Scatchard Plot and Heterogeneity in Binding Affinity of Labeled and Unlabeled Ligand

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In saturation analysis the Scatchard plot is a generally accepted method for calculation of the affinity constant, \( K \), and the molar concentration, \( q \), of the binder. However, in a system where the \( K \)'s for the labeled and unlabeled ligand are unequal, a nonlinear plot can be obtained from which incorrect values for \( K \) and \( q \) may be calculated. This paper mathematically explains how the plot may deviate and under which conditions there will be a maximum in the curve. When the binding sites are homogeneous, the coordinates of this maximum can be used to calculate \( K \) and \( q \). A general mathematical expression is derived on the basis of which a linear curve can be constructed for calculation of \( q \) and \( K \), which is valid even when affinity for the labelled and unlabelled ligand is not identical.

In 1949 Scatchard (1) proposed a graphic method for calculating the association constant for small molecule/protein interactions. Later this plot was introduced into the field of radioimmunoassay by Berson and Yalow (2). With this type of assay, more generally termed "saturation analysis" (3), aliquots of a fixed amount of binder (e.g., antibody) are equili-

\[ K = K_a = \frac{[AQ]}{[A][Q]} \]  \hspace{1cm} (1)

\[ K_h = \frac{[HQ]}{[H][Q]} \]  \hspace{1cm} (2)

The total molar concentration of binder, labeled li-

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2 Terms, nonstandard abbreviations, and symbols used:

Binder: the specific binding agent—i.e., antibody, binding protein, tissue receptor protein, etc.

Ligand: the substance to be bound by the binder.

\( A \): the labeled ligand to be bound by the binder.

\( H \): the unlabelled ligand.

\( [A] \): molar concentration of the free labeled ligand.

\( [H] \): molar concentration of the free unlabelled ligand.

\( Q \): the binder (antibody, receptor protein, binding protein, specific binding agent).

\( [Q] \): molar concentration of the binding sites.

\( [AQ] \): molar concentration of the free binding sites.

\( [HQ] \): molar concentration of the bound unlabelled ligand.

\( K = K_a \): the affinity constant for the reaction between \( Q \) and \( A \).

\( K_h \): the affinity constant for the reaction between \( Q \) and \( H \).

\( A \): total molar concentration of the labeled ligand.

\( H \): total molar concentration of the unlabelled ligand.

\( q \): total molar concentration of the binding sites.

\( h/q \): the ratio of unlabelled to labeled ligand as present in the tracer material.

\( a \): \( K_h/K_a \). (In an earlier paper (5) this quotient has been symbolized as \( a \); because \( x \) is used in this paper to designate the values on the abscissa, the quotient \( K_h/K_a \) is here symbolized as \( a \). This symbol will be maintained as such in forthcoming publications.)

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The Basic Model

We consider the system in which only one order of reactive sites is associated with the binder \( Q \) in its reaction with the unlabelled ligand \( H \) or the labeled ligand \( A \). According to the law of mass action, in state of equilibrium the following equations hold:
gand, and unlabeled ligand, respectively, can be expressed as

\[ q = [Q] + [AQ] + [HQ] \]  
\[ a = [A] + [AQ] \]  
\[ h = [H] + [HQ] \]

The ratios of bound-to-total and free-to-total radioactivity are expressed as:

\[ R = \frac{[AQ]}{a} \]  
\[ 1 - R = \frac{[A]}{a} \]

Substitution of 6 and 7 in 1 results in

\[ [Q] = \frac{R}{K_a(1 - R)} \]  
Substitution of 5 and 8 in 2 gives

\[ [HQ] = \frac{K_h R}{K_a(1 - R) + K_h R} \]

Substitution of 6, 8, and 9 in 3 further gives

\[ q = \frac{R}{K_a(1 - R) + K_h R} \]

By putting \( K_h/K_a = \alpha \) and \( K_a = K \), equation 10 can be rearranged as

\[ \frac{Kq}{R} \frac{1}{1 - R} = K \left( 1 + \frac{\alpha h}{1 + R(\alpha - 1)} \right) \]  
(11)

The ratio between the concentration of unlabeled and labeled ligand in the tracer is

\[ \frac{h}{a} = n \]

11 can be written as

\[ \frac{Kq}{R} \frac{1}{1 - R} = K_a \left( 1 + \frac{n\alpha}{1 + R(\alpha - 1)} \right) \]  
(12)

In the situation where \( K_h = K_a \) (\( \alpha = 1 \)), equation 12 can be written as

\[ \frac{R}{1 - R} = K[q - Ra(1 + n)] \]  
(13)

By plotting the ratio bound-to-free radioactivity, \( R/(1 - R) \), against the amount of bound ligand, \( Ra(1 + n) \), a linear relationship is obtained (cf. Figure 1). From the intercepts of this line with ordinate and abscissa, respectively, the values of \( Kq \) and \( q \) can be determined.

When the labeled and unlabeled ligand are not identical with respect to their affinity for the binder (\( K_a \neq K_h \)), equation 13 does not apply. Now the question arises how \( Ra(1 + n) \) will behave as a function of \( R/(1 - R) \) in this situation.

For the experimental determination of this relationship it will be necessary to know the numerical value of \( n \), which can be evaluated from the specific activity of the tracer material.

**Evaluation of the Specific Activity of the Tracer**

This is generally done by comparing the quantities of tracer \( a_i \) and unlabeled ligand \( h_i \), which lower the initial binding \( R_0 \) to the same level, \( R_i \). Then

\[ \frac{h_i}{a_i} = n + 1 \]

However, this equation does not apply when \( K_h \neq K_a \).

If the initial binding \( R_0 \) is obtained in the equilibrium between a fixed amount of binder, \( q \), and a certain amount of tracer (consisting of labeled ligand,
of unlabeled ligand, \( h_0 = n a_0 \), equation 12 can be written as

\[
\frac{Kq}{R_0} \left( 1 + \frac{n a}{1 + R_0(\alpha - 1)} \right) = K a_0 \]  

(14)

\( R_0 \) can be reduced to \( R_1 \) by an additional amount of the same tracer material, consisting of labeled ligand, \( a_1 \) (radioactivity, expressed in molar units as explained in footnote 2) and of unlabeled ligand, \( n a_1 \),

\[
\frac{Kq}{R_1} \left( 1 + \frac{n a}{1 + R_1(\alpha - 1)} \right) = K (a_0 + a_1) \]  

(15)

or by adding unlabeled ligand \( h_1 \)

\[
\frac{Kq}{R_1} \left( 1 + \frac{n a}{1 + R_1(\alpha - 1)} \right) = K a_0 \left( 1 + \frac{1 + R_1(\alpha - 1)}{1 + R_1(\alpha - 1)} \right) + \frac{K h_1 a}{1 + R_1(\alpha - 1)} \]  

(16)

Combination of equations 15 and 16 gives

\[
\frac{h_1}{a_1} = n + \frac{1 + R_1(\alpha - 1)}{\alpha} \]  

(17)

With this formula, the correct value of \( n \) can be determined by plotting \( h_0 \) vs. \( R_1 \), as pointed out elsewhere (5). This plot also makes the estimation of \( \alpha \) possible, if the binder is homogeneous. It should be kept in mind that for correct application of equation 17, \( a_1 \) has to be expressed in the same unit of concentration as \( h_1 \) is expressed.3

Effect of Unequality of \( K_r \) and \( K_a (\alpha \neq 1) \) on the Shape of the Uncorrected Scatchard Plot

When \( \alpha \neq 1 \), rearrangement of equation 12 results in

\[
\frac{R}{1 - R} = K [q - Ra(1 + n)] - \frac{K Ran(\alpha - 1)(1 - R)}{1 + R(\alpha - 1)} \]  

(18)

Comparison of equation 18 with equation 13 demonstrates that when \( R/(1 - R) \) is plotted vs. \( Ra(1 + n) \) in a situation where \( \alpha \neq 1 \), a deviation from linearity will occur, equal to

\[
\Delta \frac{R}{1 - R} = \frac{K Ran(\alpha - 1)(1 - R)}{1 + R(\alpha - 1)} \]  

(19)

Equation 19 shows that the resulting curve runs to the right of the straight line corresponding to equation 13, when \( \alpha < 1 \), and to the left when \( \alpha > 1 \) (see Figure 1).

Extrapolation of the curve to \( R/(1 - R) \) approaches 0 results in an intersection with the abscissa equal to

\[
x_y = \frac{q(n + 1)}{an + 1} \]  

which can be seen by substitution of \( y \to 0 \) in equation 21 (vide infra), which is essentially equal to equation 18.

The situation \( \alpha < 1 \) can be considered rather exceptional, because this would imply that the iodinated ligand would fit the binder better than does the unlabeled ligand, which is usually the natural counterpart of the binder. A curve similar in shape to the one obtained under the condition that \( \alpha < 1 \) can occur in case of heterogeneity of the binding sites.

The Maximum for \( Ra(1 + n) \)

In practice, it will often occur that the plot of \( y = R/(1 - R) \) vs. \( x = Ra(1 + n) \) not only deviates from the straight line corresponding with equation 13 but may even regress in the direction of the ordinate, thus rendering a maximum value for \( x \), as shown in Figure 1.

After substitution of \( R/(1 - R) = y \) and of \( Ra(1 + n) = x \), equation 18 can be rearranged to

\[
x = \frac{y - q}{K} - \frac{Ra(1 - R)(\alpha - 1)}{1 + R(\alpha - 1)} \]  

(20)

and since \( Ra = x/(1 + n) \) and \( R = y/(1 + y) \), equation 20 can be written as

\[
x = \frac{(1 + y\alpha)(q - y)}{(1 + y\alpha) + (\alpha - 1)n/(1 + n)} \]  

(21)

In case of a maximum value for \( x \), \( dx/dy = 0 \), so that

\[(1 + n)\alpha^2 y_{max}^2 + 2\alpha(1 + n)\alpha y_{max} + 1 + n\alpha - K q\alpha(\alpha - 1) = \]  

(22)

and

\[y_{max} = \sqrt{\frac{(1 + n\alpha) + \sqrt{n(\alpha - 1)[K q\alpha(n + 1) + n\alpha + 1]}}{\alpha(1 + n)}} \]  

(23)

\[x_{max} = \frac{1}{K}\left[K q + \frac{2an + 1 - n - 2\sqrt{n(\alpha - 1)[K q\alpha(n + 1) + n\alpha + 1]}}{\alpha(n + 1)} \right] \]  

(24)

Since \( y > 0 \), \( \alpha > 0 \), and \( n > 0 \), it can be seen from 22, that

\[1 + n\alpha - K q\alpha(\alpha - 1) \leq 0 \]  

or

\[K q(\alpha - 1) \geq 1 + \frac{1}{n\alpha} \]  

(25)

A maximum for \( x \) in the Scatchard plot will be obtained only when \( n, \alpha \), and \( K_q \) meet the conditions as given in 25. This maximum will lie on the abscissa when \( y_{max} = 0 \). In that case

\[n = \frac{1}{\alpha[K q(\alpha - 1) - 1]} \]  

(26)

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3 In a chemically pure, monoidindoped compound, labeled with 125I, 1000 cpm corresponds to 2.075 × 10⁻¹⁸ mole. With a counting efficiency of 50% and a mol. wt. of 1125, 1000 cpm/ml represent 0.466 pg/ml or 4.15 × 10⁻¹³ mol/liter. In terms of a formula, suppose the radioactivity in \( v \) ml of a sample is \( t \) cpm, counted with an efficiency of \( e \% \); then

\[a = \frac{t}{v} \times 2.075 \times 10^{-14} \text{ mol/liter.}\]
as can be seen by substitution of $y_{\text{max}} = 0$ in equation 22.

Since $Kq$, $\alpha$, and $n$ are $\geq 0$, it can be seen from rearrangement of 25 that

$$\alpha \geq 1 + \frac{n\alpha + 1}{Kq(na)} \quad (27)$$

Consequently, another condition for reaching a maximum value of $Ra(1 + n)$ is $\alpha > 1$.

**Calculation of $K$ by Variation of the Specific Activity of the Tracer**

With a fairly homogeneous population of binding sites it may be possible to determine the correct value of $K$ from the coordinates of the maxim in the $R/(1 - R)$ vs. $Ra(1 + n)$ plots obtained by varying the ligand concentration at different specific activities of the tracer.

Combination of 23 and 24 results in

$$y_{\text{max}} = -\frac{K}{2} x_{\text{max}} + \frac{1}{2} \left( Kq - \frac{1}{\alpha} \right) \quad (28)$$

This equation indicates that when $Kq$ and $\alpha$ are constant the coordinates of the maxima, obtained at different specific activities of the tracer, fit a straight line (Figure 2). The slope of this line is $-K/2$, and its intersection with the abcissa is

$$x_{\text{max}} = \frac{1}{K} \left( Kq - \frac{1}{\alpha} \right) \quad (29)$$

When $x_{\text{max}}$ approaches 0, then by extrapolation

$$y_{\text{max}} = \frac{1}{2} \left( Kq - \frac{1}{\alpha} \right) \quad (30)$$

The coordinates of this point cannot be measured, because there is a minimum value for $x_{\text{max}}$ when $n \to \infty$. From equations 23 and 24 it can be calculated that when $n \to \infty$

$$y_{\text{max}} = -1 + \sqrt{(Kq + 1)(1 - 1/\alpha)} \quad (31)$$

$$y_{\text{max}} = \frac{1}{K} \cdot \sqrt{(Kq + 1 - \sqrt{1 - 1/\alpha})^2} \quad (32)$$

**Modified Way of Plotting When $\alpha \neq 1$**

Resulting in a Straight Scatchard Plot

When in saturation analysis labeled and unlabeled ligand differ in affinity for the binder, the Scatchard plot will deviate from linearity as described above. The question now arises how a linear plot can be obtained from which $K$ and $q$ can be calculated under the condition $\alpha \neq 1$. Rearrangement of equation 12 results in

$$\frac{R}{1 - R} = -KRa \left( 1 + \frac{n\alpha}{1 + R(\alpha - 1)} \right) + Kq \quad (33)$$

From equation 33 it can be seen that a plot of $R/(1 - R)$ on the ordinate vs. $Ra(1 + n\alpha/[1 + R(\alpha - 1)])$ on the abscissa will be linear. The slope of this line is $-K$ and it intercepts with the ordinate and abscissa equal $Kq$ and $q$, respectively, if the binding sites are homogeneous.

In fact, equation 33 is the general mathematical expression of the Scatchard plot in saturation analysis, which is valid for all values of $\alpha$ under the conditions outlined in the introduction (see also ref. 4).

From combination of equations 6 and 9 it can be derived that $Ra(1 + n\alpha/[1 + R(\alpha - 1)]$ represents the total molar concentration of bound (labeled and unlabeled) ligand:

$$[AQ] + [HQ] = Ra + \frac{Rh\alpha}{(1 - R) + \alpha R}$$
While \( a(1 + n) \) represents the total ligand concentration, \( a(1 + na/[1 + R(\alpha - 1)]) \) can be considered as an apparent concentration, the numerical value of which can be correlated with the tendency of the labeled plus unlabeled ligand to react with the binder. Thus \( a(1 + na/[1 + R(\alpha - 1)]) \) can be designated as the apparent immunoreactive concentration, symbolized as

\[
p = a\left(1 + \frac{n\alpha}{1 + R(\alpha - 1)}\right)
\]

The degree to which the unlabeled ligand \((h = na)\) expresses its immunoreactivity is determined by the factor

\[
z = \frac{\alpha}{1 + R(\alpha - 1)}
\]

For calculation of \( p \) it is necessary to know the values of \( n \) and \( \alpha \). This information can be obtained from a plot, which is based on equation 17 and described in more detail elsewhere (5). Probably the best approach to this evaluation of \( n \) is to plot the two standard curves (obtained by varying the amount of labeled (\( a \)) and unlabeled (\( h \)) ligand, respectively) according to Hales and Randle (6)—\( R/R \) vs. \( a \) or \( h \), respectively. From these two curves the quotient \( h_i/a_i \) can be calculated at various values for \( R_i \). When \( h_i/a_i \) is plotted vs. \( R_i \), a straight line will be obtained with a slope equal to \( 1 - 1/\alpha \) (cf. Figure 1 in ref. 5); extrapolation of this line to \( R = 1 \) will render

\[
[h_i/a_i]_{R = 1} = n + 1
\]

Discussion

Nonlinearity in the Scatchard plot, manifesting itself in a hyperbolic appearance, is generally recognized as a resultant of binder heterogeneity (e.g., ref. 2). A deviation in the opposite direction, which might occur when the affinity for the labeled and unlabeled ligand is unequal \((K_h > K_a)\), will be observed only when the plot is drawn over the full range of \( R/(1 - R) \). When in such a situation the plot is drawn through a rather restricted number of points, the deviation may not be observed or appear to be negligible, and this may lead to erroneous conclusions in the sense that

- the value of \( Kq \) will be slightly overestimated
- the value of \( q \) will be underestimated, and consequently
- the value of \( K \) will be overestimated.

The extent to which the calculated values of \( K \) and \( q \) will deviate from their real values depends very much on the magnitude of \( \alpha \) and \( n \). Even when only a small amount of unlabeled ligand is present in the tracer (e.g., \( n = 0.25 \) in Figure 2), this might markedly interfere with the linearity of the plot and with correct calculation of \( K \) and \( q \). With tracer of high specific activity \((n \) is small but not negligible), the underestimation of \( q \) (and consequently the overestimation of \( K \)) will be increased by the fact that the assumed value of \( n \)—and therefore the value of \( Ra(1 + n) \)—will be underestimated, as pointed out elsewhere (5).

When a fairly homogeneous population of binder is present (e.g., antibodies after affinity chromatography, or binding proteins after gel filtration), the set of maxima obtained by variation of the specific activity of the tracer may provide the correct values of \( K \) and \( q \), as indicated in Figure 2 and equation 28. However, this method cannot be applied generally, because binder heterogeneity will too often interfere in this approach.

A more advisable procedure for constructing the Scatchard plot in case \( \alpha \neq 1 \) is to plot on the abscissa the apparent immunoreactive concentration, \( p \), represented by equation 34, rather than the (assumed) bound ligand concentration \( Ra(1 + n) \).

In any case it is advisable, when radioiodinated label is used, first to attempt to determine the values of \( n \) and \( \alpha \) according to equation 17, then to apply these numbers in the formulas given above (e.g., equation 33) for determination of \( K \) and \( q \). When exact determination of \( \alpha \) is not possible, the set of maxima as in Figure 2, obtained by variation of the specific activity of the tracer, may enable the determination of the values of \( K \), \( q \), and \( \alpha \).

In fact, for its reaction with radioiodinated label, a binder is only fully characterized by \( K \) and \( \alpha \).

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