LUCENZ II

Software for use in the teaching of enzyme kinetics.

(suitable for introductory biochemistry classes).

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Introduction

This programme contains routines that are intended for use in undergraduate classes for an introduction to the quantitative analysis of enzyme kinetic data.

The programme allows one first to enter new data or to retrieve previously stored data. It then permits the selection of a rate equation to which the data may be fitted. The fitting method used is a weighted linear regression on the reciprocal rate data - this has been chosen, in spite of theoretical drawbacks, because of its simplicity, robustness and speed.

Results may be presented graphically, using any of four common methods of graphical analysis. These options may be explored more fully in this help manual.

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Main Window

On first starting the programme you are confronted with the main window , which offers you a number of choices. The window occupies a central position in the programme and the various procedures will return you here so that you may make your next choice.

There are several key controls. There is a grid for data entry and modification. The number of rows and columns active in the grid is controlled by one (uninhibited) or two (two-substrate or inhibited) sliders. Data may be added directly to the grid or retrieved from stored files. The choice of routines for uninhibited data or inhibited is controlled by two option buttons above the sliders. To the right of the option buttons is a menu which allows you, when working with inhibited data, to choose the model of inhibition that you wish to select or to choose the appropriate mechanistic model when using two substrates.

The Files menu allows you to open stored data files (extension ".ktn") or to store data that you have just entered into the grid or to re-save files data files that you have modified. You can quit the programme from the Files menu.

The button marked **GO** initiates the regression procedure, fitting the experimental data to the theoretical model you have chosen.

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Data entry:

Prominent in the main window is a grid into which you may enter your data. Substrate concentrations are to be entered into the cells in the extreme left-hand column. Inhibitor concentrations (if appropriate) may be entered into the top row. Reaction rates may be entered into the appropriate cells in the body of the grid. The number of active rows and columns in the grid may be altered by means of the slider(s) immediately above the grid. After data have been entered into the grid, they may be corrected and stored at any stage.

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Data Correction

After you have checked your data, corrections may be made to the data in the grid by moving to the appropriate cell and entering the correct value. During the course of the analysis, you may wish to remove aberrant data altogether. This can be done by replacing such points with a zero value. The fitting routine will ignore zero rates. Note that the routine will not tolerate zero substrate concentrations.

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Data Storage:

Use of the ***Save*** option in the ***Files*** menu enables you to save data in a random access file, named as appropriate. The stored data set will be given the default extension of "ktn". These stored data may be recalled by using the ***Open*** option in the **Files** menu. They may be **corrected** after recall, if necessary and then resaved using the ***Save*** option in the ***Files*** menu. Note that files are saved with the information as to the mechanism, number of substrate concentrations employed, whether the experiment includes inhibition data and, if so, how many inhibitor concentrations were employed. When opening a stored file there should therefore be no need to give this information.

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Kinetic Models

After loading your data, you need to select a model to fit your data to. You need to stipulate whether the experiment involves inhibition or not. In the absence of inhibition, you are offered only a single model. This is for the simple, single substrate model, conforming to Michaelis-Menten kinetics . See option 1 below. Other options are for three different mechanisms of inhibition. Descriptions of these models are found by following the links below. Rate equations derived from these models may also be viewed.

- Uninhibited - One substrate
- Competitive inhibition
- Non-competitive inhibition
- Uncompetitive inhibition

If inhibition is involved, you will be offered the choice of the three basic mechanisms of inhibition - options ii) - iv) above. These options will be displayed as a menu to the right of the option buttons and clicking on the one you wish will select the corresponding rate equation for the fitting procedure.

Data are fitted directly to double reciprocal (1/v vs 1/[s]) curves (assumed to be linear) derived for the se simple models. Once you have selected the model you want, press the button marked GO and the fitting to the corresponding equation is performed automatically by a **sum of squares** minimization routine.

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Uninhibited Kinetics:

The default choice in this programme is for uninhibited kinetics involving only one substrate.

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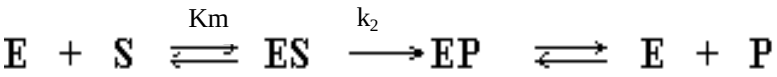
One - substrate, Uninhibited

This is the simple , single substrate Michaelis-Menten equation. This equation involves determining the best value for two parameters, the limiting velocity (V_m and the Michaelis constant, K_m) .

The simple mechanism may be represented as below:



Where K_m is the dissociation constant of the ES complex and $V_m = [E]^* k_2$

Rate equation:

$v = V_m / (1 + K_s/[S])$

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Inhibition:

If you select the option button for inhibition, a menu will appear which gives you the choice of three mechanisms
These are :

- Competitive
- Noncompetitive
- Uncompetitive

Select the mechanism that you wish to test by clicking on it. If you data are entered properly you will then be able to fit to your chosen mechanism by clicking the GO button.

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Competitive Inhibition

Competitive inhibition may involve physical competition between the substrate and the inhibitor for a common binding site on the enzyme. Double-reciprocal plots will intersect at a single point on the vertical axis. Three parameters have to be determined, V_m, K_m and the inhibition constant, K_{is}, the dissociation constant of the enzyme-inhibitor complex.

$$v = V_m/(1 +(K_s/[S])*(1 + [I]/K_{is}))$$

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Noncompetitive inhibition

Noncompetitive inhibition involves an interaction of the inhibitor with the enzyme which may or may not affect the binding of the substrate but which completely prevents it undergoing catalytic conversion in the active centre of the enzyme.

Double-reciprocal plots generated by this mechanism will intersect to the left of the vertical axis. In contrast with Competitive Inhibition, four parameters have to be determined, V_m, K_m and K_{is} and K_{ii}. These last two constants are dissociation constants defining the dissociation of the inhibitor either from the EI complex or from the EIS complex.

See equation

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Uncompetitive inhibition

In this mode of inhibition, the inhibitor binds only to the ES complex, preventing its breakdown. It will generate sets of parallel double reciprocal plots. It will be defined by the kinetic constants, V_m, K_m and K_{ii}. The last constant is the dissociation constant for the EIS complex.

See equation

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Sum of Squares

The routine used attempts to find those values for V_m, K_m and Ki in the selected rate equation, which give the minimum overall discrepancy between experimental points and the points predicted by the rate equation as they are plotted in a double reciprocal plot. These values are the so-called "best fit" values. The discrepancy is measured by means of the parameter ES

$$ES = \frac{\sum wi(1/Vci - 1/Vei)^2}{(N - P)}$$

Where N = no. of points and P = no. of parameters

and where Vci is the calculated value for the velocity in the ith data pair, Vei is the experimentally determined value and wi is a weighting factor . The weighting factor used in this version of the programme is the square of Vei. This factor is employed assuming that the standard deviation on experimental measurements is approximately proportional to the magnitude of the measurement itself. The routine in the programme finds the values of the kinetic parameters (Vm, Km , Ki etc) which give the minimum value of ES. It does this essentially by solving simultaneous equations for the optimum values of the kinetic constants.

A major feature of using this programme is that it estimates standard deviations on the final best fit values of the parameters. A clear indication as to the reliability of these parameters may thus be obtained. This is often invaluable information in helping one to appreciate the worth of one's experimental data.

The ES parameter be may used to test how well different equations predict a given set of data. The equation that generates the lowest value of ES may be selected, on an objective basis, as giving the best fit.

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Output

After fitting to the selected model has been completed, you will see a summary of the results of the fitting procedure. This will give you the best fit values for V_m, K_m and (if appropriate) a K_i. Standard deviations are given for each, expressed as coefficients of variation

The goodness of fit can be seen by the value of the parameter "RMS fractional residual". This gives the root mean square of the deviation of experimental points from calculated values, calculated as a fraction of the theoretical value. Thus an RMS fractional residual estimates how well, on average, the theoretical lines predict the values of the experimental points. A value of 0.05 suggests that your experiment agrees with the theory to about 5% - a good result. If you get values of 0.2 or greater, you need to consider a different model, or to improve your lab technique.

At the same time graphical output will be seen in another window. The default display plots your crude experimental data. Other methods of plotting can be accessed from the ***Plot*** menu found in the graphical display window. You can print graphical data from the graphics window by copying to the clipboard. Note that the graphics window can be re-sized to give an appropriately dimensioned printed graph.

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Plotting Methods

In the Graphics Display window you will see a menu tab labelled "Plotting". Click this and you will be presented with a menu of different plotting methods with which you can convert your raw data into forms that will give linearized plots. The options are :

- Velocity vs substrate concentration
- Lineweaver-Burke
- Hanes Plot
- Eadie-Hofstee Plot
- Dixon Plot

In the graphical displays, holding the cursor over any point, however transformed, will highlight the original data point in the data screen.

Select from the above options for a more detailed discussion of these plotting methods.

(See Webb, 1963, Cornish Bowden, 1974):

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Velocity vs Substrate

This simply plots values of initial velocity versus substrate concentration. Hyperbolic curves are the expected result.

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Lineweaver-Burke

This is the so-called double reciprocal plot in which the reciprocal velocity is plotted against the reciprocal of the substrate concentration. It generates straight line graphs of positive gradient. This plotting method exaggerates errors inherent in small velocity measurements and, although very commonly used, is not the best plotting method.

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Hanes Plot

In this method the ratio s/v is plotted against s, the substrate concentration. Straight line graphs of positive gradient are generated. This method does not exaggerate errors in low velocity measurements and is the preferred method for graphical analysis of enzyme kinetic data (Wilkinson, 1961).

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Eadie-Hofstee Plot

In this method, the velocity, v_i, is plotted against the ratio v_i/s. Straight lines of negative gradient are the result. This method is very sensitive for detecting departures from simple kinetic behaviour. However, since the experimentally measured velocity appears in both independent and dependent variables, analysis of errors is difficult.

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Dixon Plot

This method plots the reciprocal velocity against the concentration of inhibitor - it can therefore be used only when inhibition is being examined.

Its value is greatest for indicating whether there are departures from the simple models of inhibition discussed above. The simple models will generate straight lines, intersecting at the negative value of the Ki. Hyperbolic curves indicate a more complex class of inhibition known as partial inhibition.

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Hard Copy of Graphs

On the graphics window there is a menu tab ***Copy.*** Clicking this will copy the graphical display to the Clipboard. From there you may paste it into any suitable graphics application and print from there.

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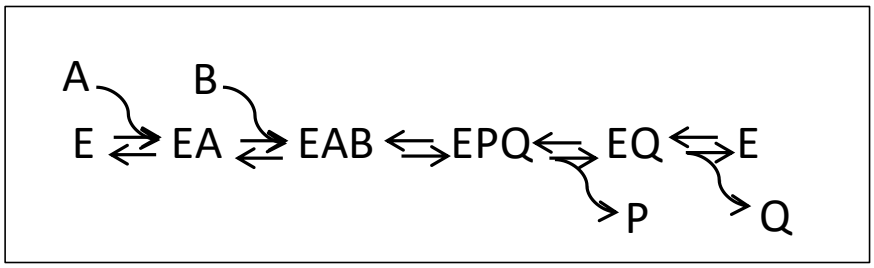
MODELS USED FOR FITTING IN THIS PROGRAMME:

Two substrates - Sequential

This is an uninhibited reaction involving two substrates rather than just one. It describes the reaction of two substrates via a "sequential reaction" (Ordered or Rapid Equilibrium Random) in which no covalently bound complex is formed. The nature of these reactions is that all substrates must bind before any products may be released. The sequence of events in these two types of sequential reaction are shown below.

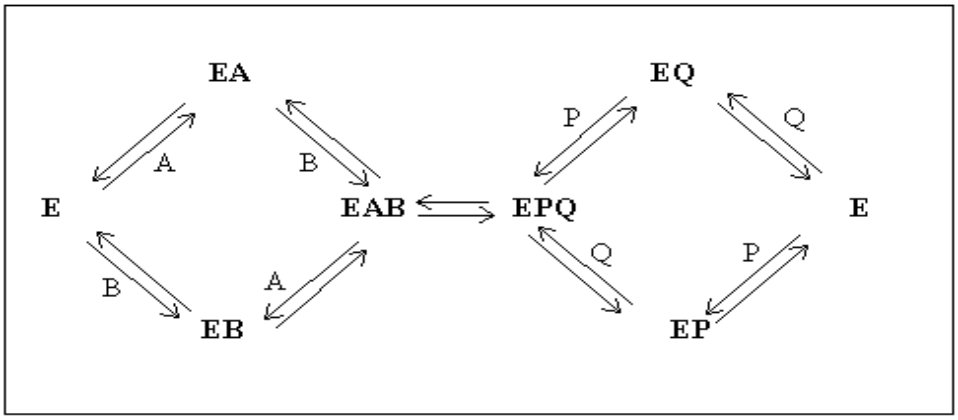
I. Ordered Bi-Bi

The order of binding of substrates and release of products is obligatory. Binding of the first substrate (A) is followed by the second (B). The two substrates then react on the enzyme leading to the formation of the products P and Q, which are then released in strict order.



II. Rapid Equilibrium Random:

In this mechanism, the order of binding of substrates and release of products is random. For hyperbolic kinetics to be obtained (i.e. to get linear double-reciprocal plots) it is necessary that the rate of conversion of the EAB complex is small relative to any of the other processes.



Double-reciprocal plots generated by either of these types of mechanism will be intersecting straight lines. This reaction scheme requires that four kinetic constants be determined; V_m, Michaelis constants with respect to both substrates, K_a and K_b, and an "inhibition constant" (Cleland, 1963) K_{ia} relating to the first-bound substrate.

The equation for both mechanisms maybe represented as:

$$v = Vm/(1 + K_a/[A] + K_b/[B] + K_{ia}/([A].[B])$$

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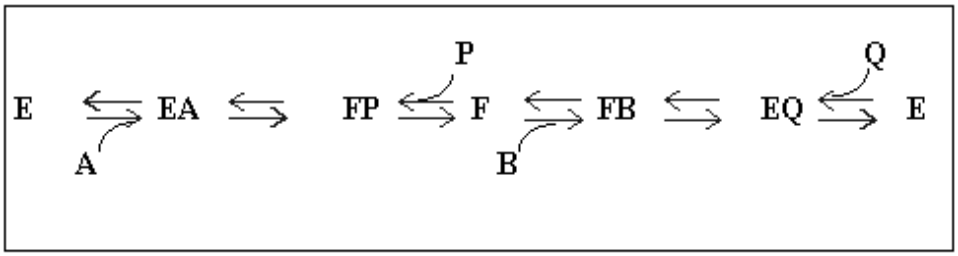
Uninhibited - Ping-Pong

The "Ping-Pong" reaction proceeds through the formation of a covalent complex between enzyme and substrates. The enzyme thus alternates between covalently modified and unmodified forms. Binding of substrate is followed by release of a product. Three kinetic parameters have to be determined; the Vm and two Michaelis constants, one relating to each substrate. This mechanism produces parallel double-reciprocal plots.



Two substrates, A and B:

Rate Equation: $v = V_m/(1 + K_a/[A] + K_b/[B])$

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INHIBITION MECHANISMS:

The equations used are derived from the model in Fig. 1.

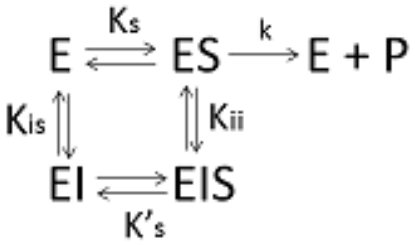

Fig. 1. General model for inhibition in a simple enzyme system.

K_s, K_{is} and K_{ii} are dissociation constants of the ES, EI complexes and EIS respectively and k is the rate constant governing the rate of breakdown of the ES complex. Overall equilibrium is assumed and as a consequence the constant $K'_s = K_s * K_{ii}/K_{is}$.

This model generates the following equations for the different types of inhibition:

- Competitive Inhibition
- Noncompetitive Inhibition
- Uncompetitive Inhibition

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Inhibition equations

i) Competitive inhibition

$v = V_m/(1 +(K_s/[s])*(1 + [i]/K_{is}))$

This mode of inhibition corresponds to the general case of inhibition (see below) when the inhibitor has no affinity for the ES complex ($K_{ii} = \infty$)

ii) Non-competitive inhibition:

This is the general model for inhibition. The inhibitor can bind to both free enzyme and the ES complex and K_{ii} and K_{is} both have finite values.

$v = V_m/((1 + [i]/K_{ii} + (K_s/[s])*(1 + [i]/K_{is}))$

So-called "Classical non-competitive inhibition, which generates double reciprocal plots intersecting on the horizontal axis, is seen when K_{ii} and K_{is} are equal in value.

iii) Uncompetitive inhibition

$v = V_m/(1 + [i]/K_{ii} + K_s/[s])$

In this case, the inhibitor can bind only to the ES complex ($K_{is} = \infty$)

Go to Inhibition Mechanisms

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References

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Quit

Select **Exit** from the Files Menu.

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