Alcohol dehydrogenase

An enzyme kinetic study

Protein Chemistry and Enzyme Kinetics

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Biotechnology program

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Introduction

If there is a magical component to life, surely the enzymes could be one of the things close to that. Thanks to enzymes, reactions that could take hundreds of years to complete in the "real world," occur in seconds, when they present.
Chemical catalysts, like platinum, speed reactions, but enzymes (which are simply super-catalysts) definitely win against them. Enzyme-catalyzed reactions can be studied in a variety of ways. Kinetic analysis of enzyme-catalyzed reactions, is though one of the most commonly used methods for studying an enzyme mechanism.

Theory

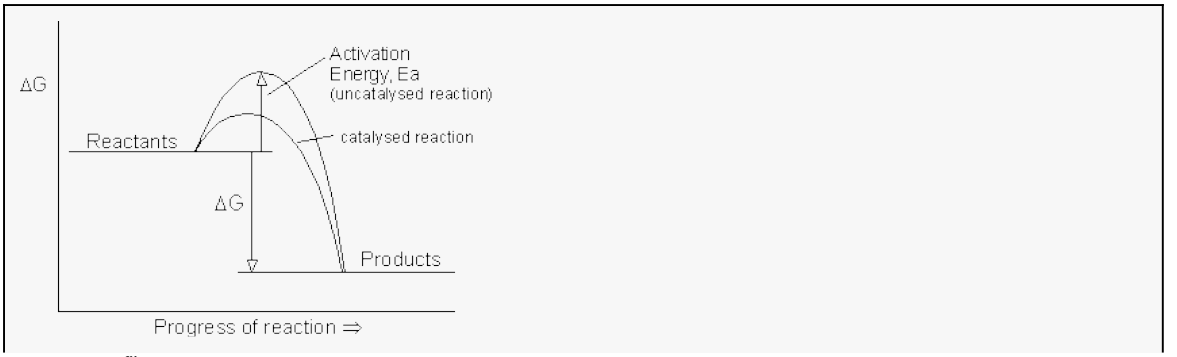
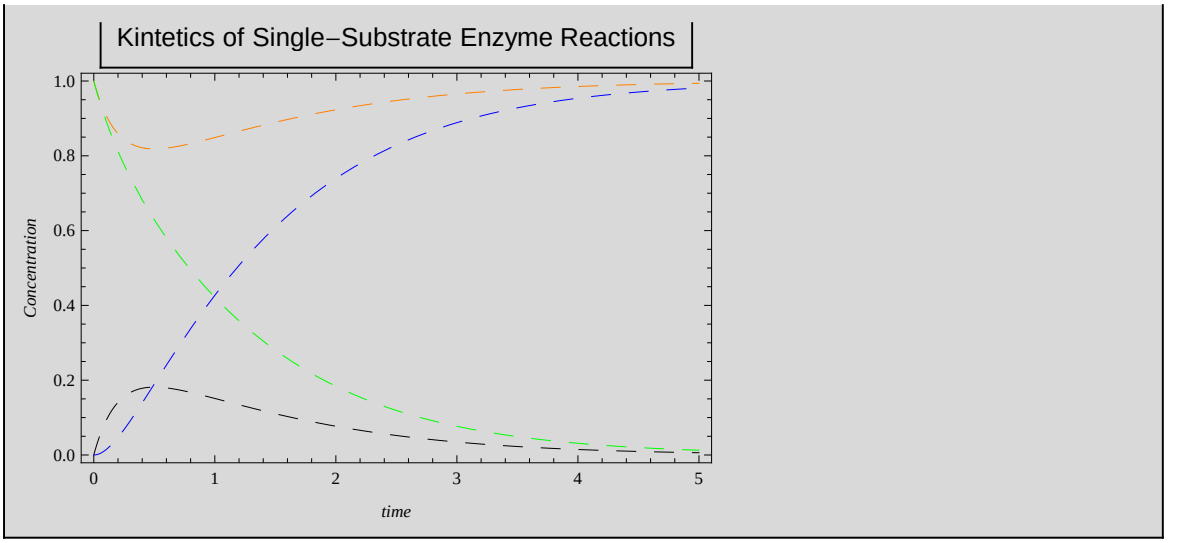


Figure 1; Free energy profiles.
Source http://www.avogadro.co.uk/h_and_s/entropy.htm

The first important rule to understand any kind of catalysis is that, it is a substance that speeds up a reaction without being consumed itself. It does no change the overall energy of a reaction. Given enough time, a non-catalyzed reaction will get to the same equilibrium as a catalyzed one. Another feature to note is; reducing the activation energy needed to reach the transition state of the catalyzed reaction. Lowering activation energies of reactions helps the molecules more easily to reach the energy necessary to get to the point where the reaction occurs.
So catalyst ends up after a reaction just the way it started so it can catalyze other reactions, as well. Enzymes share this property, but in the middle, during the catalytic action, an enzyme is transiently changed. It is also important to recognize that enzymes are not fixed, rigid structures, but rather flexible. Flexibility allows movement, and movement facilitates alteration of electronic environments necessary for catalysis. Enzymes are, thus, much more efficient than rigid chemical catalysts as a result of their abilities to facilitate the changes necessary.



orange - the enzyme concentration
blue - the product concentartion
green - the reactant concentration
black - SE complex concentration

Figure 2; Reaction progress curves for the loss of substrate [S] and production of product [P] during an enzyme-catalyzed reaction. (Sorce:Workbooks-Mathematica, Physical Chemistry-Kinetics by Horia Metiu, year 2006, university of California-Santa Barbara

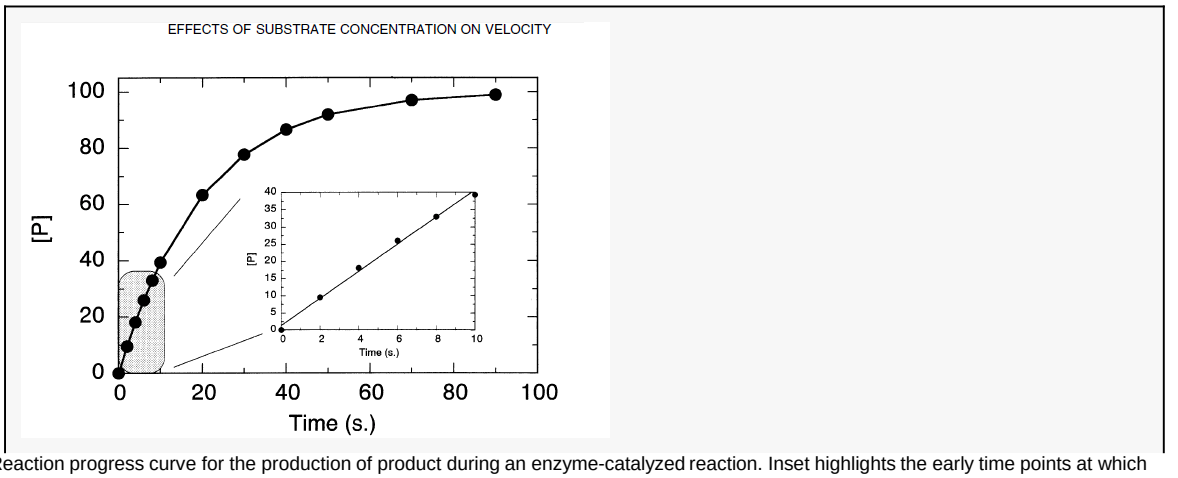


Figure 3; Reaction progress curve for the production of product during an enzyme-catalyzed reaction. Inset highlights the early time points at which the initial velocity can be determined from the slope of the linear plot of [P] versus time.

To understand how the enzymes do their job and what the characterizations they have, we must first understand a few parameters. For example, how fast the enzyme works and how much affinity the enzyme has for its substrate. To perform this analysis, we could perform the following experiment. Into X different tubes, we put enzyme buffer (to keep the enzyme stable),

the same amount of enzyme, and then a different amount of substrate in each tube, ranging from tiny amounts in the first tubes to large amounts in the last tubes.
Then the reaction is aloud to proceed for a fixed, short amount of time and the amount of product contained in each tube is measured. For each reaction, the velocity of the reaction is determined as the concentration of the product found in each tube divided by the time through plotting the results and using the slope for the first several points measured, because we are interested only of the initial speed Vo, when the reaction behaves as a first-order reaction.
When Vo is determined for all X tubes, the data is plotted on a graph using velocity on the Y-axis, and the concentration of substrate used during the seven different tries on the X-axis. (see Figure 3)

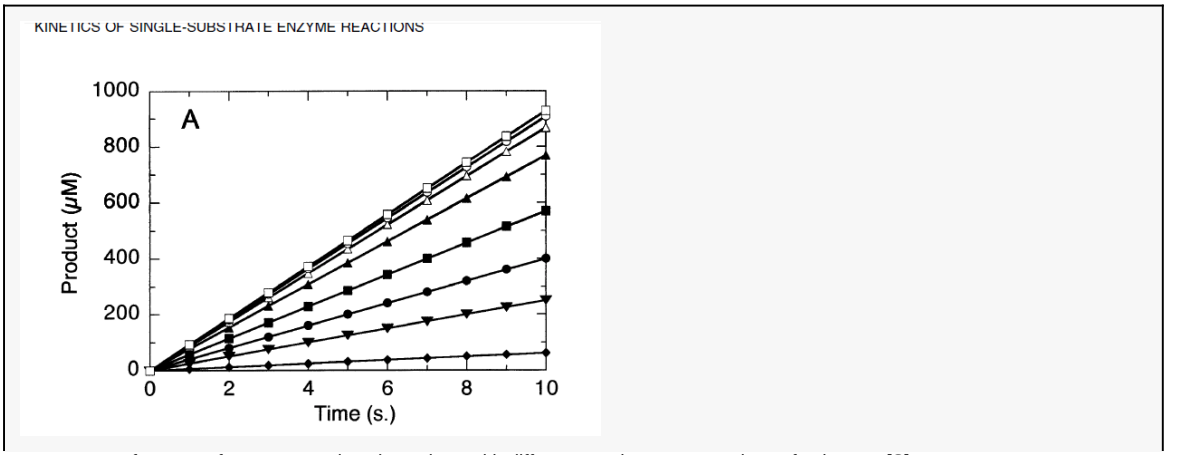


Figure 4; Progress curves for a set of enzyme-catalyzed reactions with different starting concentrations of substrate [S].

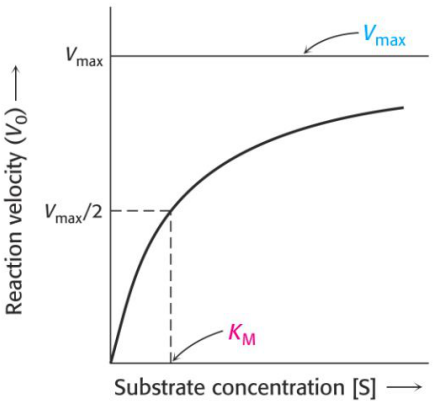


Figure 5; Plot of the reaction velocities, measured as the slopes of the lines from Figure 3, as a function of [S].
Source: http://cellbiologyolm.stevegallik.org/node/55

Typically, a curve, like Figure 5, is generated. The velocity increases almost linear in the tubes with the lowest amounts of substrate. This indicates that substrate is limiting, and the enzyme converts it into a product as soon as it can bind it. As the substrate concentration increases, however, the velocity of the reaction in tubes with higher substrate concentration ceases to increase linearly and instead begins to flatten out, indicating that as the substrate concentration gets higher and higher, the enzyme can not menage converting the substrate to product linearly anymore. What is happening is the enzyme is becoming saturated with a substrate at higher concentrations of the latter. When the enzyme becomes completely saturated with a substrate, it will not have to wait for substrate to diffuse to it and will therefore be operating at maximum velocity.

Before the mathematical introduction of how to handle with different enzyme-kinetics variables it is good to determine certain conditions that have to be fulfilled, for correct calculations;

- **Conditions Michaelis-Menten Equation;**

  1. The reaction involves only one substrate..

  2. [S]o >> [E]t and [E]t is held constant/unchanged.

  3. [P]=0 when, time=0
  4. All other variables that might influence the rate of the reaction (temperature, pH, ionic strength, and so on) are constant.

  5. Vo-initial velocity

  Source: http://online.redwoods.edu/instruct/darnold/DEProj/sp02/SauceGent/Kinetics_Presentation.pdf

Summarized form of the mechanism for an enzyme - catalyzed reaction.

Here k_1 k_{-1}, k_2 and k_M are the following rate constants

k_1 is the rate constant of E+S →ES
k_{-1} is the rate constant of ES→E+S
$k_2 = K_{CAT}$ is the rate constant of ES→E+P
$k_M \equiv \frac{k_{-1}+k_2}{k_1}$ is the so Michaelis-Menten constant

$$V = \frac{K_{CAT}[E]\,t\ [S]}{k_M+[S]}$$ **(Michelis-Menten Equation)**

The equation can be rewritten as;

$$V = \frac{V\,\mathrm{max}\ [S]}{k_M+[S]}$$

We are going to call k_2 , Kcat instead in this lab.

Vmax and Kcat

On the plot of Velocity vs. Substrate Concentration (Figure 5), the maximum velocity (Vmax) is the value on the Y axis that the curve asymptotically approaches. It should be noted that the value of Vmax depends on the amount of enzyme used in a reaction. Double the amount of enzyme, double the Vmax. If one wanted to compare the velocities of two different enzymes, it would be necessary to use the same amounts of enzyme in the different reactions they catalyze. In the initial stages of the reaction, so little product is present that no reverse reaction of ES→ E+P need to be taken into account. So in the Michelis-Menten model, the initial rate V, of the product formation could be described only through the rate of breakdown of the [ES] complex, so;

$V = k_2$ [ES]

When the value of [S] >>[E], so the enzyme is completely saturated with substrate ([ES] = [E]t),

basically the reaction reach Vmax, and the equation above could be rewritten as;

$V_o = V_{max} = k_2 [E]_t$ **or** $V_{max} = K_{CAT} [E]_t$

Kcat is a constant and does not depend on enzyme concentration the same way as Vmax.To determine Kcat, one must know the Vmax at a particular concentration of enzyme. Kcat is thus a constant for an enzyme under given conditions. The value of Kcat is sometimes referred to as the turnover number for the enzyme, since it defines the number of catalytic turnover events that occur per unit time. The units of Kcat are reciprocal time (e.g., min^{-1}, s^{-1}).
So Kcat is the number of substrate molecules converted to product by a single enzyme molecule per unit time.

Km

Another parameter of an enzyme that is useful is known as Km, so called Michaelis-Menten constant. It measures, the affinity an enzyme has for its substrate. Affinities of enzymes for substrates. Knowing Km helps us to understand how well an enzyme is suited to the substrate being used. Measurement of K_m depends on the measurement of V_{max}. K_m is determined as the value that gives V_{max} /2. K_m is a substrate concentration and is the amount of substrate it takes for an enzyme to reach V_{max} /2. On the other hand, V_{max} /2 is a velocity and is nothing more than that. The value of Km is inversely related to the affinity of the enzyme for its substrate. High values of Km correspond to low enzyme affinity for substrate (it takes more substrate to get to Vmax). Low K_m values for an enzyme correspond to high affinity for substrate. As a unit Vo it has often been used (M/s). It should be remembered that velocity measurements can be made using any convenient unit of change per unit of time. K_m is by definition, a substrate concentration (when Vo= V_{max}/2), so its value does not reflect how the velocity is measured. While Km is a constant for each enzyme, V_{max} is not really a constant, because it depends on the enzyme concentration.

Kcat/Km

When [S]<<Km the reaction has more second-order character. The catalytic efficiency of an enzyme is best defined by the ratio of the kinetic constants, Km/Kcat. That means;

$V = \frac{K_{CAT}[Et]\ [S]}{k_M + [S]}$ **(Michelis-Menten Equation) , can be modified as** $V = \frac{K_{CAT}[Et]\ [S]}{k_M}$

**Enzymatic reactions are, in principle, reversible, although for many enzymes, the reverse reaction is thermodynamically unfavorable, Kcat /Km is also used to compare the efficiency with which an enzyme catalyzes a particular reaction in the forward and reverse directions.
So simply said, specificity constant Kcat/Km is showing us how often enzyme and substrate encounter one another in the solution, when, [S]<<Km.**

Perfect Enzymes

If we think about what an ideal enzyme might be, it would be one that has a very high velocity and a very high affinity for its substrate. That is, it wouldn't take much substrate to get to Vmax/2 and the Kcat would be very high. Such enzymes would have values of Kcat / Km that are maximum. There are several enzymes that have this property, and their maximal values are all approximately the same. Why should there be a maximum possible value of Kcat / Km? The answer is the diffusion-controlled rate of between 10^8 and 10^9 M^{-1} s^{-1}.

Low Km vs. high Kcat

We know already that low Km, high Kcat and Kcat/Km are defining good substrates in enzyme kinetics. But what go first?
When comparing different substrates for en enzyme, the substrates with the highest Kcat/Km value should be considered the better substrate, not the one with the lowest Km. Further more at a constant Kcat/Km it is better to have a high Kcat rather than a low Km.

Experimental Measurement of Kcat and Km

Graphical Determinations from Untransformed Data

As we already said the kinetic constants Vmax and Km are estimated graphically with initial velocity measurements obtained at varying substrate concentrations.
The first and most straightforward way of graphing the data is as a direct plot of velocity as a function of [S]; we shall refer to such a plot as a Michaelis—Menten plot (Figure 5).

The accuracy of such estimates will depend on the range of substrate concentrations over which the initial velocity has been
determined. If measurements are made only at low substrate concentrations, the data will appear to be first-ordered. In this concentration range, the enzyme active sites never reach saturation, and graphically, both Vo and Km appear to be infinite.

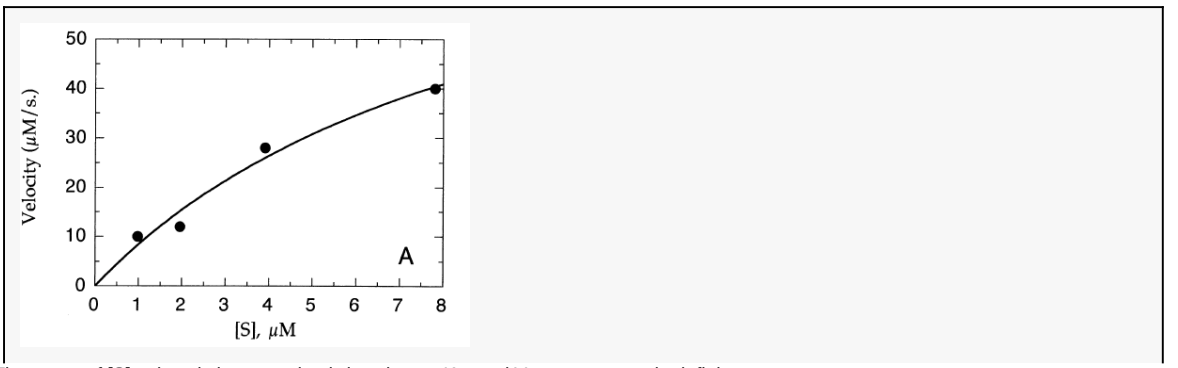


Figure 6; The range of [S] values is inappropriately low, hence Km and Vmax appear to be infinite.

On the other hand, when measurements are made at very high saturating substrate concentration range, the velocity appears to be almost independent of substrate concentration. While a rough estimate of Vo might be obtained from these data, there is no way to determine the Km value here.

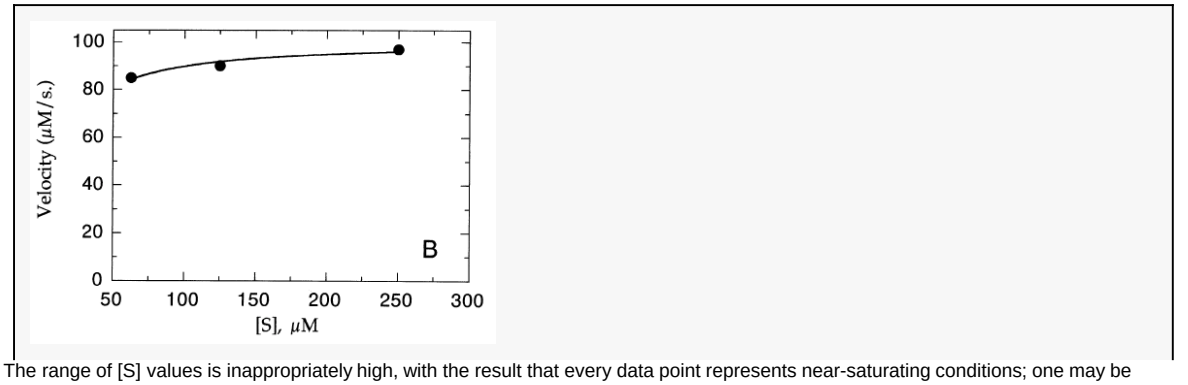


Figure 7; The range of [S] values is inappropriately high, with the result that every data point represents near-saturating conditions; one may be able to approximate Vmax, but Km cannot be determined.

Figure 6,7 emphasize the need for exploring a broad range of substrate concentrations to accurately determine the kinetic constants for the enzyme of interest.

Lineweaver-Burk Plots of Enzyme Kinetics

The most commonly used method for linearized enzyme kinetic data is that of Lineweaver and Burk. Without going into to much details about the derivation of the formulas it is known that;

V = $\frac{\text{Vmax [S]}}{k_M+[S]}$ (Michelis-Menten Equation)

Now we simply take the reciprocal of this equation and rearrange to obtain:

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

We could visualize the formula through a plot.

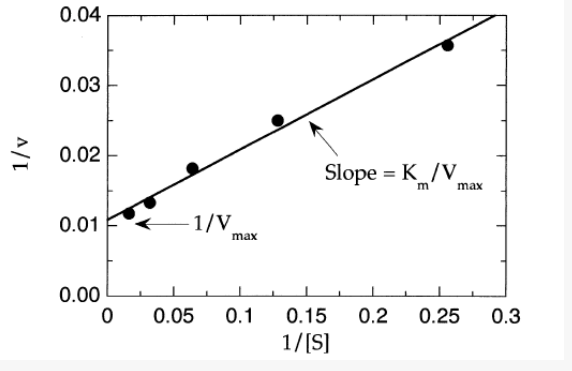


Figure 8; Lineweaver—Burk plot

The kinetic constants Km and Vo can be determined from the slope and intercept values of the linear fit of the data in a Lineweaver—Burk plot. Alternatively, we could extrapolate our linear fit to the point of intersecting the x axis. This x intercept is equal to -1/Km;
thus we could determine Km from the absolute value of the reciprocal of the x intercept of our plot. (this method is going to be used in this lab).

Eadie-Hofstee Plots of Enzyme Kinetics

If we multiply both sides of:

V = $\frac{\text{Vmax [S]}}{k_M+[S]}$ (Michelis-Menten Equation)

by Km+[S], we obtain:

$$v(K_m + [S]) = V_{max}[S]$$

If we now divide both sides by [S] and rearrange, we obtain:

$$v = V_{max} - K_m\left(\frac{v}{[S]}\right)$$

We could visualize the formula through a plot.

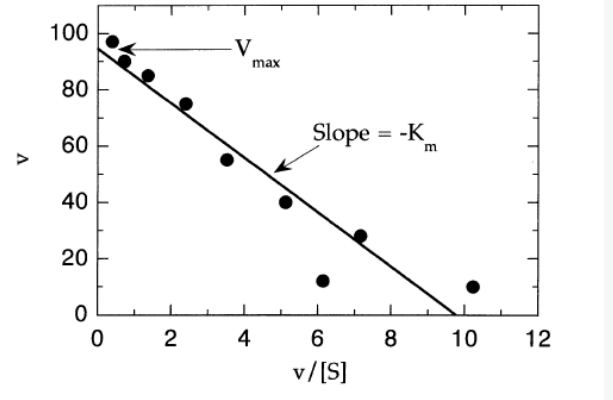


Figure 9; Eadie—Hofstee plot

Source Figure 3, 4, 6,7,8,9: Enzymes - A Practical Introduction to Structure, Mechanism and Data Analys by Robert Copeland; year 2000; ed.2; ch.5.

Sources: Chemistry by Zumdahl ed.8; page 571
Textbook of Biochemistry with Clinical Correlations by Thomas M. Devlin; ed. 7; year: 2010; page 383,384
Inroduction to Bioorganic Chemistry and Chemical Biology by David Van Vranken and Gregory Weiss; year 2013; pages 238-240
Enzymes - A Practical Introduction to Structure, Mechanism and Data Analys by Robert A. Copeland; year 2000; ed.2; ch.5
Principles of Boichemistry ed. 4 by Voet, Voet, Pratt; page 363
Lehninger PRINCIPLES OF BIOCHEMISTRY ed.4 David L. Nelson, Michael M. Cox page 207-209
Lippincott's Illustrated Reviews: Biochemistry ed.5 by Richard A. Harvey, page 55-61

ADH

In this lab, it has been investigated the kinetic properties of Alcohol Dehydrogenase using ethanol as a substrate. ADH catalyze the oxidation of alcohols to aldehydes.

Alcohol dehydrogenase (ADH) is an important enzyme involved in a detoxification mechanism of alcohols in many living organisms, thus regulating the alcohol concentration. For some species, alcohol could be lethal if they lack an active ADH detoxification system. For example, fruit fly.
***Many if not all ADH have an active site with greater affinity for propanols and butanols than for ethanol, often by a large factor.**

*Source: Isozymes V2: Physiological Function by Clement Markert, Volume 2, page 733.

ADH activity is measurable: the extinction coefficient at 340 nm of NADH is much higher than that of NAD. If the only substrates added to the reaction are NAD and alcohol, the change in absorbance at 340 nm should be proportional to the change in NADH concentration due to the ADH activity present in the cuvette.

It is an Oxidation-Reduction Reactions (electrons gained/lost);
Metal ions can serve as electrophilic reactants in catalysis, as in the case of the active site zinc ions of ADH. Most commonly metals are bound to the protein portion of the enzyme by formation of coordinate bonds with certain amino acid side chains. NAD activates alcohol dehydrogenase. Alcohol dehydrogenase removes a hydride from ethanol and transfers it to

NADH.

Sources: Principles of Boichemistry ed. 4 by Voet, Voet, Pratt; page 319

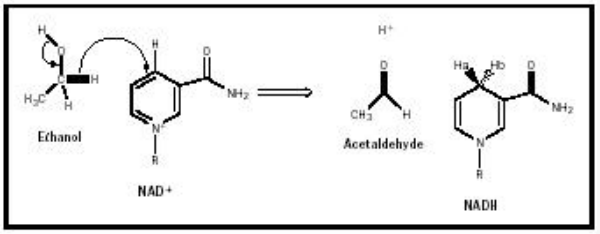


Figure 10; Alcohol dehydrogenase removes a hydride from ethanol and transfers it to NAD+.
Source Figure http://www.chemistryexplained.com/Ne-Nu/Nicotinamide-Adenine-Dinucleotide.html

- This how Vo is going to be calculated in this lab.

```
ξ = 6220;(*M^-1 cm^-1*)
```

```
l = 1.0;(*cm*)
```

$$V_0 = \frac{\Delta[P]}{\Delta t} = \left(\frac{\Delta A340 / \Delta sec}{\xi\, l}\right) * 10^9 \,(*nM/s*)$$

From the A340 data gained for the reaction ADH with ethanol, it could be calculated Vmax, Km, Kcat, Kcat/Km.

The second part of the lab is;
from an already given data(Vmax measured with different [S]) it has been calculated Km, Kcat, Kcat/Km for a reaction ADH with;
1-propanol, 2-propanol, 1-butanol.

Material and Methods

Materials:

- **0.1 M phosphate buffer**
- **2.5 mM NAD+, neutralized with NaOH**
- **Alkoholdehydrogenase, 0.012 mg/ml in phosphate buffer**
- **Ethanol 2M**
- **Spectrophotometer**
- **Pipettes**
- **Cuvette**

Methods:

The values used during the lab; constant Nad+, ADH and a total volume of the cuvete 3ml. Different [S] and buffer volume.

Before the measurements of the initial reaction rate was started, seven different substrate concentrations in the range of 0.002-0.1M were determined. The values that was used are shown in the table below. Then, these concentrations were used to make the seven different measurements of the initial reaction rate. Before each measurement was done, the spectrophotometer was reset at 340 nm with phosphate buffer, NAD+ and ethanol. Then ethanol was added to a total volume of 3 ml and the absorbance change was recorded for 5 minutes for

each measurement. The data was saved and transferred *Mathematica* - software for further analysis and calculations.

```
Ethvolym = 3(*kyvetvol ml*) [Ethanol] / [EthanolStock = 2.0(*M*)]
```

[Ethanol](M)	NAD (ml)	ADH (ml)	Vol.ethanol (ml)	Vol.phosphate (ml)	Kyvetvol.Tot(ml)
0.002	1.	0.05	0.003	1.947	3.
0.006	1.	0.05	0.009	1.941	3.
0.012	1.	0.05	0.018	1.932	3.
0.02	1.	0.05	0.03	1.92	3.
0.03	1.	0.05	0.045	1.905	3.
0.06	1.	0.05	0.09	1.86	3.
0.1	1.	0.05	0.15	1.8	3.

- Table 1 - Volumes used and [S] during the lab.

Results

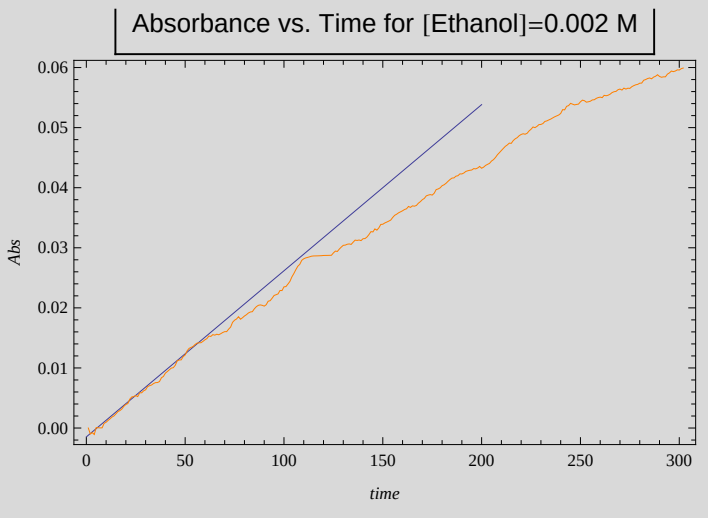
Ethanol

In this section "Absorbance(A340) vs. Time" - graphs and the tangents to them, when the reaction is initiated, for all seven measurements done with different concentrations of the substrate (ethanol).

The function for the tangent, is represented under every graph, for each and every [S]. They are calculated by Mathematica software.

The slope of that tangent determines ΔA340/Δs for every [S]., from which Vo is going to be calculated.

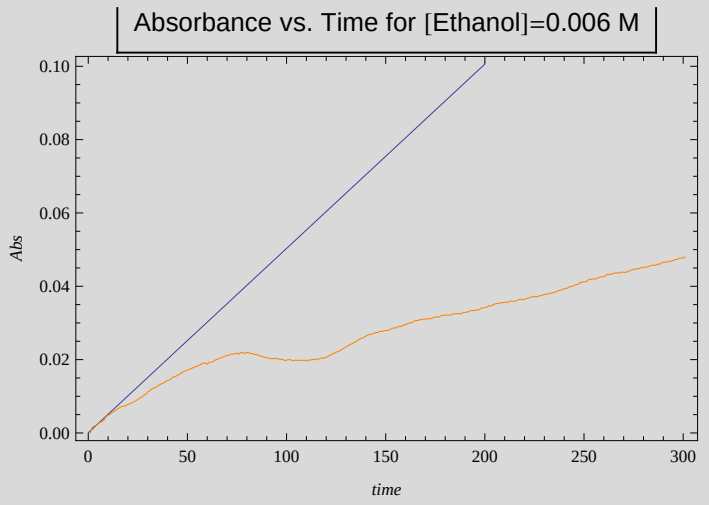
After Vmax, Km, Kcat and Kcat/Km are calculated for both "Eadie—Hofstee plot" and "Lineweaver - Burk plot". The values are represented in a table.



- Graph 1 - [S]=0.002 M; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ -0.00147576 + 0.000276506 x ]
```

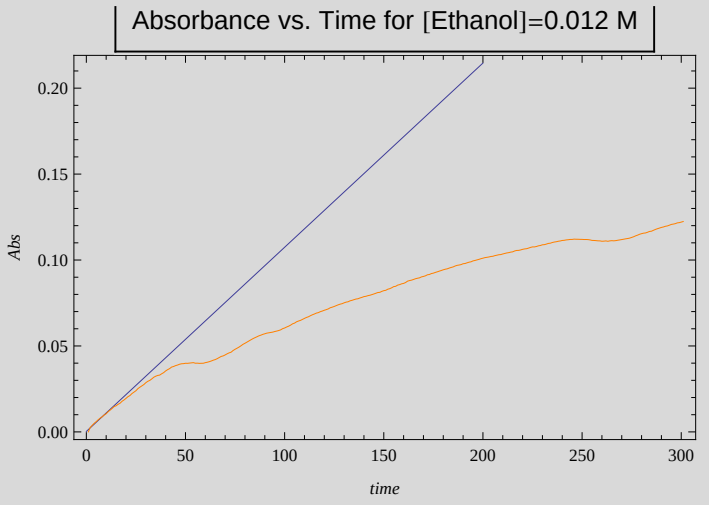
- $\Delta A340/\Delta sec = 0.000276506\ (sec^{-1})$



- Graph 2 - [S]=0.006 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ -0.00008012 + 0.00056158 x ]
```

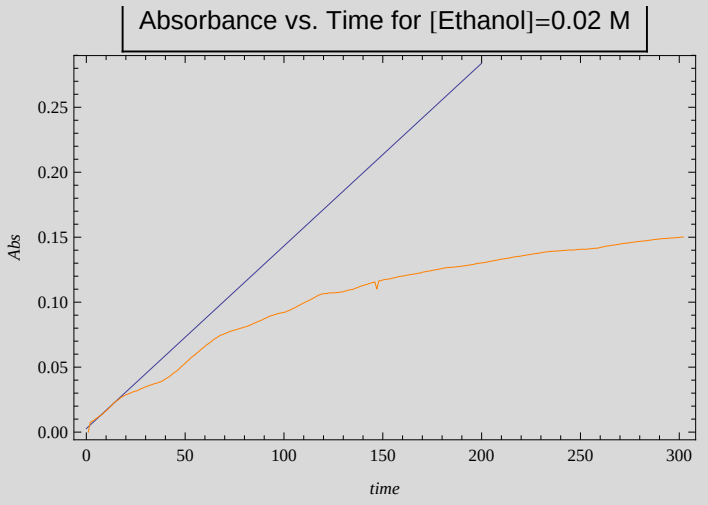
- $\Delta A340/\Delta sec = 0.00056158\ (sec^{-1})$



- Graph 3 - [S]=0.012 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ 0.000259964 + 0.00107172 x ]
```

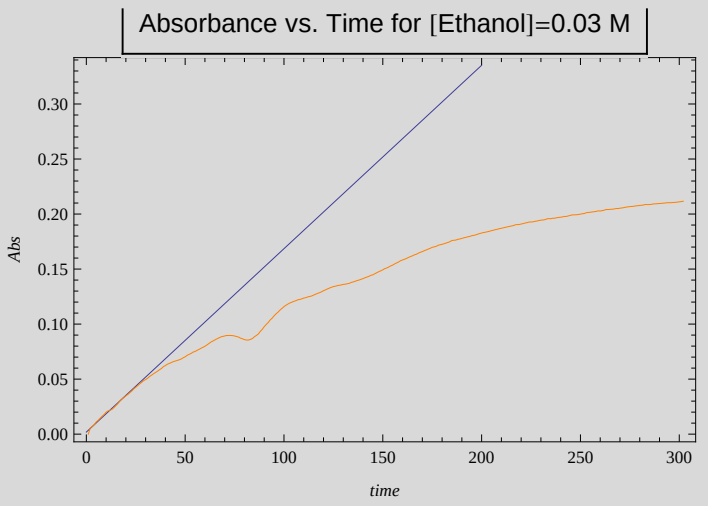
- $\Delta A340/\Delta sec = 0.00107172\ (sec^{-1})$



- Graph 4 - [S]=0.02 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ 0.00273053 + 0.00140565 x ]
```

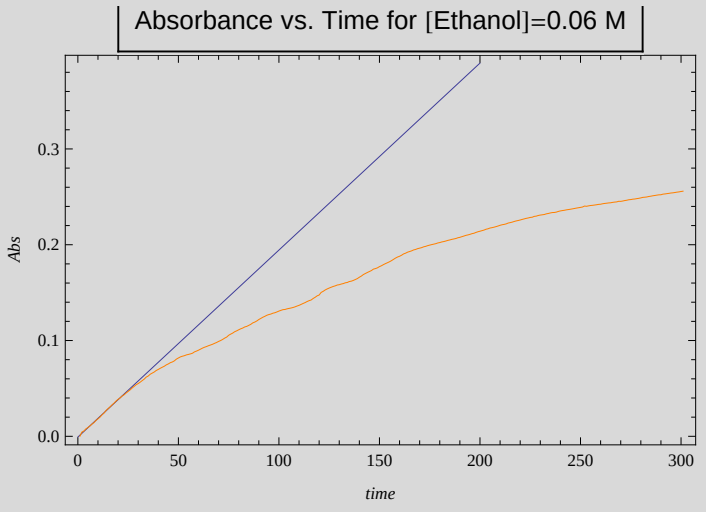
- ΔA340/Δsec = 0.00140565 $\left(\text{sec}^{-1}\right)$



- Graph 5 - [S]=0.03 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ 0.00187078 + 0.0016665 x ]
```

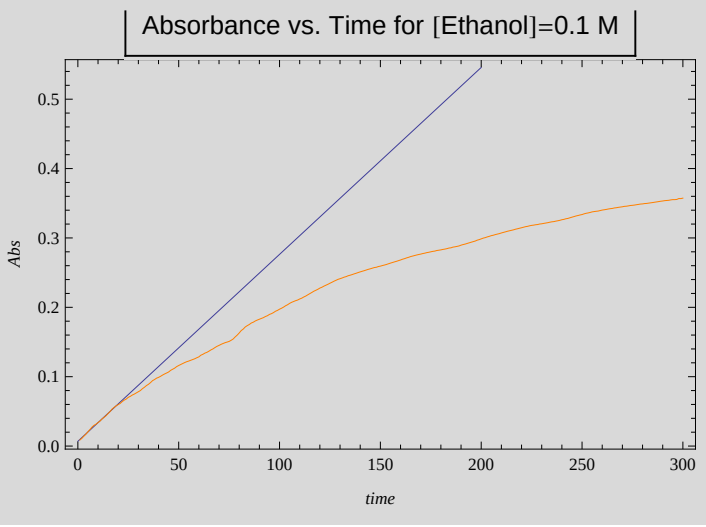
- ΔA340/Δsec = 0.0016665 $\left(\text{sec}^{-1}\right)$



- Graph 6 - [S]=0.06 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ -0.000781529 + 0.00195206 x ]
```

- ΔA340/Δsec = 0.00195206 $\left(\text{sec}^{-1}\right)$



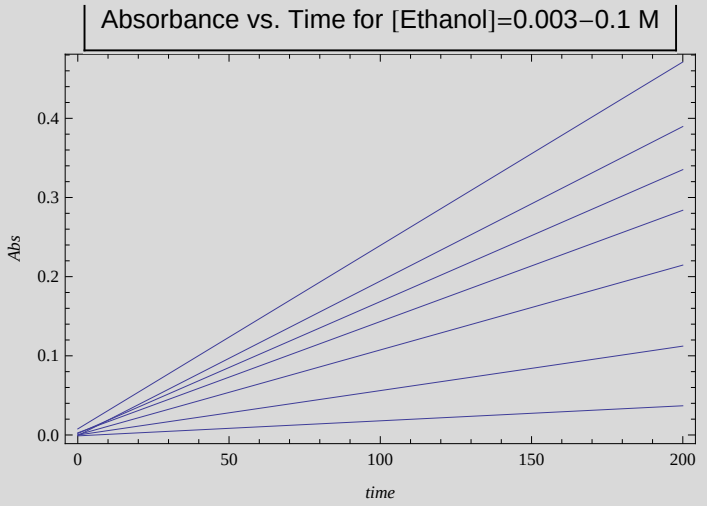
- Graph 7 - [S]=0.1 M ; time is in seconds; Absorbance at 340 nm.

```
FittedModel[ 0.00765952 + 0.00231758 x ]
```

- ΔA340/Δsec = 0.00231758 $\left(\text{sec}^{-1}\right)$

All tangents on the same graph are represented bellow.

```
ΔA340 / Δsec = {0.0002765, 0.00056158, 0.00107172,
    0.00140565, 0.0016665, 0.00195206, 0.00231758} (sec^-1)
```



- Graph 8 - all tangents ΔA340/Δsec at initial stage of the reaction.

```
ξ = 6220;(*M^-1 cm^-1*)
```

```
l = 1.0;(*cm*)
```

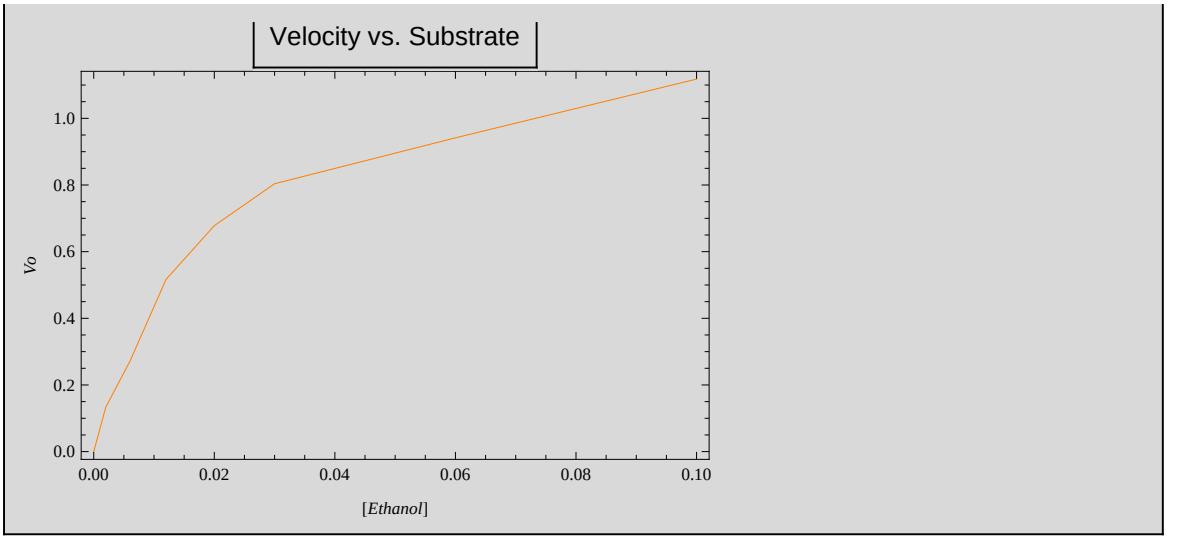
$$V_0 = \left(\frac{\Delta A340 / \Delta sec}{\xi\, l}\right) * 10^9 \; (*nM/s*)$$

```
volumeKyvet = 0.003(*L*) ;
```

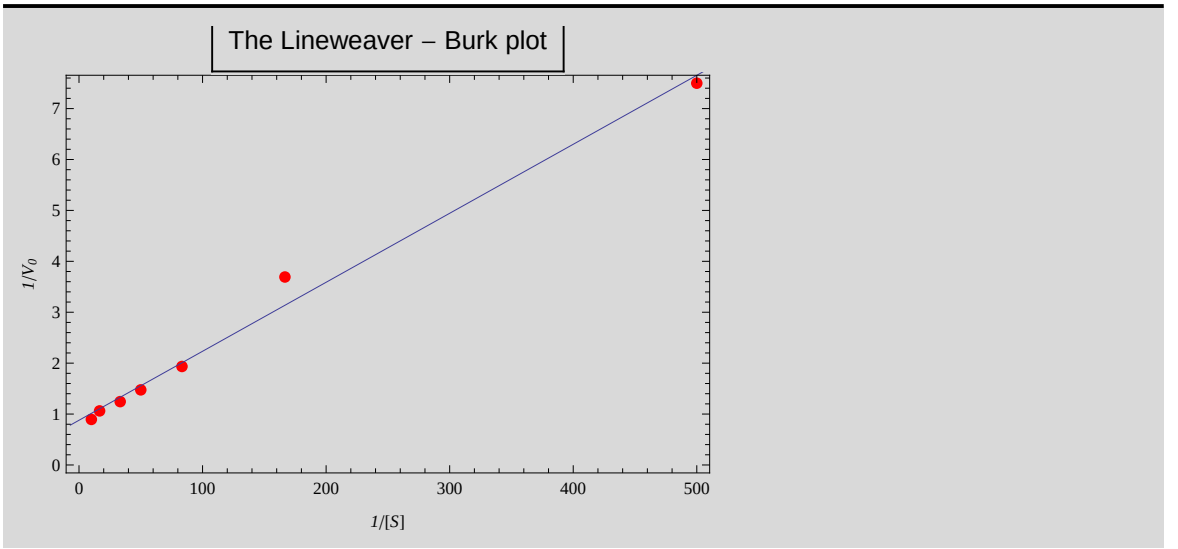
```
Vo = V0(*nM/sec*) * volumeKyvet    (*Vo changes to nmol/sec*)
```

[Ethanol](M)	△A340/△s	V_0(nmol/s)	1/[S]	$1/V_0$	V_0/[S]
0.002	0.0002765	0.13336	500.	7.49849	66.6801
0.006	0.00056158	0.270859	166.667	3.69196	45.1431
0.012	0.00107172	0.516907	83.3333	1.93458	43.0756
0.02	0.00140565	0.677966	50.	1.475	33.8983
0.03	0.0016665	0.803778	33.3333	1.24412	26.7926
0.06	0.00195206	0.941508	16.6667	1.06213	15.6918
0.1	0.00231758	1.1178	10.	0.894611	11.178

- Table 2-Initial velocity as a function of substrate concentration, used for enzymatic reaction calculations.



- Graph 9 - Michaelis—Menten plot for the velocity data.



- Graph 10 - Lineweaver—Burk double-reciprocal plot for selected data from Table 2.

```
FittedModel[ 0.878065 + 0.0135517 x ]
```

```
f1[x_] := 0.87806461 + 0.01355168 x
```

```
Solve[f1[x] == 0, x]
```

```
{{x → -64.793782}}
```

```
Solve[-(1 / Km1) == 64.793782, Km1]   (*M*)
```

```
{{Km1 → -0.0154336}}
```

```
Solve[1 / Vm == 0.878065, Vm]   (*nmol/s*)
```

```
{{Vm → 1.13887}}
```

So to summarize;

```
Km = 0.0154 (*M*)
```

```
Vmax = 1.1389(*nmol/s*) ;
```

```
(*Massa enzyme in grams*)
mEnz = (0.05(*ml*) * 0.012(*mg/ml*)) / 1000   = 6.0 * 10^-7(*g*)
```

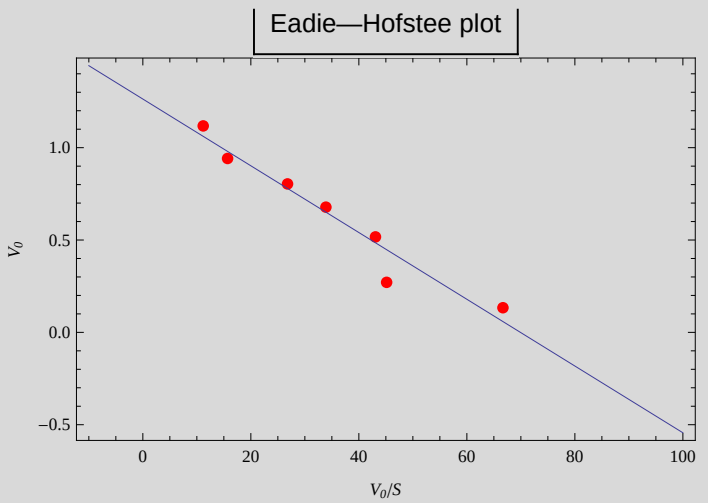
```
(*Molecular weight*)
MwEnz = 73 000(*Da*) ;
```

```
(*Quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9   = 0.00822 (*we change it to nmol*)
```

So we have everything needed to calculate Kcat .

```
Kcat = Vmax / nEnz (*1/s*) = 168.978 (*1/s*)
```

```
Kcat / Km = 10 972 (*M^-1 s^-1*)
```



- Graph 11 - Eadie—Hofstee plot of enzyme kinetic data. Data taken from Table 2.

```
Eadie–Hofstee plot LinearModelFit :
```

```
FittedModel[ 1.26332 – 0.0180692 x ]
```

For Km see "FittedModel" Eadie-Hofstee plot:

```
Km = 0.018 (*M*)
```

For Vmax see "FittedModel" Eadie-Hofstee plot:

```
Vmax = 1.26332(*nmol/s*) ;
```

We remember from above;

```
(*Massa enzyme in grams*)
mEnz = (0.05(*ml*) * 0.012(*mg/ml*)) / 1000   = 6.0 * 10^-7 (*g*)
```

```
(*Molecular weight*)
MwEnz = 73 000(*Da*) ;
```

```
(*Quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  = 0.00822 (*we change it to nmol*)
```

So we have everything needed to calculate Kcat .

```
Kcat = Vmax / nEnz (*1/s*) = 153.70  (*1/s*)
```

```
Kcat / Km = 8500 (*M^-1 s^-1*)
```

In the table bellow are compared the values obtained by the two methods;

	V_{max} (nmol/s)	Km (M)	Kcat (s^{-1})	$\frac{Kcat}{Km}$ (M^{-1} s^{-1})
Lineweaver–Burk	1.389	0.0154	168.98	10 972
Eadie—Hofstee	1.26332	0.018	153.7	8500

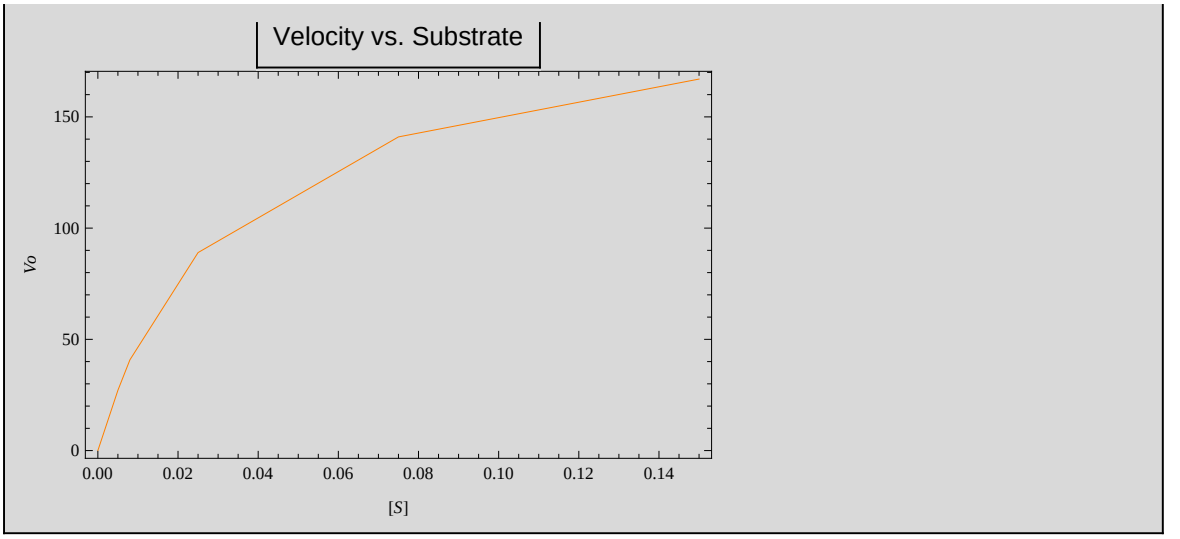
- Table 3 - comparison of values obtain by Lineweaver—Burk and Eadie—Hofstee method.

1-propanol

In this section from given values [S],[Enzyme] and Vo it is calculated Vmax, Km, Kcat and Kcat/Km for both "Eadie—Hofstee plot" and "Lineweaver - Burk plot". The values are represented in a table. Further down together with all the other substrates.

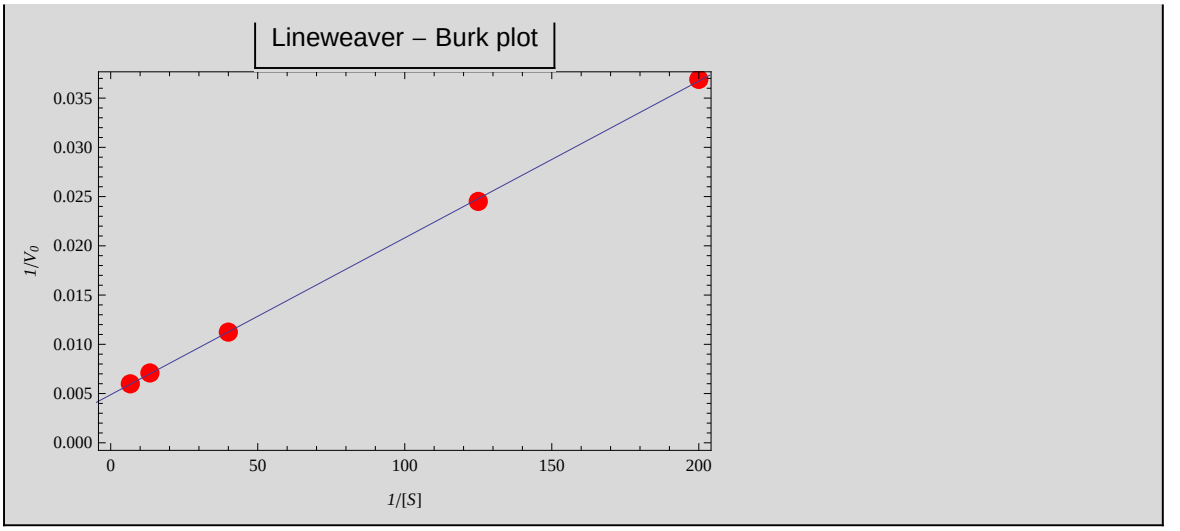
[Substrate] (M)	V_0 (nmol/s)	1/[S]	$1/V_0$	$V_0/[S]$
0.005	27.1	200.	0.0369004	5420.
0.008	40.8	125.	0.0245098	5100.
0.025	89.	40.	0.011236	3560.
0.075	141	13.3333	$\frac{1}{141}$	1880.
0.15	167	6.66667	$\frac{1}{167}$	1113.33

- Table 4-Initial velocity as a function of substrate concentration, used for enzymatic reaction calculations.



- Graph 12 - Michaelis—Menten plot for the velocity data.

Lineweaver - Burk plot



- Graph 11 - Lineweaver—Burk double-reciprocal plot for selected data from Table 4.

```
FittedModel[ 0.00488499 + 0.000159224 x ]
```

```
Solve[0.00488499 + 0.000159224 x1 == 0, x1]
```

```
{{x1 → -30.68}}
```

```
Solve[-1 / Km == -30.68, Km]      (*M*)
```

```
{{Km → 0.0325945}}
```

```
Solve[1 / Vmax == 0.00488499, Vmax]     (*nmol/s*)
```

```
{{Vmax → 204.709}}
```

```
(*massa enzyme in grams*)
mEnz = (0.1(*ml*) * 0.012(*mg/ml*)) / 1000  (*g*)
```

1.2×10^{-6}

```
(*molecular weight*)
MwEnz = 73 000(*Da*) ;
```

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

0.0164384

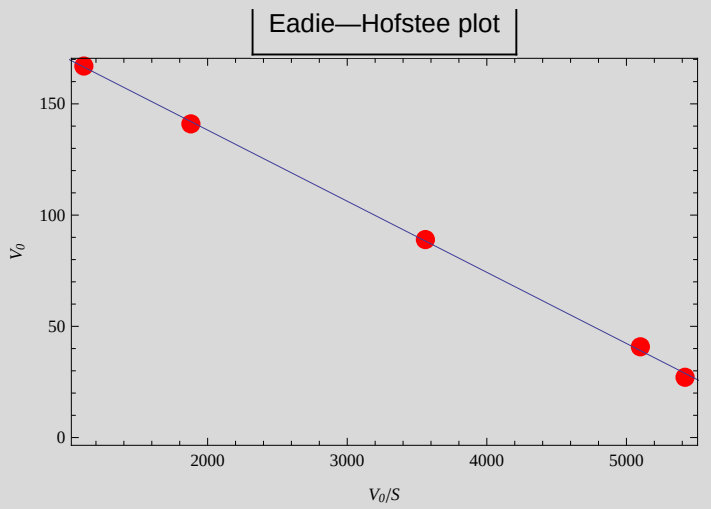
```
Kcat = Vmax / nEnz(*1/s*)
```

12 453.1

```
Km = 0.03259452  (*M*);
Kcat / Km          (*M^-1 s^-1*)
```

382 061.

Eeadie - Hofstee plot



- Graph 12 - Eadie—Hofstee plot of enzyme kinetic data. Data taken from Table 4.

FittedModel[202.15 – 0.0319709 x]

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

0.0164384

For Vmax see "FittedModel" Eadie-Hofstee plot:

```
Vmax = 202.15;       (*nmol/s*)
Kcat = Vmax / nEnz   (*1/s*)
```

```
12 297.5
```

For Km see "FittedModel" Eadie-Hofstee plot:

```
Km = 0.03197       (*M*);
Kcat / Km          (*M^-1 s^-1*)
```

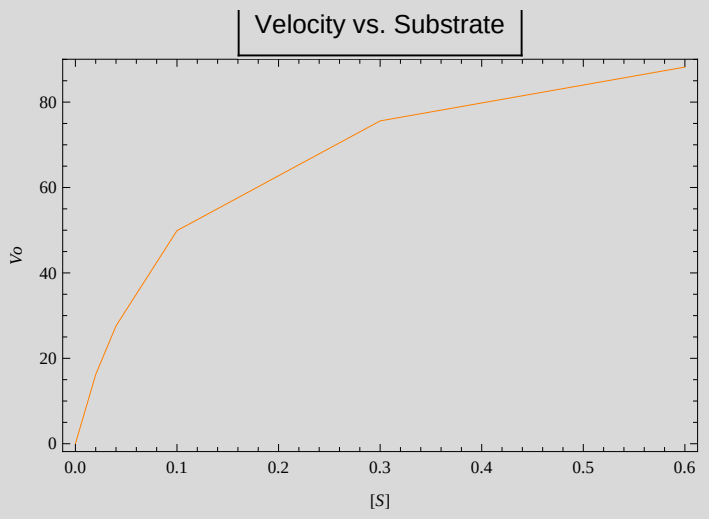
```
384 656.
```

2-propanol

In this section from given values [S],[Enzyme] and Vo it is calculated Vmax, Km, Kcat and Kcat/Km for both "Eadie—Hofstee plot" and "Lineweaver - Burk plot". The values are represented in a table. Further down together with all the other substrates.

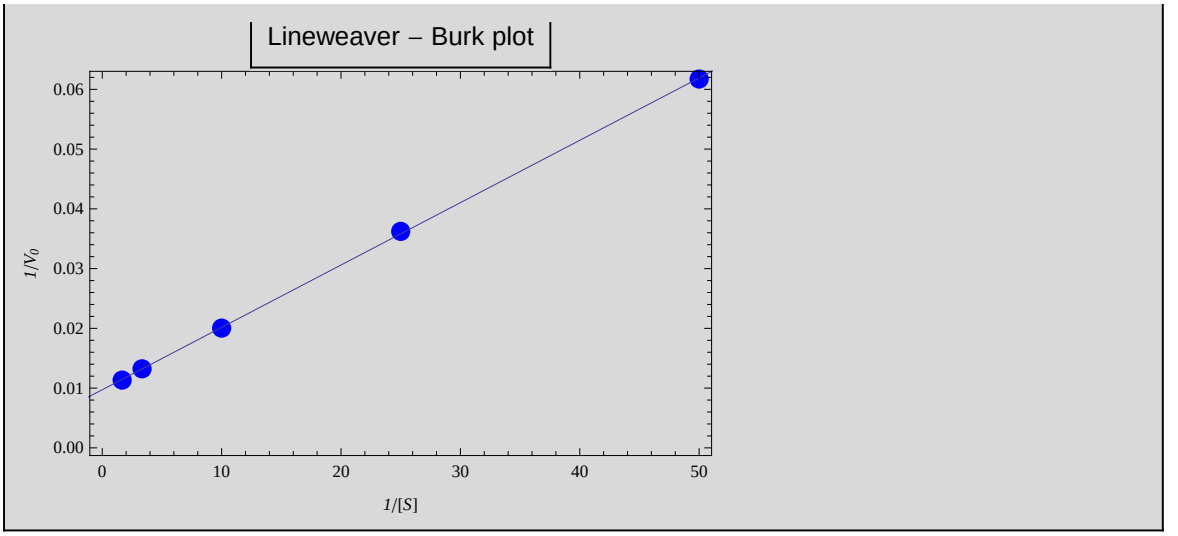
[Substrate] (M)	V_0 (μM/s)	1/[S]	$1/V_0$	V_0/[S]
0.02	16.2	50.	0.0617284	810.
0.04	27.6	25.	0.0362319	690.
0.1	49.9	10.	0.0200401	499.
0.3	75.6	3.33333	0.0132275	252.
0.6	88.2	1.66667	0.0113379	147.

- Table 5-Initial velocity as a function of substrate concentration, used for enzymatic reaction calculations.



- Graph 13 - Michaelis—Menten plot for the velocity data.

Lineweaver - Burk plot



- Graph 14 - Lineweaver—Burk double-reciprocal plot for selected data from Table 5.

```
FittedModel[ 0.0097289 + 0.00104357 x ]
```

```
Solve[0.0097289 + 0.00104357 x2 == 0, x2]
```

```
{{x2 → -9.32272}}
```

```
Solve[-(1 / Km) == -9.32272, Km]   (*M*)
```

```
{{Km → 0.107265}}
```

```
Solve[1 / VmaX == 0.0097289, VmaX]    (*nmol/s*)
```

```
{{VmaX → 102.787}}
```

```
(*massa enzyme in grams*)
mEnz = (0.5(*ml*) * 0.012(*mg/ml*)) / 1000  (*g*)
```

```
6. × 10^-6
```

```
(*molecular weight*)
MwEnz = 73 000(*Da*) ;
```

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

```
0.0821918
```

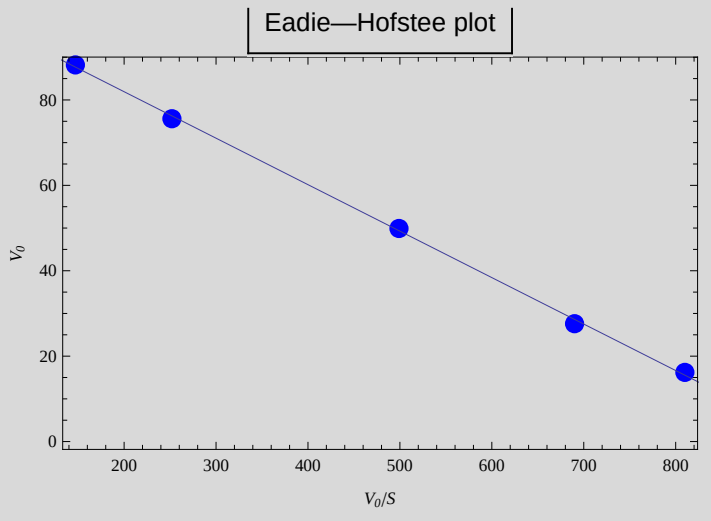
```
Kcat = Vmax / nEnz   (*1/s*)
```

```
1250.57
```

```
Km = 0.107265  (*M*)
Kcat / Km      (*M^-1 s^-1*)
```

```
11658.7
```

Eeadie - Hofstee plot



- Graph 15 - Eadie—Hofstee plot of enzyme kinetic data. Data taken from Table 5.

```
FittedModel[ 103.693 – 0.108826 x ]
```

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

```
0.0821918
```

For Vmax see "FittedModel" Eadie-Hofstee plot:

```
Vmax = 103.693;     (*nmol/s*)
Kcat = Vmax / nEnz  (*1/s*)
```

```
1261.6
```

For Km see "FittedModel" Eadie-Hofstee plot:

```
Km = 0.108826    (*M*);
Kcat / Km        (*M^-1 s^-1*)
```

```
11592.8
```

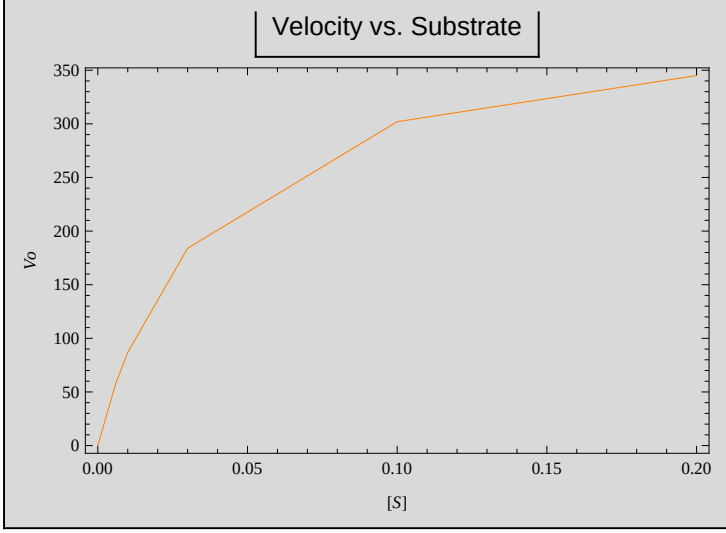
1-butanol

In this section from given values [S],[Enzyme] and Vo it is calculated Vmax, Km, Kcat and Kcat/Km for both "Eadie—Hofstee plot" and "Lineweaver - Burk plot". The values are

represented in a table. Further down together with all the other substrates.

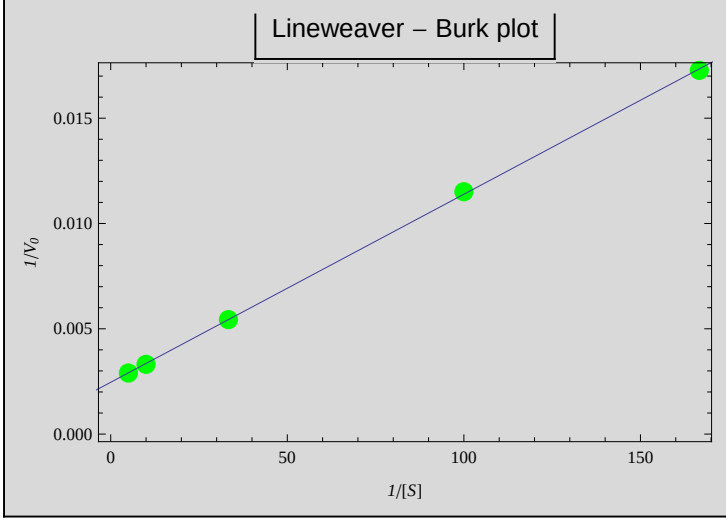
[Substrate](M)	V_0(nmol/s)	1/[S]	$1/V_0$	V_0/[S]
0.006	57.9	166.667	0.0172712	9650.
0.01	86.9	100.	0.0115075	8690.
0.03	184	33.3333	$\frac{1}{184}$	6133.33
0.1	302	10.	$\frac{1}{302}$	3020.
0.2	345	5.	$\frac{1}{345}$	1725.

- Table 6-Initial velocity as a function of substrate concentration, used for enzymatic reaction calculations.



- Graph 16 - Michaelis—Menten plot for the velocity data.

Lineweaver - Burk plot



- Graph 17 - Lineweaver—Burk double-reciprocal plot for selected data from Table 6.

FittedModel[0.00246008 + 0.0000892788 x]

```
Solve[0.00246008 + 0.0000892788 x3 == 0, x3]
```

```
{{x3 → -27.555}}
```

```
Solve[-(1 / Km) == -27.555, Km]   (*M*)
```

```
{{Km → 0.036291}}
```

```
Solve[1 / Vmax == 0.00246008, Vmax]   (*nmol/s*)
```

```
{{Vmax → 406.491}}
```

```
(*massa enzyme in grams*)
mEnz = (0.5(*ml*) * 0.012(*mg/ml*)) / 1000  (*g*)
```

```
6. × 10^-6
```

```
(*molecular weight*)
MwEnz = 73 000(*Da*) ;
```

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

```
0.0821918
```

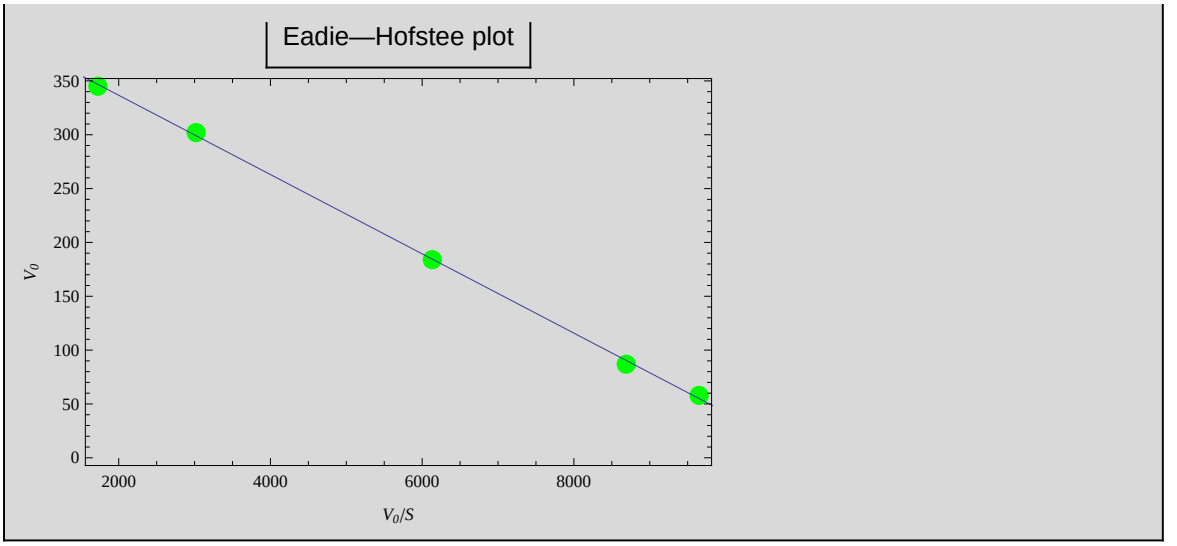
```
Kcat = Vmax / nEnz     (*1/s*)
```

```
4945.64
```

```
Km = 0.036291   (*M*)
Kcat / Km          (*M^-1 s^-1*)
```

```
136 277.
```

Eeadie - Hofstee plot



- Graph 18 - Eadie—Hofstee plot of enzyme kinetic data. Data taken from Table 6.

```
FittedModel[ 410.281 – 0.0368126 x ]
```

```
(* quantity of subsatance enzyme*)
nEnz = mEnz / MwEnz(*mol*) * 10^9  (*nmol*)
```

```
0.0821918
```

For Vmax see "FittedModel" Eadie-Hofstee plot:

```
Vmax = 410.281;          (*nmol/s*)
Kcat = Vmax / nEnz  (*1/s*)
```

```
4991.75
```

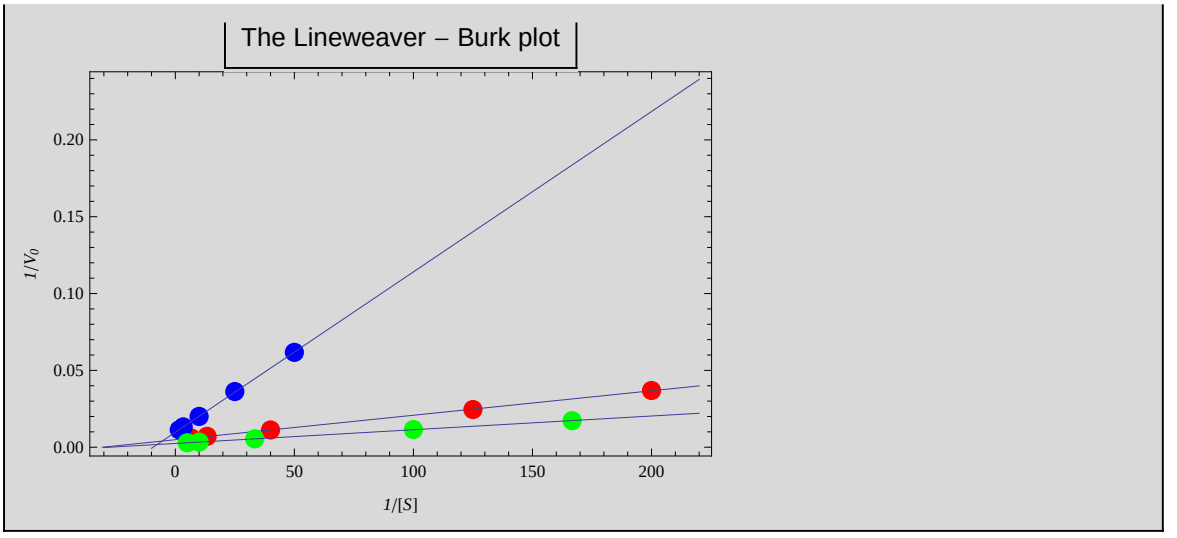
For Km see "FittedModel" Eadie-Hofstee plot:

```
Km = 0.0368126    (*M*);
Kcat / Km         (*M^-1 s^-1*)
```

```
135 599.
```

Lineweaver - Burk plot summary and results table

1-Propanol, 2-Propanol, 1-Butanol at once. “Lineweaver - Burk” plot.



- Graph 19 - Lineweaver—Burk double-reciprocal plot for selected data from Table 4,5,6.

red - 1 -propanol
blue - 2 - propanol
green - 1 - butanol

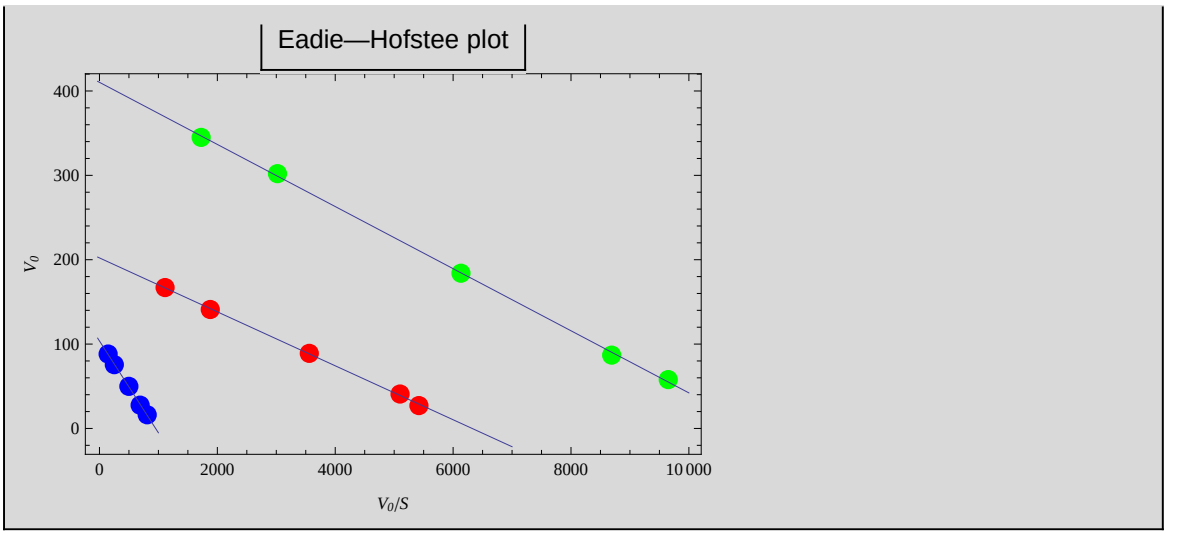
Ethanol is not shown on the plot because it almost disappears compared to the others.
The values bellow confirm this, and especially Vmax shows it clearly.

		Quantity Enzyme(nmol)	Vmax(nmol/s)	Km(M)	Kcat(1/s)	Kcat/Km($M^{-1}s^{-1}$)
1	Ethanol	0.008221	1.1389	0.0154	168.98	10 972
2	1-propanol	0.0164384	204.709	0.0326	12 453.1	382 061
3	2-propanol	0.0821918	102.787	0.10725	1250.57	11 658.7
4	1-butanol	0.0821918	406.491	0.036291	4945.64	136 277

- Table 7- Lineweaver—Burk plot, ADH - kinetic values for four different alcohols.

Eadie - Hofstee plot summary and results table

The three alcohols at once. “Eadie-Hofstee” plot



- Graph 20 - Eadie—Hofstee plot of enzyme kinetic data. Data taken from Table 4, 5, 6.

red - 1 -propanol
blue - 2 - propanol
green - 1 - butanol

		Quantity Enzyme(nmol)	Vmax(nmol/s)	Km (M)	Kcat(1/s)	Kcat/Km($M^{-1}s^{-1}$)
1	Ethanol	0.008221	1.26332	0.018	153.7	8500
2	1-propanol	0.0164384	202.15	0.03197	12297.5	384656
3	2-propanol	0.0821918	103.693	0.108826	1261.6	11592.8
4	1-butanol	0.0821918	410.281	0.0368126	4991.75	135599

- Table 8- Eadie—Hofstee plot, ADH - kinetic values for four different alcohols.

Discussion

Comparison of Km;

Let us start by comparing the values of Km for the four substrates. If we look a bit closer to the data, we could realize that primary alcohols have lower Km, which tells us that ADH-enzyme apparently has higher affinity to them. Recalling from the theory part; Km is the amount of substrate needed for reaching Vmax/2 for the enzyme. Lower Km values higher affinity.

Comparison of these numbers tells us a lot about alcohol metabolism. Clearly, ADH handles with ethanol, 1-propanol and 1-butanol faster, than 2-propanol. Furthermore, ADH reaches Vmax/2 rather fast for ethanol compared to 1-propanol and 1-butanol. This tells us that the amount of ethanol is balanced almost rapidly in the body. This though does not give as the whole picture, how the four substrates are "taken care" of ADH. It is not only Km that is important when the properties of enzymes vs. substrates are compared.

Comparison of Turnover Number - Kcat

It can be seen on Table 7,8, that the catalytic rate for 1-propanol is really elevated, because it has the highest Kcat. This high number shows the importance in detoxifying 1-propanol and preventing damages in the tissues as a whole. We know from above that the affinity (low Km) for ethanol was highest. However, at the end more 1-propanol would be oxidized. 1-butanol has rather high Kcat as well and shows similar properties as 1-propanol. Even 2-propanol has higher Kcat compared to ethanol. We though, have no background information about what type of ADH we were dealing with, and how the data given to us was obtained. It could be ADH from another specie.

Comparison Kcat/Km

Recalling from the theory, Kcat/Km is showing us how often enzyme and substrate encounter one another in the solution. Then it behaves as a second-order rate constant of the enzymatic reaction and especially when, [S]<<Km.
Here we could think the same way as only for Kcat. Obviously 1-propanol is the best substrate, followed by 1-butanol.
Now we see that Kcat/Km for ethanol and 2-propanol are very close to each other.

Recall from the theory part;
When comparing different substrates for en enzyme, the substrates with the highest Kcat/Km value should be considered the better substrate, not the one with the lowest Km. Further more at a constant Kcat/Km it is better to have a high Kcat rather than a low Km.
Which means that 2-propanol is a better substrate than ethanol. Which was a bit surprising to us.
Again to emphasize that we do not have any background information about how the data given

to us was obtained.

Enzyme concentration and substrate concentration

We know from the theory part that higher [Enzyme] gives higher Vmax. We made our lab at constant [Enzyme], so we can not say that much, about it, by using experimental data. If we speculate though, this pattern is followed for all the primary alcohols, because we get higher Vmax with higher [Enzyme]. But againg to remind, it is different substrates. We usually say that it is for a specific enzyme with, a specific substrate, that with highering of the [Enzyme], Vmax is elevated.

It is interesting that 2-propanol shows far from high Vmax compared to how much [Enzyme] it has. We saw from its Km that it has low affinity as well.

Data modification

When mathematical transformation of taking the reciprocal is done, the greatest percent error is usually associated with velocity values at low substrate concentration.
Unfortunately, in the reciprocal plot, the lowest values of [S] corresponds to the highest values of 1/[S], and because of the details of linear regression, these data points affect more heavily in the analysis compared to the other observations. This could result in poor estimates of the kinetic constants even when the experimental error is relatively small. This cause certain limitations of using linear transformations of the primary data for determining the values of the kinetic constants.

The line drawn by the reciprocal method data on the Lineweaver—Burk plot (graph 10) may not appear to fit well as a linear regression, but it gave us much more accurate reflection of the kinetic behavior of the enzyme, compared to if we just relied on Michaelis—Menten plot for the velocity data. If we used Michaelis-Menten plot at most, we could never be sure what Vmax is. Km would be also uncertain. When we use a double reciprocal plot, it is desirable to choose substrate concentrations that will be evenly spaced along a reciprocal x axis (i.e., 1/[S]). This is accomplished when one picks a maximum value of [S]), and diluting the original stock solution by ~ 1:2, 1:3, 1:4, 1:5, and so on. In this way, the data points will fall along the 1/[S] axis at intervals of 1, 2 , 3, 4, 5, . . . , units. We chose our [S]-data according to this principle though not exactly the same dilution factors.

Otherwise, we would expect Lineweaver—Burk plot to differ more, from the real, Vmax and Km, compared to Eadie—Hofstee plot, simply because in the second case, we have Vo that is not reciprocal, but unmodified on the Y-axis.
We can not say that with certainty for all the alcohols in this lab though.
It is the case only if we consider specifically ethanol.

References

Chemistry by Zumdahl ed.8; page 571

Textbook of Biochemistry with Clinical Correlations by Thomas M. Devlin; ed. 7; year: 2010; page 383,384

Inroduction to Bioorganic Chemistry and Chemical Biology by David Van Vranken and Gregory Weiss; year 2013; pages 238-240

Enzymes - A Practical Introduction to Structure, Mechanism and Data Analys by Robert A. Copeland;

year 2000; ed.2; ch.5

Principles of Boichemistry ed. 4 by Voet, Voet, Pratt; page 363

Lehninger PRINCIPLES OF BIOCHEMISTRY ed.4 David L. Nelson, Michael M. Cox page 207-209

Lippincott's Illustrated Reviews: Biochemistry ed.5 by Richard A. Harvey, page 55-61

Extra discussion questions

- **Factors affecting the enzymes activity;**

  The protein is felxible. It sometimes changes during the reaction. Changing one amino acid in the protein, at a remote location from the active site, causes substantial changes in catalytic activity. This demonstrates that in some cases the overall structure of the protein and its ability to change shape are important.

  Source: Textbook of Biochemistry with Clinical Correlations by Thomas M. Devlin; ed. 7; year: 2010; page 402

  The rate of an enzymatic reaction depends on enzyme and reactant concentration. It can be strongly inhibited or activated by the presence of specific substances (called effectors). It depends strongly on temperature, through the usual ΔG^{+} dependence of the rate constant and through additional factors. The protein is destroyed by high temperature, while low temperature slows down the protein motion needed for performing the reaction. Different enzymes have different range of pH values. The bonds within the enzyme are affected by H+ and OH- ions in such a way that the shape of their active site is the most complementary to the shape of their substrate.

  Source: http://alevelnotes.com/Factors-affecting-Enzyme-Activity/146?tree=

- **The Substarte Binds at the Active Site of an Enzyme;**

  An enzyme molecule is often alot larger than its substrate. The active site of an enzyme is called the place where the Substrate binds. The conformation of the active site is structured to form a large scarffold whose three-dimensional architecture is complemetary to the structure of the substrate. The enzyme and the substrate find each other through this structural complementarity. The active site is specific for the substrate.They bound to each other through relatively weak forces - H bonds, ionic bonds, (salt bridges), and van der Waals interactions.

  Source: Textbook of Biochemistry with Clinical Correlations by Thomas M. Devlin; ed. 7; year: 2010; page 387

- **Conditions Michaelis-Menten Equation;**

  1. The reaction involves only one substrate, because each enzyme can act on only one other molecule at a time.

  2. [S]o >> [Et] and [Et] is held constant/unchanged.

  3. [P]=0 when, time=0
  3. All other variables that might influence the rate of the reaction (temperature, pH, ionic strength, and so on) are constant.

  Source: Lippincott's Illustrated Reviews: Biochemistry ed.5 by Richard A. Harvey, page 58

- **Is Km dependent of the enzyme concentration;**

  On this questions we choose to answer only by mathematical terms.

Et= [E] =total concetration of Enzyme

$$\mathbf{Km} = \frac{\mathbf{Vmax}}{\mathbf{2}}$$

It is actually not exsactly right. It is better if we say that Km is the substrate when we have reached Vmax/2

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Again; the value of Vmax is not related to the value of Km. Km is the [S] when Vmax / 2.
Km is not a rate.
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We know also;

$$V_{max} = E_t K_{cat}$$

But we can not write the following fromula;

$$K_m = \frac{E_t K_{cat}}{2}$$

So we apply opposite proving to prove our thesis.

Answer: Km and [E] are linearly independent of each other. Km is a constant describing an ezyme. It does not change.