Overview of the Enzyme Kinetics Block of Laboratories

In previous course work, the activity of an enzyme was used to follow the purification of the enzyme and to quantitate the amount of an enzyme present in a solution. In CHEM331, you have also been exposed to "one substrate" kinetics where you can determine the Km and Vmax from a single set of experiments by varying the concentration of the substrate. The experiments in this course focus on using measurements of the rate of enzyme activity to characterize the interaction of the substrates and inhibitory ligands with the enzyme, and to ascertain the overall rate limiting step in the enzyme catalyzed pathway for enzymes with two substrates.

The first experiment will illustrate how the basic kinetic parameters, Km and Vmax, for an enzyme are determined and how careful analysis of the data obtained in such experiments can be used to determine whether

and enzyme obeys "normal" behavior or might be subject to some type of "homotropic" regulation, where the activity is disproportionate to the substrate concentration and the activity of the enzyme is "regulated" in some way by changing substrate concentrations.

As an important corollary to the actual experiments, the data analysis discussed in this chapter, both for the actual experiments and for the problem sets, illustrates how replicate measurements and appropriate data analysis can be used to determine whether the assumed equation used for the analysis is appropriate and you can maximize the information obtainable from your data.

Introduction

There are many different types of enzymes, categorized using the so-called "Enzyme Commission," E.C., numbers into classes based upon the types of chemistry that they utilize in their reactions. What is it about enzymes that make them so attractive for study? Enzymatic activities play crucial roles in every type of life process in addition to being increasingly important in both drug design and synthesis and biotechnology. This is easily seen by considering the types of enzymes that might be found in a typical cell.

Some enzymes are involved in the basic biochemistry and molecular biology of virtually every cell, whether they are associated with metabolism [the enzyme Glyceraldehyde-3-Phosphate Dehydrogenase is an excellent example: virtually all cells have a constant level of expression of the gene for Glyceraldehyde-3-Phosphate Dehydrogenase as it is involved in a critical step in glycolysis, common to virtually all cells], or the machinery of RNA transcription and protein synthesis: such enzymes are often referred to as "house-keeping" enzymes.

Particular enzymes are sometimes associated only with specific tissues or cell types, or only with certain subcellular organelles and can be used as "marker enzymes" for that tissue or organelle [for example the Heart Isoenzyme of Lactate Dehydrogenase: LDH-H, is found only in aerobic muscle such as the heart rather than skeletal muscle which is designed to operate under anaerobic conditions: measurement of the amount of H-LDH can be used to determine whether a person has had a heart attack.] Similarly, the various Glycosyl transferases used in glycoprotein biosynthesis are found only in the Golgi membrane and their activity can be used as a marker for that subcellular organelle. Table.1 summarizes several tissue or organelle specific enzymes the measurement of whose activity has been useful in defining the tissue or organelle.

Marker Enzymes:

Membrane or Location	Marker Enzyme
Mitochondrial: Inner Membrane	Succinate-Cytochrome c Reductase Rotenone Sensitive NADH Cytochrome c Oxidase
Mitochondrial: Outer Membrane	MonoAmine Oxidase Rotenone Insensitive NADH Cytochrome c Oxidase
Endoplasmic Reticulum	RNA Protein Synthesis Enzymes NADPH Cytochrome c Reductase
Plasma Membrane	5' Nucleotidase Lectin Binding Oxytocin [or Hormone] Binding
Golgi Apparatus	Glycosyl Transferases
Mitochondrial Matrix	Glutamate Dehydrogenase

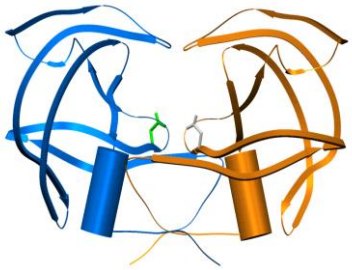
Cytosol	Glyceraldehyde 3 Phosphate Dehydrogenase

Enzymes in both these first two categories are not only necessary for the everyday functions of a particular cell but often have their activity regulated to allow the cell to respond to normal changes in its environment.

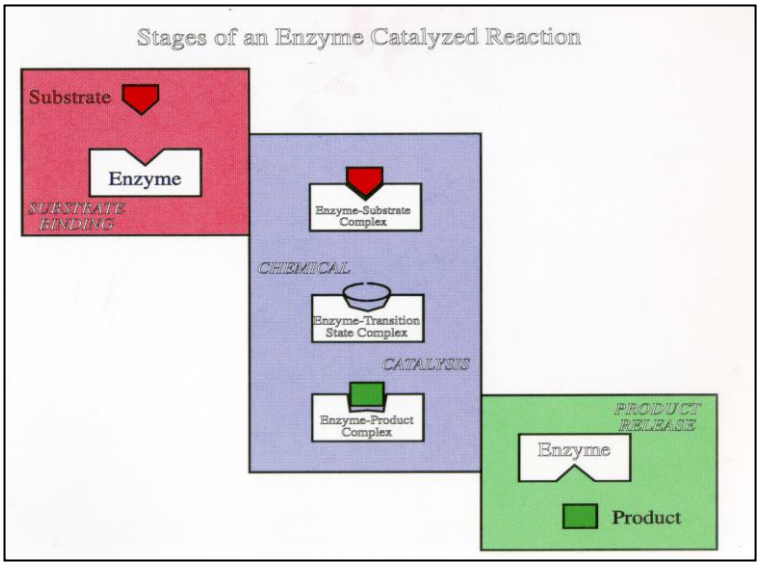
Virtually all cell types in eukaryotic biology differentiate during their life time and usually certain enzyme activities can be associated with the stage of differentiation of the cell: for example chondrocytes: [cells that help create bone formation] differentiate to produce specific types of collagen and the enzyme alkaline phosphatase [which is directly involved in releasing the phosphate that will be used in hydroxyapatite-the main mineral in bone or teeth formation]. The quantitation of the activity of alkaline phosphatase can be used to follow the level of differentiation of these cells.

Finally, the activity of some enzymes indicates the presence of an invading organism. During the early stages of the AIDS crisis scientists measured the activity of the enzyme reverse transcriptase in infected tissue to show that a retrovirus was involved in the infection. Enzymes specific for an invading organism are often targets for drug design: again the AIDS crisis offers many examples. The reverse transcriptase and the HIV protease have both been targets of intensive research in recent years to find specific inhibitors to block their activity.

Pepsin [left] and the HIV Protease [right] are both Aspartyl proteases. The catalytic aspartate groups are shown on the ribbon diagram of pepsin, which is a monomeric enzyme, and as can be seen are similarly located in the HIV protease although each group is contributed by a different subunit. The two subunits of the HIV protease are identical and together the dimer shows overall structural similarity to pepsin.



So, what type of questions do we want to explore with enzymes? Any enzyme catalyzed reaction can be broken down into three simple phases, see scheme 1:



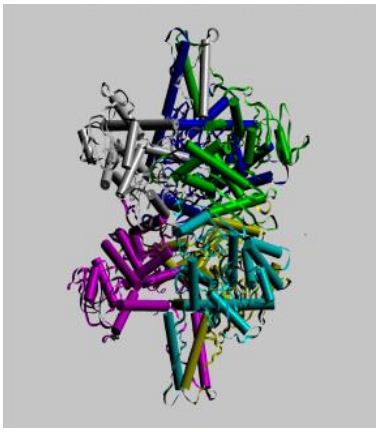
1] **substrate binding**,

2] **chemical catalysis,** and

3] **product release**.

To understand an enzyme, we must be able to examine each of these phases and document their individual contributions to the overall activity of the enzyme.

In the first phase, we want to know what is the order of addition of substrates if there are more than one substrate, and how tightly do the substrates bind. In the second phase, we want to be able to ask



Eukaryotic Glutamate Dehydrogenases, the structure of bovine glutamate dehydrogenase is shown on the left, are multisubunit enzymes which show complex allosteric behavior involving both substrates [homotropic regulation] and a variety of other ligands [heterotropic regulation] such as ADP, GTP, Leucine, Zinc and a wide variety of other ligands.
Enzyme kinetic studies have played an important role in elucidating how substrates and regulatory ligands interact with this complex enzyme.
The enzyme has six identical subunits [each subunit is shown in a different color] with complex subunit interactions mediated by the "antennae" [top and bottom] which connect three subunits within each half of the enzyme

questions about the nature of the overall rate limiting step of the reaction. In the third phase, like the first phase, the order of product release is important. Since an enzyme is often present with a mixture of both substrates and products, we may also want to know whether complexes where, for example, one substrate and one product can bind simultaneously to the enzyme exit. Such complexes may well play important roles in the regulation of the activity of the enzyme if they are more stable than the "normal" enzyme-substrate or enzyme-product complexes.

In the first two classes of enzymes considered above (housekeeping, cell type/organelle specific), in addition to understanding these three phases of the enzyme's activity, it may be important to understand how the activity of the enzyme is regulated: Does regulation of the enzyme involve alterations in substrate or product binding, or does it involve changes in the rate of the chemical catalytic step? Does the regulation involve substrate molecules themselves: homotropic regulation, or does it involve molecules separate from the normal substrates and products of the reaction: heterotropic regulation? What information can the study of enzyme kinetics give us that let us understand these aspects of an enzyme catalyzed reaction?

In the third class of enzyme, those that are differentiation specific or invading organism specific, the most important question we can ask may well be: how tightly does a certain molecule inhibit the enzyme? Understanding what chemical and structural features of the substrate [or product] are important in providing interactions with the enzyme in the binding site are important in the rational design of drugs that will bind tightly and specifically to the active site of the enzyme. Remember also that understanding the chemistry of the catalytic step can also provide valuable information for the design of drugs since the transition state of the chemical step binds to the active site far more tightly than does either the substrate or the product: transition state analogs often are very high affinity inhibitors of enzymes.

In these laboratories, we will explore how enzyme kinetic measurements can contribute to our understanding of how enzymes perform. In each section, the experiments progress from how to accurately measure the initial rate of an enzyme and characterize its basic properties to more sophisticated laboratories where questions about how substrates, products and inhibitors bind. Since these experiments involve two substrates, we will also explore experiments that can give us an indication as to the order of addition of the substrates to the enzyme: is there a compulsory order or can either substrate bind first followed by the other [a so called random order of substrate addition]? Many of the experiments described here also give an indication as to where the overall rate limiting step of the enzyme catalyzed reaction is - usually catalysis or product release – and, as appropriate, this will be discussed.

What type of experimental measurements will allow us to study and understand the activity, regulation, and potentially inhibition of enzymes in addition to simply showing that they are present and how much is present?

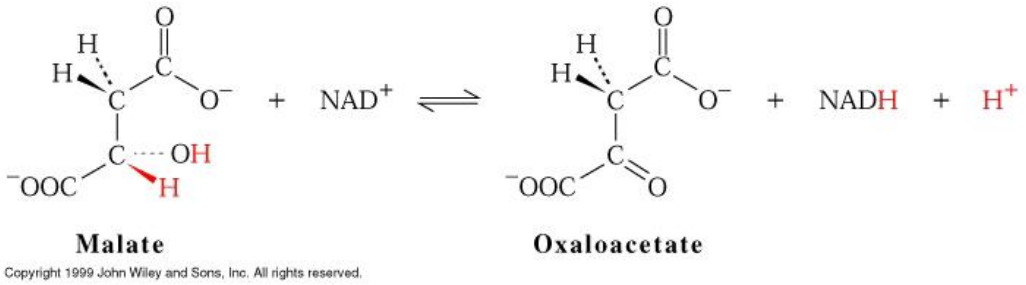
It is often not sufficient to simply show that a protein is present. Mutant forms of a protein may be present in "normal" amounts but show very different activity or regulation of its activity. The experiments described are designed to show the types of experiments that can be used to fully investigate the activity of enzymes.

Measurement of the activity of enzymes also plays a critical role in many other types of experiments that can be used to investigate "structure-activity" relationships in proteins and fully understanding the types of information that apparently simple measurement of enzyme activities can provide is the underpinning of much of modern biochemistry, molecular biology and biotechnology.

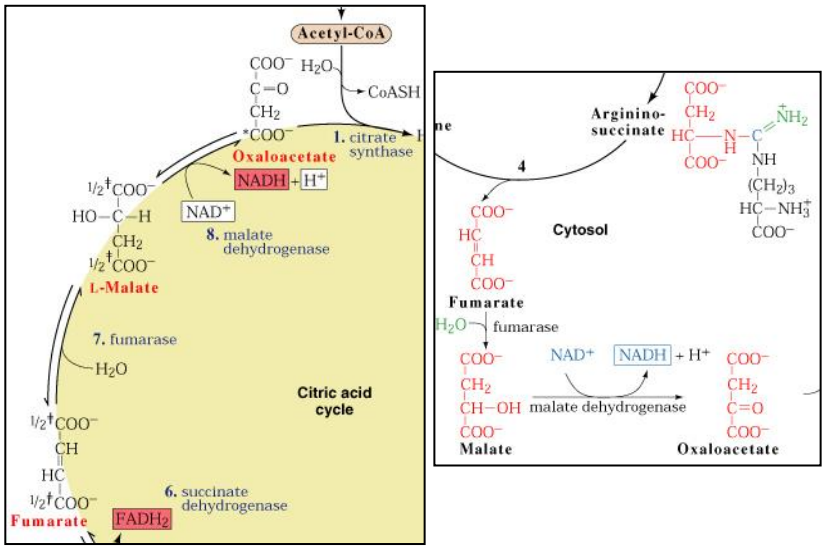
Review of the Roles of Malate Dehydrogenase
Malate Dehydrogenases catalyze the reaction:

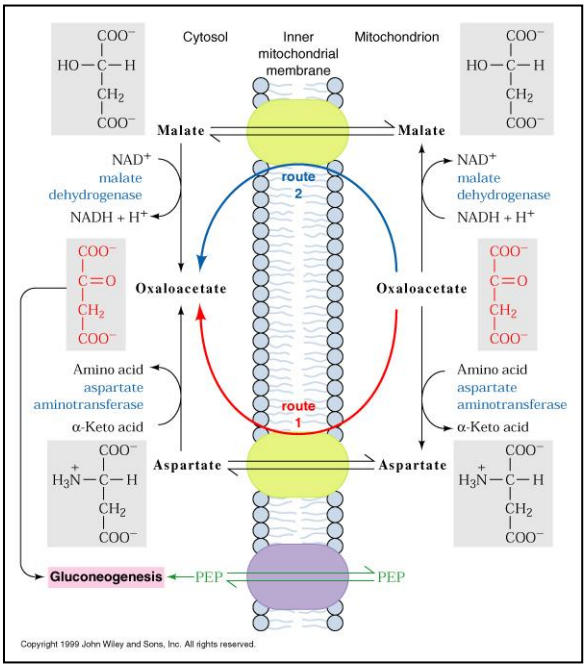
Malate + NAD^+ ←→ Oxaloacetate + NADH

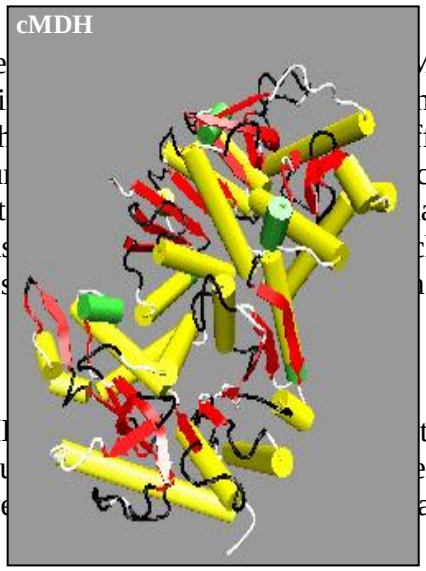
Involving a simple hydride transfer from the 2 position of Malate to the nicotinamide ring of NAD^+ to give NADH. During the process a proton is also released to the solvent.

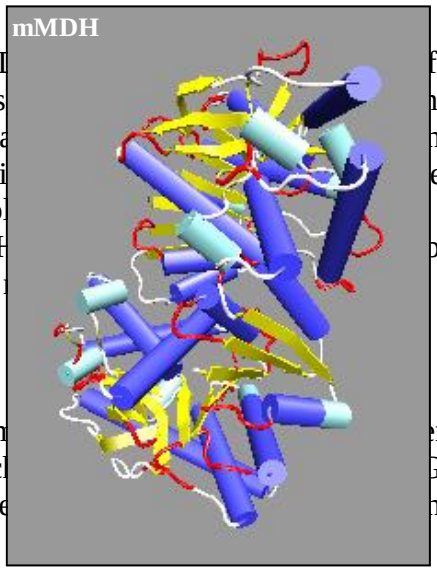


This reaction plays a number of important roles in metabolism, illustrated by a reaction in the Tricarboxylic acid cycle (left), a reaction critical to the Urea Cycle (center), and a reaction playing a role in the shuttling of reducing equivalents into mitochondria (right):



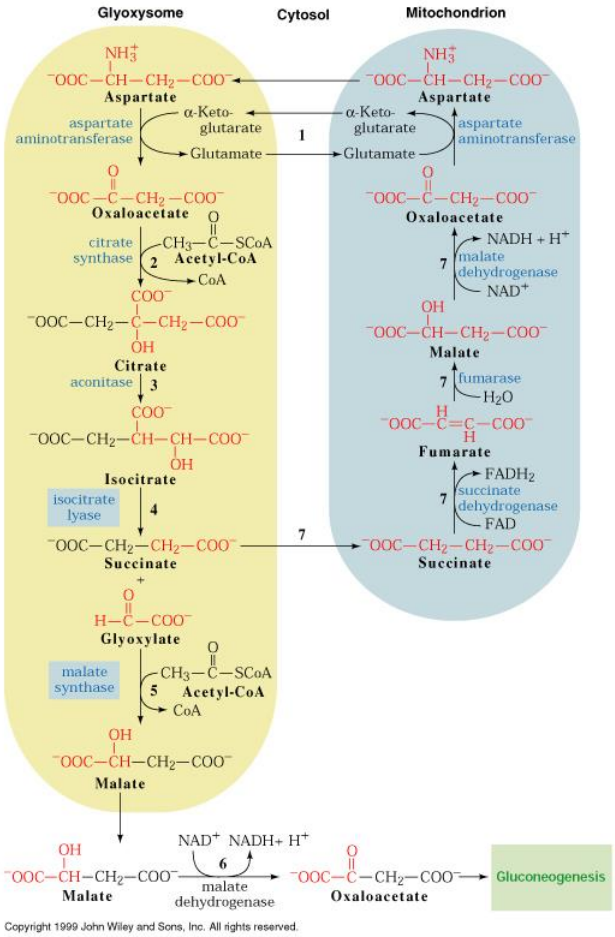






In the later example Malate I ferent locations within the cell and i nzymes nd a mitochondrial MDH [mMDH] in h fferent a nt three dimensional structu chondri each of the major types of experiment at invo of cytoplasmic and mitochondrial forms ch MDH unit contains two domains, with a clas and a

In mitochondria, MI tienzym enzymes sharing substrates, in particu es whic Glutamate and Oxaloacetate to give a key re nd Gluconeogenesis:



In the case of an enzyme such as Malate dehydrogenase, which catalyzes the reaction:

Malate + NAD^+ ←→ Oxaloacetate + NADH

where two substrates are involved, NAD^+ and Malate in the forward direction, NADH and Oxaloacetate in the "reverse" of the reaction, there is yet another consideration: what concentration of the non-varied substrate to use. The answer, when the aim of the experiment is to determine the Km of one of the substrates, in the first experiment here, that is the K_m of Oxaloacetate, is to fix the concentration of the non-varied substrate as close to saturation as is practical. In the experiment described below, the concentration of NADH will be fixed at 0.1mM and the concentrations of Oxaloacetate varied.

You will have noticed that in all of the discussion of Malate Dehydrogenase the utilization of NADH has been used in the assay. Why not use the experimentally simpler NAD^+ → NADH direction of the reaction?

The answer is actually quite simple if you remember that a reaction can reach an equilibrium position, and in the case of MDH the equilibrium position significantly favors Malate/NAD^+ and as a result the oxidation of Malate goes rapidly to equilibrium making it very difficult to determine an initial rate in the NAD^+ → NADH direction.

Experiment 1
Determination of the Basic Kinetic Parameters, K_m and V_{max}
Does the Enzyme Obey Simple Michaelis-Menten Kinetics?

In experiments concerning the measurement of initial rates, a number of critical features involved with accurately measuring the initial rate of an enzyme catalyzed reaction and some of the uses of such measurements must be considered. In this experiment, we start to answer some of the questions about what sort of information do we want to know about enzymes: specifically, how well do they bind their substrates [as assessed by their Michaelis Constant, K_m], what is the maximum rate of the catalyzed reaction [as assessed by the Maximum Rate of the catalyzed reaction with saturating substrate concentrations, V_{max}] and how efficiently does the enzyme operate, as estimated by V_{max}/K_m.

Why do we want to know these things about an enzyme? Knowing each of the above parameters can help us understand how the enzyme may operate in vivo, how changes in the enzyme as a result of mutation may contribute to a clinical condition, or how an enzyme might be engineered to be more useful in a biotechnological setting. Knowing the K_m for Glutamate of the enzyme N-AcetylGlutamate Synthase and realizing that the in vivo concentrations of Glutamate are much lower helps us understand how the Urea Cycle is regulated [small changes in Glutamate concentrations have a direct and proportional effect on the concentration of N-AcetylGlutamate, an absolutely required allosteric effector of the Enzyme Carbamoyl Synthase- the key first step in Urea Synthesis]. Understanding how fast an enzyme can go at saturating substrate concentrations can help elucidate whether or not the enzyme plays an overall rate limiting role in a pathway, and hence may be subject to regulation. Isocitrate Dehydrogenase in the Krebs Cycle has the lowest V_{max} of any enzyme in the cycle and hence is the rate limiting enzyme of the cycle. As a result it is the major point of activation of the cycle [you must speed up the overall rate limiting step if you want to speed up the cycle]. The catalytic efficiency of an enzyme can give not only information about its evolutionary status [for example the enzyme Triose Phosphate Isomerase, a very primitive enzyme whose catalytic efficiency has evolved to near perfection], but also the potential to improve the enzyme for biotechnological purposes.

Introduction to the Design of the Experiment.

The Michaelis-Menten Equation:

$$v_o = V_{max}[S]/\{K_m + [S]\}$$

Together with its linear transformation, the LineWeaver Burk Equation:

$$1/v_o = 1/V_{max} + \{K_m/V_{max}\} \times 1/[S]$$

are the basic equations of enzyme kinetics which allows us to not only calculate values for K_m and V_{max} from the appropriate experiments, but helps us to understand the design of such experiments. These equations are used to analyze simple one substrate kinetics and calculate Km and Vmax. The basic equations also apply to multi-substrate enzyme kinetics except that the values for Km and Vmax obtained from the simple experiment used earlier do not take into account the possibility that the second substrate may have some influence on the values for each parameter. For a given two substrate experiment where values for Km and Vmax are obtained from a single experiment with one of the substrate concentrations varied, they are the values determined at a fixed concentration of the other substrate or substrates and are usually referred to as "apparent" values. Furthermore, they are values obtained assuming that the enzyme obeys Michaelis-Menten kinetics. This, too, is not always the case.

Using the Predictions of the Equation to Design the Experiment:
Both equations predict a particular response of the initial rate, v_o, to changes in the concentration of a substrate. The parameters Km and Vmax are obtained from experiments where the initial velocity is measured as a function of the substrate concentration. In these experiments, it is important to keep in mind how you plan to display and analyze the resultant data. If you are using the Michaelis Menten equation, which gives a plot of v_o vs [S], the experiment should be designed to have regular spacing along the [S] axis. If you plan to use the LineWeaver Burk plot to display the data, which utilizes a plot of 1/vo vs 1/[S], then 1/[S] should be regularly spaced. Are there any other considerations in the design of the experiment? Two. First, the most accurate data is likely to be obtained from $0.5K_m$ to $5K_m$ and hence a preliminary experiment to give a rough value of K_m can be useful. Second, you are testing the hypothesis that the enzyme obeys the fit to the appropriate equation, and to test this assumption as thoroughly as possible you need as wide a range of substrate concentrations as possible.

Why the Data May Not Fit the Predictions of the Equations
The activity of many enzymes is "regulated" by the concentration of their own substrates. In such cases, simple Michaelis Menten kinetics are not observed. Three scenarios are possible: First, the enzyme could have a regulatory binding site for the substrate in addition to the active site. At concentrations of substrate where there was significant binding to the "regulatory" site, the activity could be increased or decreased. Think what this would do to the simple assumption made in the Michaelis Menten or LineWeaver Burk equations. If we assume that the "regulatory" site has lower affinity than the active site, the apparent activity of the enzyme would disproportionally increase or decrease depending upon whether the binding to the regulatory site increased or decreased the activity of the enzyme.

Second, if the enzyme has multiple subunits with active sites, there could be some type of allosteric interaction between the sites such that substrate affinity or catalytic activity increased or decreased as a higher degree of saturation was achieved. Such "homotropic" allosteric interactions could give rise to sigmoidal saturation curves. Like those obtained with oxygen saturation of hemoglobin in the case where the substrate binding was enhanced as saturation increased-positive cooperativity. Or, saturation curves which decreased substrate binding-so called negative cooperativity.

While both of these mechanisms require either a second binding site for the substrate or allosteric interactions between subunits, there is an explanation for both the above types of "deviation" from Michaelis-Menten behaviour that requires no such causes. Consider a two substrate enzyme with subsites for each substrate. In many cases, the products of a reaction closely resemble the substrates- Malate Dehydrogenase for example where the substrate NAD^+ is very similar to the product NADH and similarly the substrate Malate is very similar to the product Oxaloacetate. If in a two substrate-two product system such as malate dehydrogenase, malate were the first product to leave, but the rate limiting step in the overall reaction was the release of NAD^+ from the resultant Enzyme-NAD^+ complex, it is conceivable that at high concentrations of oxaloacetate, oxaloacetate would bind to the E-NAD^+ complex before NAD^+ was released to form an E-NAD^+-Oxaloacetate Complex. If this complex released NAD^+ faster than the E-NAD^+ complex, the result would be that oxaloacetate would speed up the overall rate limiting step and cause "substrate activation." If the E-NAD^+-oxaloacetate complex released NAD^+ more slowly that the E-NAD^+ complex, oxaloacetate would slow the overall reaction, and a "substrate inhibition" effect would be observed. Such complexes, containing one product and one substrate, often called "abortive" complexes, play key roles in many enzyme catalyzed reactions and result in significant deviations at high substrate concentrations from expected Michaelis-Menten behavior. As a result, there is a necessity to decide whether or not the collected data has in fact obeyed the predictions of the Michaelis Menten or LineWeaver Burk equations. As discussed in the "Quantitative Analysis of Data" section this involves an analysis of the residuals of the fit to the appropriate equation to determine whether in fact the data obeys the predicted equation.

Experiment

Determination of the Km for Oxaloacetate in the Reaction Catalyzed by Malate Dehydrogenase.

The aims of this laboratory are to show how an experiment is designed and conducted to a] obtain the most appropriate values for the Km of the enzyme Malate Dehydrogenase for its substrate Oxaloacetate and for its apparent Vmax, and b] to decide whether or not the enzyme obeys the assumptions made in the Michealis Menten or LineWeaver Burk equations: ie does the enzyme follow "normal" kinetic behavior. This will entail not only carefully conducting the experiment, but also learning how to appropriately analyze and present the data that you obtain.

Set Up of the Experiment

We will assume that the Km for Oxaloacetate is in the range 0.1 to 0.5mM and hence as discussed above we will want to vary the concentration of Oxaloacetate from 0.02mM to at least 2.5mM. The NADH concentration will be fixed at 0.1mM. We will replicate each data point three times to give sufficient data for reasonable statistical analysis. Six to twelve Oxaloacetate concentrations over our range would give a total of 18-36 assays and since each assay takes a minute or so, this can be easily achieved during the limited time available for the laboratory.

So, what 12 Oxaloacetate Concentrations should we choose?

If you examine the concentrations listed in Table 1.1 you will find that they are reasonably spaced throughout the concentration range, but weighted toward the low end of the spectrum. When you examine the 1/[S] values in the same table you will see that they are reasonably spaced over the whole range, although a little bunched towards the high concentration range. These concentrations represent a compromise between the needs of a Michaelis Menten plot and a LineWeaver Burk plot, and you will use both types of plots to analyze the data.

Table 1.1. Concentrations to Be Used to determine the K_m for Oxaloacetate

Concentration, mM	1/[S], mM^{-1}	Diluted Stock*	Original Stock	H_2O added
0.025	40	0.05mL		0.95mL
0.05	20	0.1mL		0.9mL
0.075	13.3	0.15mL		0.85mL
0.1	10	0.2mL		0.8mL
0.15	6.67	0.3mL		0.7mL
0.25	4	0.5mL		0.5mL
0.5	2		0.1mL#	0.9mL
0.75	1.33		0.15mL	0.85mL
1.0	1.0		0.2mL	0.80mL
1.5	0.67		0.3mL	0.7mL
2.0	0.5		0.4mL	0.6mL
2.5	0.4		0.5mL	0.5mL

How do we achieve these concentrations in the actual assays?

In this experiment we were provided with a series of solutions:
0.04M Phosphate Buffer
15mM Oxaloacetate in H_2O
0.6mM NADH in H_2O and

an enzyme solution, in 0.1M Phosphate Buffer, of approximately 0.1mg/mL
You will use a total of 3mL in the cuvette before addition of the enzyme, and again to achieve the least chance for pipetting errors you will premix everything that can be mixed: in this case, the buffer and the NADH can be premixed. As before since you want a final buffer concentration of 0.02M you will need 1.5mL buffer per cuvette.

How much NADH do you need to give a final concentration of 0.1mM?

You have a stock of 0.6mM, you want 0.1mM: the dilution Factor is 0.6/0.1 = 6
Simply divide the total volume, ie 3mL, by the dilution factor, 6, to give 3.0/6 = 0.5mL of the stock NADH solution added per 3mL of total volume.

Thus for each cuvette you can premix 1.5mL of the stock 0.04M buffer and 0.5mL of the stock 0.6mM NADH and add 2.0mL per cuvette. You will have a total of 36 cuvettes but using the principle of making up a bit more that you need, you should plan for 40 cuvettes.

Measure out 40 x 1.5mL = 60mL of the Stock Buffer
Add 40 x 0.5mL = 20mL of the stock NADH
Mix
Add 2.0mL to each cuvette that you will need

What else do you need in each cuvette?
Oxaloacetate: added to give the concentrations in Table 1.1, and H_2O to make up the volume to 3.0mL

Consider the 1mM concentration of Oxaloacetate. You have a 15mM stock solution: the dilution factor is 15/1 = 15. Thus you will need to add 3/15 = 0.2mL of the stock to give 1mM

If you make the same calculation for the 0.025mM you will get a dilution factor of 15/0.025 = 600 and would need to add 3.0/600 = 0.005mL of the stock to give 0.025mM Oxaloacetate. This is not very practical from the accuracy standpoint and so you will make a 10 fold dilution of the stock solution [5mL of the stock plus 45mL H_2O] which will give a second stock of 1.5mM. Now calculating a dilution factor for the 0.025mM point you get 1.5/0.025 = 60 which would mean that you add 0.05mL of the diluted stock to give 0.025mM oxaloacetate in the cuvette. As a general rule of thumb, for accurate work try not to add less than 0.05mL since pipetting errors are magnified as the volume pipetted decreases.

In table 1.1, the volumes of oxaloacetate to add to the appropriate cuvettes are indicated, with * indicating that it is the diluted stock rather than the original stock that is being used.

The final column in table 1.1-ib shows the amount of H_2O to add to the cuvette to bring the total volume to 3.0mL.

Set Up of the Experiment that You Will Perform
In the experiment that you will perform you are provided with the following solutions:

50mL of 10mM Oxaloacetate in H_2O
30mL of 0.5mM NADH in H_2O
200mL of 0.04M Phosphate
2mL of Malate Dehydrogenase at approximately 0.1mg/mL [you will be provided with the exact concentration].

Using the above worked example of experimental set up and design as a guide, design the experiment that you will perform, using the above solutions that you will be provided with, to determine the Km for Oxaloacetate at pH 8.0. Fill in the necessary information on Experimental Set Up WorkSheet 1.1

You will not be able to calculate the actual enzyme concentration that you will use but will add this information to your worksheet at the start of the actual experiment.

Experiment 1.1
Experimental Set Up Work Sheet

		Components of Assay Mix		
Abs of Enzyme at 280nm:	[enzyme]:	mL H_2O:	mL Buffer :	mL NAD^+:
Cuvette #	[Oxaloacetate]	mL Oxaloacetate	mL Assay Mix	mL H_2O
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
# of Replicates				
Volume of Enz to initiate:				

Preequilibrate the cuvettes to the desired temperature, room temperature for example

While the cuvettes are pre-equilibrating, determine the enzyme concentration in the stock enzyme solution and decide what volume you wish to add per assay. To determine the enzyme concentration take a 100μL aliquot and add to a cuvette containing 1.0mL of 0.1M Phosphate buffer at pH 7.0, that has been zeroed in the spectrophotometer at 280nm, and read the resultant absorbance after careful mixing. For example, in the quantitative analysis data set given below, utilizing Glutamate Dehydrogenase, a value of 0.152 for the absorbance was obtained when 200μL of stock enzyme solution was added to give a total of 1.2mL buffer. The concentration of the enzyme was calculated by multiplying the measured absorbance by the dilution factor [1.2/0.2 = 6] and dividing by the extinction coefficient for glutamate dehydrogenase, 0.93mg/mL cm-1 at 280nm. Thus the stock enzyme solution was:

{0.152 x 6}/ 0.93 = 0.98mg/mL

10μL of the stock was added to initiate the reaction with each cuvette as appropriate.

Depending upon the exact concentration you obtain for the stock enzyme solution you may want to dilute to no more than 0.1mg/mL since adding 10μL of a higher enzyme concentration will give problems in determining an initial rate for the cuvettes in the experiment. A concentration of the stock solution in the range 0.05 to 0.1mg/mL should give good data in this experiment.

Conduct the Experiment.

Since you need no more than about 30 seconds of data to determine an initial rate, there is no point in collecting more data than that for each cuvette. Be sure to mix thoroughly after the enzyme addition. Record the absorbance at 340nm for 30-40 seconds for each cuvette and calculate the ΔA/minute for each cuvette. Remember that the absorbance will go down as NADH is consumed. This means that you will need to "blank" each cuvette with a buffer blank, not the actual cuvette you will use to make the rate measurement. Remember to switch to the actual cuvette before adding the enzyme!

At the end of the measurements, calculate the initial rate for each trace, calculate the averages and standard deviations for each set of replicates. Record the values that you obtain in the data analysis work sheet:

Data Analysis Work Sheet 1.1-I:

[OAA],mM	***Rate 1 ΔA/min***	***Rate 2 ΔA/min***	***Rate 3 ΔA/min***		***Average ΔA/min***		***St. Dev. ΔA/min***

Convert your ΔA/minute values into μM NADH utilized/minute and enter into Data Analysis Work Sheet 1.1-II

Data Analysis Work Sheet 1.1-II

[OAA], mM	***Average Rate*** *μM NADH Utilized/minute*	***Standard Deviation*** *μM NADH Utilized/minute*	***%Standard Deviation***

Determine the K_m and V_{max} for the reaction using both the Michaelis Menten equation and the LineWeaver Burk equation. From the experimentally determined V_{max}, calculate the specific activity of the enzyme.

Experiment

Determining the K_m for NADH

Design Your Own Experiment:

Using the above experiment as a guide, you should design an experiment to determine the V_{max} and K_m values for NADH at pH 8.0, using a fixed concentration of Oxaloacetate of 1mM. Assume that the Km for NADH is in the range of 20 to 200μM. You should use 10 concentrations of NADH.

You will be provided with the following solutions to conduct the experiment:
0.04M Phosphate Buffer, pH 8.0
6mM Oxaloacetate in H_2O
Assume 1.5mM NADH in H_2O [you can check the actual concentration using a millimolar extinction coefficient for NADH at 340nm of 6.22cm^{-1}]
Approximately 0.1mg/mL Malate Dehydrogenase in 0.1M Phosphate Buffer at pH 7.0 [check the actual concentration as before].

Experiment 1.2

Experimental Set Up Work Sheet

		Components of Assay Mix		
Abs of Enzyme at 280nm:	***Concentration of enzyme:***	***mL H_2O:***	***mL Buffer :***	***mL Oxaloacetate:***
Cuvette #	[NADH]	mL NADH	mL Assay Mix	mL H_2O
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
# of Replicates				
Volume of Enz to initiate:				

At the end of the measurements, calculate the initial rate for each trace and calculate the averages and standard deviations for each set of replicates. Record the values that you obtain in the data analysis work sheet:

Data Analysis Work Sheet 1.2-I:

[NADH], μM	***Rate 1 ΔA/min***	***Rate 2 ΔA/min***	***Rate 3 ΔA/min***		***Average ΔA/min***		***St. Dev. ΔA/min***

Convert your ΔA/minute values into μM NADH utilized/minute and enter into Data Analysis Work Sheet 1.2-II.

Data Analysis Work Sheet 1.2-II

[NADH], μM	***Average Rate*** ***μM NADH Utilized/minute***	***Standard Deviation*** ***μM NADH Utilized/minute***	***%Standard Deviation***

Determine the K_m and V_{max} for the reaction using both the Michaelis Menten equation and the LineWeaver Burk equation. From the experimentally determined V_{max}, calculate the specific activity of the enzyme.

EXPERIMENT 2

Characterization of the Kinetic and Binding Properties of the Enzyme

In the previous experiments with Malate Dehydrogenase, experiments 1.1-2, the apparent values for K_m for either substrate, and for V_{max} were determined in experiments where one of the substrate concentrations was fixed and the other varied. Since in a two substrate enzyme it is possible that the binding of one substrate influences the binding of the other substrate, it is necessary to establish whether one substrate binding affects the Km for the other substrate. Similarly, in the experiment with one substrate concentration fixed and the other varied you are in effect extrapolating the rate to saturation of the varied substrate [the $1/v_o$ intercept is $1/V_{max}$(apparent) since you have extrapolated to saturation by going to 0 on the 1/[S] axis]. The true V_{max} of course would be obtained by extrapolation to saturation with both substrates. This is achieved by conducting a series of experiments where one substrate concentration is varied at a series of fixed concentrations of the other substrate. This experiment is described by the generalized rate equation for a two substrate enzyme catalyzed reaction:

$$\mathbf{e/v_o = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2] + \Phi_{12}/[S_1 x S_2]}$$

where the Φ parameters are the so-called initial rate parameters and, as we shall see, are related to the K_m and V_{max} parameters of a standard LineWeaver Burk equation. This equation is of course in the format of a LineWeaver Burk equation and can be easily rearranged to give a LineWeaver Burk equation for either substrate:

For Substrate 1, S1 varied: we get:

$$\mathbf{e/v_o = \{\Phi_0 + \Phi_2/[S_2]\} + \{\Phi_1 + \Phi_{12}/[S_2]\} x\ 1/[S_1]}$$

intercept **slope**

or for Substrate 2, S_2 varied: we get:

$$\mathbf{e/v_o = \{\Phi_0 + \Phi_1/[S_1]\} + \{\Phi_2 + \Phi_{12}/[S_1]\} x\ 1/[S_2]}$$

intercept **slope**

These equations predict that both the slope and the intercept of a LineWeaver Burk plot with S_1 as the varied substrate will vary as a function of S_2, and similarly for the slopes and intercepts of LineWeaver Burk plots with S_2 as the varied substrate when different S_1 values are used.

These equations also show how the initial rate parameters are related to the true K_m and V_{max} values for the enzyme in question. From the equation above, the apparent K_m for substrate 1 will be slope/intercept:

$K_m = \{\Phi_1 + \Phi_{12}/[S_2]\} / \{\Phi_0 + \Phi_{12}/[S_2]\}$ and is clearly a function of S_2. At saturating S_2 however the terms $\Phi_{12}/[S_2]$ and $\Phi_{12}/[S_2]$ go to zero and the Km becomes Φ_1/Φ_0. Likewise, the Km for S_2 becomes Φ_2/Φ_0. From either equation it should be apparent that the true V_{max} for the enzyme is $1/\Phi_0$.

The generalized rate equation for a two substrate enzyme also shows how the individual initial rate parameters can be experimentally determined to allow calculation of the true kinetic parameters for the enzyme. As shown in the problem set below, the slope of a primary LineWeaver Burk plot with S_1 as the varied substrate is a

function of S_2, as is the intercept. A secondary plot of either slope or intercept against $1/S_2$ allows for the four initial rate parameters to be calculated. An experiment to determine the values of the initial rate parameters would consist of varying one substrate concentration in the presence of a series of fixed concentrations of the second substrate. A typical experiment might involve five different concentrations of each substrate, in a 5 x 5 grid, as illustrated in the problem set at the end of this section.

Determine the True Kinetic Parameters for 3 Phosphoglycerate Dehydrogenase at pH 7.5
Rationale to the Set Up the Experiment: Worked Example

What considerations do you need to take into account to design an experiment to determine the initial rate parameters for a two substrate enzyme? Consider the experimental set up for determining the initial rate parameters for the enzyme Glutamate dehydrogenase at pH 7.0 outlined below. How were the concentration ranges for Glutamate and for NAD^+ chosen? Preliminary experiments such as those illustrated earlier in this section established the approximate values for Km for each substrate. From such an experiment, we know that the Km for Glutamate is approximately 3-4mM and hence a good range of Glutamate concentrations to use would be from 1mM to 20mM. From such an experiment, we expect that the LineWeaver Burk plots with Glutamate as the varied substrate will be linear in this range. Remembering that we will plot LineWeaver Burk plots to analyze the data, we will choose five concentrations of Glutamate, as shown in the table below: why were these concentrations chosen?

It is known that with NAD^+ as the varied substrate, a non-linear LineWeaver Burk plot is generated when the NAD^+ concentration is varied from 5μM to 1000μM, but that the data is approximated by two linear regions, one with NAD+ up to about 45μM and the other with NAD+ concentrations between 100 and 1000μM. We can choose to determine the initial rate parameters in either of these two NAD^+ concentration ranges.

In the ***example*** here we will use the 5-45μM range, and choose five NAD^+ concentrations, as shown in the table below.

Experiment Set Up Work Sheet 2.1:

[Glutamate], mM	***Volume of Glutamate Added***	***[NAD^+], μM***	***Volume of NAD^+ Added***	***Volume of Buffer Added***	***Volume of Water Added***
1.0		5			
		7.5			
		10			
		20			
		45			
1.5		5			
		7.5			
		10			
		20			
		45			
2.5		5			
		7.5			
		10			
		20			
		45			
5.0		5			
		7.5			

		10			
		20			
		45			
20.0		5			
		7.5			
		10			
		20			
		45			

Pre-Laboratory Problem:
Assuming that you are provided with the following solutions, how would you make up each cuvette if you plan to use a final buffer concentration of 0.1M?

0.2M Phosphate Buffer containing 20μM EDTA
120mM Glutamate in H_2O
270μM NAD^+ in H_2O

If you are planning to make each measurement in quintuplet [five] how much of each solution would you plan to make up?

Note: After you have made up the stock solution of NAD^+, you would check its concentration by absorbance measurements at 260nm using a millimolar extinction coefficient of $17.8cm^{-1}$.

Performing the Experiment:
Make up three cuvettes each with the following contents:

[Glutamate], mM	***Volume of Glutamate Added***	***[NAD^+], μM***	***Volume of NAD^+ Added***	***Volume of Buffer Added***	***Volume of Water Added***
1.0	0.1*	5	55μL	1.5mL	1.345mL
	0.1	7.5	83	"	1.317
	0.1	10	110	"	1.290
	0.1	20	220	"	1.180
	0.1	45	500	"	0.900
1.5	0.15*	5	55	"	1.295
	0.15	7.5	83	"	1.267
	0.15	10	110	"	1.240
	0.15	20	220	"	1.130
	0.15	45	500	"	0.850
2.5	0.25*	5	55	"	1.195
	0.25	7.5	83	"	1.167
	0.25	10	110	"	1.120
	0.25	20	220	"	1.030
	0.25	45	500	"	0.750
5.0	0.5*	5	55	"	0.945
	0.5	7.5	83	"	0.917
	0.5	10	110	"	0.890

	0.5	20	220	"	0.780
	0.5	45	500	"	0.500
20.0	0.5	5	55	"	0.945
	0.5	7.5	83	"	0.917
	0.5	10	110	"	0.890
	0.5	20	220	"	0.780
	0.5	45	500	"	0.500

When you have pre-equilibrated the cuvettes at whatever temperature you choose, initiate the reaction by the addition of 10μL of the approximately 0.5mg/mL solution of Glutamate dehydrogenase and determine the initial rate for each cuvette.

Using Data Analysis work sheets 2.2 and 2.3 calculate the initial rate parameters for Glutamate Dehydrogenase under these conditions. You would calculate the actual NAD^+ concentrations from the experimentally determined concentration of the stock NAD^+ solution.

Data Analysis Work Sheet 2.2

Glutamate, mM	***NAD^+, μM***	***Rate #1 ΔA/min***	***Rate #2***	***Rate #3***	***Average Rate***	***Standard Deviation***

Convert the ΔA/minute to nM NADH produced per minute and enter the results into Data Analysis Work Sheet 2.3

Data Analysis Work Sheet 2.3

Glutamate, mM	***NAD^+, μM***	***Average Rate nM NADH produced per minute***	***Standard Deviation***

Plot the primary plots as illustrated in the section on quantitative analysis of the data and enter the values for the slopes and intercepts into Data Analysis Work Sheet 2.4

Data Analysis Work Sheet 2.4

Glutamate, mM	***Intercept from LWB plot with NAD^+ Varied***	***Intercept Standard Deviation***	***Slope from LWB plot with NAD^+ Varied***	***Slope Standard Deviation***

Construct the secondary plot and enter the values for the appropriate initial rate parameters in Data Analysis Work Sheet 2.5 together with the calculated values for the Km values for each substrate and the V_{max}

Data Analysis Work Sheet 2.5

Parameter	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}
Value				
Standard Deviation				
Units				
K_m	Substrate 1=		Substrate 2=	
V_{max}				

Experimental Set Up WorkSheet
Design Your Own Experiment:
Design and conduct an experiment to determine the initial rate parameters for either Malate Dehydrogenase. Use NADH concentrations in the range 10-100μM, and oxaloacetate concentrations in the range 0.025-0.4mM.

Assuming that you can make up the stock solutions of NADH and Substrate as you desire:

NADH Stock Concentration_____
Substrate Stock Concentration_____

Using an 0.04M Phosphate Buffer stock as before.

Experiment Set Up Work Sheet 2.6:

[Substrate], mM	***Volume of Substrate Added***	***[NADH], μM***	***Volume of NADH Added***	***Volume of Buffer Added***	***Volume of Water Added***

Initiate the reaction with the addition of 10-20μL of 0.1mg/mL MDH.

Using Data Analysis work sheets 2.7-2.9 calculate the initial rate parameters for the enzyme under these conditions. You would calculate the actual NADH concentrations from the experimentally determined concentration of the stock NADH solution.

Data Analysis Work Sheet 2.7-I

Substrate, mM	***NADH, μM***	***Rate #1 ΔA/min***	***Rate #2***	***Rate #3***	***Average Rate***	***Standard Deviation***

Convert the ΔA/minute to nM NADH utilized per minute and enter the results into Data Analysis Work Sheet 1.8-II

Data Analysis Work Sheet 2.7-II

Substrate, mM	***NADH, μM***	***Average Rate nM NADH utilized per minute***	***Standard Deviation***

Plot the primary plots as illustrated in the section on quantitative data analysis and enter the values for the slopes and intercepts into Data Analysis Work Sheet 2.8.

Data Analysis Work Sheet 2.8

Substrate, mM	***Intercept from LWB plot with NADH Varied***	***Intercept Standard Deviation***	***Slope from LWB plot with NADH Varied***	***Slope Standard Deviation***

Construct the secondary plot and enter the values for the appropriate initial rate parameters in Data Analysis Work Sheet 2.9 together with the calculated values for the Km values for each substrate and the V_{max}.

Data Analysis Work Sheet 2.9

Parameter	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}
Value				
Standard Deviation				
Units				
K_m	Substrate 1=		Substrate 2=	
V_{max}				

Quantitative Analysis of the Data

The enzyme 3-Phosphoglycerate Dehydrogenase has a subunit molecular weight of 42,000 and utilizes 3-phosphoglycerate and NAD^+ as substrates. The data in the table below was obtained in an experiment where S1 is NAD+ and S2 is 3-phosphoglycerate. The reaction was initiated by the addition of 20μL of a 0.23mg/mL solution of the enzyme to a reaction mixture with a total volume of 1mL.

The following data was obtained:

Concentration of S1	***Concentration of S2***	***Average Initial Rate μM NADH per minute***	***Standard deviation***
10μM	0.05mM	0.156	0.006
4		0.130	0.007
2		0.104	0.007
1.25		0.084	0.011
0.83		0.0667	0.012
10	0.133mM	0.189	0.006
4		0.164	0.005
2		0.137	0.007
1.25		0.113	0.009
0.83		0.0926	0.011
10	0.2mM	0.247	0.007
4		0.219	0.007
2		0.193	0.006
1.25		0.164	0.008
0.83		0.135	0.007
10	0.5mM	0.303	0.009
4		0.285	0.008
2		0.257	0.008
1.25		0.23	0.006
0.83		0.196	0.006
10	2.0mM	0.337	0.005
4		0.3106	0.006
2		0.290	0.007
1.25		0.263	0.008
0.83		0.230	0.008

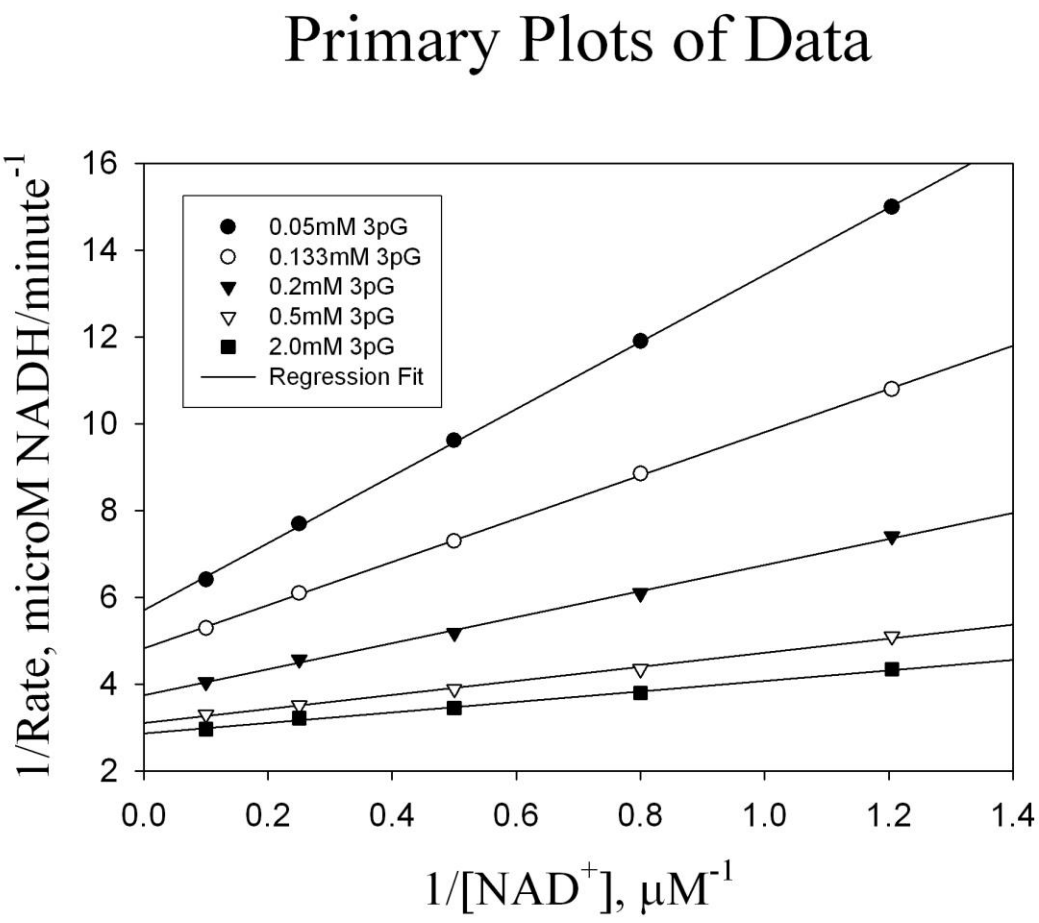
From the above data, determine the values of the 4 initial rate parameters for the reaction and determine the Km values for each substrate and the overall maximum rate of the reaction.

For Substrate 1, S1, varied, we get:

$$e/v_o = \{\Phi_0 + \Phi_2/[S_2]\} + \{\Phi_1 + \Phi_{12}/[S_2]\} \times 1/[S_1]$$

intercept slope

If we plot the above data in double reciprocal LineWeaver Burk format we obtain a graph:



If the slopes and intercepts obtained from this plot are then plotted according to:

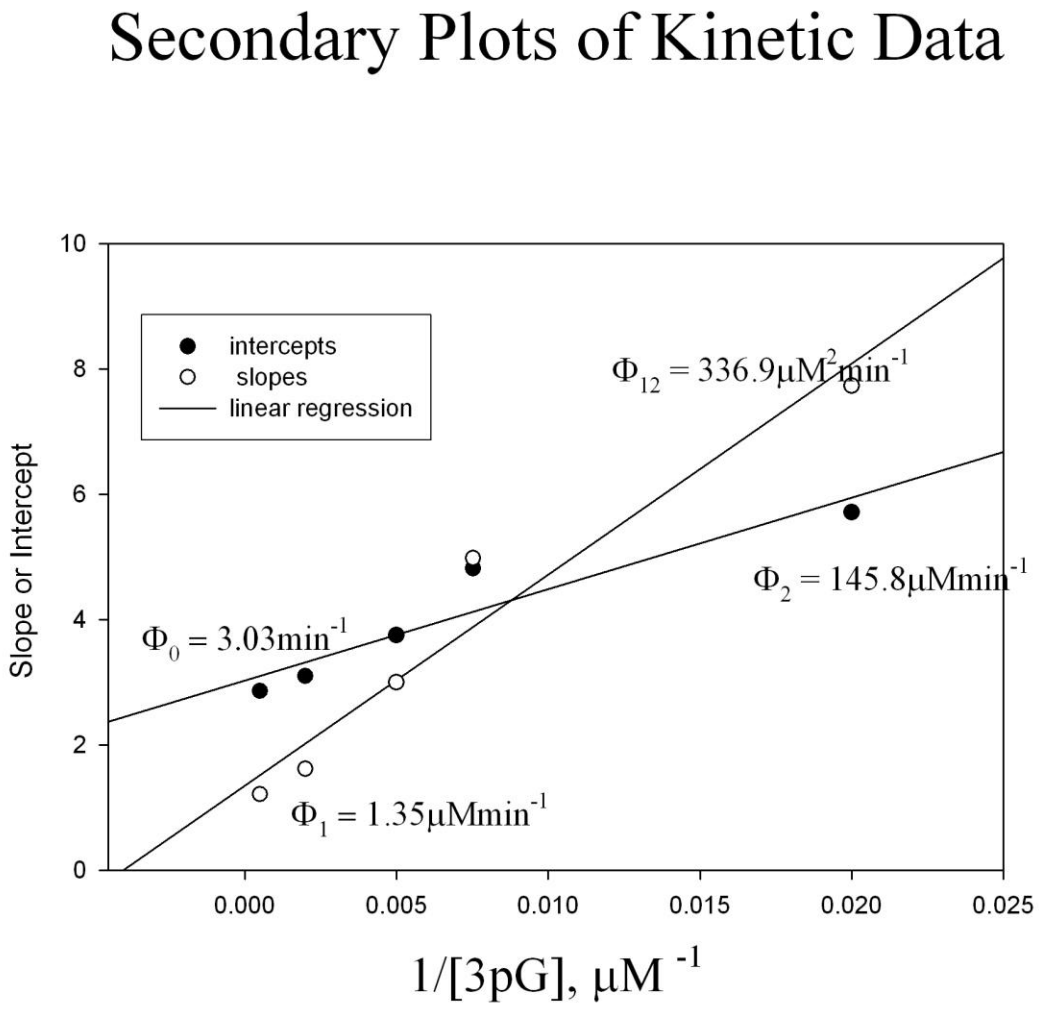
Slope = $\{\Phi_1 + \Phi_{12}/[S_2]\}$

And

Intercept = $\{\Phi_0 + \Phi_2/[S_2]\}$

It is clear that plots of slope or intercept versus $1/[S_2]$ should be linear. For the slopes plot, we obtain an intercept of Φ_1 and a slope of Φ_{12}. For the plot of the intercepts versus $1/[S_2]$, the slope is Φ_2 and the intercept is Φ_0.

The so-called secondary plot of the primary data allows calculation of all of the initial rate parameters as shown below



Km for S_1 is $\Phi_1/\Phi_0 = 1.35/3.03 = 0.446\mu M$. Likewise, the Km for S_2 becomes $\Phi_2/\Phi_0 = 48.1\mu M$. From either equation, it should be apparent that the true V_{max} for the enzyme is $1/\Phi_0 = 1/3.03 = 0.33 min^{-1}$. But what are the units? In reality, but rarely in practice, we would have plotted e/Vo, where e is the active site concentration in μM, and hence the units of Vmax would be min^{-1}. The units of Φo are currently $\mu M/min^{-1}$, hence if we convert the enzyme concentration to μM and divide we will obtain the correct units.

To calculate the μM of the enzyme in the reaction mix, we must take into account the fact that 20μL of stock enzyme was added to 1mL reaction mix to initiate the reaction and that the stock enzyme was 0.23mg/mL [with an active site molecular weight of 42,000].

First, to calculate the stock enzyme concentration:

42,000mg/mL = 1M
42mg/mL = 1mM

1mg/mL = 31.7μM, thus 0.23mg/mL = 31.7 x 0.23 = 7.28μM

To obtain the concentration in the reaction mix:

Stock concentration x Volume Added/Total volume:

(7.28 x 20)/1020 = 0.143μM

Thus the value for Φ_0 would have been 0.143/3.03 = 0.0472 and Vmax becomes 1/0.0472 = 21.2min^{-1}.

Since the Km values are obtained from ratios of initial rate parameters we do not need to make the same type of correction since the enzyme concentration would cancel out.

Group Projects
Comparison of Initial Rate Parameters for Cytoplasmic and Mitochondrial Malate Dehydrogenases.

Problem Set 1
The enzyme Glucose-6-Phosphate Dehydrogenase was examined using NADP+ as substrate 1 and Glucose 6 Phosphate as substrate 2. Initial rates were measured at varying concentrations of both substrates using a protein concentration of 0.034μM in the cuvette

Concentration of S1	***Concentration of S2***	***Average Initial Rate μM NADPH per minute***	***Standard deviation***
5mM	1.0mM	0.1232	0.0081
2.5		0.0969	0.0063
1.67		0.0732	0.0055
1.11		0.0577	0.0051
0.83		0.0444	0.0042
5mM	2.0mM	0.1942	0.0087
2.5		0.1493	0.0071
1.67		0.1190	0.0034
1.11		0.0939	0.0055
0.83		0.0758	0.0051
5mM	3.0mM	0.2985	0.0093
2.5		0.2326	0.0092
1.67		0.1887	0.0080
1.11		0.1493	0.0066
0.83		0.1212	0.0079
5mM	5.0mM	0.5263	0.0070

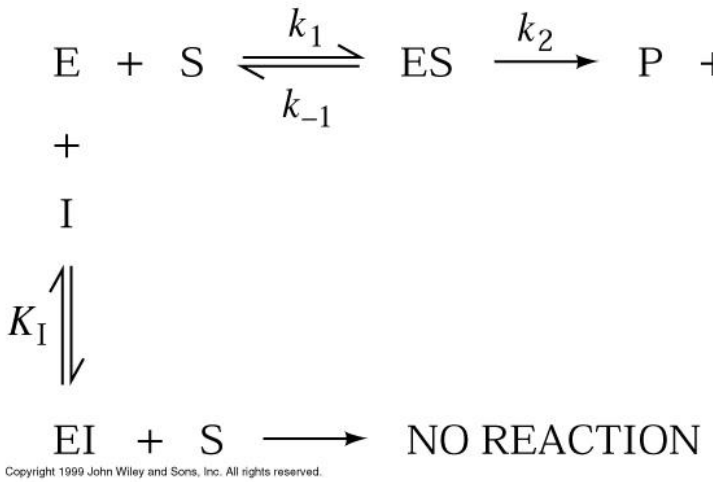
2.5		0.4082	0.0065
1.67		0.3390	0.0071
1.11		0.2632	0.0080
0.83		0.2151	0.0081
5mM	10.0mM	0.8333	0.0063
2.5		0.5882	0.0089
1.67		0.4651	0.0088
1.11		0.3984	0.0081
0.83		0.3279	0.0066

Calculate the initial Rate Parameters for Glucose 6 Phosphate Dehydrogenase and Vmax, and Km for each substrate.

EXPERIMENT 3

Competitive Inhibitors

Many drugs and therapeutic agents are based on the principle that they look like the normal substrate [or ligand] for an enzyme or protein and hence will bind specifically to that enzyme or protein and block the effects of the normal substrate binding. In the case of an enzyme catalyzed reaction, this results in an inhibition of the activity of the enzyme. However, since the normal substrate and the "inhibitor" compete for the normal binding site on the enzyme, the inhibition can be overcome at a fixed concentration of the inhibitor by saturating with the normal substrate: the inhibitor is a "competitive" inhibitor of substrate binding. The more tightly the inhibitor binds to the active site of the enzyme, the more of the normal substrate is required to displace the inhibitor. The tightness of inhibitor binding is assessed by the inhibition constant, Ki, which is the dissociation constant for the inhibitor binding to the enzyme in the scheme:



Enzyme-Inhibitor Complex

The inclusion of this pathway in the presence of the inhibitor alters the Michaelis Menten Equation for the reaction:

$$vo = Vmax[S]/\{\alpha Km + [S]\}$$

Where $\alpha = 1 + [I]/Ki$.

This has an effect also on the LineWeaver Burk equation for the system:

$$1/vo = \{\alpha Km/Vmax\} \times 1/[S] + 1/Vmax$$

slope intercept

The value of Ki can be obtained by determining the effect of the presence of a known inhibitor concentration on the slope of the LineWeaver Burk plot obtained with the "normal" substrate as the varied substrate:

Slope (with inhibitor present) = slope (no inhibitor present) x α (that is $1 + [I]/Ki$)

Thus the Dissociation Constant (Ki) for inhibitor binding can be readily determined experimentally.

Ki = [E][I]/[EI] is related to the equilibrium constant for the binding of inhibitor to enzyme, Keq = [EI]/[E][I] = 1/Ki, and hence is a measure of the Free Energy of Binding of the inhibitor to the enzyme:

$\Delta G^o = -RT\ln(1/Ki)$

for example, an inhibition constant (dissociation constant) of 1mM (10^{-3}M) at 25°C (298°K) results from a ΔG^o of -4.096kcal/mol:

$\Delta G^o = -1.99 \times 298 \times \ln(1/10^{-3}) = -4.096$kcal/mol

These values must be considered in light of the strengths of the various bond types that could be involved in the non-covalent interaction of an inhibitor with an enzyme, shown in table

Table 3.1.

Type of Bond	Example	Bond Strength, $kJ \cdot mol^{-1}$	Bond Strength, $kcal \cdot mol^{-1}$
Ionic Interaction	$-COO^{-} \cdots {}^{+}H_3N-$	86	20.5
Hydrogen Bond	-O-H$\cdots$O<	20	4.78
Van der Waals Forces			
Dipole-Dipole	>C=O$\cdots$>C=O	9.3	2.22
London Dispersion	$-CH_2-H\cdots H-CH_2-$	0.3	0.072

Consider if the structure of an inhibitor was changed so that it made one additional hydrogen bond to its target, this would increase the free energy of binding by 4.78kcal mol-1. If the original inhibitor bound with a dissociation constant of 1mM, as in our example above, we can calculate what the new inhibitor would have as a dissociation constant:

ΔG^o for the new inhibitor would be -8.88kcal mol-1: $-8{,}800 = 1.99 \times 298 \times \ln(1/K_i^{new})$

thus $14.97 = \ln(1/K_i^{new})$ and the new dissociation constant would be 0.000315mM, over 3000-fold tighter binding than the original compound.

The ratio of the increase in the affinity can be calculated directly using:

$\Delta\Delta G^o = -RT \times \ln(K_1/K_2)$, where (K_1/K_2) is the ratio of the two constants:

$4{,}780 = 593 \times \ln(K_1/K_2)$ and hence $(K_1/K_2) = 3{,}167$

Note that this gives the same ratio as comparing the calculated dissociation constants. The gain or loss of a hydrogen bond can have a very significant effect on the affinity of a protein for a compound. The relative contributions of enthalpy and entropy to the binding of the inhibitor can be assessed by examining the temperature dependence of Ki and the relationship:

$\ln(1/Ki) = \{-\Delta H^o/R\} \times 1/T + \Delta S^o/R$

where H^o and S^o represent enthalpy and entropy in the standard state and R is the Gas Constant, = $8.3145 J \cdot K^{-1} \cdot mol^{-1} = 1.99 cal \cdot K^{-1} \cdot mol^{-1}$

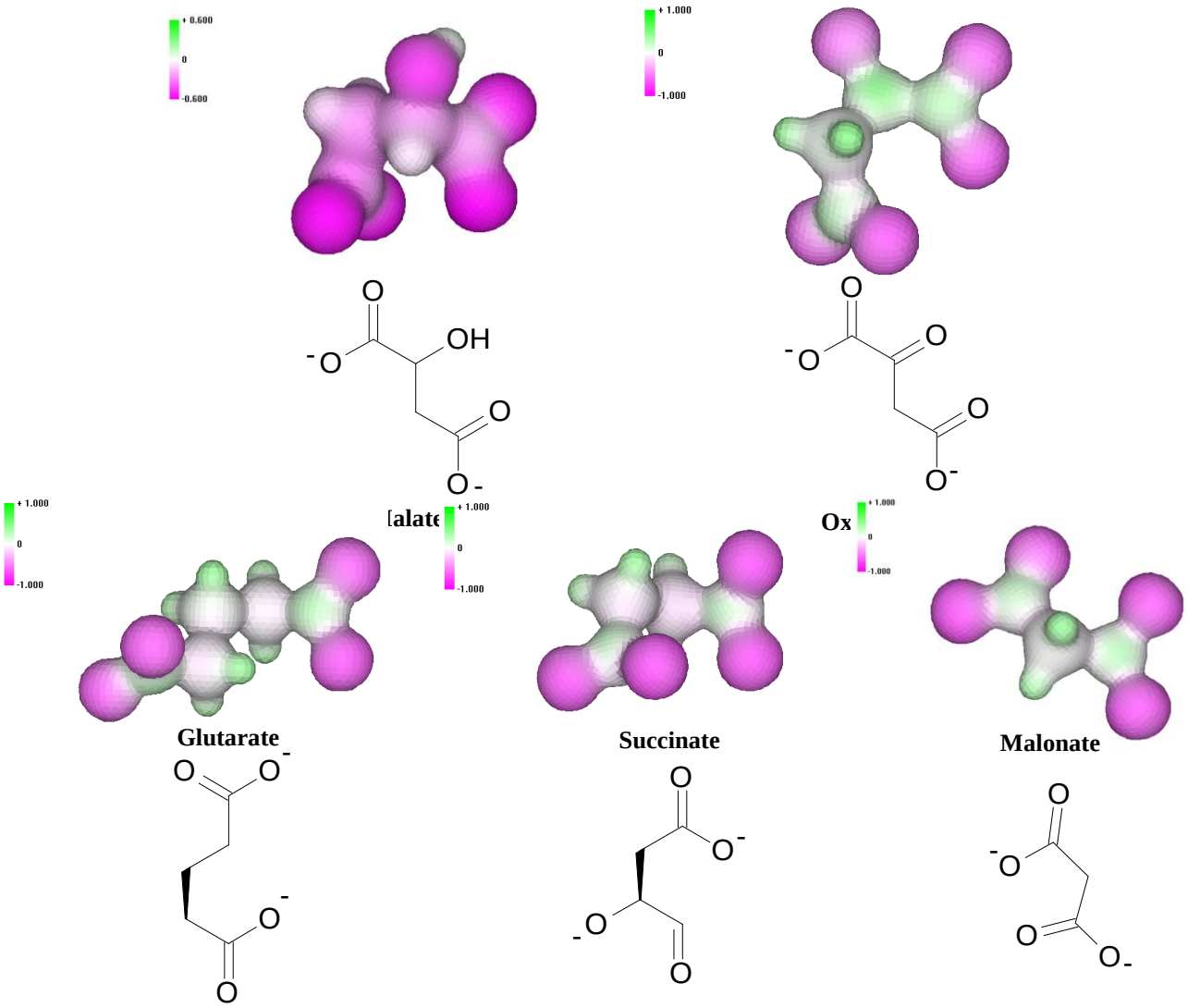
Experiment 3.1

Experiments to Investigate the Behavior of Structural Analogs and Competitive Inhibitors on the Activity of Malate Dehydrogenase

Design Your Own Experiments:

Using the problem set below as a guide, design an experiment to investigate the relationship between structure and affinity for one or more of the potential 3, 4 and 5 carbon analogs of malate shown in scheme 3.1. Fill in Experimental Set Up Work Sheet 3.1 for the experiment where you investigate the effects of the inhibitor(s) you choose as a function of varied oxaloacetate concentration. Fill in Experimental Set Up Work Sheet 3.2 for the experiment where you investigate the effects of the inhibitor(s) you choose as a function of varied NADH concentration. *You may choose to do the experiment either with two different inhibitors and either NADH or oxaloacetate varied,* ***OR*** *you can choose one inhibitor and examine its effects with a) NADH varied and b) oxaloacetate varied.* Remember the data that you collected in experiment 2 when deciding upon the NADH concentrations to use. Your laboratory notebook should contain a detailed discussion of your experimental design and should include some discussion about the structures of the analogs you choose to use. They, together with the substrate-product pair, are shown here both as structures and as electrostatic surface potential diagrams.

Scheme 3.1



You are provided with the following solutions:

10mM Oxaloacetate in H_2O
1mM NADH in H_2O
0.04M Phosphate Buffer containing, pH 8.0
0.1mg/mL Malate Dehydrogenase in 0.1M Phosphate Buffer, pH 7.0
30mM Glutarate, in H_2O
30mM Malonate, in H_2O
30mM Succinate, in H_2O

Decide upon the varied concentrations of Oxaloacetate [5 concentrations] that you want to use, calculate the volumes to add to a total of 3mL and enter the volumes in the Experimental Set Up Work Sheet. Second, choose two different concentrations of each inhibitor that you choose [each of them has Ki values in the range 1-5mM as a guide to choosing your concentrations] and calculate how much of the inhibitor solution you need to add to achieve those concentrations: enter these volumes in the appropriate parts of the work sheet. Finally calculate how much NADH, buffer and water that you will add to each cuvette so that the total volume is 3mL

Experimental Set Up Work Sheet 3.1:

Cuvette #	[OAA], mM	mL OAA	mL Inhibitor	mL H_2O	mL Buffer	mL NADH
NO INHIBITOR						
[ ] Inhibitor 1						
[ ] Inhibitor 1						
[ ] Inhibitor 2						

[ ] Inhibitor 2						

Make up the cuvettes, in triplicate, as detailed above and one cuvette at a time initiate the reaction by the addition of 10μL of the stock Malate Dehydrogenase solution.

Calculate the ΔA/minute and enter the values into the Data Analysis Work Sheet below

Data Analysis Work Sheet 3.2:

[OAA]	Rate #1	Rate #2	Rate #3	Average	Standard Deviation
NO INHIBITOR					
[ ] mM Inhibitor 1					
[ ] mM Inhibitor 1					
[ ] mM Inhibitor 2					
[ ] mM					

Inhibitor 2					

Convert the average rates and standard deviations into rates in terms of nM NADH utilized per minute and enter into Data Analysis Work Sheet 3.3.

Data Analysis Work Sheet 3.3

[OAA], mM	Average Rate nM NADH/minute	Standard Deviation nM NADH/minute	% Standard Deviation
NO INHIBITOR			
[ ] mM Inhibitor 1			
[ ] mM Inibitor 1			
[ ] mM Inhibitor 2			
[ ] mM Inhibitor 2			

Construct LineWeaver Burk Plots for each set of Oxaloacetate Varied Data and analyze each line to obtain a slope and intercept and the associated standard deviations. Enter these values into Data Analysis Work Sheet 3.4.

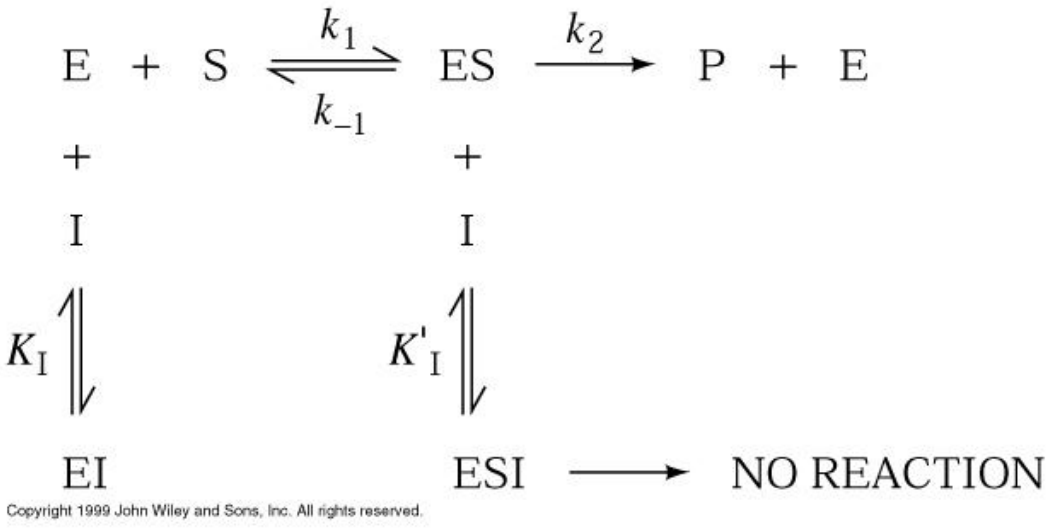
Data Analysis Work Sheet 3.4

Condition	Slope	Std. Deviation	Intercept	Std. Deviation
No Inhibitor				
mM Inhibitor 1				
mM Inhibitor 1				
mM Inhibitor 2				
mM Inhibitor 2				

From the effects of the inhibitor on the slope and intercept determine whether the inhibitor obeys competitive inhibitor behavior and calculate the Ki values form the slope effects.

Mixed Inhibitors

Sometimes the inhibitory molecule can bind not only to free enzyme but also to the enzyme-substrate complex, causing an additional mode of inhibition:



The inclusion of this pathway in the presence of the inhibitor alters the Michaelis Menten Equation for the reaction:

$Vo = \beta Vmax[S]/\{\alpha Km + [S]\}$

Where $\alpha = 1 + [I]/K_i$
And $\beta = 1 + [I]/K_i'$

This has an effect also on the LineWeaver Burk equation for the system:

$1/vo = \{\alpha Km/Vmax\} \times 1/[S] + \beta/Vmax$

slope intercept

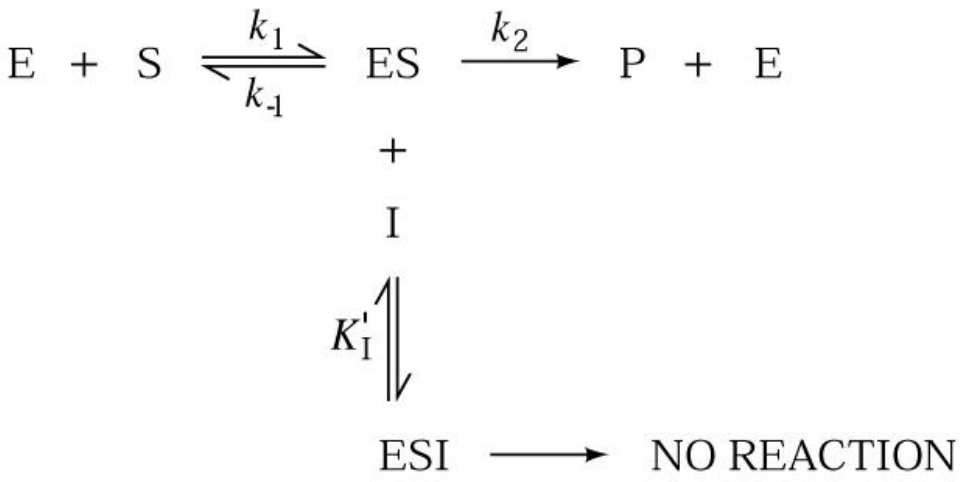
The value of K_i can be obtained by determining the effect of the presence of a known inhibitor concentration on the slope of the LineWeaver Burk plot obtained with the "normal" substrate as the varied substrate:

Slope (with inhibitor present) = slope (no inhibitor present) x α (that is $1 + [I]/K_i$)

While the value of Ki' can be obtained by determining the effect of the presence of a known inhibitor concentration on the intercept of the LineWeaver Burk plot obtained with the "normal" substrate as the varied substrate.

Thus the two Dissociation Constants (Ki and K_I') for inhibitor binding can be readily determined experimentally and have quite different physical significance: Ki represents the tightness of binding to free enzyme while Ki' represents the tightness of binding to the Enzyme-Substrate Complex. When both modes of binding can occur the inhibition is said to be "mixed" type inhibition.

Occasionally an inhibitor will bind only to the Enzyme-Substrate complex and the LineWeaver Burk equation will contain the β term and not the α term in the equation above:



$1/vo = \{Km/Vmax\} \times 1/[S] + \beta/Vmax$

slope intercept

As a result, the intercept of the resultant LineWeaver Burk plot, not the slope, will be altered in the presence of the inhibitor and the inhibition will be "uncompetitive."

When reporting Ki values it is important to indicate whether the Ki value was calculated from a slope effect or from an intercept effect.

Experiment 3.2
Determination of the Inhibition Patterns of Malate Dehydrogenase by Oxaloacetate Analogs, with NADH as the Varied Substrate

In the previous experiment, 3.1, a series of structural analogs of Oxaloacetate/Malate were used as potential inhibitors of Malate Dehydrogenase, with Oxaloacetate as the varied substrate. In this experiment, you will utilize the same inhibitors, but with NADH as the varied substrate. In this case, it is important that you decide what Oxaloacetate concentration to use. If it is too high, you may swamp out the effect of the inhibitors, at least in terms of the "competitive" component of any inhibition [ie the slope effect]. You should choose a concentration that is about the same as the Km concentration for Oxaloacetate that you found in experiment 3.1, ie around 50μM.

The design of the experiment will be essentially the same as in the experiments with Oxaloacetate as the varied substrate and you are provided with the same stock solutions of reagents that you used in that part of the experiment. Design the experiment using Experimental Set Up Work Sheet 3.5

Experimental Set Up Work Sheet 3.5:

Cuvette #	[NADH], mM	mL NADH	mL Inhibitor	mL H_2O	mL Buffer	mL OAA
NO INHIBITOR						
[ ] Inhibitor 1						
[ ] Inhibitor 1						
[ ] Inhibitor 2						

[ ] Inhibitor 2						

Make up the cuvettes, in triplicate, as detailed above and one cuvette at a time initiate the reaction by the addition of 10μL of the stock Malate Dehydrogenase solution.

Calculate the ΔA/minute and enter the values into the Data Analysis Work Sheet 3.6 below.

Data Analysis Work Sheet 3.6:

$[NAD^+]$	Rate #1	Rate #2	Rate #3	Average	Std Deviation
NO INHIBITOR					
[ ] mM Inhibitor 1					
[ ] mM Inhibitor 1					
[ ] mM Inhibitor 2					
[ ] mM Inhibitor 2					

Convert the average rates and standard deviations into rates in terms of nM NADH utilized per minute and enter into Data Analysis Work Sheet 3.7.

Data Analysis Work Sheet 3.7

[NADH], μM	Average Rate nM NADH/minute	Standard Deviation nM NADH/minute	% Standard Deviation
NO INHIBITOR			
[ ] mM Inhibitor 1			
[ ] mM Inibitor 1			
[ ] mM Inhibitor 2			
[ ] mM Inhibitor 2			

Construct LineWeaver Burk Plots for each set of NADH Varied Data and analyze each line to obtain a slope and intercept and the associated standard deviations. Enter these values into Data Analysis Work Sheet 3.8.

Data Analysis Work Sheet 3.8

Condition	Slope	Std. Deviation	Intercept	Std. Deviation
No Inhibitor				
mM Inhibitor 1				
mM Inhibitor 1				
mM Inhibitor 2				
mM Inhibitor 2				

From the effects of the inhibitor on the slope and intercept determine whether the inhibitor obeys competitive or mixed inhibitor behavior and calculate the appropriate Ki values.

Quantitative Analysis of the Data

In the following data set, initial rates were obtained, using 17nM GDH, with Glutamate as the varied substrate at a fixed concentration of NAD^+ (500μM) in the absence or presence of 5mM of the substrate analog, Glutarate, at pH 7.0 in 0.1M Phosphate Buffer containing 10μM EDTA, at 25°C.

Glutamate Concentration, mM	**Initial Rate, No Glutarate, ΔA/minute**	**Standard deviation**	**Initial Rate, 5mM Glutarate Present, ΔA/minute**	**Standard Deviation**
1.0	0.0172	0.00047	0.00687	0.00073
1.5	0.0222	0.00121	0.0098	0.0009
2.5	0.0301	0.0020	0.0146	0.0023
5.0	0.0428	0.0017	0.0253	0.0021
20.0	0.0684	0.0023	0.0461	0.0016

Using this data determine whether Glutarate is a competitive inhibitor with respect to the substrate Glutamate and determine a value for the Inhibition Constant, K_i. From the value for K_i calculate the free energy of binding for Glutarate. The double reciprocal plot of the data is constructed: take the reciprocals of column 1 (gives 1/[S]), column 2 (gives 1/Rate-no inhibitor) and column 4 (gives 1/Rate+ Inhibitor).

From the regression fits of the data, the slopes of the lines are 46.89 +/-3.1 and 135.65+/-3.7 in the absence or presence of inhibitor, respectively, and the intercepts of the lines are 12.69+/-1.8 and 11.27+/-2.1 in the absence or presence of inhibitor, respectively.

First, it is clear that this is competitive inhibition- there is no significant effect on the intercept.

Second, the Ki for the inhibitor can be calculated as follows:

Slope(+ inhibitor) = slope - inhibitor)(1 + [I]/Ki)

Hence:

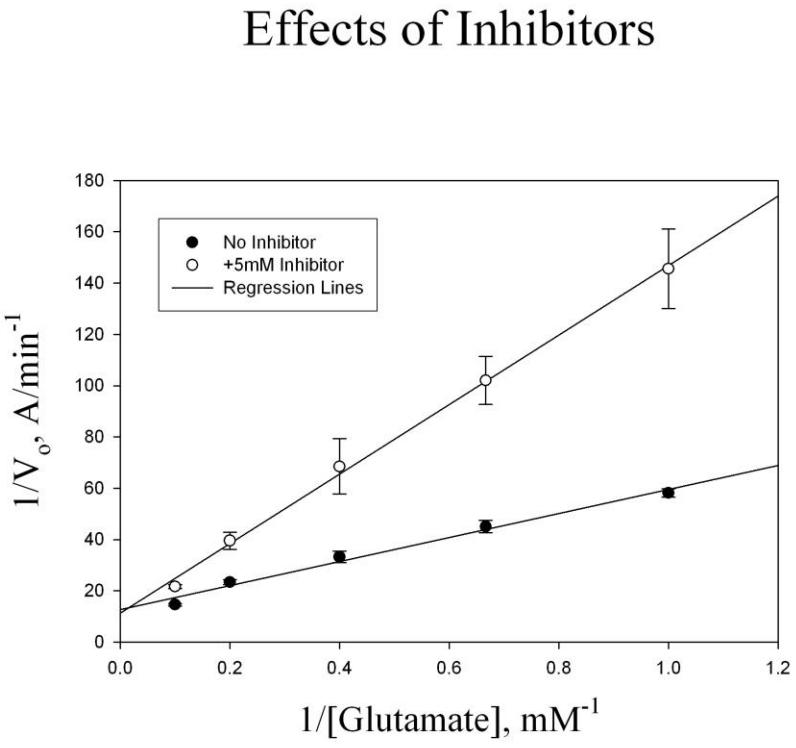
135.65 = 46.89(1 +5/Ki)

Solving for Ki gives a Ki value of 2.6mM. This can be compared to the Km for the uninhibited data set (slope/intercept) which equals 3.7mM. Thus, stating that you are assuming that Km is a reasonable reflection of the affinity of the substrate, it is reasonable to conclude that the inhibitor binds with approximately equal affinity to the substrate.

Problem Set 2

In an experiment with varied concentrations of one substrate in the presence or absence of a fixed concentration of an inhibitor [at 0.45mM] the following data were obtained:

Substrate, mM	Rate (No Inhibitor), ΔA/min	Rate (Plus Inhibitor), ΔA/min



2.0	0.606	0.247
1.0	0.513	0.192
0.5	0.435	0.149
0.33	0.345	0.118
0.25	0.278	0.093
0.2	0.241	0.078
0.17	0.213	0.077

How tightly does the inhibitor bind relative to the substrate?

In a separate experiment, the pH dependence of Km for the substrate was examined and the following data obtained:

pH	**Km, mM**
6.0	5.1
7.0	5.2
8.0	3.9
9.0	0.9
10.0	0.6

What would you conclude about the nature of the interaction between the substrate and the enzyme?

EXPERIMENT 4
Determination of Thermodynamic Parameters from Kinetic Experiments:

Experiment 4.1
Determining the Thermodynamic Parameters of Inhibitor Binding to Malate Dehydrogenase.
As discussed in the introduction to this section on Inhibitor effects, the Ki for an inhibitor is directly related to the free energy of binding of the inhibitor, ΔG. By studying the effects of temperature on Ki, values for both the enthalpy changes and entropy changes associated with inhibitor binding to the enzyme can be determined. Using the above experiment as a guide, a class experiment, where each pair of students determines a Ki value for the inhibitor at a particular temperature can be conducted. At the end of the experiment the class pools their data for the values of Ki at whatever temperature they used and each student can calculate the appropriate thermodynamic parameters. For example by using a range of temperatures from 15^{o}C to 40^{o}C the relative contributions of enthalpy and entropy to the binding of each of the above inhibitors can be determined.

Problem Set 2:
The Following data was collected during studies of the inhibition of Aspartate Aminotransferase by a series of structural analogs of L-Aspartate:

Parameter	**23^{o}C**	**30^{o}C**	**37^{o}C**	**45^{o}C**
Succinate	0.054mM	0.2mM	0.45mM	1.37mM

Glutarate	0.42mM	0.48mM	0.53mM	0.58mM
D-Glutamate	1.0mM	0.8mM	1.0mM	0.9mM
L-OH-Glutarate	5.8mM	6.5mM	4.6mM	3.2mM
Km (Asp)	2.1mM	1.2mM	0.8mM	0.7mM
Vmax	0.23nM/sec	0.31nM/sec	0.64nM/sec	1.49nM/sec

Calculate the appropriate thermodynamic parameters from this data and draw what conclusions you can from the complete set of results.