Version 1.4

**\*\* ONLY TEXT AND IMAGES APPEARING INSIDE THE RED BOXES WILL BE GRADED\*\***

1. Consider the reaction catalyzed by an enzyme (E), where S is the substrate, ES is the Michaelis Complex, P is the product, and k1, k-1, and k2 are rate constants:

the initial velocity of a reaction () is given by the Michaelis-Menten Equation

1. In this assignment you will analyze the initial reaction velocity () obtained with various substrate concentrations ([S]) for three different enzymes. You will determine the basic descriptors of enzyme kinetics for each enzyme/substrate combination.

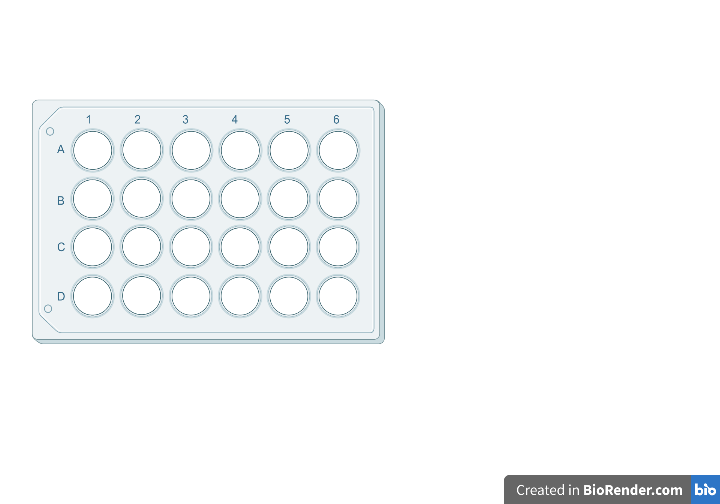
is the maximum reaction velocity, which gives kcat, if you know (you do).

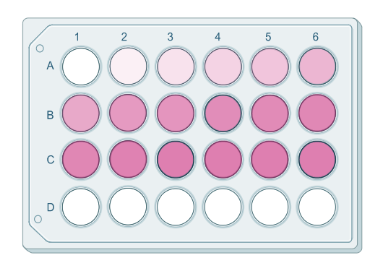
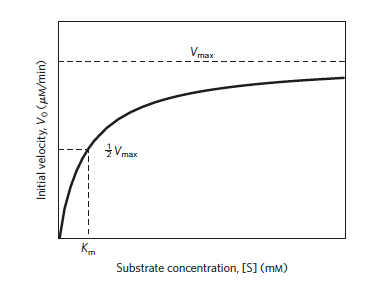
is the turnover number (maximum number of reactions catalysed per second per enzyme)

is the Michaelis-Menton constant

is the catalytic efficiency aka the pseudo second order rate constant.

1. An enzyme kinetics experiment in the laboratory might proceed as follows:
   1. A very small amount of enzyme (E) is contained in buffer in an Eppendorf tube.
   2. A series of dilutions of substrate (S) is made in wells A2-A6, B1-B6, and C1-C6 of the microplate shown below, where A2 contains the least substrate and C6 contains the most substrate. In addition, a ‘no substrate’ control solution is made in well A1. In this example, the microplate wells appear clear after substrate addition because the substrate does not emit or absorb light in the visible range.



* 1. A small and fixed volume of enzyme (E) is then added to each of the 18 microplate wells. With the exception of the ‘no substrate’ control well A1, the amount of substrate in each well is very large relative to the amount enzyme (i.e., [S] >> [E]). The product absorbs light at 650 nM and makes the solution red. A microplate reader can be used to quantitate product formation, which in this example is measured by an increase in the absorbance of light at a 650 nM over time.
  2. After addition of the enzyme, the plate is quickly put into the reader. Product formed by the enzyme + substrate mixtures is measured. It is necessary to measure quickly because our goal is to measure the ***initial velocity*** of product formation (*v*o) in each well.
  3. In this example, *v*o is measured as an increase in the absorbance of light over time in each well at a specific wavelength. Since each well contains a different amount of substrate (and a fixed amount of enzyme), measuring the absorbance of all wells in the plate at one time point (simultaneously) allows one to determine *v*o (initial velocity) at many different [S]. At a given time point, the microplate might look like the image below, where well A1 contains the ‘no substrate’ control and has no color change compared to the clear plate above (meaning that there is no increase in absorbance therefore no product formation), and wells A2-A6, B1-B6, and C1-C6 contain increasing amounts of product. The darkest colored wells correspond to the greatest absorbance and the greatest amount of product. 
  4. In a Michaelis-Menton plot, the x-axis is the substrate concentration (not time) and the y-axis the initial rate of product formation (or initial velocity, *v*o). In this assignment, the substrate concentration is in mM (millimolar) and the velocity in mM/sec. This plot allows you to estimate Vmax.
  5. Open the spreadsheet called ‘Michaelis-Menten and Lineweaver-Burk.xlsx.
  6. Go to the ‘Data’ tab. Here, there are three sets of substrate and velocity data corresponding to Enzyme 1, Enzyme 2, and Enzyme 3. Below the tabular data are scatter plots of the data, with substrate concentration, [S] (mM), on the x-axis and the initial velocity of product formation, *v*o (mM/sec), on the y-axis.
  7. Obtain the three descriptors of enzyme kinetics, Vmax, Km, and kcat,from the data for each enzyme. The first way to do this is to estimate these values from the Michaelis-Menten ([S] vs Vo) plot above. You need [E]TOT (=1.0 mM to estimate kcat). Sketch your parameters on each graph and take a screen shot of each.

|  |  |
| --- | --- |
| MM Plot |  |
| Screen Shot  Enzyme 1 |  |
| Screen Shot  Enzyme 2 |  |
| Screen Shot  Enzyme 3 |  |

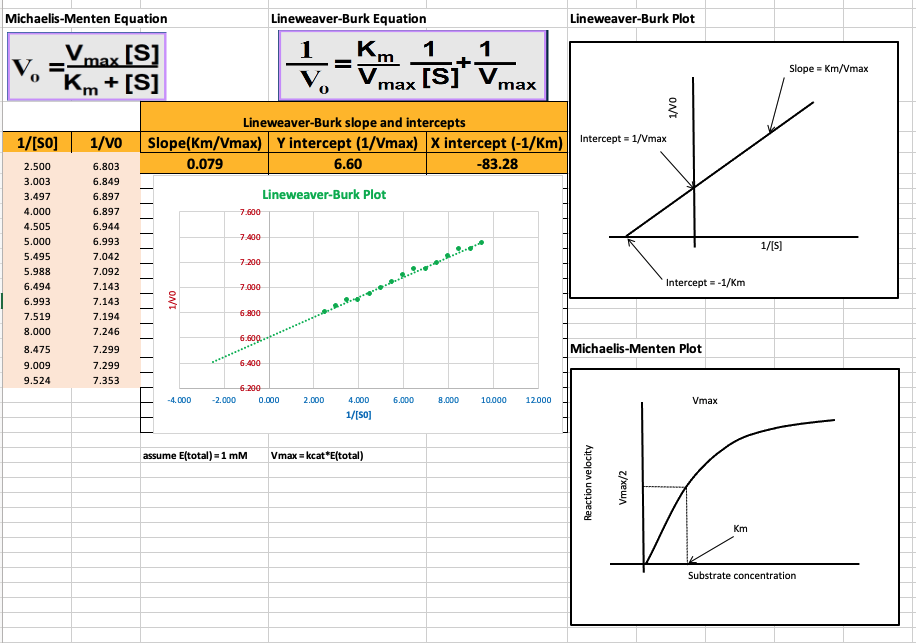
For reference, see Lehninger Section 6.3. It illustrates estimation of these kinetic parameters using idealized data.

* + 1. Look at the idealized data in Lehninger.
       1. When [S] is small, the initial *v*o is proportional to [S].
          1. The initial velocity is limited by the enzyme’s ability to bind substrate when [S] is small.
       2. When [S] is large, *v*o does not increase with increasing [S].
          1. At high [S], all enzyme is saturated with substrate and the enzyme cannot generate product faster as [S] increases.
    2. Look at the estimated kinetic parameters
       1. vmax is the maximum velocity and asymptote of *v*o as a function of [S]. Notice that it will also have units of concentration per unit time.
       2. Km (the Michaelis Constant) is the concentration of substrate that gives an initial velocity of half of vmax. Km is specific to a given enzyme-substrate pair. It is independent of substrate and enzyme concentration and can also be derived from the rate constants for the enzyme-substrate pair (i.e., Km = (k-1 + k2)/k1)).

1. Enzymatic Parameters Estimated from the Michaelis-Menten ([S] vs Vo) plot.   
   Include units

|  |  |  |  |
| --- | --- | --- | --- |
|  | Vmax | Km | kcat |
| Enzyme 1 |  |  |  |
| Enzyme 2 |  |  |  |
| Enzyme 3 |  |  |  |

* + 1. The beauty of the Michaelis-Menten Equation is that once Vmax and Km are known for an enzyme-substrate pair, the velocity of the reaction can be predicted for any substrate concentration (assuming the same buffer conditions, of course).
  1. Another way to obtain Vmax, Km, and kcat from real data is to use a double-reciprocal plot (also known as Lineweaver-Burk plot; 1/[S] v 1/Vo).
     1. Determining VMAX and KM for these data accurately using the Michaelis-Menten ([S] vs Vo) plot is not easy. Use a Lineweaver-Burk plot to validate (or not) the Michaelis-Menten parameters.
     2. On the ‘Sample Data’ tab, for Enzyme 1, calculate the reciprocal of [S] (i.e., 1/[S]) in cells D4:D18 and the reciprocal of Vo (i.e., 1/Vo) in cells D4:E18. Type this as an Excel function that references the value of [S] or Vo in columns B or C (i.e., =1/B4). Do this for all values of [S] and Vo.
     3. Copy the resulting values in cells D4:E18 of the ‘Sample Data’ tab and paste them into cells A6:B20 of the ‘Calculations’ tab. You may need to use Edit>Paste Special>Values from the toolbar Edit menu.
     4. Your ‘Lineweaver-Burk’ tab should then look something like this:



1. The Lineweaver-Burk equation transforms the hyperbolic Michaelis-Menten equation into a linear form. The slope, Y-intercept, and X-intercept are used to calculate Km, and Vmax.
2. The slope (Km/Vmax), Y-intercept (1/Vmax) and X-intercept (-1/Km) are automatically calculated from the data for Enzyme 1 in the orange boxes just above the plot.
3. Calculate Km, Vmax and kcat for Enzyme 1 and enter these values (including units!) into the box below. Hint: kcat = Vmax / [E]TOT. [E]TOT = 1.0 mM in this experiment.

Repeat the calculations for Enzyme 2 and Enzyme 3. **Don’t forget the units!**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Vmax | Km | kcat |
| Enzyme 1 |  |  |  |
| Enzyme 2 |  |  |  |
| Enzyme 3 |  |  |  |

1. The rate constant kcat,also called turnover number, can be thought of as the number of substrate molecules converted to product per unit time per enzyme molecule. Is kcat a zero order, first-order, or second order rate constant?

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| INSERT HERE |

1. Calculate the catalytic efficiency (or specificity constant, kcat/Km) of Enzyme 1, Enzyme 2, and Enzyme 3. Include units.Then, rank the enzymes from 1 to 3, where 1 is the most efficient enzyme, and 3 is the least efficient. Assume [S] >> Km (i.e., specificity constant = kcat/Km). More information about this assumption can be found in Lehninger Section 6.3.

|  |  |  |
| --- | --- | --- |
|  | kcat/Km | Rank |
| Enzyme 1 |  |  |
| Enzyme 2 |  |  |
| Enzyme 3 |  |  |

1. Is the catalytic efficiency a zero order, first-order, or second order rate constant?

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| --- |
| INSERT HERE |

1. What is the maximum theoretical catalytic efficiency of any enzyme? Hint: see Lehninger Section 6.3.

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| INSERT HERE |

1. Go the ‘Experiments’ tab of the spreadsheet. Study the double-reciprocal plots for each type of inhibition. Which type of inhibition does not affect Vmax?

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| INSERT HERE |

Chem 3521, stop here

1. Get your protein’s Enzyme Commission (EC) number from the list of Protein Assignments under course webpage. Enter the EC number below

|  |
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| INSERT HERE |

1. Search the [SABIO-RK Biochemical Reactions Kinetics Database](http://sabio.h-its.org/newSearch/index) with your protein’s EC number.



1. Expand the triangle under the first hit you receive. Briefly describe any results received. Under Substrates, which substrates are used? Under Products, which products are generated? Note: ignore ions and molecules listed as both Substrates and Products (e.g., Na+, K+, H2O). Which kinetic parameters are reported (e.g., kcat, KM)? Under Experimental Conditions, note the pH of the reaction and the divalent metal concentration. If your search does not return any results, search ‘atpase’ and answer the questions with the first hit.

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