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:

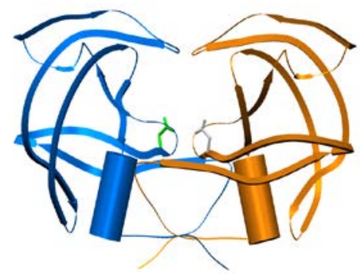
Membrane or Location	Marker Enzyme
Mitochondrial: Inner Membrane	Succinate-Cytochrome c Reductase Rotenone Sensitive NADH Cytochrome c Oxidase
Mitochondrial: Outer Membrane	MonoAmine Oxidase Rotenone Insensitive NADH Cytochrome c Oxidase
Endoplasmic Reticulum	RNA Protein Synthesis Enzymes NADPH Cytochrome 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.

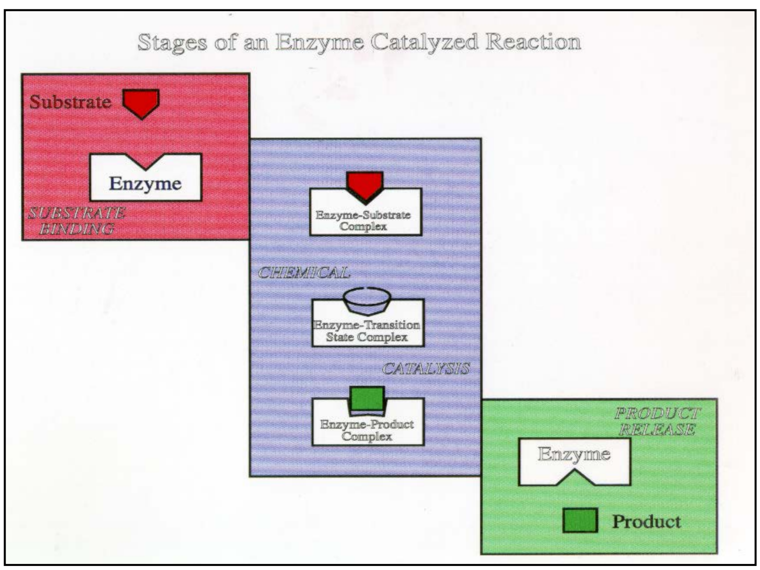
Virtually all cell types in eukaryotic biology differentiate during their life time and usually certain enzyme activities can be associated with the stage of differentiation of the cell: for example chondrocytes: [cells that help create bone formation] differentiate to produce specific types of collagen 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:



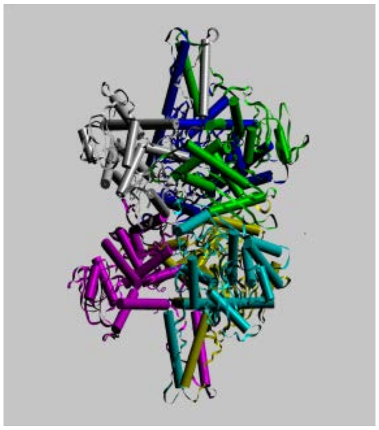
1] **substrate binding**,

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 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,



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

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.

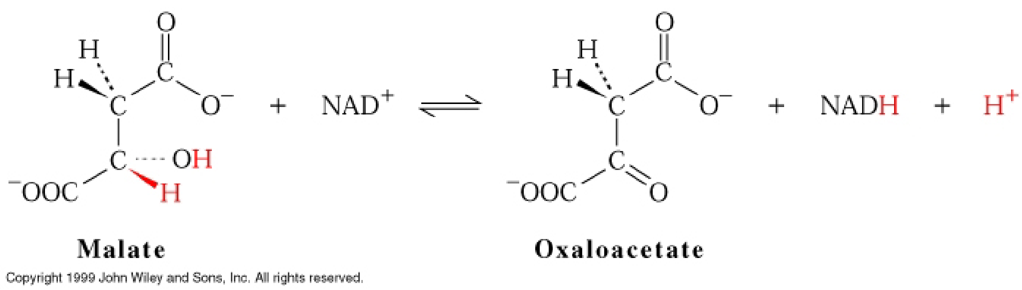
Measurement of the activity of enzymes also plays a critical role in many other types of experiments that can be used to investigate "structure-activity" relationships in proteins and fully understanding the types of information that apparently simple measurement of enzyme activities can provide is the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

Review of the Roles of Malate Dehydrogenase

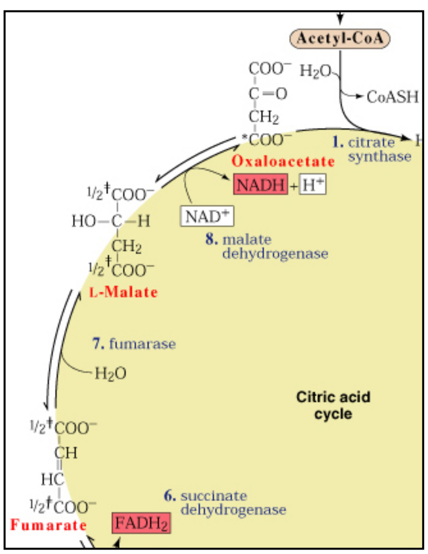
Malate Dehydrogenases catalyze the reaction:

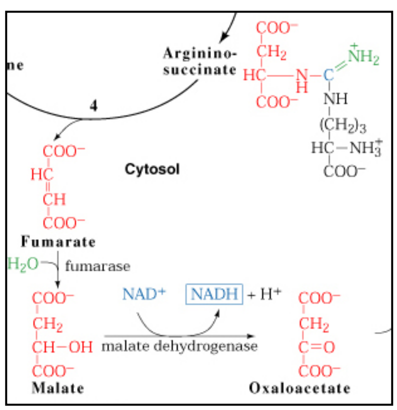
$$\textbf{Malate} + \textbf{NAD}^{+} \leftrightarrow \textbf{Oxaloacetate} + \textbf{NADH}$$

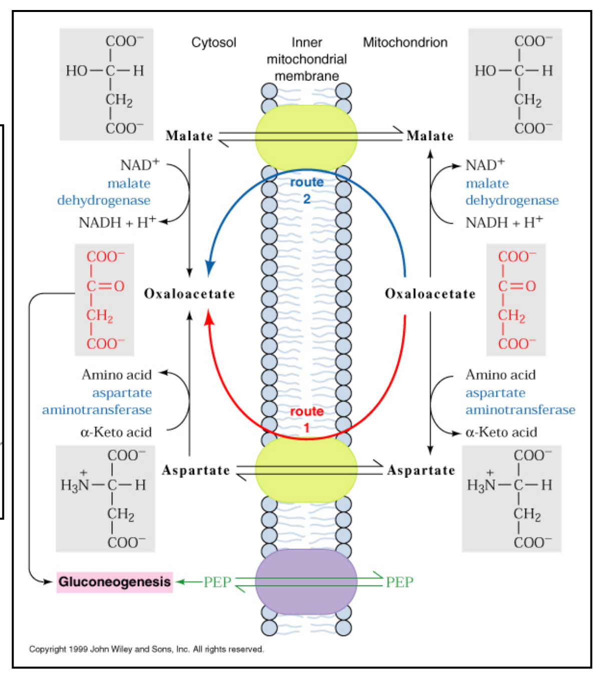
Involving a simple hydride transfer from the 2 position of Malate to the nicotinamide ring of NAD^+ to give NADH. During the process a proton is also released to the solvent.



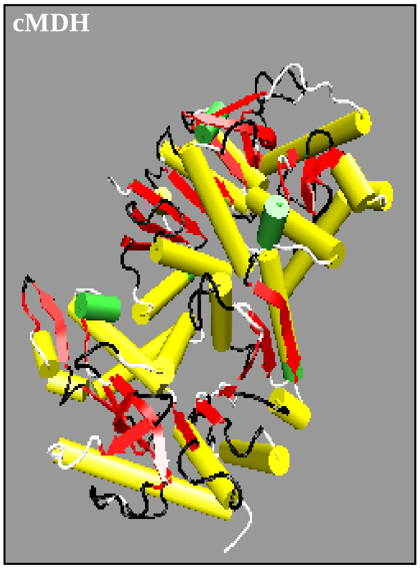
This reaction plays a number of important roles in metabolism, illustrated by a reaction in the Tricarboxylic acid cycle (left), a reaction critical to the Urea Cycle (center), and a reaction playing a role in the shuttling of reducing equivalents into mitochondria (right):

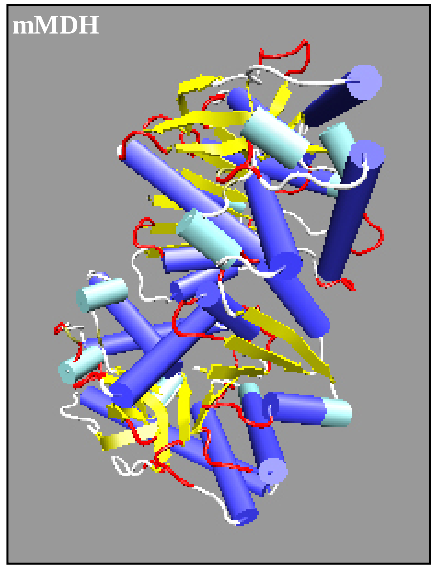






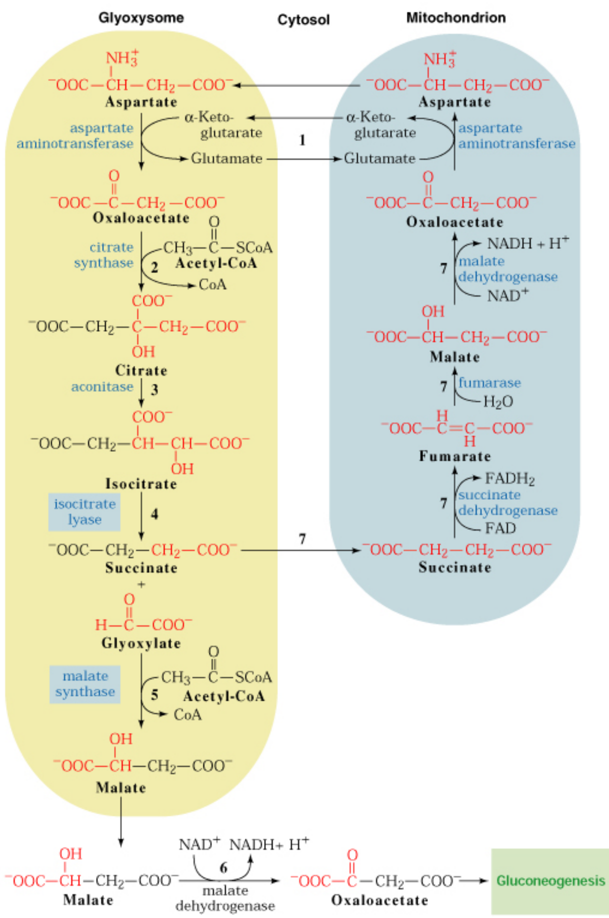
In the later example it is clear that there must exist Malate Dehydrogenase in at least two different locations within the cell and in fact there are two distinct isoenzymes, a cytoplasmic MDH [cMDH] and a mitochondrial MDH [mMDH] in higher eukaryotes which have different amino acid sequences and different three dimensional structures: cytoplasmic MDH and mitochondrial MDH. [In the final section of each of the major





types of experiments described are group projects that involve comparisons of the properties of cytoplasmic and mitochondrial forms of malate dehydrogenase.] Each MDH isoform is a dimer, and each subunit contains two domains, with a classic dinucleotide binding domain and a malate binding domain.

In mitochondria, MDH is thought to form loose multienzyme complexes with several other enzymes sharing substrates, in particular Aspartate AminoTransferases which catalyze the transamination of Glutamate and



Oxaloacetate to give Aspartate and 2-Oxoglutarate, a key reaction in the Glyoxylate Cycle and 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:

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

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

For glyoxosomal malate dehydrogenase, 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. (If using mMDH from porcine, assume the Km for Oxaloacetate is in the range of 15-35 micromolar.) 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:
0.05M 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 0.025M 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 0.05M 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 0.05M 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:

$\{0.152 \times 6\}/ 0.93 = 0.98$mg/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:
0.05M 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:

$$\mathbf{e/v_o = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2] + \Phi_{12}/[S_1xS_2]}$$

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:

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

or for Substrate 2, S_2 varied: we get:

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

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:
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 0.05M Phosphate Buffer stock as before.

Experiment Set Up Work Sheet 2.2:

[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 2.3-2.5 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 2.3-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 2.3-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 2.4

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 2.5

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 S_1 is NAD and S_2 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

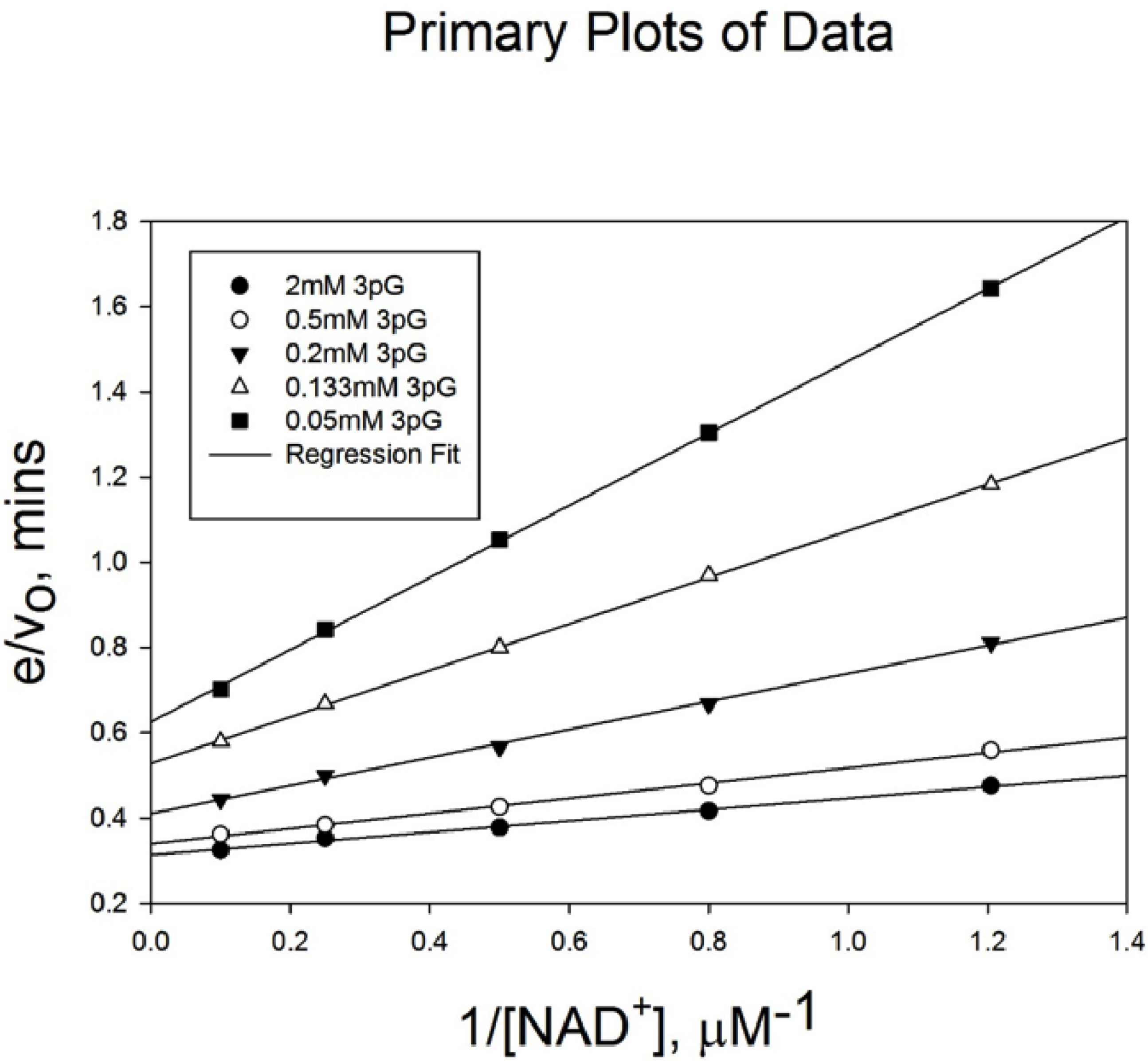
From the above data, determine the values of the 4 initial rate parameters for the reaction and determine the Km values for each substrate and the overall maximum rate of the reaction.

For Substrate 1, S1, varied, we get:

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

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:

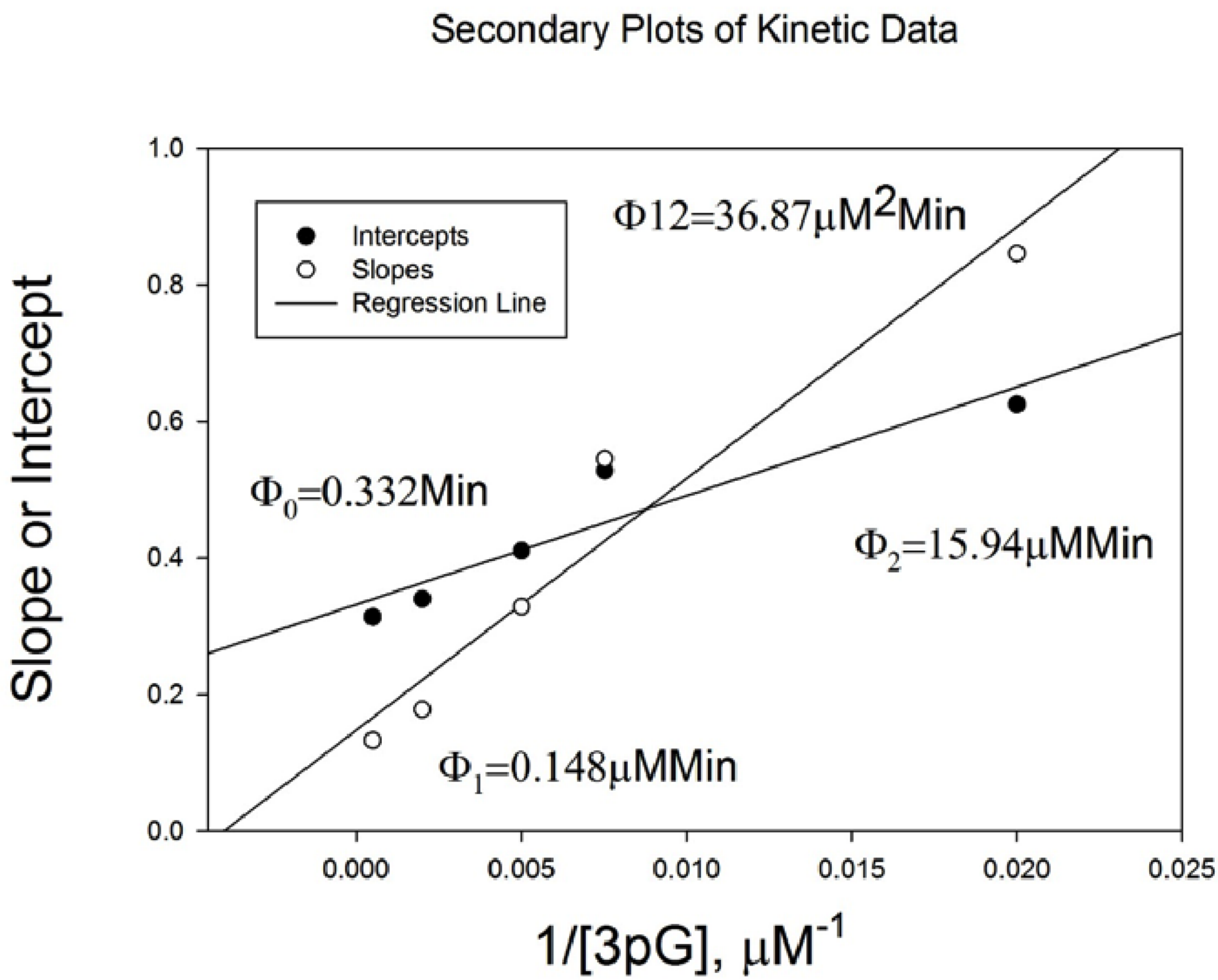
Slope = $\{\Phi_1 + \Phi_{12}/[S_2]\}$

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 Φ_1/Φ_0. = 0.148/0.332 = 0.446μM Likewise, the Km for S_2 becomes Φ_2/Φ_0. = 48.0μM. From either equation, it should be apparent that the true V_{max} for the enzyme is $1/\Phi_0$. = 1/0.332 = 3.01min^{-1}. But what are the units. Here, but rarely in practice, we plotted e/Vo, where e is the active site concentration in μM, and hence the units of Vmax are min^{-1}. If we had plotted 1/vo, as is often done, the units of Φo would have been $\mu M/min^{-1}$, hence if we then converted the enzyme concentration to μM and divide we would obtain the correct units.

If primary plots were not e/v_o but rather 1/v_o, you will need to correct for enzyme. 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 = 23.8 μM, thus 0.23mg/mL = 23.8 x 0.23 = 5.5μM

To obtain the concentration in the reaction mix:

Stock concentration x Volume Added/Total volume:

(5.5x 20)/1020 = 0.108 μM

Using data from primary plots that included 1/v_o instead of e/v_o, the value for Φ_0 would then need to be corrected for enzyme concentration by: [e/((1/Φ_0□] and V_{max} will now have the units min^{-1} instead of $\mu M min^{-1}$.

Since the Km values derived from either a primary plot containing 1/v_o or e/v_o 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 1.1
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
Determining Thermodynamic Parameters for Enzyme Catalyzed Reactions

Synonyms
Activation energy, enthalpy, entropy, Gibb's free energy, energy diagrams

Synopsis
A variety of thermodynamic parameters can be obtained from temperature dependence studies of various kinetic and equilibrium parameters for enzyme catalyzed reactions. These include the activation energy for the overall reaction as well as activation energies for discrete steps in the enzyme catalyzed reaction which are obtained using Arrhenius plots constructed for the temperature dependence of appropriate rate constants. Temperature dependence of various equilibrium constants (for the overall chemical reaction or for discrete binding steps in the enzyme cycle) and van't Hoff plots give the enthalpy and entropy contributions to the various steps. For either type of plot constructed using initial rate data, it is necessary to understand the formal kinetic mechanism and the nature of the rate limiting step in the overall reaction to fully interpret the data. Non-linear effects in either type of plot can result from changing rate limiting steps or phase transitions as the temperature varies.

Introduction and Background: Connection to Foundational Concepts of Reactions
The effects of temperature
Increased temperature, in general, leads to increased reaction rates as a result of increased collisional frequency (small effect - frequency of collisions is proportional to square root of T in K) and an increase in the proportion of the molecules that have sufficient energy to react (large effect due to shift in Maxwell Boltzmann distribution) when they collide correctly. Increased reaction rates, reflected in the rate constants for the reaction may affect forward and reverse reactions differently resulting in equilibrium constant effects of temperature. In the energy diagram for a reversible reaction, increased temperature in effect "raises" the energy of the reactants while decreased temperature lowers the "energy" of the reactants. Temperature changes have little effect on the energy of the transition state. The effects of temperature on reaction rates or equilibrium constants are described quantitatively by the Arrhenius and Van't Hoff equations, respectively, and allow the appropriate thermodynamic data to be calculated from the temperature dependence of either rate or equilibrium constants.

As a general rule of thumb in biological systems a 10 K rise in temperature approximately doubles the rate of a reaction.

All enzyme catalyzed reactions proceed through three phases (Figure 4.1), substrate binding, chemical catalysis and product release. Each of these phases can be characterized by some type of energy barrier (Figure 4.2), and the magnitude of the energy changes involved experimentally determined by measuring the effects of temperature on the appropriate parameter of the reaction.

In essence, the thermodynamics that govern these steps come down to some type of transition state energy and some type of overall free energy change, and are governed by the Arrhenius and van't Hoff relationships, respectively.

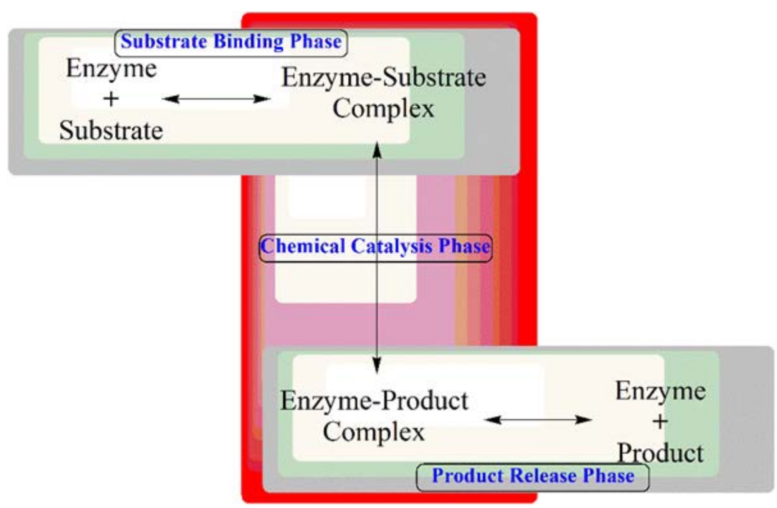


Figure 4.1. Scheme of the three phases of any enzyme reaction including the substrate binding phase, the chemical catalysis phase and the product release phase.

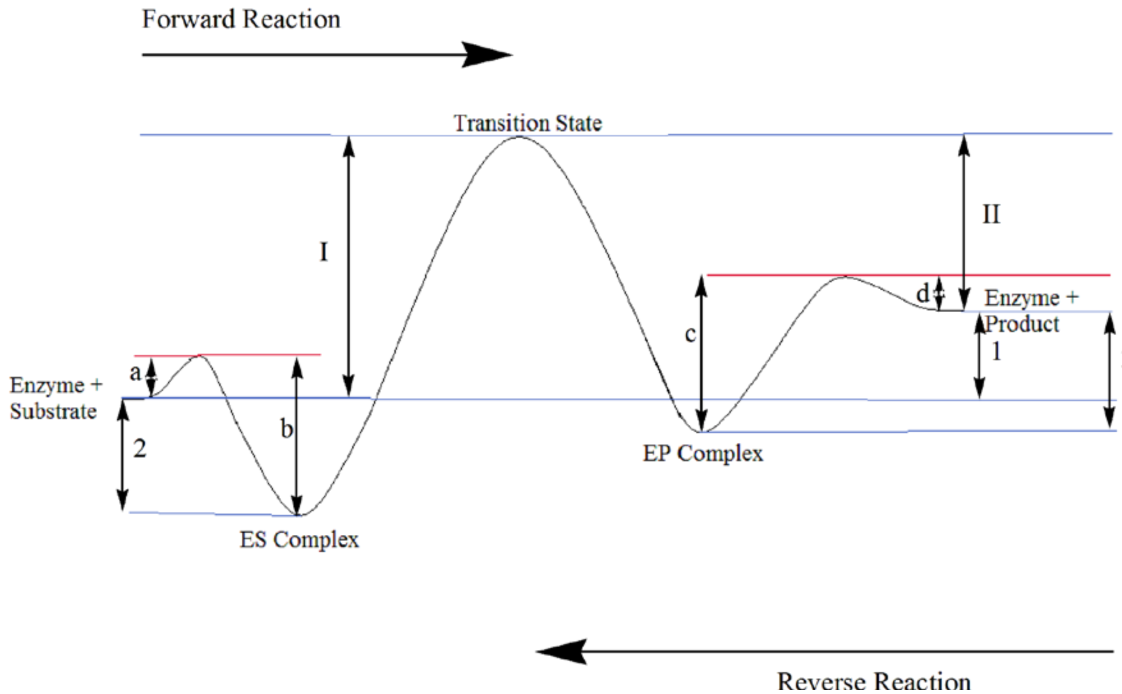


Figure 4.2. Energy diagram for forward and reverse reactions of a simple one substrate enzyme catalyzed reaction defining the relationship of the energy to various thermodynamic parameters: I, the activation energy of the forward reaction, related to k_{cat} (forward), II, the activation energy of the reverse reaction, related to k_{cat} (reverse)1, ΔG for the reaction S←→P, 2, ΔG for substrate binding, related to the dissociation constant for the substrate from the ES complex, 3, ΔG for product binding, related to the dissociation constant for the product from the EP complex, a, the activation energy for substrate binding, related to the on velocity constant for substrate binding, b, the activation energy for substrate release from the ES complex, related to the off velocity constant for substrate from the ES complex, c, the activation energy for product release from the EP complex, related to the off velocity constant for product from the EP complex, and d, the activation energy for product binding, related to the on velocity constant for product binding.

The Arrhenius Equation:

The Arrhenius equation:

$$k = Ae^{-E_a/RT}$$

where k is the rate constant for the process, Ea is the activation energy, R the gas constant and T the temperature in degrees Kelvin.

If we take the log of both sides of this equation and separate the pre exponential term we get:

$$\ln k = \ln(Ae^{-E_a/RT}) = \ln A + \ln(e^{-E_a/RT})$$

and:

$$\ln k = \ln A - E_a/RT$$

allowing lnk to be plotted versus 1/T, the Arrhenius plot with a slope of -Ea/RT and an intercept of ln A (Figure 4.3)

For a chemical reaction, whether catalyzed or not, k is a chemical reaction rate constant, and is usually expressed in units of s^{-1}(for a 1st order rate constant) or $M^{-1}s^{-1}$ (for a 2nd order rate constant).

The pre-exponential factor or frequency factor, A, is related to molecular collisions, and reflects the frequency of molecules that collide in the correct orientation and have enough energy for the reaction to occur. A is

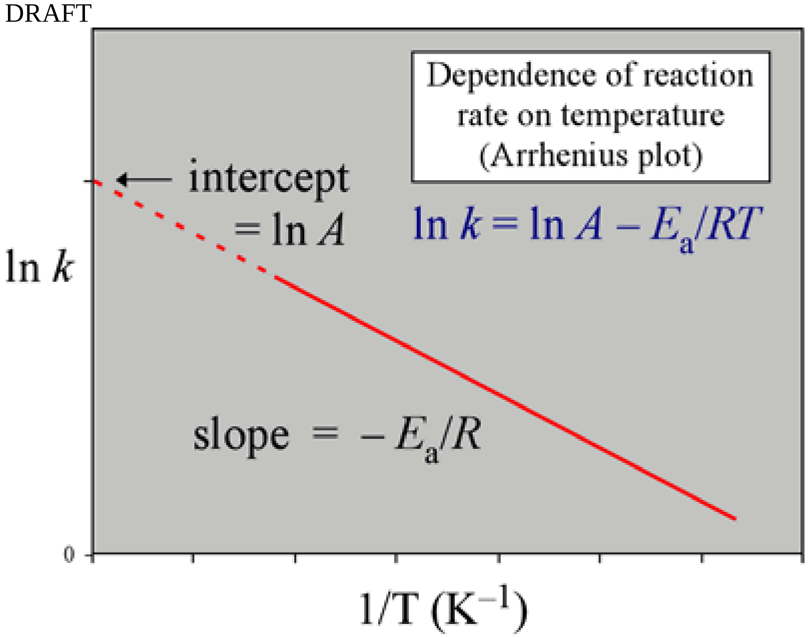


Figure 4.3. Arrhenius plot indicating the parameters that can be derived from the slope and intercept.

determined experimentally, and varies for different reactions. It has units of L $mol^{-1}s^{-1}$ or $M^{-1}s^{-1}$ (for 2nd order rate constants) and s^{-1} (for 1st order rate constants), and is temperature dependent. The pre-exponential factor is hard to extrapolate since lnk is often only linear over a narrow range of temperatures.

The activation energy, Ea, is the minimum energy that the reactant(s) must acquire to reach the transition state, and once the transition state is reached, the reaction can proceed in the forward direction towards product(s), or in the opposite direction towards reactant(s). The units of the activation energy are kJ/mol.

R is the gas constant, and has a value of 8.314 J/mol K.

T is the absolute temperature and is expressed as degrees Kelvin (K).

For an enzyme catalyzed reaction, the rate of the reaction may or may not be limited by the chemical step in the reaction since either the chemical step or the product release step is often the rate limiting step. While the "activation energy" can be experimentally determined using the Arrhenius equation, the identity of the step involved may not be obvious unless the formal kinetic mechanism of the enzyme is known.

The van't Hoff Equation

The equilibrium constant for a process, K_{eq}, is related to the Gibbs free energy, ΔG^o, by the relationship:

i) $$\Delta G^o = -RT\ln K_{eq}$$

and ii) $$K_{eq} = e^{-\Delta G/RT}$$

Since

$$\Delta G^o = \Delta H^o - T\,\Delta S^o$$

Taking the natural log of equation ii and substituting ($\Delta H^o - T\,\Delta S^o$) for ΔG^o gives:

$$\ln Keq = (-\Delta H^o/R)\,1/T + \Delta S^o/R$$

which gives rise to the van't Hoff plot, Figure 4.4, with a slope of $-\Delta H^o/R$ and an intercept of $\Delta S^o/R$ allowing thermodynamic parameters for the equilibrium to be calculated from measurements of the effects of temperature on the equilibrium constant for a process.

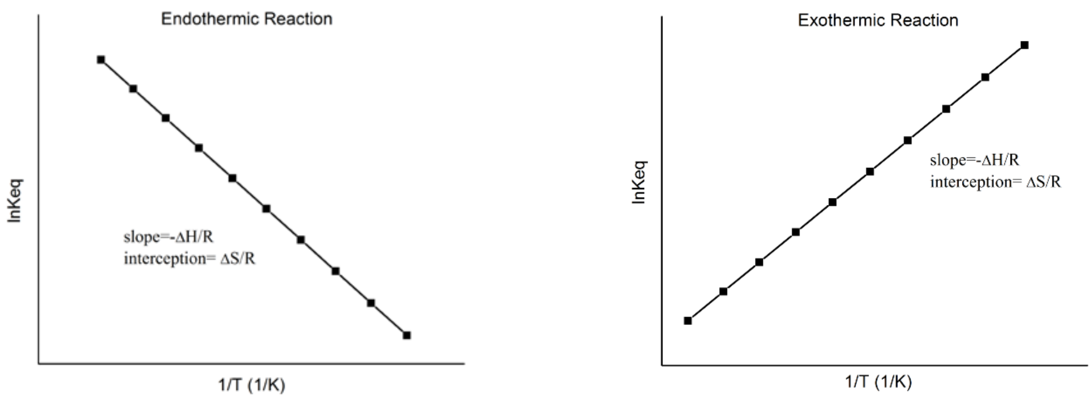


Figure 4.4. van't Hoff plots for endothermic reaction (left) and exothermic reaction (right) indicating the parameters that can be derived from the slope and intercept. Plots reproduced from: http://commons.wikimedia.org/wiki/File:Endothermic_Reaction_van%27t_Hoff_Plot.png#mediaviewer/File:Endothermic_Reaction_van%27t_Hoff_Plot.png & http://commons.wikimedia.org/wiki/File:Exothermic_Reaction_van%27t_Hoff_Plot.png#mediaviewer/File:Exothermic_Reaction_van%27t_Hoff_Plot.png

In terms of an enzyme catalyzed reaction, the equilibrium constant can be for the overall chemical reaction catalyzed (remember the enzyme simply speeds the rate of attainment of equilibrium but does not affect the position of the chemical equilibrium), or can be for one of the substrate or product binding steps in the reaction.

Examples with NAD(P)$^+$ linked Dehydrogenases

Experiments to determine thermodynamic parameters can be done with any enzyme provided there is an experimental way to follow either overall rate or binding, and chemical equilibrium. The following examples with NAD(P)+ linked dehydrogenases will illustrate the utilization of these approaches.

Enzymes such as Malate Dehydrogenase or Glutamate Dehydrogenase catalyze reactions involving the conversion of NAD(P)+ to NAD(P)H which can be easily monitored spectrophotometrically since NAD(P)H absorbs light at 340nm while NAD(P)+ does not. Figure 4.5 illustrates the definitions of initial rate and equilibrium position that apply to any enzyme catalyzed reaction. In each case, the resultant data gives a measure of the amount of product [or loss of substrate] as a function of time, which is plotted as in Figure 5 to give the initial rate of the reaction. You will note that, as the reaction proceeds, it will eventually slow down and, in time, reach an equilibrium position. For the study of enzyme kinetics, it is critically important that you measure the "initial rate" of the reaction. How do you know that the rate measured is the initial rate? First, three or more of the data points used to determine the rate must be strictly linear, and second, the extrapolation to t=0 should give the starting point of whatever concentration is being measured and should be consistent with the condition before the reaction was initiated. To determine the equilibrium position of the reaction much more enzyme can be used since, unless the enzyme concentration becomes stoichiometric with the reactants and products, it will not perturb the equilibrium position.

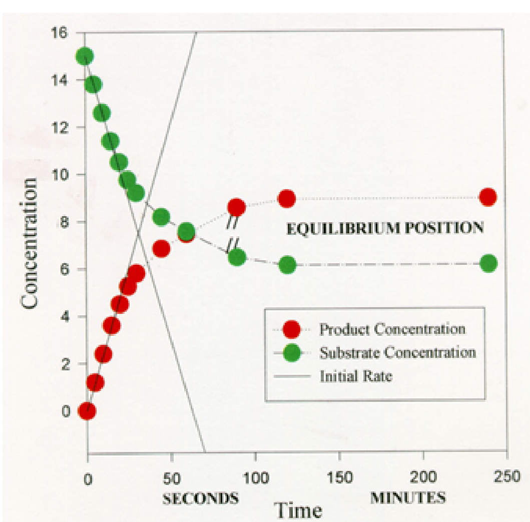


Figure 4.5. Illustration graphically of the initial rate and equilibrium positions for an enzyme catalyzed reaction.

The initial rate can be calculated from the first 20-30 seconds of the reaction while the final equilibrium position is obtained when the absorbance at 340nm no longer changes. If such experiments are repeated at a series of temperatures, say between 288K (15°C) and 313K (40°C), data for either an Arrhenius or van't Hoff plot can be obtained. Remember that enzymes can denature often at temperatures around 40-50°C so extended temperature ranges cannot be used unless the enzyme is particularly thermostable.

Using Initial Rate Data in an Arrhenius Plot
It is important to remember that for the determination of the activation energy for an enzyme catalyzed reaction you should perform the experiments at saturating substrate concentrations otherwise you may also measure effects of temperature on the equilibrium of binding the substrates- Under these conditions you are, in effect, measuring the turnover number of the enzyme (units: time^{-1}) at different temperatures

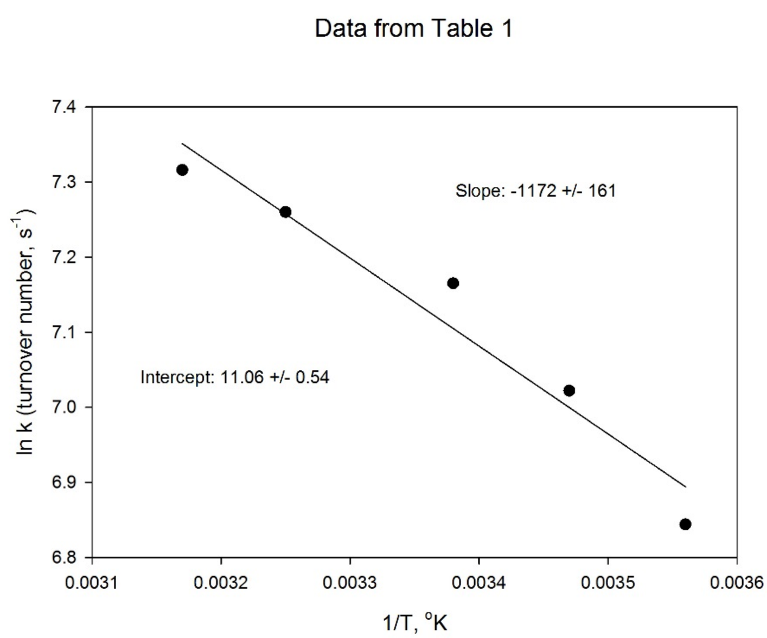
If you collected the following data (Table 4.1) using an enzyme concentration of 0.01micrograms/mL and 1mM Oxaloacetate and 0.1mM NADH:

Table 4.1

Temperature K (^{o}C)	Measured Initial Rate ΔA_{340nm}/min	Rate, mM/minute	Turnover Number, seconds^{-1}
281 (8)	0.103	0.0166	939
288 (15)	0.123	0.0198	1121
296 (23)	0.142	0.0228	1294
308 (35)	0.156	0.0251	1422
315 (42)	0.165	0.0265	1504

From the resultant Arrhenius Plot, Figure 4.6, an activation energy of 9.74KJ/Mole is calculated

Figure 4.6



Using Equilibrium Data for the van't Hoff Plot
Using the same substrate concentrations as above for the Arrhenius experiments, but with a larger enzyme concentration to allow the reaction to proceed to equilibrium in a reasonable period of time, an absorbance of 0.076 was obtained at 25^{o}C (298K), indicating that 0.0122mM NADH remained at equilibrium.

Since the starting concentration of NADH was 0.1mM, 0.1-0.0122= 0.0878mM NAD+ was produced, hence the Malate concentration at equilibrium is also 0.0878mM. By subtraction from the 1mM oxaloacetate at the start of the reaction, the final oxaloacetate concentration is 0.9122mM.

Since the overall reaction is:

$$\text{Malate} + \text{NAD} + \leftrightarrow \text{Oxaloacetate} + \text{NADH} + H^+$$

$$K_{eq} = ([\text{Oxaloacetate}][\text{NADH}][H^+])/([\text{Malate}][\text{NAD}^+])$$

Using molar concentrations this becomes:

$$(0.0009122 \times 0.0000122 \times 0.00000001)/(0.0000878 \times 0.0000878)$$

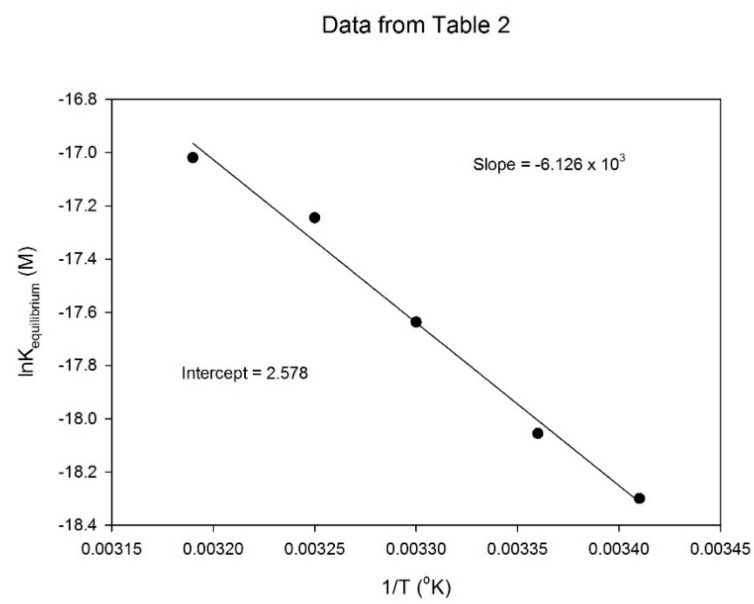
$K_{eq} = 1.44 \times 10^{-8}$M, and since $\Delta G^o = -RT\ln K_{eq} = 44.7$kJ/mole

Using different temperatures the data in Table 4.22 was obtained

Temperature, ^oK	$K_{equilibrium}$ (M)	$\ln K_{eq}$
293	1.13×10^{-8}	-18.300
298	1.44×10^{-8}	-18.056
303	2.19×10^{-8}	-17.637
308	3.24×10^{-8}	-17.245
313	4.06×10^{-8}	-17.019

From the resultant van't Hoff plot, Figure 4.7, $\Delta H^o = 0.737$kJ/mol and $\Delta S^o = 0.310$eu/mol.

Figure 4.7



Application to Individual Steps in the Reaction

While the examples discussed above relate to the overall reaction catalyzed by an enzyme , the same approaches can be applied to individual steps in the reaction. For example, the binding of a single substrate where the rate constant for binding or dissociation can be followed by rapid reaction approaches, or the dissociation constant (K_D) for binding can be determined using some type of ligand binding measurement. The temperature dependence of such parameters yields the data for either an Arrhenius plot (rate constants) or van't Hoff plot (remembering that $K_D = 1/K_{eq}$).

Depending upon the formal kinetic mechanism of the enzyme, individual rate constants or dissociation constants can also be obtained from enzyme kinetics (initial rate) data as shown in Table 3.

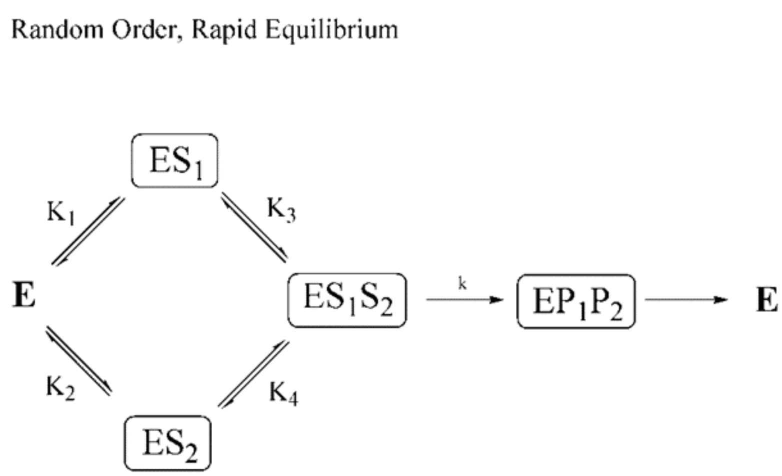
Table 4.3. Relationships involving initial rate parameters and kinetic mechanisms.

Mechanism	Φ_0	Φ_1	Φ_2	Φ_{12}	V_{max} ($1/\Phi_0$)	$K_m^{S_1}$ (Φ_1/Φ_0)	$K_m^{S_2}$ (Φ_2/Φ_0)
COSS	$1/k_9+1/k_7+(k_6+k_7)/k_5k_7$	$1/k_1$	$\frac{k_4k_6+k_4k_7+k_5k_7}{k_3k_5k_7}$	$\frac{k_2(k_4k_6+k_4k_7+k_5k_7}{k_1k_3k_5k_7}$	complex	complex	complex
CORE	$1/k$	0	k_2/k	K_1K_2/k	k		k_2
RORE	$1/k$	K_4/k	K_3/k	K_1K_3/k	k	K_4	K_3
Enzyme Substituted	$1/k_3 + 1/k_7$	$(k_2+k_3)/k_1k_3$	$(k_6+k_7)/k_5k_7$	0	complex	complex	complex

COSS: Compulsory Order, Steady State
CORE: Compulsory Order, Rapid Equilibrium
RORE: Random Order, Rapid Equilibrium

Either individual initial rate parameters or combinations of initial rate parameters give rise to rate constants or dissociation constants for certain mechanisms. For example in a rapid order, rapid equilibrium mechanism (Figure 8), the Km for each substrate is the dissociation constant for dissociation of that substrate from the ternary complex while Vmax is the rate constant for the interconversion of substrate ternary complex to product ternary complex (or as discussed earlier the rate limiting release of products from the ternary complex.

Figure 8.



Non Linear Arrhenius or van't Hoff Plots

While Arrhenius and van't Hoff analysis have been used with many enzymes to give appropriate thermodynamic data (1-4), certain factors such as enzymes in a membrane environment, or ones that undergo some type of allosteric transition may produce non-linearity in Arrhenius plots or van't Hoff plots. Such effects can be classified into two categories: involving (i) thermodynamic factors that modify the elementary steps of the reaction, and (ii) changes in rate-limiting steps that occur in the experimental temperature range (5-7).

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 group of students will determine a Ki value for the inhibitor at a particular temperature will be conducted. At the end of the experiment, the class will pool their data for the values of Ki at whatever temperature they used and each group 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 3.1:

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.

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