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ENZYME KINETICS

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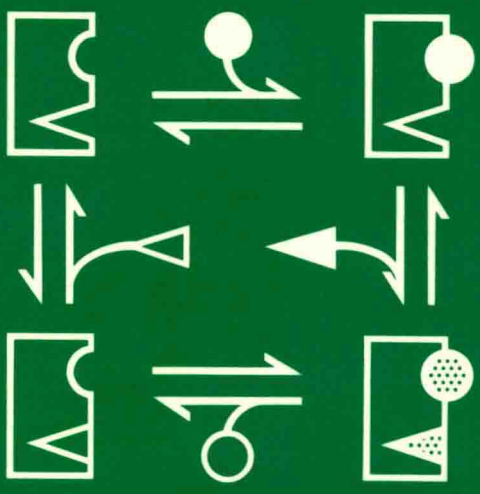

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ENZYME KINETICS

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Preface

To describe the whole of enzyme kinetics in the compass of a relatively short book would clearly be an impossible task, and we have not attempted it. The serious student of enzymes will need to look to more advanced texts. Nonetheless, there is a core of enzyme kinetics that every biochemist needs to be familiar with, and every student of biochemistry needs to be taught; it is this core that we have tried to describe in this book. We believe that there is nothing here that is inappropriate in an undergraduate course, though not all of the topics are elementary. This is especially true of the last chapter, where we have tried to show that despite changes in fashion there is still plenty of life in enzymology, and exciting work still to be done.

We are grateful to Marilú Cárdenas for helpful comments.

Athel Cornish-Bowden
Christopher W.Wharton

Dedications

To Isadora

To Amanda and Debbie

Abbreviations and symbols

a_0	total enzyme concentration
A (or B)	substrate
Ala	alanine
Asp	aspartate
Cys	cysteine
dx/dt	rate of change of EA concentration
e_0	total enzyme concentration
E	enzyme
EA(B)	enzyme – substrate complex
EAI	enzyme – substrate – inhibitor complex
Glu	glutamate
Gly	glycine
h	hydrogen ion concentration
His	histidine
I	inhibitor
Ile	isoleucine
IR	infrared
k_0	catalytic constant
k_A	specificity constant
k_n	rate constant
k_{obs}	observed rate constant
K_a	acid dissociation constant
K_{eqm}	equilibrium constant
K_i	competitive inhibitor constant
K_m	Michaelis constant
K_m^{app}	apparent Michaelis constant
K_s	equilibrium dissociation constant
Lys	lysine
NMR	nuclear magnetic resonance
p	concentration of product
P (or Q)	product
Pro	proline
Ser	serine
Thr	threonine

Tyr	tyrosine
UV	ultraviolet
v	rate
V	limiting rate
Val	valine
x	concentration of EA

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4. Inhibition of enzyme activity

5. The pH-dependence of enzyme-catalysed reactions

6. Enzyme mechanisms

1

Simple enzyme kinetics

1. Enzyme saturation

The kinetic feature that most distinguishes enzyme-catalysed reactions from simple chemical reactions is that they show *saturation*. Nearly all enzyme-catalysed reactions show a first-order dependence of rate on substrate concentration at very low concentrations, but instead of increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no dependence of rate on concentration and the reaction becomes of *zero order* with respect to substrate. This behaviour, which was known from the earliest studies of enzymes, is illustrated in *Figure 1.1*. Note especially that, despite what is shown in some textbooks, the curve does not reach the limit at any finite concentration, but rather remains far from it at even the highest concentrations that can realistically be achieved. We shall consider the reasons and consequences of this below.

The first investigators to provide a reasonably clear interpretation of enzyme saturation were A.J.Brown and V.Henri, but the somewhat later work of Michaelis and Menten (1) is usually taken as the starting point when discussing enzyme kinetics. Their claim to be regarded as the founders of enzyme kinetics rests not so much on their interpretation of saturation as on the fact that they were the first to carry out experiments in a modern way, with proper control of pH, use of initial rates rather than whole time courses, and allowances for non-enzymic processes. Their mechanism supposes that the first step in the reaction is the binding of the substrate (A) to the enzyme (E) to form an *enzyme–substrate complex* (EA) which then reacts to give the product (P) with the regeneration of the free enzyme:

$$\begin{array}{ccccccc} & & & & \xrightarrow{k_0} & & \\ \mathrm{E} & + & \mathrm{A} & = & \mathrm{EA} & \longrightarrow & \mathrm{E} + \mathrm{P} \\ & & & \overset{K_s}{} & & & \\ e_0 - x & & a & & x & & p \end{array} \qquad (1.1)$$

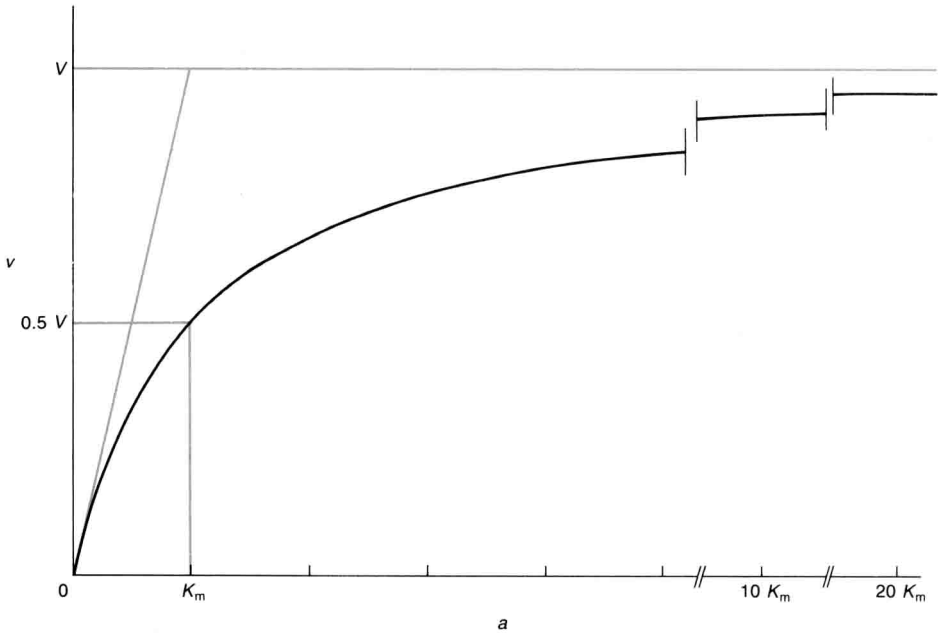

Figure 1.1. Dependence of rate v on substrate concentration a for a typical enzyme-catalysed reaction. The coloured additions illustrate the meanings of the limiting rate V and the Michaelis constant K_m.

If the *total* enzyme concentration is e_0 and the concentration of EA is x, the concentration of *free* enzyme must be e_0 minus x, as indicated under the equation, because all of the enzyme can only exist in one of the two forms. We can apply the same argument to the substrate, so that if the total substrate concentration is a_0 the free concentration must be a_0 minus x, at least until there has been sufficient time for the conversion of significant amounts of A into P. However, although it is possible to derive a rate equation in this way it is unnecessarily complicated because of the presence of square roots. Experimentally, one nearly always makes measurements with substrate concentrations that are very large compared with enzyme concentrations (e.g. 1 mM as compared with 1 nM), so that the analysis can be simplified by assuming that a_0 is so much larger than e_0 that a is effectively equal to a_0 minus x and so a is not significantly different from a_0. Hence, it is not necessary to distinguish between the free and total concentrations of substrate, and one can write both just as a. *In this book we shall always make this assumption*, even if it is not stated explicitly.

Michaelis and Menten assumed that the first step was an *equilibrium*, where equilibrium dissociation constant K_s equals [E][A]/[EA] which in turn is equal to $(e_0 - x)a/x$. This definition can easily be rearranged to express x in terms of e_0, a and K_s as follows:

$$x = e_0 a/(K_s + a) \tag{1.2}$$

Now, as product is released only in the second step of the reaction, the rate of the overall reaction is the rate of this step. As it is a first-order conversion of EA into E and P, with rate constant k_0 as indicated in the mechanism, the rate v is equal to k_0x or, taking into account Equation 1.2:

$$v = k_0e_0a/(K_s + a) \tag{1.3}$$

This, then, is a modern formulation of the equation obtained by Michaelis and Menten. Confusingly, however, it is *not* the equation known to modern biochemists as the 'Michaelis–Menten equation': this name is reserved for the steady-state equation (Equation 1.12) that we shall consider in Section 2.

Van Slyke and Cullen (2) considered the problem at about the same time as Michaelis and Menten, but in a different way. They also imagined a two-step process, but instead of treating the first as an equilibrium they treated it as irreversible, and they argued that the total time required could be treated as the sum of the times for the two steps:

$$\begin{array}{ccccccc} & & & \overset{k_1}{\longrightarrow} & & \overset{k_2}{\longrightarrow} & \\ \mathrm{E} & + & \mathrm{A} & & \mathrm{EA} & & \mathrm{E} + \mathrm{P} \\ e_0 - x & & a & & x & & p \end{array} \tag{1.4}$$

At the instant of mixing enzyme with substrate, before any EA or P has been produced, all of the enzyme exists as free enzyme, and so the initial rate of the first step must be k_1e_0a. If this rate were maintained until all of the enzyme was converted into EA the time required would be e_0/k_1e_0a, or $1/k_1a$. If this conversion were then followed by the complete conversion of EA back into E at a rate k_2e_0, the time required would be e_0/k_2e_0, or $1/k_2$. In practice, of course, the two processes occur simultaneously, but for any one enzyme molecule they are consecutive, and we can still regard these times as the average times required to convert a single molecule of E into EA and to convert a molecule of EA into E. Thus the average time required to carry out both steps, with concomitant conversion of a molecule of A into P, is the sum of the two. It follows that the rate of reaction is the concentration of molecules able to perform the transformation divided by this total time:

$$v = e_0/[(1/k_1a) + (1/k_2)] \tag{1.5}$$

Arranged more conventionally, this is of the same form as Equation 1.3:

$$v = k_2e_0a/[(k_2/k_1) + a] \tag{1.6}$$

with k_2 replacing k_0 and k_2/k_1 replacing K_s. Replacing k_0 by k_2 is just a change in symbol for what is really the same quantity, the rate constant for conversion of EA into products. However, replacing K_s, an equilibrium constant, by k_2/k_1,

the ratio of two rate constants for consecutive reactions, is more than just a change in symbol, as the two quantities have conceptually different meanings. However, this does not mean that they can be easily distinguished experimentally, as behaviour obeying Equation 1.3 can equally well be interpreted in terms of Equation 1.6. As we shall see in the next section, both equations are special cases of a more general treatment.

2. The steady-state assumption

Briggs and Haldane (3) showed that the equilibrium-binding mechanism of Michaelis and Menten and the irreversible-binding mechanism of Van Slyke and Cullen were special cases of a more general mechanism in which the binding of substrate was assumed to be reversible, but not necessarily at equilibrium during the reaction:

$$\begin{array}{ccccccc} \mathrm{E} & + & \mathrm{A} & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} & \mathrm{EA} & \xrightarrow{k_2} & \mathrm{E} + \mathrm{P} \\ e_0 - x & & a & & x & & p \end{array} \tag{1.7}$$

The rate of change of the concentration of intermediate ($\mathrm{d}x/\mathrm{d}t$), is the difference between the rate at which it is being produced from E and A and the sum of the rates at which it is being converted back into E and A and forward into E and P:

$$\mathrm{d}x/\mathrm{d}t = k_1(e_0 - x)a - k_{-1}x - k_2x \tag{1.8}$$

Briggs and Haldane postulated that although at the instant of mixing $\mathrm{d}x/\mathrm{d}t$ must be positive, because no EA would then exist at that instant, the rates of removal of EA would rapidly increase and, after a very short time, they would balance its rate of production. A *steady state* would be established, in which $\mathrm{d}x/\mathrm{d}t$ is zero, so

$$k_1(e_0 - x)a - k_{-1}x - k_2 = 0 \tag{1.9}$$

This may be rearranged to express x in terms of e_0 and a:

$$x = \frac{k_1e_0a}{k_{-1} + k_2 + k_1a} \tag{1.10}$$

The reaction rate is the rate of the step in which P is produced, that is k_2x, or

$$v = \frac{k_1k_2e_0a}{k_{-1} + k_2 + k_1a} \tag{1.11}$$

Although this is not immediately of the form of Equations 1.3 and 1.6 it may be written so that it is by dividing all terms by k_1, that is:

$$v = \frac{k_0 e_0 a}{K_m + a} \tag{1.12}$$

where k_2 is written as k_0 (for reasons that will be considered shortly) and $(k_{-1} + k_2)/k_1$ as K_m, the Michaelis constant. This is the *Michaelis–Menten equation*, the fundamental equation of enzyme kinetics. This name is *not* normally applied to the equation derived with the equilibrium assumption of Michaelis and Menten, but to the equation obtained with the steady-state assumption.

3. The Michaelis–Menten equation

The two-step mechanism shown in Equation 1.7 is only the simplest of an infinite range of mechanisms that give rate equations of the form of the Michaelis–Menten equation, Equation 1.12. Thus, although the two-step mechanism is useful for discussion, one cannot be certain that it is the true mechanism simply because one observes adherence to the Michaelis–Menten equation experimentally. It is therefore best to represent its parameters as in Equation 1.12, with symbols k_0 and K_m that do not imply that they refer to particular steps of a mechanism, even though in the two-step case k_0 is in fact identical to k_2.

Although k_0 may not refer to a single step of a mechanism, it does have the properties of a first-order rate constant, defining the capacity of the enzyme–substrate complex, once formed, to form the product, P. It is commonly called the *catalytic constant* of the enzyme (and often symbolized as k_{cat}); the alternative name *turnover number* is sometimes used, though it is becoming less common. Values of about 10^3 s^{-1} are typical for k_0, and some enzymes show much larger values. It is a useful measure of catalytic activity, but one cannot always measure it, especially in the early stages of characterizing an enzyme, because the enzyme concentration is often unknown or difficult to measure. For this reason $k_0 e_0$ is often replaced by V (or V_{max}), a quantity known as the *limiting rate*.

The other parameter of the Michaelis–Menten equation is called the *Michaelis constant* (K_m). It corresponds to K_s in Equation 1.3, and resembles it in having the dimensions of concentration, that is of a dissociation constant, and in specifying the relative concentrations of free enzyme, free substrate and enzyme–substrate complex. However, unlike an equilibrium constant, it defines these concentrations under steady-state reacting conditions, not at equilibrium. Its value, K_m, is equal to $(k_{-1} + k_2)/k_1$ and it approximates to k_{-1}/k_1, that is to the equilibrium dissociation constant K_s, only if k_{-1} is large compared with k_2; it approximates to k_2/k_{-1}, the corresponding constant in Equation 1.6, if k_2 is large compared with k_{-1}.

Although it has often been suggested that K_m can in practice be assumed to be similar in magnitude to K_s, there is no good reason for this to be true and it is much more likely that enzymes will evolve with values of k_2 that are larger than k_{-1}. This is because once the enzyme–substrate complex is formed it is in the interests of catalytic efficiency to convert it into products as fast as possible (cf. Chapter 6, Section 5).

If the substrate concentration a is much smaller than K_m, it can be ignored by comparison with K_m in Equation 1.12, which thus simplifies to:

$$v \simeq (k_0/K_m)e_0a \tag{1.13}$$

that is with first-order dependences on both the enzyme and substrate, or second-order kinetics overall. It is evident that k_0/K_m is an important quantity and is more than just a ratio of two other quantities. It is sometimes called the 'second-order rate constant' for the reaction, but as it is the parameter that defines enzyme specificity, as we shall discuss in Chapter 4, Section 5, it is also known as the *specificity constant* and symbolized as k_A, where the subscript specifies which substrate is considered.

As a increases it becomes similar in magnitude to K_m, surpasses it, and eventually makes it insignificant. In the limit at high substrate concentrations, therefore, Equation 1.12 simplifies to:

$$v \simeq k_0e_0 = V \tag{1.14}$$

This is the reason for giving V the name 'limiting rate'. It is not reached at any finite value of a and is, moreover, approached rather slowly, so that, for example when a is 10 times the K_m then v is only 0.91 V, still 9% less than V. There is no maximum value of v in the mathematical sense, that is, there is no point at which the slope of the plot of v against a is zero, and thus the name 'maximum rate' (or 'maximum velocity') is not an accurate description, though it is very common.

The concentration at which an enzyme obeying Michaelis–Menten kinetics is 'half-saturated', that is, where v is equal to 0.5 V, may readily be shown to be when a is equal to K_m by substituting this value of a in Equation 1.12. This emphasizes that the Michaelis constant is a concentration, and gives physical meaning to it as the half-saturation concentration. It is often believed that the K_m value for an enzyme with its natural substrate is similar to the physiological concentration of the substrate. Substrate concentrations *in vivo* are difficult to measure and so there is no extensive evidence for the truth or otherwise of this supposition.

It follows that the plot of v against a has the shape shown in *Figure 1.1*, that is, the experimental behaviour observed for many enzymes is, in fact, explained by the Briggs–Haldane mechanism.

Although the Michaelis–Menten equation is usually written as Equation 1.12, that is, in terms of k_0 (or V) and K_m, there is nothing fundamental or necessary