Opinion TRENDS in Pharmacological Sciences Vol.28 No.12

Why the Schild method is better than Schild realised

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It is almost 60 years since Heinz Schild devised a method that allowed measurement of a genuine physical quantity, the equilibrium constant for binding of a competitive antagonist. The clever bit was that the measurements could be made using responses from tissues despite the fact that little or nothing was known about how the agonist worked. Since then, attempts have been made to generalise the Schild equation, but they are all based on false premises. It turns out that generalisation is usually not needed. His original simple result is still valid in cases where several agonist molecules must be bound to produce a response, even if the agonist binding sites interact or are not identical.

Introduction

It is almost 60 years since Heinz Schild, working in the late-lamented Pharmacology Department of University College London (UK) [1], discovered a method for measuring the affinity of a competitive antagonist for its receptor [2–4]. His enormous achievement was to show how it was possible to obtain a genuine physical constant, the equilibrium constant for binding of an antagonist to a receptor, from measurements of tissue responses, even when the mechanism of action of the agonist was both complex and unknown. Schild was generous in his acknowledgement of Clark and Raventos (1937) [5], who mentioned that "An alternative method of estimating antagonistic power is to determine the concentration of B, which alters by a selected proportion (e.g. tenfold) the concentration of A needed to produce a selected effect". But they failed to realise the potential of this approach, in particular that this 'proportion' (in modern terms: the dose ratio) is a function of the affinity constant of B for the receptor only. That was Schild's achievement. If a concentration of agonist A_0 produced a certain response in the absence of antagonist, and an increased agonist concentration A_1 evoked the same response in the presence of antagonist, then the factor by which the agonist concentration had to be increased, A_1/A_0, was defined as the dose ratio (denoted r here).

Schild's discovery was made many years before radioligand binding was invented by Paton and Rang (1965) [6]. In many cases, the results given by binding experiments and by the Schild method agree well: for example, there is excellent agreement [7] for a large number of muscarinic-receptor antagonists, with affinities that vary over six orders of magnitude. The Schild approach remains as important now as when it was first discovered, for several reasons. One reason is that there are often not enough receptors to do direct binding experiments. For example, receptors at a synapse or recombinant receptors on a single cell might be too few in number to be measurable. Another reason is that receptors might be too heterogeneous to enable unambiguous interpretation of ligand-binding experiments; but, if a specific agonist is available, the Schild method can be used to examine only those receptors that elicit the response. Recently, Wyllie and Chen [8] have discussed the enduring importance of the Schild approach in contemporary research.

The purpose of this review is to examine the conditions under which the Schild equation is valid. Schild's original derivation considered a single binding site. It was shown in 1973 [9] that the Schild equation gives the right answer under far wider conditions than that. The question continues to be misunderstood widely in the literature, so it seems worthwhile to clarify and expand the 1973 results.

The early work

The Schild equation states that:

$$r = 1 + c_B \qquad \text{[Equation 1]}$$

where r denotes the dose ratio (or, better, the concentration ratio) and c_B is defined as the concentration of the antagonist, B, expressed as a multiple of its equilibrium constant (K_B) for binding to its site. Thus, we define the normalised concentration as:

$$c_B = \frac{[B]}{K_B} \qquad \text{[Equation 2]}$$

Use of these dimensionless, normalised concentrations reduces greatly the amount of writing to be done.

The great beauty of this result is that the agonist does not appear at all. The nature of the agonist, its concentration, affinity and efficacy, are all irrelevant. The dose ratio, that is, the extent of the rightward shift of the equilibrium log-concentration-response curve produced by the antagonist, should be the same regardless of the nature of the agonist and the amplitude of response chosen for the measurement. The simplicity of Schild's result is the more remarkable because it does not need knowledge of the relationship between agonist occupancy and response. The experiments shown by Arunlakshana and Schild (1959) [4] were with muscarinic and histamine (H1) receptors. These are G-protein-coupled receptors (GPCRs) and, at the time, nothing at all was known about transduction

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 0165-6147/$ – see front matter doi:10.1016/j.tips.2007.09.011

mechanisms for these receptors. Even now, the mechanism cannot be written as a set of equations with any certainty, which would be needed to predict the effect of an antagonist. Nevertheless, the predictions of the simple theory were verified with impressive accuracy. They observed that equilibrium log-concentration-response curves showed a parallel rightward shift when antagonist was added. The extent of this shift, log(r), was independent of the nature of the agonist and a plot of log($r-1$) against log(B) was a straight line with a slope close to one, as the Schild equation predicts. Schild therefore felt justified in interpreting the concentration of antagonist that produces $r = 2$ as being the equilibrium constant for the binding of antagonist to the receptor, K_B. Arunlakshana and Schild (1959) also showed that the K_B so estimated was the same for muscarinic receptors in different tissues. The same was true for H1 receptors. They proposed the estimation of K_B as a method of classifying receptors and this approach remains useful even in the days of recombinant DNA. James Black gives great credit to the role of Schild's methods in helping his own discovery of β-blockers and H2-receptor antagonists [10].

Schild's derivation of his famous equation was based on the simplest case of a single binding site for the agonist. He assumed that, if the occupancy of this site by agonist was kept the same in the presence and absence of antagonist (by raising the agonist concentration by a factor r), then the measured response would also be the same, regardless of the details of the relationship between occupancy and response. That relationship was entirely unknown at the time.

The Schild factor, $(1 + c_B)$, occurs also in the 'Cheng-Prusoff' correction but, because that is merely a correction for having fitted the wrong equation in the first place, it will not be discussed here.

The scope of the Schild equation

The Schild method will give the correct equilibrium constant for the binding of an antagonist under conditions that can be put into words, as shown in Box 1. The term 'binding site' is used here to mean the area of a receptor that binds to a single agonist or antagonist molecule. A receptor might contain any number of such sites.

Under these conditions, the Schild equation will hold exactly, regardless of the number of binding sites, regardless of how these binding sites interact with each other in the presence of the agonist and regardless of whether all the binding sites have the same affinity for A.

Why is this so? If we have some postulate about the reaction mechanism, we can just write down the equilibrium equations in any particular case and show that the Schild equation is predicted to hold. Explicit examples are given in Box 2. But it would be good to have a more general argument, for three reasons. First, there are many sorts of receptor for which no explicit reaction mechanism exists. Second, even when there are reasonable postulates about the mechanism, the derivation has to be done separately for each different mechanism. Third, it would be good to have an argument that gives a more pictorial feel for what is happening that can be obtained by doing the algebra. We wish to ask which class of mechanisms will obey the Schild equation.

Box 1. Necessary and sufficient conditions for the Schild equation to be exact

(1) The antagonist, B, is a true antagonist that, alone, does not change the conformation of the receptor.
(2) Binding of agonist, A, and antagonist, B, is mutually exclusive at every binding site.
(3) B has the same affinity for every binding site.
(4) The observed response is the same if the occupancy of each site by A is the same, regardless of how many sites are occupied by B.
(5) Measurements are made at equilibrium.

First, consider a single binding site at which A and B compete. In this case, the fraction of binding sites (p_A) that is occupied by A at equilibrium is:

$$p_A = \frac{c_A}{1 + c_A + c_B} \qquad \text{[Equation 3]}$$

where the concentrations of A and B are expressed as a multiple of their equilibrium constants for binding to the site. Thus, we define normalised concentrations as:

$$c_A \equiv \frac{[A]}{K_A} \quad \text{and} \quad c_B \equiv \frac{[B]}{K_B} \qquad \text{[Equation 4]}$$

The result in Equation 3 is attributed commonly to Gaddum [11], but it is actually much older and dates back to Michaelis [12] in 1914.

Although Equation 3 is the simplest and most elegant way of expressing competition between A and B at a single class of sites, for our purposes, it will be better to divide top and bottom by $(1 + c_B)$ to put the occupancy by A in to the form:

$$p_A = \frac{c_A}{1 + c_A + c_B} = \frac{\left(\frac{c_A}{1+c_B}\right)}{1 + \left(\frac{c_A}{1+c_B}\right)} \qquad \text{[Equation 5]}$$

The form of Equation 5 is less elegant but it shows that the occupancy will be kept the same in the presence of antagonist if the agonist concentration is increased by a factor $(1 + c_B)$ and this is simply what the Schild equation states. In every place where the agonist concentration appears on the right-hand side, it is invariably divided by the Schild factor, $(1 + c_B)$ and that is sufficient to imply that the Schild equation is obeyed exactly, however complex the mechanism.

The Schild equation is valid for receptors with more than one binding site

All agonist-activated ion channels have more than one binding site for agonist and antagonist. GPCRs might function as dimers with two binding sites. Schild's original derivation does not deal with cases like these.

The approach through the Hill equation

The idea that several antagonist molecules might combine with the receptor was discussed even before Schild, by Gaddum (1943) [13]. His approach was through the Hill equation and was essentially based on what was known about the competition between oxygen and carbon monoxide for binding to haemoglobin. Gaddum was clearly not very happy with this approach because it was already

Box 2. Exact analysis for a complex mechanism: the glycine receptor flip mechanism

Figure I shows a 'flip' mechanism [30] but for a receptor with two binding sites rather than three (two is sufficient for generality of the results). It differs from standard mechanisms because it postulates the existence of a 'flipped' conformation, intermediate between the resting state and the open state.

In Figure I, A represents an agonist molecule, and B represents a molecule of the competitive antagonist. The receptor has three different conformations. The resting state of the receptor is denoted R, the flipped (but still shut) conformation, F and the open conformation, F*. In the presence of a competitive antagonist, there are 18 states in which the receptor can exist. The key to getting the right answer is to include all the states. One way of looking at why the Hill equation approach fails is that it does not do so. For example, non-liganded openings are included in the mechanism because, in principle, they must occur. In practice, with real data, the rate of spontaneous openings might be so low that they cannot be fitted in practice, but the argument now is about principles.

The rules in Box 1 imply the existence of all 18 states and, as long as all are included, the result comes out simply. They imply, for example, the existence of complexes that have both agonist and antagonist bound (ABR, ABF and ABF*) and the assumption that the binding of antagonist does not change the conformation means that such mixed complexes should behave like the same complexes without antagonist bound (AR, AF, AF*). Because we are assuming that true equilibrium is reached, all the cycles in the mechanism are assumed to obey microscopic reversibility [31].

Interaction between agonist binding sites

The mechanism shown in Figure I is more general than that described before [30]. One of the elegant features of the original mechanism was that a good fit to the data could be obtained when it was assumed that agonist-binding sites were independent of each other. In other words, for any given conformation (R, F or F*) of the receptor, binding of the agonist to one site was independent of whether the other site as occupied, so $K_{A1} = K_{A2}$ etc. In this case, the 18 states of the receptor could be divided into three different affinity classes, each containing six states. These are the top, middle and bottom planes in Figure I. In Figure I, however, we have allowed for the possibility that agonist-binding sites might interact, to justify the claim that this does not invalidate the Schild equation. The equilibrium constant for binding if the first agonist to the resting state of the receptor is denoted K_{A1}, but once one site is occupied, the second binding can occur with a different affinity, denoted K_{A2}. The notation is similar for the flipped (F) and open (F*) conformations.

The equilibrium fraction of receptors in each of the 18 states can be found easily, as for any other mechanism. Simply use the law of mass action to express the equilibrium occupancy for each state, p, as a multiple of the occupancy of, say, the resting state, R, which, according to the numbering of states in Figure I, is denoted p_{18}. For example, $p_{17} = 2c_{A1}\ p_{18}$ and $p_{16} = 0.5\ c_{A2}\ p_{17} = c_{A1}\ c_{A2}\ p_{18}$ and so on. This, together with the fact that the occupancies add up to 1, generates the 18 terms in the denominator. Thus, we can write, after rearranging the 18 terms in the denominator:

$$p_{18} = 1/d$$

where

$$d = (1 + c_B)^2(1 + F_0 + E_0F_0) + 2c_{A1}(1 + c_B)(1 + F_1 + E_1F_1) + c_{A1}c_{A2}(1 + F_2 + E_2F_2) \quad \text{[Equation I]}$$

and we define normalised concentrations as

$$c_{A1} = \frac{[A]}{K_{A1}} \quad \text{and} \quad c_{A2} = \frac{[A]}{k_{A2}} \quad \text{[Equation II]}$$

The equilibrium fraction in the open states (states 1 to 6 in Figure I), for example, can be written,

$$p_{open} = \frac{E_0F_0(1 + c_B)^2 + 2E_1F_1c_{A1}(1 + c_B) + E_2F_2c_{A1}c_{A2}}{d} \quad \text{[Equation III]}$$

The terms in the numerator are the same as those in the denominator but for the six open states only.

Notice that states 4, 5 and 6 (BF*, ABF* and B_2F^*), which have either one or two antagonist molecules bound, are counted as open states. This is implicit in our assumption (Box 1) that binding of antagonist has no effect, other than excluding agonist.

If we now divide top and bottom by $(1 + c_B)^2$ (compare Equation 5), we get:

$$p_{open} = \frac{E_0F_0 + 2E_1F_1\frac{c_{A1}}{(1+c_B)} + E_2F_2\frac{c_{A1}}{(1+c_B)}\frac{c_{A2}}{(1+c_B)}}{(1 + F_0 + E_0F_0) + 2\frac{c_{A1}}{(1+c_B)}(1 + F_1 + E_1F_1) + \frac{c_{A1}}{(1+c_B)}\frac{c_{A2}}{(1+c_B)}(1 + F_2 + E_2F_2)} \quad \text{[Equation IV]}$$

We now see that every time the agonist concentration, [A], occurs, it is divided by the Schild factor $(1 + c_B)$. Therefore, p_{open} must obey the Schild equation exactly and give the correct estimate of the equilibrium constant, K_B, for binding of the antagonist.

These results are written in terms of the affinities for the resting state only (K_{A1} and K_{A2}). The affinities for the other states are implicit in the constraints implied by microscopic reversibility. These are as follows:

$$F_1 = F_0\frac{K_{A1}}{K_{F1}} \quad F_2 = F_1\frac{K_{A2}}{K_{F2}} \quad E_1 = E_0\frac{K_{F1}}{K_{o1}} \quad E_2 = E_1\frac{K_{F3}}{K_{o2}} \quad \text{[Equation V]}$$

With these relationships, the denominator in Equation IV, $d_1 = d/(1 + c_B)^2$, say, can be written in a form that has terms that depend on the affinities for resting, flipped and open states.

$$d_1 = \underbrace{\left(1 + 2\frac{c_{A1}}{(1 + c_B)} + \frac{c_{A1}c_{A2}}{(1 + c_B)^2}\right)}_{Resting} + F_0\underbrace{\left(1 + 2\frac{c_{F1}}{(1 + c_B)} + \frac{c_{F1}c_{F2}}{(1 + c_B)^2}\right)}_{flipped} + E_0F_0\underbrace{\left(1 + 2\frac{c_{o1}}{(1 + c_B)} + \frac{c_{o1}c_{o2}}{(1 + c_B)^2}\right)}_{open} \quad \text{[Equation VI]}$$

The three major terms now correspond to the three horizontal planes in Figure I, as indicated in Equation VI.

In the case where the agonist binding sites do not interact, so $K_{A1} = K_{A2}$, these results simplify to the more elegant form:

$$d_1 = \underbrace{\left(1 + \frac{c_A}{(1 + c_B)}\right)^2}_{Resting} + F_0\underbrace{\left(1 + \frac{c_{AF}}{(1 + c_B)}\right)^2}_{flipped} + E_0F_0\underbrace{\left(1 + \frac{c_{Ao}}{(1 + c_B)}\right)}_{open} \quad \text{[Equation VII]}$$

If we denote the occupancy of state i as p_i, then the overall fractional occupancy of sites by agonist is given by:

$$p_{occ} = 0.5(p_2 + p_5 + p_8 + p_{11} + p_{14} + p_{17}) + p_1 + p_7 + p_{16} \quad \text{[Equation VIII]}$$

Writing this explicitly again shows that it has the Schild form: every occurrence of the agonist concentration. [A] occurs in the form $[A]/(1 + c_B)$.

Affinity classes

It is helpful in trying to understand the reason for these results to consider separately the top (resting), middle (flipped) and bottom (open) planes in Figure I.

The fraction of the six resting states (top plane) that is occupied by agonist is given by:

$$p_{rest} = \frac{0.5(p_{14} + p_{17}) + p_{16}}{p_{13} + p_{14} + p_{15} + p_{16} + p_{17} + p_{18}} = \frac{\left(\frac{c_{A1}}{(1+c_B)} + \frac{c_{A1}c_{A2}}{(1+c_B)^2}\right)}{\left(1 + 2\frac{c_{A1}}{(1+c_B)} + \frac{c_{A1}c_{A2}}{(1+c_B)^2}\right)} \quad \text{[Equation IX]}$$

Once again, this is seen to obey the Schild equation exactly.

If the agonist binding sites do not interact, so $K_{A1} = K_{A2}$, then Equation IX reduces to simply:

$$p_{rest} = \frac{c_A}{1 + c_A + c_B} = \frac{\left(\frac{c_A}{1+c_B}\right)}{1 + \left(\frac{c_A}{1+c_B}\right)} \quad \text{[Equation X]}$$

This is identical with the simple expression for a single sort of site (Equation 5), as might be expected because now all six resting states have the same affinity for the agonist.

Exactly similar expressions hold for the flipped states (middle plane) and open states (bottom plane) if the appropriate affinities are used.

These results show that the occupancies in each separate class of affinity states are kept constant in the presence of an antagonist, if the agonist concentration is raised by the Schild factor, $(1 + c_B)$. Furthermore, the fraction of all receptors that is in each affinity class follows directly from Equation VI or Equation VII and this is clearly also kept constant.

This result obviously generalises to any number of affinity classes, each containing any number of states.

Analysis of a mechanism in which conformation changes are not concerted

It might be stated that the mechanism in Figure I is not entirely general because all of the conformation changes are concerted. Consider another example in which subunits are not assumed to all have the same conformation. Figure II shows a simple example. The receptor is supposed to consist of two subunits, each of which can bind agonist separately and change conformation, according to the simple scheme of del Castillo and Katz (1957) [32] and each can be blocked by a competitive antagonist, B.

If the two subunits are identical and independent, then it is trivial to show that the Schild equation is obeyed exactly. But what happens if the subunits are not identical in their ability to bind agonist and/or in their ability to change conformation? And what happens if the subunits are not independent, in the sense that the behaviour of one subunit depends on whether or not the other subunit is occupied by agonist or not? Both of these possibilities are encompassed in Figure II. The equilibrium constants for the binding of agonist are denoted K_{1j} for subunit 1 and K_{2j} for subunit 2, with $j = 0$ if the other subunit is not occupied by agonist and $j = 1$ if the other subunit is occupied by agonist. The same notation is used for the efficacies, $E_{i\,j}$. Normalised agonist concentrations are defined in the obvious way, $c_{i\,j} = [A]/K_{ij}$.

In general, the scheme in Figure II has 16 distinct states of the receptor. Each of the four states of subunit one can be combined with any of the four states of subunit 2. After rearranging the 16 terms in the denominator, we can write, as in Equation I:

$$p_{16} = 1/d$$

where

$$d_1 = \frac{d}{(1+c_B)^2} = 1 + \frac{c_{10}}{(1+c_B)}(1+E_{10}) + \frac{c_{20}}{(1+c_B)}(1+E_{20}) + \frac{c_{10}c_{21}}{(1+c_B)^2}(1+E_{10}) \times (1+E_{21}) \quad \text{[Equation XI]}$$

Thus, if, for example, the only open state is that with both subunits in state 1, AR* (Figure II), then the equilibrium response is:

$$p_{open} = \frac{E_{10}E_{21}\frac{c_{10}c_{21}}{(1+c_B)^2}}{d_1} \quad \text{[Equation XII]}$$

Once again, the agonist concentration occurs only in terms with the form $[A]/(1 + c_B)$, so the Schild equation is obeyed exactly and gives the correct K_B.

If the subunits are identical and independent, then the subscripts are not needed, all $c_{ij} = c_A$ say, and Equation XII reduces to:

$$p_{open} = \left[\frac{Ec_A}{1 + c_B + c_A(1+E)}\right]^2 \quad \text{[Equation XIII]}$$

This is the square of the expression for a single subunit (because both must be in state 1 to get a response in this example).

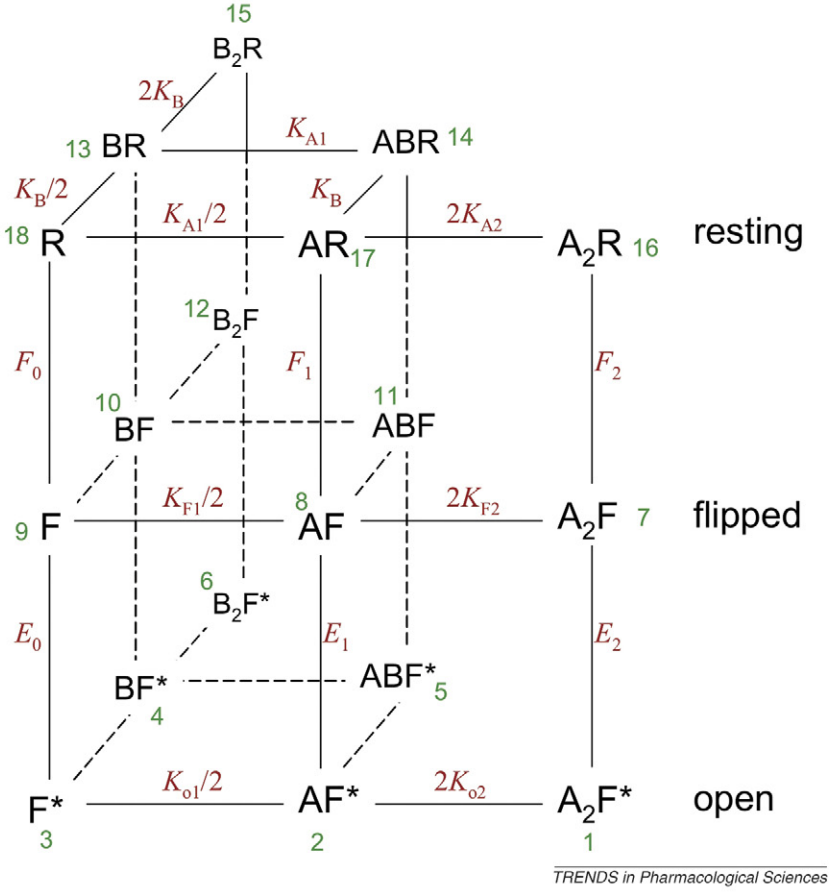

Figure I. Representation of the flip mechanism [30] for a receptor that contains two interacting and non-identical binding sites, in the presence an agonist (A) and a competitive antagonist (B). The 18 states are numbered arbitrarily.

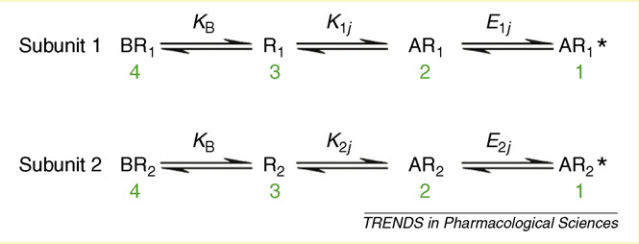

Figure II. A receptor that consists of two subunits of the del Castillo Katz (1957) type.

known in 1943 that haemoglobin binds four oxygen molecules but has a Hill slope of only about 2.5.

This had already been explained by Adair (1925). It happens because some molecules of haemoglobin have only one, two or three oxygen molecules bound, rather than four, and these cannot be neglected, By contrast, the Hill equation is essentially a limiting case in which all molecules are either unoccupied or have all four sites occupied.

Arunlakshana and Schild (1959) mention a similar approach but do not use it. It is clear that Schild thought that a real equilibrium constant for the antagonist could be estimated only when the Hill slope was 1 [3]. In his 1973 review, Schild [14] pointed out that genuine equilibrium constants were needed to classify receptors and said "It is doubtful whether valid data, suitable for receptor classification can be derived, unless the [Schild plot]... is linear with slope = 1".

Hill himself said "There can, however, be little doubt now that my equation $y = Kx^n/(1 + Kx^n)$, based on the aggregation theory, is wrong, or at least a serious oversimplification". He added later, "In 1962, when I wrote the preceding commentary, I had supposed that 'Hill's equation' and all its works had been dead and buried for more than a third of a century. To my astonishment, in 1963, I found it had been resurrected in 1961... The equation originally deduced in 1910 from the aggregation theory had been laid decently to rest in the 1920s; its body lay mouldering in the grave, but apparently its soul goes marching on" ([15], pp. 105–106).

In the same spirit, Bernard Katz [16] wrote, in his obituary for A.V. Hill: "It is generally realised that the Hill equation is strictly applicable only if all n molecules react simultaneously (which is unlikely), and it is theoretically unjustified if intermediate steps cannot be ignored. Nevertheless the formula has been widely used by many investigators. It has been a typical case of a useful theoretical 'half-truth', cutting corners and oversimplifying the real situation, but still enabling one to gain some insight and to make practical, if only approximate, calculations."

Gaddum and Schild, then, realised that the approach through the Hill equation would not give the right results and Gaddum (1943) [13] explained clearly what was wrong with it. Although they cited Hill forms from time to time, they did not use them. But others were less cautious and the incorrect approach, through the Hill equation, has persisted to the present day. The Hill-equation approach is described in standard textbooks [17,18] and also in many papers [19–22].

For example, in Ref. [17], a "general form of the Gaddum equation" is proposed, which (in the notation used here) can be written as:

$$p_A = \frac{c_A^n}{1 + c_A^n + c_B^m} \qquad \text{[Equation 6]}$$

where "n and m represent the numbers of agonist and antagonist molecules, respectively, that interact with the receptor". From this, a 'modified Schild equation' can be obtained [17]:

$$r^n = 1 + c_B^m \qquad \text{[Equation 7]}$$

As was known even in the 1920s, and discussed by Gaddum (1943) [13], n and m in this sort of equation are Hill slopes that cannot be equated with the "number of molecules", so this result is clearly baseless.

Likewise, it has been said [18] that "Schild analysis cannot give a precise estimate of K_B e.g. when two molecules of agonist must bind to two cooperatively linked sites for receptor activation to occur (Sine and Taylor, 1981 [23]). An example is the nicotinic cholinergic receptor on skeletal muscle linked to Na^+ channel [sic] opening. In this case the pA_2 value calculated from the Schild analysis does not correspond to the K_B for receptor antagonist interactions". Well, actually, it does. In fact, Sine and Taylor were concerned with the case in which there are two binding sites that have different affinities for the antagonist and do not claim that Schild analysis is affected by interaction between the agonist binding sites.

Others [22] use an equation similar to Equation 7 but do not make the mistake of calling n and m the number of molecules that combine with the receptor. They are referred to properly as Hill slopes. The analysis that follows still makes no sense, nevertheless, because the Hill equation is empirical. Therefore, it cannot be used to estimate real physical-equilibrium constants.

The reason that this Hill-equation approach is misleading is because it has not got a proper physical basis. Reactions of order n do not occur and n in the Hill equation does not represent the number of binding sites. Therefore, it cannot be used to represent the action of a competitive antagonist. Equation 7 is therefore derived from an incorrect premise and cannot be used for anything other than empirical curve fitting.

Fortunately, when the problem is analysed properly, the result turns out to be much simpler. It is a pity that neither Gaddum nor Schild tried to analyse competitive antagonism using the Adair (1925) [24] mechanism. If they had done so, they would have found that it predicted that the Schild equation would be obeyed exactly. Indeed, this is true for a wide class of mechanisms and so empirical coefficients, such as those in Equation 7, are unnecessary. It was pointed out in 1973 [9] that a mechanism of the Monod-Wyman-Changeux type obeyed the Schild equation exactly, but in that paper, there was no explicit consideration of the case in which the binding sites for agonist interact with each other. It turns out that this makes no difference to the conclusions.

Why do complicated mechanisms obey the Schild equation?

Under the conditions outlined in Box 1, the Schild equation is obeyed exactly, whether or not the agonist-binding sites interact.

The justification for this statement is given in Box 2, by the explicit analysis of particular mechanisms. The mechanisms chosen are sufficiently complicated that it is clear that results of the same form will be obtained however many states with different agonist affinities might exist and however agonist-binding sites interact.

For now, though, a much abbreviated form of the argument in Box 2 will be given with the aim of conveying the flavour of the reasoning without getting bogged down in the detail.

Consider, for example, a glycine-activated ion channel. This sort of receptor needs to bind three agonist (glycine) molecules for full activation [30]. The activation of the receptor can be described by a mechanism in which the binding sites interact strongly, so that the more molecules are bound, the higher the affinity of the remaining vacant sites. It can also be described by a mechanism that makes more physical sense (the flip mechanism), in which the binding sites are independent (do not interact) but in which the receptor can undergo a conformational change to a higher affinity state ('flipped' state) while still shut.

The assumption made in the Schild analysis is that, whenever we have identical occupancies by the agonist of every sort of binding site, the observed response will be the same.

Consider each individual binding site, rather than the whole receptor. In fact, it suffices to consider all states of the receptor that have the same affinity of agonist. For each such class, the occupancy by agonist of the sites in that class will be described by the simple competitive Equation 3. Different affinity classes will have different values for K_A, and so different c_A values, but it remains true that, every time the agonist concentration occurs on the right-hand side, it is in the form $[A]/(1 + c_B)$. It follows that the effect of adding an antagonist can be compensated by raising the agonist concentration by a factor $r = 1 + c_B$ at every class of site. The fraction of sites in each affinity class also stays constant under these circumstances. Thus, the Schild equation is obeyed exactly and gives a correct estimate of the real physical equilibrium constant for the binding of the competitive antagonist.

In the case of the glycine receptor examples, all the conformational changes are concerted (all the subunits change simultaneously), so that, at any instant, each receptor is in one or another of its affinity states. But this is not an essential part of the argument. Box 2 shows that the Schild equation is obeyed exactly if subunits are not all in the same conformation and that this is still true, even if the subunits interact and if they are not identical (for agonist binding) (Box 2).

What happens if the antagonist is actually an inverse agonist or a weak partial agonist?

All that has been said so far relies explicitly on the assumption that we are dealing with a pure competitive antagonist. In other words, the antagonist has no effects other than to occlude the binding site and so prevent binding of the agonist. It follows that a pure antagonist has exactly the same affinity for both active and inactive conformations of the receptor.

Is this condition verified for most antagonists? And does it matter if it is not?

In fact, at least for GPCRs, most competitive antagonists seem to be inverse agonists (e.g. [25]). This is shown by the fact that, when they are applied to a preparation in which receptors are active in the absence of agonist, this activity is inhibited. From the two-state model point of view, this is what would be expected; it is impossible that two ligands should have exactly the same relative affinity for active and inactive conformations.

It is also possible that a very weak partial agonist might be mistaken for a pure antagonist but, if it were so weak that the response was undetectable, the conclusions drawn here should be valid to a good approximation.

It makes little difference to any of the conclusions drawn here if the 'antagonist' is actually an inverse agonist [9], provided that the tests are done with an efficacious agonist and the spontaneous level of activity of the receptor, in the absence of agonist, is near zero. That is the case for most agonist-activated ion channels and also for most GPCRs. Mutant receptors might show much increased amounts of spontaneous activity and, for them, the theory presented here would have to be modified accordingly.

Some practical problems

It is the primary purpose of this article to explore the conditions under which the Schild equation is obeyed exactly and gives the correct equilibrium constant for the binding of a competitive antagonist. Nevertheless, it is worth mentioning briefly some practical matters that can arise in real experiments. Some of the hazards are as follows.

Problems in attaining genuine equilibrium

The assumption that equilibrium has been reached is important. It cannot be expected that the Schild equation will give good results with responses that are inherently not at equilibrium, such as synaptic currents or FLIPR (fluorescence imaging plate reader) assays. In the case of a high-affinity antagonist, it is expected that it will take a long time for equilibrium to be attained. In the absence of agonist, the time constant for the exponential approach towards equilibrium can be written as $k_{-B}(1 + c_B)$, where k_{-B} is the dissociation-rate constant for the antagonist [26]. This will usually be slow for a high-affinity antagonist. This means that the Schild method might not be feasible for antagonists with equilibrium constants in the picomolar range, for which equilibration might take hours rather than minutes to occur. This is a question that needs to be tested experimentally, if only because re-equilibration of antagonist occupancy when agonist is added may be faster than expected [27]. Clearly, the Schild method cannot be used at all for antagonists that bind irreversibly ($k_{-B} = 0$).

Desensitisation

Desensitisation is almost universal and it is often a problem when attempting to measure equilibrium responses.

There are two obvious solutions. One is to apply agonist for sufficiently long that desensitisation has reached equilibrium. Although there are no satisfactory explicit mechanisms for desensitisation, the considerations discussed here suggest that the extra-high affinity-desensitised states will not invalidate the Schild approach if they have reached equilibrium. The other solution is to apply agonist sufficiently fast that a plateau response can be obtained before much desensitisation has occurred. Of course, it is essential that the agonist and antagonist have equilibrated with the receptor by the time this plateau is reached, so this method is not going to work if the antagonist is slow or desensitisation is too fast, or both. It is worth remembering also that attainment of a plateau, with no visible sag in the response, is not sufficient reason to believe that desensitisation has been avoided [28].

Antagonists might have more than one effect

For example, competitive neuromuscular-blocking agents can also, as a separate effect, block the ion channel itself. In this type of case, methods have to be devised to separate the two effects.

Conclusion

Even 60 years after Gaddum pointed out that the Hill equation was inappropriate for the analysis of competitive antagonism, it continues to be used widely. It is wrong and the right analysis gives a much simpler result. Most receptor mechanisms, under the rather weak assumptions summarised in Box 1, predict that the Schild equation will be obeyed exactly by a competitive antagonist and that the Schild analysis will give the correct equilibrium constant of binding of the antagonist to the receptor.

Acknowledgements

I am grateful to Lucia Sivilotti and Donald Jenkinson for critical and helpful comments. And I am particularly grateful to Heinz Otto Schild [29], who gave me my first job and who failed to fire me despite my low productivity.

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