Enzyme Technology

III. Enzyme kinetics

III.1. Enzymes are catalysts

An enzyme is a catalyst and is responsible for rate acceleration of a reaction that is inherently slow.
Enzymes are catalysts that increase the rate of chemical reactions without being consumed.
Enzymes accelerate reaction rates phenomenally.

Catalytic efficiency of enzymes

Enzymes speed up reaction rates by reducing the activation energy:

- enzymatic reactions take place following a mechanism of organic reactions (acid-base, nucleophilic, electrophilic) for which the enzyme provides the catalytic groups
- the factors of proximity and orientation are preponderant. Without an enzyme, the encounters of two molecules occur randomly: this is no longer the case here
- some substrate bonds are tightened by the enzyme

However, the reaction "S is transformed into P" must first be possible from a thermodynamic point of view. However, before a substrate molecule is transformed into a product, it must have a minimum amount of energy to pass through a transition state. This activated state represents a sort of midpoint where substrate bonds are changed sufficiently that conversion to product is possible. There are two ways to speed up this reaction. The first is to raise the temperature such that a significant number of molecules reach the transition state. Another way is to decrease the activation energy.

Energetics of enzyme-catalyzed reactions

In any reaction, converting substrate to product requires intermediate states. Those intermediates are always less stable – and thus of higher energy – than either the substrate or the product. In the simplest theoretical case of one intermediate, we can speak of the transition state, although invariably more than one exists. The difference in energy level between the substrate and the transition state is called **the activation energy**. Energy input is needed to move reactants up to the transition state's energy level.

Living cells exist at relatively low temperatures (between 0 and 100°C). At these temperatures of life, virtually none of the intermediate metabolism reactions can proceed at a sufficient rate to allow growth or maintenance of the cell. Furthermore, even if the cell could significantly increase its temperature, there would be no favored reaction over another. It is therefore the enzymes that the cells possess which reduce the activation energy. These enzymes selectively reduce the **activation energy** of a given reaction which can then take place at a low temperature.

Enzymes are therefore catalysts that increase the speed of chemical reactions without being consumed. The equilibrium constant of the reaction is not changed, just the rate of the reaction is affected by the enzyme.

There is a transition state S*
How to accelerate the transformation reaction from S to P?
- raise the temperature
- reduce the activation energy

Enzymes selectively decrease energy activation of a given reaction.
The ambient temperature is then sufficient for a significant number of $S \rightarrow P$.

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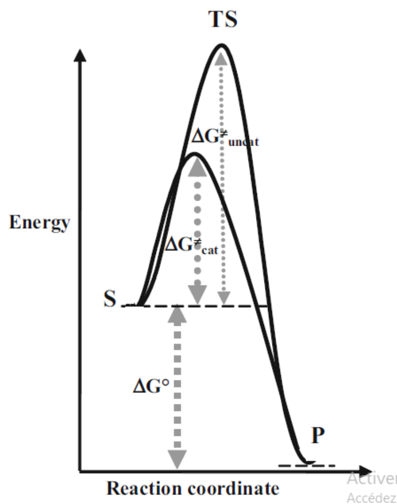


Fig. Free energy diagram – schematic comparing the catalyzed versus uncatalyzed reactions. The standard free energy of reaction ($\Delta G°$) and free energy of activation for uncatalyzed ($\Delta G^{\#}$uncat) and catalyzed ($\Delta G^{\#}$cat) reactions are shown (Punekar, 2018)

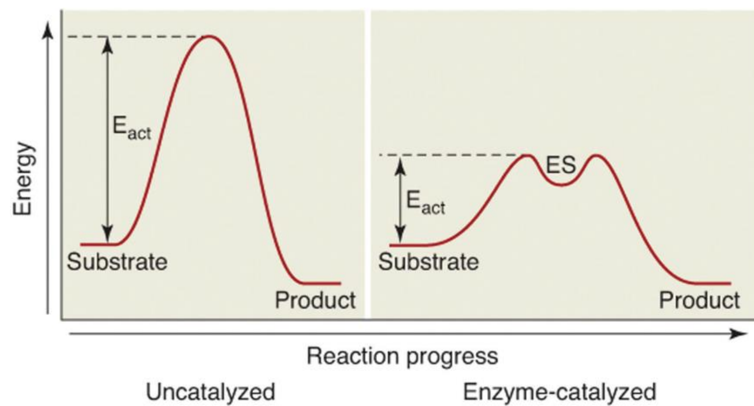


Fig. Energy of activation in uncatalyzed and catalyzed reactions. On the left is the high energy intermediate of an uncatalyzed reaction between substrate and product. On the right, an enzyme-catalyzed reaction shows an additional high-energy but stable region, the intermediate enzyme–substrate complex (ES). The enzymatic reaction course has higher reaction barriers on either side; the highest barrier that defines the E act is lower than that of the uncatalyzed reaction (McDonald & Tipton, 2023).

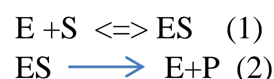
III.2. Kinetic Foundations

As early as 1898, the reversibility of an enzyme reaction was reported. The enzymatic synthesis of a glucoside (maltose from glucose) by the yeast maltase established some key features:
(a) an enzyme being a catalyst speeds up the reaction in both directions of a reversible reaction,
(b) at least some steps in metabolism may go in either direction, and
(c) enzymes may be involved in the cellular biosynthetic processes.

A more general form of the Henri-Michaelis-Menten equation to describe enzyme kinetics was derived by Briggs and Haldane via the steady-state approach in 1925. We continue to use this fundamental equation even today to describe the substrate saturation phenomenon of an enzyme reaction. A very popular linear form of this hyperbolic relation between initial velocity and substrate concentration is attributed to Lineweaver and Burk (1934).
Leonor Michaelis and others emphasized the importance of pH on enzyme activity and routinely controlled it in all their studies. The ES complex formation was a kinetic concept to begin with. First direct observation of an enzyme substrate complex of catalase was made by KG Stern (1935).
Emil Fischer, as early as 1894, observed that substrates for invertin (now the invertase or sucrose hydrolase) are not substrates for emulsin (a -glucosidase) and vice versa. Fischer opined that “enzymes are fussy about the configuration of their object of attack.” For example, the enzyme and the glucoside on which it acts must fit each other like a “lock and key” to be able to catalyze the chemical reaction. This laid the foundation for describing fundamental properties of enzyme like specificity, stereoselectivity, and the famous lock-and-key analogy for enzyme– substrate interactions.

The simplest possible reaction is S → P, where S is the substrate, and P is the reaction prod-uct. The enzymatic route consists of three steps: the enzyme binds the substrate, the enzyme transforms the substrate into the product, and the enzyme releases the product.
The entire process may be written as two chemical reactions:

$$E + S \Leftrightarrow ES \quad (1)$$



$$ES \longrightarrow E + P \quad (2)$$

In Equation (1), E and S reversibly combine to form the enzyme–substrate complex ES. In Equation (2), the complex ES irreversibly breaks down to form P and regenerate E. The net reaction – the sum of these two – is just S → P, the same as the nonenzymatic reaction.

Note that the enzyme assumes two forms over the course of the enzymatic reaction: the free enzyme forms E and the bound enzyme forms **ES**.

We can also rewrite Equations (1) and (2) in a single line and assign rate constants for each chemical step:

.3)

The rate constants are proportionalities between substrate concentrations and reaction rate.

The rate constants in Equation (3) are:

kf: the forward rate constant for the formation of **ES** from **E** and **S**

kr: the reverse rate constant for the formation of **E** and **S** from **ES**

kcat: the rate constant for the breakdown of **ES** to **E** and **P**

The kcat rate constant, which leads to product formation, is also known as the **catalytic rate constant** indicated by the subscript *cat*.

Assumptions

Equation (3) is our model for enzyme kinetics. It is possible to derive an equation from this model that relates the initial velocity *v* to the substrate concentration [S].

To accomplish this, we make three assumptions:

1. Steady-State

The first assumption is that the system is in a steady-state. Both experimental enzyme assays and pathways in living cells achieve a steady-state, at least when considered for longer than a fraction of a second. As **ES** is the only intermediate in the process, the concentration of this species remains constant with time as the reaction proceeds.

2. Enzyme Conservation

Second, we assume that the total amount of enzyme is unchanged throughout the reaction. This assumption will always be true of the *in vitro* enzyme assay because the total amount of enzyme, [E]tot, is fixed by the analyst. *In vivo*, the total amount of enzyme is also constant over reasonably short periods, usually for hours to days. It is subject to fluctuation at more extended periods as the enzyme protein may be increased in production (protein synthesis) or decreased (protein degradation). Here, we are concerned exclusively with the shorter time frame. Enzyme conservation is expressed as:

$$[E]\ tot = [E] + [ES]$$

3. Initial Velocity is The Product Formation Step

Third, we assume that the initial velocity *v* can be identified as the step in the mechanism that leads to product formation. In our mechanism, this is the reaction governed by the catalytic rate constant (kcat):

$$v_o = kcat\ [ES]$$

III.3. Effect of enzyme concentration

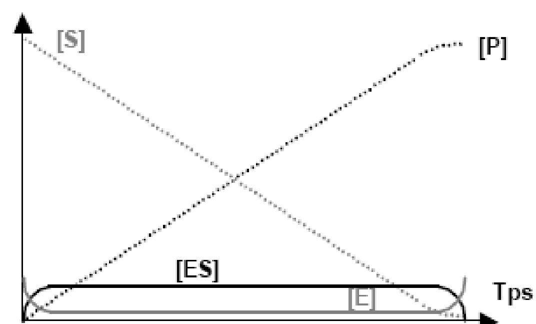
Michaelis - Menten Equation (1913)

Leonor Michaelis and Maud Menten deduced in 1913 an equation which predicts the initial velocity (of P formation) as a function of the substrate concentration:

Initial velocity of a réaction:

v_o **= d[P]/dt = k_2.[ES]--------** (1)

The reaction velocity depends on the concentration of the enzyme-substrate (**ES)** complex.



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It is therefore necessary to calculate the concentration of **ES**:

We start by expressing the steady-state assumption as:

Rate of ES formation = Rate of ES destruction

ES is formed from **E** + **S** and decomposed into either **E** + **S** or **E** + **P**.

Formation rate of **ES** : $v_f = k_1 \cdot [E] \cdot [S]$

Destruction velocity of **ES** : $v_d = (k_{-1} + k_2) \cdot [ES]$

[ES] Quickly reaches a constant value (***steady state conditions***)

So: $v_d = v_f$

$(k_{-1} + k_2) \cdot [ES] = k_1 \cdot [E] \cdot [S]$

$[ES] = [E] \cdot [S]\, k_1 / (k_{-1} + k_2) = [E] \cdot [S] / K_M$ -------- (2)

$K_M = (k_{-1} + k_2) / k_1$ = The Michaelis (-Menten) constant

The second assumption is enzyme conservation. We start the reaction with a fixed, known amount of enzyme and finish it with the same amount.

We must now know the concentration of the free enzyme **[E]**

$$[E] = [E]_T - [ES]$$

Where $[E]_T$ is the total concentration of enzyme, free or bound. So we have:

$[ES] = ([E]_T - [ES]) \cdot [S] / K_M = ([E]_T \cdot [S] / K_M) - ([ES] \cdot [S] / K_M)$

$$[ES] + [ES] \cdot [S] / K_M = [E]_T \cdot [S] / K_M$$

$$[ES] \cdot (1 + [S] / K_M) = [E]_T \cdot [S] / K_M$$

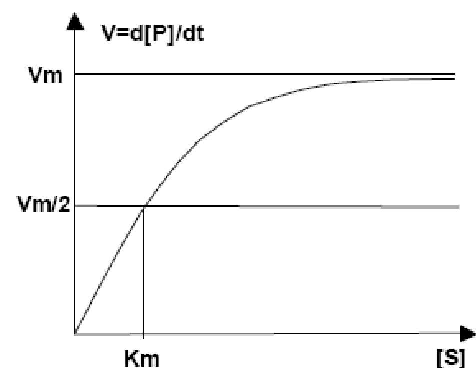
$$[ES] \cdot (K_M + [S]) / K_M = [E]_T \cdot [S] / K_M$$

$$[ES] \cdot (K_M + [S]) = [E]_T \cdot [S]$$

$$[ES] = [E]_T \cdot [S] / (K_M + [S])$$

We now introduce our third assumption: **the overall velocity of the reaction** involves the step leading to product formation.

We can introduce this term in the equation for the initial velocity (1):



$v_o = k_2 \cdot [ES] = k_2 \cdot [E]_T \cdot [S] / (K_M + [S])$

$v_o = v_{max} \cdot [S] / (K_M + [S])$

The Michaelis-Menten Equation

Special cases

For high values of *[S], much higher than that of Km, that is to say that Km is negligible compared to [S], and the equation simplifies:*

$[S] >> K_M$ $\quad v_o = v_{max} \cdot [S] / [S]$

$v_o = vmax$

For low values of *[S], well below Km; in other words, [S] is negligible compared to Km and the velocity v is directly proportional to [S]:*

$[S] << K_M$ $\quad v_o = v_{max} \cdot [S] / K_M$

Km represents the substrate concentration for which the velocity is equal to half the velocity limit

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$[S] = K_M$ $\quad v_o = v_{max} \cdot K_M / 2 K_M$

$v_o = v_{max} / 2$

V_{max} is the maximum velocity that the reaction can reach when the enzyme is saturated with substrate, that is to say when **[E] T = [ES].** The Vmax is the maximal velocity at saturating concentrations of substrate.
K_M is the substrate concentration that saturates the enzyme by half. It is a measure of the affinity of the enzyme for the substrate: **the higher the affinity is, the smaller K_M becomes**.

$k_2 = (v_{max}/[E]_T)$ is often called the catalytic constant **kcat,** and represents the number of catalytic cycles per second (number of rotations) of which the enzyme is capable.
Kcat = number of moles of P formed per second and per mole of enzyme. It is also known as turnover number – and it defines the number of turnovers (catalytic cycles) – the enzyme can undergo in unit time when the enzyme is fully saturated with substrate.
Whereas Vmax depends on the enzyme concentration ([Et]), kcat does not.

The kcat is therefore a direct measure of the catalytic activity of an enzyme: the greater the kcat, the faster the catalytic events within the enzyme-substrate complex.
The kcat is expressed in s^{-1} and its inverse has the dimension of a time: it is the time required by an enzyme molecule to transform a substrate molecule. The catalytic constant of most enzymes is between 10^2 and 10^4 s^{-1}.
The **kcat/K_M** ratio is often also given as a measure of **catalytic efficiency**, because it corresponds to a velocity constant for low substrate concentrations **($[S] \ll K_M$, so $[E] = [E]_T$)**

III.4. Enzyme Unit

With the help of a robust and reliable assay method, reaction rates can be recorded for any enzyme sample. Assay conditions like concentration of the substrate, pH, and temperature should be well defined to obtain a reproducible initial velocity (v). Since the rate of the reaction varies with the enzyme concentration, it is useful to define a quantity which is independent of the concentration of enzyme used.
To facilitate comparison of enzyme activities from various samples (and from values reported in the literature), an international unit is recommended.

The standard enzyme unit (U) is the amount that catalyzes the formation of one micromole of product per minute, under defined assay conditions. This unit has the dimensions of μmol x min^{-1}. The more the number of units in a sample means the more enzyme catalyst present in that sample.
While one U of enzyme in a standard assay produces one μmol of product per min, two U of the same enzyme gives 2.0 μmolxmin-1 of the product – and so on. The enzyme concentration in a given sample is then expressed in terms of U x ml^{-1}. A sample containing 2.0 U x ml^{-1} is four times more concentrated enzyme than a sample with 0.5 U x ml^{-1}. We should note that, as defined, the catalysis unit by itself does not indicate anything about the purity of the enzyme sample.

The enzyme activity unit may also be expressed in terms of μmol substrate consumed per min.
The International Union of Biochemistry has recommended the use of katal – according to SI units. A katal corresponds to the amount of enzyme that produces one mole of product per second.
From the calculations (see box below), it is obvious that katal is a very large unit and hence is not in common use.

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$$
\begin{aligned}
1 \text{ katal} &= 1 \text{ mol} \times \sec^{-1} \\
&= 10^6 \mu\text{mol} \times 60 \text{ min}^{-1} \\
&= 6 \times 10^7 \mu\text{mol} \times \text{min}^{-1} \\
&= 6 \times 10^7 \text{U}
\end{aligned}
$$

Similarly, $1 \text{ U} = 16.67 \text{ nkatal}$

Specific Activity A way to express the amount and concentration of enzyme is through U and U x ml^{-1}, respectively. These units reflect on the enzyme content of the given sample but do not tell us anything about the purity of the enzyme. The units of enzyme in a sample can be same regardless of the quantity and diversity of other proteins present. We could however present the quantity (U) of enzyme present in a known amount of protein.

Specific activity is thus defined as the number of units per mg of protein. It is an index of the purity of the enzyme sample – the higher the proportion of enzyme protein in a given protein sample, the greater will be its specific activity.

The purer the enzyme sample, the higher is its specific activity. If this is extended logically to the stage of highest enzyme purity, then that sample must have every protein molecule representing only that enzyme.

Turnover Number The specific activity of an enzyme sample is expressed as U x mg^{-1} protein (note that 60 U x mg^{-1} corresponds to 1 katal x kg^{-1}). This is nothing but velocity per unit amount of catalyst protein – i.e., µmol product formed per min per mg protein.

With a pure enzyme (possessing highest limiting specific activity), the amount of enzyme protein (say in mg) can also be expressed as number of moles of that enzyme (say in µmol).

However, to do this we need to know one additional bit of information – the molecular mass of the enzyme. When this is available, we can present the specific activity (see box) of the pure enzyme.

$$
\begin{aligned}
\text{Specific activity} &= \text{U} \times \text{mg}^{-1} \\
&= \mu\text{mol product formed} \times \text{min}^{-1} \times \text{mg}^{-1} \\
&= \mu\text{mol product formed} \times \text{min}^{-1} \times \mu\text{mol}^{-1} \text{of enzyme}
\end{aligned}
$$

This quantity – called the turnover number – has the units of dimension "$time^{-1}$" (more commonly, sec^{-1}). It indicates the number of times a single enzyme molecule converts substrate into product in 1 min. In this definition it is assumed that substrate is saturating and that the enzyme has one active site per molecule.

III.5. Graphical representations

Experimentally, it is not possible to determine Vmax because only an approximate value is known. We can find the two important values Vmax and Km using different mathematical treatments.

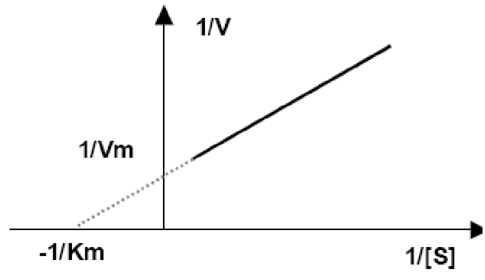
1- Double-reciprocal or lineweaver–burk plot

The hyperbolic Michaelis–Menten curve can be transformed into a straight line by plotting 1/v versus 1/[S]. This graph is known as the double–reciprocal or Lineweaver–Burk plot. The values of Vmax and Km appear at the intersections of its axes.

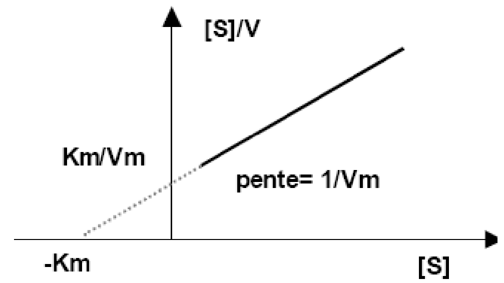
LINEWEAVER–BURK PLOT (1934): 1/V =f (1/S)

$$\mathbf{1 / v_o = 1 / v_{max} + (K_M / V_{max}) \cdot 1 / [S]}$$

The main problem with this method is the preponderance given to the lowest values of S and vo, for which the uncertainty is nevertheless the greatest. It can lead to false estimates. It only becomes interesting for high values of [S], therefore in "saturating" conditions.



2. Woolf–Hanes Plot



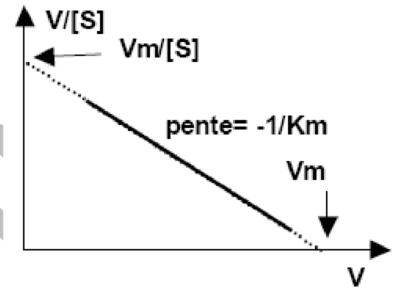
[S]/v is plotted against [S].

It is derived simply by multiplying the Lineweaver–Burk transformation by [S]. The following linear transform of the Michaelis–Menten equation is thus obtained:

[S]/vo = (1/Vmax) [S]+ Km/Vmax

The kinetic parameters are extracted from the slope (1/Vmax) and intercept (KM/Vmax) of such a plot. extrapolation gives –Km at the intersection with the abscissa axis.

3. Eadie–Hofstee Plot



By dividing both sides by [S] and rearranging, we obtain,

$$\frac{v}{[S]} = -\frac{1}{K_M}v + \frac{V_{max}}{K_M}$$

The slope is –1/Km, the intersections with the axes give Vm/[S] and Vmax.

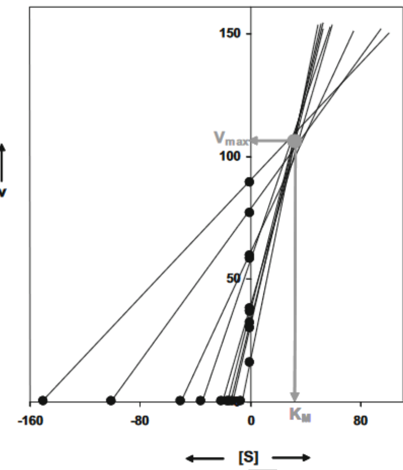
Linear transformation of Henri-Michaelis- Menten equation: a summary (Punekar, 2018)

Plot	Lineweaver–Burk (1934)	Eadie–Hofstee (1942)	Scatchard (1949)	Woolf–Hanes (before 1932)
Plot of (Y → X)	$1/v \rightarrow 1/[S]$	$v \rightarrow v/[S]$	$v/[S] \rightarrow v$	$[S]/v \rightarrow [S]$
Y-axis intercept	$1/V_{max}$	V_{max}	V_{max}/K_M	K_M/V_{max}
X-axis intercept	$-1/K_M$	V_{max}/K_M	V_{max}	$-K_M$
Slope	K_M/V_{max}	$-K_M$	$-1/K_M$	$1/V_{max}$
Features	Individual display of v and [S] on two axes; rate constants directly visualized from intercept and slope	View nonlinearity due to cooperativity and departure from hyperbolic kinetics	Same as Eadie–Hofstee but axes interchanged; ligand binding studies	Weighting of errors from original data is least distorted

Note V_{max} and V_{max}/K_M, respectively, correspond to zero-order and first-order rate constants in the Michaelis–Menten formalism

4. Direct Linear Plot of EISENTHAL and CORNISH-BOWDEN

This plot was suggested by Eisenthal and Cornish–Bowden (1974) where a series of “v-[S]” data pairs are directly plotted. For each “v-[S]” pair of data, we can generate a straight line by marking v on the Y-axis (the Vmax axis) and [S] on the negative side of the X-axis (the KM axis). All these lines must intersect at a point in the first quadrant with KM and Vmax as its coordinates.



$$\frac{1}{v} = \frac{K_M + [S]}{V_{max}[S]}$$

$$\frac{V_{max}}{v} = \frac{K_M}{[S]} + 1$$

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