, the depletion must be accounted for in the analyses. There are several approaches.

1. Measure the free concentration of ligand in every tube.
2. Calculate the free concentration in each tube by subtracting the number of cpm (counts per minute) of total binding from the cpm of added ligand. This method works only for saturation binding experiments, and cannot be extended to analysis of competition or kinetic experiments. One problem with this approach is that experimental error in determining specific binding also affects the calculated value of free ligand concentration. When fitting curves, both x and y would include experimental error, and the errors will be related. This violates the assumptions of nonlinear regression. Using simulated data, Swillens (1995) has shown that this can be a substantial problem. Another problem is that the free concentration of radioligand will not be the same in tubes used for determining total and nonspecific binding. Therefore specific binding cannot be calculated as the difference between the total binding and nonspecific binding.
3. Fit total binding as a function of added ligand to a model (equation) that accounts both for nonspecific binding and for ligand depletion.

SATURATION BINDING EXPERIMENTS

Saturation binding experiments determine receptor number and affinity by determining binding at various concentrations of the radioligand. Because this kind of experiment can be graphed as a Scatchard plot (more accurately attributed to Rosenthal, 1967), they are sometimes called "Scatchard experiments."

The analyses depend on the assumption that the incubation has reached equilibrium. This can take anywhere from a few minutes to many hours, depending on the ligand, receptor, temperature, and other experimental conditions. Since lower concentrations of radioligand take longer to equilibrate, use a low concentration of radioligand (perhaps 10% to 20% of the estimated K_d) when measuring how long it takes the incubation to reach equilibrium. Experimenters typically use 6 to 12 concentrations of radioligand, since data with fewer than 6 concentrations are usually insufficient to provide accurate estimates of the binding parameters.

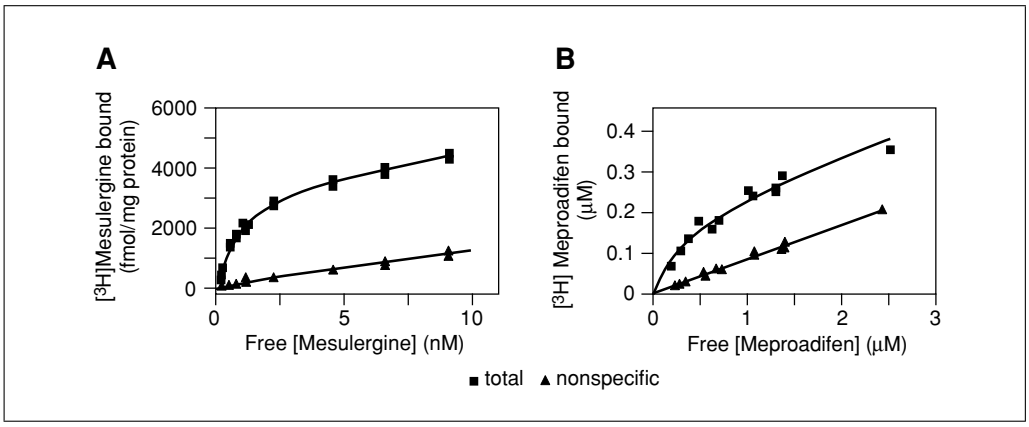

Figure 7.5.2 Examples of nonspecific binding. (**A**) [^{3}H]Mesulergine binding to serotonin receptors has low nonspecific binding (<25% of total binding at the highest concentrations). (**B**) [^{3}H]Meproadifen binding to the ion channel of nicotinic receptors has high nonspecific binding (>50%).

Theory of Saturation Binding

Nonspecific binding

Analysis of saturation binding curves requires accounting for nonspecific binding. The left panel of Figure 7.5.2 shows data from a nearly ideal system, where nonspecific binding is less than 25% of total binding. The right panel shows a less ideal system where nonspecific binding is over 50% of total binding at high ligand concentrations. If nonspecific binding were much higher than this, it would be very difficult to get reliable results.

The best approaches to accounting for nonspecific binding take advantage of the fact that nonspecific binding is generally proportional to the concentration of radioligand (within the concentration range used in the experiment). This means that a graph of nonspecific binding as a function of radioligand binding is generally linear, as shown in Figure 7.5.2. This assumption is reasonable if the nonspecific binding is due to general binding to membranes, but may not be reasonable if some of the nonspecific binding represents binding to receptors or transporters other than the one being studied.

Specific binding

Specific binding at equilibrium equals fractional occupancy times the total receptor number (B_{max}), and depends on the concentration of free radioligand ([L]):

$$\text{specific binding} = \text{fractional occupancy} \times B_{max} = \frac{B_{max} \times [L]}{K_d + [L]}$$

Equation 7.5.6

This equation describes a *rectangular hyperbola* or a *binding isotherm*. [L] is the concentration of free radioligand, the value plotted on the x axis (see Fig. 7.5.3). B_{max} is the total number of binding sites and is expressed in the same units as the y values (i.e., cpm, sites/cell, or fmol/mg protein). K_d is the equilibrium dissociation constant (expressed in the same units as [L], usually nM). Figure 7.5.3 shows the total binding, specific binding, and nonspecific binding for a hypothetical experiment.

Total binding

Total binding is the sum of specific and nonspecific binding.

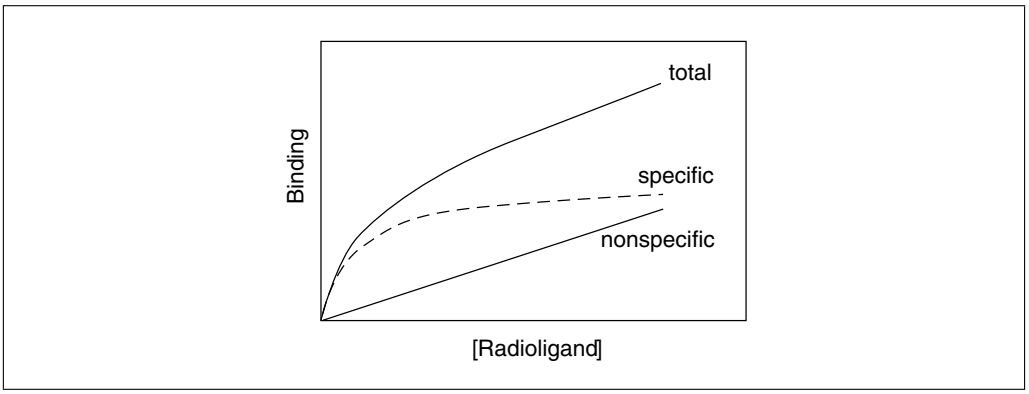

Figure 7.5.3 Total binding, specific binding, and nonspecific binding for a saturation binding experiment.

Analysis of Saturation Binding Curves

Before fitting the data to a model, transform the data to convenient units. Convert the total and nonspecific binding data from counts per minute to more useful units such as fmol/mg protein or sites per cell.

Fitting the data to determine B_{max} and K_d can use three different strategies as follows.

Strategy 1: Fit specific binding only

Before fitting a model with nonlinear regression, calculate specific binding at each concentration of ligand. If you have measured nonspecific binding at each concentration of ligand, simply subtract nonspecific from total. If you are willing to accept the assumption that nonspecific binding is linear with radioligand concentration, use linear regression to find the best-fit line through the nonspecific binding data. Specific binding is calculated by subtracting the nonspecific binding predicted by that line from the total binding measured at each concentration of radioligand. With this approach, it is only necessary to experimentally measure nonspecific binding experimentally at a few concentrations of radioligand.

Fit the data to this equation:

$$y = B_{max} \times [L] / (K_d + [L])$$

Equation 7.5.7

If the curve-fitting program does not provide initial values (sometimes called estimated values) automatically, estimate B_{max} as the largest value of y and estimate K_d as 0.2 times the largest value of [L].

Strategy 2. Fit total binding only

For systems where nonspecific binding is linear with ligand concentration, one can fit total binding only. Nonspecific binding is imputed from the shape of the total binding curve, but not actually measured experimentally. Use this model, where NS is the slope of the nonspecific binding line:

$$\text{total binding} = \text{specific binding} + \text{nonspecific binding} = \frac{B_{max} \times [L]}{K_d + [L]} + NS \times [L]$$

Equation 7.5.8

The strategy of fitting total binding only is also used when a high fraction of ligand binds to receptors, so one cannot assume that the free concentration of ligand equals the concentration of ligand added. In this case, it is necessary that [L] and y both be expressed in the same concentration units. Then, fit to the model derived by Swillens (1995).

Strategy 3. Globally fit total and nonspecific binding

Assuming that nonspecific binding is linear with radioligand concentration, the best way to analyze saturation binding data is to globally fit total and nonspecific binding at once. Fit total binding to the same model used in Strategy 2, above.

Fit nonspecific binding using the model: $y = \text{NS} \times [\text{L}]$, where NS is the slope of the nonspecific binding line.

Fit the two data sets globally (simultaneously), sharing the value of NS between the two data sets, so there is only one best-fit value for that parameter.

This approach takes full advantage of all the information in your data, and gives the most accurate values of B_{max} and K_d.

Are the results reasonable?

Before accepting the results of the curve fit, ask the questions listed in Table 7.5.1 to determine whether the results are reasonable.

Table 7.5.1 Evaluating the Results of Saturation Binding Curve Analysis

Question	Comment
Does the calculated curve go near the data points?	If the curve doesn't go near the data, then something went wrong with the curve fit, and the "best-fit" values of B_{max} and K_d should be ignored.
Were sufficient concentrations of radioligand used?	Ideally, the highest concentration should be at least 10 times the K_d. Calculate the ratio of the highest radioligand concentration used divided by the K_d reported by the program (both in nM or pM). The ratio should be greater than 10.
Is the B_{max} reasonable?	Typical values for B_{max} are 10 to 1000 fmol binding sites per milligram of membrane protein, 1000 sites per cell, or 1 receptor per square micron of membrane. If using cells transfected with receptor genes, then the B_{max} may be 10 to 100 times larger than these values.
Is the K_d reasonable?	Typical values for K_d of useful radioligands range between 10 pM and 100 nM. If the K_d is much lower than 10 pM, the dissociation rate is probably very slow and it will be difficult to achieve equilibrium. If the K_d is much higher than 100 nM, the dissociation rate will probably be fast, and may result in the loss of binding sites during separation of bound from free radioligand.
Are the standard errors too large? Are the confidence intervals too wide?	Nonlinear regression programs report the uncertainty of the best-fit values for B_{max} and K_d as standard errors and 95% confidence intervals. Divide the SE of the B_{max} by the B_{max}, and divide the SE of the K_d by the K_d. If either ratio is much larger than ~20%, look further to determine why.
Is the nonspecific binding too high?	Divide the nonspecific binding at the highest concentration of radioligand by the total binding at that concentration. Nonspecific binding should usually be less than 50% of the total binding.

Table 7.5.2 Evaluating the Assumptions of Saturation Binding Analysis

Assumption	Comment
Binding has reached equilibrium.	It takes longest for the lower concentrations to equilibrate, so test equilibration time with the lowest concentration of radioligand.
There is only one population of receptors.	See Theory: Comparing One- and Two-Site Models, below.
Only a small fraction of the radioligand binds, therefore the free concentration is essentially identical to the concentration added.	Compare the cpm obtained for total binding to the amount of ligand. If the ratio is greater than 10% at any concentration, this assumption has been violated. Increase the volume of the reaction but use the same amount of tissue.
There is no cooperativity. Binding of a ligand to one binding site does not alter the affinity of another binding site.	See Cooperativity, below.

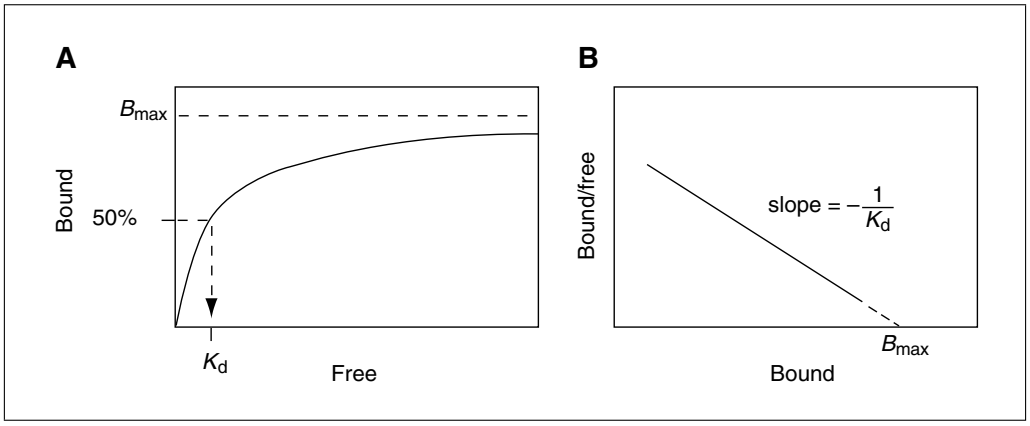

Figure 7.5.4 Displaying results as a Scatchard plot. (**A**) Specific binding as a function of free radioligand. (**B**) Transformation of Scatchard data to a plot.

If the results are not reasonable, the experimental protocol may need revision. Also check that the data are being analyzed correctly. In addition, it is possible that the system is more complex than the simple one-site binding model. To determine whether the system follows the assumptions of the simple model, consider the points in Table 7.5.2.

Displaying results as a Scatchard plot

Before nonlinear regression programs were widely available, scientists transformed data to make a linear graph and then analyzed the transformed data with linear regression. There are several ways to linearize binding data, but Scatchard plots (more accurately attributed to Rosenthal, 1967) are used most often. As shown in Figure 7.5.4, the *x* axis of the Scatchard plot represents specific binding (usually labeled "bound") and the *y* axis is the ratio of specific binding to concentration of free radioligand (usually labeled "bound/free"). B_{max} is the *x* intercept; K_d is the negative reciprocal of the slope.

When making a Scatchard plot, there are two ways to express the *y* axis. One choice is to express both free ligand and specific binding in cpm so the ratio bound/free is a unitless fraction. The advantage of this choice is that you can interpret *y* values as the fraction of radioligand bound to receptors. If the highest *y* value is large (>0.10), then the free

concentration of radioligand will be substantially less than the added concentration, and (as discussed earlier) the standard analyses will yield inaccurate values for B_{max} and K_d.

An alternative is to express the *y* axis as the ratio of units used to display bound and free on the saturation binding graph (i.e., sites/cell/nM or fmol/mg/nM). While these values are hard to interpret, they simplify calculation of the K_d, which equals the negative reciprocal of the slope. The specific binding units cancel when calculating the slope. The negative reciprocal of the slope is expressed in units of concentration (nM), which equals the K_d.

The problem with using Scatchard plots to analyze saturation binding experiments

While Scatchard plots are very useful for visualizing data, they are not the most accurate way to analyze data. The problem is that the linear transformation distorts the experimental error. Linear regression assumes that the scatter of points around the line follows a Gaussian distribution and that the standard deviation is the same at every value of *x*. These assumptions are not true with the transformed data. A second problem is that the Scatchard transformation alters the relationship between *x* and *y*. The value of *x* (bound) is used to calculate *y* (bound/free), and this violates the assumptions of linear regression.

Since these assumptions are violated, the B_{max} and K_d values determined by linear regression of Scatchard-transformed data are likely to be far from the actual values than the B_{max} and K_d determined by nonlinear regression. Nonlinear regression produces the most accurate results, whereas a Scatchard plot produces only approximate results.

Figure 7.5.5 illustrates the problem of transforming data. The left panel shows data that follow a rectangular hyperbola (binding isotherm). The solid curve was determined by nonlinear regression. The right panel is a Scatchard plot of the same data. The solid line shows how that same curve would look after a Scatchard transformation. The dotted line shows the linear regression fit of the transformed data. The transformation amplified and distorted the scatter, and thus the linear regression fit does not yield the most accurate values for B_{max} and K_d. In this example, the B_{max} determined by the Scatchard plot is ~25% too large and the K_d determined by the Scatchard plot is too high. The errors could just as easily have gone in the other direction.

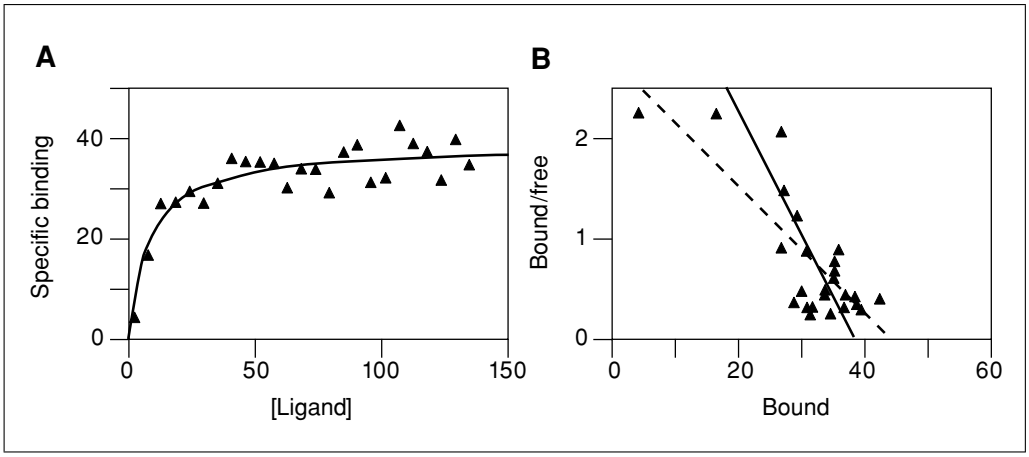

Figure 7.5.5 Why Scatchard plots (though useful for displaying data) should not be used for analyzing data. (**A**) Experimental data with best-fit curve determined by nonlinear regression. (**B**) Scatchard plot of the data. The solid line corresponds to the B_{max} and K_d determined by nonlinear regression in panel A. The dashed line was determined by linear regression of transformed data in panel B. The results of linear regression of the Scatchard plot are not the most accurate values for B_{max} (*x* intercept) or K_d (negative reciprocal of the slope).

The experiment in Figure 7.5.5 was designed to determine the B_{max} with little concern for the value of K_d. Therefore, it was appropriate to obtain only a few data points at the beginning of the curve and many in the plateau region. Note, however, how the Scatchard transformation gives undue weight to the data point collected at the lowest concentration of radioligand (the lower left point in panel A, the upper left point in panel B). This point dominates the linear regression calculations on the Scatchard graph. It has "pulled" the regression line to become shallower, resulting in an overestimate of the B_{max}.

Again, although it is inappropriate to analyze data by performing linear regression on a Scatchard plot, it is often helpful to display data as a Scatchard plot. Many people find it easier to visually interpret Scatchard plots than binding curves, especially when comparing results from different experimental treatments or trying to detect complex binding behavior.

Example of a Saturation Binding Experiment

Raw data

Figure 7.5.6 shows duplicate values for total binding of six concentrations of a radioligand to angiotensin receptors on membranes of cells transfected with an angiotensin receptor gene (R. Neubig, unpub. observ.). The figure also shows nonspecific binding (assessed with 10 μM unlabeled angiotensin II) at three concentrations of radioligand.

Converting units

Convert from cpm to fmol/mg using the amount of protein in each tube (0.01 mg), the efficiency of the counting (90%), and the specific radioactivity of the ligand (2190 Ci/mmol).

$$\text{fmol/mg} = \frac{\text{cpm}}{2.22\times10^{12}\ \text{dpm/Ci} \times 0.90\ \text{cpm/dpm} \times 2190\ \text{Ci/mmol} \times 10^{-12}\ \text{mmol/fmol} \times 0.01\ \text{mg}}$$

Equation 7.5.9

For this example, the equation simplifies by dividing the cpm by 43.756 (see Table 7.5.3).

Notes to help understand the equation:

1. Receptors in membrane preparations are often expressed as fmol of receptor per milligram of membrane protein. One fmol is 10^{-15} mol.

[Radioligand] (nM)	Total binding (cpm)		Nonspecific binding (cpm)	
0.125	818	826		
0.25	1856	1727	94	88
0.5	3452	3349		
1.0	6681	6055	354	375
2.0	10077	9333		
4.0	13715	13277	1573	1525

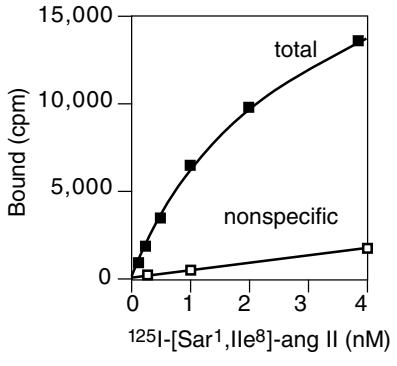

Figure 7.5.6 Sample saturation binding experiment. The ligand binding to angiotensin receptors in a membrane preparation was measured. Total and nonspecific binding are shown.

Table 7.5.3 Calculating Specific Binding

[Radio-ligand] (nM)	Total binding (cpm)		Computed nonspecific binding (cpm)	Calculated specific binding (cpm)		Specific binding (fmol/mg)	
	Duplicate 1	Duplicate 2		Duplicate 1	Duplicate 2	Duplicate 1	Duplicate 2
0.125	818	826	34	784	792	17.9	18.1
0.25	1856	1727	82	1774	1645	40.5	37.6
0.5	3452	3349	180	3272	3169	74.8	72.4
1.0	6681	6055	375	6306	5680	144.1	129.8
2.0	10,077	9333	766	9311	8567	212.8	195.8
4.0	13,715	13,277	1547	12,168	11,730	278.1	268.1

2. Counting efficiency is the fraction of the radioactive disintegrations that are detected by the counter. This example uses a radioligand labeled with ^{125}I, so the efficiency (90%) is very high.
3. The Curie (Ci) is a unit of radioactivity and equals 2.22×10^{12} radioactive disintegrations per minute.
4. The value 2190 Ci/mmol is worth remembering. It is the specific activity of ligands iodinated with ^{125}I, when every molecule is labeled with one atom of iodine.

Calculate and fit specific binding

If you want to use Strategy 1 (fit specific binding), first compute specific binding. Since nonspecific binding was only determined at three concentrations of radioligand, the standard method of subtracting each nonspecific value from the corresponding total value cannot be used. Instead, the fact (confirmed in other experiments) that nonspecific binding is proportional to radioligand concentration is relied upon, and the best-fit value of nonspecific binding is subtracted from each total binding value. This can be done in one step by choosing "remove baseline analysis" in GraphPad Prism software (see below). Alternatively:

1. Use linear regression. The best fit line through the nonspecific binding data is:

$$\text{nonspecific binding in cpm} = -15.25 + 390.5\left(\left[\text{radioligand}\right] \text{ in nM}\right)$$

Equation 7.5.10

2. Use this equation to calculate nonspecific binding at each of the six radioligand concentrations.
3. Subtract that calculated value from the observed total binding to compute specific binding (Table 7.5.3).

Fitting with nonlinear regression

When fitting the example data to a curve, one must decide whether to enter the data as six points or twelve. Entering each replicate individually is better, as it provides more data to the curve fitting procedure, and helps you spot any outliers. This should be avoided only when the replicates are not independent (i.e., when experimental error in

Table 7.5.4 Fitted Parameters Determined from Data in Table 7.5.3

Strategy	B_{max} (fmol/mg)	95% CI	K_d (nM)	95% CI
1. Specific	430.7	388.4 to 473.0	2.265	1.821 to 2.709
2. Total only	464.6	−256.8 to 1845	3.812	−0.1953 to 7.819
3. Global	433.2	394.3 to 472.1	2.289	1.922 to 2.656

one value is likely to affect the other value as well). In this case, each replicate was determined in a separate tube poured over a separate filter, and all the data were obtained from one membrane preparation. Except for errors in preparing the radioligand dilutions, experimental errors will affect each value independently.

If you are using GraphPad Prism (*http://www.graphpad.com*) or some other program that understands the concept of duplicates, then enter the data with radioligand concentration as six *x* values and the duplicate values of specific binding at each concentration. If the program does not understand how to deal with duplicates, enter each concentration value twice in the *x* column, to fill twelve rows. Enter the specific binding data as a column of twelve *y* values.

If the chosen nonlinear regression program does not provide initial values automatically, estimate values for B_{max} and K_d. For B_{max}, enter a value a bit higher than the highest value in the data, perhaps 300 fmol/mg for this example. For K_d, estimate the concentration of radioligand that binds to half the sites, perhaps 2 nM. These estimated values do not have to be very accurate. Results are shown in Table 7.5.4.

For this example, Strategy 2 (fit total binding only) does not work well. The 95% confidence intervals for both B_{max} and K_d are very wide, and even include negative values. With so few data points, it is impossible to reliably determine the B_{max} and K_d from analyzing only total binding data. With more data points, including some at higher concentrations, this strategy would be more useful.

For this example, the results of Strategy 1 (compute, then fit, specific binding) are almost the same as those of Strategy 3 (globally fit total and nonspecific binding). Strategy 1 requires more work from you, as you must compute the specific binding. Strategy 3 requires that you learn how to use a program that can do global fitting, but then makes analysis much quicker.

Figure 7.5.7 shows the best-fit curves from strategy I (left panel) and Strategy 3 (right panel).

Scatchard plot

As described above, a Scatchard plot is a graph of specific binding versus the ratio of specific binding to free radioligand. For specific binding, the two replicates are averaged (individual replicates could have been shown). For the example in Figure 7.5.8, bound/free is expressed as fmol/mg divided by nM.

Figure 7.5.8 shows the Scatchard transformation of the specific binding data. Since it is not appropriate to determine the K_d and B_{max} from linear regression of a Scatchard plot, derive the solid line on the graph from the best-fit values using nonlinear regression:

1. The *x* intercept of the Scatchard plot is B_{max}, which equals 431 by nonlinear regression, so one end of the line is at $x = 431$, $y = 0$.

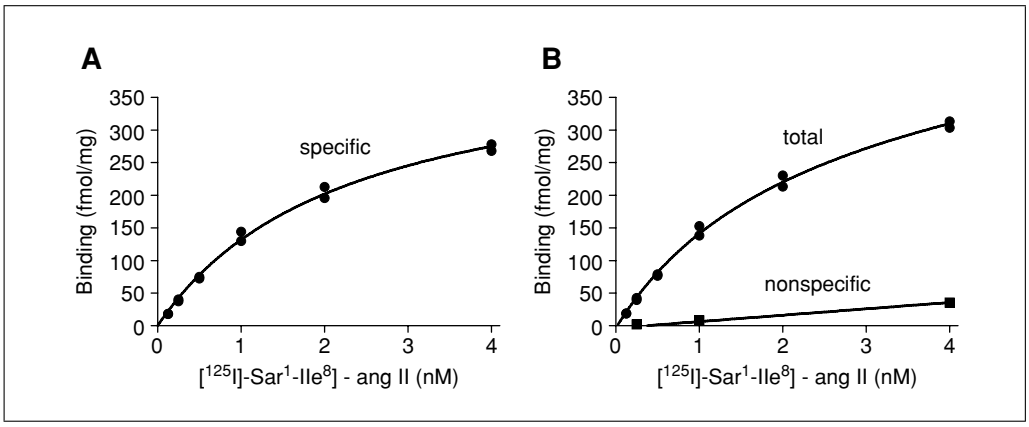

Figure 7.5.7 These data are the same as those shown in Figure 7.5.6. The left panel (**A**) fits a curve through the specific binding data (Strategy 1). The right panel (**B**) globally fits total and nonspecific binding data (Strategy 3).

Specific binding (fmol/mg protein)	Bound/free fmol/mg/nM
18	143.5
39	155.5
73	146.5
136	136.3
203	101.7
272	68.0

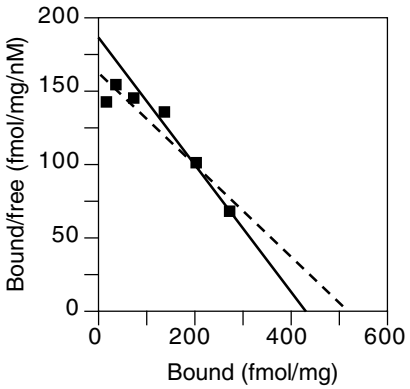

Figure 7.5.8 Scatchard transformation of the data from Figure 7.5.7. The solid line was created (as explained in the text) from the best-fit values of B_{max} and K_d determined from nonlinear regression. This is the correct line to show on a Scatchard plot. The dashed line was determined by linear regression of the Scatchard-transformed data. It is shown here for comparison only; it is not informative or helpful.

2. The slope of the line is the negative reciprocal of the K_d. Since the K_d is 2.27 nM, the slope must be $-1/2.27$, which equals -0.4405 nM^{-1}.
3. The y intercept divided by the x intercept equals the negative slope. We know the slope and the x intercept, so can derive the y intercept. It equals $-$slope $\times$ x intercept $= 0.4405 \times 431 = 189.8$.
4. Draw the line from $x = 0$, $y = 189.8$ to $x = 431$, $y = 0$, as in Figure 7.5.8.

Figure 7.5.8 also shows the dotted line derived by linear regression of the Scatchard transformed data. This is shown only to emphasize the difference between the curve derived by linear regression of the Scatchard transformed data and the best-fit line derived from nonlinear regression. The linear regression line should not be used for data analysis and does not aid data presentation.

Critiquing the experiment

This example is not an ideal experiment. Consider these points:

The highest concentration of radioligand used (4 nM) is not even twice the K_d (2.27 nM). Ideally the highest concentration of radioligand should be ten times the K_d. In addition,

the specific binding of the first few points lies below the best fit curve. There are many possible explanations for this, including chance, but it may be because the system is not at equilibrium or because a large fraction of ligand is bound (depleted) at those low concentrations. The lowest concentrations take the longest to equilibrate, so it is possible that the first few concentrations had not equilibrated, resulting in an underestimate of specific binding at equilibrium.

Two Classes of Binding Sites

If the radioligand binds to two classes of binding sites, use this equation:

$$\text{specific binding} = \frac{B_{\max 1} \times [\text{L}]}{K_{\text{d}1} + [\text{L}]} + \frac{B_{\max 2} \times [\text{L}]}{K_{\text{d}2} + [\text{L}]}$$

Equation 7.5.11

This equation assumes that the radioligand binds to two independent noninteracting binding sites, and that the binding to each site follows the law of mass action. A comparison of the one-site and two-site fits will be addressed later in this unit (see Theory: Comparing One- and Two-Site Models, below).

Meaningful results will be obtained from a two-site fit only if you have ten or more data points spaced over a wide range of radioligand concentrations. Binding should be measured at radioligand concentrations below the high-affinity K_{d} and above the low-affinity K_{d}.

Homologous Competitive Binding Curves

Some investigators determine the K_{d} and $B_{\max}$ of a ligand by holding the concentration of the radioligand constant and competing with various concentrations of the unlabeled ligand. This approach will be discussed below.

COMPETITIVE BINDING EXPERIMENTS

Theory of Competitive Binding

Using competitive binding curves

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations (often twelve to sixteen) of unlabeled ligand. Competitive binding experiments are used to:

1. Pharmacologically identify a binding site. Perform competitive binding experiments with a series of drugs whose potencies at potential receptors of interest are known from functional experiments. Demonstrating that these drugs bind with the expected potencies, or at least the expected order of potency, helps prove that the radioligand has identified the correct receptor. This kind of experiment is crucial, because there is usually no point studying a binding site unless it has physiological significance.
2. Determine whether a drug binds to the receptor. Thousands of compounds can be screened to find drugs that bind to the receptor. This can be faster and easier than other screening methods.
3. Investigate the interaction of low-affinity drugs with receptors. Binding assays are usually only useful when the radioligand has a high affinity (K_{d} <100 nM or so). A radioligand with low affinity generally has a fast dissociation rate constant, and so will not stay bound to the receptor while washing the filters. To study the binding of a low-affinity drug, use it as an unlabeled competitor.

4. Determine receptor number and affinity by using the same compound as the labeled and unlabeled ligand (see Homologous Competitive Binding Curves, below).

Performing the experiment

Competitive binding experiments use a single concentration of radioligand and require incubation until equilibrium is reached. That raises two questions: how much radioligand should be used, and how long does it take to equilibrate?

There is no clear answer to the first question. Higher concentrations of radioligand result in higher counts and thus lower counting error, but these experiments are more expensive and have higher nonspecific binding. Lower concentrations save money and reduce nonspecific binding, but result in fewer counts from specific binding and thus more counting error. Many investigators choose a concentration approximately equal to the K_d of the radioligand for binding to the receptor, but this is not universal. In general, you should aim for a minimum of 1000 cpm from specific binding in the absence of competitor.

Many investigators' first thoughts are that binding will reach equilibration in the time it takes the radioligand to reach equilibrium in the absence of competitor. It turns out that this may not be long enough. Incubations should last four to five times the half-life for receptor dissociation as determined in a dissociation experiment.

Equations for competitive binding

Competitive binding curves are described by this equation:

$$\text{total radioligand binding} = \text{NS} + \frac{(\text{total} - \text{NS})}{1+10^{\log x - \log(\text{IC}_{50})}}$$

Equation 7.5.12

The x axis of Figure 7.5.9 shows varying concentrations of unlabeled drug (x in Equation 7.5.12) on a log scale. The y axis can be expressed as cpm or converted to more useful units like fmol bound per milligram protein or number of binding sites per cell. Some investigators like to normalize the data from 100% (no competitor) to 0% (nonspecific binding at maximal concentrations of competitor).

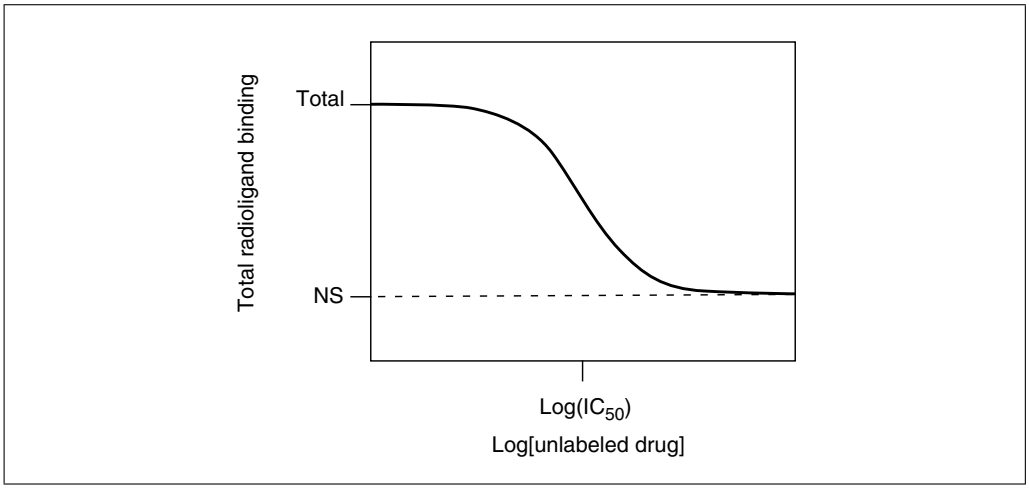

Figure 7.5.9 Schematic of a competitive binding experiment.

The top of the curve shows a plateau at the amount of radioligand bound in the absence of the competing unlabeled drug. This equals the parameter *total* in the equation. The bottom of the curve is a plateau equal to nonspecific binding; this is *nonspecific* (NS) in the equation. These values are expressed in the units of the *y* axis. The difference between the top and bottom plateaus is the specific binding. Note that this not the same as B_{max}. When using a low concentration of radioligand (to save money and avoid nonspecific binding), only a fraction of receptors will be bound (even in the absence of competitor), so specific binding will be lower than the B_{max}.

The concentration of unlabeled drug that results in radioligand binding halfway between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%), also called the EC_{50} (effective concentration 50%). The IC_{50} is the concentration of unlabeled drug that blocks half the specific binding, and it is determined by three factors:

1. The K_i of the receptor for the competing drug. This is what is to be determined. It is the equilibrium dissociation constant for binding of the unlabeled drug—the concentration of the unlabeled drug that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors. The K_i is proportional to the IC_{50}. If the K_i is low (i.e., the affinity is high), the IC_{50} will also be low.
2. The concentration of the radioligand. If a higher concentration of radioligand is used, it will take a larger concentration of unlabeled drug to compete for the binding. Therefore, increasing the concentration of radioligand will increase the IC_{50} without changing the K_i.
3. The affinity of the radioligand for the receptor (K_d). It takes more unlabeled drug to compete for a tightly bound radioligand (small K_d) than for a loosely bound radioligand (high K_d). Using a radioligand with a smaller K_d (higher affinity) will increase the IC_{50}.

Calculate the K_i from the IC_{50}, using the equation of Cheng and Prusoff (1973).

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{radioligand}]}{K_d}}$$

Equation 7.5.13

Remember that K_i is a property of the receptor and unlabeled drug, while IC_{50} is a property of the experiment. By changing experimental conditions (changing the radioligand used or changing its concentration), the IC_{50} will change without affecting the K_i.

This equation is based on the following assumptions:

1. Only a small fraction of either the labeled or unlabeled ligand has bound. This means that the free concentration is virtually the same as the added concentration.
2. The receptors are homogeneous and all have the same affinity for the ligands.
3. There is no cooperativity—binding to one binding site does not alter affinity at another site.
4. The experiment has reached equilibrium.
5. Binding is reversible and follows the law of mass action.
6. The K_d of the radioligand is known from an experiment performed under similar conditions.

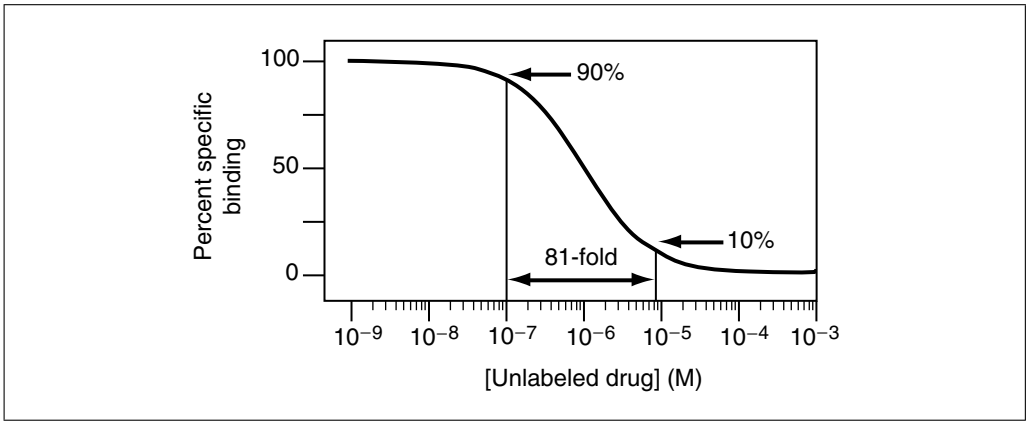

Figure 7.5.10 Steepness of a competitive binding curve. This graph shows the results at equilibrium when radioligand and competitor bind to the same binding site. The curve will descend from 90% binding to 10% binding over an 81-fold increase in competitor concentration.

If the labeled and unlabeled ligands compete for a single binding site, the steepness of the competitive binding curve is determined by the law of mass action (see Fig. 7.5.10). The curve descends from 90% specific binding to 10% specific binding with an 81-fold increase in the concentration of the unlabeled drug. More simply, nearly the entire curve will cover two log units (100-fold change in concentration).

Analyzing Competitive Binding Data

Using nonlinear regression to determine the K_i

Follow these steps to determine the K_i with nonlinear regression.

1. Enter the x values as the logarithm of the concentration of unlabeled compound, or enter the concentrations, and use the program to convert to logarithms. Since log(0) is undefined, the log scale cannot accommodate a concentration of zero. Instead enter a very low concentration. For example, if the lowest concentration of unlabeled compound is 10^{-10} M, then enter -12 for the zero concentration.
2. Enter the y values as cpm total binding. There is little advantage to converting to units such as fmol/mg or sites/cell. There is also little advantage to converting to percent specific binding.
3. Select the competitive binding equation (TOP is binding in the absence of competitor, BOTTOM is binding at maximal concentrations of competitor, $\log IC_{50}$ is the logarithm base 10 of the IC_{50}):

$$y = \mathrm{NS} + \frac{\mathrm{TOTAL} - \mathrm{NS}}{1 + 10^{x - \log \mathrm{IC}_{50}}}$$

Equation 7.5.14

4. If the chosen nonlinear regression program doesn't provide initial estimates automatically, enter these values. For NS, enter the smallest y value. For TOTAL, enter the largest y value. For $\log(IC_{50})$, enter the average of the smallest and largest x values.
5. If the data do not form clear plateaus at the top and bottom of the curve, consider fixing top or bottom to constant values. TOTAL can be fixed to the binding measured in the absence of competitor and NS to binding measured in the presence of a large concentration of a standard drug known to block radioligand binding to essentially all receptors.
6. Start the curve fitting to determine TOTAL, NS, and $\log(IC_{50})$.

7. Calculate the IC_{50} as the antilog of log(IC_{50}).

8. Calculate the K_i using this equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{radioligand}]}{K_d}}$$

Equation 7.5.15

When to set total and NS constant

In order to determine the best-fit value of IC_{50}, the nonlinear regression program must be able to determine the 100% (total) and 0% (nonspecific) plateaus. If there are data over a wide range of concentrations of unlabeled drug, the curve will have clearly defined bottom and top plateaus and the program should have no trouble fitting all three values (both plateaus and the IC_{50}).

With some experiments, the competition data may not define a clear bottom plateau. If data are fit in the usual way, the program might stop with an error message, or it might find a nonsense value for the nonspecific plateau (it might even be negative). If the bottom plateau is incorrect, the IC_{50} will also be incorrect. To solve this problem, determine the nonspecific binding from other data. All drugs that bind to the same receptor should compete for all specific radioligand binding and reach the same bottom plateau value. When running the curve-fitting program, set the bottom plateau of the curve (NS) to a constant equal to binding in the presence of a standard drug known to block all specific binding.

Similarly, if the curve doesn't have a clear top plateau, set the total binding to be a constant equal to binding in the absence of any competitor.

Fitting the K_i directly

Rather than fit the logIC_{50} and then compute the K_i, it is possible to fit the K_i directly. Simply replace the IC_{50} in the competitive binding equation, with this equation:

$$\text{Total radioligand binding} = \text{NS} + \frac{(\text{total} - \text{NS})}{1 + 10^{\log x - \log\left[K_i \cdot \left(1 + \frac{[L]}{K_d}\right)\right]}}$$

Equation 7.5.16

The parameters [L] and K_d represent the concentration of radioligand and its affinity for the receptors. These parameters must be constrained to constant values based on other experiments. The variable x represents the concentration of unlabeled drug. This approach will give exactly the same results as fitting the IC_{50} and then computing the K_i, but it is a bit more convenient to fit the K_i directly.

Interpreting the Results of Competitive Binding Curves

Are the results reasonable?

Table 7.5.5 presents some questions to consider when determining whether the results are reasonable and logical.

Do the data follow the assumptions of the analysis?

Table 7.5.6 lists the assumptions.

Table 7.5.5 Evaluating the Results of Competitive Binding Curve Analyses

Question	Comment
Is the log(IC_{50}) reasonable?	The IC_{50} should be near the middle of the curve, with at least several concentrations of unlabeled competitor on either side of it.
Are the standard errors too large? Are the confidence intervals too wide?	The SE of the log(IC_{50}) should be <0.5 log unit (ideally much less).
Are the values of TOTAL and NS reasonable?	TOTAL should be near the binding observed in the absence of competitor. NS should be near the binding observed in the presence of a maximal concentration of competitor. If the best-fit value of NS is negative, consider fixing it to a constant value equal to nonspecific binding.

Table 7.5.6 Evaluating the Assumptions of Competitive Binding Analyses

Assumption	Comment
Binding has reached equilibrium.	Competitive binding incubations take longer to incubate than saturation binding incubations. Incubate for 4 to 5 times the half life for radioligand dissociation.
There is only one population of receptors	See Theory: Comparing One- and Two-Site Models
Only a small fraction of the radioligand binds, therefore the free concentration is essentially identical to the concentration added.	Compare the total binding in the absence of competitor in cpm, to the amount of ligand added in cpm. If the ratio is >10% at any concentration, then you've violated this assumption.
There is no cooperativity. Binding of a ligand to one binding site does not alter the affinity of another binding site.	See Cooperativity.

Why determine log(IC_{50}) rather than IC_{50}?

The equation for a competitive binding curve (Equation 7.5.12) looks a bit strange since it combines logarithms and antilogarithms (10 to the power). A bit of algebra simplifies it:

$$y = \text{nonspecific} + \frac{(\text{total} - \text{nonspecific})}{1 + \frac{[\text{drug}]}{IC_{50}}}$$

Equation 7.5.17

Fitting data to this equation results in the same best-fit curve and the same IC_{50}. However, the confidence interval for the IC_{50} will be different.

Which confidence interval is correct? With nonlinear regression, the standard errors of the parameters are only approximately correct. Since the confidence intervals are calculated from the standard errors, they too are only approximately correct. The problem is that the real confidence interval may not be symmetrical around the best-fit value. It may extend further in one direction than the other. However, nonlinear regression programs always calculate symmetrical confidence intervals (unless you use advanced techniques).

Log [competitor](M)	Binding (cpm) in triplicate		
–12.0	4549	4380	4554
–10.0	4604	4803	4213
–9.5	4353	4278	4508
–9.0	4192	4156	3972
–8.5	4053	4420	4191
–8.0	3453	3018	3024
–7.5	2587	2946	2367
–7.0	1295	1405	1402
–6.5	886	880	888
–6.0	591	612	603
–5.5	580	559	555
–5.0	521	545	555

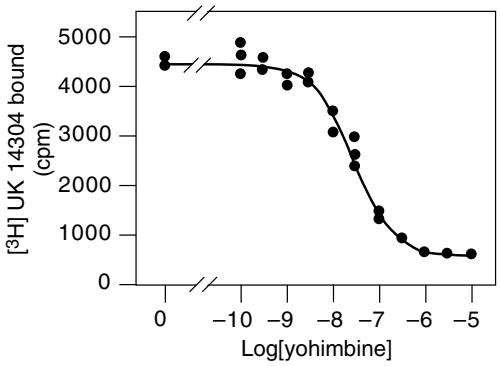

Figure 7.5.11 Example of a competitive binding experiment. Yohimbine competes for radioligand binding to α_2 receptors on membranes.

Therefore, when writing the equation for nonlinear regression, choose parameters so the uncertainty is as symmetrical as possible. Because data are collected at concentrations of unlabeled drug equally spaced on a log axis, the uncertainty is symmetrical when the equation is written in terms of the log(IC_{50}), but is not symmetrical when written in terms of IC_{50}. Thus, confidence intervals are more accurate when the equation is written in terms of the log(IC_{50}).

Figure 7.5.11 (R. Neubig, unpub. observ.) shows competition of unlabeled yohimbine for labeled UK14341 (an α_2 adrenergic agonist).

1. Enter the data into a nonlinear regression program. Enter the logarithm of concentration of the unlabeled ligand in the *x* column, and the triplicate values of total binding in the *x* columns. If the selected program does not allow entry of triplicate values, enter each log of concentration three times.
2. Fit the data to a one-site competitive binding curve. If necessary, enter it in this format:

$$y = \mathrm{NS} + \left[\mathrm{Total} - \frac{\mathrm{NS}}{1 + 10^{\left(x - \log \mathrm{IC}_{50}\right)}} \right]$$

Equation 7.5.18

3. If the nonlinear regression program does not provide initial values automatically, estimate the values of the parameters. TOTAL is the top plateau of the curve, so estimate its value from the highest data values, perhaps 4500. NS is the bottom plateau, so estimate its value from the lowest data values, perhaps 500. Log(IC_{50}) is the *x* value in the middle of the curve. From looking at the data, estimate its value as −7. None of these estimates has to be very accurate, and the nonlinear regression will probably work fine even if the estimates are fairly different than the values listed here.
4. Note the best-fit results: NS = 530.3, TOTAL = 4418, and log(IC_{50}) = −7.532.
5. Convert the log(IC_{50}) to the IC_{50} by taking the antilog. IC_{50} = 29.4 nM.

6. Convert the IC_{50} to K_i. To do this, the concentration of radioligand used (2.0 nM) and its K_d for the receptors (0.88 nM, determined in a separate saturation binding experiment not shown here) must be known:

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{radioligand}]}{K_d}} = \frac{29.4\ \text{nM}}{1 + \frac{2.0\ \text{nM}}{0.88\ \text{nM}}} = 8.98\ \text{nM}$$

Equation 7.5.19

Homologous Competitive Binding Curves

A competitive binding experiment is termed *homologous* when the same compound is used as the hot and cold ligand. The term *heterologous* is used when the hot and cold ligands differ. Homologous competitive binding experiments can be used to determine the affinity of a ligand for the receptor and the receptor number. In other words, the experiment has the same goals as a saturation binding curve. Because homologous competitive binding experiments use only one or two concentrations of radioligand (which can be low), they consume less radioligand and thus are more practical than saturation experiments when radioligands are expensive or difficult to synthesize.

Analyses of homologous competitive binding curves depend on the following assumptions:

1. The receptor has identical affinity for the labeled and unlabeled ligand. If you choose an iodinated radioligand, you should also use an iodinated unlabeled compound (using nonradioactive iodine), because iodination often changes the binding properties of ligands.
2. There is no cooperativity.
3. There is no ligand depletion. The methods in this section assume that only a small fraction of ligand binds. In other words, the method assumes that free concentration equals the concentration added.
4. There is only one class of binding sites. It is difficult to detect a second class of binding sites unless the number of lower-affinity sites vastly exceeds the number of higher-affinity receptors (because the single low concentration of radioligand used in the experiment will bind to only a small fraction of low affinity receptors).

Homologous competition follows this model, where B_{max} is the total number of binding sites (in the same units as y), H is the concentration of radioligand (hot), C is the concentration of unlabeled (cold) ligand, K_d is the dissociation constant you are trying to determine, and NS $\times$ H is the amount of nonspecific binding in the same units as y. H, C, and K_d must all be expressed in the same concentration units. Note that B_{max} is the total number of binding sites, which exceeds the number bound by radioligand in this experiment.

$$y = \frac{B_{max} - H}{H + C + K_d} + \text{NS} \cdot H$$

Equation 7.5.20

To get reliable data, it is best to use two different concentrations of radioligand, and fit the two curves globally—sharing the values of B_{max}, K_d, and NS so that there is only one

log(unlabeled)	5	2
-12.0	10,101	6520
-10.0	10,254	6008
-9.0	9857	5014
-8.5	7450	3214
-8.0	4512	2215
-7.0	2254	1105
-6.0	2195	989

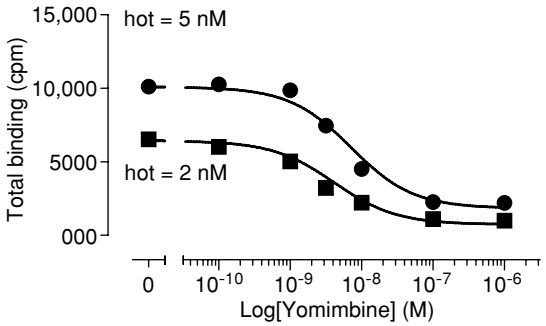

Figure 7.5.12 Example of homologous competitive binding experiment. The hot and cold ligands are identical.

best-fit value for the entire experiment. Note that the amount of nonspecific binding is the product of the concentration of hot ligand, *H*, and the parameter NS. That product is different for each concentration of ligand, even though NS is shared:

$$B_{\max} = \frac{\text{TOP} - \text{BOTTOM}}{\text{fractional occupancy}} = \frac{\text{TOP} - \text{BOTTOM}}{\dfrac{[\text{radioligand}]}{\left(K_{\text{d}} + [\text{radioligand}]\right)}}$$

Equation 7.5.21

Example of homologous competitive binding

Figure 7.5.12 shows data from a binding experiment using [^{3}H]yohimbine to quantify α_2 adrenergic receptors to compete with unlabeled yohimbine. There is no reason to think that adding a tritium label will alter yohimbine's affinity for the receptor, so it seems safe to assume that hot and cold yohimbine bind with the same affinity.

1. Enter the data into a nonlinear regression program. Enter the logarithm of concentration as *x* and cpm bound as *y*. The first point represents a control with no yohimbine. Since the log of zero is undefined, this cannot be shown on a log scale. Instead enter this value as −12 (the exact value is a bit arbitrary but any value much smaller than the K_d will work.).
2. Fit the data using global nonlinear regression to this equation, sharing all three parameters and setting HotnM to a different constant value for each data set.

```
ColdNM=10^ (x+9); Cold concentration in nM
KdNM=10^ (logKD+9); Kd in nM
Bottom=NS*HotnM
Y=(Bmax*HotnM)/(HotnM + ColdNM + KdNM) + Bottom
```

The first line converts the *x* values in log(molar) to concentrations in nM.

The next line converts the log of K_{d} (which the program will fit) to the K_{d} in nM.

The third line calculates the bottom plateau of the curve.

The final line matches Equation 7.5.20.

If you don't have access to a program that can do global nonlinear regression, fit each data set individually.

Table 7.5.7 Fitted Parameters Determined from Data in Figure 7.5.12

Parameter	Global fit		Only fit 5 nM data		Only fit 2 nM data	
	Best fit	95% CI	Best fit	95% CI	Best fit	95% CI
B_{max} (cpm)	11753	8812 to 14734			6820	4353 to 9286
Log K_d	−8.67	−8.93 to −8.41	Did not converge		−9.28	−10.02 to −8.545
K_d (nM)	2.14	1.19 to 3.87			0.54	0.009 to 2.84

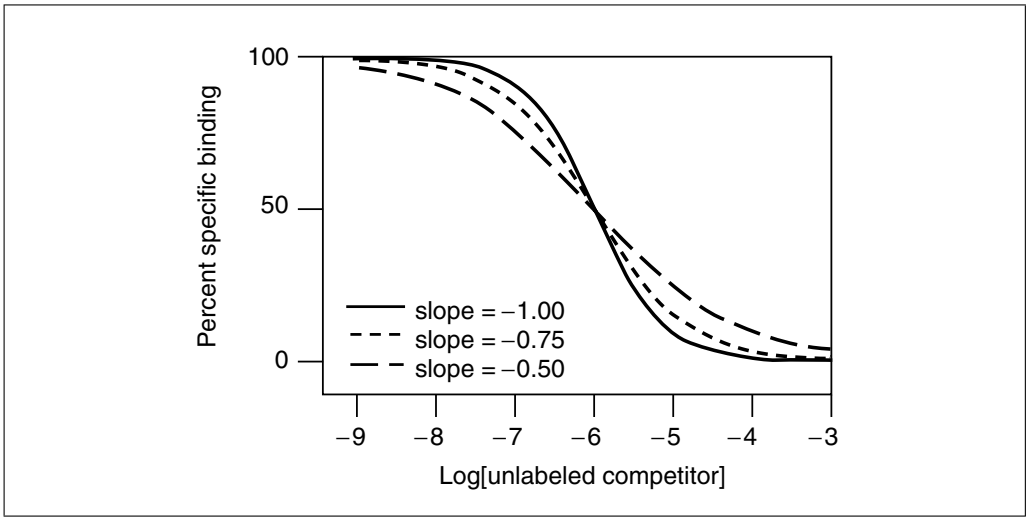

Figure 7.5.13 Examples of slope factors. The slope factor quantifies the steepness of the curve, and is determined by nonlinear regression of competitive binding data. It is not the same as the slope of the curves at the midpoints.

3. The best-fit results are shown in Table 7.5.7.

   It was not possible to fit the 5 nM data. The fit simply did not converge. The data do not define unique values for the B_{max} and K_d. It was possible to fit the 2 nM data, but the K_d was poorly determined with wide confidence interval. The global fit worked much better, giving useful results.

4. Finally convert B_{max} to more useful units. In this example there were 6×10^4 cells per well, the specific activity of the [^{3}H]yohimbine was 78 Ci/mmol, and the scintillation counting efficiency was 33%. Calculate receptors/cell using the equation:

$$\frac{11{,}753\ \text{cpm} \times 6.02 \times 10^{20}\ \text{receptors/mmol}}{2.22 \times 10^{12}\ \text{dpm/Ci} \times 0.33\text{cpm/dpm} \times 78\text{Ci/mmol} \times 60{,}000\ \text{cells}} = 2.06 \times 10^6\ \text{receptors/cell}$$

Equation 7.5.22

The Slope Factor or Hill Slope

Many competitive binding curves are shallower than predicted by the law of mass action for binding to a single site. The steepness of a binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of −1.0. If the curve is shallower, the slope factor will be a negative fraction (i.e., −0.85 or −0.60; see Fig. 7.5.13). The slope factor is negative because the curve goes downhill.

To quantify the steepness of a competitive binding curve (or a dose-response curve), fit the data to this equation:

$$\text{total radioligand binding} = \text{NS} + \frac{(\text{total} - \text{NS})}{1 + 10^{(\log \text{IC}_{50} - \log x) \times \text{slope factor}}}$$

Equation 7.5.23

The slope factor is a number that describes the steepness of the curve. In most situations, there is no way to interpret the value in terms of chemistry or biology. If the slope factor differs significantly from −1.0, then the binding does not follow the law of mass action with a single site.

Explanations for shallow binding curves include:

1. *Heterogeneous receptors*. Not all receptors bind the unlabeled drug with the same affinity. This can be due to the presence of different receptor subtypes, or due to heterogeneity in receptor coupling to other molecules such as G proteins. In Fig. 7.5.13, the slope factor equals −0.78.
2. *Negative cooperativity.* Binding sites are clustered (perhaps several binding sites per molecule) and binding of the unlabeled ligand to one site causes the remaining site(s) to bind the unlabeled ligand with lower affinity.
3. *Curve fitting problems*. If the top and bottom plateaus are not correct, then the slope factor is not meaningful. Don't try to interpret the slope factor unless the curve has clear top and bottom plateaus.

KINETIC BINDING EXPERIMENTS

Dissociation Experiments

A dissociation binding experiment measures the "off rate" of radioligand dissociating from the receptor. Perform dissociation experiments to fully characterize the interaction of ligand and receptor and confirm that the law of mass action applies. Such experiments may also be used to help design the experimental protocol. If the dissociation is fast, filter and wash the samples quickly so that negligible dissociation occurs. Lowering the temperature of the buffer used to wash the filters, or switching to a centrifugation or dialysis assay, may also be required. If the dissociation is slow, then the samples can be filtered at a more leisurely pace, because the dissociation will be negligible during the wash.

To perform a dissociation experiment, first allow ligand and receptor to bind, perhaps to equilibrium. At that point, block further binding of radioligand to receptor using one of these methods:

1. If the tissue is attached to a surface, remove the buffer containing radioligand and replace with fresh buffer without radioligand.
2. Centrifuge the suspension, decant supernatant, and resuspend pellet in fresh buffer.
3. Add a very high concentration of an unlabeled ligand (perhaps 100 times its K_i for that receptor). It will instantly bind to nearly all the unoccupied receptors and block binding of the radioligand.
4. Dilute the incubation by a large factor, perhaps a 20- to 100-fold dilution. This will reduce the concentration of radioligand by that factor. At such a low concentration, new binding of radioligand will be negligible. This method is only practical when

using a fairly low radioligand concentration so its concentration after dilution is far below its K_d for binding.

After initiating dissociation, measure binding over time (typically 10 to 20 measurements) to determine how rapidly the ligand dissociates from the receptors.

Using nonlinear regression to determine k_{off}

1. Enter the *x* data as time in minutes.
2. Enter the *y* data as total binding in cpm.
3. Choose the exponential dissociation equation. Total binding and nonspecific binding (NS) are expressed in cpm, fmol/mg protein, or sites/cell. Time (*t*) is usually expressed in minutes. The dissociation rate constant (k_{off}) is expressed in units of inverse time, usually min^{-1}:

$$\text{total binding} = \text{NS} + (\text{total} - \text{NS}) \times e^{-k_{\text{off}} t}$$

Equation 7.5.24

4. If the chosen nonlinear regression program does not provide initial estimates of the parameters, enter these values. Total is the total binding at time zero and is estimated as the first *y* value. NS is the binding after a long time, and reflects nonspecific binding. Estimate it as the last *y* value. *K* is the dissociation rate constant (k_{off}). Estimate it by dividing 0.69 by an estimate of the half-time of dissociation.
5. Start the nonlinear regression procedure.
6. Calculate the half-life of dissociation from the rate constant.

$$\text{half-life} = \ln(2)/k_{\text{off}} = 0.693/K$$

Equation 7.5.25

In one half-life, half the radioligand will have dissociated (see Fig. 7.5.14). In two half-lives, three quarters of the radioligand will have dissociated, etc.

Typically the dissociation rate constant of useful radioligands is between 0.001 and 0.1 min^{-1}. If the dissociation rate constant is any faster, it will be difficult to perform radioligand binding experiments, as the radioligand will dissociate from the receptors

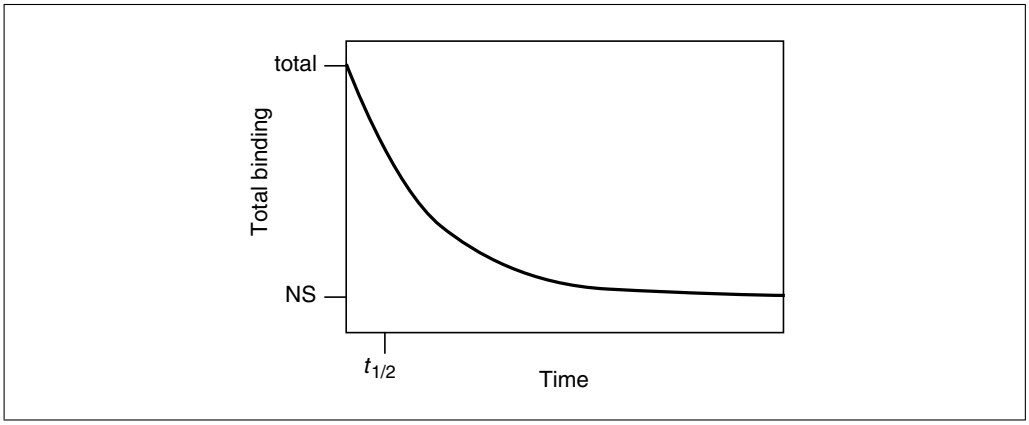

Figure 7.5.14 Schematic of a dissociation kinetic experiment.

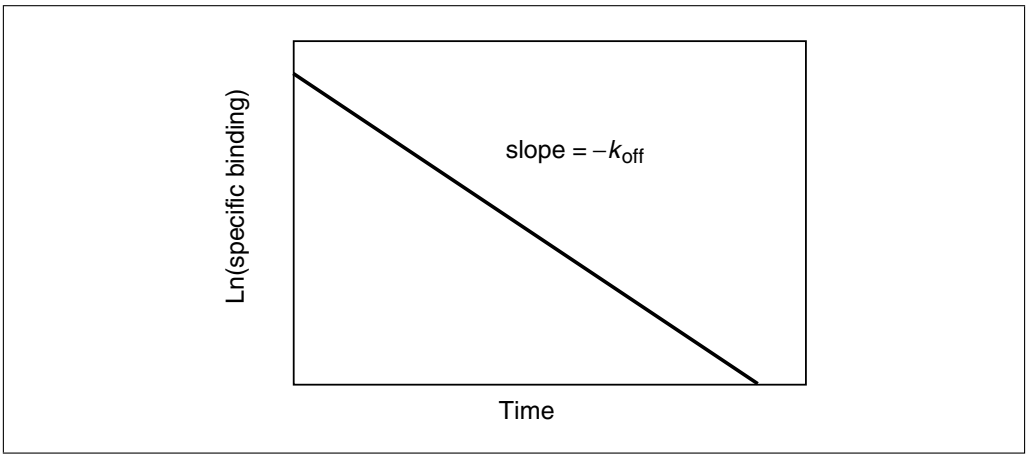

Figure 7.5.15 Schematic of a dissociation kinetic experiment shown on a log scale. The *y* axis plots the natural log of specific binding.

while you wash the filters. If the dissociation rate constant is any slower, it will be hard to reach equilibrium.

Displaying dissociation data on a log plot

Figure 7.5.15 shows a plot of $\ln(B_t/B_0)$ versus time. The graph of a dissociation experiment will be linear if the system follows the law of mass action with a single affinity state. B_t is the specific binding at time t; B_0 is specific binding at time zero. The slope of this line will equal $-k_{off}$.

The log plot will only be linear when taking the logarithm of specific binding as a fraction of binding at time zero. Don't use total binding.

Use the natural logarithm, not the base ten log in order for the slope to equal $-k_{off}$. If you use the base 10 log, then the slope will equal -2.303 times k_{off}.

Use the log plot only to display data, not to analyze data. A more accurate rate constant will be obtained by fitting the raw data using nonlinear regression.

Association Binding Experiments

Association binding experiments are used to determine the association rate constant. This value is useful to characterize the interaction of the ligand with the receptor. It is also important as it permits the determination of the time it takes to reach equilibrium in saturation and competition experiments.

To perform an association experiment, add a single concentration of radioligand and measure specific binding at various times thereafter. You can also do the experiment with several different concentrations of radioligand.

Association of ligand to receptors (according to the law of mass action) follows the equation:

$$\text{specific binding} = \text{max} \times (1 - e^{-k_{ob} \times t})$$

Equation 7.5.26

In Figure 7.5.16, note that the maximum binding (max) is not the same as B_{max}. The maximum (equilibrium) binding achieved in an association experiment depends on the concentration of radioligand. Low to moderate concentrations of radioligand will bind

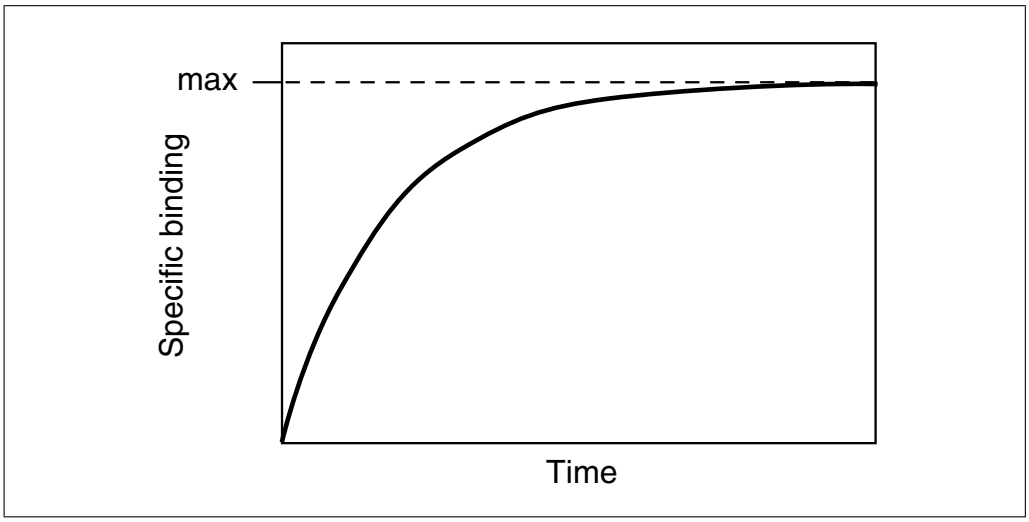

Figure 7.5.16 Schematic of an association kinetic experiment.

to only a small fraction of all the receptors no matter how long binding is allowed to proceed.

Note that the equation used for fitting does not include the association rate constant, k_{on}, but rather contains the observed rate constant, k_{ob}, which is expressed in units of inverse time (usually min^{-1}). The k_{ob} is a measure of how quickly the incubation reaches equilibrium and in the case of a simple bimolecular binding reaction is defined by this equation:

$$k_{ob} = k_{off} + k_{on} \times [\text{radioligand}]$$

Equation 7.5.27

The equation defines k_{ob} as a function of three factors:

1. The association rate constant, k_{on}. This is what is to be determined. If k_{on} is larger (faster), k_{ob} will be larger as well.
2. The concentration of radioligand. When using more radioligand, the system will equilibrate faster and k_{ob} will be larger.
3. The dissociation rate constant, k_{off}. It may be surprising to discover that the observed rate of *association* depends in part on the *dissociation* rate constant. This makes sense because an association experiment does not directly measure how long it takes radioligand to bind, but rather measures how long it takes the binding to reach equilibrium. Equilibrium is reached when the rate of the forward binding reaction equals the rate of the reverse dissociation reaction. If the radioligand dissociates quickly from the receptor, equilibrium will be reached faster, but there will be less binding at equilibrium. If the radioligand dissociates slowly, equilibrium will be reached more slowly and there will be more binding at equilibrium.

To calculate the association rate constant, usually expressed in units of M^{-1} min^{-1}, use the following equation. Typically ligands have association rate constants of $\sim 10^8$ M^{-1} min^{-1}.

$$k_{on} = \frac{k_{ob} - k_{off}}{[\text{radioligand}]}$$

Equation 7.5.28

Determining k_{on} when you already know k_{off}

1. Enter the *x* data as time in minutes.
2. Enter the *y* data as total binding in cpm.
3. Fit to this equation, constraining k_{off} to a constant value based on other experiments and HotnM to the concentration of radioligand in nM.

```
Kd=koff/kon
L=Hotnm*1e-9
kob=kon*L+koff
Occupancy=L/(L+Kd)
Ymax=Occupancy*Bmax
Y=Ymax*(1 - exp(-1*kob*X)) + NS
```

4. Provide the nonlinear regression program with initial estimates of the parameters. B_{max} is the maximum specific binding at equilibrium with high ligand concentration, expressed in the same units as `Y`, so it can be estimated as 2 to 5 times the last *y* value. NS is the nonspecific binding in the same units as *y*, so is estimated by the smallest *y* value. k_{on} is the association rate constant. There is no straightforward rule to use for its initial value, so use a standard value of `1E8` (the units are min^{-1} M^{-1}).
5. Start the nonlinear regression procedure to determine k_{on}, NS, and B_{max}. Note that K_d, `L`, `kob`, `Occupancy`, and `Ymax` are all intermediate variables used to make the equation more clear. Their value is computed from the two constants (hotnm and k_{off}) and the two parameters you are fitting (k_{on} and B_{max}).

Determining k_{on} and k_{off} in one experiment

After running an association experiment, add cold ligand to initiate dissociation. This lets you fit both the association and dissociation rate constants in one experiment.

1. Enter the *x* data as time in min.
2. Enter the *y* data as total binding in cpm.
3. Fit to this equation, constraining HotnM to the concentration of radioligand in nM and Time0 to the time at which dissociation was initiated.

```
Radioligand=HotNM*1e-9
kob=[Radioligand]*kon+koff
Kd=koff/kon
Eq=Bmax*radioligand/(radioligand + Kd)
Association=Eq*(1-exp(-1*kob*X))
YatTime0 = Eq*(1-exp(-1*kob*Time0))
Dissociation= YatTime0*exp(-1*koff*(X-Time0))
Y=IF(X<Time0, Association, Dissociation) + NS
```

4. Provide the nonlinear regression program with initial estimates of the parameters. B_{max} is the maximum specific binding at equilibrium with high ligand concentration, expressed in the same units as `Y`, so can be estimated as a few times the largest *y* value. k_{on} is the association rate constant. There is no straightforward rule to use for its initial value, so use a standard value of `1E8` (the units are min^{-1} M^{-1}).
5. Start the nonlinear regression procedure to determine k_{on}, k_{off}, and B_{max} . Note that `Kd`, `L`, `kob`, `Association`, `YatTime0`, and `Dissociation` are all intermediate variables used to make the equation more clear. Their value is computed from the two constants (hotnm and k_{off}) and the two parameters you are fitting (k_{on} and B_{max}).

Table 7.5.8 Evaluating the Results of Association Binding Analyses

Question	Comment
Were data collected over a long enough period of time?	Dissociation and association data should plateau, so the data obtained at the last few time points should be indistinguishable.
Is the value of k_{on} reasonable?	The association rate constant, k_{on}, depends largely on diffusion, so the value is similar for many ligands. Expect a result of $\sim 10^8$ M^{-1} min^{-1}.
Is the value of k_{off} reasonable?	If the k_{off} is >1 min^{-1}, the ligand has a low affinity for the receptor. Most likely, dissociation will occur during separation of bound and free ligands. If k_{off} is <0.001 min^{-1}, attaining equilibrium will be difficult as the half-time of dissociation will be greater than 10 hr! Even if one waits that long, other reactions may occur that ruin the experiment.
Are the standard errors too large? Are the confidence intervals too wide?	Examine the SE and the confidence intervals to gauge the level of confidence to give the rate constants.
Does only a tiny fraction of radioligand bind to the receptors?	The standard analyses of association experiments assume that the concentration of free radioligand is constant during the experiment. This will be approximately true only if a tiny fraction of the added radioligand binds to the receptors. Compare the maximum total binding in cpm to the amount of added radioligand in cpm. If that ratio exceeds $\sim 10\%$, revise the experimental protocol.

Interpreting Kinetic Results

Are the results reasonable?

Table 7.5.8 presents some questions that should be addressed when determining if the results are reasonable.

Using Kinetic Data to Test the Law of Mass Action

Standard binding experiments are usually fit to equations derived from the law of mass action. Kinetic experiments provide a more sensitive test than equilibrium experiments to determine whether the law of mass action actually applies for the system of interest. To test the law of mass action, ask the following questions.

Does the K_d calculated from kinetic data match the K_d calculated from saturation binding?

According to the law of mass action, the ratio of k_{off} to k_{on} is the K_d of receptor binding:

$$K_d = \frac{k_{off}}{k_{on}}$$

Equation 7.5.29

The units are consistent: k_{off} is in units of min^{-1} and k_{on} is in units of $M^{-1}min^{-1}$, so K_d is in units of M.

If binding follows the law of mass action, the K_d calculated in this way should be the same as the K_d calculated from a saturation binding curve.

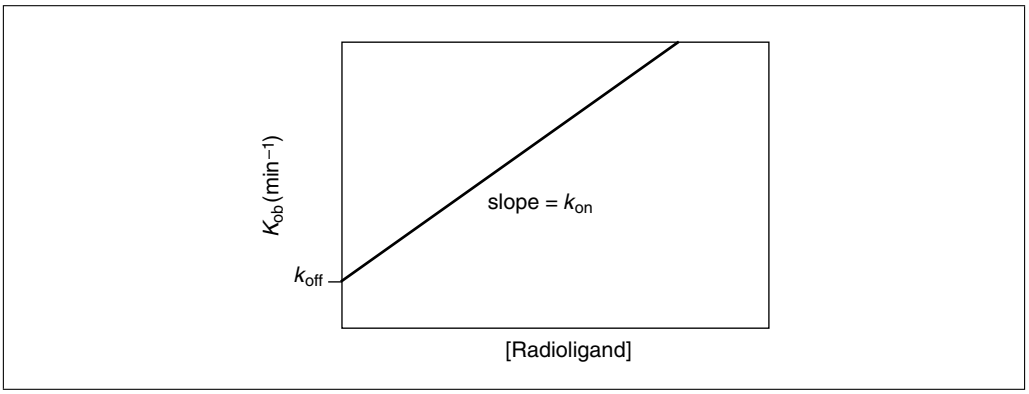

Figure 7.5.17 Schematic of observed association rate constants as a function of radioligand concentration. Higher concentrations of radioligand equilibrate more quickly. The slope of the line equals the association rate constant (k_{on}); the *y* intercept is the dissociation rate constant (k_{off}).

Does k_{ob} increase linearly with the concentration of radioligand?

The observed association rate constant, k_{ob}, is defined by this equation:

$$k_{ob} = k_{off} + k_{on} \times [\text{radioligand}]$$

Equation 7.5.30

Therefore, association rate experiments performed at various concentrations of radioligand should look like Figure 7.5.17. As the concentration of radioligand is increased, the observed rate constant increases linearly. If the binding is more complex than a simple mass action model (such as a binding step followed by a conformational change), the plot of k_{ob} versus [radioligand] may plateau at higher radioligand concentrations. The *y* intercept of the line equals k_{off}. If the law of mass action applies to the system, the k_{off} determined in this way should correspond to the k_{off} determined from a dissociation experiment. Finally, this kind of experiment provides a more rigorous determination of k_{on} than the value obtained with a single concentration of radioligand.

Is specific binding 100% reversible, and is the dissociated ligand chemically intact?

Nonspecific binding at "time zero" should equal total binding at the end (plateau) of the dissociation. In other words, all of the specific binding should dissociate after a sufficiently long period of time. Use chromatography to analyze the radioligand that dissociates to prove that it has not been altered.

Is the dissociation rate the same when dissociation is initiated from different amounts or times of receptor occupation?

If the ligand binds to a single site and obeys the law of mass action, the dissociation rate constant is independent of the amount of radioligand used or the time before initiating dissociation.

Is there cooperativity?

If the law of mass action applies, binding of a ligand to one binding site does not alter the affinity of another binding site. This also means that dissociation of a ligand from one site should not change the dissociation of ligand from other sites. To test this assumption, compare dissociation initiated by adding an unlabeled ligand with dissociation initiated by infinite dilution. The two rate constants should be identical (see Competitive Binding with Two Sites, below).

Kinetics of Competitive Binding

The standard methods of kinetic binding determine the k_{on} and k_{off} for a labeled ligand. Competitive binding can be used to determine the k_{on} and k_{off} of an unlabeled ligand. Add the two ligands at the same time, and measure radioligand binding over time. Use the following information to set up the equation (Motulsky and Mahan, 1984).

Define the following parameters:

k1 association rate constant of radioligand (M^{-1} min^{-1})
k2 dissociation rate constant of radioligand (min^{-1})
k3 association rate constant of unlabeled ligand (M^{-1} min^{-1})
k4 dissociation rate constant of unlabeled ligand (min^{-1})
[radioligand] concentration of labeled drug (M)
[unlabeled drug] concentration of unlabeled drug (M)
S an arbitrary designation of an intermediate variable
B_{max} total number of binding sites (same units as specific binding, usually cpm)
t time (min)

$$K_A = k_1 \times [\text{radioligand}] + k_2$$

Equation 7.5.31

$$K_B = k_3 \times [\text{unlabeled ligand}] + k_4$$

Equation 7.5.32

$$S = \sqrt{(K_A - K_B)^2 + 4 \times k_1 \times k_3 \times [\text{radioligand}] \times [\text{unlabeled ligand}]}$$

Equation 7.5.33

$$K_F = \frac{K_A + K_B + S}{2} \qquad K_S = \frac{K_A + K_B - S}{2}$$

Equation 7.5.34

specific binding =

$$\frac{B_{max} \times k_1 \times [\text{radioligand}]}{K_F - K_S}\left[\frac{k_4 \times (K_F - K_S)}{K_F \times K_S} + \frac{k_4 - K_F}{K_F} \times e^{-K_F \cdot t} - \frac{k_4 - K_S}{K_S} \times e^{-K_S \cdot t}\right]$$

Equation 7.5.35

Many data points are needed at early time points for this method to work. When fitting the data, set k_1 and k_2 to constant values determined from standard kinetic experiments with the radioligand. Set B_{max} to a constant value determined in a saturation binding experiment. The concentrations of labeled and unlabeled compound are also constants, set by your experimental design. Fit the data to determine k_3 and k_4.

To get reliable results, perform the experiment with two concentrations of radioligand, and fit both data sets globally, sharing all the parameters.

TWO BINDING SITES

Several receptor molecules frequently evolve for a single hormone or neurotransmitter. Also, many ligands bind to more than one receptor subtype. Consequently, it is often necessary to fit binding data with a model that includes two or more binding sites.

Saturation Binding Experiments with Two Sites

When the radioligand binds to two classes of receptors, analyze the data by using this equation:

$$\text{specific binding} = y = \frac{B_{\max 1} \times [\text{L}]}{K_{\text{d}1} + [\text{L}]} + \frac{B_{\max 2} \times [\text{L}]}{K_{\text{d}2} + [\text{L}]}$$

Equation 7.5.36

Panel A of Figure 7.5.18 shows specific binding to two classes of receptors present in equal quantities, whose K_d values differ by a factor of ten. Panel B shows the transformation to a Scatchard plot. In both graphs the dotted and dashed lines show binding to the two individual receptors; the sum in each graph is represented by a solid curve.

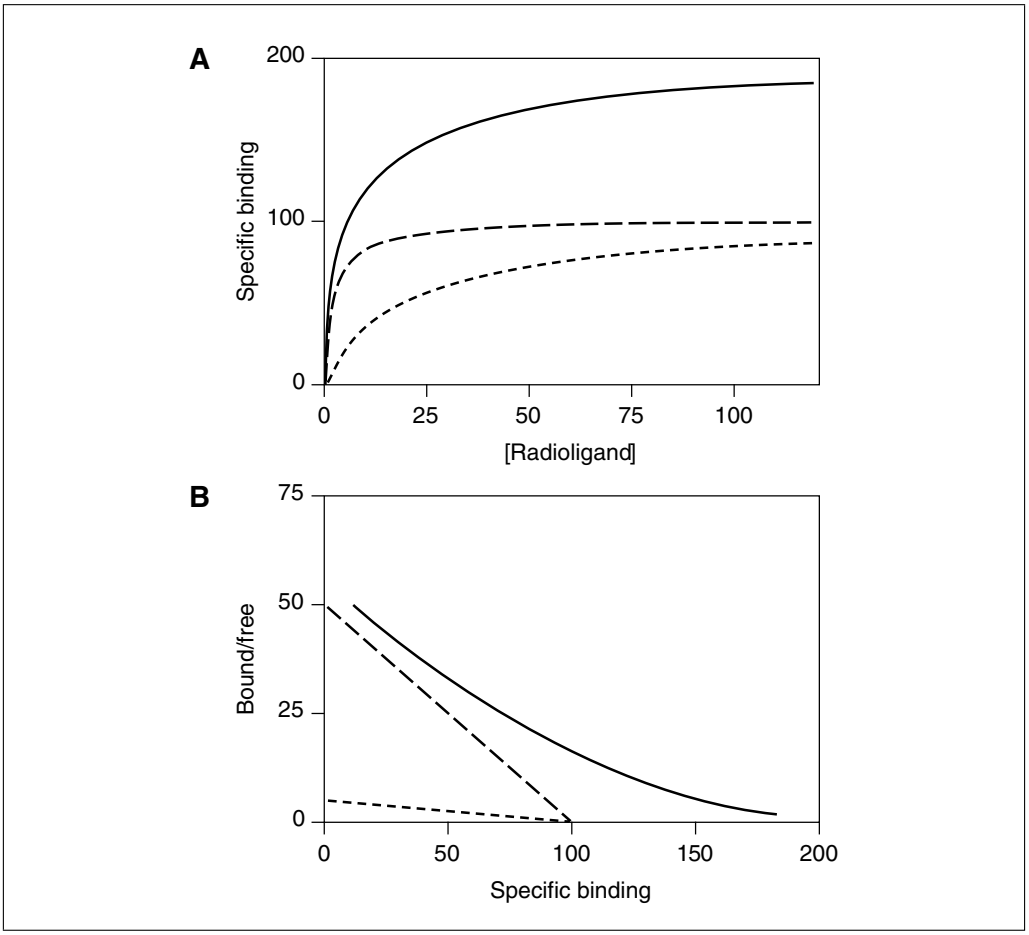

Figure 7.5.18 Saturation binding to two classes of receptors. The two receptor types are present in equal quantities, but have K_d values that differ by a factor of ten. (**A**) Binding to the two individual receptor types are shown as dashed curves. The sum (observed experimentally) is shown as a solid curve. It is not obviously biphasic. (**B**) Scatchard transformation. The curvature of the overall Scatchard plot (solid) is subtle, and it would be easy to miss the curvature if the data were scattered. Note that the Scatchard plots for the individual receptors (dashed) are *not* asymptotes of the two-site Scatchard plot (solid).

Note that the graph of specific binding is not obviously biphasic. It is very hard to see the presence of two binding affinities by just looking. The best way to detect the second site is to fit data to one- and two-site curves, and let the nonlinear regression program compare the two fits (see Theory: Comparing One- and Two-Site Models, below). The curvature of the Scatchard plot is not dramatic and can easily be obscured by experimental scatter. Note the location of the solid and dashed line in the Scatchard plot. The two components of a biphasic Scatchard are not the asymptotes of the curve.

Competitive Binding with Two Sites

Competitive binding experiments are often used in systems where the tissue contains two classes of binding sites (e.g., two subtypes of a receptor). Analysis of these data is straightforward if the following assumptions are met:

1. There are two distinct classes of receptors. For example, a tissue could contain a mixture of β_1 and β_2 adrenergic receptors.
2. The unlabeled ligand has distinct affinities for the two sites.
3. The labeled ligand has equal affinity for both sites or the affinity of the radiological for the two sites is known from other experiments.
4. Binding has reached equilibrium.
5. A small fraction of both labeled and unlabeled ligand bind. This means that the concentration of labeled and unlabeled ligand added is very close to the free concentration in all tubes.

Based on these assumptions, binding follows the equation:

$$y = \mathrm{NS} + (\mathrm{Total} - \mathrm{NS})\left[\frac{F}{1+10^{\log x - \log(\mathrm{IC_{50}A})}} + \frac{1-F}{1+10^{\log x - \log(\mathrm{IC_{50}B})}}\right]$$

Equation 7.5.37

This equation has five parameters: the total and nonspecific binding (the top and bottom binding plateaus), the fraction of binding to receptors of the first type of receptor (F), and the IC_{50} of the unlabeled ligand for each type of receptor. If the K_d and concentration of the labeled ligand is known, the IC_{50} values can be converted to K_i values (see Analyzing Competitive Binding Data, above).

Since there are two different kinds of receptors with different affinities, a biphasic competitive binding curve might be expected. In fact, a biphasic curve is seen only in unusual cases where the affinities are extremely different. More often, the two components are blurred together into a shallow curve. For example, Figure 7.5.19 shows competition for two equally abundant sites with a ten-fold (one log unit) difference in IC_{50}. Careful observation will reveal that the curve is shallow (it takes more than two log units to go from 90% to 10% competition), but two distinct components are not visible.

Cooperativity

In the standard mass action model, each binding site is independent. The standard mass action model assumes that there is no cooperativity. Cooperativity occurs when binding of a ligand to one binding site affects binding to adjacent sites. Usually these binding sites are on the same molecule. If binding of one ligand increases the affinity of an adjacent site, this is positive cooperativity. This results in a Hill slope with an absolute value greater than 1. If binding of one ligand decreases the affinity of an adjacent site, this is

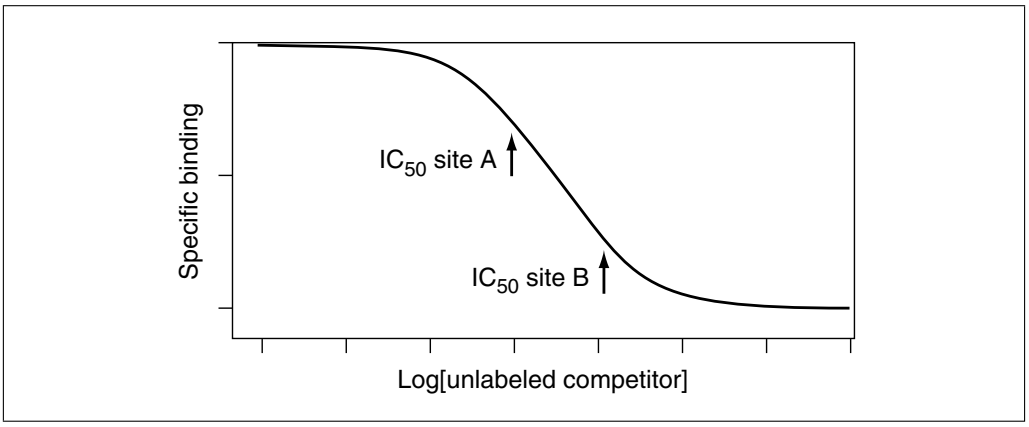

Figure 7.5.19 Two-site competitive binding curve. The radioligand binds identically to two kinds of receptors, but these two receptors have a ten-fold difference in affinity for the competitor. The curve is shallow, but not obviously biphasic.

negative cooperativity. This results in a Hill slope with an absolute value less than 1. It is impossible to distinguish negative cooperativity from multiple independent binding sites (with different affinities) from data collected at equilibrium. Kinetic experiments are needed.

To distinguish between multiple independent binding sites and negative cooperativity, compare the dissociation rate after initiating dissociation by infinite dilution with the dissociation rate when initiated by addition of a large concentration of unlabeled drug. If the radioligand is bound to multiple noninteracting binding sites, the dissociation will be identical in both experimental protocols as shown in panel A of Figure 7.5.20. Note that the *y* axis is shown using a log scale. If there were a single binding site, the dissociation data would be expected to appear linear on this graph. With two binding sites, the graph is curved, even on a log axis (assuming the radioligand is present at high enough concentration to bind appreciably to both sites).

Panel B shows ideal dissociation data when radioligand is bound to interacting binding sites with negative cooperativity. The data are different depending on how dissociation was initiated. If dissociation is initiated by infinite dilution, the dissociation rate will change over time. The dissociation of some radioligand will leave the remaining ligand bound more tightly. When dissociation is initiated by addition of cold drug, all the receptors are always occupied by ligand (some hot, some cold) and dissociation occurs at its maximal unchanging rate.

Theory: Comparing One- and Two-Site Models

Why not just compare sum of squares or R^2?

In a least-squares analysis of data (either linear or nonlinear), the computer program will give an R^2 value and the sum of the squared deviations from the theoretical fit in the experimental result. The smaller the sum of squares (SS) value and the higher the R^2, the better the theory fits the data. However, a two-site model will almost always fit the data better than a one-site model. A three-site model fits even better, and a four-site model better yet! As more parameters (sites) are added to the equation, more inflection points are added to the curve, so it gets closer to the points. The sum of squares gets smaller and R^2 gets higher. Statistical calculations (such as the *F* test described below) should be used to see whether these changes are larger than expected by chance.

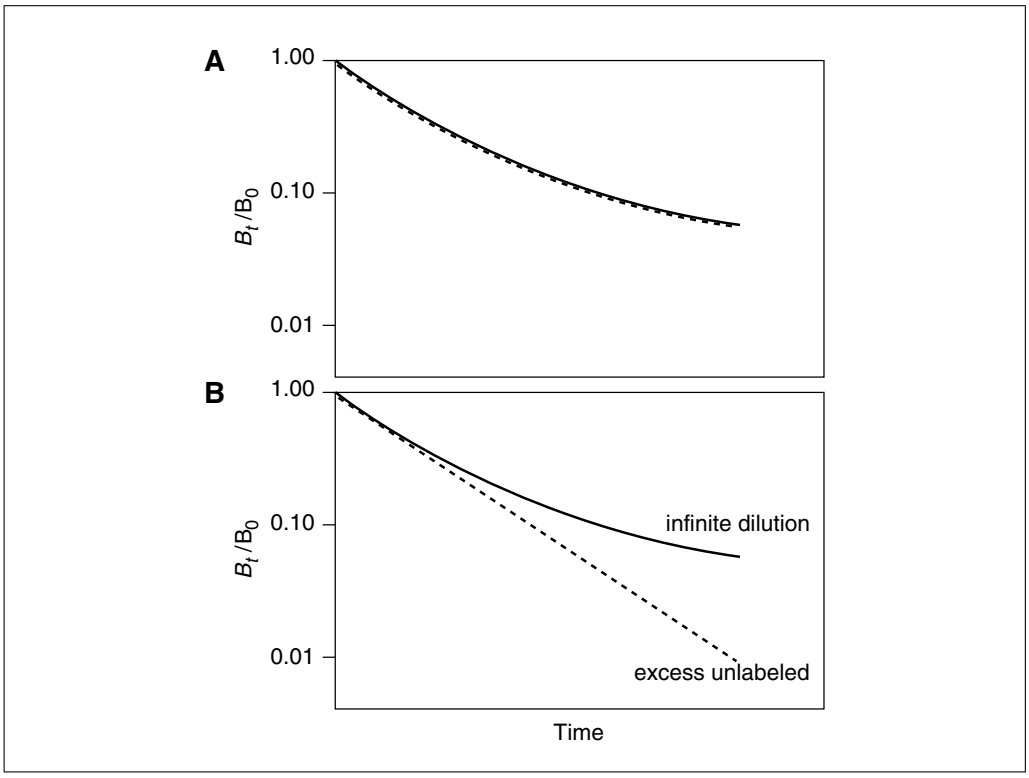

Figure 7.5.20 Discriminating between binding to two (or more) binding sites (**A**) and negative cooperativity (**B**). With negative cooperativity, dissociation will be faster when initiated by adding excess unlabeled ligand than when initiated by infinite dilution.

Reality check

Before performing statistical comparisons, however, look at whether the results make sense. Sometimes the two-site fit gives results that are clearly nonsense. Disregard a two-site fit when:

1. The two IC_{50} or K_d values are almost identical—the data probably fit quite well by a single-site model.
2. One of the IC_{50} or K_d values is outside the range of data.
3. One of the sites has a very small fraction of the receptors —if there are too few sites, the IC_{50} or K_d cannot be determined reliably.
4. The best-fit values for the bottom and top plateaus are far from the range of y values observed in the experiment (applies to competitive binding curves only).

If the two-site fit seems reasonable, test whether the difference between the one- and two-site fit is statistically significant.

Using the F test to compare one- and two-site fits

Even if the simpler one-site model is correct, the fit is expected to be worse (have the higher sum of squares) because it has fewer inflection points (more degrees of freedom; DF). In fact, statisticians have proven that the relative increase in the sum of squares is expected to equal the relative increase in degrees of freedom. In other words, if the one-site model is correct it would be expected that:

$$(SS1 - SS2)/SS2 \approx (DF1 - DF2)/DF2$$

Equation 7.5.38

If the more complicated two-site model is correct, then the relative increase in sum of squares (going from two-site to one-site) is expected to be greater than the relative increase in degrees of freedom:

$$(SS1 - SS2)/SS2 > (DF1 - DF2)/DF2$$

Equation 7.5.39

Follow these steps to compare the two models:

1. Fit the data to the simpler (one-site) model and record the sum of squares (SS1) and degrees of freedom (DF1). Degrees of freedom equal the number of data points minus the number of fitted parameters.
2. Fit the data to the more complicated (two-site) model and record the sum of squares (SS2) and degrees of freedom (DF2).
3. Look at whether the two-site model makes sense. If the best-fit values don't make sense (or the values for the two sites are almost the same), then discard the two-site model and accept the one-site model.
4. Compare SS2 with SS1. If for some reason SS2 is larger than SS1, then the two-site fit is worse than the one-site fit and should be discarded. Accept the one-site fit. In most cases SS1 is larger, and further calculations will be needed.
5. Calculate the *F* ratio, which quantifies the relationship between the relative increase in sum of squares and the relative increase in degrees of freedom.

$$F = \frac{(SS1 - SS2)/SS2}{(DF1 - DF2)/DF2}$$

Equation 7.5.40

The equation for calculating *F* is usually presented in this equivalent form (see Table 7.5.9 for corresponding ANOVA table).

$$F = \frac{(SS1 - SS2)/(DF1 - DF2)}{SS2/DF2} \qquad DF_n = (DF1 - DF2), \quad DF_d = DF2$$

Equation 7.5.41

Table 7.5.9 ANOVA Table for Comparison of One- and Two-Site Fits[a]

Source of variation	Sum of squares	DF	Mean square
Difference	SS1 - SS2	DF1 - DF2	SSI - SS2 DF1 - DF2
Model 2 (complicated)	SS2	DF2	SS2/DF2
Model 1 (simple)	SS1	DF1	

[a] ANOVA, analysis of variance.

6. Use a table or program to determine the *P* value. When doing so, degrees of freedom should be entered for both the numerator (DF_n) and denominator (DF_d). The numerator has (DF1 − DF2) degrees of freedom. The denominator has DF2 degrees of freedom.

If the one-site model is correct, an *F* ratio near one and a large *P* value are expected. If the two-site fit is correct, a large *F* ratio and a small *P* value would be seen. The *P* value can be small for two reasons. One possibility is that the two-site model is correct. The other possibility is that the one-site model is correct, but random scatter led the two-site model to fit better by chance. The *P* value tells how rarely this coincidence would occur. More precisely, the *P* value answers the following question: if the one-site model is really correct, what is the chance that data would randomly fit the two-site model so much better?

If the *P* value is smaller than a preset threshold (set to the arbitrary value of 0.05 by tradition), conclude that the two-site model is significantly better than the one-site model.

Figure 7.5.21 compares a one-site and two-site competitive binding curve. The results are shown in Table 7.5.10.

In going from the two-site to the one-site model, two degrees of freedom are gained, because the one-site model has two fewer parameters. Since the two-site model has 10 degrees of freedom (15 data points minus 5 parameters), the degrees of freedom increased 20%. If the one-site model were correct, the sum of squares would be expected to increase ~20% just by chance. In fact, the sum of squares increased 91%. The percent increase was 4.56 times higher than expected (91.1/20.0 = 4.56). This is the *F* ratio (*F* = 4.56), and it corresponds to a *P* value of 0.039. If the one-site model is correct, there is only a 3.9% chance that randomly obtained data would fit the two-site model so much better. Since this is below the traditional threshold of 5%, conclude that the two-site model fits significantly better than the one-site model.

Table 7.5.10 Comparison of One-Site and Two-Site Competitive Binding Curve

	Two-site	One-site	% increase
Degrees of freedom	10	12	20.00
Sum of squares	129,800	248,100	91.14

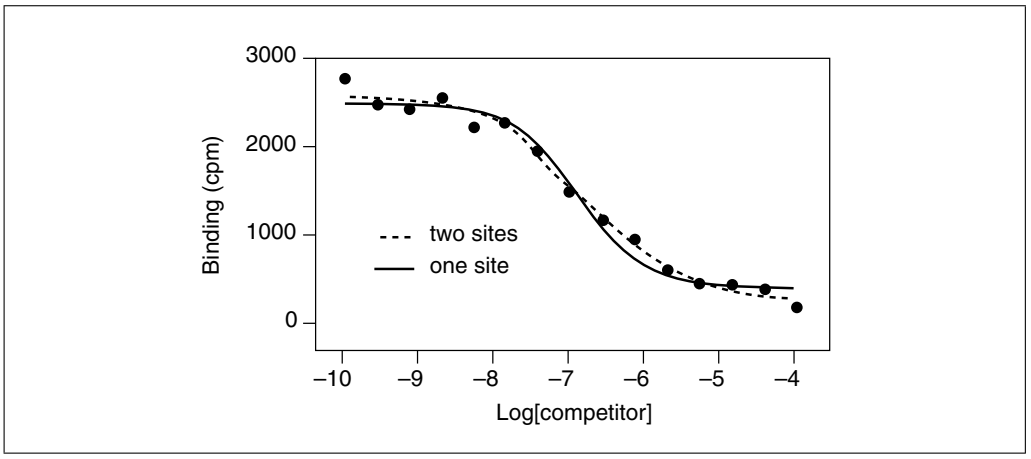

Figure 7.5.21 The solid curve shows the fit to an equation describing competition for a single class of receptors. The dashed curve shows the fit to an equation describing competition for binding to two classes of receptors.

AGONIST BINDING

Receptors Linked to G Proteins

The most studied example of agonist binding is the interaction of agonists with receptors that are linked to G proteins. This is studied by comparing the competition of agonists with radiolabeled antagonist binding in the presence and absence of GTP (or its analogs). These experiments are done in membrane preparations to wash away endogenous intracellular GTP. Without added GTP, the competitive binding curves tend to be shallow. When GTP or an analog is added, the competitive binding curve is of normal steepness. Figure 7.5.22 shows the results of an idealized experiment.

The extended ternary complex model can partially account for these findings (and others). In this model, receptors can exist in two states, R and R*. The R* state has a high affinity for agonist and preferentially associates with G proteins to form an R*G complex. Although some receptors may exist in the R* state in the absence of agonist, the binding of agonist fosters the transition from R to R*, and thus promotes interaction of the receptor with G protein to form the ternary complex HR*G. The extended ternary complex model is shown in Figure 7.5.23.

The agonist binding curve is shallow (showing high- and low-affinity components) in the absence of GTP because some receptors interact with G proteins and others do not.

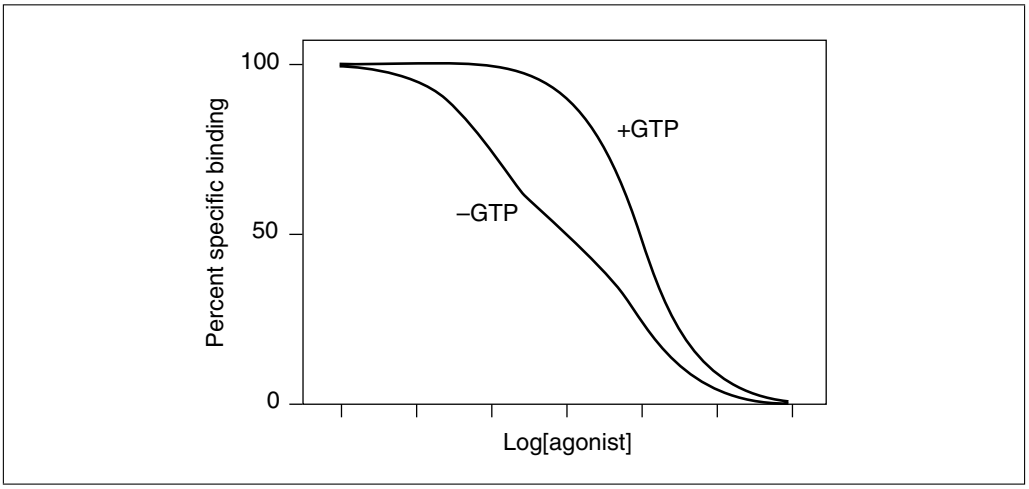

Figure 7.5.22 Schematic of agonist competition for binding to a receptor linked to a G protein. In the absence of GTP (left) the curve is shallow (and in this extreme case, biphasic). In the presence of GTP (or an analog) the curve is shifted to the right and is steeper.

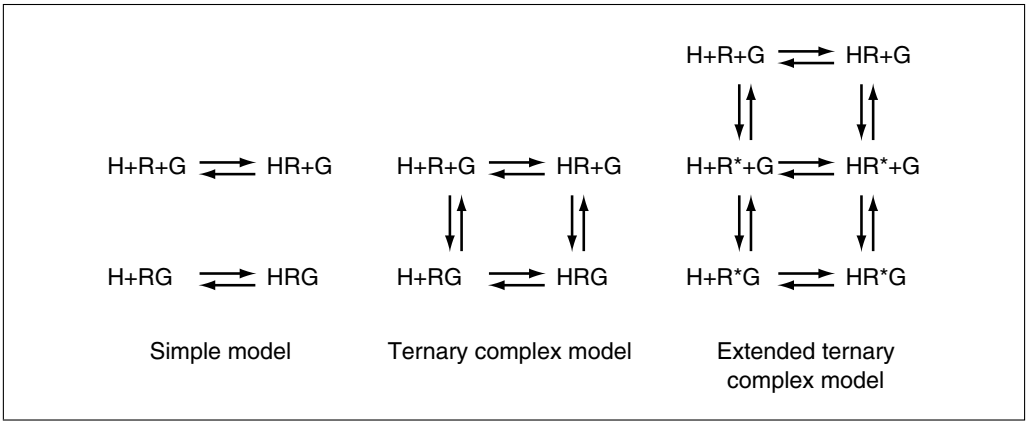

Figure 7.5.23 Models for agonist binding to receptors linked to G proteins. H, hormone or agonist; R, receptor; G, G protein.

The receptors that do interact with G proteins bind agonist with high affinity, while those that do not interact bind with low affinity. Not all receptors can bind to G proteins because either the receptors are heterogeneous, the G proteins are limiting, or membrane compartmentalization prevents some receptors from interacting with G proteins. If all the receptors could interact with G proteins, the expectation would be a high affinity, competitive binding curve in the absence of GTP. In the presence of GTP (or an analog), the HR*G complex is not stable, so the G protein dissociates into its α_{GTP} and $\beta\gamma$ subunits, and is uncoupled from the receptor. With GTP present, only a tiny fraction of receptors are coupled to G at any given time, so the agonist competition curves are of low affinity and normal steepness as if only R was present and not RG.

Although the extended ternary complex model is very useful conceptually, it is not very useful when analyzing data. There are simply too many parameters. The simpler ternary complex model shown in Figure 7.5.23 has fewer parameters, but still too many to reliably fit with nonlinear regression. For routine analyses, most investigators fit data to the much simpler two-state model shown in the figure. This model allows for receptors to exist in two affinity states (R and RG), but does not allow conversion between them. It is easy to fit data to this simpler model using a two-site competition curve model. Since the model is too simple, the high- and low-affinity dissociation constants derived from the model should be treated merely as empirical descriptions of the data and should not be thought of as true molecular equilibrium constants.

Other Kinds of Receptors

By definition, the binding of agonists to receptors makes something happen. So it is not surprising that agonist binding is often more complicated than the simple mass action model. For example, binding of agonists to nicotinic acetylcholine receptor causes a conformational change characterized by a high-affinity binding of the agonist and desensitized receptors, and insulin binding to its receptor shows negative cooperativity due to dimerization of the receptors.

USE OF FLUORESCENCE OR OTHER SPECTROSCOPIC METHODS IN BINDING EXPERIMENTS

Advantages of Fluorescent Ligands

Recently, many investigators have begun to use nonradioactive methods to detect ligand binding to receptors or macromolecules. In most cases, these methods use fluorescent ligands.

An advantage of fluorescent methods is elimination of both the dangers of radioactivity and the expenses of radioactive waste disposal. Additionally, fluorescent methods are easier to perform and automate—a big plus for high throughput screening studies by the pharmaceutical industry. Fluorescent studies work very well for kinetics, since binding in a single sample can be measured at many time points.

Disadvantages of Fluorescent Ligands

One disadvantage (compared to radioligands) is that fluorescence measurements are expressed in arbitrary fluorescence units. While one can compare values between samples in a single experiment, it is rarely possible to convert the fluorescent measurements into the actual number of binding sites in the incubation. This problem, however, does not affect determinations of equilibrium or kinetic constants.

Another disadvantage of fluorescent methods is that the signal-to-noise ratio tends to be low.

Fluorescence methods rarely use a washing step to reduce nonspecific binding (as radioligand methods do). Instead, fluorescence is measured in the experimental incubation. The background signal includes not only binding to nonspecific sites but primarily fluorescence from the free ligand. The signal-to-noise problem is reduced when the concentration of binding sites is high, as found in studies of purified proteins or cell lines genetically engineered to overexpress binding sites, and these are the kinds of systems where fluorescence methods are most useful.

In order to detect a change in fluorescence on ligand binding without physical separation, a significant fraction of the ligand often needs to be bound. This means that analyses of fluorescent binding need to account for ligand depletion, as discussed earlier.

How Fluorescence Binding Measurements Work

Fluorescent ligand binding studies measure some change in the spectral properties of a fluorescent ligand upon binding to the receptor. There are at least three different methods used for fluorescent ligand binding studies: fluorescence intensity (FI), fluorescence polarization (FP), and fluorescence resonance energy transfer (FRET). The methods of analysis are similar for FI and FRET but because FP measures a ratio of two differently polarized emission values, the analysis of FP results is significantly different (see below).

A less frequently used method is to use a fluorescent receptor (either natural fluorescence such as tryptophan or a labeled protein) and detect changes in the spectral properties of the receptor as the small molecule binds. Finally, some other approaches make use of a change in localization of the fluorescent ligand upon binding which concentrates it in the vicinity of an excitation light source [e.g., total internal reflection fluorescence (TIRF), or bead-based methods such as flow cytometry protein interaction assay (FCPIA) (Roman et al., 2007)]. In some cases, standard separation methods like filtration are used with fluorescence detection of a bound ligand, but those methods entail the same considerations as standard radioligand methods, and will not be considered here.

Fluorescence Intensity

Fluorescent intensity changes when the fluorophore moves to a different environment on binding, for example moving from an aqueous environment for the free ligand to a hydrophobic protein surface for the bound ligand. As increasing amounts of a fluorescent ligand are added to a biological preparation containing a receptor or binding site, fluorescence is detected from both the free ligand L and the bound ligand LR. If the fluorescence of the bound ligand is higher (or lower) than the fluorescence of the free ligand, then the system can be used to measure binding. In general, however, a significant fraction of the ligand must be bound (e.g., 2% to 50%) in order to detect a clear signal in FI studies. Saturation binding experiments lead to results similar to those for a radioligand saturation study (see Fig. 7.5.24), in which there is a linear component due primarily to the fluorescence from the free ligand and a saturable component due to the receptor binding.

Depending on the concentration of the receptor binding sites (R_{tot}) relative to the K_d for the binding interaction, this may follow either a simple hyperbolic binding isotherm:

$$\text{Fractional occupancy} = \frac{[\text{ligand}]}{[\text{ligand}] + K_d}$$

Equation 7.5.42

or that for a saturation curve with ligand depletion. As a rule of thumb, if R_{tot} is $< K_d/5$ then it is appropriate to use the simple saturation isotherm with a linear nonspecific

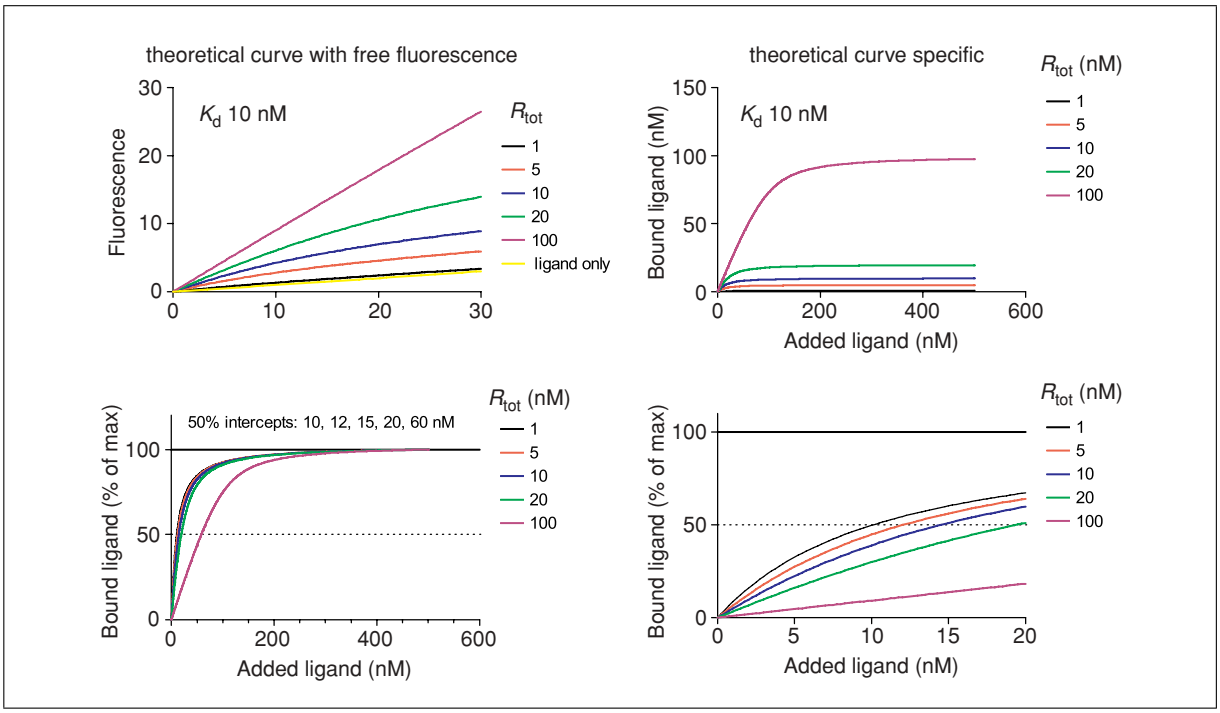

Figure 7.5.24 Fluorescence intensity at various concentrations of receptor as a function of added ligand concentration. The upper left panel shows the total fluorescence intensity. The upper right panel converts to nM bound. The lower left panel normalizes the curve for each receptor concentration so they all plateau at the same maximum, and compares the EC_{50} values. The lower right panel zooms in on the lowest ligand concentrations.

component. Regardless of whether the receptor concentration is much below the K_d or not, an apparent K_d can be calculated and this will equal approximately $K_d + R_{tot}/2$ (Fig. 7.5.24, bottom left). When R_{tot} is much greater than the K_d the binding curve becomes a titration curve with the ligand concentration at half-maximal binding equal to $R_{tot}/2$ and a sharp transition from increasing binding to a plateau at R_{tot}. Indeed, this has been used as a means of calculating the concentration of binding sites based on the known concentrations of a fluorescent ligand (Neubig and Cohen, 1979).

The first study usually done to assess fluorescent ligand binding is a time course. For example, the binding of a fluorescent guanine nucleotide (BODIPY FL-GTPγS) to a binding site on the $G\alpha_o$ G protein was measured by McEwen et al. (2001) using a fluorescence intensity readout. First, a near saturating amount of "receptor," in this case G protein, was added to a low concentration of ligand and the increase in fluorescence intensity was measured over time (see Fig. 4 in McEwen 2001). The increase in fluorescence intensity was between 3- and 6-fold depending on which G protein is used and half-times varied from 2 to 10 min for $G\alpha_o$, $G\alpha_s$, and $G\alpha_{i1}$.

BASIC PROTOCOL

FLUORESCENCE SATURATION BINDING OF BODIPY FL-GTPγS TO $G\alpha_O$

Quantification of nucleotide binding to heterotrimeric G proteins has traditionally used radioactive nucleotides such as [^{35}S]GTPγS. With the development of BODIPY-labeled nucleotides, including BODIPY-FL-GTPγS, it became possible to assess binding by fluorescence methods. In particular, the fluorescence of BODIPY-FL-GTPγS free in solution is quenched by intramolecular energy transfer. Upon binding to the G protein, quenching is relieved and fluorescence intensity is increased (McEwen et al., 2001). This protocol illustrates the basic method to perform a saturation binding experiment using 96-well plates and serial dilutions, with determination of both total and nonspecific fluorescence.

Materials

Ligand (BODIPY-FL-GTPγS; Invitrogen) stock solution: 10 μl of 100 μM frozen in 1 mM DTT
G protein ($G\alpha_o$) stock solution: 20 μM snap frozen in HED buffer
Binding buffer: HED buffer (see below) containing 10 mM $MgCl_2$
HED buffer: 50 mM HEPES, pH 8, containing 1 mM EDTA and 1 mM DTT
Competing ligand (e.g., 100 μM GTPγS)

Costar 3915 black 96-well microplate
Fluorescence plate reader

1. Thaw ligand and G protein stocks on ice.
2. Prepare ligand dilutions on ice using serial dilutions.
   a. Prepare a working stock solution of BODIPY-FL-GTPγS (1 μM) by adding 990 μl of binding buffer to the 10 μl stock tube on ice.
   b. Prepare a solution with 60 nM BODIPY-FL-GTPγS (2× the maximum final ligand concentration) in binding buffer.
   c. Add 100 μl to the top row of the desired number of replicate samples in a 96-well plate (usually two to three samples per condition studied).
   d. Add 50 μl of binding buffer to each of the lower wells.
   e. Prepare serial dilutions by transferring 50 μl from the top wells using a multichannel pipettor to the next lower wells. Mix by pipetting up and down two to three times, and again transfer 50 μl to the next lower well.
   f. Stop before the last well to leave it as a blank, with no fluorophore.

   This will provide concentrations of 60, 30, 15, 7.5, 3.75, 1.88, 0.94, and 0 nM fluorophore. Final concentrations will be half of this.
3. Prepare 1 to 2 ml of a 2× stock solution (e.g., 40 nM $G\alpha_o$) of G protein in binding buffer, and store on ice.
4. Prepare nonspecific binding samples—either leave G protein out of half of the fluorophore wells by adding 50 μl of binding buffer or add excess competing ligand (e.g., 1 μM GTPγS, 1 μl of a 100 μl stock) to half of the wells.
5. Prewarm the plate with ligand for 3 min at room temperature.
6. Initiate binding by adding 50 μl of 2× Gα subunit to the appropriate wells with an automatic pipettor.
7. Incubate at room temperature for 20 min (or the appropriate time determined in previous time course studies).
8. Read fluorescence intensity in a fluorescence plate reader (excitation 490 nm, emission 510 nm)
9. Plot Total and Nonspecific fluorescence as shown in Figure 7.5.25.
10. Subtract Nonspecific fluorescence from Total to determine Specific fluorescence
11. Fit Specific fluorescence to a hyperbolic binding function to calculate an apparent K_d (i.e., not corrected for depletion).

    If apparent K_d is more than 3× greater than the protein concentration, it represents a good estimate of the true K_d. If the apparent K_d is equal to the protein concentration (or even less than 3× greater), fit the data with a depletion equation as described above.

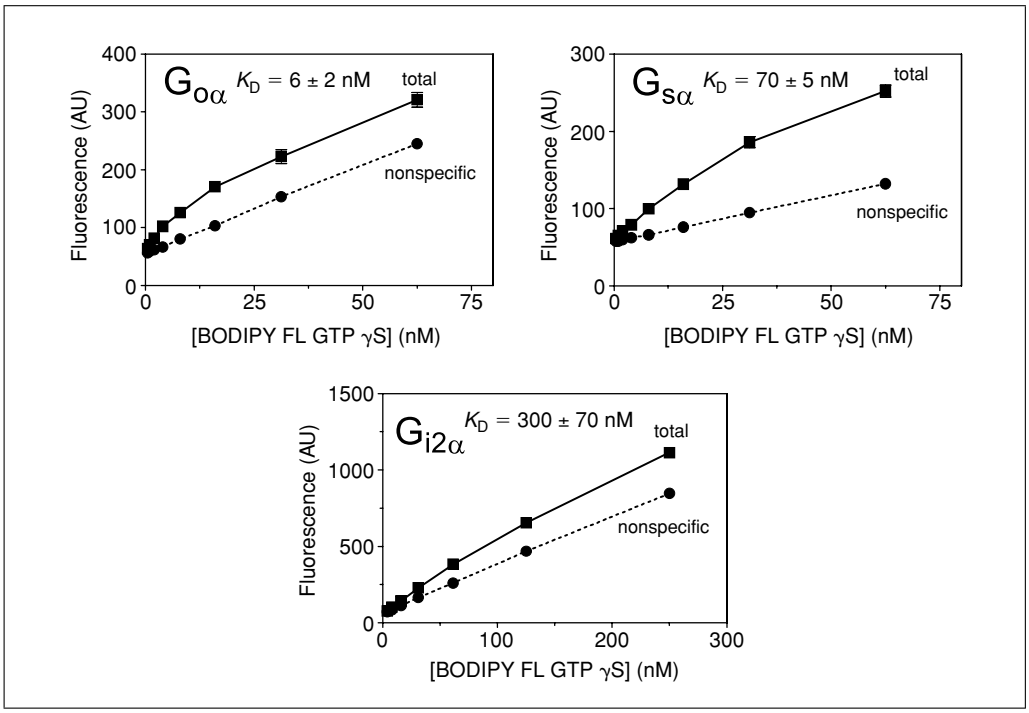

Figure 7.5.25 Fluorescence saturation curves for binding of BODIPY-FL-GTPγS to three different G protein alpha subunits. Binding was measured as described in the Basic Protocol with a 20 nM final concentration of $G\alpha_o$ and $G\alpha_s$, incubated at room temperature for 20 min. For $G\alpha_{i1}$, a final concentration of 100 nM was used with incubations for 60 min at 30°C (reprinted with permission from McEwen et al., 2001).

Example data are shown (in Fig. 7.5.25) for $G\alpha_o$ as well as similar data for $G\alpha_s$ and $G\alpha_{i1}$. In this case, the "nonspecific" fluorescence was determined in the absence of G protein, since the background signal is essentially all due to the presence of free fluorophore. Similar results were obtained when an excess of unlabeled GTPγS was used to determine the "nonspecific" binding. Thus, this type of study is analyzed in a manner that is virtually identical to that used for a standard radioligand binding assay.

Fluorescence Polarization

Fluorescence polarization (FP) measures changes in the mobility of the ligand upon binding.

The basis of the method is that small ligands rotate or tumble rapidly when free in solution, with a time scale similar to the lifetime of the fluorescent excited state (Lakowicz, 2006). When a fluorophore is excited by polarized light, the emission will also be highly polarized if the fluorophore is immobile. But if the fluorophore rotates quickly, the polarization will be low.

An advantage of FP over FI is that it is easier to create ligands. Since there is no need for the fluorescence properties of the fluorophore per se to change when it binds, chemists have much more choice in how to design a ligand. For example, FP is often used with peptide ligands where the fluorophore is on the opposite end of the peptide from the binding site. It is essential, of course, that the fluorophore does not block ligand binding.

FP measurements rely upon a substantial difference in molecular size between the ligand and binding site. This limits the method to the use of small ligands (i.e., less than ~5000 Da) and large binding sites (or binding sites attached to cells or membrane fragments).

The fluorophore is excited with polarized light and the emission is measured with a polarizer parallel to the excitation polarization ($F_{\parallel}$), and also with a polarizer perpendicular to the excitation polarizer ($F_{\perp}$). The difference between these two measurements, usually calculated as a *polarization* (P) value, is a measure of how rapidly the fluorophore is tumbling.

$$P = (F_{\parallel} - F_{\perp}) / (F_{\parallel} + F_{\perp})$$

Equation 7.5.43

Because polarization is computed as a ratio, its value does not vary directly with the concentration of ligand and it will not be affected by small variations in ligand concentration or by the presence of compounds that might absorb the excitation or emission light. This leads to excellent reproducibility of the signal.

In theory, polarization can run from −0.33 (rapidly tumbling ligand) to 0.5 (immobile ligand) (Lakowicz, 2006). In practice, measured values of polarization tend to range from 0.02 to 0.30. This is usually expressed as milli-polarization units (mP = P/1000), with a range of 20 to 300 mP.

Experimentally measured fluorescent polarization is the sum of polarization from unbound ligand (P_L) and polarization from the receptor-ligand complex (P_{RL}). The relative amounts of each depends on the fraction of ligand bound (F). Measured polarization (P), therefore equals:

$$P = (1 - F) \times P_L + F \times P_{RL}$$

Equation 7.5.44

When the ligand concentration is low, a high fraction of ligand is bound to receptors (although a low fraction of receptors are bound to ligand). Thus F is greatest at low ligand concentrations (i.e., well below the K_d). Fluorescence polarization is greatest when ligands are immobile, so P_{RL} is greater than P_L. Therefore measured polarization, P, is highest at low ligand and high receptor concentrations. As shown in Fig. 7.5.26, P increases as the total receptor concentration, R_T, is increased. At a fixed receptor concentration, as the ligand concentration (L_T) increases, the free fraction of ligand increases and P decreases (Fig. 7.5.27).

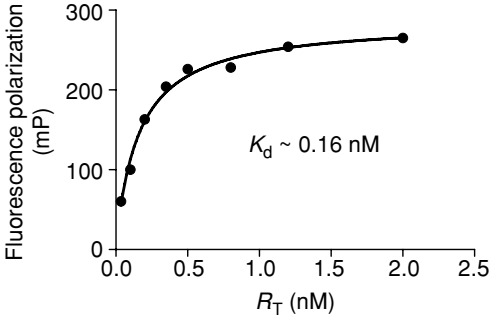

Figure 7.5.26 Fluorescence polarization measurements of αMSH binding. Fluorescent ligand (0.5 nM BODIPY-NDP-αMSH) was incubated with increasing amounts of membrane containing the indicated concentrations of MC_5 receptor. Fluorescence polarization was measured and plotted versus concentration of added receptor. Data from Nosjean et al. (2006).

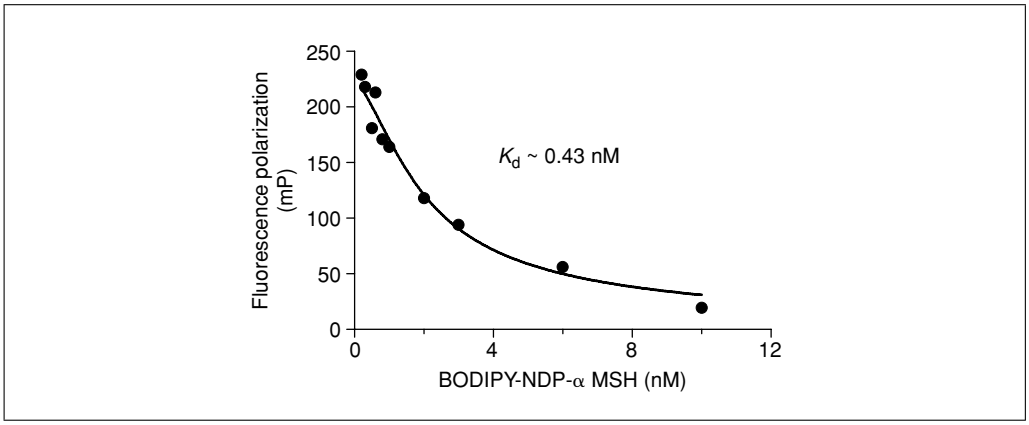

Figure 7.5.27 Fluorescence polarization measurements of αMSH binding with varying ligand. Membranes containing 1 nM MC_5 receptor were incubated with increasing amounts of fluorescent ligand (BODIPY-NDP-αMSH). Fluorescence polarization was measured and plotted versus concentration of added ligand. As indicated in the text, the fraction of bound ligand (and the polarization) is highest at the low ligand concentrations. These data were fitted using NLLSQ analysis with Equation 7.5.20, with a fixed value of $R_T = 1.0$ nM (Nosjean et al., 2006). Although the concentration of ligand at which the polarization falls to a half-maximal value is about 2 nM, the calculated K_d is 0.43 nM due to ligand depletion that occurs because $R_T > K_d$.

Analysis of FP measurements: Varying receptor

Given that the binding equation L+R ⇆ LR is symmetrical for ligand and receptor, one can determine the K_d for a binding reaction by varying the concentration of either ligand or receptor. In fact, FP binding experiments are often performed with a single low ligand concentration (below the K_d) and various receptor concentrations (Fig 7.5.26). The half maximal amount of ligand bound occurs when free R is equal to the K_d. Since free ligand has low polarization and bound ligand has high polarization, the curve (Fig 7.5.26) looks a lot like a standard saturation experiment. The added receptor binds the free ligand, leading to increased polarization. In this type of study, the fraction of bound ligand is linear with the value of P from P_L (polarization of ligand alone i.e., zero ligand bound) to P_{RL} (polarization with all ligand bound at a saturating receptor concentration). Data from Nosjean et al. (2006) were fit to a standard one-site binding hyperbola to estimate a K_d value of 0.16 nM (Fig 7.5.26). One difficulty with this approach is that one needs to know the receptor concentration from some other method (e.g., protein concentration or radioligand binding) to get a K_d using these data alone. Also, the estimated K_d from this study does not take ligand depletion into account. Thus, it is surprising that half-saturation occurs at 0.16 nM, which is less than half of the concentration of the ligand ($L_T = 0.5$ nM so half saturation should not occur at less than 0.25 nM, since you need at least that much receptor to bind half of the ligand).

Analysis of FP measurements: Varying ligand

An alternative approach is to vary ligand concentrations. To analyze this type of data, Nosjean et al. (2006) developed an approach to fit polarization values from the primary experimental parameters (L_T, R_T, and K_d). The following equation can be used for this purpose (either to fit data from single experiments or to globally fit data from a series to determine K_d and R_T).

$$P = P_L + \left[(P_{RL} - P_L)/L_T \right] \times \frac{1}{2}\left[(L_T + R_T + K_d) - \sqrt{(L_T + R_T + K_d)^2 - (4 \times R_T \times L_T)} \right]$$

Equation 7.5.45

This equation combines the polarization equations and ligand binding functions, taking into account the ligand depletion which is often present in fluorescence binding methods.

With this equation, one can fit raw polarization data obtained with variable L_T with several different amounts of receptor to define both K_d and R_T.

In Figure 7.5.27, this method is applied to FP measurements of a fluorescent ligand for the MC_5 receptor similar to those shown in Figure 7.5.26. Since the concentration of receptor (1 nM) is greater than the K_d, it is essential to account for depletion. So the data were fitted to the preceding equation using L_T as the independent variable, keeping R_T constant at 1 nM, and permitting all other parameters (K_d, P_L, P_{RL}) to be optimized by the nonlinear least-squares program. The importance of including ligand depletion is clearly illustrated here. If depletion were ignored, the estimated K_d would be 1.6 nM, while the true calculated K_d based on the depletion due to the 1 nM MC_5 receptor present is only 0.43 nM.

Analysis of FP measurements: Competition

Perhaps the most useful application of FP measurements is in determining the binding of nonfluorescent competing ligands. Since polarization provides a direct measure of the fraction of labeled ligand bound, the reduction in P by a competing ligand is directly analogous to a radioligand competition study. Indeed, one of the most common uses of FP is in high-throughput screening for compounds that can bind to a receptor. The analysis is identical to that for a radioligand competition assay, as explained earlier. If the K_i value estimated using standard competition equations is less than 2 to 3 times the value of R_T, one should include ligand depletion in the analysis.

FRET

Fluorescence resonance energy transfer (FRET) detects changes in the proximity of the ligand to the receptor. It is widely used in detecting protein-protein interactions, but it can also be used for measurements of ligand binding. The principle of FRET involves nonradiative transfer of energy from an excited donor fluorophore (on either the ligand or receptor) to an acceptor fluorophore within about 100 Å on the other binding partner. Extensive information on the theory and analysis of FRET is available (Gordon et al., 1998; Lakowicz, 2006), so that will not be repeated here. Measurements typically involve three readings, Donor, FRET, and Acceptor, where the first two use excitation at the donor wavelength and measure emission at the donor and acceptor wavelengths, respectively. The Acceptor signal is measured with both excitation and emission at the acceptor wavelength. These three measurements are combined to calculate "corrected FRET." This corrected FRET signal is directly proportional to the amount of ligand bound and can be analyzed using the standard ligand binding equations described above for FI and for radioligand binding methods. Because the initial analysis of the raw data (as described in Gordon et al., 1998) requires appropriate corrections for the fluorescence from free donor and acceptor, the nonspecific binding is essentially removed during that correction and fitting can proceed as for specific binding measurements.

ANALYZING DATA USING NONLINEAR REGRESSION

Radioligand binding data are best analyzed using nonlinear regression to fit curves through the data.

The Problem with Using Linear Regression on Transformed Data

Before the age of microcomputers, scientists transformed their data to make a linear graph, and then analyzed the transformed data with linear regression. Examples include Lineweaver-Burke plots of enzyme kinetic data, Scatchard plots of binding data, and logarithmic plots of kinetic data.

These methods are outdated, and should not be used to analyze data. The problem is that the linear transformation distorts the experimental error. Linear regression assumes that the scatter of points around the line follows a Gaussian distribution and that the standard deviation is the same at every value of *x*. These assumptions are usually not true with transformed data. A second problem is that some transformations alter the relationship between *x* and *y*. For example, in a Scatchard plot, the value of *x* (bound) is used to calculate *y* (bound/free), and this violates the assumptions of linear regression. For an example of this, see Saturation Binding Experiments, Analysis of Saturation Binding Curves, The problem with using Scatchard plots to analyze saturation binding experiments).

Since the assumptions of linear regression are violated, the results of linear regression are incorrect. The values derived from the slope and intercept of the regression line are not the most accurate determinations of the receptor number, rate constants, or dissociation constants. Considering all the time and effort put into collecting data, the best possible analysis technique should be used, and nonlinear regression produces the most accurate results.

Although linear regression is usually inappropriate for analyzing transformed data, it is often helpful for displaying transformed data because many people find it easier to visually interpret linear data. This makes sense because the human eye and brain evolved to detect edges (lines), not to detect rectangular hyperbolas or exponential decay curves.

Comparison of Linear and Nonlinear Regression

A line is described by a simple equation that calculates *y* from *x*, slope, and intercept. The purpose of linear regression is to find values for the slope and intercept that define the line that best fits the data. More precisely, it finds the line that minimizes the sum of the squares of the vertical distances of the points from the line.

The goal of minimizing the sum of squares in linear regression can be achieved quite simply. A bit of algebra (shown in many statistics books) derives equations that define the best-fit slope and intercept. Enter the data, perform a few calculations, and the answers come out. There is no chance for ambiguity.

Nonlinear regression fits data to any equation that defines *y* as a function of *x* and one or more parameters. Like linear regression, it finds the values of those parameters that minimize the sum of the squares of the vertical distances of the points from the curve. With the exception of a few special cases (like linear regression), it is not possible to solve the equations directly to find the best-fit values of the parameters. Instead, nonlinear regression requires an iterative approach that requires use of a computer.

To analyze data with nonlinear regression, the program will require an equation (model) that defines *y* as a function of *x* and one or more parameters (i.e., K_d, B_{max}, or rate constants). It will also require an estimate (or guess) for the best-fit value for each parameter in the equation (some programs provide the initial estimates automatically).

Every nonlinear regression program follows these steps:

1. Using the initial values provided, the program calculates a predicted value of *y* for each value of *x*. It then compares the actual *y* values with the predicted *y* values, and calculates the sum of the squares of the differences between observed and predicted *y* values.

2. The program then adjusts the parameters to improve the fit and reduce the sum of squares. There are several algorithms for adjusting the parameters. The most commonly used method was derived by Levenberg and Marquardt (often called simply the Marquardt method), but the details of how this method works cannot be understood without matrix algebra. However, nonlinear regression can be used to analyze data without knowing anything about these algorithms.
3. Step 2 is repeated. Each time the parameters are adjusted by a smaller amount. The fitting stops when the adjustments make virtually no difference in the sum of squares. This typically requires 5 to 20 iterations.
4. Best-fit results are reported. The precise values obtained will depend in part on the initial values and the stopping criteria. This means that repeat analyses of the same data will not always give exactly the same results.

Decisions That Need to be Made When Fitting Curves with Nonlinear Regression

When using a program for nonlinear regression, the following decisions must be made.

Which equation?

An equation must be chosen that defines y as a function of x and one or more parameters. This equation should represent a model, usually the law of mass action.

In many cases, the best approach is to globally fit several data sets (to the same, or different, models) sharing some parameters. Global nonlinear regression finds one best-fit value for all shared parameters, and finds separate best-fit values for each data set for the other parameters.

Which units?

In pure mathematics, it does not matter whether data are entered as 1 pM or 10^{-12} M, as 100 fmol/mg or 60,000,000,000 receptors/mg. When computers do the calculating, however, it can matter. Calculation problems such as round off errors are far more likely when the values are very high or very low. Scale data to avoid values $<10^{-4}$ or $>10^{4}$.

Global nonlinear regression only makes sense if the y values for all data sets are expressed in the same units.

Which estimated initial values?

Nonlinear regression is an iterative procedure. The program must start with estimated values for each parameter that is in the right "ball park"—usually within a factor of five of the actual value. It then adjusts these initial values to improve the fit and repeats the adjustments until the improvement is no longer significant.

Later sections of this unit explain how to choose initial values for various kinds of experiments. The estimates do not need to be extremely accurate. Nonlinear regression will usually work fine as long as the estimates are within 3 to 5 times their actual values.

Some programs (including GraphPad Prism) automatically choose initial values for you. This is a great convenience most of the time. But occasionally, the automatic initial values are not appropriate for the situation. The first step in troubleshooting problem fits is to plot the curve defined by the initial values and make sure it goes near the data. If not, you can manually adjust the initial values to make the curve better approximate the data.

Fix one or more parameters to a constant value?

In some situations it makes sense to fix some of the parameters to constant values. For example, when analyzing specific (rather than total) binding, the bottom plateau of a dissociation experiment should be defined as a constant equal to zero.

Weighting

In general, the goal of nonlinear regression is to find the values of the parameters in the model that make the curve come as close as possible to the data points. Usually this is done by minimizing the sum of the squares of the vertical distances of the data points from the curve. This is appropriate when the scatter of points around the curve is expected to be Gaussian and unrelated to the *y* values of the points.

With many experimental protocols, the experimental scatter is not expected to be the same for all points. Instead, the experimental scatter is expected to be a constant percentage of the *y* value. If this is the case, points with high *y* values will have more scatter than points with low *y* values. When the program minimizes the sum of squares, points with high *y* values will have a larger influence while points with smaller *y* values will be relatively ignored. This problem may be avoided by minimizing the sum of the square of the relative distances. This procedure is termed *weighting* the values by $1/y^2$. Because it prevents large points from being over-weighted, the term *unweighting* seems more intuitive but *weighting* is the usual term used.

Data may be weighted in other ways. The goal is to obtain a measure of goodness-of-fit that values all the data points equal to their reliability.

With binding data, scatter is often proportional to the amount of binding, so relative weighting may be appropriate. With good-quality data, results are usually very similar whether or not you choose to use relative weighting.

Average replicates?

If replicate *y* values are collected at every value of *x*, there are two ways to analyze the data: (1) treat each replicate as a separate point, or (2) average the replicate *y* values and treat the mean as a single point.

With radioligand binding data, the first approach is usually best, because all the data are obtained from one tissue preparation and the sources of experimental error are independent for each tube. If one value happens to be a bit high, there is no reason to expect the other replicates to be high as well. Each replicate can be considered an independent data point.

Do not treat each replicate as a separate point when the experimental error of the replicates is related. Instead, average the replicates and analyze the averages. This situation does not come up often with radioligand binding data, but here is one example. Assume that you perform an experiment with only a single replicate at each value of *y* (concentration or time) but count each tube three times. It is not fair to enter the three counts as triplicates, and then analyze each triplicate as a separate value. As the replicates are not independent, any experimental error would appear in all the replicates.

Assumptions of Nonlinear Regression

The results of nonlinear regression are meaningful only if the following assumptions are true (or nearly true):

1. The model is correct. Nonlinear regression adjusts the parameters in the equation you chose to minimize the sum of squares. It does not attempt to find a better equation.

2. The variability of values around the curve follows a Gaussian distribution. Even though no biological variable follows a Gaussian distribution exactly, it is sufficient that the variation be approximately Gaussian.
3. The standard deviation (SD) of the residuals is the same everywhere, regardless of the value of x. In other words, the average scatter of the points around the curve is the same at all parts of the curve. The assumption is termed *homoscedasticity*. If the SD is not constant but rather is proportional to the value of y, weight the data to minimize the sum of squares of the relative distances.
4. The model assumes that x is known exactly. This is rarely the case, but it is sufficient to assume that any imprecision in measuring x is very small compared to the variability in y.
5. The errors are independent. The deviation of each value from the curve should be random, and should *not* be correlated with the deviation of the previous or next point. If there is any carryover from one sample to the next, this assumption will be violated.

EVALUATING RESULTS OF NONLINEAR REGRESSION

Before accepting the results of nonlinear regression, the following questions should be asked.

Did the Program Converge on a Solution?

A nonlinear regression program will stop its iterations when it cannot improve the fit by adjusting values of any of the parameters. At that point, the program is said to have *converged* on the best fit. In some cases, the program gets stuck. It does not know whether the fit would improve by increasing or decreasing the value of a parameter. When this happens, the program stops. The exact wording of the error message is unlikely to be helpful. In this situation, some programs may still apparently show results, but these "results" do not represent a best-fit curve.

Are the Results Scientifically Plausible?

The mathematics of curve fitting sometimes yields results that make no scientific sense. For example, noisy or incomplete data can lead to negative rate constants, fractions greater than 1.0, and negative K_d values.

If the results make no scientific sense, they are unacceptable, regardless of R^2 and of how close the curve comes to the points. Try a simpler equation, or try fixing some parameters to constant values.

Also check that the best-fit values of the parameters are reasonable compared to the range of the data. Do not trust the results if the top plateau of a sigmoid curve is far higher than the highest data point. Do not trust the results if an EC_{50} value is not within the range of the x values.

How Wide are the Confidence Intervals?

In addition to reporting the values of the parameters that make the equation fit the data best, nonlinear regression programs also express the uncertainty as a 95% confidence interval (CI) for each parameter. If all the assumptions of nonlinear regression are true, there is a 95% chance that the interval contains the true value. More precisely, if a nonlinear regression is performed many times (on different data sets), the confidence intervals will include the true value 95% of the time, but exclude the true value the other 5% of the time (but you will not know when that happens).

Three factors can make the confidence interval too narrow (i.e., narrower than the true range of parameter values):

1. The CI is based only on the scatter of data points around the curve within this one experiment. If the data points happen to fall right on a curve that agrees with the model, the CI may be very small. If the experiment is repeated many times, the scatter between the results is likely to be greater than predicted from the CI determined in one experiment.
2. If any of the assumptions of nonlinear regression are violated, the confidence intervals will probably be too narrow.
3. The confidence intervals from nonlinear regression are calculated using mathematical shortcuts and so are referred to as *asymptotic confidence intervals* or *approximate confidence intervals*. In some cases, these intervals can be too narrow (too optimistic).

Because of these problems, the confidence intervals should not be interpreted too rigorously. Rather than focusing on the CI reported from analysis of a single experiment, repeat the experiment several times.

If the confidence interval is extremely wide (e.g., > 50% of the parameter value), do not trust the results. Confidence intervals are wide when the data are very scattered, data have not been collected over a wide enough range of *x* values, or you are fitting too many parameters and should constrain one or more parameters to a constant value.

The data in Figure 7.5.28 were fit to a dose-response curve, and the 95% CI for the EC_{50} extends over six orders of magnitude. The explanation is simple. Since the data do not define plateaus at either the top or the bottom, zero and one hundred are not defined. This makes it impossible to determine the EC_{50} with precision.

In this example, it might make scientific sense to set the bottom plateau to 0% and the top plateau to 100% (if the plateaus were defined by other controls not shown on the graph). If this were done, the equation would fit fine and the confidence interval would be narrow.

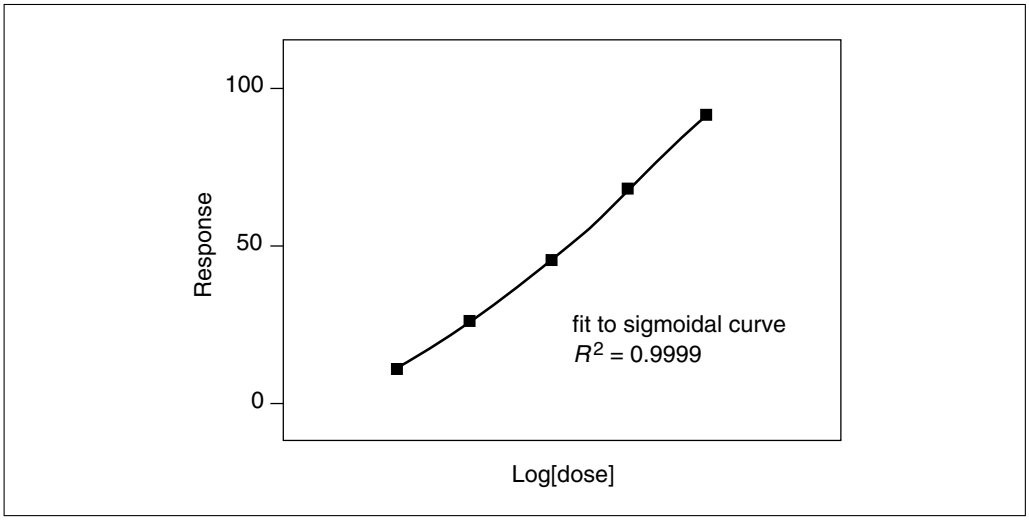

Figure 7.5.28 A dose-response curve with data collected over a narrow range of concentrations. When a nonlinear regression program tries to fit the top and bottom plateaus as well as the EC_{50} and slope, the resulting confidence intervals are very wide. Since there is no data to define zero and one hundred, the program will be very uncertain about the EC_{50}. If the nonlinear regression program is told to set the top and bottom plateaus to constant values (from controls), then it can determine the EC_{50} with precision.

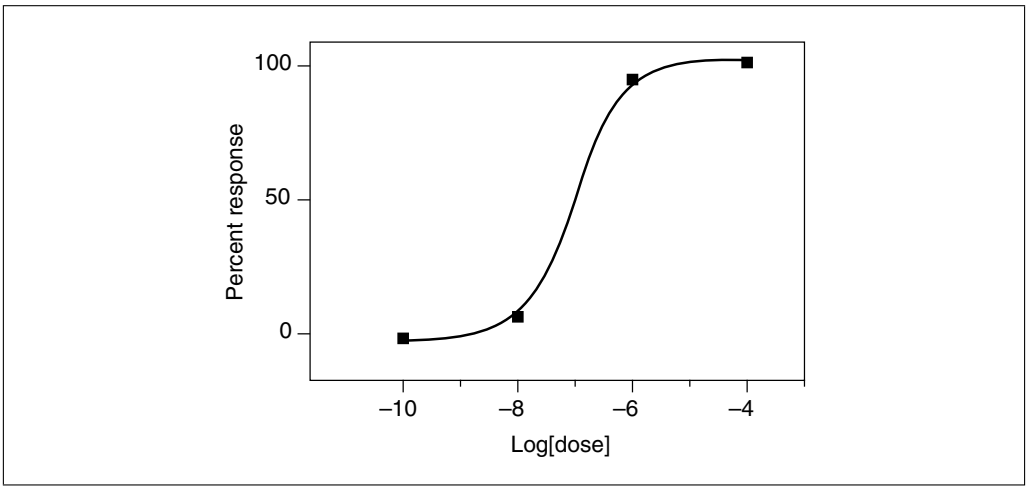

Figure 7.5.29 A dose response curve with no data in the middle of the curve. Since there are no data points in the middle of the curve, the best-fit value of the EC_{50} will be uncertain with a wide confidence interval.

Note that the problem with the fit is not obvious by inspecting a graph, because the curve goes very close to the points. The value of R^2 (0.9999) is also not helpful. That value also indicates that the curve comes close to the points, but does not indicate whether the fit is unique.

The CI is also wide when data in an important part of the curve has not been collected. The dose-response curve in Figure 7.5.29 has wide confidence intervals. Even when constraining the bottom to be zero, the top to be 100, and the slope to equal 1.0, the 95% CI for the EC_{50} extends over almost an order of magnitude. The problem is simple. The EC_{50} is the concentration at which the response is half-maximal, and this example has no data near that point.

Finally, the CI is wide if one tries to fit data to a two-site model when the data really follow a one-site model. In this case, the program might report very wide confidence intervals, as it will report that the two sites are very similar.

Some programs report the standard error of each parameter, instead of (or in addition to) the confidence interval. It is not entirely straightforward to interpret the standard error values. The best use of the standard error values is to calculate 95% confidence intervals (CI), if the nonlinear regression program does not calculate one. The 95% confidence interval extends from approximately two standard errors below the best-fit value to approximately two standard errors above the best-fit value (the number 2.0 is approximate; the exact multiplier comes from the *t* distribution and depends on the number of degrees of freedom, which equals the number of data points minus the number of parameters fit by the program).

Does the Curve Come Close to the Points?

In rare cases, the fit may be far from the data points. This may happen, for example, if the wrong equation is chosen. Look at the graph to make sure this did not happen.

Goodness of fit can also be evaluated by looking at the value of R^2 (known by statisticians as the *coefficient of determination*). R^2 is the fraction of the total variance of y that is explained by the model (equation). Mathematically, it is defined by the equation: $R^2 = 1.0 - SS/s_y^2$, where s_y^2 is the variance (standard deviation squared) of y values. The value of R^2 is always between 0.0 and 1.0, and it has no units.

When R^2 equals 0.0, the best-fit curve fits the data no better than a horizontal line going through the mean of all y values. In this case, knowing x does not help you predict y. When $R^2 = 1.0$, all points lie exactly on the curve with no scatter; if x is known, y may be calculated exactly.

If R^2 is high, the curve comes closer to the points than would a horizontal line through the mean y value, but a high R^2 should not be overinterpreted. It does not mean that the chosen equation is the best to describe the data. It also does not mean that the fit is unique—other values of the parameters may generate a curve that fits just as well.

When comparing one- and two-site models, it is not sufficient to simply compare R^2 values.

It is easy to put too much importance on the value of R^2. It only quantifies how close the curve comes to the points. Evaluating nonlinear regression results requires looking at more than the R^2.

Do the Data Systematically Deviate from the Curve?

If the data really follow the model described by the chosen equation, the data points should be randomly scattered above and below the curve. The distance of the points from the curve should also be random, and not be related to the value of x.

The best way to look for systematic deviations of the points from the curve is to inspect a graph of the residuals and to look at the runs test.

Residuals

A residual is the distance of a point from the curve. A residual is positive when the point is above the curve, and is negative when the point is below the curve. The residual table has the same x values as the original data, but each y value is replaced by the vertical distance of the point from the curve. An example is shown in Figure 7.5.30. As shown in panel A, the data points are not randomly distributed above and below the curve. There are clusters of points all above or all below. This is much easier to see on the graph in panel B. The points are not randomly scattered above and below the x axis.

The runs test

The runs test determines whether the data deviate systematically from the equation you selected. A run is a series of consecutive points that are either all above or all below the regression curve. Another way of saying this is that a run is a series of points whose residuals are either all positive or all negative.

If the data points are randomly distributed above and below the regression curve, it is possible to calculate the expected number of runs. If there are fewer runs than expected, it may mean that the regression model is wrong. If the data really follow the equation used to create the curve, the P value from the runs test may be used to determine the chance of obtaining as few (or fewer) runs as observed in the experiment. If the P value is small, it indicates that the data really do not follow the model.

In the example in Figure 7.5.30, the equation does not adequately match the data. There are only six runs, and the P value for the runs test is very small. This means that the data systematically deviate from the curve, and the data were fit to the wrong equation.

The replicates (or lack of fit) test

When evaluating a nonlinear fit, one question you might ask is whether the curve is "too far" from the points. The answer, of course, is another question: too far compared to

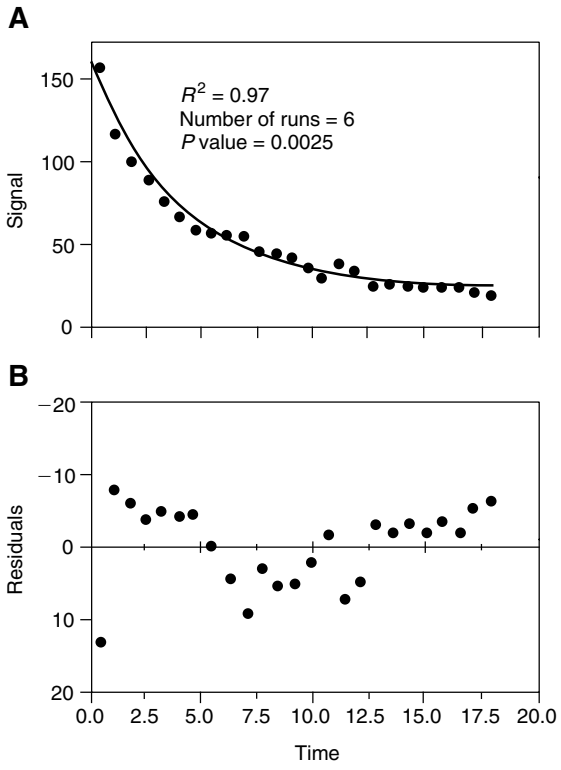

Figure 7.5.30 Residuals. The top panel (**A**) graphs dissociation kinetic data. The bottom panel (**B**) shows the residuals (i.e., the *y* axis plots the distance between the point and the curve from the top panel).

what? If you have collected one *y* value at each *x* value, you can't really answer that question (except by referring to other similar experiments). But if you have collected replicate *y* values at each *x*, then you can ask whether the average distance of the points from the curve is "too far" compared to the scatter among replicates.

If the *P* value is low, it means that the scatter of the points around the best-fit curve is much greater than predicted by the scatter among the replicates. This is evidence that the data may actually follow a model that is different than the model that you chose (or that the replicates are not fully independent from one another).

If the *P* value is high, it means the scatter of points from the curve is entirely consistent with the scatter among the replicates. There is no reason (based on this test) to question the validity of the model. You should not conclude that you have chosen the best possible model. But you can conclude that the fit of the model is entirely consistent with expectations from the scatter among replicates within the range of *x* values you chose to use.

Is the Fit a Local Minimum?

The nonlinear regression procedure adjusts the parameters in small steps in order to improve the goodness of fit. If GraphPad Prism converges on an answer, altering any of the parameters a little bit will make the fit worse. But it is theoretically possible that large changes in the parameters might lead to much better goodness of fit. Thus, the curve that Prism decides is the "best" may really not be the best.

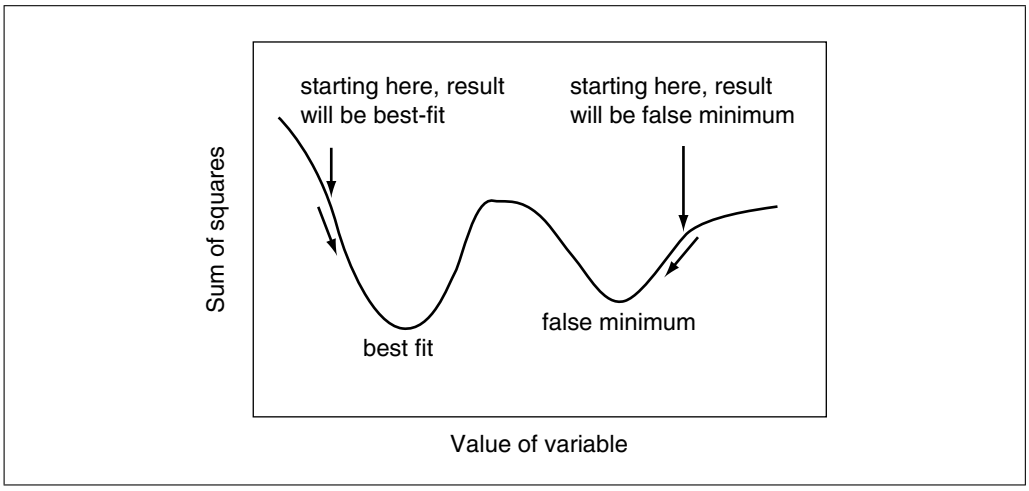

Figure 7.5.31 What is a false minimum? A nonlinear regression program stops when making any small change to a parameter will worsen the fit and thus raise the sum of squares. In rare cases, this may happen at a false minimum rather than the true best fit value.

Think of latitude and longitude as representing two parameters Prism is trying to fit. Now think of altitude as the sum of squares. Nonlinear regression works iteratively to reduce the sum of squares. This is like walking downhill to find the bottom of the valley. When nonlinear regression has converged, changing any parameter increases the sum of squares. When at the bottom of the valley, every direction leads uphill. But there may be a much deeper valley over the ridge that is unknown (see Fig. 7.5.31). In nonlinear regression, large changes in parameters might decrease the sum of squares.

This problem (called finding a local minimum) is intrinsic to nonlinear regression, no matter what program is used. A local minimum will rarely be encountered if the data have little scatter, the data are collected over an appropriate range of x values, and an appropriate equation is chosen.

To continue the analogy, the confidence intervals for the parameters are very wide when the bottom of the valley is very flat. A great distance can be traveled without changing elevation. The values of the parameters can be changed a great deal without changing the goodness of fit.

To test for the presence of a false minimum:

1. Look at the fitted curve and see if it comes close to the data or if it deviates systematically (also check runs and replicates tests). Such systematic deviations may result either from an incorrect model or from convergence to a local minimum.
2. Note the values of the parameters and the sum of squares from the first fit.
3. Make a large change to the initial values of one or more parameters and run the fit again. Repeat several times.
4. Ideally, nonlinear regression will report nearly the same sum of squares and same parameters regardless of the initial values. If the values are different, accept the ones with the lowest sum of squares.

What to Do When the Fit Is No Good?

The previous sections explained how to identify a bad fit. If any of these situations are encountered, Table 7.5.11 describes some things to try.

Table 7.5.11 Troubleshooting Guide to Evaluating Results of Nonlinear Regression

Potential problem	Solution
The equation simply does not describe the data.	Try a different equation.
The initial values are too far from their correct values.	Enter different initial values. If using a user-defined equation, check the rules for initial values.
The range of x values is too narrow to define the curve completely.	If possible, collect more data. Otherwise, hold one of the variables to a constant value.
There is not enough data collected in a critical range of x values.	Collect more data in the important regions.
The data are very scattered and don't really define a curve.	Try to collect less scattered data. If combining several experiments, normalize the data for each experiment to an internal control.
The equation includes more than one component, but the data don't follow a multicomponent model.	Use a simpler equation.
The numbers are too large.	If the y values are very large, change the units. Do not use values greater than $\sim 10^4$.
The numbers are too small.	If your y values are very small, change the units. Do not use values less than $\sim 10^{-4}$.

Table 7.5.12 Log(K_i) Values for a Sample Competitive Binding Experiment

Experiment	Control	Treated
1	−6.13	−6.53
2	−6.39	−6.86
3	−5.92	−6.31

COMPARING TREATMENT GROUPS

The results of radioligand binding experiments will often be compared between treatment groups. There are three ways to do this.

Compare the Results of Repeated Experiments

After repeating the experiment several times, compare the best-fit value of a parameter for each fit of control and treated preparations using a paired t test (or the analogous Wilcoxon nonparametric test).

For example, in Table 7.5.12, the log(K_i) values of results from a competitive binding curve performed with two groups of cells are shown. Compare the results using a paired t test. The t ratio is 16.7, and the P value is 0.0036 (two-tail). If the treatment did not alter the log(K_i), there is only a 0.36% chance that such a large difference (or larger) between log(K_i) is by chance. Since the P value is so low, conclude that the change in K_i was statistically significant.

Note that we compare log(K_i) values rather than K_i values. When doing a paired t test, a key assumption is that the distribution of differences (treated versus control) follow a Gaussian distribution. Since a competitive binding curve (similar to a dose

response curve) is conducted with x values (concentration) equally spaced on a log scale, the uncertainty of the EC_{50} is reasonably symmetrical (and perhaps Gaussian) when expressed on a log scale. It is equally likely that the best-fit value of the log (K_i) is 0.1 log units too high or 0.1 log units too low. In contrast, the uncertainty in K_i is not symmetrical.

Compare the Results Within One Experiment: Extra Sum-of-Squares Approach

To compare two curves, first fit the two curves separately, and then fit all the data globally to fit one curve to all the data. Comparing the sum of the sum of squares with the independent fit to the sum of squares for the global fit can result in a P value that compares the fits (Munson and Robard, 1980).

For example, competitive binding curves of control and treated cells were compared in an experiment performed once. Nonlinear regression fit three parameters, Top, Bottom, and log(EC_{50}). Only the log(EC_{50}) values are of interest. In this example, the control log(EC_{50}) was −6.08 with a standard error of 0.3667. The treated log(EC_{50}) was −6.20 with a standard error of 0.0617.

GraphPad Prism can compare two fits with the extra sum-of-squares F test by checking an option. The instructions below assume that you are using a program that cannot do the comparison automatically. It is also useful to follow these steps to understand how the approach works.

1. Fit the two data sets separately as in the previous section.
2. Total the sum of squares and DF from the two fits. For this example the total sum of squares equals 19,560 + 29,320 = 48,880, and the total DF equals 12 + 12 = 24. Since these are the results of fitting the two data sets separately, label these values $SS_{separate}$ and $DF_{separate}$.
3. Combine the two data sets into one. For this example, the combined data set has 30 xy pairs, with each x value appearing twice.
4. Fit the combined data set to the same equation. Note the SS and DF. For this example, SS = 165,200, and DF = 27 (30 data points minus three parameters). Call these values $SS_{combined}$ and $DF_{combined}$.
5. $SS_{separate}$ is expected to be smaller than $SS_{combined}$ even if the curves are really identical, simply because the separate fits have more degrees of freedom. Two separate curves will always come closer to the points than one combined curve. The question is whether the SS values are more different than expected by chance. To find out, calculate the F ratio using the equation:

$$F = \left(\frac{SS_{combined} - SS_{separate}}{SS_{combined}} \right) \Bigg/ \left(\frac{DF_{combined} - DF_{separate}}{DF_{separate}} \right)$$

Equation 7.5.46

   For this example, $F = 19.03$.

6. Determine the P value from F. There are 3 degrees of freedom ($DF_{combined} - DF_{separate}$) in the numerator, and 24 degrees of freedom ($DF_{separate}$) in the denominator. Use the free QuickCalc Web calculator at *http://www.graphpad.com*, or this Excel formula:

```
= FDIST(19.03, 3, 24)
```

7. For this example, the *P* value is <0.0001. If the treatment is really ineffective, there is less than 0.01% chance that the two curves would differ as much (or more) as they differed in this experiment. Since the *P* value is low, you'll conclude that the curves are really different.

This method only uses data from one experiment. Despite the impressively low *P* value, these results should not be trusted until the experiment is repeated. This method compares the curves overall. It does not determine which parameter(s) are different. Differences might be due to something trivial such as a different baseline, rather than something important such as a different EC_{50}.

Compare the Results Within One Experiment: *t* Test Approach

Since each best-fit value of a parameter is reported with a confidence interval (CI) and a standard error, a *t* test can determine whether the difference between best-fit values is greater than what would be expected by chance.

Compare the two groups with an unpaired *t* test.

1. Calculate the *t* ratio as the difference between $\log(EC_{50})$ values divided by the standard error of that difference (calculated from the two standard errors). Since the sample size is the same in the two groups, use the equation:

$$t = \frac{\log(EC_{50})_A - \log(EC_{50})_B}{\sqrt{SEM_A^2 + SEM_B^2}} = 2.292$$

Equation 7.5.47

2. Calculate the number of degrees of freedom (DF), which equals the sum of the number of degrees of freedom in each group. This equals the number of data points minus the number of parameters fit by the nonlinear regression procedure. In this example, there were 15 data points, and three parameters were fit. So there are 12 DF in each group, and 24 DF altogether.
3. To determine the *P* value that corresponds to the values of *t* and DF, use the free QuickCalc Web calculator at *http://www.graphpad.com* or this Excel formula (the first parameter is *t*, the second is DF, and the third is always 2, since you want a two-tail *P* value):

```
=TDIST(2.292,24,2)
```

For this example, the *P* value is 0.0309. If the treatment really didn't alter the EC_{50}, there is only a 3.09% chance that this large of a difference (or more) is by coincidence. Since the *P* value is so low, it is concluded that the two EC_{50} values are statistically significantly different.

You don't need to do the calculations manually. GraphPad Prism, GraphPad InStat, and many other programs can compute *t* and the *P* value from data entered as mean, standard error of the mean (SEM), and *N*. Enter the best-fit value of the $\log(EC_{50})$ (or any other fit parameter) instead of the mean, and the standard error (SE) of that parameter instead of the SEM. The trick is figuring out what value to enter as "*N*" (sample size). Remember that:

1. For nonlinear regression, the number of degrees of freedom equals the number of data points minus the number of parameters fit.

2. For an ordinary *t* test, the number of degrees of freedom for each sample equals one less than the number of data points.
3. The *t* test calculations are based on the numbers of degrees of freedom. However, most programs ask for *N* instead, and then compute DF as $N - 1$. When comparing the results of nonlinear regression, enter *N* as the number of degrees of freedom plus 1. The program will subtract 1 to determine the DF. All the other calculations are based on the value of DF, and *N* is ignored. In this example, enter $N = 12 + 1 = 13$ for each group.

This method only uses data from one experiment. The SE value is a measure of how precisely the $\log(EC_{50})$ has been determined in this one experiment. It is not a measure of how reproducible the experiment is. Despite the impressive *P* value, these results should not be trusted until the experiment is repeated.

The *t* test assumes that the uncertainty in the values of the parameters follows a Gaussian distribution. This assumption is not necessarily true with the SE values that emerge from nonlinear regression. The only way to assess the validity of this assumption is to simulate many sets of data, fit each with nonlinear regression, and examine the distribution of best-fit values. This has been done with many commonly used equations, and it seems that the assumption is reasonable in many cases.

To avoid this problem when fitting binding data, compare $\log(EC_{50})$, not EC_{50}. You want to express the parameters in a form that makes the uncertainty as symmetrical and Gaussian as possible. In general, the uncertainty of $\log(EC_{50})$, but not of EC_{50} itself, is reasonably symmetrical (and perhaps Gaussian). It is equally likely that the observed $\log(K_i)$ is 0.1 log units too high or 0.1 log units too low. In contrast, the uncertainty in K_i is not symmetrical.

Advantages and Disadvantages of the Three Approaches

If the experiment has been repeated several times, use the first method (Comparing the Results of Repeated Experiments). There are two advantages. The first is that compared to the other methods discussed, this method is far easier to understand and communicate to others. Second, the entire test is based on the consistency of the results between repeat experiments. Since there are usually more causes for variability between experiments than within experiments, it makes sense to base the comparison on differences between experiments.

The disadvantage of the first method is that information is being discarded. The calculations are based only on the best-fit value from each experiment, and they ignore the SE of those values presented by the curve fitting program.

If the experiment has been performed only once, the experiment should be repeated. Regardless of what statistical results are obtained, results from a single experiment should not be trusted. To compare results in a single experiment, the extra sum-of-squares *F* test is a better test (but a bit more work) than the *t* test.

Generally, only one parameter is of interest (i.e., a rate constant or EC_{50}); the others are less important. The *t* test compares only the parameter you care about. The extra sum-of-squares *F* test is more general. Since that method compares the entire curve, it does not force a decision regarding which parameter(s) to compare. This is both its advantage and disadvantage.

CALCULATIONS WITH RADIOACTIVITY

Efficiency of Detecting Radioactivity

Efficiency is the fraction of radioactive disintegration that is detected by the counter. Efficiency is determined by counting a standard sample under conditions identical to those used in the experiment.

With ^{125}I, the efficiency is usually >90%, depending on the geometry of the counter. The efficiency is not 100% because the detector does not entirely surround the tube, which allows a few gamma rays (photons) to miss the detector.

With tritium (^{3}H), the efficiency of counting is much lower, and usually varies between 30% and 50%. The low efficiency is mostly a consequence of the physics of decay and cannot be improved by better instrumentation or better scintillation fluid. When a tritium atom decays, a neutron converts to a proton and the reaction emits an electron and neutrino. The energy released is always the same, but it is randomly partitioned between the neutrino (not detected) and an electron (detection attempted). When the electron has sufficient energy, it will travel far enough to encounter a fluor molecule in the scintillation fluid. This fluid amplifies the signal and gives off a flash of light detected by the scintillation counter. The intensity of the flash (number of photons) is proportional to the energy of the electron. If the electron has insufficient energy, it is not captured by the fluor and is not detected. If it has low energy, it is captured but the light flash has few photons and is not detected by the instrument. Since the decay of many tritium atoms does not lead to a detectable number of photons, the efficiency of counting is much less than 100%.

The efficiency of counting ^{3}H is reduced by the presence of any color in the counting tubes, if the mixture of water and scintillation fluid is not homogeneous, or if the radioactivity is trapped in the tissue (thus emitted electrons do not travel into the scintillation fluid).

Specific Radioactivity

Radioligand packaging usually states the specific radioactivity as Curies per millimole (Ci/mmol). Because measurements are expressed in counts per minute (cpm), the specific radioactivity is more useful when stated in cpm. Often, for radioligand binding experiments, it is useful to express the specific radioactivity as cpm/fmol (1 fmol = 10^{-15} mol).

To convert from Ci/mmol to cpm/fmol, know that 1 Ci equals 2.22×10^{12} disintegrations per minute (dpm). Use this equation to convert Z Ci/mmol to Y cpm/fmol when the counter has an efficiency (expressed as a fraction) equal to E.

$$Y\,\frac{\text{cpm}}{\text{fmol}} = Z\,\frac{\text{Ci}}{\text{mmol}} \times 2.22 \times 10^{12}\,\frac{\text{dpm}}{\text{Ci}} \times 10^{-12}\,\frac{\text{mmol}}{\text{fmol}} \times E\,\frac{\text{cpm}}{\text{dpm}}$$

Equation 7.5.48

$$Y = Z \times 2.22 \times E \quad (\text{in cpm}/\text{fmol})$$

Equation 7.5.49

For example, the specific activity will be 2190 Ci/mmol if every molecule incorporates exactly one ^{125}I atom. If the counting efficiency is 85%, then the specific activity is 2190 × 2.22 × 0.85 = 4133 cpm/fmol.

In many countries, radioligand packaging states the specific radioactivity in GBq/mmol, rather than Ci/mmol. To convert to cpm/fmol, you need to know that 1 Bq (Becquerel) is one radioactive disintegration per second (1 GBq = 10^9 dps). To convert from GBq/mmol to cpm/fmol, use this equation:

$$Y\,\frac{\text{cpm}}{\text{fmol}} = Z\,\frac{\text{GBq}}{\text{mmol}} \times 10^{9}\,\frac{\text{dps}}{\text{GBq}} \times 60\,\frac{\text{sec}}{\text{min}} \times 10^{-12}\,\frac{\text{mmol}}{\text{fmol}} \times E\,\frac{\text{counts}}{\text{disintegrations}} = Z \times 0.06 \times E$$

Equation 7.5.50

If every molecule is labeled with ^{125}I, the specific activity is 81,030 GBq/mmol. If the counting efficiency is 85%, then the specific activity can also be expressed as 81,030 × 0.06 × 0.85 = 4133 cpm/fmol.

Calculating the Concentration of the Radioligand

Rather than trust dilutions, the concentration of radioligand in a stock solution can be accurately calculated. Measure the cpm in a small volume of solution and use the following equation, in which *C* is cpm counted, *V* is volume of the solution in ml, and *Y* is the specific activity of the radioligand in cpm/fmol (calculated in the previous section).

$$\text{concentration in pM} = \frac{C\ \text{cpm}}{\frac{Y\ \text{cpm/fmol}}{V\ \text{ml}} \times \frac{0.001\ \text{pmol/fmol}}{0.001\ \text{liter/ml}}} = \frac{C/Y}{V}$$

Equation 7.5.51

Radioactive Decay

Radioactive decay is entirely random. The probability of decay at any particular interval is the same as the probability of decay during any other interval. Starting with N_0 radioactive atoms, the number remaining at time *t* is:

$$N_t = N_0 \times e^{-k_{\text{decay}}t}$$

Equation 7.5.52

The rate constant of decay (k_{decay}) is expressed in units of inverse time. Each radioactive isotope has a different value of k_{decay}. The value *e* refers to the base of natural logarithms (2.71828).

The half-life ($t_{1/2}$) is the time it takes for half the isotope to decay. Half-life and the decay rate constant are related by this equation:

$$t_{1/2} = \frac{\ln(2)}{k_{\text{decay}}} = \frac{0.693}{k_{\text{decay}}}$$

Equation 7.5.53

Table 7.5.13 shows the half-lives and rate constants for commonly used radioisotopes. The table also shows the specific activity assuming that each molecule is labeled with one radioactive atom. This is often the case with ^{125}I and ^{32}P. Tritiated molecules often incorporate two or three tritium atoms, which increases the specific radioactivity.

Table 7.5.13 Half-Lives and Rate Constants for Commonly Used Isotopes

Isotope	Half-life	k_{decay}	Specific radioactivity
^{3}H	12.43 years	0.056 $year^{-1}$	28.7 Ci/mmol
^{125}I	59.6 days	0.0116 day^{-1}	2190 Ci/mmol
^{32}P	14.3 days	0.0485 day^{-1}	9128 Ci/mmol
^{35}S	87.4 days	0.0079 day^{-1}	1493 Ci/mmol

Radioactive decay can be calculated from a date where you knew the concentration and specific radioactivity using the equation:

$$\text{fraction remaining} = e^{-k_{decay}t}$$

Equation 7.5.54

For example, after ^{125}I decays for 20 days, the fraction remaining equals 79.5%. Although data appear to be scanty, most scientists assume that the energy released during decay destroys the ligand so it no longer binds to receptors. Therefore, the specific radioactivity does not change over time. What changes is the concentration of ligand. After 20 days, the concentration of the iodinated ligand is 79.5% of what it was originally, but the specific radioactivity remains 2190 Ci/mmol. This approach assumes that the unlabeled decay product is not able to bind to receptors and has no effect on the binding. Rather than trust this assumption, use newly synthesized or repurified radioligand for key experiments.

Calculations of radioactive decay are straightforward only when each molecule is labeled with a single radioactive isotope, as is usually the case. If a molecule is labeled with several radioactive isotopes, the effective half-life is shorter. If only a fraction of the molecules are labeled with a radioactive isotope, then the decay formula only applies to the labeled portion of the mixture, as the concentration of the unlabeled compound never changes.

Counting Error and the Poisson Distribution

The decay of a population of radioactive atoms is random, and therefore subject to a sampling error. For example, the radioactive atoms in a tube containing 1000 cpm of radioactivity will not give off exactly 1000 counts in every minute. There will be more counts in some minutes and fewer in others, with the distribution of counts following a Poisson distribution. This variability is intrinsic to radioactive decay (or any stochastic process) and cannot be reduced by more careful experimental controls (but can be reduced by counting for a longer time to accumulate more events). There is no way to know the "real" number of counts, but a range of counts can be calculated that is 95% certain to contain the true average value. As long as the number of counts (C) is greater than ~100, the confidence interval can be calculated using this approximation:

$$95\%\ \text{CI}:\ (C - 1.96\sqrt{C})\ \text{ to }\ (C + 1.96\sqrt{C})$$

Equation 7.5.55

Computer programs can calculate a more exact confidence interval, as becomes necessary when C is less than ~100. For example, if $C = 100$, the simple equation above calculates a 95% confidence interval from approximately 80 to 120. A more exact equation calculates an interval from 81.37 to 121.61.

Table 7.5.14 Determination of Confidence Values

	1 min	10 min	100 min
Counts per min (cpm)	100	100	100
Total counts	100	1000	10000
95% CI of counts	81.4 to 121.6	938 to 1062	9804 to 10196
95% CI of cpm	81.4 to 121.6	93.8 to 106.2	98.0 to 102.0

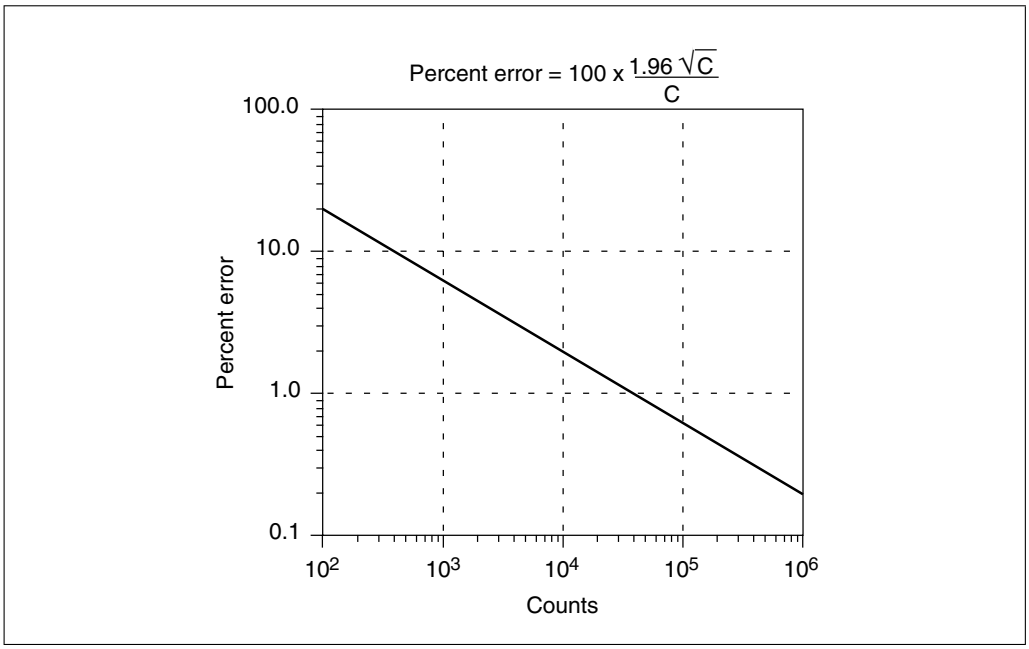

Figure 7.5.32 Counting error. With more counts, the fractional counting error decreases. The *x* axis shows the number of radioactive decays actually counted (counts per minute times number of minutes).

When calculating the confidence interval, set *C* equal to the total number of counts you measured experimentally, *not* the number of counts per minute.

For example, if a radioactive sample is placed into a scintillation counter for 10 min, the counter detects 225 counts per minute. What is the 95% confidence interval? Since the total time was 10 min, the instrument must have detected 2250 radioactive disintegrations. The 95% confidence interval of this number extends from 2157 to 2343. This is the confidence interval for the number of counts in 10 min, so the 95% confidence interval for the average number of counts per minute extends from 216 to 234. That is, there is a 95% certainty that the average cpm value lies within this range.

The Poisson distribution explains why it is helpful to count samples longer when the number of counts is small. For example, Table 7.5.14 shows the confidence interval for 100 cpm counted for various times. When longer times are used, the confidence interval is narrower.

Figure 7.5.32 shows percent error as a function of *C*. Percent error is defined from the width of the confidence interval divided by the number of counts. Of course this graph only shows error due to the randomness of radioactive decay. This is only one source of error in most experiments.

ANALYZING DATA WITH GraphPad PRISM

GraphPad Prism is a general-purpose program for scientific graphics, statistics and nonlinear regression, available for both Windows and Macintosh computers.

While Prism is not designed especially for analyses of binding data, it is very well suited for such analyses. It provides a menu of commonly used equations, including all equations listed in this unit, and can automatically compare one- and two-site models with an *F* test. When analyzing competitive binding curves, Prism calculates the K_i from the IC_{50}. It can globally fit a family of curves at once, sharing specified parameters among the curves. The program can automatically create a residual plot and calculate the runs test and replicates test. In addition, Prism's manual and help screens, like this unit, explain the principles of curve fitting.

A trial version of the Windows or Mac versions of Prism can be obtained from the GraphPad Web site at *http://www.graphpad.com*. The trial version is fully functional for 30 days.

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