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# An accurate method for determination of receptor–ligand and enzyme–inhibitor dissociation constants from displacement curves

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**ABSTRACT** **Receptor–ligand dissociation constants are usually calculated from the displacement curve of a radioactively labeled ligand bound to the receptor. The formula used is restricted to cases in which the concentration of receptor is negligible compared to the concentration of both the displacing ligand and the radioactive ligand used. In this study, we rigorously derive a simple equation that can be used for calculating receptor–ligand dissociation constants for any set of experimental conditions. A linearized form of this equation provides a convenient plot from which the dissociation constant of the displacing ligand can be directly obtained. The plot is also a test for the competitive mode of binding. This exact equation now allows us to estimate the error incurred by the conventionally used equations. Similarly, we show that for competitive inhibition in enzymology, one can derive the analogous formula. Our new formula is free of the usual restrictions—namely, that the enzyme concentration is very small compared to the concentration of both the substrate and the inhibitor. It may therefore be applied to any set of experimental conditions.**

Ligand-binding experiments were first carried out in the early 1970s and have since revolutionized the study of receptors, as it became feasible to quantify the number of receptors and to determine their affinities to a variety of ligands by simple methodologies (1–3).

A classical way of determining the dissociation constant between a receptor and a nonradioactive ligand is by performing a displacement experiment. Such experiments are based on the competition for binding sites on the receptor between the nonradioactive ligand and the radioactive ligand. In practice, a single concentration of the radioactive ligand is used, and its displacement is measured as a function of increasing concentrations of the nonradioactive ligand. In the absence of displacing ligand, the receptor occupancy is given by

$$\bar{Y}_L = \frac{[RL]}{R_T} = \frac{[RL]}{[R] + [RL]} = \frac{[L]/K_L}{1 + [L]/K_L}, \qquad [1]$$

where $[RL]$ is the concentration of the receptor–ligand (radioactive) complex, $R_T$ is the total receptor concentration, $[L]$ is the concentration of the free radioactive ligand, and $K_L$ is the receptor–ligand dissociation constant. $\bar{Y}_L$ is the fraction of the receptor occupied by the radioactive ligand in the absence of a competing ligand.

In the presence of a competing ligand ($H$), Eq. 1 takes the form of

$$\bar{Y}_H = \frac{[RL]_H}{R_T} = \frac{[RL]_H}{[R] + [RL]_H + [RH]} = \frac{[L]_H/K_L}{1 + [L]_H/K_L + [H]/K_H}, \qquad [2]$$

where $K_H$ is the dissociation constant between the receptor and the competing ligand, and $\bar{Y}_H$ and $[L]_H$ are the fraction of the receptor occupied by the ligand and the concentration of free radioactive ligand, respectively, both in the presence of the competing ligand. The quantity of interest is actually $\bar{Y}_H/\bar{Y}_L$, which is given by

$$F = \frac{\bar{Y}_H}{\bar{Y}_L} = \frac{1 + [L]/K_L}{1 + [L]_H/K_L + [H]/K_H} \times \frac{[L]_H}{[L]}. \qquad [3]$$

Eq. 3 actually defines a displacement curve, where one plots $F$ vs. $\log[H]$ or $F$ vs. $[H]$. It is common practice among investigators to calculate the value for $K_H$ from the half-displacement point—namely, $F = 1/2$. Inserting $F = 1/2$ into Eq. 3 yields (2)

$$K'_H = \frac{[H]_{0.5}}{2[L]_H/[L] - 1 + [L]_H/K_L}. \qquad [4]$$

Eq. 4 may be used provided one knows the value of $[H]_{0.5}$. It is generally assumed that the concentration of the free nonradioactive ligand is equal to its total concentration ($[H] = H_T$) and in particular that

$$[H]_{0.5} = H_{T(0.5)}. \qquad [5]$$

In other words, it is assumed that throughout the titration $H_T >> R_T$. Under such conditions, the value of $K_H$ can indeed be obtained from the midpoint of the displacement curve using Eq. 4. When both the concentration of the primary radioactive ligand ($L$) and the displacing agent ($H$) are larger than that of the receptor ($L_T >> R_T$; $H_T >> R_T$), one obtains the very familiar Cheng and Prusoff (3) equation

$$K''_H = \frac{[H]_{0.5}}{[L]/K_L + 1}. \qquad [6]$$

Since here $[L]_L \sim [L]_H \sim [L]_T$, they are commonly designated by $[L]$. Eq. 6 is therefore valid only when the receptor concentration is much smaller than both the total concentration of the radioactive ligand used and the total concentration of the displacing agent.

Often, the conditions $[L] \sim [L]_H \sim L_T$ and $H_T \sim [H]$ are not met. When such a situation arises, the analysis of $F$ vs. $\log[H]$ or $[H]$ becomes complex and cannot be achieved by simple algebraic means (4). In such cases, one needs to calculate the value of $[H]$, using the relationship

$$H_T = [H] + [RH] = [H] + \frac{[H][R]}{K_H}. \qquad [7]$$

This makes the task of defining $\bar{Y}_H$ very complicated. We have therefore sought a reliable and accurate protocol to determine $K_H$ directly from a displacement curve of $\bar{Y}_H$ vs. $\log(H_T)$ (or vs. $H_T$), without any assumptions.

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## THEORY

Eqs. 8 and 9 are the accurate expressions for $K_L$ and $K_H$, respectively,

$$K_L = \frac{[R][L]}{[RL]} = \frac{(R_T - [RL] - [RH])(L_T - [RL])}{[RL]} \quad [8]$$

$$K_H = \frac{[R][H]}{[RH]} = \frac{(R_T - [RL] - [RH])(H_T - [RH])}{[RH]}. \quad [9]$$

It follows that

$$\frac{K_L}{K_H} = \frac{(L_T - [RL])[RH]}{(H_T - [RH])[RL]}. \quad [10]$$

Rearrangement of Eq. 10 yields

$$[RH] = \frac{K_L[RL]H_T}{(L_T - [RL])K_H + K_L[RL]}. \quad [11]$$

Insertion of the above expression for $[RH]$ into Eq. 8 yields

$$K_L^2[RL]^2 + [RL](L_T - [RL])(K_HK_L + K_LH_T) = (R_T - [RL])(L_T - [RL])\{(L_T - [RL])K_H + K_L[RL]\}. \quad [12]$$

Upon rearrangement, one obtains

$$K_H = \frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{R_T}{K_L}\left(\frac{L_T}{R_T} - \bar{Y}\right) - 1} + \frac{K_L^2\bar{Y}^2 - (1-\bar{Y})\left(\frac{L_T}{R_T} - \bar{Y}\right)\bar{Y}R_TK_L}{(1-\bar{Y})\left(\frac{L_T}{R_T} - \bar{Y}\right)^2R_T - \bar{Y}\left(\frac{L_T}{R_T} - \bar{Y}\right)K_L}. \quad [13]$$

Eq. 13 may be written more concisely as follows:

$$K_H = K'_H - \frac{K_L\bar{Y}}{\frac{L_T}{R_T} - \bar{Y}}, \quad [14]$$

where $K'_H$, which is the receptor–competing ligand dissociation constant when $H \sim H_T$ (see below), is given by

$$K'_H = \frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{R_T}{K_L}\left(\frac{L_T}{R_T} - \bar{Y}\right) - 1}. \quad [15]$$

$\bar{Y}$ in Eqs. 13–15 and in all following equations unless otherwise stated is equal to $\bar{Y}_H$—i.e., it is the fraction of the receptor occupied by the radioactive ligand in the presence of the competing ligand. Eq. 14 is the accurate expression for the dissociation constant between the receptor and the competing ligand as obtained from a displacement curve.

### Limiting Cases

(*i*) $H_T >> R_T$. When the concentration of the displacing agent always exceeds that of the receptor, Eq. 9 simplifies to

$$K'_H = \frac{(R_T - [RL] - [RH])H_T}{[RH]}. \quad [16]$$

Eq. 10 now simplifies to

$$\frac{K_L}{K'_H} = \frac{(L_T - [RL])[RH]}{[RL]H_T}. \quad [17]$$

Performing the same algebraic manipulations as before, one obtains

$$K'_H = \frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{R_T}{K_L}\left(\frac{L_T}{R_T} - \bar{Y}\right) - 1}. \quad [18]$$

(*ii*) $L_T >> R_T$. When only the ligand is in excess of the receptor, Eq. 9 simplifies to

$$K'^*_H = \frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{L_T}{K_L} - 1} - \frac{R_TK_L\bar{Y}}{L_T}. \quad [19]$$

(*iii*) $H_T, L_T >> R_T$. When both the ligand displaced as well as the displacing ligand are in excess of the receptor, Eq. 13 simplifies further to

$$K''_H = \frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{L_T}{K_L} - 1}. \quad [20]$$

When, in addition, $L_T >> K_L$, Eq. 13 simplifies even further to yield

$$K'''_H = \frac{\bar{Y}H_T}{(1-\bar{Y})L_T}K_L. \quad [21]$$

Eqs. 20 and 21 have already been derived for the special case of $\bar{Y} = 1/2$ (5).

When $H_T >> R_T$ and $L_T >> R_T$, one can use the Cheng–Prusoff (3) treatment to obtain the correct result. When these conditions are not met, Eq. 13 or 14 must be used.

## DISCUSSION

In this study, a rigorous method is presented for the determination of receptor–agonist dissociation constants from ligand displacement experiments. At any degree of receptor occupancy ($\bar{Y}$) by the primary ligand ($L$) and at any concentration of the displacing ligand ($H$), one can calculate the value of $K_H$ using the formulas derived and summarized in Table 1.

### A Test for Competitive and Noncooperative Behavior

Since the determination of $K_H$ results from a pair of $\bar{Y}$ and $H_T$ values, one must verify the michaelian and competitive nature of the relationship between $H$ and $L$. Different pairs of $\bar{Y}$ and the corresponding value of $H_T$ should yield the same value of $K_H$ if the binding is noncooperative as is assumed. More complex behavior—e.g., $H$ binding with more than one affinity—requires an analysis where more than one possible value for $K_H$ is assumed (6). A further test for noncooperativity and also for the competitive nature of the binding is obtained by rearranging Eq. 14 as follows:

$$\frac{H_T\left(\frac{L_T}{R_T} - \bar{Y}\right)}{(1-\bar{Y})\frac{R_T}{K_L}\left(\frac{L_T}{R_T} - \bar{Y}\right) - \bar{Y}} = K_H\frac{\frac{L_T}{R_T} - \bar{Y}}{\bar{Y}} + K_L. \quad [22]$$

Table 1. Formulas for receptor–agonist dissociation constants

| Conditions | Notation | Formula |
|---|---|---|
| General case | $K_H$ | $\frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{R_T}{K_L}\left(\frac{L_T}{R_T}-\bar{Y}\right)-1}-\frac{K_L\bar{Y}}{\frac{L_T}{R_T}-\bar{Y}}$ |
| $H_T >> R_T$ | $K'_H$ | $\frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{R_T}{K_L}\left(\frac{L_T}{R_T}-\bar{Y}\right)-1}$ |
| $L_T >> R_T$ | $K^*_H$ | $\frac{H_T}{\frac{(1-\bar{Y})}{\bar{Y}}\frac{L_T}{K_L}-1}-\frac{R_TK_L\bar{Y}}{L_T}$ |
| $H_T >> R_T$, $L_T >> R_T$ | $K''_H$ | $\frac{H_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{L_T}{K_L}-1}$ |
| $H_T >> R_T$, $L_T >> R_T$, and $L_T >> K_L$ | $K'''_H$ | $\frac{\bar{Y}}{(1-\bar{Y})}\frac{H_T}{L_T}K_L$ |

A plot of

$$\frac{H_T\left(\frac{L_T}{R_T}-\bar{Y}\right)}{(1-\bar{Y})\frac{R_T}{K_L}\left(\frac{L_T}{R_T}-\bar{Y}\right)-\bar{Y}} \text{ vs. } \frac{\frac{L_T}{R_T}-\bar{Y}}{\bar{Y}}$$

should yield a straight line that has a slope of $K_H$ (Fig. 1) if

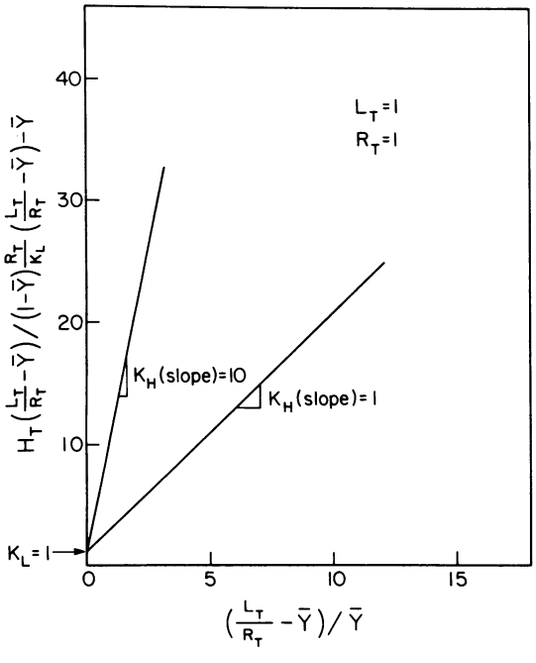


FIG. 1. A linear plot for the theoretical displacement curve. The plot was derived by rearrangement of Eq. 13 (see text) and is drawn here for different values of $K_H$ at conditions where $R_T = 1$, $L_T = 1$, and $K_L = 1$. The values of the slope and the $y$ intercept in the case of purely competitive noncooperative binding are $K_H$ and $K_L$, respectively. This plot therefore tests, in the case of actual experimental data, whether there is only one binding affinity (the plot is then linear) and whether the binding is purely competitive (the $y$ intercept is then equal to the value of $K_L$ from an independent measurement).

there is only one binding affinity. Nonlinearity would suggest more than one binding affinity. The intercept on the ordinate ($y$ axis) yields $K_L$ if the binding is purely competitive. Thus, the competitive nature of the binding can easily be verified since $K_L$ can always be determined independently by a simple binding experiment. The binding isotherm for $L$ yields both $K_L$ and $R_T$; $R_T$ is required (Eq. 22), whereas the $K_L$ obtained serves as a cross-check.

### Accuracy and Advantage

Since the value of $K_H$ may be calculated from a single $\bar{Y}$,$H_T$ pair of values with any of the equations in Table 1, it is recommended that the experiment be conducted with as many replicates as possible. The accuracy of the determination of $K_H$ is independent of the shape of the displacement curve (Fig. 2).

### Relationship Between $K_H$, $K'_H$, $K^*_H$, $K''_H$, and $K'''_H$

Table 1 summarizes the formulas for $K_H$ through $K'''_H$. A calculation of $K_H$ from the displacement curve of $\bar{Y}$ vs. log $H_T$ yields the correct value for the dissociation constant of $H$. A calculation of $K'_H$, $K^*_H$, $K''_H$, or $K'''_H$ very often yields an erroneous value (Table 2). Fig. 3 shows the relationship between $K'_H$ and $K_H$. It is clear that $K'_H > K_H$. This is also obvious

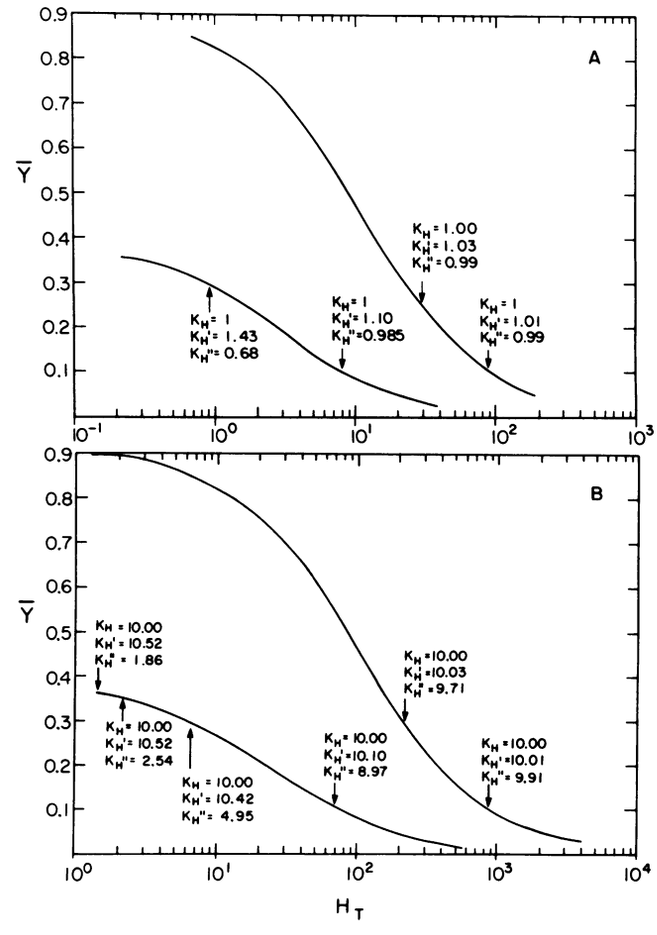


FIG. 2. Theoretical displacement curve. Receptor occupancy $\bar{Y}$ is plotted against increasing total concentrations of the displacing ligand ($H_T$). For convenience, we have used $R_T = 1.0$ and $K_L = 1.0$. (*A*) $K_H = 1$, (*B*) $K_H = 10$. The upper and lower curves were constructed for $L_T = 10$ and $L_T = 1$, respectively, using Eq. 14.

Table 2. The limits of the discrepancies between the correct and the approximate values of $K_H$ at infinitely low and high concentrations of the displacing agent ($R_T = 1$, $K_L = 1$, $H_T = 1$)

| | $L_T \to \bar{Y}\left(\frac{2-\bar{Y}}{1-\bar{Y}}\right)^*$ | $L_T \to \infty$ |
|---|---|---|
| $\frac{K'_H}{K''_H}$ | $\infty$ | 1 |
| $\frac{K_H}{K''_H}$ | $\infty$ | $\bar{Y}$ |
| $\frac{K_H}{K'_H}$ | 1 | $\bar{Y}$ |
| $\frac{K_H}{K'^*_H}$ | $\infty$ | 1 |
| $\frac{K'^*_H}{K''_H}$ | $\frac{\bar{Y}-\bar{Y}^2+1}{2-\bar{Y}}$ | $\bar{Y}$ |

*Since the value of a dissociation constant cannot be negative, it is required that $L_T > \bar{Y}\,[(2-\bar{Y})/(1-\bar{Y})]$.

from Eqs. 13 and 14 since it is always true that

$$\frac{L_T}{R_T} > \bar{Y}.$$

Fig. 3 also shows that at low $\bar{Y}$ values—i.e., at low receptor occupancy—the deviation of $K'_H$ from the accurate value of $K_H$ increases.

The plot of $1/K''_H$ vs. $1/K_H$ or vs. $1/K'_H$ shows clearly (Fig. 4) that at low $L_T$ the discrepancy between $K''_H$ and $K'_H$ or $K_H$ becomes very significant. At high $L_T$ values, $K''_H$ and $K'_H$ asymptotically approach each other. Therefore, the slope of the plot $1/K''_H$ vs. $1/K'_H$ approaches 1 at high $L_T$ values. The deviation between $K''_H$ and $K_H$ remains significant even at high $L_T$ values, but it is less serious than at low $L_T$ values. Fig. 4 shows these relationships at $\bar{Y} = 1/2$. Fig. 5 shows that

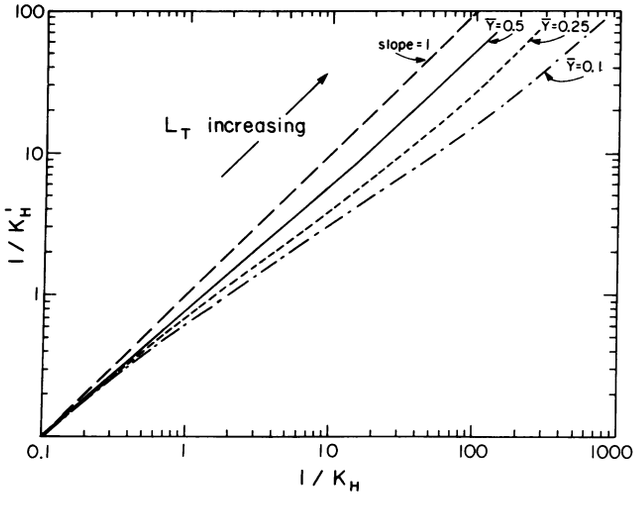


FIG. 3. The dependence of $1/K'_H$ on $1/K_H$. The relationship between $1/K'_H$ and $1/K_H$ for different values of $\bar{Y}$ is depicted. For simplicity, the conditions chosen are $R_T = 1$, $K_L = 1$, and $H_T = 1$. Eqs. 14 and 15 were used to calculate the values of $K'_H$ and $K_H$ for different values of $L_T$. All the values of $L_T$ chosen must always fulfill the conditions $(1 - \bar{Y}/\bar{Y})(R_T/K_L)(L_T/R_T - \bar{Y}) - 1 > 0$ and $(L_T/R_T) - \bar{Y} > 0$. It can be seen that the discrepancy between $K_H$ and $K'_H$ is largest at low values of $\bar{Y}$.

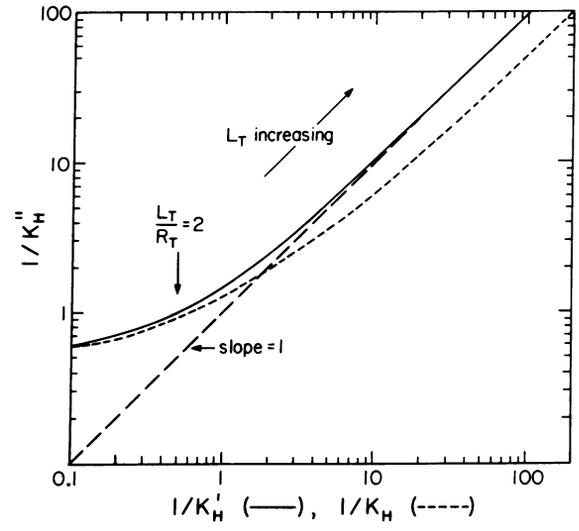


FIG. 4. The relationship of $K''_H$ with $K_H$ and $K'_H$. $1/K''_H$ is drawn as a function of $1/K_H$ and $1/K'_H$. The conditions used are the same as in Fig. 3. It can easily be seen that the largest discrepancies between $K''_H$ and $K'_H$ are at low values of $L_T$.

the deviation between $K'_H$ ($H_T >> R_T$) and $K''_H$ ($H_T >> R_T$, $L_T >> R_T$), increases with decreasing receptor occupancy $\bar{Y}$ and is most pronounced at low values of $L_T$. A summary of the discrepancies between the various values of $K_H$ at infinitely low and high values of $L_T$ is given in Table 2.

When the conditions $H_T >> R_T$, $L_T >> R_T$ are met, the Cheng–Prusoff analysis (3) is as useful and as accurate as the method presented here. In summary, the treatment here is especially useful when these conditions are not met. When only $H_T >> R_T$, the use of the present treatment is as easy as the use of Eq. 4 for the calculation of $K'_H$.

### Accurate Determination of Enzyme–Inhibitor Dissociation Constants

In enzymology, the situation is generally different from that in studies of receptors since the total enzyme concentration

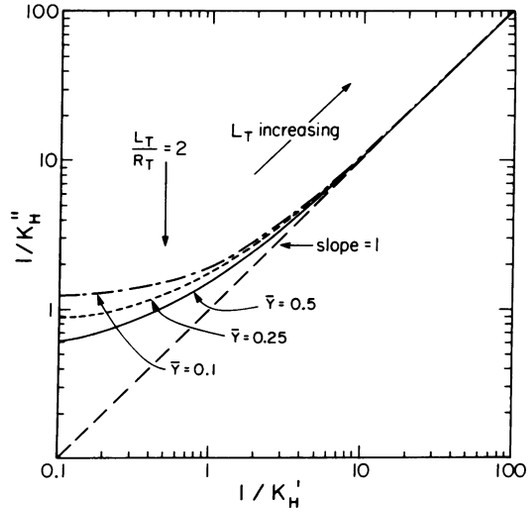


FIG. 5. $K''_H$ as a function of $K'_H$ at different receptor occupancies. The dependence of $1/K''_H$ on $1/K'_H$ for various values of $\bar{Y}$ is shown. It may be seen that for the same ratio of $L_T$ to $R_T$, the deviation of $K''_H$ from $K'_H$ is largest at low values of $L_T$. The conditions used are the same as in Fig. 3 ($R_T = 1$, $H_T = 1$, $K_L = 1$).

$E_T$ is usually very small compared to both the substrate concentration $S_T$ and the concentration of the competitive inhibitor $I_T$. Under such conditions,

$$V = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i''}\right) + [S]}, \quad [23]$$

where $[S] = [S]_{free} = S_T$, $[I] = [I]_{free} = I_T$, and $K_i''$ and $K_m$ are the enzyme–inhibitor dissociation constant and the Michaelis–Menten constant, respectively. Occasionally, however, one deals with tightly bound inhibitors that affect the enzyme at concentrations similar to those of the enzyme itself. In such cases, the classical Dixon method (7) does not apply since the concentration of the free inhibitor may not be considered equal to that of the total inhibitor. Another classical method in use is that of Green and Work (8). According to this method, one calculates the inhibition constant at the point of equivalence. There is usually an error in the determination of the point of equivalence and thus also in the binding constant itself. A further error stems from the equation used, which is based on an approximation. More recently, several other methods were devised specifically for the case of tightly bound inhibitors or substrates (9–13). These methods do not apply, however, when the concentrations of neither the substrate nor the inhibitor are in excess of the enzyme. Furthermore, these methods do not offer the possibility of a direct determination of the inhibition constant from a "titration" curve.

Using the same approach as before for receptor binding experiments where now $S_T \neq [S]_{free}$ and $I_T \neq [I]_{free}$, one can derive equations for $K_m$ and $K_i$ similar to Eqs. 8 and 9 as follows:

$$K_m = \frac{(E_T - [EI] - [ES])(S_T - [ES])}{[ES]} \quad [24]$$

$$K_i = \frac{(E_T - [EI] - [ES])(I_T - [EI])}{[EI]}. \quad [25]$$

From Eqs. 24 and 25 one obtains (see Eqs. 10–13):

$$K_i = K_i' - \frac{K_m\bar{Y}}{\frac{S_T}{E_T} - \bar{Y}}, \quad [26]$$

where

$$\bar{Y} = \frac{[ES]}{E_T} = \frac{V}{V_{max}},$$

and

$$K_i' = \frac{I_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{E_T}{K_m}\left(\frac{S_T}{E_T} - \bar{Y}\right) - 1}. \quad [27]$$

$K_i$ is the accurate value for the enzyme–inhibitor dissociation constant. For the limiting cases, one can derive the appropriate expressions as given in Table 3.

As in the hormone–receptor system, it is recommended that the data be plotted according to Eq. 26, which has been rearranged (as above) to yield

$$\frac{I_T\left(\frac{S_T}{E_T} - \bar{Y}\right)}{(1-\bar{Y})\frac{E_T}{K_m}\left(\frac{S_T}{E_T} - \bar{Y}\right) - \bar{Y}} = K_i\frac{\left(\frac{S_T}{E_T} - \bar{Y}\right)}{\bar{Y}} + K_m. \quad [28]$$

The plot of

$$\frac{I_T\left(\frac{S_T}{E_T} - \bar{Y}\right)}{(1-\bar{Y})\frac{E_T}{K_m}\left(\frac{S_T}{E_T} - \bar{Y}\right) - \bar{Y}} \text{ vs. } \frac{\frac{S_T}{E_T} - \bar{Y}}{\bar{Y}}$$

should yield a straight line in the case of one binding affinity with an intercept on the $y$ axis that has the value of $K_m$ in the case of purely competitive binding.

When $I_T >> E_T$ and $S_T >> E_T$, the classical Dixon method (7) is equally effective since it applies to such conditions. However, the protocol suggested here is more convenient since a single competition curve is used ($V/V_{max}$ vs. $I_T$).

Table 3. Formulas for enzyme–inhibitor dissociation constants

| Conditions | Notation | Formula |
|---|---|---|
| General case | $K_i$ | $\frac{I_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{E_T}{K_m}\left(\frac{S_T}{E_T} - 1\right) - 1} - \frac{K_m\bar{Y}}{\frac{S_T}{E_T} - \bar{Y}}$ |
| $I_T >> E_T$ | $K_i'$ | $\frac{I_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{E_T}{K_m}\left(\frac{S_T}{E_T} - 1\right) - 1}$ |
| $S_T >> E_T$ | $K_i'^*$ | $\frac{I_T}{\frac{(1-\bar{Y})}{\bar{Y}}\frac{S_T}{K_m} - 1} - \frac{E_T K_m\bar{Y}}{S_T}$ |
| $I_T >> E_T$ and $S_T >> E_T$ | $K_i''$ | $\frac{I_T}{\left(\frac{1-\bar{Y}}{\bar{Y}}\right)\frac{S_T}{K_m} - 1}$ |

$\bar{Y} = V/V_{max} = [ES]/E_T$.


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