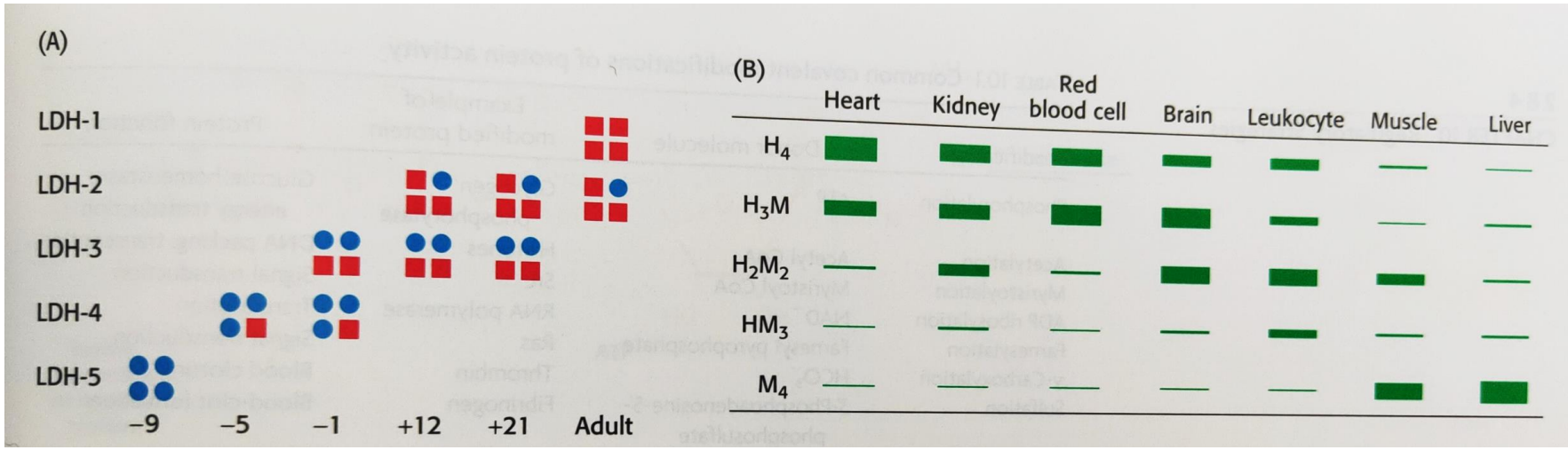
Lab 3: Lactate Dehydrogenase activity: Effect of substrate and inhibitor

- LDH (Lactate dehydrogenase) is an important enzyme in glycolysis.
- In anaerobic glycolysis
  - It prevents pyruvate build up by converting it to lactate
  - It regenerates NAD+ to continue substrate level ATP production

Isozymes provides a means of regulation specific to distinct tissues and developmental stages



Lactate dehydrogenase (LDH): H (heart) and M (muscle, liver), 75% sequence identity.

Each functional enzyme tetrameric. Many different combinations possible.

High pyruvate level inhibit H form but not M form.

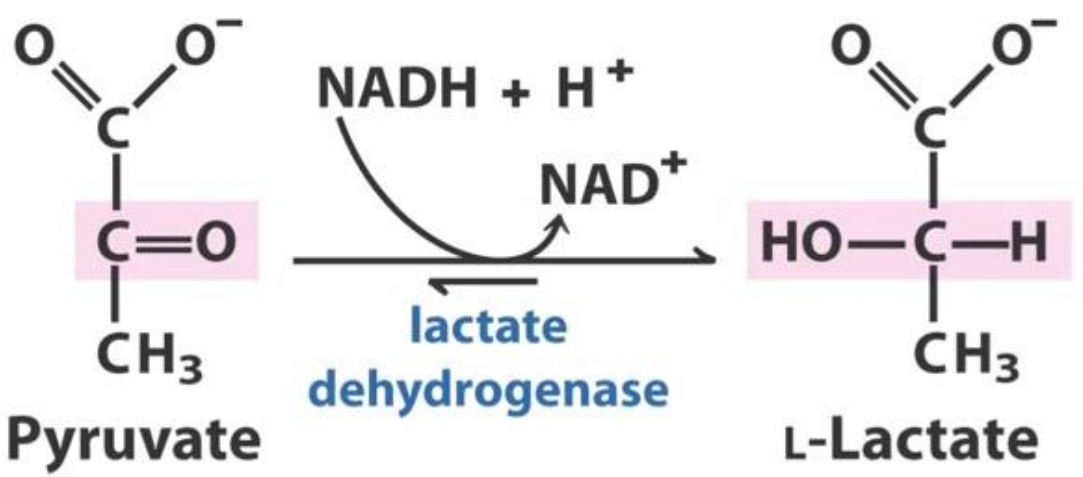
M form function optimally in anaerobic environment

H form does so in aerobic environment.

Today's Lab

- You will
  - Investigate the effect of substrate concentration (pyruvate) on LDH activity.
  - Investigate how presence of inhibitor changes the activity of the LDH

- You have
  - Buffer
  - LDH
  - Pyruvate
  - NADH



Assay Principle

- NADH absorbs light at 340 nm
- As NADH is converted to NAD, the absorbance decreases.
- Decrease (the consumption of NADH) is equivalent to the amount of lactate formed.
- The velocity of the consumption of NADH (A340) reflect the velocity of this reaction.

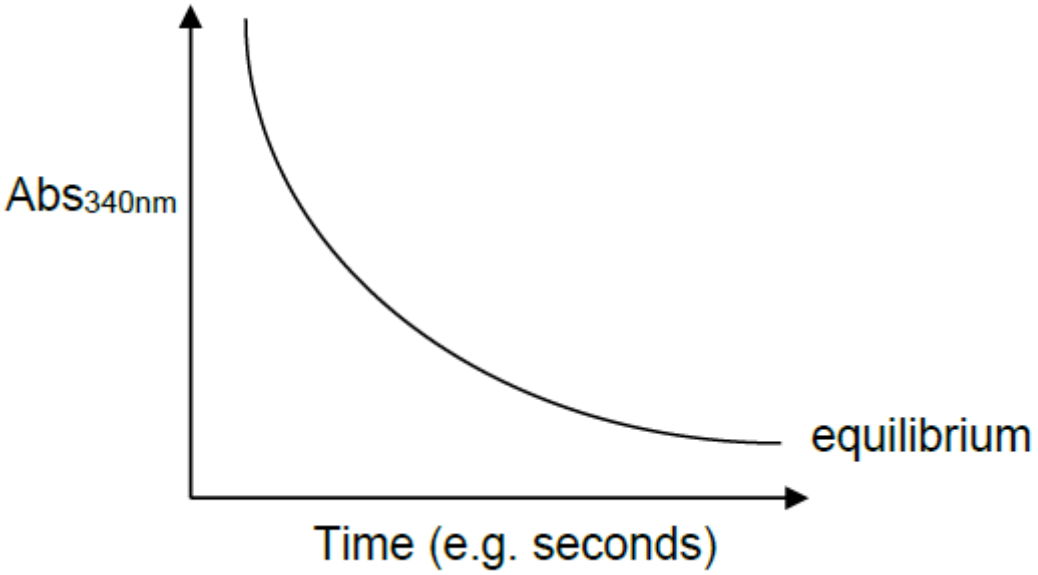
The Experiment

1. You will investigate the effect of various concentrations of pyruvate on LDH activity
2. You will investigate the activity of the same concentration of pyruvate in presence of an inhibitor, oxalate.

Things to remember

- Keep the enzyme and NADH on ice until the very end
- Prepare all the tubes except the enzyme
- Take it to the spectrophotometer
- Prepare the spectrophotometer
- Add enzyme to each sample one at time and take absorbance reading immediately
- Add enzyme one at time and take reading immediately (do not vortex).

What will the data look like



At first, the rate of absorbance change is proportional to the concentration of pyruvate. As lactate is formed, the reverse reaction becomes significant. So we only use the initial rate of change to estimate enzyme activity.

Part A

Materials

The following materials have been provided for you:

- 0.1M Sodium Phosphate Buffer pH 7.4
- 10 mM NADH (keep on ice at all times)
- 2.1 mM Pyruvate
- 10U/ml Lactate Dehydrogenase (keep on ice at all times) (Unit definition: One unit will reduce 1.0 µmole of pyruvate to L-lactate per min at pH 7.5 at 37 °C.)
- 3mM Oxalate

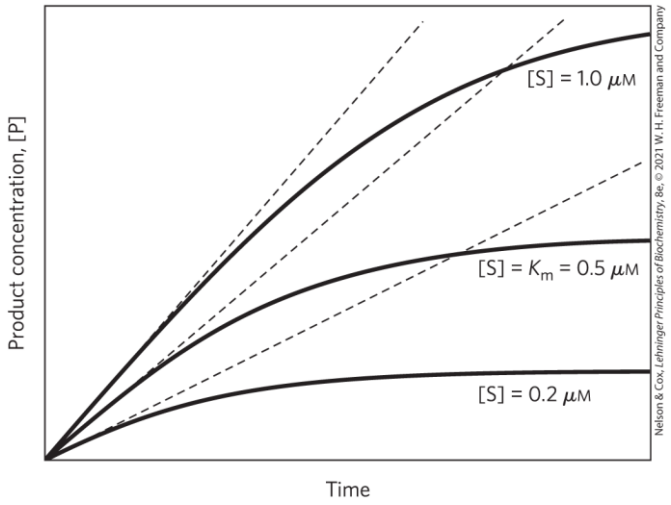
Tube #	2.0 mM Pyruvate	Na Phosphate Buffer	10 mM NADH	[Pyruvate] mM = [S]
1(A&B)	0mL	2.8 mL	0.1 mL	
2(A&B)	0.03 mL	2.77 mL	0.1 mL	
3(A&B)	0.06 mL	2.74 mL	0.1 mL	
4(A&B)	0.12 mL	2.68 mL	0.1 mL	
5(A&B)	0.24 mL	2.56 mL	0.1 mL	
6(A&B)	0.48 mL	2.32 mL	0.1 mL	
7(A&B)	0.96 mL	1.84 mL	0.1 mL	

Part B: reaction with inhibitor: oxalate

Tube #	Inhibitor 0 3mM Oxalate	2.0 mM Pyruvate	Na Phosphate Buffer	10 mM NADH	[Pyruvate] mM = [S] for your graphs
Ti1(A&B)	0.1 mL	0mL	2.7 mL	0.1 mL	
Ti2(A&B)	0.1 mL	0.03 mL	2.67 mL	0.1 mL	
Ti3(A&B)	0.1 mL	0.06 mL	2.64 mL	0.1 mL	
Ti4(A&B)	0.1 mL	0.12 mL	2.58 mL	0.1 mL	
Ti5(A&B)	0.1 mL	0.24 mL	2.46 mL	0.1 mL	
Ti6(A&B)	0.1 mL	0.48 mL	2.22 mL	0.1 mL	
Ti7(A&B)	0.1 mL	0.96 mL	1.74 mL	0.1 mL	

Calculate the final concentration of pyruvate

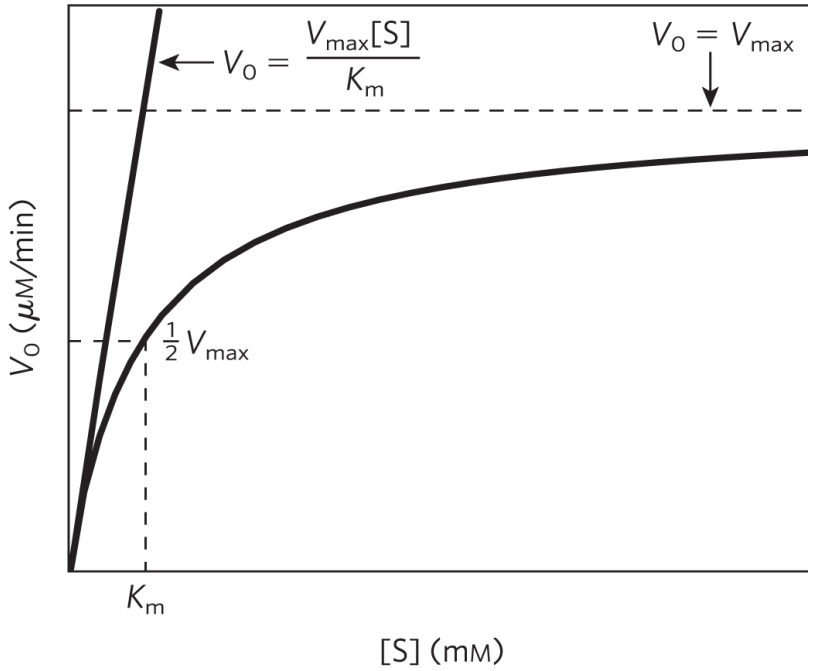
Data analysis
Revisit enzyme kinetics



A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of an in vitro reaction as substrate is converted to product. One simplifying approach is to measure the **initial rate** (or **initial velocity**), designated V_0 (**Fig. 6-11**). In a typical reaction, the enzyme may be present in nanomolar quantities, whereas [S] may be five or six orders of magnitude higher. If only the beginning of the reaction is monitored, over a period in which only a small percentage of the available substrate (<2%–3%) is converted to product, [S] can be regarded as constant, to a reasonable approximation. V_0 can then be explored as a function of [S], which is adjusted by the investigator. Note that even the initial rate reflects a steady state.

Michaelis-Menten Equation

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$



The effect on V_0 of varying [S] when the enzyme concentration is held constant is shown in **Figure 6-12**. At relatively low concentrations of substrate, V_0 increases almost linearly with an increase in [S]. At higher substrate concentrations, V_0 increases by smaller and smaller amounts in response to increases in [S]. Finally, a point is reached beyond which increases in V_0 are vanishingly small as [S] increases. This plateau-like V_0 region is close to the **maximum velocity, V_{max}**.

Lineweaver-Burk plot

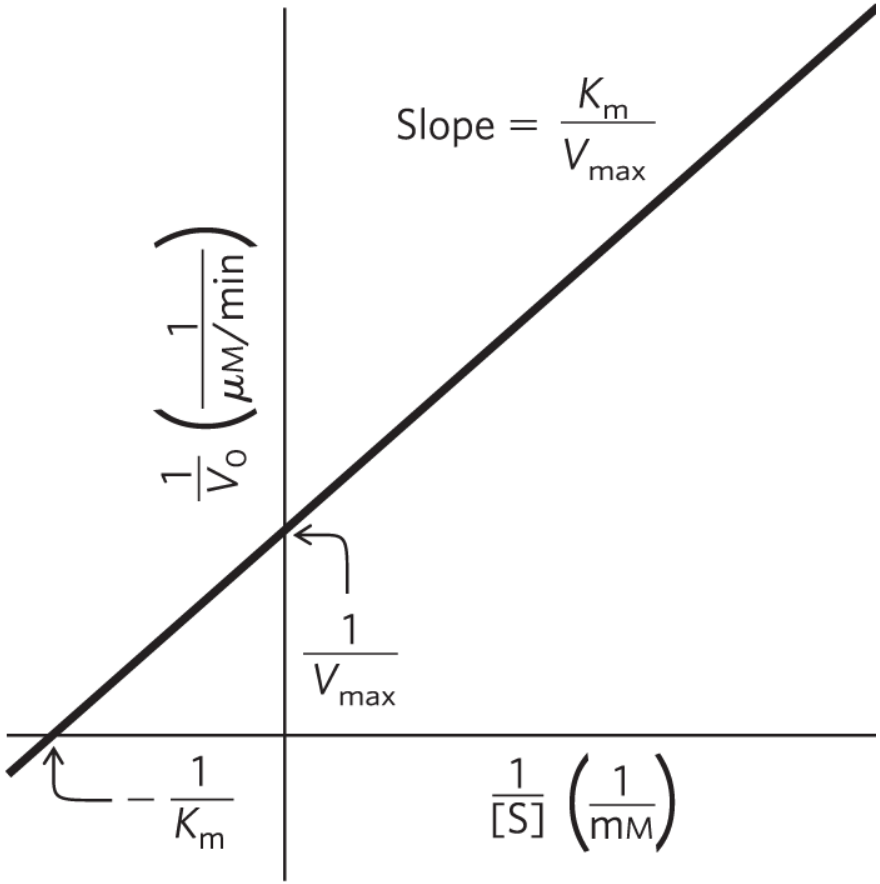
$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Y intercept= $1/V_{max}$

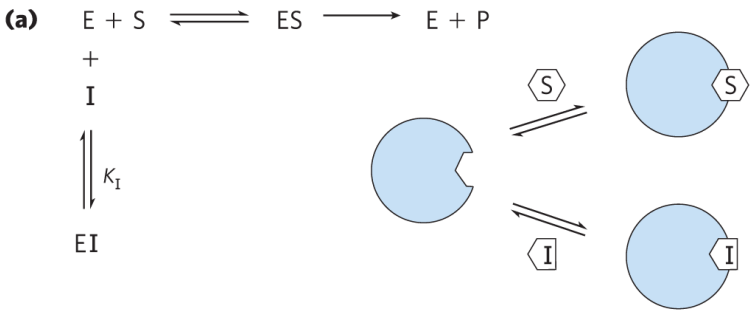
Thus: V_{max} =1/(Y intercept)

Slope=Km/V_{max}

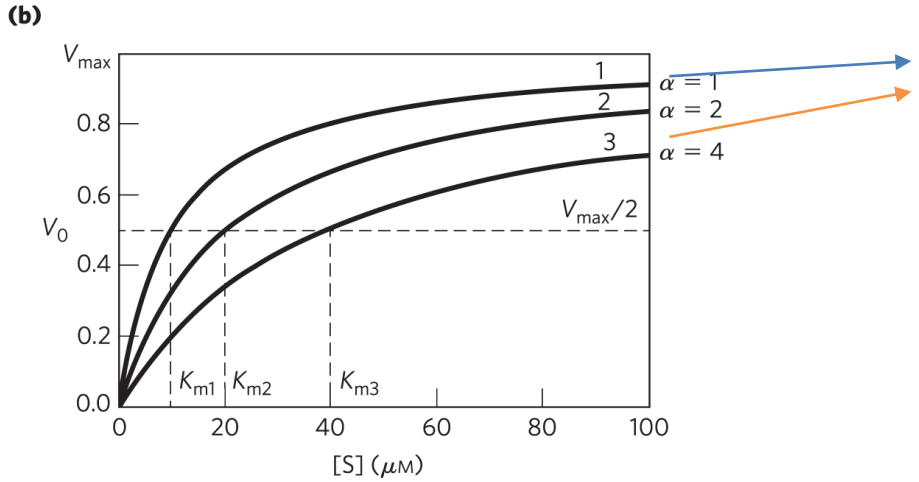
Thus: Km=slope/Y intercept



Competitive inhibition



$$V_0 = \frac{V_{max}[S]}{\alpha K_m + [S]}$$

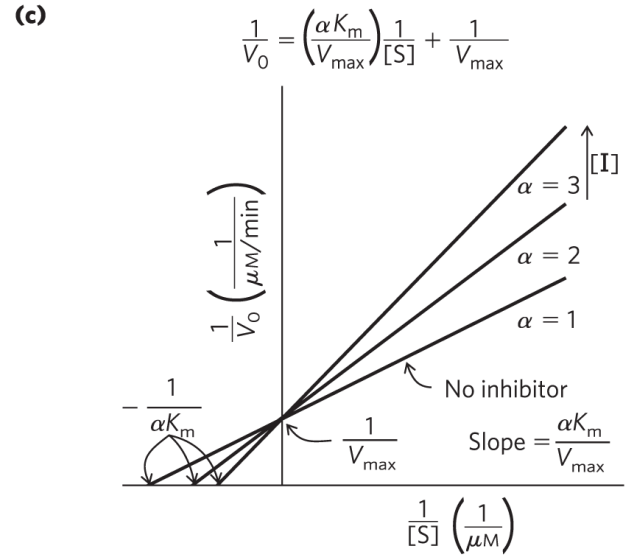


Change of slope

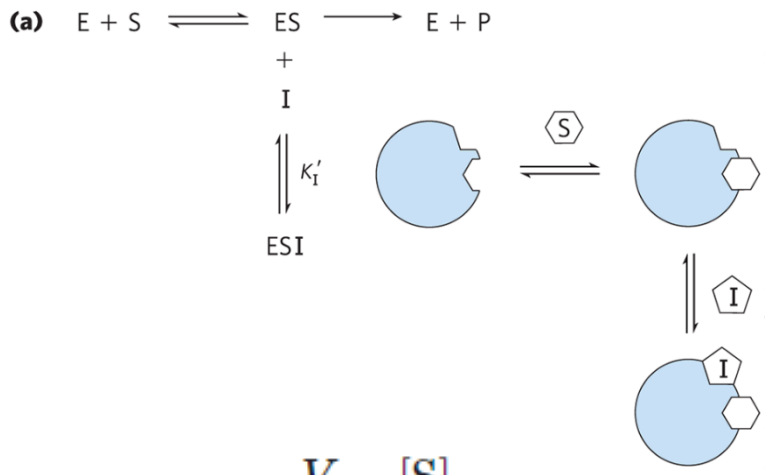
Slope= α* (K_m/V_{max})

but Y intercept keep constant

V_{max} does not change



Non-competitive inhibition



$$V_0 = \frac{V_{max}[S]}{K_m + \alpha'[S]}$$

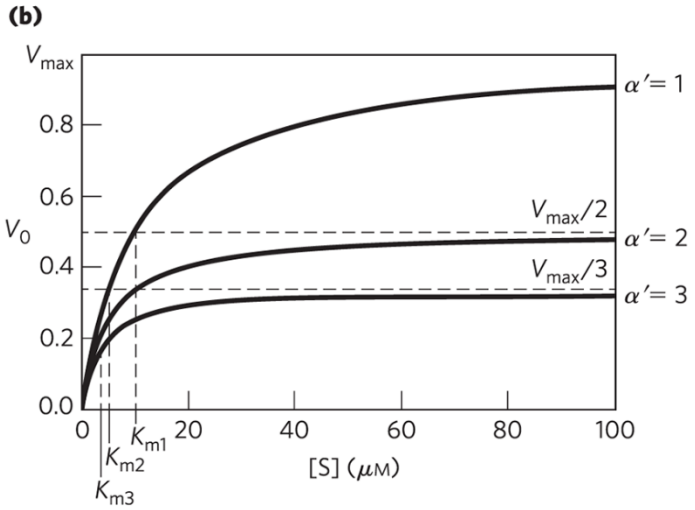
slope does not change

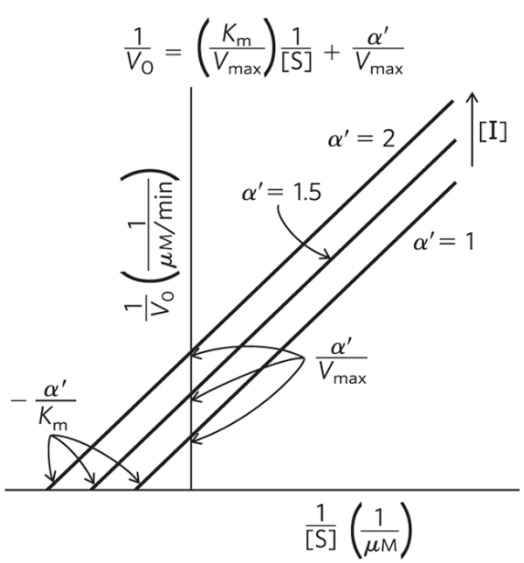
Slope= (K_m/V_{max})

Y intercept=$1/V'_{max} = \alpha'/V_{max}$

Maximum velocity is diminished

$V'_{max} = V_{max}/\alpha'$





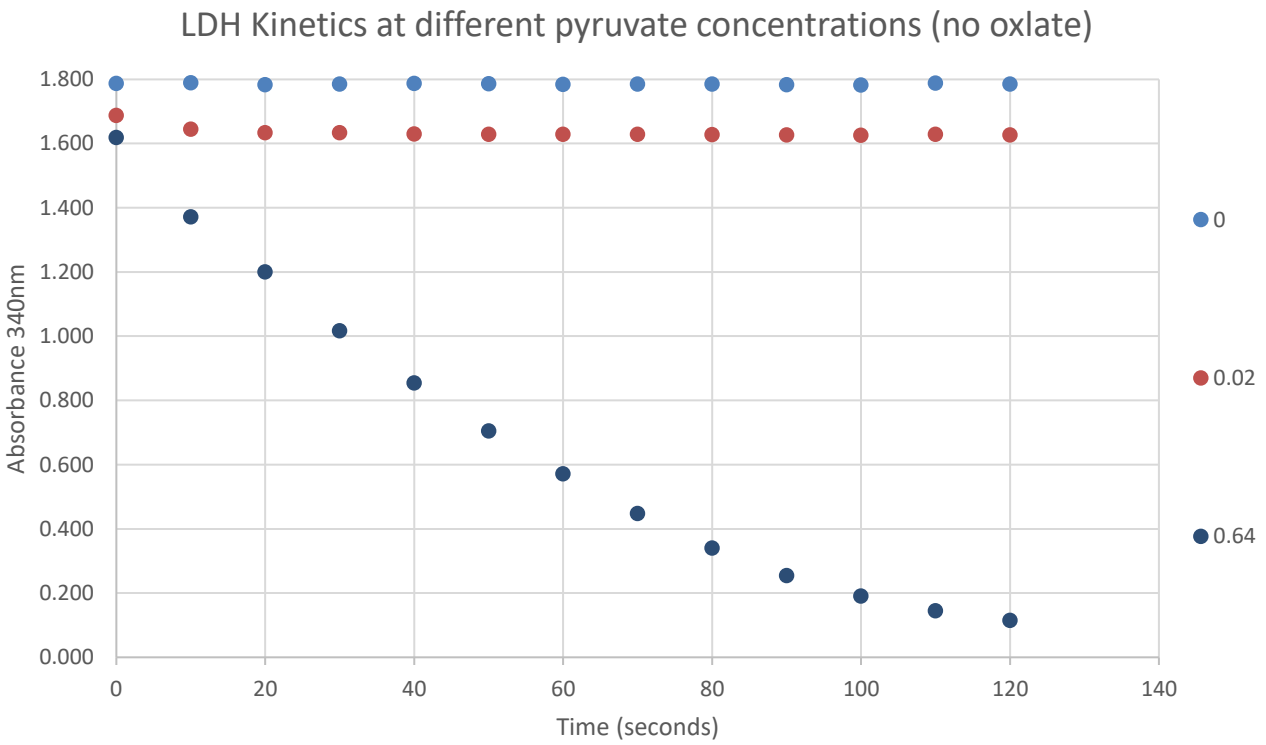
Part A Data

Experiment 1 (no inhibitor)

Time	T1A	T1B	Ave	T2A	T2B	Ave	T3A	T3B	Ave	T4A	T4B	Ave	T5A	T5B	Ave	T6A	T6B	Ave	T7A	T7B	Ave
0	1.786	1.788	1.787	1.690	1.684	1.687	1.646	1.629	1.638	1.623	1.595	1.609	1.567	1.595	1.581	1.580	1.578	1.579	1.612	1.624	1.618
10	1.789	1.788	1.789	1.649	1.640	1.645	1.547	1.546	1.547	1.418	1.393	1.406	1.273	1.309	1.291	1.290	1.280	1.285	1.353	1.390	1.372
20	1.785	1.781	1.783	1.637	1.629	1.633	1.528	1.530	1.529	1.345	1.318	1.332	1.082	1.124	1.103	1.099	1.064	1.082	1.179	1.221	1.200
30	1.784	1.786	1.785	1.636	1.631	1.634	1.526	1.525	1.526	1.319	1.296	1.308	0.934	0.974	0.954	0.904	0.850	0.877	0.985	1.048	1.017
40	1.790	1.784	1.787	1.634	1.624	1.629	1.522	1.524	1.523	1.308	1.285	1.297	0.858	0.878	0.868	0.686	0.652	0.669	0.818	0.891	0.855
50	1.784	1.788	1.786	1.632	1.625	1.629	1.520	1.523	1.522	1.303	1.278	1.291	0.821	0.835	0.828	0.511	0.490	0.501	0.660	0.749	0.705
60	1.783	1.785	1.784	1.631	1.626	1.629	1.519	1.518	1.519	1.299	1.273	1.286	0.808	0.817	0.813	0.353	0.352	0.353	0.536	0.606	0.571
70	1.784	1.786	1.785	1.632	1.624	1.628	1.518	1.518	1.518	1.296	1.268	1.282	0.800	0.808	0.804	0.226	0.236	0.231	0.423	0.472	0.448
80	1.788	1.781	1.785	1.633	1.621	1.627	1.517	1.513	1.515	1.293	1.268	1.281	0.795	0.802	0.799	0.138	0.144	0.141	0.320	0.359	0.340
90	1.783	1.783	1.783	1.631	1.622	1.627	1.516	1.516	1.516	1.293	1.267	1.280	0.791	0.797	0.794	0.087	0.087	0.087	0.231	0.277	0.254
100	1.785	1.779	1.782	1.632	1.619	1.626	1.514	1.515	1.515	1.293	1.265	1.279	0.787	0.793	0.790	0.060	0.059	0.060	0.165	0.215	0.190
110	1.786	1.789	1.788	1.633	1.624	1.629	1.515	1.515	1.515	1.291	1.264	1.278	0.783	0.791	0.787	0.046	0.047	0.047	0.126	0.164	0.145
120	1.782	1.788	1.785	1.630	1.623	1.627	1.514	1.514	1.514	1.291	1.265	1.278	0.783	0.789	0.786	0.041	0.043	0.042	0.100	0.130	0.115
ΔA			0.000																		
Time			0																		
ΔA/sec			0.000																		
ΔA/min			0.000																		

You are supposed to calculate the key statistics below.
No need to fill the grey area.

Make curves with ascending concentration of pyruvate



Using the curve to decide the time period to calculate the ΔA/min data

Note: we need to estimate the initial velocity not average velocity.

Key parameter calculations

Experiment 1--Key enzymatic parameters

[Pyruvate] mM = [S]	ΔA/min	Velocity (Amount of lactate formed) (µM/min)	1/[pyruvate], 1/mM	1/velocity, min/µM	Y intercept of double reciprocal plot 1/Vmax, min/µM	Vmax (µM/min)	Km (µM)
0.00							
0.02							
0.04							
0.08							
0.16							
0.32							
0.64							

Transform from ΔA/min to amount of lactate formed using Beer-Lamber law

Transformations to prepare for Lineweaver-Burk plot

Key data gained from linear regression of Lineweaver-Burk plot

How to transform from ΔA to molar concentration?

You should observe a linear (straight line) change in absorbance during at least the first 30-40 seconds if you have properly done your steps.

2. Calculate the change in Abs_{340}/min =ΔA = Abs at 0 – Abs at the point where the curve starts to taper off (should be within the first 30-40 seconds) enter these values into the table on the following page.

   If you calculated Abs_{340}/sec, you will need to convert this into Abs_{340}/min first.

3. Calculate the amount of lactate formed in each assay. Enter these values into the table on the following page. This is equal to the amount of NADH used up in µmoles/3mL/min, using the Beer Lambert Law:

Beer-Lambert Law

A (absorbance) = ε_m (extinction coefficient) x c_m (concentration in M) x 1

[ε_m ,Extinction co-efficient: how strongly a substance absorbs light at a given wavelength, per mass density or per molar concentration]

So $\Delta A = \varepsilon_m \times \Delta c_m \times l$

So $\Delta c_m = \Delta A / (\varepsilon_m \times l)$

we know that ε_m for NADH = 6.22×10^3 M^{-1} cm^{-1} at 340 nm, l=1 cm

So $\Delta c_m = \Delta A\ min^{-1} / (6.22 \times 10^3)\ M^{-1}\ cm^{-1}$

= _____ M min^{-1}

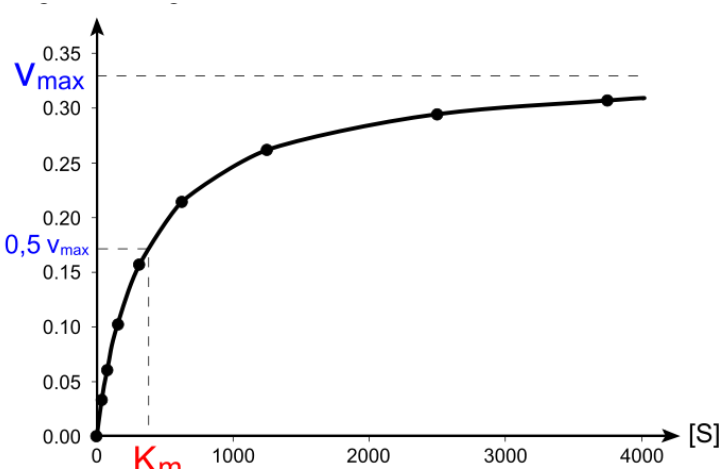
= _____ µM min^{-1}

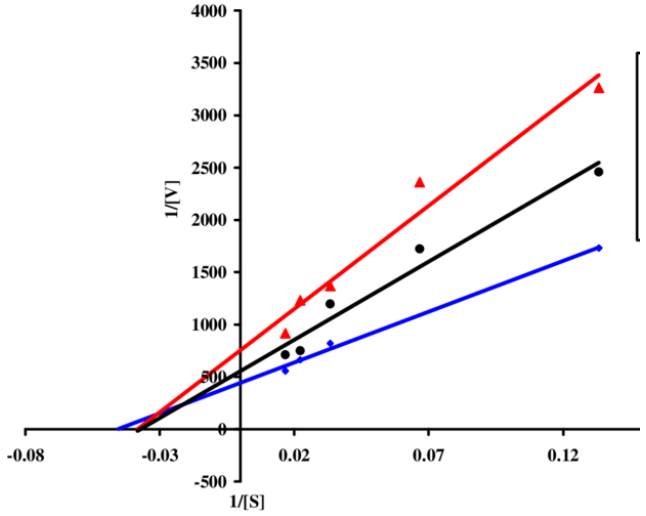
= amount of NADH consumed in the assay

= amount of lactate formed in each assay (WHY?)

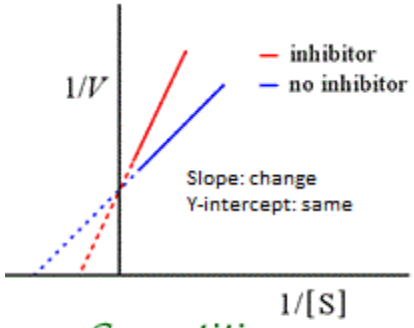
4. Express the activity of LDH in the above assays as Units, and record these values in the table on the following page. This represents the velocity (v, µM min^{-1}) of the reaction.

Plot Michaelis-Menten and Lineweaver-Burk plot with/without inhibitor (two curves in one plot)

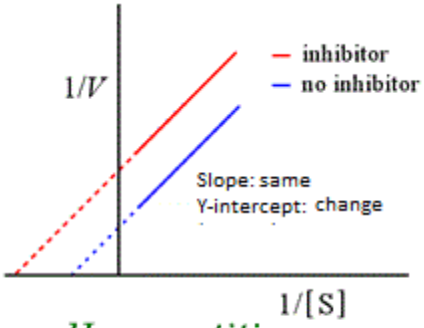




Determine whether oxalate is a competitive or non-competitive (uncompetitive) inhibitor based on the linear regression curve



Competitive inhibition
K_M increased
V_{max} unaffected



Uncompetitive inhibition
K_M reduced
V_{max} reduced