An easy method for deriving steady-state rate equations

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The scope and limitations of a simple and satisfactory method of deducing steady-state rate equations is described. This method (called the Flux Method) consists in writing down the flux in successive steps of the reaction, and calculating the relative concentration of enzyme forms and thence the turnover time. Kinetic mechanisms for linear and branched pathways are used as examples of this method.

INTRODUCTION

One of the commonest tasks in enzymology is the deduction of steady-state rate equations from kinetic mechanisms. Moreover, the relationship between a postulated mechanism and an experimentally measured rate is the first step in expressing quantitatively the effect of an enzyme. Remarkably, there is a simple, but rigorous, method that seems not to be widely known. This method, which is referred to here as the Flux Method, is an application of the 'Method of Intermediates' described (Christiansen, 1953) nearly 40 years ago. The Flux Method turns out to be well suited to the deduction of initial rate steady-state equations. The purpose of the present paper is merely to demonstrate the simplicity and usefulness of this method. The main requirement is for one step, not necessarily the last, to be effectively irreversible. This is not a very severe limitation, since steady-state rate equations are usually being deduced to fit initial-rate measurements. The answer is obtained in the form of the reciprocal rate at unit concentration of enzyme, a form introduced by Dalziel (1957). There are several advantages, explained later, in the form in which the answer is obtained.

LINEAR PATHWAYS

Single-substrate reactions

One intermediate. For the first example, consider the simplest one-substrate reaction (Scheme 1). The last step is taken to be effectively irreversible, as is the case when the initial rate is being considered, or when the reaction is coupled to another reaction that removes the product, or in many hydrolytic reactions. The concentrations of the species are denoted by lower-case symbols. We are going to use the condition that, in the steady state, the flux in the two steps must be equal (Christiansen, 1953), to find the concentrations of the two forms in which the enzyme is present, E and X. Now the steady-state rate, or flux (v), of the second step in Scheme 1 is:

$$v = k_{+2}x$$

and so, dividing by vk_{+2} , we obtain eqn. (1):

$$\frac{x}{v} = \frac{1}{k_{+2}} \quad (1)$$

The rate for the first step is:

$$v = k_{+1}e \cdot s - k_{-1}x$$

and so, again dividing throughout by v , we obtain eqn. (2):

$$1 = k_{+1}s \cdot \frac{e}{v} - k_{-1} \cdot \frac{x}{v} \quad (2)$$

$$\frac{e}{v} = \left(\frac{1}{k_{+1}s} \right) \left(1 + k_{-1} \cdot \frac{x}{v} \right)$$

Now the value of x/v is substituted from eqn. (1) into eqn. (2), and the equations for x/v and e/v are added to give e_0/v , where e_0 is the total concentration of enzyme; thus the reciprocal of the rate at unit enzyme concentration (e_0/v) is given by eqn. (3):

$$\frac{e_0}{v} = \left(\frac{1}{k_{+1}s} \right) \left(1 + \frac{k_{-1}}{k_{+2}} \right) + \frac{1}{k_{+2}} \quad (3)$$

The independent kinetic constants are k_{cat} and k_{cat}/K_m (Cornish-Bowden & Wharton, 1988; Cleland, 1990) and e_0/v is given in terms of the reciprocals of k_{cat}/K_m and k_{cat} , as in eqn. (4):

$$\frac{e_0}{v} = \left(\frac{1}{s} \right) \cdot \left(\frac{K_m}{k_{\text{cat}}} \right) + \frac{1}{k_{\text{cat}}} \quad (4)$$

The function being given in the form e_0/v has the following advantages. (1) Since the equation gives the total time taken for one turnover, and the two terms on the right-hand side give the transit times for the stages (Fersht, 1985), the physical meaning of the two terms is transparent. It is interesting that one of the original (but infrequently cited) deductions of the hyperbolic rate equation was based on the idea that the time for a turnover could be regarded as the sum of the times taken for combination of enzyme and substrate and the time taken for regeneration of free enzyme from the complex (Van Slyke & Cullen, 1914). (2) The last term in eqn. (4) gives the intercept ($1/k_{\text{cat}}$) in a reciprocal plot (at unit enzyme concentration), and the first term gives the slope (K_m/k_{cat}). This close relationship to a reciprocal plot is particularly advantageous for understanding more complex reactions (see below). The reciprocal plot is, of course, a poor way of obtaining numerical estimates of the parameters (Dowd & Riggs, 1965; Tipton, 1985). (3) Integration to obtain progress curves requires the reciprocal rate. (4) Considerations of enzyme efficiency utilize this form (Burbaum *et al.*, 1989). (5) The significance of the relative magnitudes of k_{-1} and k_{+2} is clear because k_{-1}/k_{+2} is directly compared with unity.

Scheme 1. Simplest one-substrate reaction

E, S and P denote enzyme, substrate and product, and X is the enzyme-substrate complex.

Scheme 2. Mechanism for two-intermediate reaction

Two intermediates. When the mechanism includes a second intermediate (Y), the method is just as simple. Thus, for the mechanism shown in Scheme 2 there are three forms in which the enzyme is present, E, X and Y. The following equations hold:

$$\begin{aligned}v &= k_{+3}y \\v &= k_{+2}x - k_{-2}y \\v &= k_{+1}e \cdot s - k_{-1}x\end{aligned}$$

As before, the steady-state approximation implies that v has the same value for all three steps.

Next we solve the three equations for y/v , x/v and e/v . Note that this is done successively, starting with the last reaction.

$$\begin{aligned}\frac{y}{v} &= \frac{1}{k_{+3}} \\v &= k_{+2}x - k_{-2}y\end{aligned}$$

$$1 = k_{+2}\frac{x}{v} - \frac{k_{-2}}{k_{+3}}$$

$$\frac{x}{v} = \left(\frac{1}{k_{+2}}\right)\left(1 + \frac{k_{-2}}{k_{+3}}\right)$$

Similarly:

$$\frac{e}{v} = \left(\frac{1}{k_{+1}s}\right)\left[1 + \frac{k_{-1}}{k_{+2}}\left(1 + \frac{k_{-2}}{k_{+3}}\right)\right]$$

The three equations are then added; since $x + y + e + e_o$, eqn. (5) holds:

$$\frac{e_o}{v} = \left(\frac{1}{k_{+1}s}\right)\left[1 + \frac{k_{-1}}{k_{+2}}\left(1 + \frac{k_{-2}}{k_{+3}}\right)\right] + \left(\frac{1}{k_{+2}}\right)\left(1 + \frac{k_{-2}}{k_{+3}}\right) + \frac{1}{k_{+3}} \quad (5)$$

Whether the term for $k_{cat.}/K_m$ excludes k_{+3} depends on whether or not k_{-2} is zero. If k_{-2} becomes zero in eqn. (5), then the term in square brackets no longer contains k_{+3} , and comparison with eqn. (4) shows that $k_{cat.}/K_m$ will not contain k_{+3} ; it is well known that the influence of $k_{cat.}/K_m$ extends only to the first irreversible step.

Product inhibition. When the concentration of product (p) is not negligible, so that the last step is reversible (with rate constant k_{-3}), then, in the special case that $k_{-2} = 0$, the reciprocal rate is readily deduced to be:

$$\frac{e_o}{v} = \left(\frac{1}{k_{+1}s}\right)\left[\left(1 + \frac{k_{-1}}{k_{+2}}\right)\left(1 + \frac{k_{-3}p}{k_{+3}}\right)\right] + \frac{1}{k_{+2}} + \frac{1}{k_{+3}}$$

Here the product is seen to affect the first term, i.e. to act as a competitive inhibitor. It is clear that a competitive inhibitor has no effect when the first term is made zero by the concentration of substrate becoming infinite. This is, of course, equivalent to $k_{cat.}/K_m$ being altered, but $k_{cat.}$ being unaltered. There is, however, no worthwhile simplification in the more general case that k_{-2} is not zero, and when the presence of product prevents any step being effectively irreversible.

Two-substrate reactions

The example taken here is a ternary complex mechanism with dead-end inhibition, a scheme that has been used (Cornish-Bowden & Wharton, 1988) to demonstrate the customary (King & Altman) method. The kinetic mechanism is given in Scheme 3, i is the concentration of inhibitor I. The equations for this Scheme are written down just as in the first example, starting with the last reaction.

$$\begin{aligned}v &= k_{+4} \cdot eq \\v &= k_{+3} \cdot eab \\v &= k_{+2} \cdot b \cdot ea - k_{-2} \cdot eab \\v &= k_{+1} \cdot e \cdot a - k_{-1} \cdot ea\end{aligned}$$

Scheme 3. Two-substrate (ternary complex) reaction mechanism

Scheme 4. Reaction mechanism for branched pathway

Scheme 5. Reaction mechanism for branched pathway with a binding site for the nucleophile on the enzyme

$$\begin{aligned}\frac{e_o}{v} &= \left(\frac{1}{k_{+1}a}\right)\left[1 + \left(\frac{k_{-1}}{k_{+2}b}\right)\left(1 + \frac{k_{-2}}{k_{+3}}\right)\right] + \left(\frac{1}{k_{+2}b}\right)\left(1 + \frac{k_{-2}}{k_{+3}}\right)\left(1 + \frac{i}{K_i}\right) \\ &\quad + \frac{1}{k_{+2}} + \frac{1}{k_{+3}}\end{aligned}$$

The 'dead-end' branch reaction leading to EAI is an equilibrium reaction in the steady state and so $eai = ea(i/K_i)$, where K_i is the dissociation constant. The reciprocal rate at unit enzyme concentration may be written down as before by summing eq/v , eab/v , ea/v , eai/v and e/v as usual, to give the expression shown above. This expression agrees with that given (Cornish-Bowden & Wharton, 1988) (after correction of a misprint of k_2 for k_3 in their eqn. 2.1).

The expression in this form makes it easy to see that, as a tends to infinity, the first term drops out, and the reciprocal of $k_{cat.}/K_m$ for B depends on i , and I is a competitive inhibitor for B. However, I is uncompetitive with respect to A because the term in i is still present as the concentration of A tends to infinity.

The Flux Method is seen to lead to equations readily related to the familiar and convenient form put forward by Dalziel (1957):

$$\frac{e_o}{v} = \Phi_0 + \frac{\Phi_1}{a} + \frac{\Phi_2}{b} + \frac{\Phi_{12}}{ab}$$

Branched pathways

Branched pathways may present difficulties in deducing rate equations. One example is the 'nucleophilic diversion' pathway in which an intermediate Y (here an acyl-enzyme) may react with a nucleophile N to give enzyme (E) and second product (P_2) or with water to give enzyme and first product (P_1) (Scheme 4). The procedure is as before, the flux in the last step being the sum of the two parallel steps:

$$v = k_{+3}Y + k_{+4}[N] \cdot Y$$

This leads to the following expression for the $k_{cat.}$ relating to the disappearance of substrate, and the formation of both products:

$$k_{cat} = \frac{k_{+2}(k_{+3} + k_{+4}[N])}{k_{+2} + k_{+3} + k_{+4}[N]}$$

Table 1. Values of parameters for branched-pathway equations

Parameter	Given by:
A	$k_{+2}k_{+4}k_{+5}[N]$
B	$k_{+2}k_{+3}(k_{-4} + k_{+5})$
C	$(k_{+2} + k_{+3})(k_{-4} + k_{+5}) + (k_{+2} + k_{+5})k_{+4}[N]$

Now this expression for k_{cat} can be used to find k_{cat} when either product P_1 or product P_2 is measured, by multiplying by the appropriate partition ratio. Thus, for product P_1 , k_{cat} is multiplied by $k_{+3}/(k_{+3} + k_{+4}[N])$, and for P_2 by $k_{+4}[N]/(k_{+3} + k_{+4}[N])$:

$$(k_{\text{cat}})_{P_1} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3} + k_{+4}[N]}$$

$$(k_{\text{cat}})_{P_2} = \frac{k_{+2}k_{+4}[N]}{k_{+2} + k_{+3} + k_{+4}[N]}$$

When there is a binding site on the enzyme for the nucleophile (Scheme 5) the kinetic mechanism may be written, and the calculation of the flux down each pathway is not as obvious. The answers may be given in the following form:

$$k_{\text{cat}} = \frac{A+B}{C}$$

$$(k_{\text{cat}})_{P_1} = \frac{A}{C}$$

$$(k_{\text{cat}})_{P_2} = \frac{B}{C}$$

where the values of A , B and C are given in Table 1. How these solutions were obtained is explained as follows (the equations agree with those deduced by Jamin *et al.*, 1991).

The fluxes from Y (v) and from Z (v') are given by:

$$v = (k_{+3} + k_{+4}[N])y - k_{-4}z$$

$$v' = k_{+5}z$$

and the steady-state condition $dz/dt = 0$ gives:

$$z = \frac{k_{+4}[N]y}{k_{-4} + k_{+5}}$$

When this value of z is substituted into the equation for v'/v , we obtain:

$$v' = v \left(\frac{k_{+4}k_{+5}[N]}{k_{+3}k_{-4} + k_{+3}k_{+5} + k_{+4}k_{+5}[N]} \right)$$

which is used, essentially as above, to obtain:

$$\frac{e_o}{v} = \left(\frac{1}{k_{+1}s} \right) \left(1 + \frac{k_{-1}}{k_{+2}} \right) + \frac{k_{-4} + k_{+4}[N] + k_{+5}}{k_{+3}k_{-4} + k_{+3}k_{+5} + k_{+4}k_{+5}[N]} + \frac{1}{k_{+2}}$$

CONCLUSIONS

The Flux Method is an easy and effective way of deducing steady-state initial-rate equations. The method resembles, but seems easier to use than, the method (Cleland, 1975) which employs net rate constants.

I thank Dr. C. Crawford, Dr. H. B. F. Dixon, Professor J.-M. Frère, Dr. D. A. Harris, Professor K. Tipton and Professor S. G. Withers for encouragement and criticisms.

REFERENCES

- Burbaum, J. J., Raines, R. T., Alberly, W. J. & Knowles, J. R. (1989) *Biochemistry* **28**, 9293–9305
- Christiansen, J. A. (1953) *Adv. Catal.* **5**, 311–353
- Cleland, W. W. (1975) *Biochemistry* **14**, 3220–3224
- Cleland, W. W. (1990) in *The Enzymes*, 3rd. edn. (Sigman, D. S. & Boyer, P. D., eds.) vol. 19, pp. 99–158, Academic Press, London
- Cornish-Bowden, A. & Wharton C. W. (1988) *Enzyme Kinetics*, IRL Press, Oxford
- Dalziel, K. (1957) *Acta Chem. Scand.* **11**, 1706–1723
- Dowd, J. E. & Riggs, D. S. (1965) *J. Biol. Chem.* **240**, 863–869
- Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd edn., pp. 118–119, W. H. Freeman, New York
- Jamin, M., Adam, M., Damblon, C., Christiaens, L. & Frère, J.-M. (1991) *Biochem. J.* **280**, 499–506
- Tipton, K. F. (1985) in *Techniques in the Life Sciences*, vol. B1/II Supplement, BS113, pp. 1–61, Elsevier, Ireland
- Van Slyke, D. D. & Cullen, G. E. (1914) *J. Biol. Chem.* **19**, 141–180

Received 12 December 1991/5 February 1992; accepted 12 February 1992