*Tutorials and worked examples for simulation, curve fitting, statistical analysis, and plotting.*
*http://www.simfit.org.uk*

Cooperative ligand binding models are used in the situation where a protein or receptor has more than one type of binding site and these are linked in such a way as to display deviations from normal hyperbolic binding. If a receptor has $n > 1$ binding sites that differ in binding constants but are independent this can only give rise to apparent negative cooperativity. If the sites are linked in that the binding to one site influences the subsequent binding of further ligands then positive or mixed cooperativity can be exhibited. These terms will be defined subsequently.

From the main SIMFIT menu choose [A/Z] then open program **sffit** and examine the default test file `sffit.tf4` which contains the following data.

| *x* | *y* | *se* |
|---|---|---|
| 0.085504 | 0.10022 | 0.0026739 |
| 0.085504 | 0.10533 | 0.0026739 |
| 0.085504 | 0.10142 | 0.0026739 |
| 0.11434 | 0.14319 | 0.010065 |
| 0.11434 | 0.16178 | 0.010065 |
| 0.11434 | 0.14578 | 0.010065 |
| 0.15291 | 0.24510 | 0.012043 |
| 0.15291 | 0.22191 | 0.012043 |
| 0.15291 | 0.22786 | 0.012043 |
| 0.20449 | 0.30735 | 0.019939 |
| 0.20449 | 0.32957 | 0.019939 |
| 0.20449 | 0.28978 | 0.019939 |
| 0.27346 | 0.43824 | 0.0071355 |
| 0.27346 | 0.43342 | 0.0071355 |
| 0.27346 | 0.44746 | 0.0071355 |
| 0.36569 | 0.57197 | 0.014359 |
| 0.36569 | 0.56004 | 0.014359 |
| 0.36569 | 0.58863 | 0.014359 |
| 0.48903 | 0.64381 | 0.030621 |
| 0.48903 | 0.63820 | 0.030621 |
| 0.48903 | 0.69382 | 0.030621 |
| 0.65398 | 0.75455 | 0.017667 |
| 0.65398 | 0.78973 | 0.017667 |
| 0.65398 | 0.77504 | 0.017667 |
| 0.87456 | 0.81456 | 0.030889 |
| 0.87456 | 0.82605 | 0.030889 |
| 0.87456 | 0.76774 | 0.030889 |
| 1.1695 | 0.95153 | 0.029772 |
| 1.1695 | 0.89315 | 0.029772 |
| 1.1695 | 0.91216 | 0.029772 |

The columns contain data in the following format.

1. **Column 1**: the non–negative ligand concentration $x$ which must be in non-decreasing order.
2. **Column 2**: the non–negative response $y$ presumed to be dependent on fractional saturation of receptor or binding site at the concentration in column 1.
3. **Column 3**: the positive sample standard deviation of the replicate response measurements.
   This column can be omitted or set to 1 if unweighted regression is required.

The model $f(x)$ fitted by SimFIT program **sffit** for $n$ binding sites in the presence of ligand at concentration $x$ is based on a binding polynomial $p(x)$ and fractional saturation function $y(x)$ expressed using overall binding constants $K_i$ as follows

$$\begin{aligned} p(x) &= 1 + K_1 x + K_2 x^2 + \cdots + K_n x^n \\ y(x) &= \frac{1}{n}\frac{d \log p(x)}{d \log x} \\ &= \frac{1}{n}\frac{x p'(x)}{p(x)} \\ f(x) &= Z y(x) + C. \end{aligned}$$

Here $Z$ is an arbitrary factor relating the observed response to fractional saturation, while $C$ is a possible background noise in the absence of any ligand. It is supposed that the number of sites $n$ would be known in advance while the arbitrary scaling factor $Z$ and background noise $C$ would be estimated in a preliminary run and used to normalize the data so that $0 \le f(x) \le 1$ for $x \ge 0$.

Before proceeding to discuss the results from analyzing the test data two things should be noted.

1. When the data have been normalized so that $Z = 1$ and $C = 0$, as with the data in test file `sffit.tf4`, program **sffit** begins by scaling the data internally, performing a $L_1$ norm fitting procedure for starting estimates followed by a refinement by random searching. For low order models with accurate data over a large range of $x$ this will often mean that the starting estimates are very close to the best-fit parameters, and the quasi-Newton constrained regression program will draw attention to the fact that only a small percentage reduction in the objective function $WSSQ$ has been achieved. This simply indicates how good the **sffit** algorithm has been in calculating starting estimates.

2. Users are given the option to fit several values of $n$ in sequence with statistical tests for model discrimination and goodness of fit. This facility is provided for preliminary analysis in the case where $Z$ is estimated and/or $n$ is not known in advance, and also so that **sffit** can be used as an empirical model for data smoothing

Proceeding to start optimization at $n = 2$ and end optimization at $n = 2$ with $Z = 1$ and $C = 0$ means that the following model was fitted

$$f(x) = \left(\frac{1}{2}\right)\frac{K_1 x + 2K_2 x^2}{1 + K_1 x + K_2 x^2}$$

leading to this table of results.

For best-fit order 2 function ($f$ for fixed parameter)

| Number | Parameter | Value | Std. error | Lower95%cl | Upper95%cl | $p$ | |
|---|---|---|---|---|---|---|---|
| 1 | $K_1$ | 1.0734 | 0.063088 | 0.94414 | 1.2026 | 0.0000 | |
| 2 | $K_2$ | 10.042 | 0.17298 | 9.6881 | 10.397 | 0.0000 | |
| 3 | $Z$ | 1.0000 | 0.0000 | 1.0000 | 1.0000 | ... | $f$ |
| 4 | $C$ | 0.0000 | 0.0000 | 0.0000 | 0.0000 | ... | $f$ |

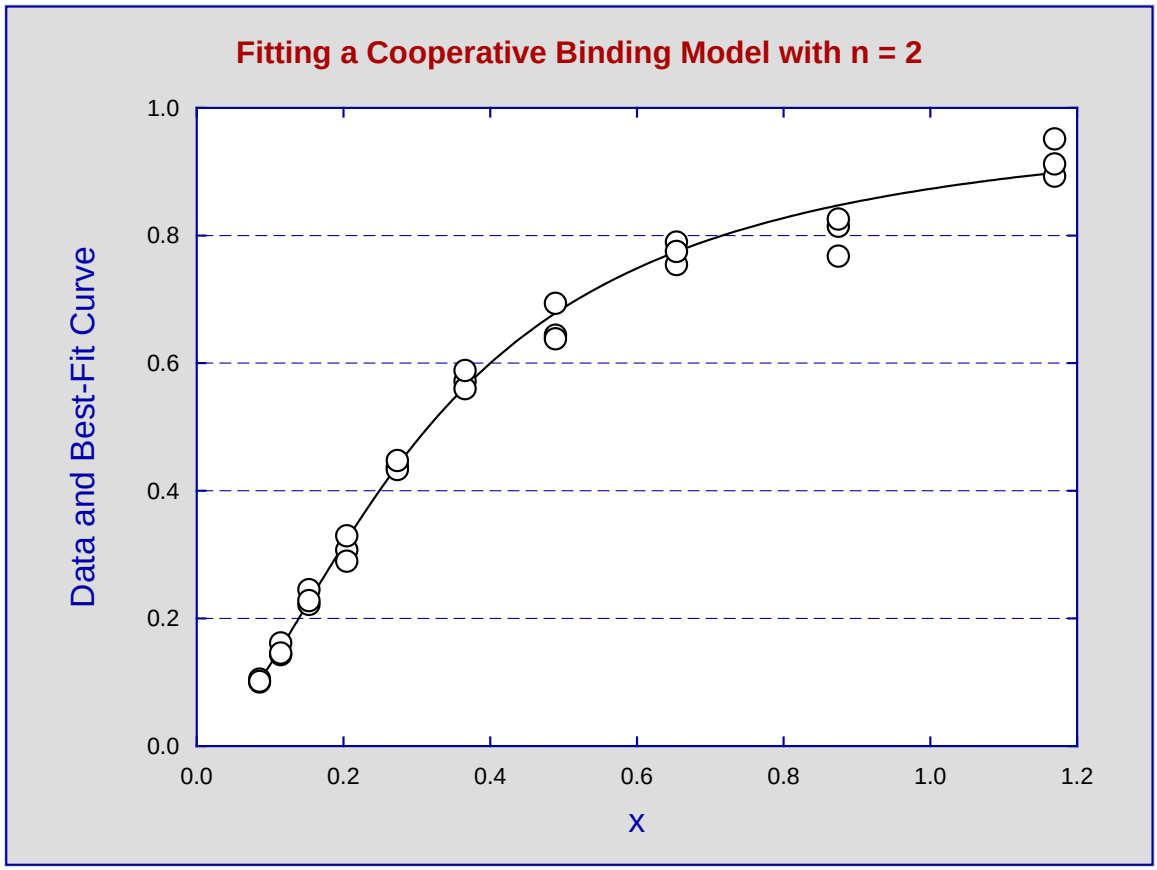
Apparent $V_{max}$ (i.e. $Z$ or $f(\infty) - C$) = 1.0000
Apparent $K_m$ (i.e. $x$ where $f(x) - C = Z/2$) = 0.31554

Parameter correlation matrix ($f$ for fixed parameter)

| | | | |
|---|---|---|---|
| 1 | | | |
| -0.5562 | 1 | | |
| $f$ | $f$ | $f$ | |
| $f$ | $f$ | $f$ | $f$ |

The excellent fit will be clear from the analysis of residuals and plot of data and best-fit curve as shown next.

| Goodness of fit for model with $n = 2$ | |
|---|---|
| Analysis of residuals: $WSSQ$ | 33.100 |
| $P(\chi^2 \geq WSSQ)$ | 0.2321 |
| $R^2, cc(theory, data)^2$ | 0.9928 |
| Largest Absolute relative residual | 10.87% |
| Smallest Absolute relative residual | 0.04% |
| Average Absolute relative residual | 3.47% |
| Absolute relative residuals in range 0.1-0.2 | 3.33% |
| Absolute relative residuals in range 0.2-0.4 | 0.00% |
| Absolute relative residuals in range 0.4-0.8 | 0.00% |
| Absolute relative residuals > 0.8 | 0.00% |
| Number of negative residuals ($m$) | 17 |
| Number of positive residuals ($n$) | 13 |
| Number of runs observed ($r$) | 22 |
| $P$(runs $\leq r$ : given $m$ and $n$) | 0.9957 |
| 5% lower tail point | 10 |
| 1% lower tail point | 9 |
| $P$(runs $\leq r$ : given $m$ plus $n$) | 0.9959 |
| $P$(signs $\leq$ least number observed) | 0.5857 |
| Durbin-Watson test statistic | 2.441 |
| Shapiro-Wilks $W$ statistic | 0.9781 |
| Significance level of $W$ | 0.7729 |
| Akaike $AIC$ (Schwarz $SC$) stats | 6.9509 (9.7529) |
| Verdict on goodness of fit: incredible | |



At this point various options are available for further study of the best fitting order two saturation function. Choosing cooperativity analysis we first observe the percentage saturation points given the $K_i$ values, which allows users to see at a glance the range of saturation spanned by the range of the data.

| Overall association constants and % saturation points | | |
|---|---|---|
| $K_1$ | 1.0734 | |
| $K_2$ | 10.042 | |
| $X$ start | at $x = 0.085504$ | |
| $X$ stop | at $x = 1.695$ | |
| $y = 0.05$ | at $x = 0.0051378$ | The 5% saturation point |
| $y = 0.10$ | at $x = 0.084086$ | The 10% saturation point |
| $y = 0.50$ | at $x = 0.31556$ | The 50% saturation point |
| $y = 0.90$ | at $x = 1.1843$ | The 90% saturation point |
| $y = 0.95$ | at $x = 1.9381$ | The 95% saturation point |

Evidently the range of these experimental data spans the range from around 10% saturation to just over the point of 90% saturation. Perhaps it will not be often that experimentalists will be able to achieve such a wide span.

The next table displays the values of the association constants and reciprocals for overall association constants $K_i$, Adair constants $A_i$ and Adair constants corrected for statistical factors $B_i$ so that the results can be compared between alternative computer packages. Note that SimFIT program **qnfit** can be used to fit these alternative model formulations if confidence limits on parameter estimates and parameter correlation matrices are required. All other goodness of fit and model discrimination results are the same irrespective of the model formulation.

| Alternative expressions for binding constants | | | | | | |
|---|---|---|---|---|---|---|
| Number | $K$ | $1/K$ | $A$ | $1/A$ | $B$ | $1/B$ |
| 1 | 1.0734 | 0.93165 | 1.0734E+00 | 0.93165 | 0.53668 | 1.8633 |
| 2 | 10.042 | 0.099577 | 9.3560E+00 | 0.10688 | 18.712 | 0.053442 |

| Intrinsic cooperativity coefficient | Value | Sign |
|---|---|---|
| $B_2 - B_1$ | 18.175 | + |

Intrinsic cooperativity coefficients are particularly easy to interpret in molecular terms. For instance, if $B_i > B_{i-1}$ this indicates that when ligand is bound to $i-1$ sites the affinity increase for when the the next ligand binds, whereas when $B_i < B_i$ the affinity decreases. So, when there are only two binding sites, the condition $B_2 > B_1$ is equivalent to positive cooperativity and the condition $B_2 < B_1$ is correctly referred to as negative cooperativity. Unfortunately, for more than two sites this argument breaks down due to the additional complication of species fractional populations $S_i$ defined as

$$S_i = \frac{K_i x_i}{K_0 + K_1 X + K_2 X^2 + \cdots + K_n x^n}.$$

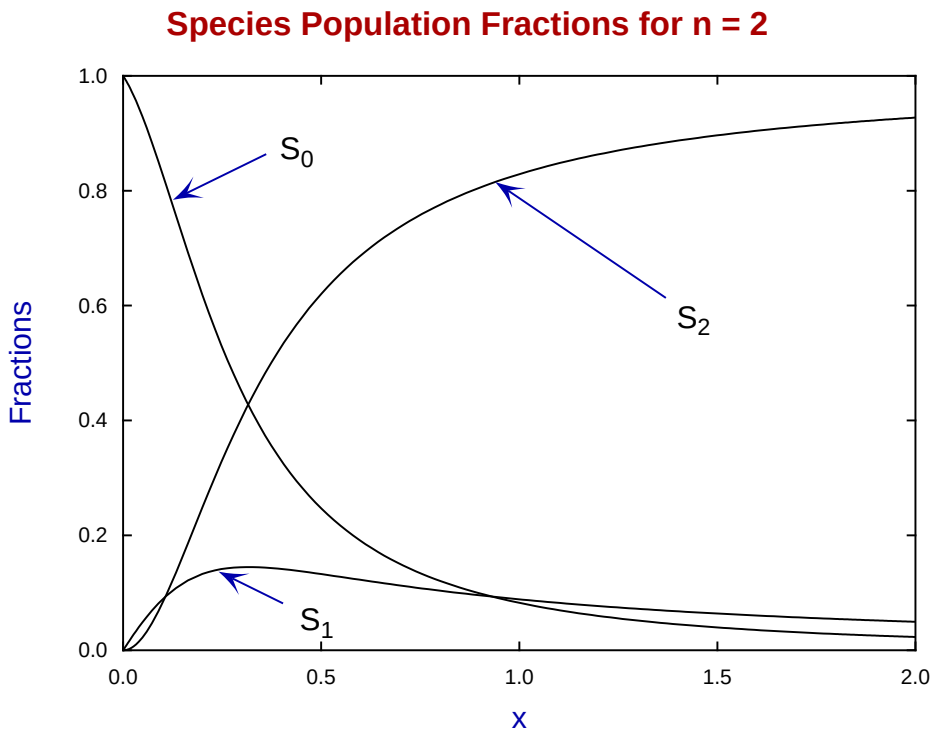
for $i = 0, 1, 2 \ldots, n$. Here $K_0 = 1, \quad 0 \le S_i \le 1, \quad S_0 + S_1 + S_2 + \cdots + S_n = 1$ and the $S_i$ measure the proportion of the macromolecule with $i$ ligands bound as the concentration of ligand $x$ varies. A similar measure, the species fraction $S_{fi}$, defined as

$$S_{fi} = iS_i/n$$

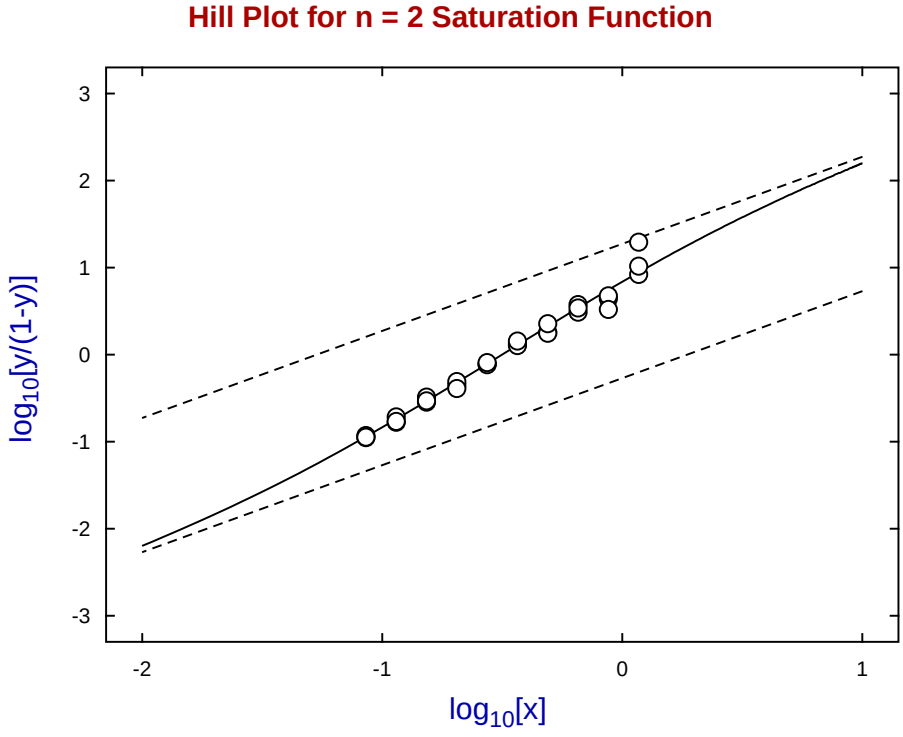
for $i = 1, 2, \ldots, n$ takes account of the stoichiometry and measures the contribution of the species with $i$ ligands attached to the overall fractional saturation $y(x)$ as

$$S_{f1} + S_{f2} + \ldots, +S_{fn} = y(x).$$

The next plot displays the population fractions for the current best-fit model showing that all the macromolecule is free from ligand at $x = 0$, then the macromolecule with one ligand appears then disappears as $x$ increases until eventually as $x \to \infty$ all the binding sites are occupied. It is this fact that makes the interpretation of the sign of cooperativity ambiguous when the order exceeds 2 which is where the Hill plot slope is a less ambiguous measure of the sign and extent of cooperativity when viewed as a function of ligand activity.




The Hill plot slope shown next is very simple to interpret in the case of fitting an order $n = 2$ saturation function but, as will be discussed subsequently, the situation is not so simple for order $n > 2$, and this is an area of unnecessarily great confusion.



## Theory

Ligand binding theory will be presented under the following headings.

1. Historical introduction
2. Binding polynomials
3. Definition of cooperativity
4. Factorability of the binding polynomial
5. Statistical interpretation of saturation functions
6. Cooperativity analysis

## Historical Introduction

In 1910 Hill [1] proposed that the sigmoid binding curve for oxygen binding to haemoglobin could be analyzed in terms of the binding of $n$ ligands in one step with no appreciable intermediates, i.e. the mass action description

$$Hb + nX \rightleftharpoons HbX_n.$$

This leads to the Hill equation describing the fractional saturation $y$ as a function of concentration $x$, and the Hill plot of $\log[y/(1-y)]$ as a function of $\log x$ as follows

$$y = \frac{Kx^n}{1 + Kx^n}$$
$$\log\left(\frac{y}{1-y}\right) = n \log x + \log K.$$

It is now realized that the Hill equation is simply an empirical equation that is at best a poor approximation to any real binding situation since:

1. it is only an appropriate representation for a one-site binding process, i.e. for $n = 1$ ;
2. when $n < 1$ it has an infinite slope at the origin and cannot model any realistic binding situation;
3. when $n > 1$ it has zero slope at the origin and cannot model any realistic binding situation;
4. when $n$ is not a positive integer it is pure nonsense; and
5. using it to discuss the effect of cooperativity on graphical features such as sigmoidicity in the $y(x)$ curve, or convexity in Lineweaver-Burke or Scatchard space, has resulted in considerable confusion.

Of course, before the days of computers and nonlinear regression, fitting a straight line to a Hill plot to get a non-integer value for the estimated slope was all that could be done, and this non-integer value was correctly taken to mean that this was a result of the model being incorrect.

Nowadays no one would dream of discussing cooperative binding in terms of the Hill equation or fitting a straight line to a Hill plot but, by a serendipitous coincidence, it turns out that the variable slope of the curve obtained by transforming a saturation curve into Hill space still provides an unambiguous definition of the sign and magnitude of cooperativity that has got nothing at all to do with the Hill equation. That is because, to use receptor terminology,

$$\frac{y}{1-y} = \frac{[\text{Bound}]}{[\text{Free}]}.$$

## Binding polynomials and their Hessians

In 1925 Adair [2] improved the description of binding isotherms by defining binding constants for the individual binding events, and later it came to be appreciated that these have to be normalized by statistical factors in order to discuss the affinity of receptor for ligand in adjacent binding events. In 1967 Wyman [3] rationalized the situation by pointing out that, for a non-aggregating macromolecule with $n$ binding sites and only one ligand $x$ varied, there would be binding polynomial which would act like a partition function in that successive terms of degree $i$ in the polynomial are proportional to the amount of macromolecule with $i$ ligands attached.

So now the binding of ligands to receptors can be defined for all possible cooperative binding schemes in terms of a binding polynomial $p(x)$ in the free ligand activity $x$, as follows

$$\begin{aligned} p(x) &= 1 + K_1 x + K_2 x^2 + \cdots + K_n x^n \\ &= 1 + A_1 x + A_1 A_2 x^2 + \cdots + \prod_{i=1}^{n} A_i x^n \\ &= 1 + \binom{n}{1} B_1 x + \binom{n}{2} B_1 B_2 x^2 + \cdots + \binom{n}{n} \prod_{i=1}^{n} B_i x^n, \end{aligned}$$

where the only difference between these alternative expressions concerns the meaning and interpretation of the binding constants. The fractional saturation is just the scaled derivative of the log of the polynomial with respect to $\log(x)$, and an important auxiliary function is $h(x)$, the Hessian of the binding polynomial defined as follows

$$\begin{aligned} y(x) &= \left(\frac{1}{n}\right) \frac{d \log p(x)}{d \log x} \\ &= \left(\frac{1}{n}\right) \frac{x p'(x)}{p(x)} \\ h(x) &= n p p'' - (n-1) p'^2. \end{aligned}$$

## Definition of cooperativity

Given a binding polynomial of degree $n$ there are $n - 1$ cooperativity coefficients $c_i$ defined as

$$c_i = B_{i+1} - B_i \text{ for } i = 1, 2, \ldots, n-1,$$

or alternatively as $\log(B_{i+1}/B_i)$, and the interpretation of these is perfectly clear: in a situation where $c_i > 0$ the macromolecule has greater affinity for binding the $i + 1$th ligand after the $i$th ligand has been bound and it is perfectly reasonable to describe this as mechanistic positive cooperativity. Hence every binding situation for $n$ ligands can be summarized by a succession of $n - 1$ signs and it might be thought that during the actual saturation of macromolecule with ligand there would be a succession of phases with possibly differing cooperativity. For instance, the sequence $+ - +$ might be supposed to give a saturation curve with positive, then negative, then positive cooperativity. Unfortunately the cooperativity coefficients cannot be interpreted in this way and they are not a unique indicator of the sign and magnitude of the type of cooperativity exhibited during the saturation process. The reason for this is simply that binding does not occur in a succession of isolated steps and at every stage for $0 < x < \infty$ every species that is possible is present, that is no ligands bound, one ligand bound, two ligands bound, etc. up to $n$ ligands bound.

At every point in the range $0 < x < \infty$ there is a one site binding curve $y_{app}$ with a uniquely defined apparent binding constant $K_{app}$ according to the scheme

$$[\text{Free sites}] + X \rightleftharpoons [\text{Occupied sites}]$$

that is

$$y_{app}(x) = \frac{K_{app}x}{1 + K_{app}x}.$$

Surely all would agree that the sign and magnitude of cooperativity at that point in the saturation curve would depend on whether $K_{app}$ is increasing or decreasing as a function of $x$. It turns out that

$$K_{app} = \frac{p'(x)}{np(x) - xp'(x)} \text{ and}$$
$$\frac{dK_{app}}{dx} = \frac{h(x)}{(np(x) - xp'(x))^2}$$

so that increasing affinity (i.e. positive cooperativity) requires $h(x) > 0$, decreasing affinity (i.e. negative cooperativity) requires $h(x) < 0$ while at a point where $h(x) = 0$ cooperativity changes sign. Bardsley and Wyman [4] emphasized that the magnitude of the Hill slope with respect to 1 is the unambiguous indicator of cooperativity which also depends on the sign of the Hessian as follows

$$\frac{d\log[y/(1-y)]}{d\log x} = 1 + \frac{xh(x)}{p'(x)(np(x) - xp'(x))}.$$

and Wood and Bardsley [5] proved that the Hessian can have at most $n - 2$ positive zeros.

## Zeros of the binding polynomial

If the $n$ zeros of the binding polynomial are $\alpha_i$ then the fractional saturation $y$ can be expressed as

$$y = \left(\frac{x}{n}\right)\sum_{i=1}^{n}\frac{1}{x - \alpha_i},$$

but further discussion depends on the nature of the zeros.

First observe that, for a set of $m$ groups of receptors, each with $n_i$ independent binding sites and binding constant $k_i$, then the zeros are all real and

$$p(x) = \prod_{i=1}^{m}(1 + k_i x)^{n_i},$$
$$\text{and } y = \frac{1}{\sum_{i=1}^{m} n_i}\sum_{i=1}^{m}\frac{n_i k_i x}{1 + k_i x},$$

so $y$ is just the sum of simple binding curves, giving concave down double reciprocal plots, etc.

Actually Bardsley et al [6] and [7] proved that, if a binding polynomial factorizes into $m$ polynomials $p_i$ with positive coefficients according to

$$p(x) = p_1(x)p_2(x)\ldots p_m(x)$$

then the Hill plot slope cannot exceed that of the Hill plot slope for any of the individual factors. As a binding polynomial can always be factorized into a product of linear factors with real negative zeros and complex conjugate pairs forming quadratic factors it might be supposed that the Hill slope can never exceed two. However, if a binding polynomial of degree > 2 has complex conjugate zeros, the Hill slope may exceed two and there may be evidence of strong positive cooperativity. That is why Hill plot slopes up to a maximum of the degree of the binding polynomial can be achieved if there are quadratic factors with negative coefficients, corresponding to a group of at least three linked binding sites.

For instance, the binding polynomial for a four site Monod-Wyman-Changeux model is

$$p(\alpha) = \frac{1}{1 + L}\left((1 + \alpha)^4 + L(1 + c\alpha)^n\right)$$

and this can factorize into the form

$$q(x) = (1 + a_1 x + b_1 x^2)(1 - a_2 x + b_2 x^2)$$

with $a_1 > 0, a_2 > 0, b_1 > 0, b_2 > 0$ under certain constraints so that the meaningless quadratic factor with a negative term allows Hill slopes greater than two.

Edelstein and Bardsley [8] subsequently explored the relationship between the Hill slope at half-saturation and the Hessian of the binding polynomial.

## Statistical interpretation of saturation functions

The species fractional populations $s_i$ which are defined for $i = 0, 1, \ldots, n$ as

$$s_i = \frac{K_i x^i}{K_0 + K_1 x + K_2 x^2 + \cdots + K_n x^n}$$

with $K_0 = 1$, are interpreted as the proportions of the receptors in the various states of ligation as a function of ligand activity. The species fractions defined as $y_i = i s_i / n$ for $i = 1, 2, \ldots, n$ are the contributions of the species to the overall saturation. Note that

$$\sum_{i=0}^{n} s_i = 1, , \text{ while}$$
$$\sum_{i=1}^{n} y_i = (1/n) d \log p / d \log x.$$

Such expressions are very useful when analyzing cooperative ligand binding data and they can be generated from the best fit binding polynomial after fitting binding curves with program **sffit**, or by interactive input of binding constants into program **simstat**. At the same time other important analytical results like factors of the Hessian and minimax Hill slope are also calculated.

The species fractional populations can be also used in a probability model to interpret ligand binding in several interesting ways. For this purpose, consider a random variable $U$ representing the probability of a receptor existing in a state with $i$ ligands bound. Then the the probability mass function, expected values and variance are

$$\begin{aligned}
P(U = i) &= s_i \ (i = 0, 1, 2, \ldots, n), \\
E(U) &= \sum_{i=0}^{n} i s_i, \\
E(U^2) &= \sum_{i=0}^{n} i^2 s_i, \\
V(U) &= E(U^2) - [E(U)]^2 \\
&= x \left( \frac{p'(x) + x p''(x)}{p(x)} \right) - \left( \frac{x p'(x)}{p(x)} \right)^2 \\
&= n \frac{dy}{d \log x},
\end{aligned}$$

as fractional saturation $y$ is $E(U)/n$. In other words, the slope of a semi-log plot of fractional saturation data indicates the variance of the number of occupied sites, namely; all unoccupied when $x = 0$, distribution with variance increasing as a function of $x$ up to the maximum semi-log plot slope, then finally approaching all sites occupied as $x$ tends to infinity. You can input binding constants into the statistical calculations procedure to see how they are mapped into all spaces, cooperativity coefficients are calculated, zeros of the binding

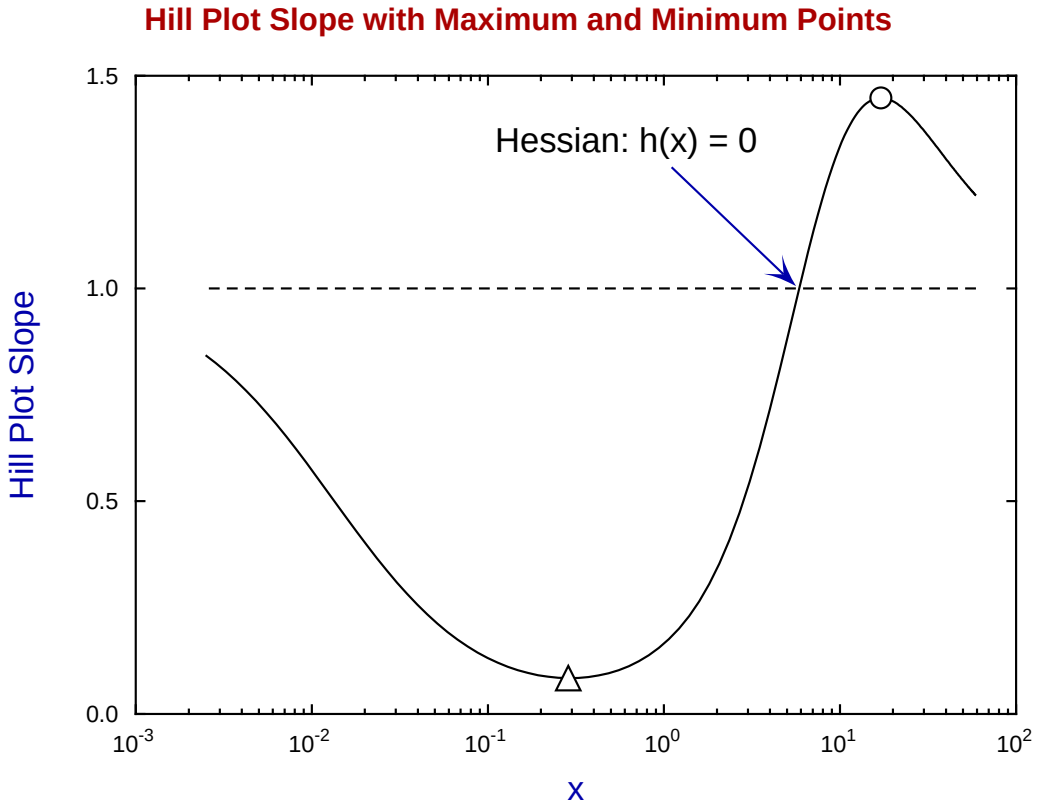
polynomial and Hessian are estimated, Hill slope is reported, and species fractions and binding isotherms are displayed, as is done automatically after every $n > 1$ fit by program **sffit**.

## Cooperativity analysis

After fitting a model, program **sffit** outputs the binding constant estimates in all the conventions and, when $n > 2$ it also outputs the zeros of the best fit binding polynomial and those of the Hessian of the binding polynomial $h(x)$. The positive zeros of $h(x)$ indicate points where the theoretical one-site binding curve coinciding with the actual saturation curve at that $x$ value has the same slope as the higher order saturation curve, which are therefore points of cooperativity change. The SimFIT cooperativity procedure allows users to input binding constant estimates retrospectively to calculate zeros of the binding polynomial and Hessian, and also to plot species population fractions.

For instance, for 4 sites with $K_1 = 100, K_2 = 10, K_3 = 1,$ and $K_4 = 0.1$, the Hessian has a positive zero at $x = 5.86139$, the minimum Hill slope in the range plotted is $0.0842$, at $x = 0.28607$, the maximum is $1.44479$, at $x = 17.059$, and the slope at half saturation is $1.0847$, at $x = 6.5808$.

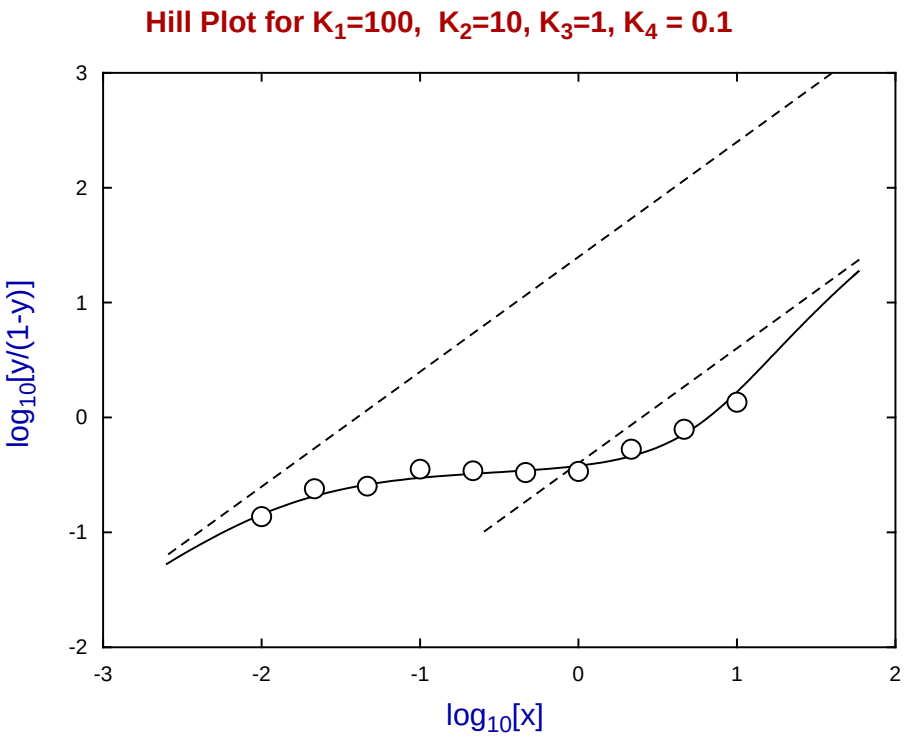
The next graph shows how the Hill plot slope varies with the maximum and minimum slopes indicated along with the point where the positive zero of the Hessian occurs.




The following graph shows the sort of complicated Hill plots that can be obtained when there are more than two cooperatively linked sites. The asymptotes are for the equation

$$y = \frac{kx}{1 + kx}$$

with $k = K_1/n$ as $x \to 0$ and $k = nK_n/K_{n-1}$ as $x \to \infty$.

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