EXPERIMENTAL DETERMINATION OF ENZYME KINETIC PARAMETERS

Table of contents

Objectives

At the end of the lesson the participants should be able to:
1. Define the conditions for application of the Michaelis-Menten model, its advantages and limitations.
2. Interpret the statistic distribution of the experimentally determined kinetic parameters.
3. Solve biologically relevant problems with the help of enzyme kinetic parameters.

Recommended order of the simulated experiments

1. Adjust the experimental conditions using a continuous assay of enzyme activity. ATTENTION! The results contain experimental errors. Always pay attention to the numeric values of the results (due to the scaling of the axes variations as low as 10 % of the measured values may seem large in the graphic presentation of the results)!

a) select a reaction (a pair of enzyme *E* and substrate), co-ordinate your selection with your colleagues, because at the end of the exercise data on all enzymes are necessary for the solution of the two application examples (if even a single enzyme is not characterized it is not possible to solve these).

b) examine the pH and temperature dependence of the reaction rate and make a record of the range, in which the experiment yields measurable results. This part of the work provides only preliminary data on the pH and temperature effects and illustrates the relation between the planned conditions for the next experiment (pH 7.2 and 37 °C) and the "optimal conditions" (check the apparent nature of these so called optimal conditions). Planned duration not more than 15 min.

c) examine the effect of total enzyme concentration on the reaction rate (using Fig. 1. of this menu point select an enzyme concentration, for which the product formation is linear in time). For relevant initial guesses see the reported *in vivo* concentration values.

d) examine the effect of initial substrate concentration on the reaction rate (using Fig. 1. of this menu point select a range of substrate concentrations, for which the product formation is linear in time). For relevant initial guesses see the reported *in vivo* concentration values. The well selected range of substrate concentrations should include values below the K_m as well as saturating values.

e) using the virtual possibility to visualize the time-course of the enzyme-substrate complex concentration check the validity of the steady-state assumption for the selected experimental conditions

2. Using the experimental setting satisfying the assumptions of the Michaelis-Menten model identify the kinetic parameters of the enzyme (in order to prepare the solution of the task in Application 2. perform the determination at pH 7.2 and 37 °C). For reliable evaluation of the experimental error use at least 5 replicas (too many repeats result in unacceptably long experimentation time). The Lineweaver-Burk plot provides a helpful check-point. If the kinetic parameters are determined from linear regression in a double-reciprocal plot, these are valid only if the conditions of the Michaelis-Menten model are perfectly satisfied and there are no experimental errors. Any deviation from the model conditions and experimental errors distort the parameters after reciprocal transformation. Thus, the difference between the two lines in Fig. 3 of this menu indicates

poorly adjusted experimental conditions. In such a situation you should go back to the "Adjustment of experimental conditions using a continuous assay" and check the validity of the model conditions (the most common reason for the deviation is the lack of steady state at low substrate concentrations which can be corrected with changes in the incubation time or enzyme concentration).

3. Determine the confidence intervals of the identified parameters. This step should be preceded by generation of multiple sets (300 is the recommended number) of kinetic parameters from simulated experimental data with the same experimental error (Computer simulation menu point). If you use less than 10 replicas for each experimental point, the recommended simulation procedure is the Monte Carlo. This step takes approximately 10 min, the end of the simulation is indicated by the return of the menu for selection of simulation procedure. The generated Monte Carlo data can be plotted under the "Confidence intervals and areas of the kinetic parameters" point of the main menu, which offers also an option to compare your results with those of your colleagues (if the parameters are correctly determined in independent experiments, the best guess of others should lie within your 95 % confidence area).

4. Repeat steps 2 and 3 at pH 6.3, so that you can answer the question from Application 2 (Is the metabolic control the same in two different metabolic states?). If you have time, check also the effect of fever on metabolic control (determine the kinetic parameters at 40 °C).

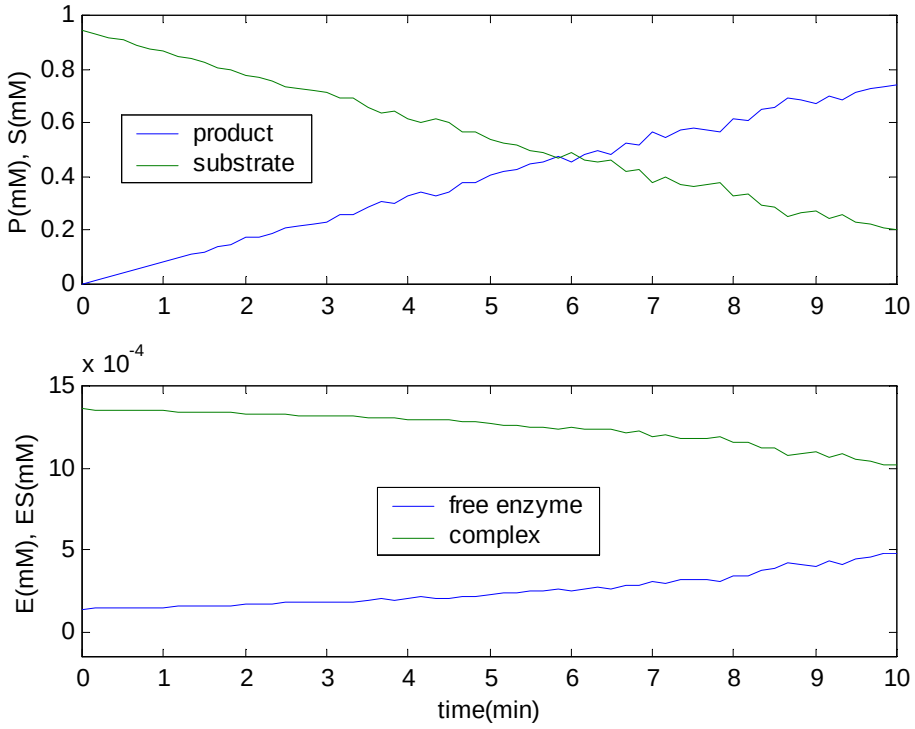
5. Perform the tasks in Applications 1. and 2.

Please, make records of your results at each stage of the experiment!

Theoretical and experimental issues addressed in the computer simulation

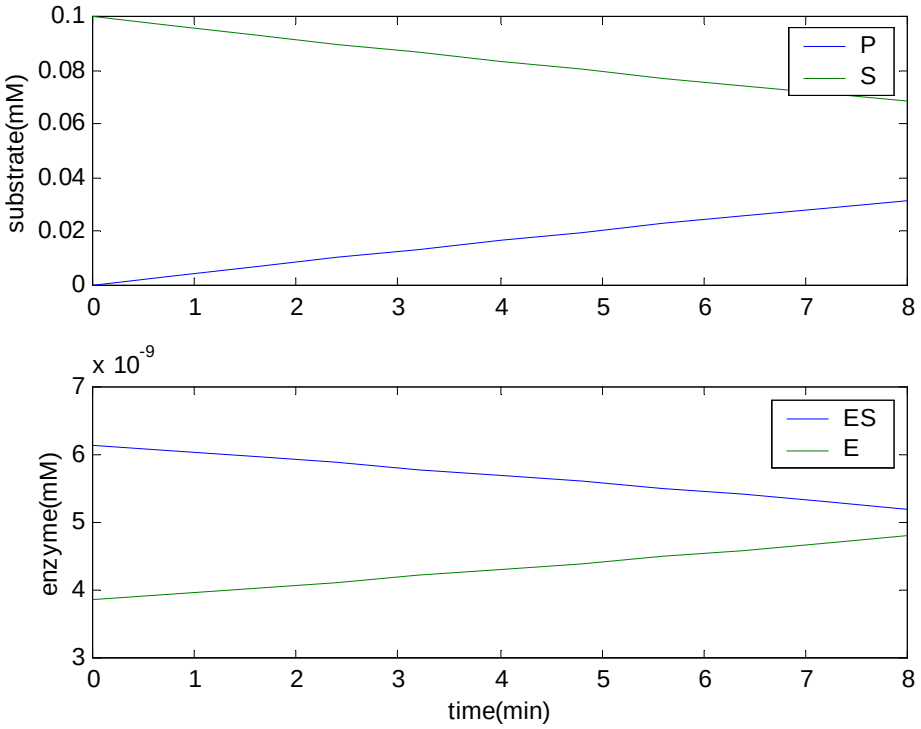
1. Time dependence of velocity

The rate of a reaction S→P catalysed by enzyme E is expressed as $v = \frac{dP}{dt}$, which is the first derivative of a product (P) generation curve gained in a continuous enzyme assay (in continuous enzyme assays the formation of product is monitored for the whole course of the reaction):



For such an experimental design a single sample occupies the measuring instrument for the whole course of the reaction, which limits the number of samples that can be examined (or alternatively makes the experimental procedure unacceptably long). This problem can be solved with end-point enzyme assays, in which the P is measured only once at a time-point t_{max} and the reaction rate is defined as $v = \frac{P_{\max}}{t_{\max}}$ (our computer simulation refers to such an end-point assay). How should the t_{max} be chosen for the applicability of the

Michaelis-Menten equation $\left(v = \frac{V_{\max}.S}{K_m + S} \right)$, where v is "initial" rate? Examine the effects of various combinations of E and S concentrations and t_{max} on v! The equation above refers to a "steady-state" (how can this term be defined?). Using the figure below suggest which conditions should be adjusted in the experiment to satisfy the "steady-state" criterion:



Define the appropriate experimental conditions for the determination of the kinetic parameters of each of the available 5 enzymes using the Michaelis-Menten model (E, range S, t_{max})!

2. Effect of temperature

The collision theory

The velocity of any homogeneous chemical reaction depends on the frequency of fruitful collisions of the reactant molecules, which is determined by their kinetic energy and thus by the temperature (T). The Arrhenius equation $k = Ae^{\frac{-E_a}{RT}}$ expresses the relation between temperature and rate constant (k, which for enzyme-catalysed reactions is usually the catalytic constant for the ES→E+P partial process) (A and E_a are characteristic constants of the reaction).

Temperature effects on enzyme stability

The tertiary structure of an enzyme is maintained by a large number of weak noncovalent bonds. If the molecule absorbs too much energy (over a critical value), the tertiary structure will disrupt and the enzyme will be denatured (loss of activity). Temperature increases the number of molecules that get lost in the denaturing energy zone, but this effect is time-dependent (at lower temperatures the number of denaturing molecules increases slower than at higher temperatures).

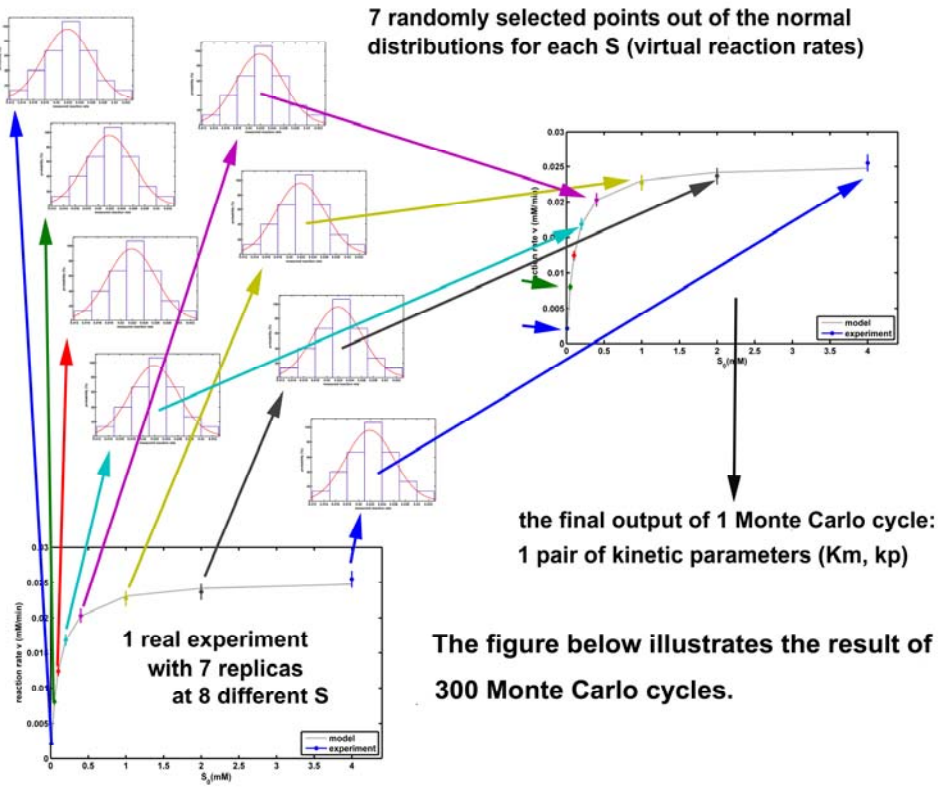
The two opposite effects of temperature on the velocity of enzyme-catalysed reactions result in a peak in the plot of v versus T, called "optimum temperature". Is this a constant characteristic for the enzyme? Address this question experimentally! (Hint: measure the "optimum temperature" using different overall reaction time!)

3. Effect of pH

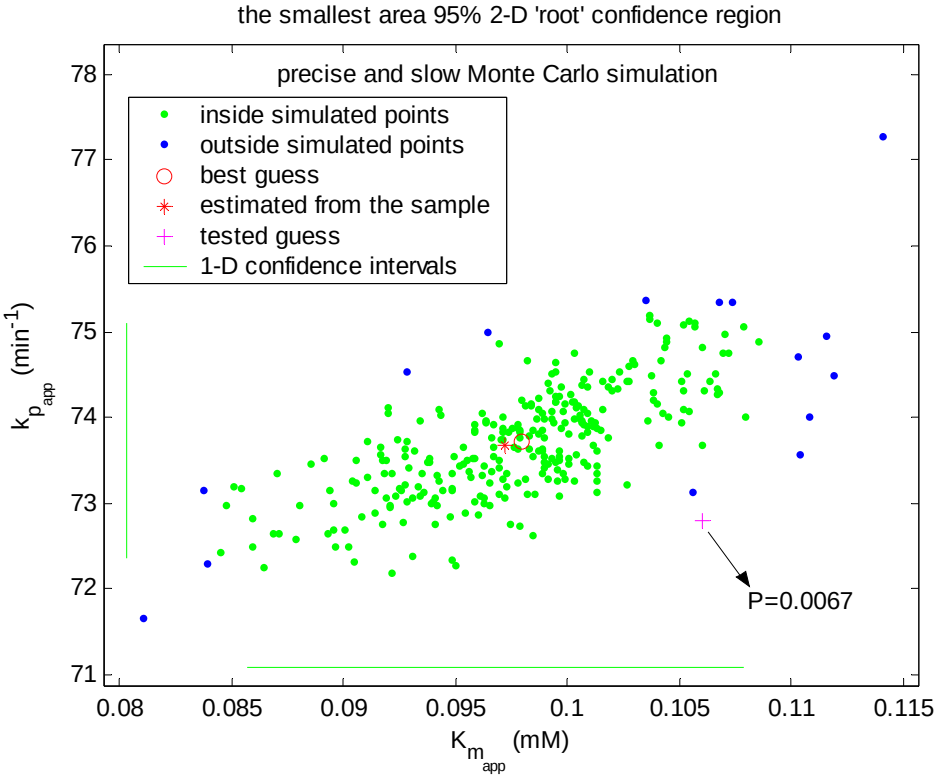
The active sites on enzymes are often composed of ionizable groups which must be in the appropriate ionic form to maintain the conformation of the active site, bind the substrates or catalyse the reaction. On the other hand one or more of the substrates may contain ionizable groups and only one ionic form of that substrate may bind to the enzyme or undergo catalysis. The pK values of the critical groups of the active site can often be determined by measuring the pH-dependence of the enzyme activity.

4. Estimation of the distribution of the parameters

With experiments we take measured samples out of the real world. The enzyme characterised with the real parameters K_m and k_p ($k_p=V_{max}/E_t$) generates a product *P* in our experiments, which we measure and calculate the reaction rate *v* for various substrate concentrations *S*. Based on these measured data we try to deduce the values of the real parameters using non-linear regression to the model equation $\left(v = \frac{V_{\max}.S}{K_m + S} \right)$ (assuming various values for the parameters, *v* is calculated multiple times, the best guess of the parameters yielding the minimal difference between the calculated and measured *v* values according to the least-square method). Because of the biological errors (e.g. variations in the enzyme preparations due to oxidation or contaminations) and experimental errors (e.g. variations in measured volume of substrates and enzymes, imprecision of the instrumentation) with the latter procedure it is impossible to identify the real parameters. What we can do is to approximate their values with the best guess from the optimisation procedure (called actual estimate) and its distribution (the range within which the real parameter is found with given probability, for biological systems typically the 95 % confidence interval is used). Nowadays Monte Carlo simulations of all types are the preferred method for parameter distribution estimation of ligand binding and kinetic problems. The main idea behind the Monte Carlo simulation is that if the way in which the true parameters generate a sample is known (it can be deduced from the distribution of the experimentally measured data), then it is possible to build parallel worlds, in which the actual estimates will play the role of true parameters. In these worlds, multiple synthetic samples can be generated with the help of computer simulation procedures and synthetic estimates of the 'true' parameters can be obtained. In the specific setting of our experiment in a real wet measurement we gain 5-10 replica values of reaction rate for each substrate concentration. Using these measured data the computer builds up their distribution (assuming normal distribution characterized by mean and standard deviation values). In the next step the computer picks up randomly the same number of replicas from each distribution as in the wet experiment and using these as measured data performs a non-linear regression according to the Michaelis-Menten equation with their mean values to determine the kinetic parameters. The outcome of one Monte Carlo cycle is one pair of K_m and k_p values. The process of Monte Carlo simulation is illustrated in the figure below.



If this virtual experiment is repeated hundreds of times, high number of kinetic parameter pairs are gained as illustrated in the figure below (in this case the computer does what the experimenter would do in practice if it were possible: repeat the experiment infinite number of times).



The Monte Carlo modification called bootstrap does not even need to know the process by which the true parameters generate experimental samples (instead, repeated drawing of random observations from the experimental sample with replacement is used to construct synthetic samples). It is possible to approximate the difference between the true parameters and the actual estimates with the difference between the actual estimates and the synthetic estimates. The confidence interval of the best guess can be approximated from the boundaries within which the desired ratio of synthetic parameters (typically 95 %) is localised (see the figure above). Using the distribution of the estimates a frequently asked question can be answered: are the values of the parameters gained in two different experiments identical or not. This question can be answered at various levels of probability (in biological systems the usual level of statistical significance is 5 %). Task: Compare the parameter estimates of your colleagues to your own best guess!

Applications

1. Determination of physiologically relevant pathway substrates

In the elucidation of a metabolic pathway it is frequently necessary to establish which one of alternative substrates plays a part. The activity of the enzyme that utilizes these substrates is usually measured at saturating concentrations to give V_{max}. For example, hexokinase from brain catalyses the phosphorylation of both glucose and fructose and the question arises if both substrates are physiologically important. The V_{max} of hexokinase from brain tissue extracts can be measured *in vitro* with the following results:

Substrate	V_{max} ($\mu mol.min^{-1}.g^{-1}$)
glucose	17
fructose	25

However, the substrate concentration *in vivo* may be much lower than that required to give the maximum activity. For glucose and fructose the reported values for their intracellular concentration in brain are 10 μM and 1 μM, respectively. Knowledge of K_m is crucial for the prediction of the physiological relevance of the two sugars in brain:

Substrate	K_m (μM)
glucose	10
fructose	1000

Which substrate is preferred by brain?

Let's consider the following metabolic pathways:

S1: $A \xrightarrow{E_1} B \xrightarrow{E_2} C \xrightarrow{E_3} D \xrightarrow{E_4} F$

S2: $G \xrightarrow{E_1} C \xrightarrow{E_3} D \xrightarrow{E_4} F$

Using your experimental data and the values for the intracellular concentrations of *A* and *G* do an educated prediction on the physiological relevance of the two metabolic pathways.

2. Prediction of the flux-generating (rate-limiting) step of a metabolic pathway

The data on the values of K_m, V_{max} and the *in vivo* concentrations of the substrates can be used for the identification of the flux-generating (rate-limiting) step of a metabolic pathway which is of primary importance for the elucidation of the physiological regulation of the pathway and for designing pharmacological agents that efficiently affect it.

Comparison of maximum activities of enzymes in a metabolic pathway can be used as one means of indicating whether a reaction is near- or non-equilibrium. A high value of the V_{max} in comparison with other enzymes indicates that the reaction is near-equilibrium ($\Delta G \cong 0$), whereas a low V_{max} indicates a non-equilibrium reaction ($\Delta G << 0$).

> Topic for discussion: How can the statement above be interpreted using the equation for the free energy changes in biochemical reactions
>
> $\Delta G = \Delta G^{0'} + RT \ln \frac{[product]}{[substrate]}$, where $\Delta G^{0'}$ is the standard free energy change at pH=7.0? Use your knowledge on the thermodynamics of metabolic pathways.

Identification of a reaction in a metabolic pathway as non-equilibrium, however, is not enough to consider it as rate-limiting for the pathway. If the substrate concentration is considerably lower than the K_m, a change in substrate concentration will cause a proportional change in catalytic activity and thus the flux through this step is determined by the substrate availability and not by the enzyme. In contrast, if the *in vivo* substrate concentration is significantly higher than the K_m, large changes in substrate concentration will have little effect on the rate of catalysis. Thus, a non-equilibrium reaction that is saturated with its pathway substrate with high probability can be expected to be the rate-limiting step of the metabolic pathway. Using this criterion identify the rate-limiting step in the *S1* pathway under varying physiological conditions. For example, at rest the intracellular pH of the skeletal muscle is 7.2, but in intensive anaerobic exercise it decreases down to 6.3. Is the metabolic control the same in these two states? Check also if any change occurs in fever.

Admittance test

The questions below are designed to check if the participant possesses the minimal level of theoretical knowledge required for successful experimentation. The correct answers are the password for entry into the experimental procedure.

1. Which statements are true for the Michaelis-Menten equation $\left(v = \frac{V_{\max}.S}{K_m + S} \right)$ widely used in the kinetic evaluation of enzymes?

A. The equation can be applied freely for any enzyme-catalysed reaction without any restrictions.
B. The value of *v* can be measured under conditions, when the decrease in *S* is negligible compared to the initial S_0 (e.g. $\Delta S < 0.1 * S_0$).
C. The value of *v* can be measured under conditions, when the decrease in *S* is substantial compared to the initial S_0 (e.g. $\Delta S > 0.1 * S_0$).
D. The equation is valid for reactions in equilibrium.
E. The equation is valid for steady-state systems.

2. Which statements are true for the rate-limiting reaction in a metabolic pathway?

A. *In vivo* all other pathway reactions always proceed at higher rate, because the V_{max} of the rate-limiting step is the lowest one in the pathway.
B. The standard free energy change of this reaction is a high positive value.
C. The *in vivo* free energy change of this reaction is a high positive value.
D. The standard free energy change of this reaction is a high negative value.
E. The *in vivo* free energy change of this reaction is a high negative value.

3. Which statements are true for the effects of factors influencing the velocity of enzyme-catalysed reactions?

A. The pH affects the reaction rate through the degree of protonation of the substrates and amino acid residues in the structure of the enzymes.
B. The pH does not affect the reaction rate for substrates with no protonizable groups, because for such reactions the substrate-enzyme interaction is independent of the electric charge of the molecules.
C. The total amount of active enzyme added to an enzyme assay should be considered constant for the whole course of the assay independently of even gross changes in the pH and temperature.
D. Increase in the temperature up to a certain value results in acceleration of the enzyme-catalysed reaction because of the increase in the number of

productive collisions, but further increase beyond this value results in decline in the reaction rate because of the disruption of the tertiary structure of the enzyme (denaturation).

E. The peak in the curve of reaction rate versus temperature gained from experiments as described above in D. (the so called optimal temperature) is a physical characteristic of the enzyme independent of the experimental conditions.