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Diagnostic Uses of the Hill (Logit and Nernst) Plots

ATHEL CORNISH-BOWDEN† AND D. E. KOSHLAND JR

Department of Biochemistry, University of California
Berkeley, Calif. 94720, U.S.A.

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Analysis of the Hill plot for ligand-binding studies shows that it can adopt a variety of shapes other than a straight line. The shape of the curve can yield valuable information about the details of the binding process. In some cases it is possible to discriminate between models of co-operativity without the need for curve-fitting by computer.

1. Introduction

"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."

A. V. HILL (1965)

For many proteins it has been convenient to express the ligand-binding properties by equation (1), given originally by Hill (1910)

$$y = K[\mathrm{X}]^{n_\mathrm{H}}/(1 + K[\mathrm{X}]^{n_\mathrm{H}}), \tag{1}$$

in which y, the "fractional saturation", is the fraction of the total number of binding sites occupied by ligand, [X] is the free ligand concentration, and K and n_H are constants. The original theoretical basis of their equation is now known to be an oversimplification, but it has been useful because it describes many experiments with fair accuracy with only two adjustable constants. It can be rearranged into the form of a straight line as follows:

$$\log[y/(1 - y)] = \log K + n_\mathrm{H}\log[\mathrm{X}]. \tag{2}$$

The resulting plot of $\log[y/(1 - y)]$ against $\log[\mathrm{X}]$ is known as a Hill plot, and the slope n_H is often called the Hill coefficient. This value is often confused with the number of ligand-binding sites, but the two quantities are related only by the restriction that the Hill coefficient cannot exceed the number of sites (Monod *et al.*, 1965). Similar plots are widely used in other disciplines. For example, the "logit plot" of pharmacology is an identical plot under a different name. The "Nernst plot" of electrochemistry is essentially the same plot with the axes reversed, as Malmström (1974) has recently pointed out, in a review that clarifies the meaning of the Nernst plot as applied to problems of biological oxidation. The theory of the Hill plot applies equally to these analogues.

† Permanent address: Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, England.

A more general approach to ligand binding is to treat each step in the binding process separately, with a separate association constant (Adair, 1925; Whitehead, 1970). For a protein with four binding sites this leads to the following expression for the fractional saturation:

$$y = \frac{K'_1[X] + 3K'_1K'_2[X]^2 + 3K'_1K'_2K'_3[X]^3 + K'_1K'_2K'_3K'_4[X]^4}{1 + 4K'_1[X] + 6K'_1K'_2[X]^2 + 4K'_1K'_2K'_3[X]^3 + K'_1K'_2K'_3K'_4[X]^4}, \quad (3)$$

in which K'_1, K'_2, K'_3 and K'_4 are the "intrinsic association constants" for the four ligand binding steps, defined as follows:

$$K'_1 = \frac{[EX]}{4[E]\,[X]}, K'_2 = \frac{2\,[EX_2]}{3[EX]\,[X]}, K'_3 = \frac{3[EX_3]}{2[EX_2]\,[X]}, K'_4 = \frac{4\,[EX_4]}{[EX_3]\,[X]}.$$

The statistical factors 1/4, 2/3, 3/2 and 4/1 included in the definitions of intrinsic constants simplify the discussion of site–site interactions, since binding sites that are identical and independent give $K'_1 = K'_2 = K'_3 = K'_4$. If the sites interact in such a way that binding at any site facilitates binding at the remaining site, then $K'_1 < K'_2 < K'_3 < K'_4$ (positive co-operativity). If the sites interact in such a way that binding at any site impedes binding at any other site (negative co-operativity), then $K'_1 > K'_2 > K'_3 > K'_4$. Finally, mixed positive and negative co-operativity occurs if more complex inequalities apply, such as $K'_1 < K'_2 > K'_3 > K'_4$.

Although equation (3) rests on a much more secure theoretical foundation than equation (1), it is not nearly so convenient to apply, because it contains four adjustable constants, which cannot readily be estimated without recourse to computation (Cornish-Bowden & Koshland, 1970*a*). Consequently the Hill plot has continued to be widely used, even though the potential utility of the plot beyond the Hill slope has been largely neglected.

If equation (3) is rearranged to yield an expression for the Hill function:

$$\log\left(\frac{y}{1-y}\right) = \log\left[\frac{K'_1[X] + 3K'_1K'_2[X]^2 + 3K'_1K'_2K'_3[X]^3 + K'_1K'_2K'_3K'_4[X]^4}{1 + 3K'_1[X] + 3K'_1K'_2[X]^2 + K'_1K'_2K'_3[X]^3}\right], \quad (4)$$

the result does not define a straight line. Indeed, only in the special case in which all the association constants are equal does it simplify to equation (2), with a Hill coefficient $n_H = 1$. It is therefore rather puzzling that straight Hill plots have so often been published in which Hill coefficients are appreciably different from unity.

In recent years there have been a number of reports of Hill plots that apparently consist of two or more straight-line segments with abrupt transitions between them (e.g. Cook & Koshland, 1970; Nagel *et al.*, 1973; Baykov & Avaeva, 1973; Teng-Leary & Kohlhaw, 1973). These plots appear to be no more readily reconcilable with equation (4) than the simple straight-line plots. But since the data of the yeast glyceraldehyde-3-phosphate dehydrogenase were shown to fit equation (3) satisfactorily (Cook & Koshland, 1970), it seemed likely that some or all of the other examples might do so as well. Thus, the properties of the Hill plot and its relation to the intrinsic binding constants K'_j should be thoroughly examined, given that the plot is convenient and widely used, and that the relations between the K'_j values can be used to discriminate between different models of co-operativity (Cornish-Bowden & Koshland,

1970*a*,*b*). We shall show in this paper that judicious examination of a Hill plot can yield a substantial amount of information about the K'_j values and their interrelations, without the need for curve-fitting by computer, and in many cases, permits a discrimination between models for co-operative effects. This is not meant to imply that curve fitting by computer is undesirable, but only that the non-mathematical biologist analyzing kinetic or binding relationships may wish to use simpler and more convenient methods.

2. Theory and Results

(a) *Properties of the Hill plot for a tetrameric protein*

When [X] is very small, equation (4) simplifies to $\log[y/(1-y)] = \log K'_1 + \log[X]$ since all except the first term in the numerator and denominator of the fraction on the right-hand side of the equation vanish. Similarly, when [X] is very large, equation (4) simplifies to $\log[y/(1-y)] = \log K'_4 + \log[X]$. Thus the two asymptotes of the Hill plot are straight lines of unit slope (Wyman, 1964). This may be seen very clearly in the excellent data recently published by Tyuma *et al.* (1973) for the binding of oxygen to human hemoglobin. If the experimental data are confined to a narrow range of y values, e.g. from 0·25 to 0·75, as is quite common, the curvature of the plot and the trend toward unit slope at the extremes is not obvious. Asymptotes, therefore, are rarely estimated or sketched in on Hill plots. However, it follows from the above that the values of K'_1 and K'_4 can be estimated very quickly from the

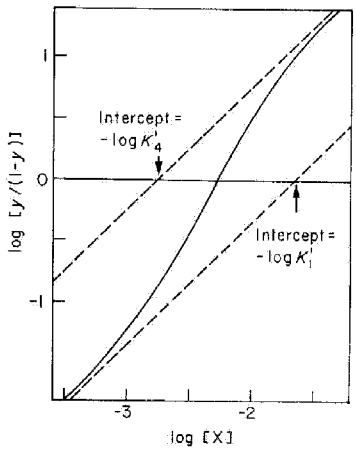

FIG. 1. Estimation of K'_1 and K'_4 for a tetrameric protein from the asymptotes of a Hill plot. The intercepts of the asymptotes on the ordinate axis have the values log K'_1 and log K'_2 as indicated. In the general case of a protein with n binding sites, the intercept of the asymptote at high ligand concentration gives log K'_n rather than log K'_4.

asymptotes of a Hill plot as illustrated in Figure 1. Even if the data are not precise or extensive enough to locate both asymptotes exactly, it will usually be possible to derive some useful information about the magnitudes of K'_1 and K'_4. Moreover, the asymptotes permit visual assessment of the slope of the curve at intermediate values of log[X]. Hence it is useful to draw the asymptotes at both extremes of ligand concentration to gain maximum information from a Hill plot.

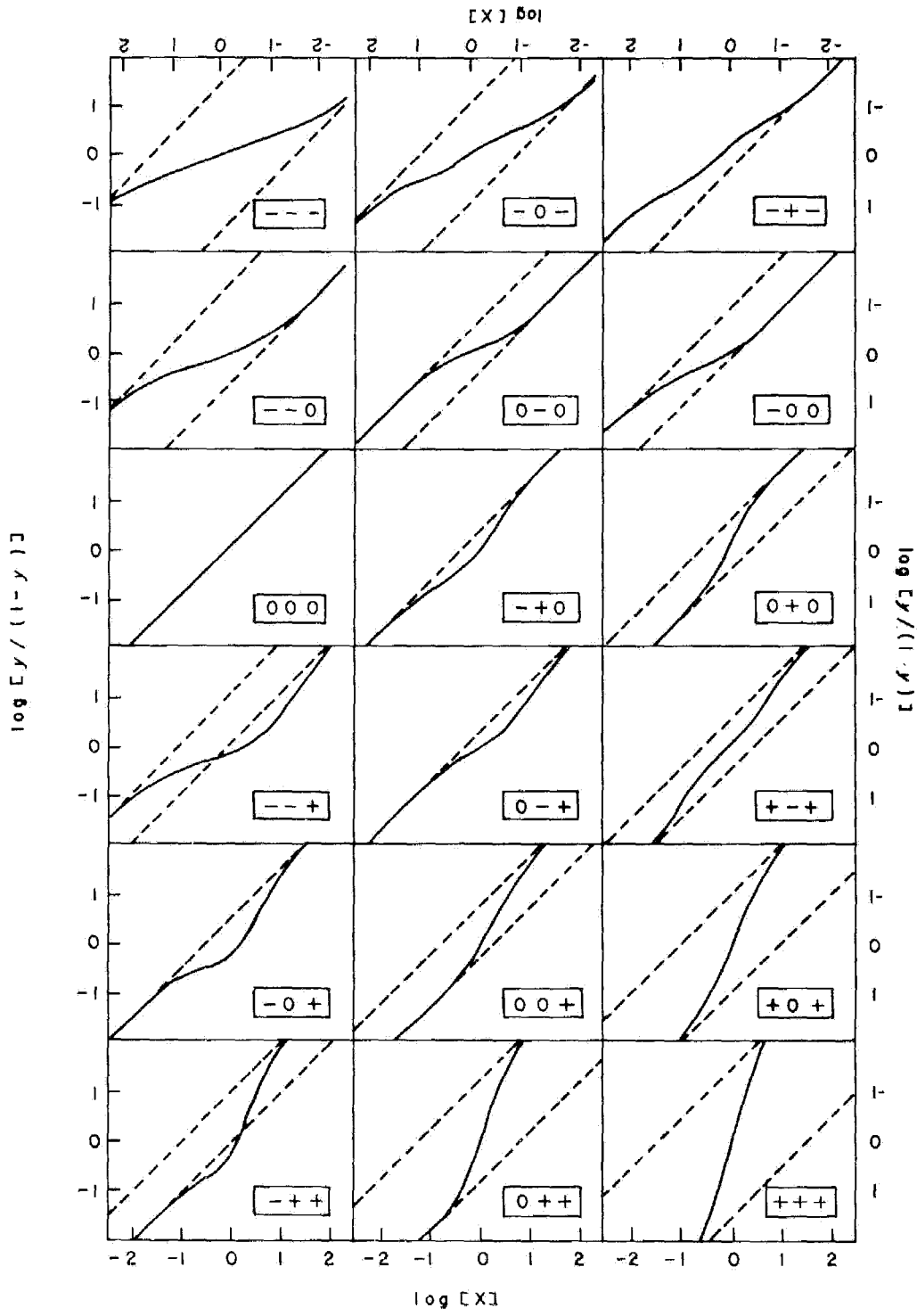

FIG. 2. Dependence of the shape of the Hill plot on the ratios of the binding constants. For each plot the 3-part symbol enclosed in a box indicates the values of K'_2/K'_1, K'_3/K'_2 and K'_4/K_3', in that order. For each ratio, the symbol + indicates positive co-operativity, with a value of 10 in this illustration; 0 indicates no co-operativity, with a value of 1; and — indicates negative co-operativity, with a value of 0·1. Of the 18 curves shown right side up, 9 are unchanged by inversion and 9 others produce new curves on inverting the Figure to complete the total of 27 possible permutations. Thus, if a comparison of an experimental curve fails to indicate a similarity with the 18 curves shown right side up, inversion of the Figure will reveal the 9 remaining permutations.

To obtain information about the values of K'_2 and K'_3 it is necessary to examine the shape of the complete Hill plot. Although it is possible to derive some generalizations about the shapes of Hill plots by considering equation (4) analytically, it is not very profitable to do this. One can prove, for example, that a discontinuity in slope, as at the junction of two straight-line segments, is impossible. But this is of very little experimental significance, because it is never likely to be possible to distinguish in practice between a region of very high curvature and a true break in slope. Accordingly, the properties of equation (4) have been investigated by plotting curves calculated by inserting plausible values of the binding constants.

To explore the range of shapes of Hill plots as comprehensively as possible within manageable limits, we have selected illustrative values of consecutive binding ratios, K'_2/K'_1, K'_3/K'_2 or K'_4/K'_3. For the plot in Figure 2, the values for these ratios were kept at 10 (positively co-operative), or 1 (non-co-operative), or 0·1 (negatively co-operative). Other numerical values could of course have been chosen, but these numbers define the qualitative features of the plot sufficiently. All possible permutations for the three ratios were then taken, making 27 sets in all. The results are shown in Figure 2. (Although only 18 plots are shown, the missing nine may be found by inverting the Figure, which is labeled in such a way as to be legible and correct which ever way up it is read.)

Examination of the plots in Figure 2 clarifies some puzzling features of published Hill plots. In the first place it is clear that the apparent straightness of experimental Hill plots is an illusion: almost all of the theoretical lines are perceptibly curved. In many cases, however, the curvature is so slight that it might easily be masked by even a small amount of experimental error. This is particularly true if attention is confined to the central part of the plot, since comparatively few of the plots are significantly curved between $\log[y/(1-y)] = \pm 0{\cdot}5$. Second, there are several plots that apparently consist of two straight-lines segments, particularly where the binding constants display a mixture of positive and negative co-operativity. In this context it is apposite to remark that the impression that a plot contains straight-line segments is strongly reinforced by drawing lines through the points. Consider, for example, the plots in Figure 3. Although the curve and the pair of straight lines are distinguishable from one another at first glance, they appear to fit the same points about equally

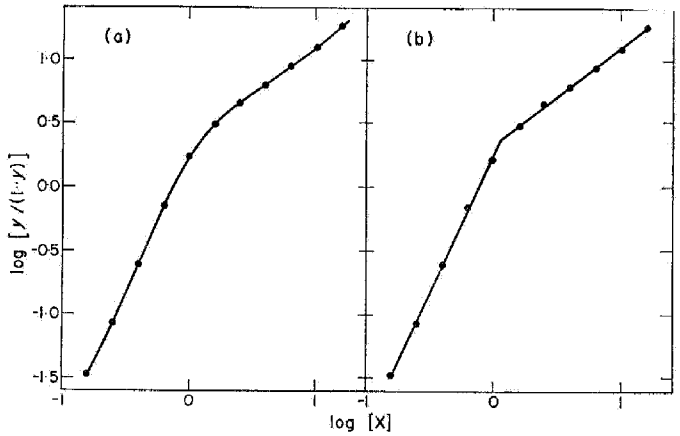

FIG. 3. Visual impact of straight lines. The points are identical in the two halves of the Figure, and the curve on the left (curve [+ + −] from Fig. 2) fits them exactly. The straight lines on the right were drawn by eye.

well, even though the curve fits exactly and the straight lines only approximately. Moreover, there is a strong predisposition to regard a pair of straight lines as "simpler" than a complex curve, notwithstanding the fact that a pair of straight lines is biochemically far more extraordinary, because of the discontinuity in behavior that it implies. It seems likely that this conclusion applies with equal force to plots other than Hill plots, for example to the breaks in double-reciprocal plots discussed by Engel & Ferdinand (1973).

In Figure 2 it is seen that the three ratios of binding constants influence different parts of the curve in a rather simple way: the value of K'_2/K'_1 mainly influences the shape of the curve at low values of $\log[y/(1-y)]$, the value of K'_3/K'_2 mainly influences the middle of the curve, and the value of K'_4/K'_3 mainly influences the shape of the curve at high values of $\log[y/(1-y)]$. To be more precise, if $K'_2/K'_1 > 1$, the slope at the low end is greater than unity; if $K'_2/K'_1 \simeq 1$, the curve tends to follow the asymptote at the low end; but if $K'_2/K'_1 < 1$, the slope at the low end is less than unity. The effect of K'_4/K'_3 on the slope at the high end is similar. The effect of K'_3/K'_2 is more subtle, because the middle part of the curve is also strongly influenced by the extremes, and has little freedom of variation. In general K'_3/K'_2 moderates the slope in the middle of the curve in the same manner as the other two ratios. These effects are illustrated in Figure 4.

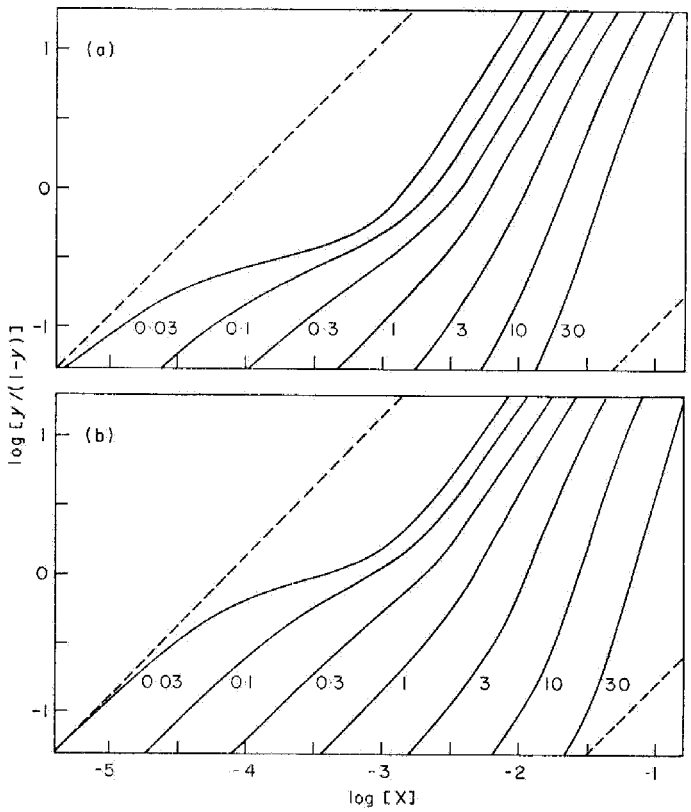

FIG. 4. Effects of the values of individual ratios of binding constants on the shape of the Hill plot. (a) For each curve, $K'_3/K'_2 = 1$ and $K'_4/K'_3 = 10$ are held constant and K'_2/K'_1 is varied from 0·03 to 30, as indicated. (b) For each curve, $K'_2/K'_1 = 1$ and $K'_3/K'_4 = 10$ are held constant and K'_3/K'_2 is varied from 0·03 to 30 as indicated.

In Figure 4(a) the values of K'_2/K'_1 are varied while holding the other ratios constant. The ratios 10, 1·0 and 0·1, which have already been used in Figure 2, have been augmented by inserting the values 30, 3·0, 0·3 and 0·03. It is evident that the main effect is in the lower region of the curve near $y = 0$. In Figure 4(b) K'_3/K'_2 is varied and similar alterations are observed, but this time near the middle portion of the curve, where K'_3/K'_2 has its major effect. A curve for the alterations in K'_4/K'_3 is not given, since it can be seen by simply inverting Figure 4(a). Altering the quantitative values of the ratios, therefore, causes some exaggeration of the shape of the curve at the extremes but leaves the qualitative conclusions unchanged.

It is useful to examine some special cases. For example, the curve labeled [+00] represents the case $K'_1 < K'_2 = K'_3 = K'_4$, which is of particular interest because it is characteristic of the symmetry model of Monod *et al.* (1965) when ligand binds exclusively to the R conformation. It may be seen that this case is readily recognizable by a region of slope close to unity that extends from high values of $\log[y/(1 - y)]$ almost to the half-saturation point.

The curve labeled [+ − +] represents the case of "half-of-the-sites reactivity" (Levitzki *et al.*, 1971) in a co-operative protein. This curve also provides an example of the dangers of basing conclusions on too narrow a range of observations. If the plot is restricted to values of the ordinate from −0·75 to +0·75 (y from 0·15 to 0·85), the curve appears to deviate away from unit slope at the extremes, in apparent violation of normal behavior. But the anomaly results from using the term "extreme" too loosely; in general the difficulty does not arise if observations extend to values of the ordinate outside the range −1 to +1.

When $K'_4/K'_3 = K'_2/K'_1$, the Hill plot is symmetrical about the half-saturation point; this is identical to the condition for a symmetrical plot of y against $\log[X]$ (Cornish-Bowden & Koshland, 1970*b*), and is characteristic of the simplest forms of the sequential model (Koshland *et al.*, 1966).

(b) *The effect of systematic error*

Systematic error is likely to create much more severe problems in the analysis of Hill plots than it does in most biochemical contexts, because of the way in which y is estimated. Since y is generally found by dividing the concentration of bound ligand by the total concentration of binding sites, the result depends heavily on accurate knowledge of the protein concentration and the number of binding sites per molecule. One cannot avoid this difficulty by estimating y as v/V_m in a kinetic experiment, where v is the measured velocity and V_m is the estimated maximum velocity, because in this case the value of y depends on V_m in exactly the same way as it depends on the protein concentration in the other case. Indeed, one may well compound the difficulties, because one requires the additional assumption that the steady-state velocity is proportional to the fractional saturation at equilibrium.

Although kinetic assays can often be used to assess the purity and concentration of an enzyme used in a binding experiment, such results must be applied with caution. Many enzymes, for example, depend for their catalytic activity on an essential sulfhydryl group, and are very sensitive to oxidation. But although such oxidation may completely remove catalytic activity it may have little or no effect on the binding capability. Thus it may often be incorrect to assume that a partially denatured enzyme necessarily lacks binding potential.

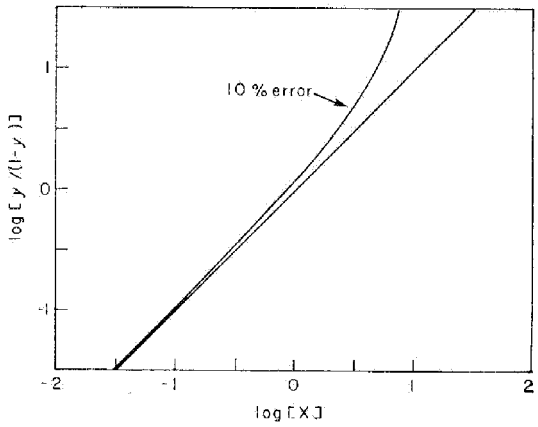

FIG. 5. Effect of systematic error. A 10% error in the protein concentration causes a 10% error in each value of y, which is transmitted in a disproportionate manner to each value of $\log[y/(1 - y)]$, with the result that the shape of the Hill plot is greatly altered.

The effect of an error in the protein concentration is to raise or lower the value of y by a constant percentage. Since $\log[y/(1 - y)]$ is not proportional to y, the resulting error in the ordinate as plotted is not a constant percentage, but is relatively much larger at large values. This can distort the true shape of the plot very considerably, illustrated in Figure 5 for a case where the true Hill plot is a straight line of unit slope. Clearly it is possible to gain a misleading impression of the true shape of the plot. Fortunately the problem can be diagnosed, at least in severe cases, by the requirement that a correct Hill plot must approach an asymptote of unit slope at high values of $\log[y/(1 - y)]$. Thus if the slope shows no tendency to approach unity at values above about 1 ($y = 0{\cdot}9$), this is a strong indication of systematic error.

Similar problems can arise at low values of $\log[y/(1 - y)]$ if the protein contains a strongly binding impurity or, in a kinetic experiment, if there is a significant uncorrected "blank" rate. These produce similar deviations from unit slope and may be diagnosed similarly.

(c) *The effect of random error*

One important aspect of the Hill plot that needs to be stressed is the effect of random experimental error. An error in the observed value of y does not produce an equal or even a proportional error in $\log[y/(1 - y)]$, and consequently the points on a Hill plot ought not to be given equal weight, even if the y values are homogeneous in variance.

The transformation of y to $\log[y/(1 - y)]$ actually results in considerable scale expansion at both ends, but very little distortion in the middle range. For values of y between about 0·15 and 0·85, $\log[y/(1 - y)]$ is a roughly linear function of y and so no great errors are likely to result from giving equal weight to points in the middle of the Hill plot. But outside this range the precision of points on the Hill plot decays very steeply, and so it is advisable to make as many observations as possible at the extremes, including some triplicates, to give an indication of the experimental error. For some methods of measuring ligand binding, notably equilibrium dialysis, measurement of high values of y is likely to be particularly imprecise, because it involves the difference of two large and almost equal numbers.

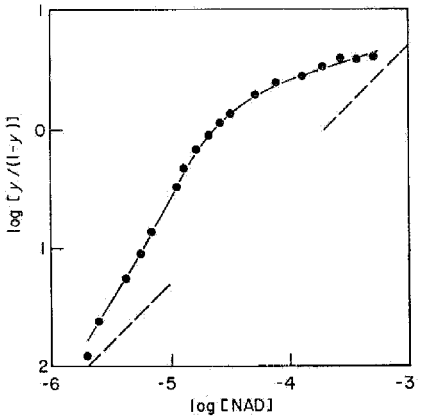

FIG. 6. Data of Cook & Koshland (1970) for the binding of NAD to yeast glyceraldehyde 3-phosphate dehydrogenase. The line was calculated from eqn (4) with the following values of the binding constants: $K'_1 = 4{\cdot}6\times10^3$ M^{-1}, $K'_2 = 1{\cdot}4\times10^5$ M^{-1}, $K'_3 = 7{\cdot}5\times10^4$ M^{-1}, $K'_4 = 3{\cdot}5\times10^3$ M^{-1}. The asymptotes were drawn with exactly unit slope but were located by eye.

The plot differs somewhat from the original plot of the same data (Cook & Koshland, 1970, Fig. 3(c)), because that was calculated after multiplying each experimental value of y by 4/3·3. This factor was originally thought necessary to correct for partial loss of binding activity on account of denaturation. But later in the paper evidence was given that the protein was fully active and that no correction was required. In reproducing the data, therefore, we have used no correction factor.

(d) *Binding of NAD to glyceraldehyde 3-phosphate dehydrogenase*

To illustrate the application of these ideas to a real Hill plot, we shall consider the plot shown in Figure 6 for the binding of NAD to glyceraldehyde 3-phosphate dehydrogenase. The curve is drawn from the experimentally determined binding constants, and it may be seen to agree very well with the points, except perhaps for the lowest point. Actually the deviation in this point corresponds to a very small error in y and is probably insignificant. The asymptotes are estimated by eye, and cannot be expected to be very precisely located, because the curve does not reach unit slope at either end. In fact the lines drawn are little better than approximate lower limits for the true asymptotes, and are useful primarily as guides to the slope of the curve. Within the limits of the experiment the asymptotes appear to coincide, and give values $K'_1 \simeq K'_4 \simeq 5\times10^3$ M^{-1}, in fair agreement with the values of $K'_1 = 4{\cdot}6\times10^3$ M^{-1}, $K'_4 = 3{\cdot}5\times10^3$ M^{-1} obtained by curve-fitting. The shape of the entire curve is very similar to the curve labeled [+0−] in Figure 4, and thus suggests the relationship $K'_1 < K'_2 \simeq K'_3 > K'_4$, which is again in good agreement with the results obtained by computer curve-fitting.

(e) *Proteins other than tetramers*

Although we have been primarily concerned with tetramers, many of the results apply to other oligomers as well. For dimers, the Hill plot has been discussed by Wyman (1964) in terms of free energies rather than binding constants. The complexity of such calculations has been discussed by Saroff & Minton (1972) and Magar & Chun (1973). The relation between the maximum slope and the ratio of binding constants has been derived by Haber & Koshland (1971), and the general characteristics of the plot are shown in Figure 7. The binding constants may be determined either from

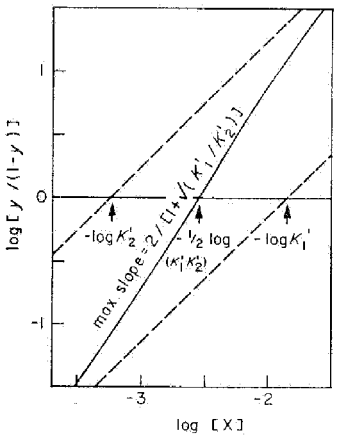

FIG. 7. Characteristics of the Hill plot for a dimeric protein. The value of the slope at the half-saturation point is taken from Haber & Koshland (1971).

the half-saturation point or the maximum slope. In the latter case it should be remembered that the maximum slope of an S-shaped curve is usually underestimated, and so bias in the results must be guarded against.

Proteins of more subunits present a greater problem, because the amount of information is too great to be accommodated in comfort by a graph in two dimensions. Nonetheless, many of the characteristics of Hill plots for tetramers will still apply to oligomers of n binding sites. If the asymptotes can be determined they define the values of K'_1 and K'_n in the same way as before (Fig. 1). Similarly, co-operativity or negative co-operativity between the first and second sites, or between the last and next to last sites, can be detected from the slope of the curve as it approaches the asymptotes. But except in very favorable cases it seems unlikely that the middle part of the curve can yield detailed information beyond a general characterization of the binding as positively co-operative, negatively co-operative, or mixed.

3. Discussion

From the above analysis it is clear that much useful information about binding systems can be obtained from a Hill plot in addition to its slope at the midpoint. In the first place it is valuable to examine more carefully the actual curve on either side of 50% saturation. This region should rarely be a straight line. Published work frequently indicates that a straight line fits the data poorly but a straight line is drawn through the data by the investigator because he is convinced *a priori* that it should be straight. In fact, a more analytical examination of this part of the curve and comparison with the qualitative features of the curves of Figure 2 can in some cases indicate relationships between the intrinsic binding constants. In addition, the plot is likely to be almost straight and the average slope will indicate whether it is positively (n_H greater than 1), negatively (n_H less than 1), or non-(n_H equals 1) co-operative. Pronounced curvature in this region generally indicates a mixture of positive and negative co-operativity and comparison with Figure 2 may indicate the type.

More complete information requires observations over a wider range, ideally from $y = 0{\cdot}05$ to $y = 0{\cdot}95$. Using these data further information is obtained by estimating the positions of the asymptotes. The asymptotes are known to be lines of unit slope and this can be useful in placing them on the plot, even though the data near the extremes are difficult to obtain. Not only will such asymptotes permit numerical values to be estimated for the extreme binding constants, i.e. K'_1 and K'_4 in the case of a tetramer, but they also permit the slope of the line in other positions to be estimated. Thus, some estimate is obtained from the approach to the asymptote of the ratios K'_2/K'_1 or K'_4/K'_3. If the slope at the low end is less than unity, K'_2/K'_1 is less than unity, and so on. The slope at the high end contains similar information about K'_4/K'_3. Although the slope in the middle region of the curve contains similar information about K'_3/K'_2, it will be more difficult to distinguish *a priori* but examination relative to the asymptotes that are drawn may be extremely valuable. It goes without saying that accurate data near the extremes are usually the most difficult to obtain experimentally.

If the actual values do not approach the asymptotes at the extremes, a systematic error may be indicated and should be tested. A common systematic error is caused by the presence of denatured protein, which is usually included in the total amount of protein but may not bind the ligand. In some cases strong negative co-operativity can lead to difficulty in the high y value regions of the curve.

Since Figure 2 is drawn with an illustrative set of values for the constant, one cannot expect any experimental curve to agree exactly with that listed. However, the variation to other values for K'_2/K'_1, K'_3/K'_2 and K'_4/K'_3 shows little change in the general appearance of the curves. Hence comparison of the shapes of the curves of Figure 2 with experimental cases may be particularly helpful. The fact that the above procedure did lead to qualitatively correct values for the constants of glyceraldehyde 3-phosphate dehydrogenase, which had also been fitted by computer fitting, does indicate it can give satisfactory results.

Finally, it should be emphasized that plots of v/V_m are frequently used for Hill plots. If v/V_m is proportional to the fraction bound over the entire range of data, it will of course give the same plot as the Hill plot and the analysis will be as described above. This is frequently true but it does not have to be (Ainslie *et al.*, 1972). Therefore, deductions reached by Hill plots obtained from kinetic data must be considered working hypotheses until binding data have been obtained. Nevertheless, the large number of cases in which the v/V_m assumption has been correct indicates that such "working hypotheses" can be a very valuable starting point.

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