

# The Hill equation revisited: uses and misuses

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**ABSTRACT** The Hill coefficient is commonly used to estimate the number of ligand molecules that are required to bind to a receptor to produce a functional effect. However, for a receptor with more than one ligand binding site, the Hill equation does not reflect a physically possible reaction scheme; only under the very specific condition of marked positive cooperativity does the Hill coefficient accurately estimate the number of binding sites. The Hill coefficient is best thought of as an "interaction" coefficient, reflecting the extent of cooperativity among multiple ligand binding sites. Several relatively simple, physically plausible reaction schemes are shown here to produce a variety of ligand dose-response curve phenotypes more appropriately suited to modeling ligand-receptor interactions, especially if independent information about the stoichiometry of the ligand-receptor interaction is available.—Weiss, J. N. The Hill equation revisited: uses and misuses. *FASEB J.* 11, 835–841 (1997)

*Key Words:* ligands · receptors · binding · ligand-receptor interaction · ion channels · enzymes

THE HILL EQUATION WAS EMPIRICALLY APPLIED by A. V. Hill in 1910 to describe the binding of oxygen to hemoglobin (1), and subsequently has been widely used in biochemistry, physiology, and pharmacology to analyze the binding equilibria in ligand-receptor interactions. In addition to providing a measure of the affinity of the ligand for the receptor, the Hill equation is commonly used to estimate the number of ligand molecules that are required to bind to the receptor in order to produce a functional effect. The latter estimate is obtained from the Hill coefficient ( $n$  in equations 1 and 2). However, as recognized by A. V. Hill, the conditions under which the Hill coefficient provides an accurate estimate of the number of binding sites are very specific: only when extreme positive cooperativity is present between the binding of the first and subsequent ligand molecules. That is, the affinity of the binding has to be very asymmetric, with a much lower affinity of binding for the first ligand molecule than for the subsequent ligand molecules. To this end, the Hill coefficient has been described more appropriately as an "interaction coefficient" reflecting cooperativity rather than as a reliable estimate of the number of binding sites (2).

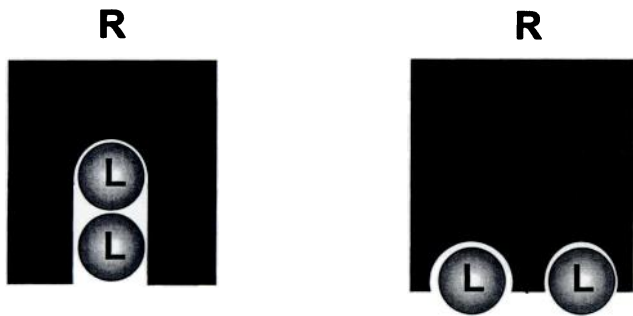
The conditions under which the Hill equation is appropriately applied to ligand binding curves, however, are poorly appreciated, based on examples in the literature and an informal poll of scientific colleagues. The condition of extreme positive cooperativity cannot be assumed, and even seems unlikely in many instances. For example, in multimeric proteins, it imposes the constraint on quaternary protein structure that the ligand binding site on one monomer has to interact cooperatively with the ligand binding sites on distant monomers, which is not easy to visualize. Even in the case of positive cooperativity, the Hill coefficient provides only a *minimum* estimate of the number of binding sites involved. For example, in the case of hemoglobin, in which four oxygen molecules are known to bind with a high degree of positive cooperativity, the measured Hill coefficient ranges from 1.7 to 3.2 rather than 4 (1).

A. V. Hill had a practical reason for using the simplified (and physically unrealistic) binding scheme 1 from which the Hill equation is derived—the computational intractability of fitting his data to a more realistic reaction scheme without the aid of modern computers. However, computational intractability is no longer a limiting factor, and numerous studies have described more realistic reaction schemes for analyzing receptor-ligand and enzyme-substrate interactions (2–5). For this reason it is timely to review the appropriateness of using the Hill equation in the analysis of receptor-ligand interactions. Accordingly, the purpose of this paper is threefold: to review the assumptions required for interpreting the Hill equation properly; to summarize equilibrium behavior of ligand-receptor interactions for physically plausible reaction schemes; and to point out the discrepancies between the two approaches.

## THE HILL EQUATION IMPLIES SIMULTANEOUS LIGAND BINDING

The Hill equation is readily derived from a binding reaction scheme in which  $n$  molecules of ligand ( $L$ ) bind to a receptor ( $R$ ), i.e.,

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### Sequential

### Independent

**Figure 1.** Schematic representation of a sequential vs. an independent ligand binding mechanism. L, ligand; R, receptor.

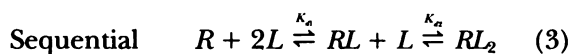


where  $K_d \equiv k_b/k_f$ . At equilibrium, the ratio of bound to total receptors is given by a familiar form of the Hill equation:

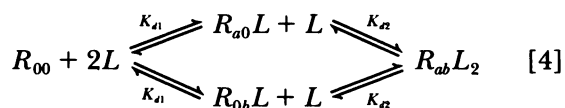
$$\frac{\text{Bound}}{\text{Total}} = \frac{[RL_n]}{[R] + [RL_n]} = \frac{\frac{[L]^n}{K_d}}{1 + \frac{[L]^n}{K_d}} = \frac{\frac{[L]^n}{K_d}}{1 + \left(\frac{[L]}{K_{0.5}}\right)^n} \quad (2)$$

where  $K_{0.5}$  is  $[L]$  at which half of the receptors are bound, and is equivalent to the  $n^{\text{th}}$  root of the dissociation constant  $K_d$ .

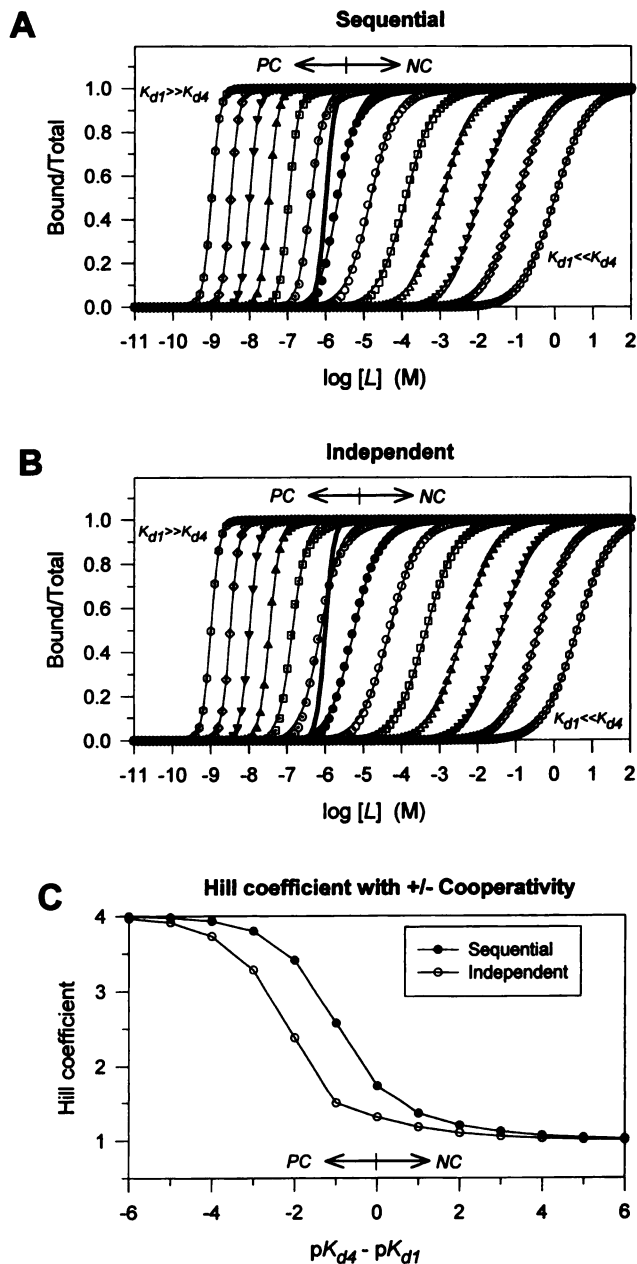
Although reaction 1 is mathematically possible, it is not physically possible in the real world except for the condition in which  $n = 1$ . For  $n = 2$ , for example, the reaction implies the *simultaneous* interaction of three molecules in a trimolecular reaction in which no intermediate-state  $RL$  occurs between the  $R$  and  $RL_2$  states. In real ligand-receptor interactions, intermediate states have to occur, as in the sequential and independent binding reaction schemes shown below (reactions 3 and 4).



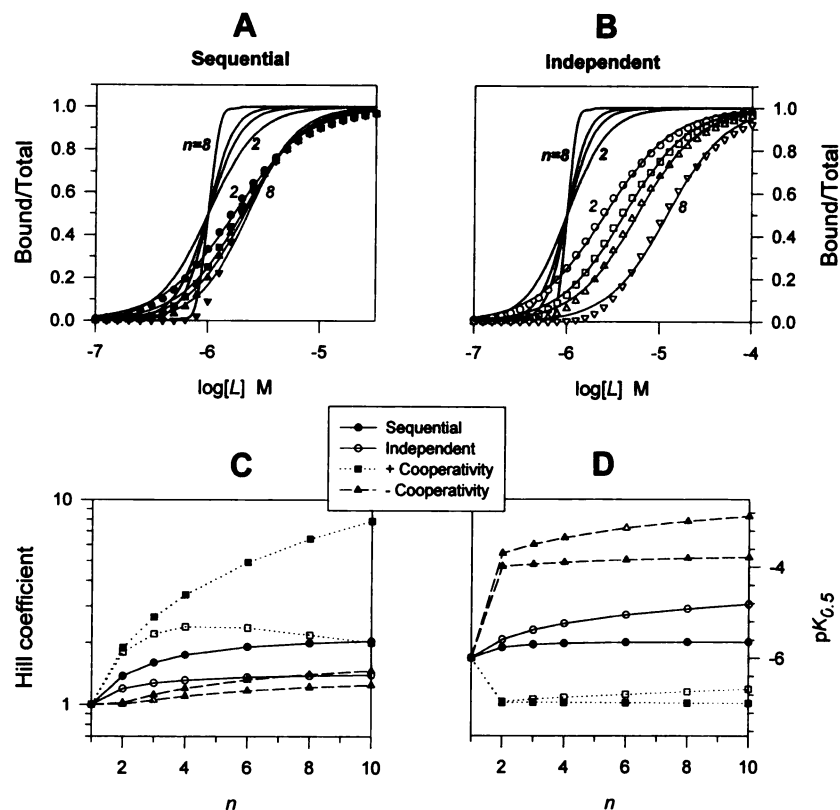
Independent



The only condition in which either the sequential or independent binding schemes are approximated by reaction 1 is when the  $RL$  state never accumulates significantly. This occurs only when marked positive cooperativity is present, i.e., when  $K_{d1} \gg K_{d2}$ .



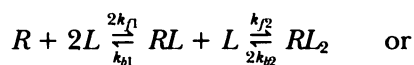
**Figure 2.** Dose-response curves for the sequential (A) and independent (B) binding reaction schemes with four binding sites, as affected by varying degrees of cooperativity. In panels A and B, thick lines show the curve for the Hill equation with  $n = 4$ . Solid symbols indicate no cooperativity ( $K_{d1} = K_{d2} = K_{d3} = K_{d4} = 10^{-6}$  M), open symbols to the left show progressive orders of magnitude positive cooperativity (i.e.,  $K_{d1}/K_{d4} = 10^{-1}, 10^{-2}, 10^{-3}$ , etc.), and to the right, progressive orders of magnitude negative cooperativity are shown (i.e.,  $K_{d1}/K_{d4} = 10^1, 10^2, 10^3$ , etc.). Thin lines show the best fits to the Hill equation. Note that positive cooperativity (PC) increases and negative cooperativity (NC) decreases the steepness of the dose-response curve, as reflected in the value of the fitted Hill coefficients, shown in panel C. In this and subsequent figures, curve-fitting routines were performed using Sigmaplot Windows software (Jandel Scientific, San Raphael, Calif.) on a Pentium PC. Numerical simulations were performed using SCoP (version 3.5, Simulation Resources, Inc., Berrien Springs, Md.).



**Figure 3.** Comparison of the dose-response curves for the Hill equation with the sequential (A) and independent (B) binding mechanisms for up to 10 binding sites. Thick lines show the curves for the Hill equation for  $n = 2, 3, 4,$  and  $8$ ; symbols show the corresponding curves for the sequential (solid symbols) or independent (open symbols) reactions with no cooperativity present ( $K_{d1}=K_{d2}=K_{d3}=K_{d4}=10^{-6}$  M). Thin lines are the fitted Hill curves to the sequential or independent reactions. Panels C and D show the values of the fitted Hill coefficients and  $pK_{0.5}$ 's as a function of the number of binding sites ( $n$ ) for the sequential (solid symbols) or independent (open symbols) reactions with no cooperativity (solid lines), positive cooperativity (dotted lines), or positive cooperativity (dashed lines). The degree of cooperativity was two orders of magnitude between the first and last ligand-bound states for all  $n$  (i.e.,  $K_{d1}/K_{dn}=10^{+2}$ ) distributed equally between the intermediate states.  $K_{d1}$  was fixed at  $10^{-6}$  M.

### PHYSICALLY MORE PLAUSIBLE REACTION SCHEMES: SEQUENTIAL OR INDEPENDENT LIGAND BINDING

Reaction 3, the *sequential* binding scheme, requires that the first binding site be filled in order for the second site to become occupied, e.g., as if the ligand molecules stack on top of each other at their binding sites (Fig. 1A). This scheme could be relevant to an ion transporter, such as the Na-K pump or Na-Ca exchanger, in which the Na ion binding sites may be located in a partial channel-like structure (6), assuming that the Na ions were to stack up in single file. In reaction 4, the *independent* binding scheme, two binding sites (a and b) are available to the ligand; either one can be occupied independently of the other (Fig. 1B). This is likely to be a relevant binding scheme for multimeric proteins with individual binding sites located on different subunits, such as ligand-gated ion channels (e.g., muscarinic G-protein-activated K channels, Ca-activated K channels, or ATP-sensitive K channels) or ligand-gated enzymes (e.g., muscarinic G-protein-activated phospholipase C). Equation 4 is equivalent to:



This is because when the receptor is unbound, either of two ligand binding sites are available to be filled, so the total "on-rate" from the empty receptor is twice the individual forward rate constant  $k_{f1}$ . In contrast, when one ligand molecule is already bound, only the other site is available, so the total on-rate is  $k_{f2}$ . The converse applies to the coefficients of the backward rate constants.

The general equilibrium solutions for binding schemes 3 and 4a, extrapolated to  $n$  binding sites, are given in the Appendix. Figure 2 compares the ligand binding curves, with  $n = 4$ , for the Hill equation, the sequential (Fig. 2A), and independent binding schemes (Fig. 2B) under conditions of no cooperativity, positive cooperativity, and negative cooperativity. The fractional concentration of the fully ligand-bound state ( $RL_4$ ) is plotted as a function of  $[L]$ . If the ligand binding curves for the sequential or independent binding schemes (shown by symbols) are fit to the Hill equation (shown

by thin lines), the Hill coefficient is only 1.74 for the sequential and 1.31 for the independent binding schemes, respectively. As shown in Fig. 2C, for the Hill coefficient to approach 4 with either scheme requires marked positive cooperativity, in this case  $K_{d1} > 10^4 K_{dn}$ ; that is, the  $K_d$  for each successive ligand-bound state must decrease by an order of magnitude for the Hill coefficient to estimate accurately the number of binding sites.

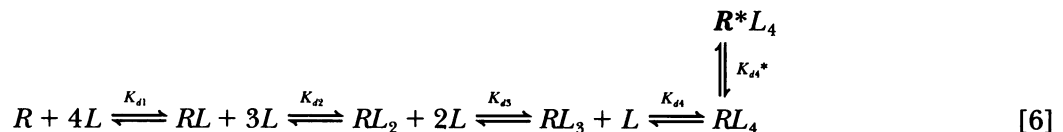
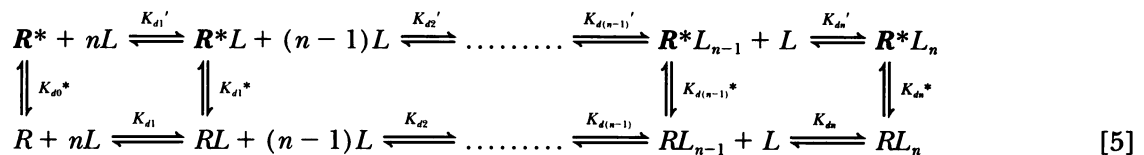
Close inspection of Fig. 2A, B also shows that the fits of the Hill equation to the ligand binding curves for either the sequential or independent binding schemes are close, but not perfect. The  $[L]$  at which half of receptors are fully ligand bound ( $K_{0.5}$ ) also varies with the degree of cooperativity.

Figure 3 illustrates the ligand-receptor saturation curves for the Hill equation and for the sequential and independent binding schemes for up to 10 binding sites. In the case of no cooperativity (i.e.,  $K_{d1} = K_{d2} = \dots = K_{dn}$ ), the Hill coefficient never exceeds 2.1 for the sequential reaction and 1.4 for the independent reaction, even with 10 binding sites. Fig. 3C, D plot the values of the Hill coefficient and  $K_{0.5}$  as a function of the number of binding sites for the sequential and independent binding

schemes under conditions of no cooperativity, positive cooperativity (for a 100-fold increase in binding affinity evenly distributed between the first and the fully bound state), and negative cooperativity (for a 100-fold decrease in binding affinity evenly distributed between the first and the fully bound state). This degree of positive cooperativity increases the Hill coefficient in the sequential mechanism substantially, but has only a modest effect in the independent mechanism, especially as the number of binding sites increase (Fig. 3C). Positive and negative cooperativity also markedly affect the  $K_{0.5}$  values by more than an order of magnitude (Fig. 3D).

### EFFECTS OF INCLUDING AN ACTIVATED STATE OF THE RECEPTOR

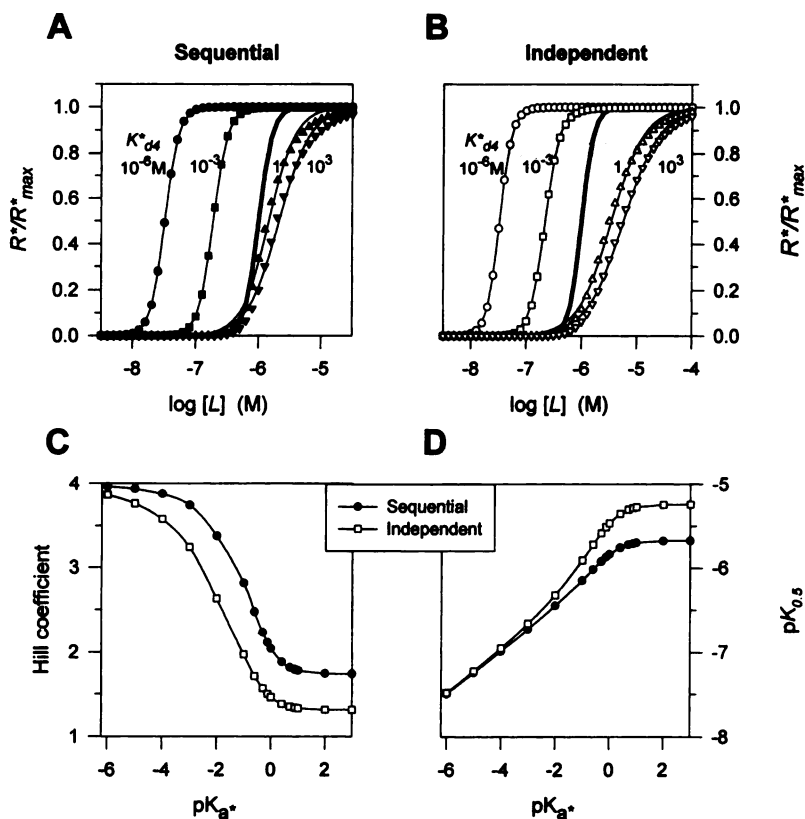
The results above illustrate the importance of positive cooperativity in determining the steepness of the saturation curve. However, the simple sequential and independent binding schemes 3 and 4 leave out an important feature present in ligand-activated receptors: a conversion of the receptor from an inactive ( $R$ ) to an activated form ( $R^*$ ) promoted by ligand binding (reaction 5).



This binding + activation reaction scheme leads to a wide range of possible dose-response relationships, limiting its practical usefulness for determining a unique model from experimental data. However, it has been treated previously for  $n = 4$  under special conditions (in which symmetries in the various equilibrium constants were assumed) to model oxygen binding to hemoglobin and allosteric regulation of various enzymes (2). A similar model has also been used to explain inverse agonist effects of receptors, such as the  $\beta$ -adrenergic receptor, by assigning different  $K_d$  values for binding of the ligand to the  $R$  vs. the  $R^*$  state of the receptor (7). Consider two physiologically relevant simplifications of reaction 5, which have interesting effects on the steepness and shape of the ligand binding curves. The first is reaction 6, shown for  $n = 4$ , in which only the fully bound receptor can undergo a transition to the activated state

and the ligand cannot unbind from activated receptor until it deactivates.

This case might apply, for example, to a partial reaction of an ion transporter such as the Na-K pump (with  $n=3$  or 2): after the cation binding sites are filled with 3 Na or 2 K ions, the pump undergoes a conformational change in which the ions become occluded within the protein (8) and cannot unbind until the conformation reverts and releases the ions (at the other side of the membrane). Figure 4 illustrates the saturation curves for reaction 6. If the  $RL_4$  conformation is highly favored over the  $RL_4$  state (i.e.,  $K_{d4}^* < 10^{-4}$ ), the effect is similar to that of positive cooperativity: the dose-response curve becomes much steeper and the Hill coefficient approaches 4 for either the sequential or independent binding scheme. This effect occurs because the  $RL_4$  state, when highly favored, depletes ligand-bound states of

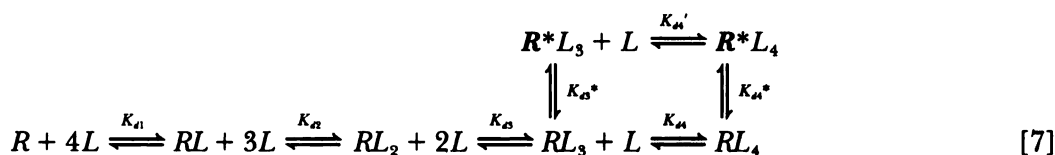


**Figure 4.** Dose-response curve for reaction 6, when the fully bound receptor ( $RL_4$ ) undergoes a transition to an active state ( $R^*L_4$ ), for either the sequential (A) or independent (B) binding mechanism with four binding sites. Different symbols show the effect of increasing the dissociation constant ( $K^*_{d4}$  in reaction 6) for the transition to the active state; the thin lines are the Hill fits to the symbols, and the thick line is the Hill curve for  $n = 4$ . As the active state becomes more favored, the steepness of the dose-response curve increases and the Hill coefficient approaches 4 for either model (C). The  $K_{0.5}$  is also affected (D). For comparison, the ordinate axes in panels A and B plot  $R^*$  as a fraction of the maximal activation of the receptor ( $R^*_{max}$ ) rather than total  $R + R^*$ . No positive cooperativity has been assumed ( $K_{d1} = K_{d2} = K_{d3} = K_{d4} = 10^{-6}$  M).

$R$ . This minimization of intermediates thereby approximates the Hill equation. For  $K^*_{d4} > 10^{-4}$ , however, the Hill coefficient of the dose-response curve is progressively lower (Fig. 4C). As an example, consider a ligand-gated ion channel operating according to this reaction scheme that opens only when four ligand molecules are bound: no cooperativity is present, and the open probability ( $p_o$ ) reflects the equilibrium distribution between the  $RL_4$  and  $RL_4^*$  states. The steepness of the dose-response of current flow to  $[L]$  will depend on the extent to which the  $p_o$  of the channel is increased by binding four ligand molecules. For an increase in  $p_o$  from 0 to 0.9999

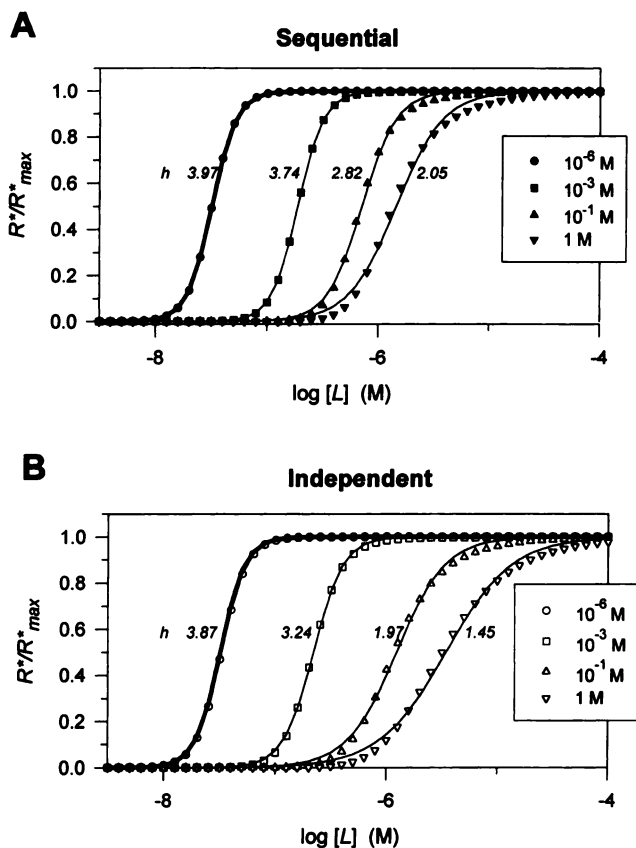
(i.e.,  $K^*_{d4} = 10^{-4}$ ), the Hill coefficient for the activation of current flow as a function of  $[L]$  will approach 4; for an increase in  $p_o$  to 0.5 (i.e.,  $K^*_{d4} = 1$ ), however, the Hill coefficient will be near 1.7 for the sequential and 1.3 for the independent binding + activation scheme (Fig. 4C), essentially equivalent to the binding reaction without the activated  $RL_4$  state (Fig. 2).

A second and perhaps more physiologically relevant special case of reaction 5 is when the ligand can unbind from the activated receptor, but this rapidly leads to deactivation of the receptor, as shown in reaction 7 for  $n = 4$ .



Because this binding + activation scheme has a cyclic component, the equilibrium behavior cannot be solved in terms of the  $K_d$ 's alone, and is influenced by the values of the individual on- and off-rate constants. An additional constraint imposed by the cyclic component are the principles of microscopic reversibility and detailed balance: the ratio of the product of rate constants in one direction around the cycle to the product of rate con-

stants around the opposite direction is proportional to the exponent of the free energy change (9). If the cyclic reaction is passively driven, so that no net energy (e.g., from ATP hydrolysis) is required to complete the cycle, the ratio must equal unity. Figure 5 illustrates examples of dose-response curves for this reaction when dissociation of the 4<sup>th</sup> ligand molecule strongly deactivates the receptor ( $K^*_{d3} = 10^3$ ) and no cooperativity is present. If



**Figure 5.** Dose-response curve for reaction 7, when the fully bound receptor ( $RL_4$ ) undergoes a transition to an active state ( $R^*L_4$ ) and dissociation of one ligand molecule strongly favors deactivation ( $K^*_{d4}=10^3$  M), for either the sequential (A) or independent (B) binding mechanism with four binding sites. Different symbols show the effect of increasing the dissociation constant ( $K^*_{d4}$  in reaction 7) for the transition to the active state; the thin lines are the Hill fits to the symbols; for comparison, the thick line is the curve for reaction 6 with  $K^*_{d4} = 10^{-6}$  M. Hill coefficients are as indicated. For comparison, the ordinate axis in panels A, B plots  $R^*$  as a fraction of the maximal activation of the receptor ( $R^*_{max}$ ) rather than total  $R + R^*$ . No positive cooperativity has been assumed ( $K_{d1}=K_{d2}=K_{d3}=K_{d4}=K_{d4}^*=10^{-6}$  M). Since there is a cyclic component to the reaction scheme, the results are dependent on the values of the forward and backward rate constants rather than the  $K_d$ 's alone; the values used were  $k_{f1} = k_{f2} = k_{f3} = k_{f4} = 10^6$   $M^{-1}\cdot s^{-1}$  and  $k_{b1} = k_{b2} = k_{b3} = k_{b4} = 1$   $s^{-1}$ ;  $k^*_{f3} = 1$   $s^{-1}$  and  $k^*_{b3} = 10^3$   $s^{-1}$ ;  $k'_{f4} = 10^6$   $M^{-1}\cdot s^{-1}$  and  $k'_{b4} = 1$   $s^{-1}$ ;  $k^*_{b4} = 1$   $s^{-1}$  and  $k^*_{f4} = 10^{-6}$  to  $1$   $s^{-1}$ . The simulation also assumed that the cyclic reaction was passively, not actively, driven, so that  $k_{f4}\cdot k^*_{f4}\cdot k'_{b4}\cdot k^*_{b4} = k^*_{f3}\cdot k'_{f4}\cdot k^*_{b3}\cdot k_{b4}$  to satisfy the requirement of detailed balance.

the cyclic reaction is passively driven, the results are similar to those in reaction 6 (Fig. 4), and depend on the value of  $K^*_{d4}$ . For  $K^*_{d4} = 10^{-6}$  to  $10^{-3}$ , the Hill coefficient is between 3 and 4 (Fig. 4). For  $K^*_{d4} = 10^{-1}$  to 1, the Hill coefficient falls below 3, and positive cooperativity between the intermediate ligand-bound states would have to be present to yield a higher Hill coefficient. Considering again the ligand-gated ion channel that opens upon binding of four ligand molecules but then markedly decreases its  $p_o$  to 0.001 if one ligand molecule unbinds ( $K^*_{d4}=10^3$ ), the Hill coefficient of the dose-response curve strongly depends on the  $p_o$  of the

fully ligand-bound channel. For  $p_o > 0.999$  ( $K^*_{d4} < 10^{-3}$ ), the Hill coefficient is 3–4 if no cooperativity is present. For  $p_o$  between 0.5 and 0.9 ( $K^*_{d4}=0.1-1$ ), however, a smaller Hill coefficient (1.4–2.8) is obtained. If the cyclic reaction is actively driven, more complex behavior can arise; the dose-response curve can even take on a biphasic shape under some conditions, requiring a sum of two Hill curves for a reasonable fit.

## SUMMARY AND CONCLUSIONS

The results above illustrate the following important points about the use of the Hill equation in the analysis of ligand–receptor interactions. 1) As emphasized previously (2), the Hill coefficient is not a reliable indicator of the number of functionally important ligand binding sites on the receptor, but provides only a minimum value. 2) For simple sequential or independent binding schemes (reactions 3 and 4), the Hill coefficient is always less than 2 for up to 10 binding sites for neutral cooperativity. 3) The only condition under which the Hill coefficient does accurately estimate the number of binding sites for a simple sequential or independent binding scheme is when marked positive cooperativity is present. Negative cooperativity reduces the Hill coefficient to a minimum value of 1 (but not less than 1 for these binding schemes). 4) Changes in cooperativity generally also affect the  $K_{0.5}$  as well as the Hill coefficient. 5) If the receptor undergoes a conformational change to an activated state when all of its  $n$  binding sites are occupied by ligand, as shown in the binding + activation reaction scheme 6, the Hill coefficient can increase to approach  $n$  without any positive cooperativity being present. However, this only occurs if the activated state ( $RL_n$ ) is strongly favored at equilibrium over the unactivated state ( $RL_n$ ). 6) The same is true when ligand unbinding from the activated receptor strongly promotes receptor deactivation (reaction 7), assuming a passively driven process. For an actively driven process, however, more complex dose-response curves with multiphasic shapes are possible.

Despite its appealing simplicity, the Hill equation is not a physically realistic reaction scheme, raising the question of whether it should be abandoned in favor of realistic schemes; at the very least, its limitations should be more widely recognized in its frequent application to ligand–receptor interactions. The Hill coefficient is best thought of as an interaction coefficient, reflecting the extent of positive cooperativity among multiple ligand binding sites. Relatively simple, physically plausible reaction schemes produce a variety of ligand dose-response curve phenotypes and are more appropriately suited to modeling ligand–receptor interactions, especially if independent information about the stoichiometry of the ligand–receptor interaction is available. In terms of the alternative binding reactions presented

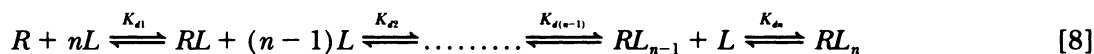
above, the independent mechanism (Fig. 1B) is likely to be the most widely relevant to multimeric receptors with binding sites located on different subunits. A "two-state" receptor model in which ligand binds to and shifts the equilibrium between an inactive ( $R$ ) and active ( $R^*$ ) form of the receptor [reaction 7]) can account for both simple and complex dose-response patterns, as well as other phenomena such as inverse agonist effects (7), and may be the most gen-

erally appropriate model for ligand-gated ion channels and enzymes.

## APPENDIX

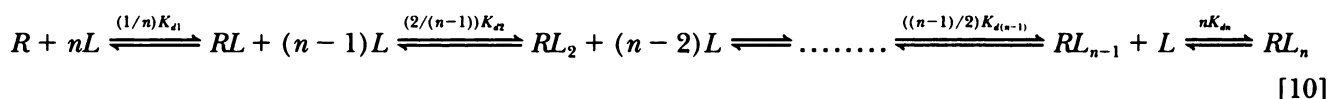
For a receptor  $R$  with  $n$  ligand binding sites, the general reaction schemes and the solutions for equilibrium conditions are:

Sequential:



$$\frac{[RL_n]}{\text{TOTAL } [R]} = \frac{[L]^n}{1 + [L] + \frac{[L]^2}{K_{d1}K_{d2}} + \dots + \frac{[L]^n}{K_{d1}K_{d2} \dots K_{d(n-1)}K_{dn}}} \quad [9]$$

Independent:



$$\frac{[RL_n]}{\text{TOTAL } [R]} = \frac{[L]^n}{1 + \sum_{i=1}^n \frac{n!}{(n-i)!i!} \frac{[L]^i}{\prod_{j=1}^i K_{dj}}} \quad [11]$$

If an activated state  $R^*L_n$  is incorporated in the sequential or independent reaction schemes, as in reaction 6, the solutions are given by:

$$\frac{[RL_n]}{\text{TOTAL } [R]} = \frac{A}{A + K_{dn}^*B}$$

where  $A$  and  $B$  represent the terms in the numerator and denominator of equations 9 and 11, respectively.

The general solutions were inferred from the pattern of the algebraically derived solutions for  $n = 2$  to 4, and subsequently confirmed to be valid by numerical methods for up to  $n = 10$ . [7]

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