Practical Lesson No 3

ENZYME KINETICS: ESTIMATION OF THE MICHAELIS CONSTANT

Key words: rate of enzyme-catalysed reaction, zero-order kinetics, saturation kinetics, the Michaelis-Menten equation, the Lineweaver-Burke equation, maximal velocity (V_{max}), the Michaelis constant (K_m).

THEORY

Effect of substrate concentration on the rate of enzyme catalysed reaction

The rate of an enzyme-catalysed reaction is defined as the amount of product P formed per unit of time:

$$\text{reaction rate} = \frac{\Delta[P]}{\Delta t}$$

The rate depends on the availability of substrate, i.e. on substrate concentration. Unless the substrate is in great excess, the reaction velocity decreases with time as the substrate is consumed in the reaction and its concentration lowers. In further consideration, we will work with *initial velocity* - v, i.e. the reaction rate in first minutes of the reaction when the substrate has not been significantly consumed yet and the effect of time is negligible.
The relationship of the *initial* velocity on substrate concentration follows the *Michaelis-Menten equation*:

$$v = \frac{V_{max} \times S}{K_m + S}$$

V_{max} maximal velocity (in unlimited availability of substrate)
S substrate concentration
K_m Michaelis constant

Mathematically the relationship takes the shape of rectangular hyperbola (see Graph I).

The <u>Michaelis constant</u> is a very important term introduced by this equation. It is defined as *<u>the concentration of substrate required to achieve half the maximum velocity of the enzyme reaction</u>*. By definition it is expressed in units of concentration. It does not depend on concentration of the enzyme in reaction mixture and remains constant for a given couple enzyme-substrate. Therefore, the Michaelis constant is actually a *<u>measure of the affinity of the enzyme to the substrate</u>*. The lower the value the higher is the affinity.

Lactate dehydrogenase assay

In this lesson, the effect of substrate concentration on reaction velocity will be demonstrated on lactate dehydrogenase (LDH), an ubiquitous glycolytic enzyme that catalyses reversible oxidation of lactate to pyruvate with concomitant reduction of NAD:

$$CH_3\text{-}CHOH\text{-}COOH + NAD^+ \longrightarrow CH_3\text{-}CO\text{-}COOH + NADH + H^+$$

The task is to obtain the value of the Michaelis constant for the enzyme-substrate couple lactate dehydrogenase-lactate. For this purpose the enzyme activity must be measured several times; the enzyme concentration is kept constant, while concentration of lactate as the substrate varies (from 12.5 to 200 mmol/l). The LDH preparation (in 0.05 mol/l Tris buffer pH 8.5 stabilised with bovine serum albumin) must be sufficiently diluted so that at all the lactate concentrations the substrate is in excess and the reaction course can be practically considered as zero-order kinetics. Obviously, the other substrate (NAD^+) must be present in excess, too.

In our LDH assay, when the enzyme is supplied with lactate and NAD^+, the LDH-catalysed reaction starts to produce pyruvate. At certain time point the reaction is terminated by addition of 2,4-dinitrophenylhydrazine, which reacts with pyruvate at acidic pH. After alkalisation (addition of NaOH) the resulting hydrazone derivative gives a brown-orange colour suitable for quantification by means of spectrophotometry at the wavelength 505 nm.

Finally, for each of the lactate concentrations used some value of absorbance at 505 nm is obtained, which could be filled into the following table:

Tube N^o	**Lactate concentration (S)** mol/l	**Velocity A_{505}**	**1/S**	**$1/A_{505}$**
1	0.2000		5	
2	0.1000		10	
3	0.0500		20	
4	0.0250		40	
5	0.0125		80	

As the measured absorbances are directly proportional to concentration of the pyruvate-related coloured product, they can be used directly to express the reaction velocity (the actual amounts of reaction product, if required, could be read from a calibration graph). The reciprocal values of absorbances (*$1/A_{505}$*) will be needed as well for further estimation of the Michaelis constant.

Instructions how to estimate the Michaelis constant

Numerical method

Michaelis constant can be calculated directly from the results using mathematical description of hyperbolic function. Your experimental data represent a set of values of v (A_{505}) and corresponding substrate concentrations S:

v_1	S_1
v_2	S_2
v_3	S_3
v_4	S_4
v_5	S_5

Taking any two pairs of corresponding values, e.g. v_1 S_1 and v_2 S_2, the Michaelis constant can be calculated from the equation:

$$K_m = S_1 \times S_2 \quad \frac{v_2 - v_1}{(v_1 \times S_2) - (v_2 \times S_1)}$$

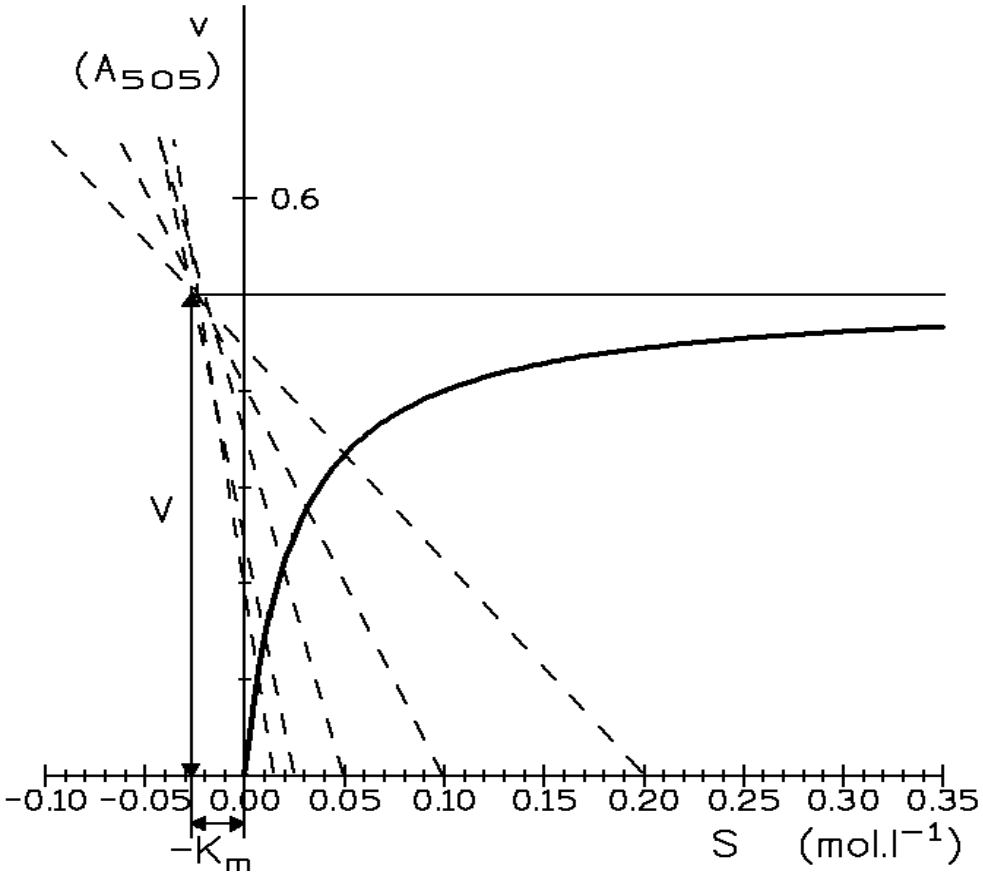
Note: For greater accuracy of the calculation, take at least <u>*three diferent pairs*</u> of the corresponding values v and S from your experimental data and present the K_m as the mean value from these results.

Graphical methods

I. <u>Direct linear plot (Eisenthal, Cornish-Bowden)</u>

Plot v (A_{505}) against substrate concentration S and try to fit a rectangular hyperbola (as in Graph I).
Then mark an S value onto the *x*-axis (horizontal) and its corresponding v onto the *y*-axis (vertical). Connect these two points by a straight line. Repeat it for all your couples of data points. Hence *n* lines are obtained for *n* pairs of points.
If data fit equation of rectangular hyperbola exactly, all the lines should intersect in one point, whose distance from *x*-axis is V_{max} and that from *y*-axis is $-K_m$.
In practice however data suffer from measurement errors and a number of different intersections is obtained. The coordinates of each intersection provide estimates of K_m and V_{max}. Medians rather than means of these estimates are the best-fit values of K_m and V_{max}. (The median is the middle one of an odd-numbered set of data or the mean of the middle pair in an even-numbered set.)

GRAPH I

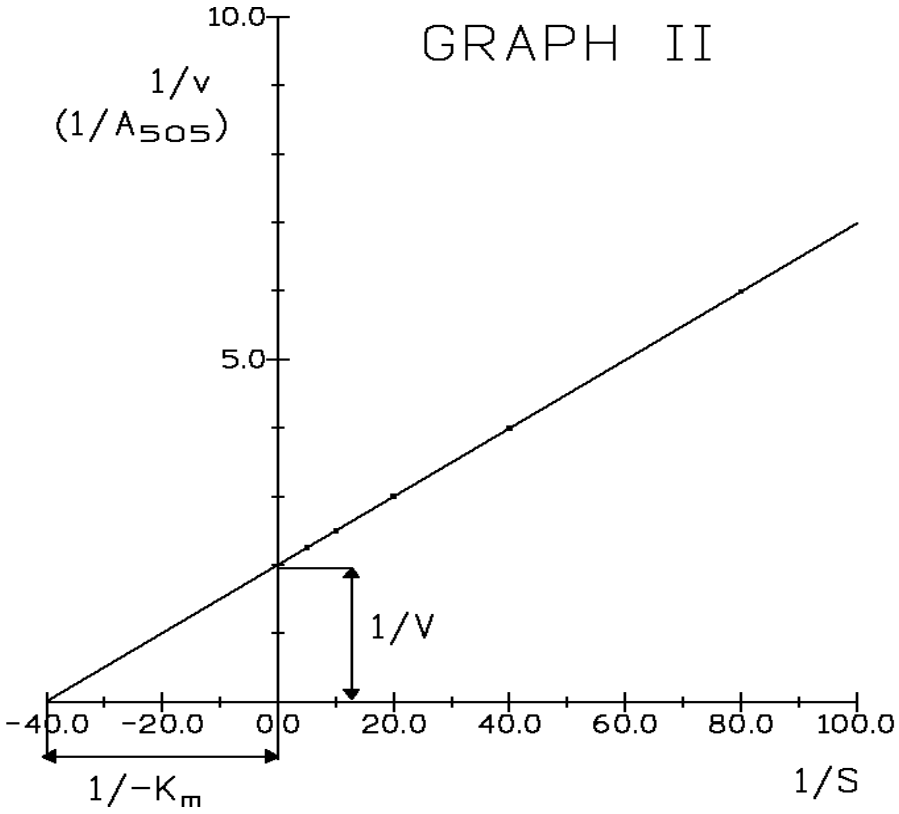


II. Double reciprocal plot (Lineweaver-Burke)

When *1/v* is plotted against *1/S*, a linear function is obtained (see Graph II). The *intercept* of the function *with x-axis* represents $-1/K_m$, while *intercept with y-axis* gives $1/V_{max}$.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}}$$

Plot $1/v$ $(1/A_{505})$ against $1/S$ as in Graph II, fit a straight line and obtain K_m and V_{max} from the intercepts.



TASK:

Estimation of Michaelis constant for LDH-lactate from supplied data

1. The LDH assay was performed by technician using several lactate concentrations and dilutions of LDH 200x, 300x, 400x, and 500x. You are given the table containing the absorbance data measured at one of LDH dilutions.
2. Use the numerical method described above for calculation of the Michaelis constant K_m.
3. Plot the data according to Eisenthal/Cornish-Bowden as described above (Graph I) and use the graph for determination of the Michaelis constant K_m and maximal velocity V_{max}.
4. Calculate the reciprocals $1/A_{505}$ and plot the data according to Lineweaver-Burke as described above (Graph II) and use the graph for determination of the Michaelis constant K_m and maximal velocity V_{max}.
5. Summarize and compare the results obtained by all three methods. Which of them appears most accurate ?
6. Finally, using one of the obtained values of the Michaelis constant and the highest substrate concentration, calculate what was the rate of LDH reaction in % of its theoretical maximal velocity.