Co-operativity and the Methods of Plotting Binding and Steady-State Kinetic Data

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(Received 8 February 1978)

The features that distinguish positive from negative co-operativity in double-reciprocal Eadie–Hofstee–Scatchard and Hanes plots, often incorrectly stated to be the sign of curvature or second derivatives, are explained. It is shown how to determine the ‘Hill exponent’ and interaction free energies from curves in these plots, and in the simple plot of ligand binding or velocity against free ligand or substrate concentration. New types of plots, where the kind of co-operative behaviour is more obvious than in the traditional ones, are proposed.

Accounts in the literature and textbooks could generate confusion about how co-operativity is manifested in the various plots used in ligand-binding and steady-state enzyme kinetic studies, such as the double-reciprocal plot, the Eadie–Hofstee–Scatchard plot and the Hanes plot. It is often stated or implied that the sign of co-operativity corresponds to a particular type of curvature or sign of second derivative in the plots (for instance, positive co-operativity to upwards-curvature or positive second derivative in the double-reciprocal plot). These correspondences do not, in fact, always hold (Bardsley, 1977*a,b,c*), and, when they do, it is for somewhat indirect reasons. The type of co-operativity could consequently sometimes be mistaken, particularly in curves of complicated shape. Some authors use intercepts of tangents to the curves to define apparent K_m and V_{max} values. These are often negative, so this is a perplexing manner of speaking.

The above does not mean that the sign of co-operativity cannot be decided by using such plots, but only that it must be inferred from some feature other than curvature or second derivative. The sign of co-operativity, and quantitative measures of it, ‘Hill’s exponent’ and interaction free energies, can be obtained from curves in the Hill plot (Wyman, 1964). A single set of data or a single equation gives rise to a curve in each plot, so it stands to reason that this same information can be obtained from curves in any usual plot. The tangents to curves in the plots can serve to measure co-operativity (see below).

Below, co-operativity of equilibrium binding of ligand of concentration x to chemically equivalent binding sites will be studied. The concentration of free

sites is $[E]$, of ligand-occupied sites is $[EX]$ and fractional saturation (fraction of sites occupied) is $\bar{X} = [EX]/([E] + [EX])$, and it is assumed that $x \gg ([E] + [EX])$. Substrate co-operativity in steady-state kinetic studies (better called ‘apparent binding co-operativity’) is obtained by substituting v/V_{max} as a fraction of that at saturating substrate concentrations, for $\bar{X}$ in all arguments below. This is purely a definition by which steady-state kinetic curves can be classified; it is equivalent to equilibrium co-operativity of substrate binding only if $\bar{X} = v/V_{max}$ at all points on a kinetic curve. This equation is satisfied in quasi-equilibrium kinetics when the effective catalytic constants at all substrate sites are equal. It cannot be satisfied where the plot of v against x has a maximum, and such cases are excluded from consideration.

A profitable, and perhaps the only, way to think about macroscopic co-operativity of equilibrium binding is to consider that the ‘constant’:

$$K_{\bar{X}} = \frac{[E]x}{[EX]} = x \left(\frac{1}{\bar{X}} - 1 \right) \quad (1)$$

of equilibrium between a ligand and its binding sites:

varies with saturation (to remind ourselves of this we write it with subscript $\bar{X}$), decreasing as $\bar{X}$ increases if co-operativity is positive, increasing if it is negative. It is obvious from eqn. (1) that the value of $K_{\bar{X}}$ at any given saturation is the slope of the chord drawn (see Fig. 1) to the corresponding point of the saturation curve in the double-reciprocal plot through the point $1/\bar{X} = 1$ on the ordinate (or through the point $v = V_{max}$ in steady-state kinetics, but see comments above). $-1/K_{\bar{X}}$ is therefore equal to the intercept of the chord on the abscissa, in accord with the usual method of estimating Michaelis constants in non-co-

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Fig. 1. Double-reciprocal plot

The curve illustrated is positively co-operative at $1/\bar{X}$ values less than that corresponding to the point Q, and negatively co-operative beyond Q. The line PC is tangent to the curve at P.

operative systems. Therefore co-operativity is positive when the chord flattens as $\bar{X}$ increases ($1/\bar{X}$ decreases) (Fig. 1) and negative when it steepens.

Curvature, tangents or second derivatives thus have nothing directly to do with the sign of co-operativity. When curvature or second derivative is of constant sign throughout the curve, then so indeed is co-operativity, so it is understandable that these features have been thought to give the sign of co-operativity. But a curve can have, for instance, downwards-curvature and negative second derivative in a region where co-operativity is, in fact, positive, as in Fig. 1 between the point Q and the inflexion point. A change in the sign of co-operativity occurs, not at an inflexion point, but where the chord is tangent to the curve (e.g. Q in Fig. 1). Binding and rate equations for non-dissociating proteins or enzymes must have degree in x greater than 2 to generate such a change. It is impossible for a curve to show positive co-operativity in any region without having a positive second derivative in some region, and negative co-operativity anywhere is likewise impossible without a negative second derivative somewhere. However, a curve can have an inflexion without a change in sign of co-operativity anywhere.

Analogous plots apply to the other plots. $-K_{\bar{X}}$ at any point, P, in the Eadie plot ($\bar{X}$ against $\bar{X}/x$) is given by the slope of a chord to P from the point $\bar{X} = 1$ on the ordinate, and the intercept of this with the abscissa is therefore $1/K_{\bar{X}}$. Co-operativity is positive if the chord flattens as we ascend the curve, negative if it steepens, and changes sign where the chord is tangent to the curve. In the Hanes plot ($x/\bar{X}$ against x) $K_{\bar{X}}$ at point P equals the intercept on the ordinate of a line of slope 1 drawn through point P. The same construction gives $-\log K_{\bar{X}}$ in the Hill plot (Whitehead, 1973). Positive co-operativity corresponds to a curve slope

of less than 1, and negative to slope greater than 1, in the Hanes plot. It is well known that a slope (h) greater than 1 in the Hill plot corresponds to positive and less than 1 to negative co-operativity (Wyman, 1964; Whitehead, 1970). One notes a family resemblance between the double-reciprocal plot and the Eadie plot on the one hand and between the Hill plot and the Hanes plot on the other. In the simple plot of $\bar{X}$ against x , $K_{\bar{X}}$ at point P is (see Fig. 4) the distance BC between the projection of P on the horizontal line $\bar{X} = 1$ and the intersection C of a chord from the origin through point P with this same line. The variation of $K_{\bar{X}}$ is less easy to spot by eye in this plot than in others.

It was mentioned above that 'Hill's exponent' (formerly symbolized by n or n_H , but now by h) can be obtained from the various plots. Thus if the definition of h :

$$h = d\{\log[\bar{X}/(1 - \bar{X})]\}/d(\log x)$$

be written in the form:

$$h = \left[\frac{(1/x)}{(1/\bar{X} - 1)} \right] \left\{ \frac{d(1/\bar{X} - 1)}{d(1/x)} \right\}$$

it becomes obvious that h at a point P equals AB/AC in Fig. 1, where A is the projection of P on a horizontal axis, B the intersection of the chord through (0,1) with the axis and C the intersection of the tangent from P with the axis. By the same type of argument it is easy to show that h at any point P in the Eadie plot equals AC/(AB · OC) (see Fig. 2), where A corresponds to the height of P, B is the point

Fig. 2. Eadie plot

It is easy to see from the criterion given in the text that binding is positively co-operative at $\bar{X}$ values less than that corresponding to Q, and negatively at higher values. The line CPE is the tangent to the curve at P.

(0,1), C the intersection of the tangent to the curve at P with the ordinate, and O the origin. [When the tangent is very steep, it will be more convenient to use a formula $h = OD/(AB \cdot OE)$ by using the intersection on the abscissa; see Fig. 2.] In the Hanes plot h is OB/OA (see Fig. 3), where the intersection of the ordinate with a line of slope 1 through P is A, and with the tangent at P is B. In the plot of $\bar{X}$ against x $h = AC/BD$ (see Fig. 4), where A is the point (0,1) and D the intersection of the tangent to the curve at P with the line $\bar{X} = 1$.

The free energy of interaction per mole site, $\Delta G_{I(1,2)}$, realized between any two saturations $\bar{X}_1$ and $\bar{X}_2$ can be obtained by using any of the plots discussed, since:

$$\Delta G_{I(1,2)} = RT(\ln K_{\bar{X}_1} - \ln K_{\bar{X}_2})$$

and $K_{\bar{X}}$ for each saturation can be determined as already explained. Finally the quantity defined as (Wyman, 1964):

$$\Delta G'_I = -d(\Delta G^0)/d\bar{X} \quad (3)$$

or interaction free-energy gradient (Whitehead, 1970), where $-\Delta G^0$ is the standard free-energy change for the equilibrium (2) above, can be determined by using the tangents in any of the plots; the Hill plot, which gives:

$$\Delta G'_I = \frac{RT[1 - (1/h)]}{\bar{X}(1 - \bar{X})} \quad (4)$$

(Wyman, 1964; Whitehead, 1970, 1973), has no privilege in this respect, other than convenience and naturalness. The following formulae can be derived either from eqn. (4) and the formulae already given for h , or else directly from eqns. (1) and (3) and $\Delta G^0 = -RT \ln(K_{\bar{X}})$. $\Delta G'_I/RT$ is equal in the double-

Fig. 3. Hanes plot
See the text for explanation.

reciprocal plot of Fig. 1 to $AP^2 \cdot BC/(DP \cdot AB)$, in the Eadie plot (Fig. 2) to $[(1/AB) - (1/AC)]$, in the Hanes plot (Fig. 3) to $OC^2 \cdot AB/(OB \cdot OA \cdot BC)$, where C corresponds to the height of P (AB is directed, i.e. negative if B is lower than A), and in the plot of $\bar{X}$ against x (Fig. 4) $[1 - (BD/AC)]/EP \cdot PB$.

It is not suggested that any of the plots discussed is as convenient or natural as the Hill plot for obtaining the quantities just discussed. This prompts the reflection that none of the plots was proposed in the first place for the purpose of exhibiting co-operative behaviour in general, but rather for that of linearizing data that conform to the Michaelis equation (or, for the Hill plot, to the Hill equation). Some of them remain useful for estimating by extrapolation $V_{\max}$ or maximum saturation in binding studies, in cases when this is difficult to do directly. But, such cases apart, is there any reason but tradition for presenting co-operative binding or kinetic data in these ways? Why not plot directly $1/K_{\bar{X}}$ or $-\log K_{\bar{X}}$, as expressed in eqn. (1), against $\bar{X}$ or x or $\log x$? Curves in all such plots would be horizontal where co-operativity is zero, slope upwards where it is positive and downwards where it is negative. A plot of $-\log(1/\bar{X} - 1) + \log x$ against $\log x$ (a modified version of the Hill plot) would give a clearer impression of co-operativity in a single curve than the Hill plot; its slope would be $(h-1)$. (In comparing $K_{\bar{X}}$ or co-operativity at constant $\bar{X}$ in different binding curves, one would have to remember that constant $\bar{X}$ corresponds to a line of slope 1.) A plot of $-\log(1/\bar{X} - 1) + \log x$ against $\bar{X}$ as suggested by Watari & Isogai (1976) would give a most vivid and direct vision of co-operativity; free energies of interaction could, of course, be read off almost directly, and the slope of a curve would be $\Delta G'_I/(2.303 RT)$.

However, experimental data are often published with the use of only the traditional plots. The relationships presented here enable a quick rough

Fig. 4. Plot of $\bar{X}$ against x
See the text for explanation.

Fig. 5. h for amphibian liver hexokinase

Double-reciprocal plots for ATP substrate redrawn from Fig. 7(b) of Ureta (1976) by kind permission of the author, with the construction for obtaining h (see also Fig. 1 and the text) at the point where v is half that at saturating ATP superimposed. (a) Curve in the absence of glucose 6-phosphate; $h = AB/AC \approx 1.6$; (b) curve in the presence of 0.5 mM-glucose 6-phosphate; $h = AB/AC \approx 1.15$.

estimate (it will usually only be a very rough estimate) of h or the other quantities to be made from the publications without replotting the data.

For instance, when the double-reciprocal plots of Ureta (1976) are treated in this way, it is found that the product glucose 6-phosphate decreases the apparent co-operativity of amphibian liver hexokinase towards substrate ATP from an h value of 1.6–1.7 in the absence of the product to about 1.15 at 5 mM-glucose 6-phosphate (see Fig. 5). Carbonell *et al.* (1973) have reported the velocity of reaction of human liver pyruvate kinase in curves against substrate (phosphoenolpyruvate) concentration in the presence of several effectors. Applying the method above, it is found that h at half-saturation, about 1.4 in the absence of modifiers, is not significantly changed by MgATP, and is slightly increased to about 1.6 by alanine and to about 1.7 by cysteine at the concentrations used (2 mM in all three cases). The greater the inhibitory effect in these curves the greater is the apparent substrate co-operativity, and this is what certain simple allosteric models predict (Whitehead, 1970, 1973). Although the additional quantitative information on apparent co-operativity that can be extracted from publications in this way will hardly ever permit any very far-reaching conclusions, it should sometimes be useful for purposes of comparisons of different data and, since different models make different predictions about the magnitude of apparent co-operativity and how this is influenced by various types of effector (see details in Monod *et al.* 1965; Koshland *et al.*, 1966; Rubin &

Changeux, 1966; Kirtley & Koshland, 1967; Whitehead, 1970, 1973), for orienting consideration towards one or another model of apparent co-operativity.

I thank Professor P. M. Fasella for comments on the manuscript and Dr. R. D. Waight for stimulating discussion. This is publication BIO-1466 of the Department of Biology, Commission of the European Communities; the work was executed under contract no. 160-76-1-BIOI.

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