Disadvantages of Double Reciprocal Plots

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In numerous applications, one desires to resolve an equilibrium constant and either some analytical measure of concentration, in the case of binding, or a rate constant, in the case of enzymes. This resolution is most often achieved by a double reciprocal Benesi–Hildebrand plot in the case of simple binding or a Lineweaver–Burk plot in the case of enzyme kinetics. The two procedures are formally similar. However, the linear double reciprocal plots suffer from a highly biased weighting of points and should *never* be used. This article recommends an equally simple and much more reliable alternative to double reciprocal plots and an even better method for those with computer access.

Consider the simple equilibrium where two reactants, E and S, combine to yield a complex, C, with association equilibrium constant K_c:

$$E + S \rightleftharpoons C \qquad K_c = [C]/[E][S]$$

where the brackets represent molar concentration. We write the mass balance equations for the total moles of starting reagents.

$$T_E = [E] + [C] \qquad T_S = [S] + [C] \approx [S]$$

where the last equality derives from the condition that reactant S remains in great excess over the complex, C: [S] >> [C]. Substituting in the equations we find

$$[C]/T_E = K_cT_S/(1 + K_cT_S) \tag{1}$$

If the concentration of complex C is assayed by absorption spectra, the observed absorptivity $A = \varepsilon_c[C]$, where ε_c is the molar absorptivity of complex C. (Alternatively, the concentration of complex may be determined from intensities in NMR spectra.) The observed molar absorptivity is given by

$$\varepsilon = A/T_E = \varepsilon_cK_cT_S/(1 + K_cT_S) \tag{2}$$

The observed molar absorptivity increases with increasing T_S concentration, finally leveling off at ε_c as the K_cT_S term in the denominator becomes much greater than unity. Our task is to resolve and obtain reliable values for the molar absorptivity of the complex, ε_c, and the association equilibrium constant, K_c.

In enzyme-catalyzed reactions a dissociation equilibrium constant, K_S, is defined as the reciprocal of the association K_c above, $1/K_c = K_S = [E][S]/[C]$, where E represents the enzyme concentration and S the substrate concentration, present in great excess. Equation 1 then becomes

$$[C]/T_E = T_S/(K_S + T_S) \tag{3}$$

The velocity of an enzyme-catalyzed reaction is represented by $v = k[C]$, where k is the first-order rate constant for decomposition of the enzyme–substrate complex, ES (C above). With the maximum attainable velocity given by $V = kT_E$ we obtain

$$v = VT_S/(K_S + T_S) \tag{4}$$

The velocity of an enzyme-catalyzed reaction increases with increasing substrate concentration, finally leveling off at V as the T_S term in the denominator becomes dominant. The task here is to resolve and obtain reliable values for the maximum rate, V, and the dissociation equilibrium constant, K_S.

With the minor complication of reciprocal equilibrium constants, the forms of eqs 2 and 4 are identical. Virtually identical resolutions of the constants have been proposed in terms of the popular linear double reciprocal plots: Benesi–Hildebrand for complexation and Lineweaver–Burk for enzyme-catalyzed reactions.

For the Benesi–Hildebrand plot (*1*) the reciprocal of eq 2 gives

$$1/\varepsilon = 1/\varepsilon_cK_cT_S + 1/\varepsilon_c \tag{5}$$

From a plot of $1/\varepsilon$ vs. $1/T_S$, one finds the intercept = $1/\varepsilon_c$ and the slope = $1/\varepsilon_cK_c$. Thus we obtain ε_c = 1/intercept and K_c = intercept/slope. Further extrapolation to the intercept with the negative abscissa also yields $-K_c$.

For the Lineweaver–Burk plot (*2*) the reciprocal of eq 4 yields

$$1/v = K_S/VT_S + 1/V \tag{6}$$

and from a plot of $1/v$ vs. $1/T_S$ one finds the intercept = $1/V$ and the slope = K_S/V. Thus we obtain V = 1/intercept and K_S = slope/intercept. Extrapolation to the negative abscissa intercept gives $-1/K_S$.

Alternatively, we may multiply eqs 5 and 6 by T_S and obtain equations for single reciprocal linear plots. For complexation we obtain

$$T_S/\varepsilon = 1/\varepsilon_cK_c + T_S/\varepsilon_c \tag{7}$$

A plot of T_S/ε vs. T_S yields an intercept of $1/\varepsilon_cK_c$ and a slope of $1/\varepsilon_c$. Thus ε_c = 1/slope and K_c = slope/intercept. Extrapolation to the negative abscissa intercept gives $-1/K_c$. This plot is sometimes termed a Scott plot (*3*).

For enzymology the analogous equation is

$$T_S/v = K_S/V + T_S/V \tag{8}$$

A plot of T_S/v vs. T_S gives an intercept of K_S/V and a slope of $1/V$. Thus we obtain V = 1/slope and K_S = intercept/slope. Extrapolation to the negative abscissa intercept also gives $-K_S$. This plot is sometimes called a Hanes plot (*4*). Although it actually predates the Lineweaver–Burk plot, unfortunately it has not become more popular. A third plot sometimes used in enzymology—the Eadie–Hofstee plot—plots v vs. v/T_S, placing the most error-prone observable on both axes; it also involves marked unequal weighting of points. It is not considered further here.

How dependable are these plots for resolving and obtaining reliable values for the paired constants? A least squares analysis minimizes the squares of the errors for all points. It is usual and easier to consider all the error to occur in one observable, the most error-prone observable. The initial concentrations of reactants can be measured more reliably than the other experimental parameters. For complexation the most error-prone observable is observed molar absorptivity (or other intensity quantity), and for enzymology it is velocity. Equations 2 and 4 are written with the most error-prone observable on the left hand side and all other variables and parameters on the right. Using these

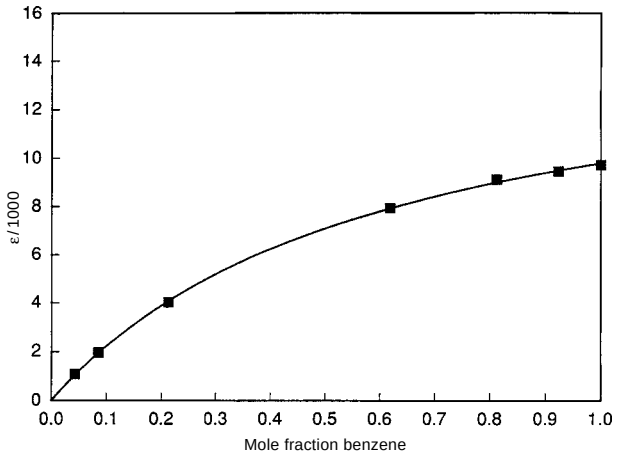

Figure 1. Molar absorptivity/1000 vs. mole fraction benzene in inert carbon tetrachloride from data of Benesi and Hildebrand. Points are experimental; curve is drawn from nonlinear least squares fit.

Table 1. Data of Benesi and Hildebrand

Benzene mol fraction	1/(Benzene mol fraction)	1000/ε	ε/1000	1000 × mol fraction/ε
0.0433	23.10	0.930	1.075	0.0403
0.0862	11.60	0.510	1.961	0.0440
0.213	4.69	0.249	4.02	0.0530
0.619	1.616	0.1259	7.94	0.0779
0.812	1.232	0.1098	9.11	0.0892
0.924	1.082	0.1058	9.45	0.0978
1.000	1.000	0.1029	9.72	0.1029

Table 2. Least-Squares Fits of Benesi and Hildebrand (B-H) Data[a]

Statistical Parameter	B-H (eq 5)	Scott (eq 7)	Nonlinear (eq 2)
K_c	1.78 (7)	1.67 (2)	1.64 (6)
$\varepsilon_c/1000$	14.9 (5)	15.6 (2)	15.8 (3)
R^2	.9998	.9991	—
SD[b]	—	—	0.07

[a]Number in parentheses is one SD in last digit listed.

[b]Apparent standard deviation of input data in ε/1000 from the fourth column of Table 1, from nonlinear least squares.

equations provides a suitable weighting of the experimental points. We would like to minimize $(d\varepsilon)^2$ from eq 2 and $(dv)^2$ from eq 4. The problem is that these equations are not linear and we cannot use linear least squares with eqs 2 and 4.

How reliable is it to apply linear least squares to the plots of eqs 5–8? Since the pairs of equations 5 and 6 on the one hand and 7 and 8 on the other are of the same form, analysis on one of each pair applies to the other. Because the symbolism is a little clearer, we work on the enzymology duo 6 and 8. In a double reciprocal plot (eq 6) the error minimization occurs in $[d(1/v)]^2$. From $d(1/v) = -dv/v^2$ we find $[d(1/v)]^2 = (dv)^2/v^4$ or $(dv)^2 = v^4[d(1/v)]^2$, for absolute or constants errors in v. For fractional or relative errors in v we obtain $(dv)^2/v^2 = v^2[d(1/v)]^2$. For eq 8 the error minimization occurs in $[d(T_S/v)]^2$. From $d(T_S/v) = T_S d(1/v) = -T_S dv/v^2$ we have $[dv(T_S/v)]^2 = T_S^2(dv)^2/v^4$ or $(dv)^2 = v^4[d(T_S/v)]^2/T_S^2$ for absolute errors in v. For fractional errors in v we find $(dv)^2/v^2 = (v^2/T_S^2)[d(T_S/v)]^2$. Usually absolute errors are considered to apply to intensity and most rate measurements.

What the derivations of the previous paragraph mean in practice is that instead of minimizing the square of the errors in the left-hand sides of eqs 2 and 4 with equally weighted points, double reciprocal plots weight the points as $1/v^4$ with absolute error and as $1/v^2$ with fractional errors. Worse is that the least reliable points in the most dilute solutions are weighted most heavily. Points at high $1/v$ (or $1/\varepsilon$) are weighted the most heavily; points near the origin need to be weighted more heavily. Compensatory v^4 weighting was actually made in an early paper by Lineweaver and Burk with a statistician (*5*). For the single reciprocal plot of eq 8 the weights are T_S^2/v^4 for absolute error and T_S^2/v^2 for fractional errors.

To see what the weightings mean in a series of experiments, assume T_S varies from $K_S/5$ to $5K_S$. Hence, in eq 4, v/V varies from 0.17 to 0.83 for a factor of 5. In a double reciprocal plot the highest $(1/v)$ point is weighted 5^4 (625) times more heavily than the lowest point with absolute errors, and 25 times more with fractional errors. As compensation with absolute errors, the lowest $1/v$ point should be weighted 625 times more heavily than the highest point in drawing a straight line. With any scatter at all, it is not possible to allow reliably for this pronounced trend in weighting when visually drawing lines on double reciprocal plots. Linear least squares programs with allowance for weightings exist (*6*), but are more complicated than the standard programs that appear on hand-held calculators and are seldom employed.

The range of weightings is less severe in the single reciprocal plots of eqs 7 and 8. With the same 25-fold concentration range and 5-fold velocity range of the previous paragraph, the lowest (T_S) point is weighted more heavily than the highest point: $25^2/5^4 = 1$ time with absolute errors and $25^2/5^2 = 25$ times with fractional errors. The weighting factor will be unity for absolute errors in a single reciprocal plot when points are paired symmetrically about K_S (or K_c). Other data ranges will yield different weightings, but the single reciprocal plots of eq 7 or 8 are clearly preferred to the double reciprocal plots of eq 5 or 6.

Table 1 presents data for the most extensive case in the classic paper of Benesi and Hildebrand, in which they used the double reciprocal plot of eq 5 to resolve ε_c and K_c. The system is that of iodine (I_2) complexation with benzene in inert carbon tetrachloride solvent. The concentrations of benzene were expressed as mole fractions (mf) and appear in the first column of the table. Because the concentration of T_S and K_c always appear as a product, the transformation of T_S to mole fractions only affects the units of K_c, and the rest of the analysis is as above. The iodine concentration, T_E, is incorporated into the reported molar absorptivity by the left equality in eq 2. The observed molar absorptivity was presented as the reciprocal, and this quantity appears in the second column with the molar absorptivity in the third column of Table 1.

A plot of the points of observed molar absorptivity versus mole fraction benzene appears in Figure 1. Typical of such plots, the absorptivity (or velocity) increases more steeply at the beginning and slowly levels off at higher concentrations. It is an interesting exercise to predict the asymptote of the limiting absorptivity plateau for the fully formed complex. Do so now.

The data in Table 1 may be used to construct linear plots according to eqs 5 and 7. For the double reciprocal Benesi–Hildebrand plot (eq 5) the third column of Table 1 is plotted against the second. For the single reciprocal Scott plot of eq 7 the last column of the table is plotted against the first. After the plots are constructed, the two parameters ε_c and K_c may be evaluated from each of them from their intercepts and slopes as outlined above. If one has access to linear least squares capability, the parameters may be determined from each plot by this more objective method, keeping in mind the extreme weighting of points in the double reciprocal plot.

Note that the range in concentration in Table 1 is a factor of 23 and that of ε a factor of 9. With absolute errors the weighting of the highest ($1/\varepsilon$) point is an astonishing 9^4 (6600) times that of the lowest point in the double reciprocal plot of eq 5 and 12 times in the single reciprocal plot of eq 7.

Table 2 presents the results of least squares fits to the data of Table 1. From their plot of eq 5 Benesi and Hildebrand offered the values $K_c = 1.72$ and $\varepsilon_c/1000 = 15.4$, close but not identical to the linear least squares fit in the second column of Table 2. Because the points are weighted differently, the fit from use of the Scott equation (eq 7) in the third column differs from that in the second column. With more scatter in the data the differences in the results may increase.

The last column of Table 2 shows the fit from a definitive nonlinear least squares calculation that directly minimizes the error $(d\varepsilon)^2$ in the most error-prone observable in the nonlinear eq 2. This fit may be viewed as the "correct" one. All three least squares fits in Table 2 are performed on the same data set in Table 1. The different fits arise from the different weighting of errors. If the data had been corrected by v^4 in the Benesi–Hildebrand treatment and by v^4/T_S^2 in the Scott treatment, the fit would have been the same as that from nonlinear least squares in the last column of Table 2. Compared to the nonlinear least squares fit, the linear Benesi–Hildebrand fit yields an equilibrium constant that is 9% too high and a molar absorptivity that is 6% too low. The fit from the Scott eq 7 in the third column agrees most closely, well within one standard deviation, with the nonlinear least squares fit. (The Scott equation fit underestimates the error in this case.) With wider scatter in the data the differences between the several fits would be greater.

Nonlinear least squares computer programs have been described (*7*, *8*), and some version is available at almost any computer center. Discoveries have been made by application of nonlinear least squares: cooperative proton as well as calcium binding was found in CaATPase (*9*).

The parameters for the nonlinear least square fit in the last column of Table 2 were used with eq 2 to construct the curve in Figure 1. Note that limiting molar absorptivity for a fully formed complex is given by $\varepsilon_c/1000 = 15.8$, to nearly the top of the ordinate in Figure 1. How close does this value compare to your earlier estimate? Probably your estimate was too low, illustrating the difficulty of determining the asymptote and hence K_c directly from such a plot. From the reciprocal $1/K_c = 1/1.64 = 0.61$ we find the benzene mole fraction on the abscissa for half of fully formed complex, corresponding to an ordinate value of $\varepsilon/1000 = 15.8/2 = 7.9$. Coincidentally, this half-way point is close to the fourth data point in Table 1 and Figure 1.

We now briefly apply an equivalent analysis to the 20 points in an enzyme-catalyzed reaction from Table III in reference 5 of Lineweaver, Burk, and Deming (*5*). The substrate concentration increases from 0.030 to 0.926 and the velocity from 0.0209 to 0.140. For absolute errors at the extremes of the range the relative weightings are 39,000! The authors of the paper appreciated and allowed for the $1/v^4$ weightings in their linear least squares analysis. With the same methods used in deriving the parameters in Table 2 we list first the Lineweaver–Burk result from eq 6, the Hanes result from eq 8, and the nonlinear least squares result for the two parameters (with one standard deviation in parentheses): $K_s = 0.202(7)$, 0.22(1), and 0.22(1), and $V = 0.162(5)$, 0.168(3), and 0.170(4). Once again, the first two results would be identical to the last with appropriate weighting, which is a factor of 39,000 across the range in the Lineweaver–Burk plot and 41 in the Hanes plot. As in the equilibrium example of Table 2, the single reciprocal Hanes plot of eq 8 yields much better agreement with the definitive nonlinear least squares result than does the double reciprocal Lineweaver–Burk plot of eq 6.

Some cautions are in order. To resolve reliably the two parameters appearing in all of these equations, it is essential to cover more than half the binding or enzyme activity curve as has just been done in Figure 1. If the data are limited to the initial straight-line portion at low concentrations, only the product K_cT_S or the quotient T_S/K_S may be determined: no technique reliably resolves the constants under such a condition. Both the double reciprocal plots of eqs 5 and 6 and the single reciprocal plots of eqs 7 and 8 are linear throughout, and a straight line drawn through a limited range of points may give a highly misleading impression of the reliability of the resolution. These and other points for more complicated cases have received discussion (*10*).

In conclusion, the best way to resolve the two parameters is to employ a nonlinear least squares fit of data expressed as in eq 2 or 4, the form of which yields an appropriate weighting of errors. Failing that, a linear least squares fit using the single reciprocal eq 7 or 8 is the next best procedure. Alternatively, the parameters may be determined from the slope and intercept of a straight line drawn through the points on a single reciprocal plot. It is as easy to use these equations as the double reciprocal plots of eq 5 or 6, which need never be used. Double reciprocal plots weight points so severely from one end to another, by factors of hundreds or even thousands, that they should never be used. Linear plots of the form of eqs 7 and 8 may be easily adapted to other situations, such as enzyme inhibition (*11*).

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