Biochem. J. (1972) **129**, 197–202
Printed in Great Britain

The Graphical Determination of K_m and K_i

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(*Received* 26 *April* 1972)

The principle of Dixon (1965) has been extended to give rapid graphical methods for determining enzyme constants for substrates (K_m) and inhibitors (K_i). It does away with the sometimes questionable assumption that the amounts of substrate or inhibitor bound by the enzyme are negligible in comparison with the total amount added, and is therefore valid even for cases of high affinity, where the usual methods fail. Besides doing away with the need for calculation, it enables the concentrations of the various components of the system to be read off directly for any point of the velocity curve.

The usual graphical methods for determining K_m or K_i make use of various plots expressing the relation between reaction velocity and concentration of substrate or inhibitor respectively. The equations on which they are based are in terms of concentrations of free substrate (s) or free inhibitor (i), but in practice the quantity that is plotted under the name of substrate or inhibitor concentration is the total added concentration (s_t or i_t), and the assumption is made (usually tacitly) that the free and total concentrations are equal. This may or may not be true; if the enzyme concentration is very small in relation to the substrate the amount of substrate bound by it is negligible and s is equal to s_t, but if the enzyme has a very high affinity for the substrate it may be necessary to use such low concentrations of substrate that a considerable portion is bound by the enzyme. The assumption is then no longer true and the methods fail. Similar considerations hold for inhibitors.

Dixon (1965) published a simple graphical method for determining equilibrium constants from plots of total concentrations, and applied it to the case of the activation of an apo-enzyme by a flavin. This method gave the constant as the distance between the two points at which the initial tangent to the curve and the line drawn from the origin through the half-maximum point on the curve cut the line for the maximum velocity V. (These are the points s_1 and s_2 in Fig. 1 of this paper.)

In the following year Kilroe-Smith (1966) suggested a modification in which the half- and three-quarter-maximum points were used (corresponding to s_2 and s_4 in Fig. 1) and the intersections were read on the line for $V/2$, to avoid possible inaccuracies in drawing the tangent.

In the present paper the theory of the method is further developed, and extended to inhibitors as well as substrates.

Substrates

For the steady-state enzyme system:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k}$$

the rates of formation and breakdown of ES will be equal, so that $k_1 e \cdot s = (k_{-1}+k)p$ where e, s and p are the concentrations of free enzyme, free substrate and ES complex respectively, and writing K_m as usual for $(k_{-1}+k)/k_1$ we have:

$$K_m = \frac{e \cdot s}{p}$$

K_m may be regarded as a 'pseudo-equilibrium constant', for which e, s and p are actual concentrations, thus differing from the equilibrium constant K_s, usually also written as $\frac{e \cdot s}{p}$ but with the understanding that e, s and p are equilibrium concentrations. We are not concerned with K_s here.

Using the subscript $_t$ to denote total concentrations, we have:

$$\left.\begin{aligned} e_t &= e+p \\ s_t &= s+p \end{aligned}\right\} \quad (1)$$

$$\left.\begin{aligned} v &= kp \\ V &= ke_t \end{aligned}\right\} \quad (2)$$

where v is the velocity at any substrate concentration and V the velocity with enough substrate to convert all the enzyme into ES. Then:

$$K_m = \frac{(e_t-p)(s_t-p)}{p} = \left(\frac{s_t}{p}-1\right)(e_t-p) \quad (3)$$

Take a point ('point n') on the curve at which:

$$V-v' = \frac{V}{n}$$

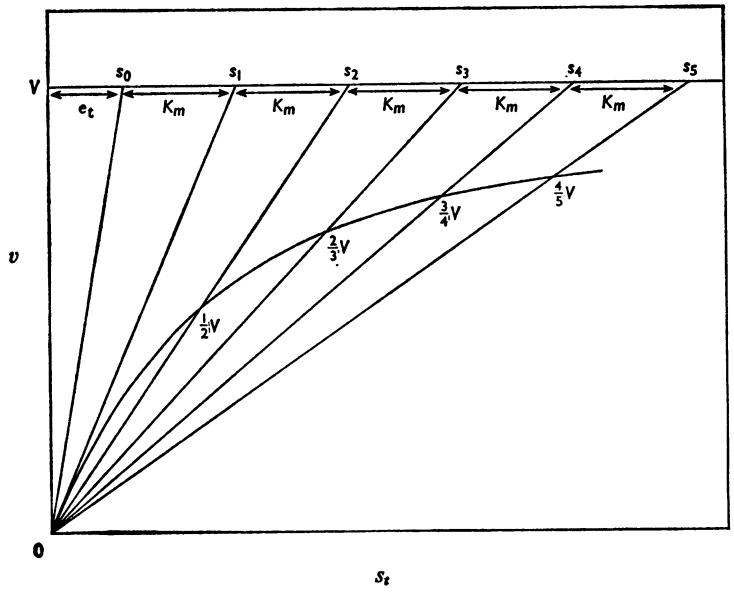

Fig. 1. *Method of determining K_m for substrate with high affinity*

The ordinate gives the velocity of the enzyme reaction and the abscissa the total concentration of the substrate.

where n is a whole number. Then, using primes to denote values at point n, we have:

$$v' = V\frac{n-1}{n}$$

and substituting from (2):

$$kp' = ke_t\frac{n-1}{n} \quad \text{and} \quad e_t - p' = \frac{e_t}{n}$$

From (3):

$$K_m = \left(\frac{s_t'}{e_t}\cdot\frac{n}{n-1} - 1\right)\frac{e_t}{n} = \frac{s_t'}{n-1} - \frac{e_t}{n} \qquad (4)$$

Draw a straight line from the origin through point n to cut the V-line at a point which we will call s_n (omitting the subscript t for simplicity). Then by simple geometry this concentration

$$s_n = \frac{n}{n-1}s_t'$$

so that:

$$K_m = \frac{s_n}{n} - \frac{e_t}{n}$$

and

$$s_n = nK_m + e_t \qquad (5)$$

It we draw a series of lines for different values of n, it follows that the distance between two neighbouring intersection points, say for n and $n-1$, will be given by:

$$s_n - s_{n-1} = K_m \qquad (6)$$

Thus the distances between the points on the V-line are all equal, and give a series of values of K_m from one curve, expressed in terms of the molarity scale used for plotting s_t.

It is easy to show (Dixon, 1965) that the line for $n = 1$ is the tangent to the curve at the origin, and the point for $n = 0$ (obtained by setting off one K_m distance to the left of s_1) gives the molarity of the enzyme, e_t.

When the plot of v against s_t has been obtained, the method is very quick, and is most easily carried out by setting off distances of $V/2$, $V/3$, $V/4$... downwards from the V-line. The accuracy of the method depends mainly on the drawing of the V-line at the correct level, which must be determined by a measurement with excess of substrate. But the constancy of the intervals gives a check on its proper positioning, for if it is drawn too high the intervals will not be constant, but will increase towards the right, whereas if it is too low they will decrease towards the right.

Competitive Inhibitors

A similar method is applicable to inhibitors, but with the following differences. Instead of rising to the maximum value V, the curve of v against i_t falls from V to the baseline. The value V may not correspond to saturation of the enzyme with substrate, for the inhibition curve may have been determined with a substrate concentration less than saturating. Thus although V is the highest point of the curve, obtained with no inhibitor and the substrate concentration

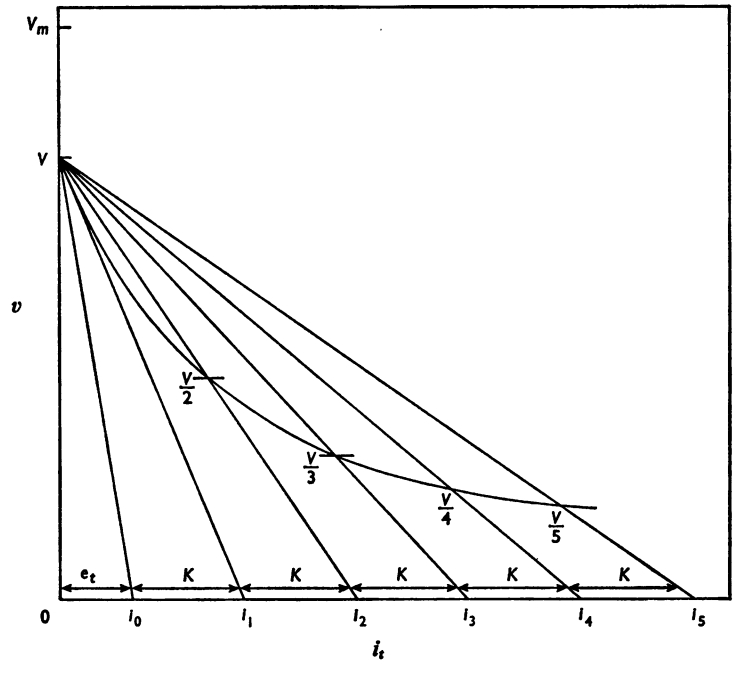

Fig. 2. *Method of determining K_i for inhibitor with high affinity*

The ordinate gives the velocity of the enzyme reaction and the abscissa the total concentration of the inhibitor. For a non-competitive inhibitor $K = K_i$; for a competitive inhibitor K is the quantity plotted in Fig. 3.

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used, we have also a limiting velocity, which we will call V_m, with no inhibitor but with excess of substrate. The enzyme combines with two substances instead of one, so that we have to consider both K_m and K_i, and we have the concentrations of the additional species I and EI (and with non-competitive inhibitors ESI also). The theory is therefore less simple, though the method is similar.

It is now the inhibitor, not the substrate, that has the high affinity and is present in low concentration, so that while we must distinguish between the concentration of free inhibitor i and the total i_t, we may assume that $s = s_t$.

We now have:

$$\begin{aligned} &E+S \rightleftharpoons ES \rightarrow \\ &E+I \rightleftharpoons EI \end{aligned}$$

$$e_t = e+p+q \tag{7}$$

$$i_t = i+q \tag{8}$$

where q is the concentration of EI.

$$\left.\begin{aligned} v &= kp \\ V_m &= ke_t \end{aligned}\right\} \tag{9}$$

$$V = V_m \Big/ \left(1+\frac{K_m}{s}\right)$$

From $K_m = e \cdot s/p$, substituting for p from (9):

$$e = \frac{K_m}{s} \cdot \frac{v}{k}$$

and from (7), substituting for e_t, e and p:

$$kq = V_m - v\left(1+\frac{K_m}{s}\right)$$

Now

$$K_i = \frac{e \cdot i}{q} = \frac{e(i_t - q)}{q} = \frac{K_m v}{sk}\left(\frac{i_t}{q} - 1\right)$$

so that:

$$\frac{i_t}{kq} = \frac{K_i}{v} \cdot \frac{s}{K_m} + \frac{1}{k}$$

and:

$$\frac{i_t}{V_m - v\left(1+\frac{K_m}{s}\right)} = \frac{K_i}{v} \cdot \frac{s}{K_m} + \frac{1}{k} \tag{10}$$

Take a point ('point n') on the curve at which:

$$v' = \frac{V}{n} = \frac{V_m}{n\left(1+\frac{K_m}{s}\right)} \tag{11}$$

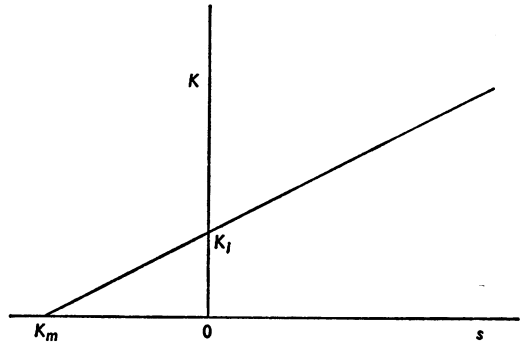

Fig. 3. *Method of determining K_t from K of Fig. 2 for a competitive inhibitor*

From (10) and (11):

$$\frac{i_t'}{V_m - v'\left(1+\frac{K_m}{s}\right)} = \frac{i_t'}{V_m\left(1-\frac{1}{n}\right)} = \frac{i_t'}{V_m}\cdot\frac{n}{n-1}$$

$$= K_t\frac{s}{K_m}\cdot\frac{n}{V}+\frac{1}{k}\Big] \qquad (12)$$

Draw a straight line from the initial point of the curve through point n to cut the base line at i_n. Then by geometry:

$$i_n = \frac{n}{n-1}i_t'$$

and so from (12) and (9):

$$i_n = nK_t\frac{s}{K_m}\cdot\frac{V_m}{V}+e_t$$

$$= nK_t\left(1+\frac{s}{K_m}\right)\Big]+e_t \qquad (13)$$

The distances between the intersection points, as before, are all equal and given by:

$$i_n - i_{n-1} = K_t\left(1+\frac{s}{K_m}\right) = K$$

and i_0 gives e_t. Because of competition by the substrate, the distances vary with the substrate concentration. If K_m is known, K_t is readily obtained. If not, the distance K should be measured at two substrate concentrations at least. A plot of K against s will be a straight line of which the intersection with the vertical axis gives K_t and that with the base line gives K_m (Fig. 3). Or, if V_m is known, we may use $K_t = K(V_m - V)/V_m$.

Non-competitive Inhibitors

In this case we have an additional component ESI, since the enzyme in such a system combines independently with S and I, and can combine with both to give an inactive complex.

We now have:

$$\begin{aligned} E+S &\rightleftharpoons ES \rightarrow \\ E+I &\rightleftharpoons EI \\ ES+I &\rightleftharpoons ESI \\ e_t &= e+p+q+r \\ i_t &= i+q+r \end{aligned}$$

where r is the concentration of ESI. Then:

$$q+r = e_t - p - e = \frac{V_m}{k} - \frac{v}{k} - \frac{vK_m}{ks}$$

$$= \frac{V_m - v\left(1+\frac{K_m}{s}\right)}{k} = \frac{U}{k}$$

writing U for $V_m - v\left(1+\frac{K_m}{s}\right)$ for convenience.

$$i = i_t - (q+r) = i_t - \frac{U}{k}$$

$$r = \frac{vi}{kK_t} = \frac{v}{kK_t}\left(i_t - \frac{U}{k}\right)$$

$$q = \frac{U}{k} - \frac{v}{kK_t}\left(i_t - \frac{U}{k}\right)$$

It is assumed that the same equilibrium constant K_t applies to the formation of ESI as well as EI, since there is no competition between S and I. Then:

$$K_t = \frac{K_m v}{sk}\cdot\frac{i_t - \frac{U}{k}}{\frac{U}{k} - \frac{v}{kK_t}\left(i_t - \frac{U}{k}\right)}$$

This simplifies to:

$$\frac{i_t}{V_m - v\left(1+\frac{K_m}{s}\right)} = \frac{K_t}{v}\cdot\frac{s}{K_m+s}+\frac{1}{k} \qquad (14)$$

This equation is the same as (10) except for the substitution of $s/(K_m+s)$ for s/K_m.

Considering point n, as in the last section, eqn. (11) still applies, and continuing the same treatment as before we obtain:

$$i_n = nK_t\frac{s}{K_m+s}\cdot\frac{V_m}{V}+e_t$$

But as $V_m/V = \frac{K_m+s}{s}$ we obtain instead of (13):

$$i_n = nK_t + e_t \qquad (15)$$

and the distance $K = i_n - i_{n-1} = K_t$.

Thus in this case the method gives K_t directly.

Concentrations of Components

The method also gives a very easy way of reading off the concentrations of the components of the system at any point on the curve. Fig. 4 shows the left-hand

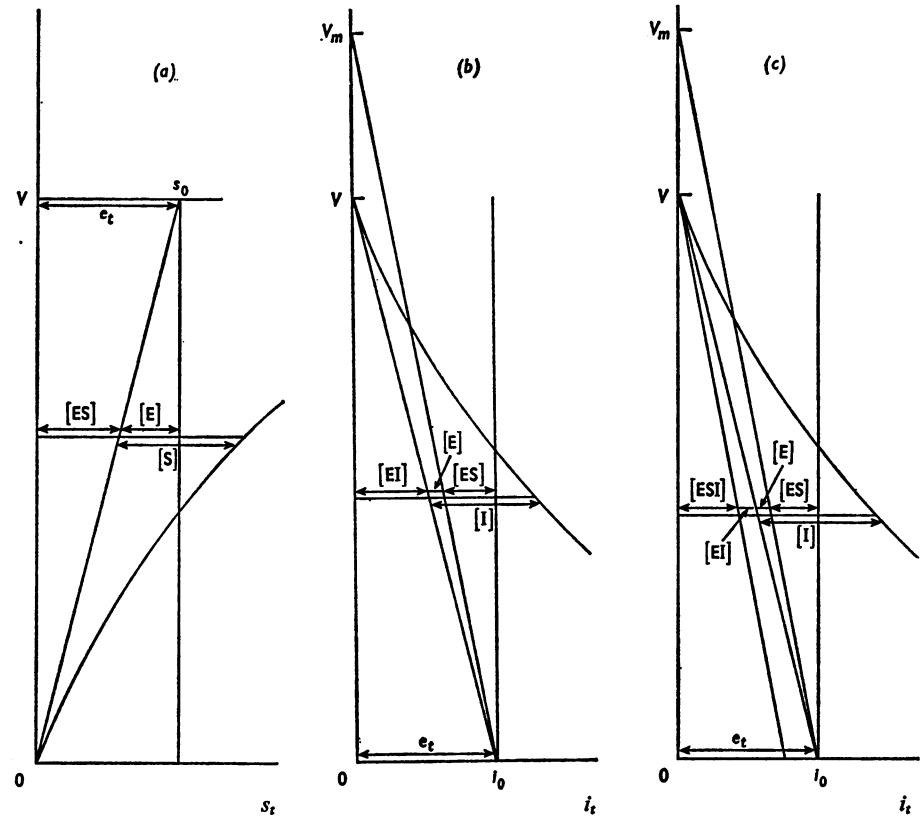

Fig. 4. *Method of determining concentrations of components at any point on the velocity curve*

The drawings show the initial parts of Figs. 1 and 2 on a slightly expanded scale for (*a*) a substrate, (*b*) a competitive inhibitor, and (*c*) a non-competitive inhibitor.

parts of the plots in the three cases, with the concentration scale somewhat expanded for clarity. Then (Fig. 4*a*) if a vertical line is drawn from the point s_0 and a line from the origin to the same point, it has already been shown by Dixon (1965) for the first case that if a horizontal line is drawn to the left from any point on the curve, the concentrations of E, ES, free S and total S at that point are given by the distances shown, using the scale of concentrations marked on the baseline. This will be obvious from the fact that the velocity is proportional to the concentration of ES.

For the case of a competitive inhibitor (Fig. 4*b*), straight lines are drawn from the point i_0 to the points for V and V_m on the vertical axis, and it can easily be shown that the five concentrations can be read off as marked.

For non-competitive inhibitors (Fig. 4*c*) an additional line must be drawn from the V-point parallel with that from the V_m-point, and then the six concentrations are as represented. The geometry is based on the fact that, as can easily be shown from the above equations:

$$\frac{[EI]}{[ESI]} = \frac{[E]}{[ES]} = \frac{K_m}{[S]} = \frac{V_m - V}{V}$$

If the selected point on the curve falls on the left of the e_t line it is of no consequence so long as the distances are measured between the same lines as before.

Discussion

The method given here has a number of advantages. It uses the quantities actually determined (velocity and total added concentrations) rather than calculated functions of them, with the possibility of arithmetical errors. It avoids the assumption that the total concentrations of substrates or inhibitors are equal to the theoretically required free concentrations, and therefore enables plots in terms of s_t and i_t to be used without restriction of range. It is valid not only for

the range where s_t and i_t are of the same order as the enzyme concentrations, where the common methods fail, but also for the more usual conditions in which the enzyme concentration is relatively minute, when the line for s_0 or i_0 effectively coincides with the vertical axis. It eliminates all calculation, except for the division of V by 2, 3, 4 etc.; the only requirements for the determination of K_m and K_i are a straight-edge and a pair of dividers! Finally it enables the concentrations of all the components of the system at any point on the curve to be read off directly; their determination would otherwise involve a rather complex calculation.

The method has the disadvantage that it is not so easy to draw a smooth curve through a number of experimental points as to draw a straight line; therefore the straight-line plots for K_m (e.g. Lineweaver–Burk) or K_i (Dixon, 1953) will probably still be used for the regions of negligible e_t, in which they are in fact straight, in spite of the extra calculation involved. Another possible disadvantage, when K_m is being determined, is that the accuracy depends on the correct placing of the line for V, which requires a measurement with the enzyme completely saturated with substrate. This does not apply to the determination of K_i, since the baseline on which the intersections are measured is fixed.

Finally, it should be pointed out that this method cannot be used for those enzymes which follow 'allosteric kinetics', with S-shaped curves of v against s, or for certain cases of complex kinetics involving higher powers of the concentrations of reactants. Such enzymes cannot be said to possess a K_m, and of course the other graphical methods also are inapplicable.

Just after this paper was written Henderson (1972) published an extensive mathematical analysis of the kinetics of 'tight-binding inhibitors', and among his equations are two which are related to equations (10) and (14) above. The present graphical method, however, is not given.

References

Dixon, M. (1953) *Biochem. J.* **55**, 170
Dixon, M. (1965) *Biochem. J.* **94**, 760
Henderson, P. J. F. (1972) *Biochem. J.* **127**, 321
Kilroe-Smith, T. A. (1966) *Biochem. J.* **100**, 334