BMOL2201/6201 PRACTICAL 5

Enzyme Kinetics

PM	AM	Outline
2:05	10:05	Introductory talk
2:15	10:15	(Go to iLearn and complete Pre-lab Quiz)
2:20	10:20	**(A) Effect of substrate structure on reaction velocity**
3:50	11:50	**(B) Determination of K_m and V_{max} (calculation only; data provided)**
		(C) Allostery in Oxidative deamination (calculation only; data provided)
4:30	12:30	Go to iLearn for upload and Prac Quiz
4:55	12:55	Prepare to leave lab

PART 1: THEORY

Practical 5 Aims

A. Determine **the effect of substrates** on the rate of the esterase hydrolysis reaction, using three different esters: **ethyl acetate, ethyl propanoate,** and **ethyl butanoate**.

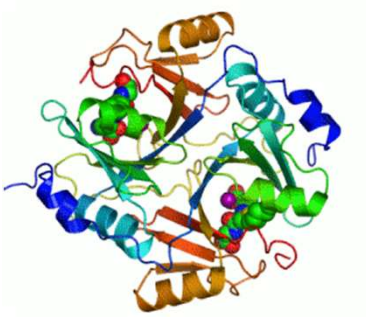
B. Determine the V_{max} for **lactate dehydrogenase (LDH)** and the K_m for **NAD^+**.

C. Investigate the **activity** of the enzyme **glutamate dehydrogenase (GDH)** in the presence of the **effector molecules, ADP** and **GTP**.

Associated Lectures for revision: 7, 8, 19.

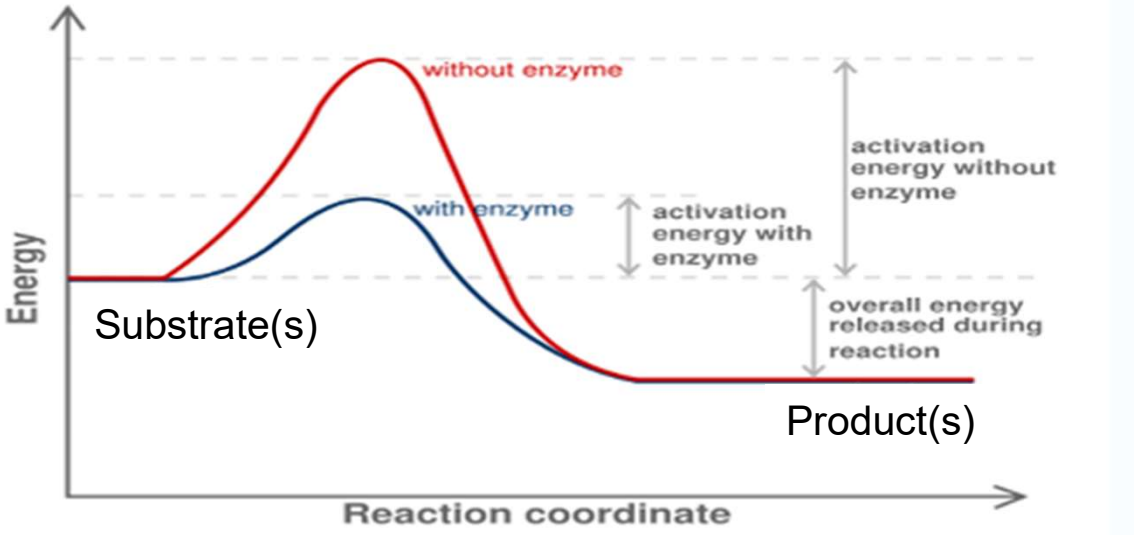
Enzymes

- Enzymes are biomolecules (commonly proteins) that catalyse chemical reactions

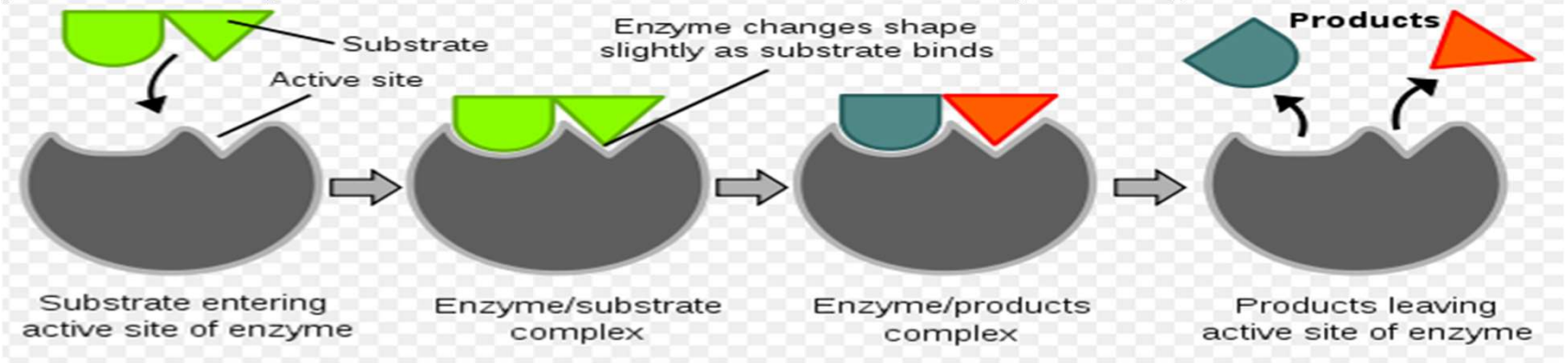


Substrate(s) → Enzyme (catalyst) → **Product(s)**

- Enzymes catalyse reactions by lowering the activation energy of the reaction

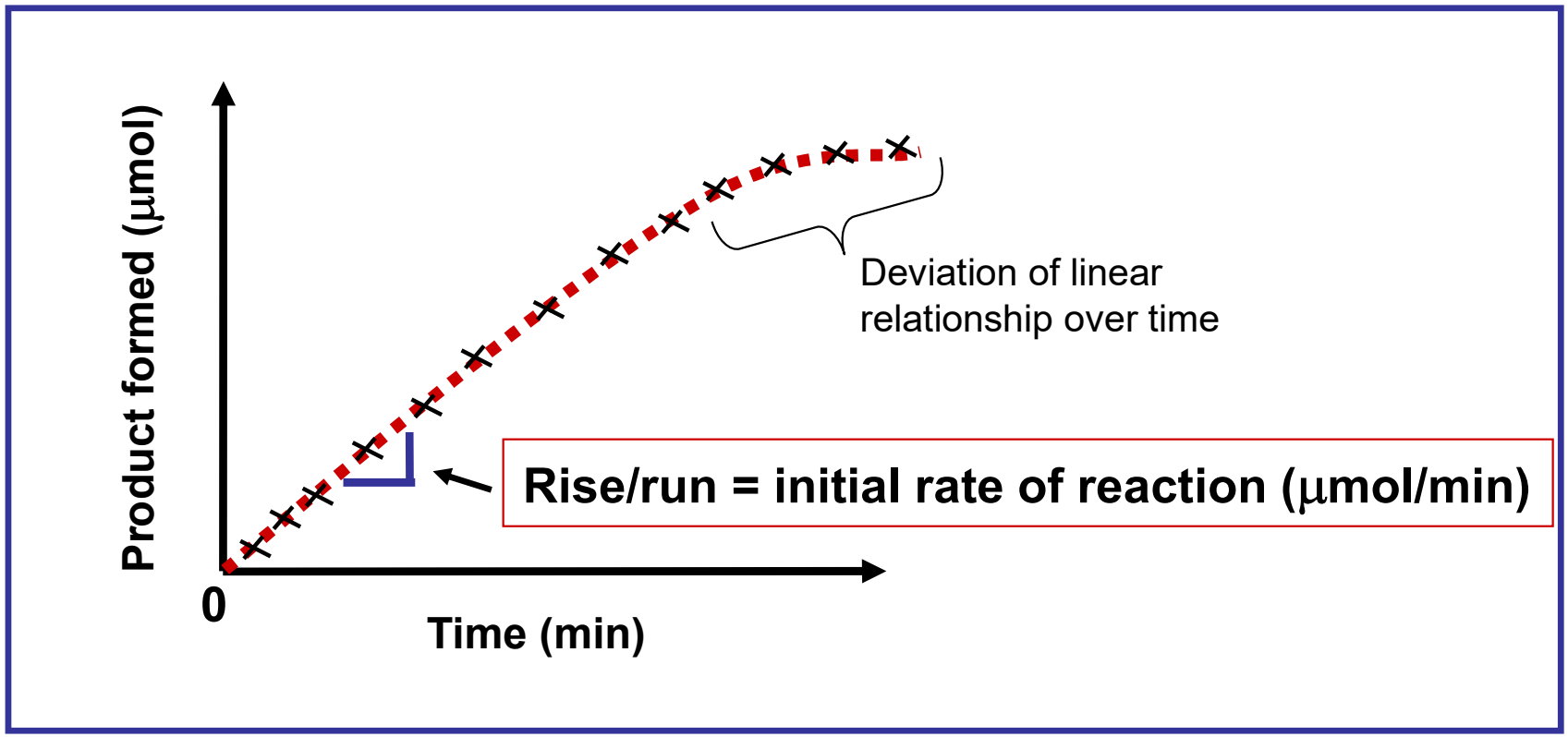


- Enzymes are specific to the reaction which they catalyse



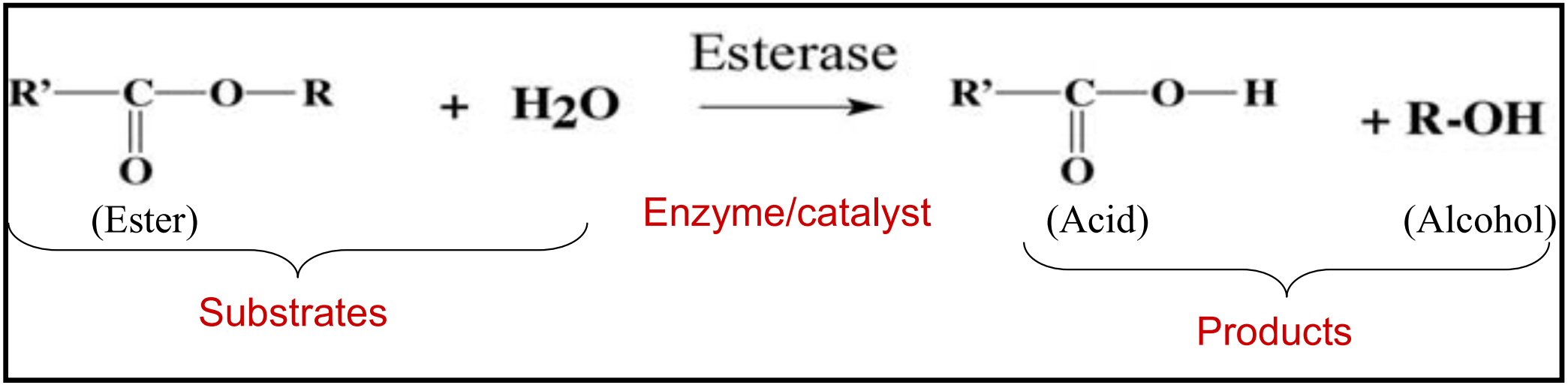
Enzyme Activity

- The activity of the enzyme can be measured as the amount of product formed per unit of time (eg. μmol/min)
- The **initial rate** of reaction is proportional to the **concentration of the enzyme** present
- Deviations from this linear relationship may occur after time due to exhaustion of substrate or denaturation of the enzyme

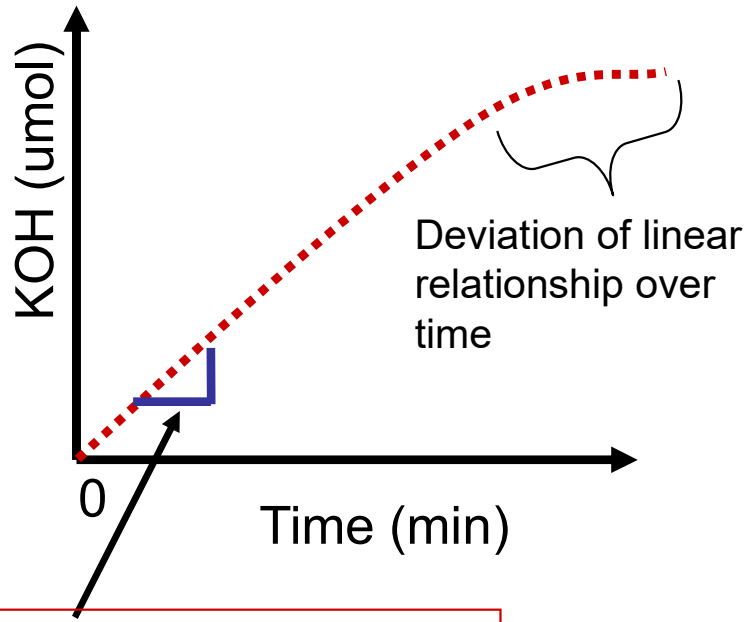


Therefore we use the initial rate of reaction to measure enzyme activity

(A) Esterase Activity



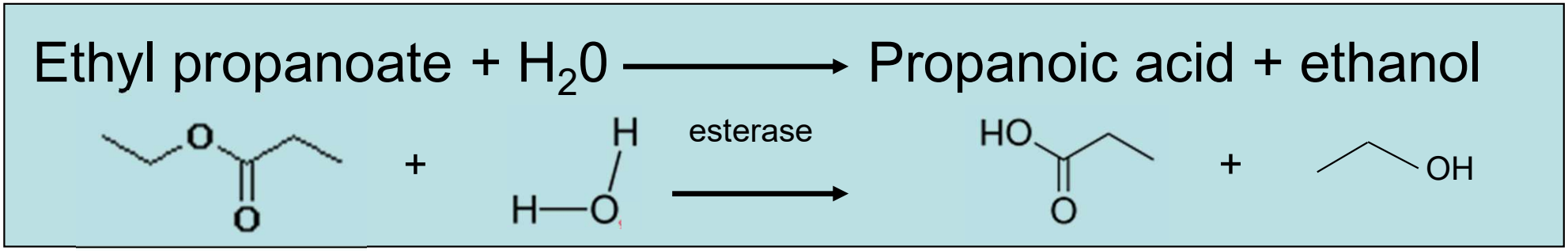
- A **product** of the esterase catalysed hydrolysis **reaction** is a **carboxylic acid**
- *Generation of acid will* ***reduce the pH of the solution***
- We can add base (KOH) to neutralise this acid and maintain a physiological **pH (7.4)**
- The **amount of base** (KOH) required to neutralise the acid being formed can be used to **calculate the rate of reaction**



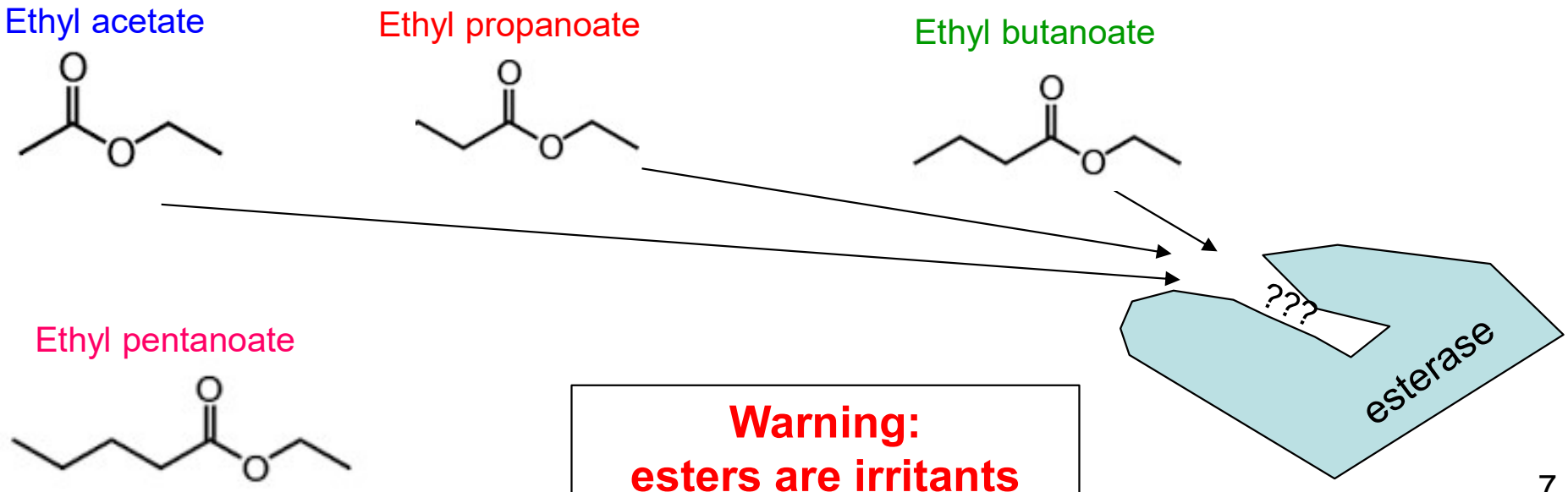
Rise/run = initial rate of reaction (μmol/min)

(A) Substrate Effect on Rate

Aim: To determine how substrate structure affects the rate of reaction



We will investigate three esters: **ethyl acetate, ethyl propanoate** and **ethyl butanoate**, where the same alcohol (ethanol) is generated by hydrolysis, but the carboxylic acid generated in each case is different.



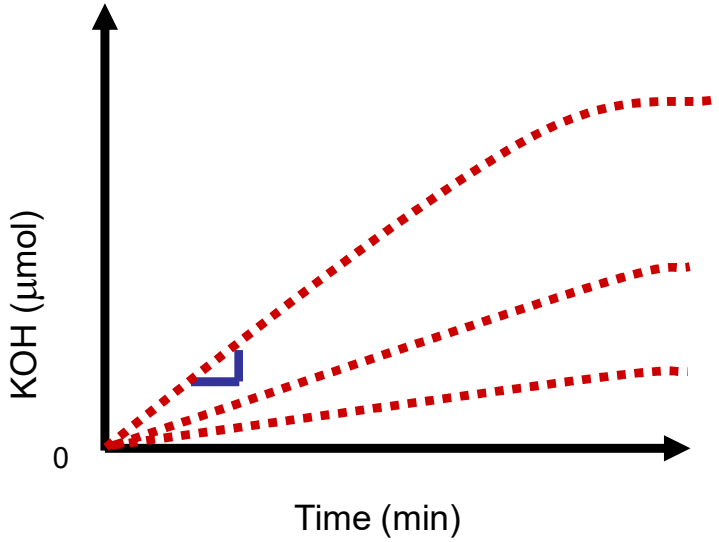
Warning:
esters are irritants

(A) Substrate Effect on Rate (done for you)

KOH IS CORROSIVE!!!

Method:

1) Dispense **50 ml phosphate buffer** and **2.5 ml ethyl acetate** into a beaker.
2) Place the stir bar and the pH electrode in a beaker.
2) Weigh 6 drops of **KOH** from the plastic pipette, in a pre-weighed weighing boat.
3) Adjust **pH** to 7.4 by adding **KOH** dropwise, with the same plastic pipette.
4) Pipette in **2 ml esterase (on ice),** **immediately** turn on **timer** and **add KOH dropwise to maintain pH = 7.4.** Start the timer.
4) Count the **number of drops added every 20 seconds** till **280 seconds.**
5) Record the results in the Table 1. Plot the results.
6) **Repeat** steps 1 to 4 with **50 ml of phosphate buffer, 2.5 ml of ethyl proprionate** and **2 ml esterase**, added at the very end. Record & Plot the results.
7) **Repeat** steps 1 to 4 with **50 ml of phosphate buffer, 2.5 ml of ethyl proprionate** and **2 ml esterase**, added at the very end. Record & Plot the results.
8) **Compare the rates of the esterase reaction**



Which substrates generate the most/least acid?

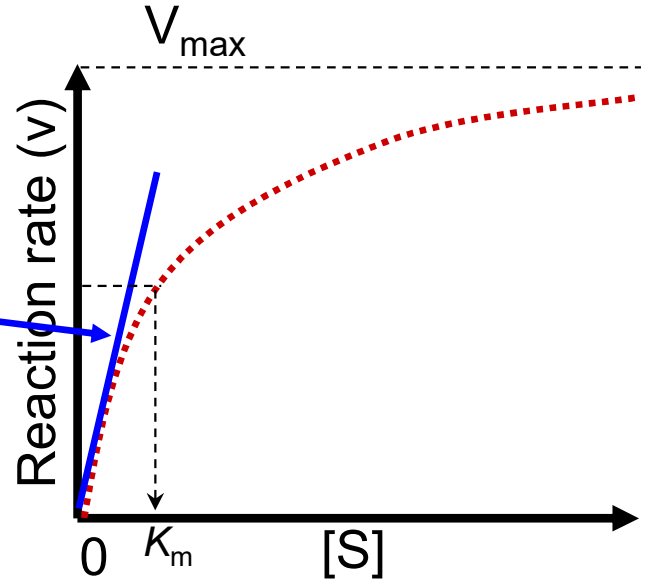
What does this tell you about the active site of esterase?

Results

1) **Weigh 6 drops of KOH**
2) **Complete Table 1 in your Prac file**
3) **Answer all questions**

Enzyme kinetics

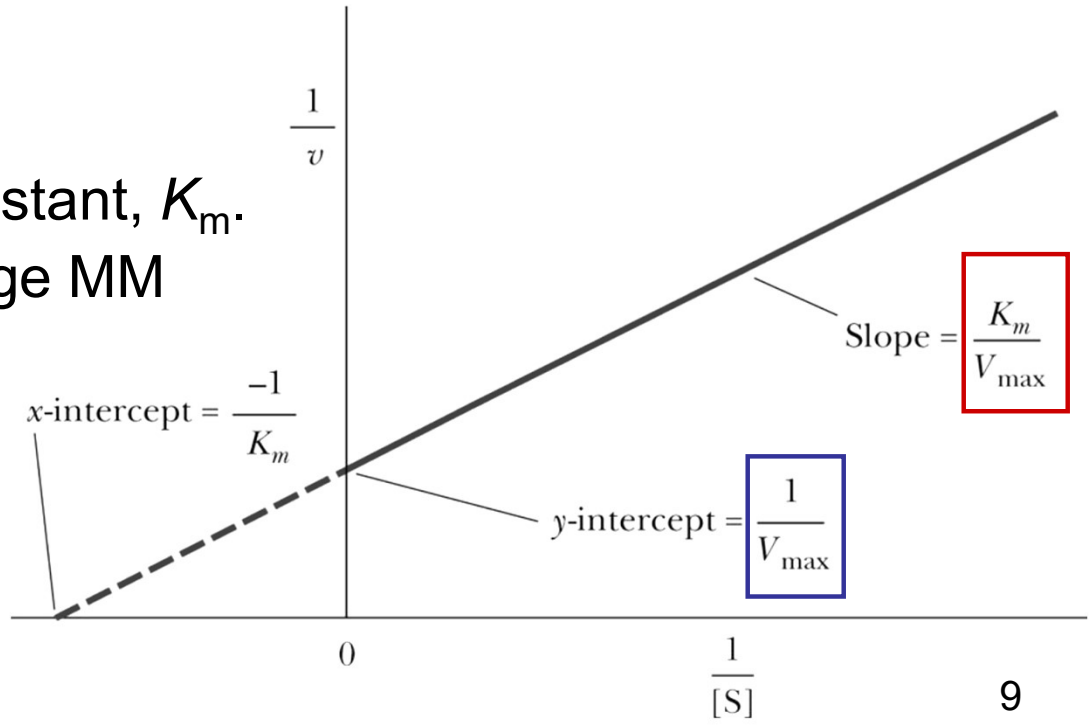
- Rate of enzyme reaction as a function of substrate concentration - Michaelis-Menten kinetics
- At low [S], v ∝ [S] – first order kinetics (straight line)
- At high [S], v is independent of [S] and approaches V_{max}.
- Michaelis-Menten equation:



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

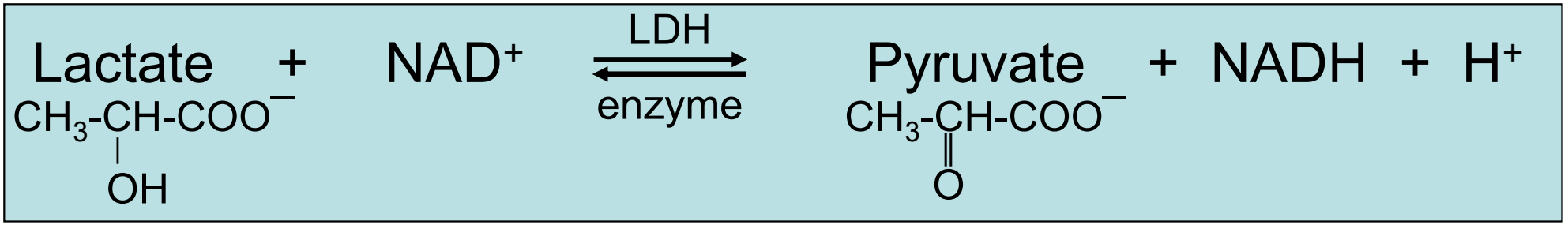
- At,1/2 V_{max}, [S] = the Michaelis constant, K_m.
- To determine V_{max} and K_m, rearrange MM equation and plot 1/v vs. 1/[S]

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



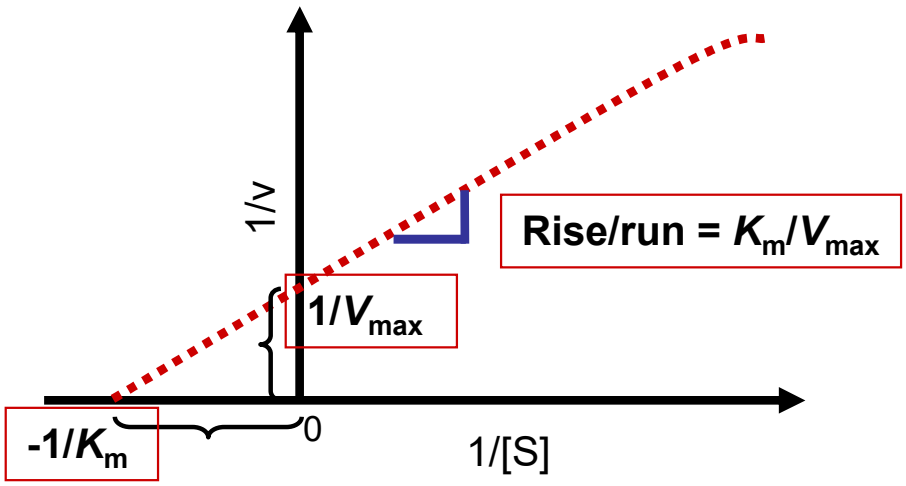
(B) K_m and V_{max} (calculation)

Aim: To determine V_{max} for lactate dehydrogenase (LDH; enzyme) and K_m for NAD^+ (substrate), from the increase in absorbance values at 340 nm due to NADH (product) formation



Method:

A. From the different concentrations of NAD^+ provided in Table 2, calculate $1/[NAD^+]$
B. From the reaction curve details provided, fill in the **ΔA** values in Table 2.
C. Complete the rest of Table 2.
D. The plot of 1/v vs. 1/[S] should refresh: from the graph, determine the values of V_{max} and K_m



What assumptions have you made?

Results

1) **Complete Table 2 in your Prac file**
2) **From the plot of 1/v vs. 1/[S] with your results, determine V_{max} and K_m**
3) **Answer all questions**

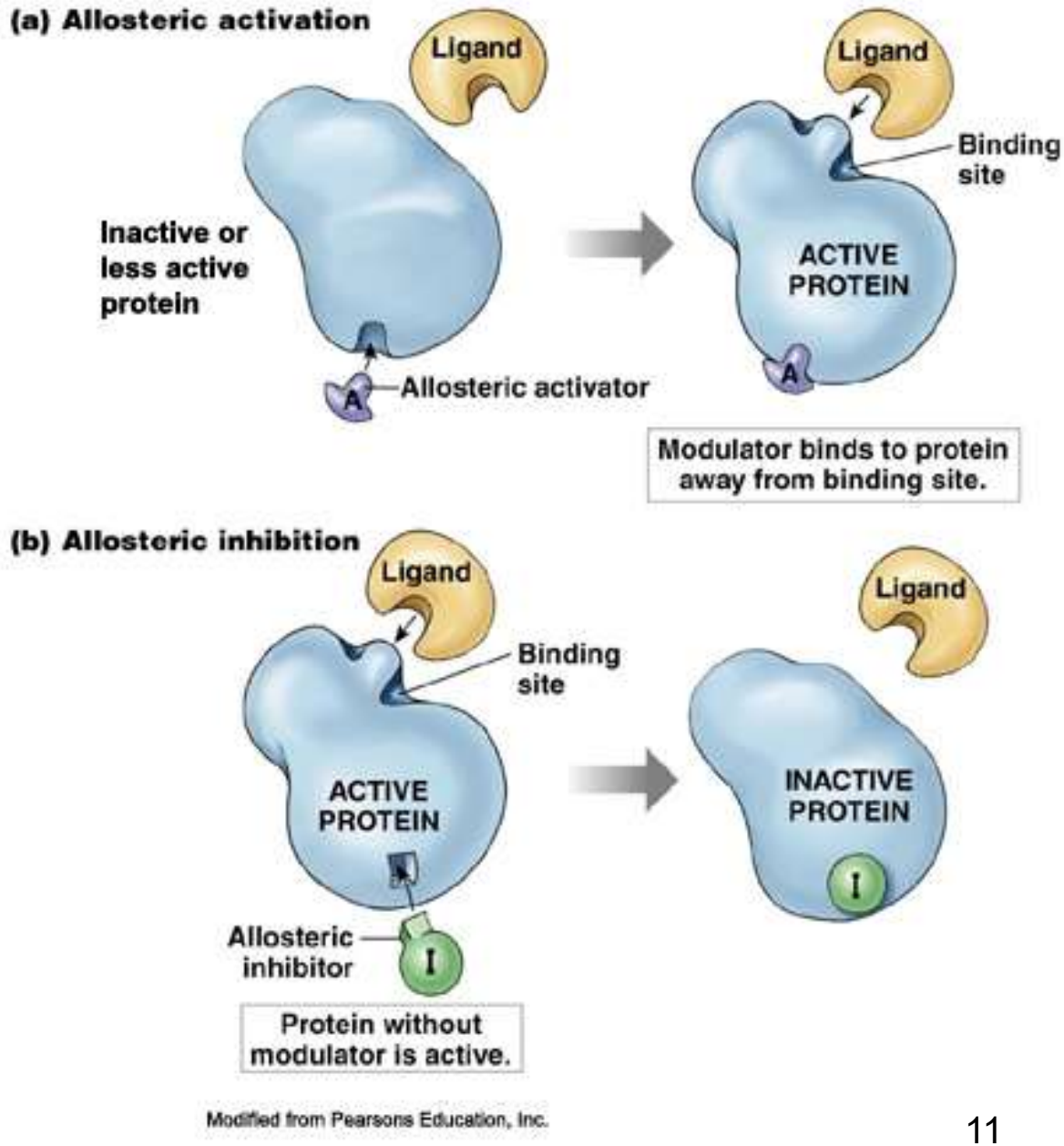
Allosteric Regulation

Allostery is the process by which **small molecules bind reversibly to a site distant from the active site** and thus, **rapidly regulate enzyme activity**.

In fact, **allosteric regulation is faster than other regulation strategies** available to the cell.

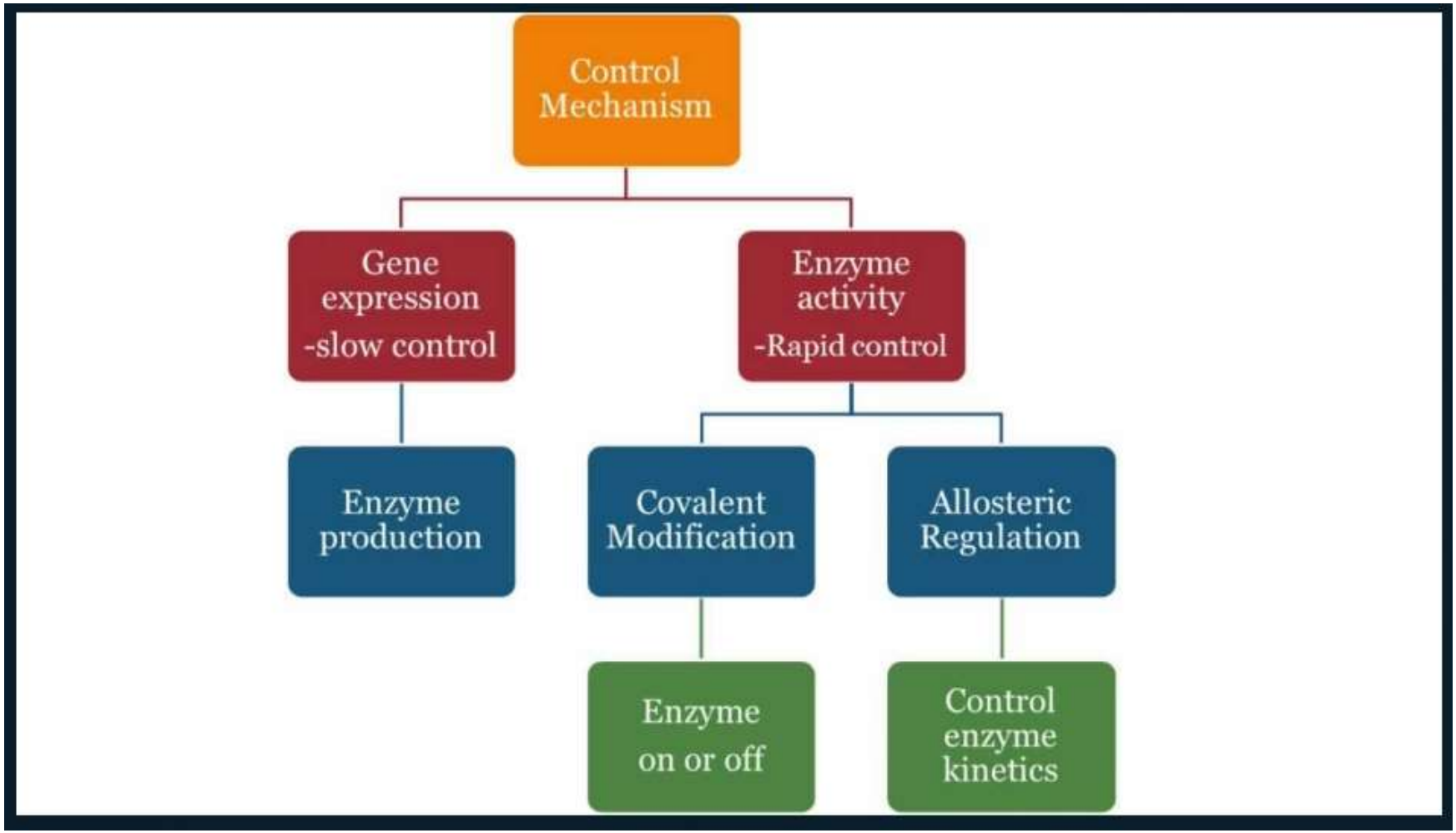
Allostery can lead to positive or negative regulation of enzyme kinetics

- **Positive: the rate is increased.**
- **Negative: the rate is decreased.**



Let's watch a Youtube video on Enzyme Regulation:

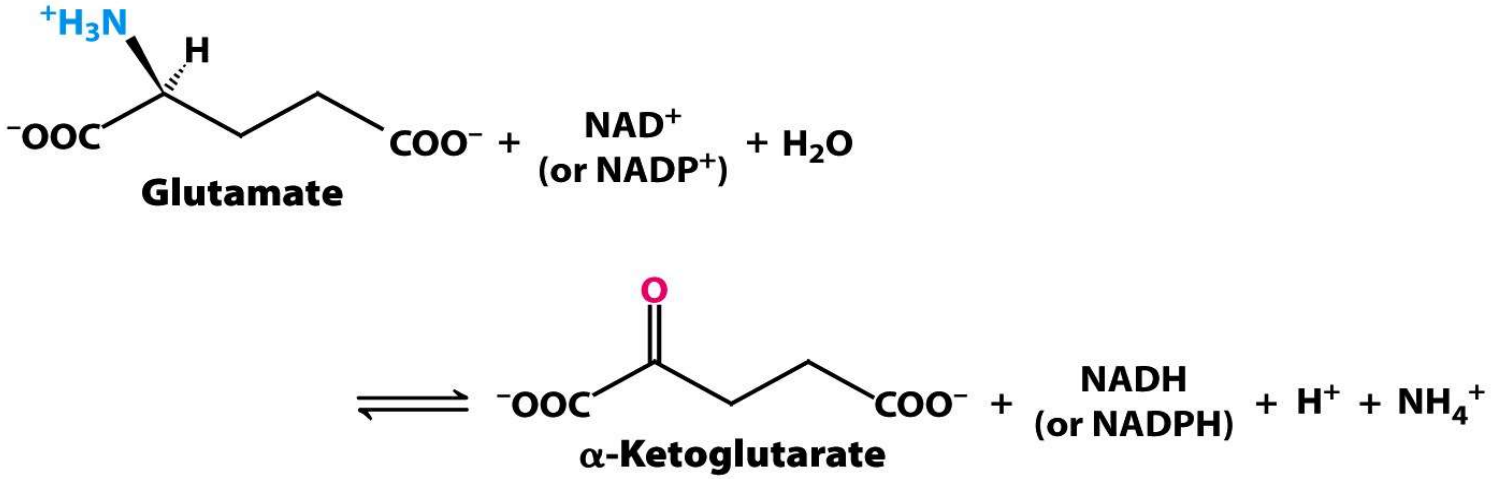
https://www.youtube.com/watch?v=hwMLhPSWIYs



(C) Allostery

Allostery is the process by which **small molecules bind to a site distant from the active site** and thus, **rapidly regulate enzyme activity**. In fact, allosteric regulation is faster than other regulation strategies available to the cell.

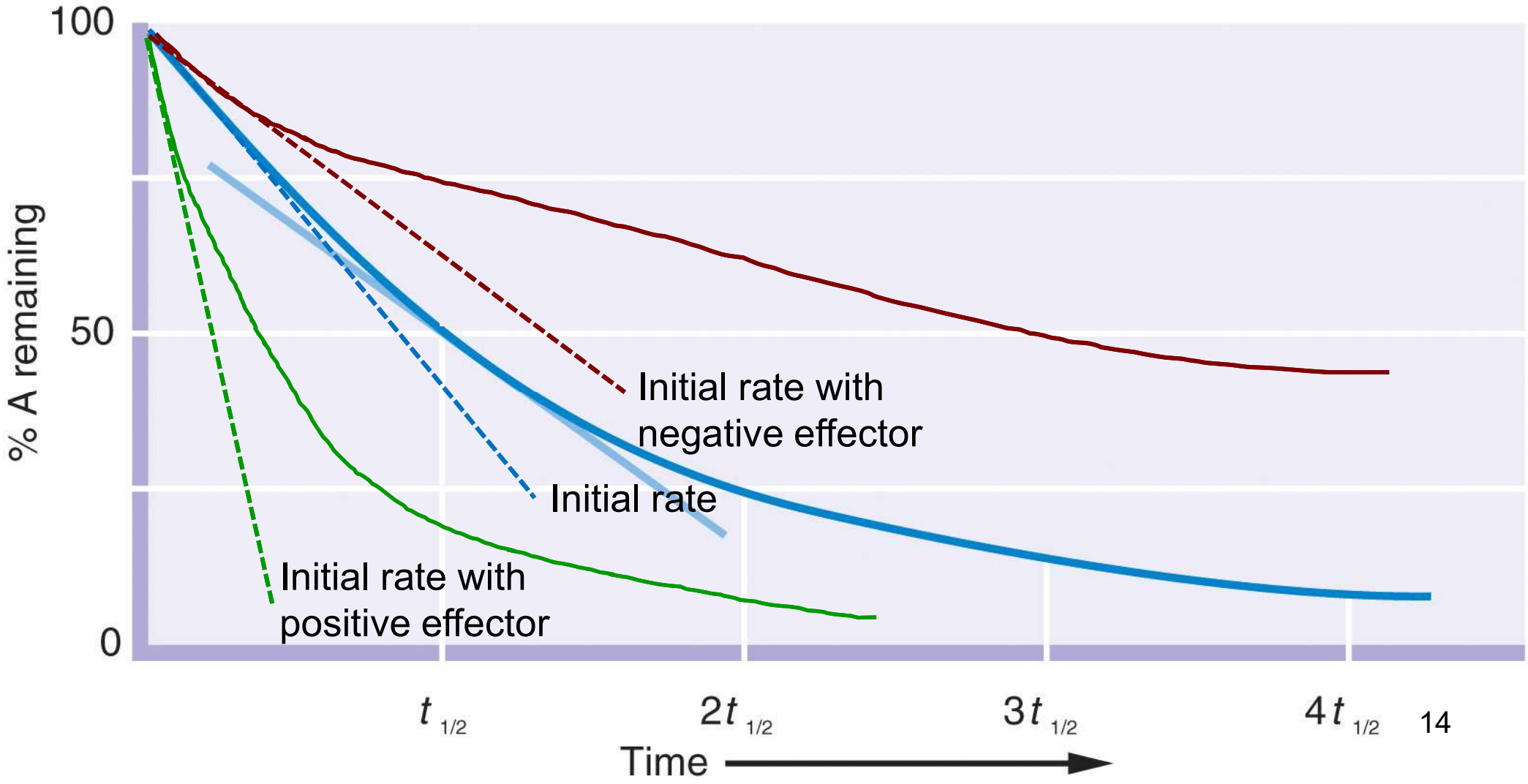
- The **oxidative deamination** reaction is catalyzed by the enzyme, **glutamate dehydrogenase (GDH)**, located in the mitochondria (Lecture 19).



- **GDH activity is allosterically affected by GTP and ADP**
- We will examine **GDH activity** using **α-ketoglutarate** as substrate, leading to **glutamate** formation (***i.e. reverse reaction shown***).
- The oxidation of **NADH** to **NAD+** results in change in absorbance which is measured with the help of spectrophotometer at **340 nm**.

(C) Allosteric Regulation Kinetics

- **Initial reaction rate** measured over **30 s**.
- *Significant deviation from linearity after this*.
- As we are following the **disappearance of substrate (NADH)**, the rate **should be negative.** This rate become *more negative with a positive effector*, and *less negative with a negative effector*.



PART 2: DETAILS OF EXPERIMENTS

(A)Substrate Effect on Rate

Method:

1) Dispense **50 ml phosphate buffer** and **2.5 ml ethyl acetate** into a beaker.
2) Place the stir bar and the pH electrode in a beaker.
2) Weigh 6 drops of **KOH** from the plastic pipette, in a pre-weighed weighing boat.
3) Adjust **pH** to 7.4 by adding **KOH** dropwise, with the same plastic pipette.
4) Pipette in **2 ml esterase (on ice),** **immediately** turn on **timer** and **add KOH dropwise to maintain pH = 7.4.** Start the timer.
4) Count the **number of drops added every 20 seconds** till **280 seconds.**
5) Record the results in the Table 1. Plot the results.

Points to note:

- **KOH IS CORROSIVE!!!**
- **Esters are irritants**
- **Enzyme will die if pH > 7.45!**

Before you start the experiment

- View the Lab Safety video
  - All laboratory technique videos have been screened before
  - PI. view them before the lab.

Prac 5 relevant videos guide for setting up and using equipment:

- pH meter video
- Magnetic stirrer video
- Micropipette video
- Waste disposal video

Procedure: Step 0

What to do - all students

1. You need covered shoes!

2. No bags on lab bench – place in under-bench storage

3. Tie up your hair, if applicable – tuck long hair into coat

4. Button up your lab coats

5. Put on safety glasses.

6. No food or drink please!

Need help?

- Lab Safety video

Procedure: Step 1: Getting started

Each team to collect one tray and fill it as follows:

Student 1 (gloves on)

A. Collect from the backbench and put into the tray:

1. a **beaker with a magnetic stirrer**
2. **5 mL Pipette** (green ring)
3. **Tips for the pipette**
4. a **bottle with 20mM KOH**
5. an **RO wash bottle**

Student 2 (gloves on)

A. Collect from the backbench and put into the tray:

1. a box of **Kimwipes**
2. a **liquid waste container**
3. a **used tip container**
4. a **permanent marker**
5. a **Plastic Pasteur pipette**

- Take the tray back to your desk
- The **esterase solution** is **on your desk** – in the esky.

Procedure: Step 2 – Part A

Student 1 (no gloves)

1. Login in with your student ID
2. Download and open Prac 5 Excel file, **save on Desktop** and fill in your team details on the first worksheet.

Need help?

Student 2 (gloves on)

- Place **50 ml** of **phosphate buffer and 2.5 ml of ethyl acetate** in the beaker using the liquid dispensers provided.
- Place the beaker on the magnetic stirrer, add in the stir bar and turn it on to stir. Rinse and dip the pHmeter electrode carefully in the beaker.
- **Add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**.

Caution: Do not go beyond pH 7.4! If pH >7.45, the enzyme will die – so please start again!

When not in use, place the dropper pipette in the tray, as it has some KOH! **CORROSIVE!!!**

- ➢ Stirrer video
- ➢ pHmeter video

Procedure: Step 3a – Part A

Student 1 (no gloves)

1. Keep the timer ready. **Turn it on** when the esterase is added by **Student 2** (*).
2. **Record** the **number of drops** counted out by Student 2 **every 20 s** in the blue column of Table 1 in the '***A. Substrate effect on rate***' worksheet, till **280 s**. Save.

Need help?

Student 2 (gloves on)

- Keep the plastic pipette and **KOH** handy.
- Pipette out **2 ml** of **esterase** enzyme (kept in ice) into the beaker (*)
- Using a **plastic pipette, add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**. ***Caution: Do not go beyond 7.4! If pH >7.45, the enzyme will die – so start again!***
- **Count out the number of drops** added **every 20 s** until **280 s**.
- Empty the beaker into the liquid waste, and rinse it out at the sink, saving the stir bar.

➢ Micropipette Video

➢ Waste disposal video

Procedure: Step 3b – Part A

This needs to done only once.
If the balance is busy, you can do this in Step 5b or Step 7b.

Student 1 (no gloves)

1. Weigh an empty weighing dish and note down its weight in your Prac file.
2. Pass it on to **Student 2**
3. Note the weight of weighting dish with 6 drops KOH from the plastic pipette in your Prac file (#).

Student 2 (gloves on)

- Add **6 drops of KOH** into the pre-weighed weighing dish, with the same plastic pipette and determine the **weight**.
- Place the weighing boat in the disposal tray and report the weight to **Student 1**(#).

1. Weight of 6 drops of KOH

- Download and open Prac 5 Data file (Excel file). Go to the tab labelled "***A. Substrate effect on rate"***.

1. Calculate the weight of 6 drops of KOH.
2. Calculate the volume of 6 drops of KOH, using the density provided.
3. Then calculate **the volume of 1 drop**! – you need this value for the next set of calculations.
4. Save the Prac 5 Data file.

2. Ethyl acetate calculations

What to do

1. Fill the pale blue cells with the **cumulative total number of drops added** in the column "**Drops**"
2. Using the volume of 1 drop calculated (see Slide 23), fill up the column "**Vol**"
3. Then, calculate the amount of KOH using ***n* = *cv***, where c is KOH conc. (20 mM) and complete the column "mmol" *Hint: use only "20" as the amount is already in mmol.* **Save.**
4. **The graph for ethyl acetate will refresh itself.**

Time (sec)	1. 50 ml phosphate buffer; 2. 2.5 ml Ethyl acetate; 3. 2 ml of esterase.			
	Drops per 20sec	Cumulative Total		
		Drops	Vol (ml)	mmol (n=cv)
0				0
20				
40				
60				
80				
100				
120				
140				
160				
180				
200				
220				
240				
260				
280				

2. Ethyl acetate calculations

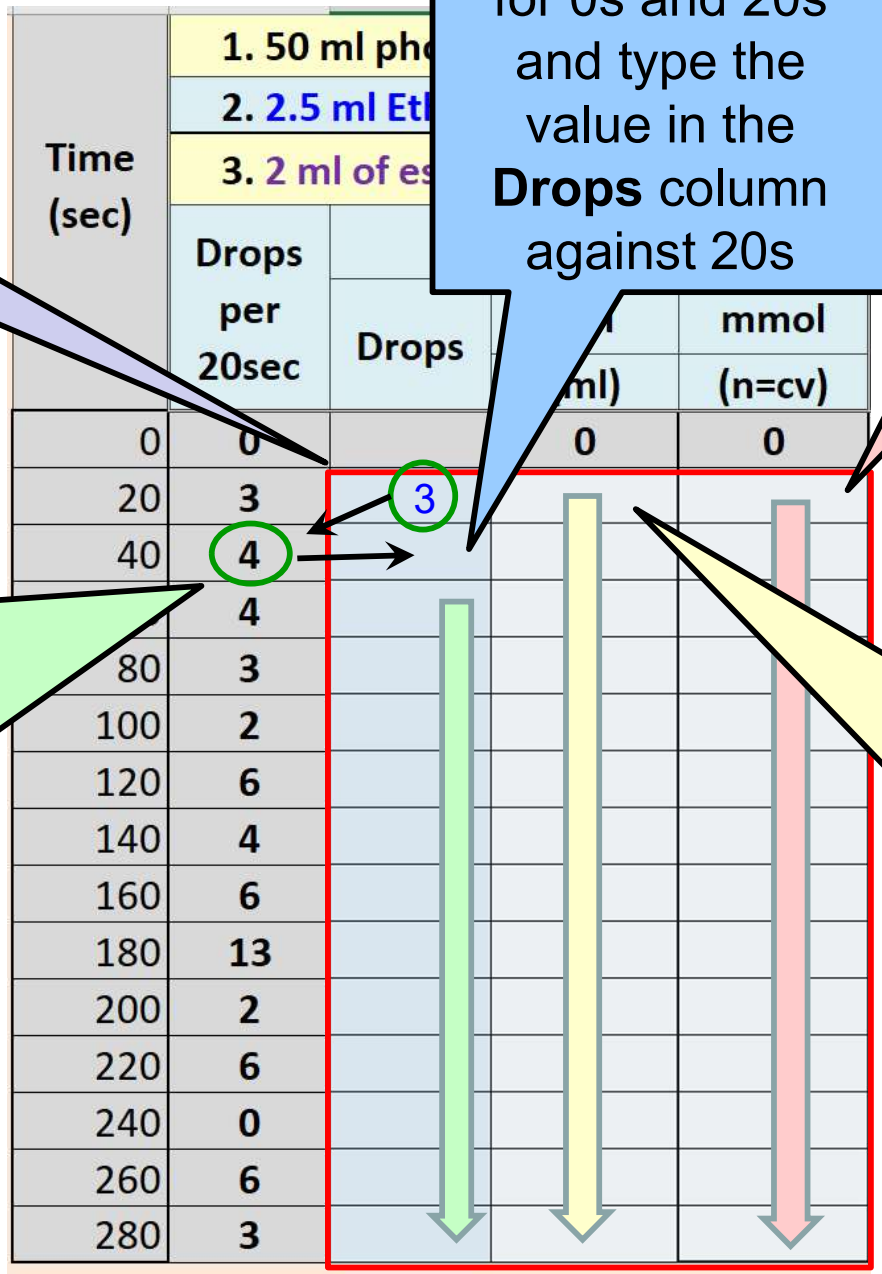
0. You need to fill the red rectangle!

1. Add the drops for 0s and 20s and type the value in the **Drops** column against 20s

4. Multiply the number in the **Vol** column with the concentration of the ester (= 20) and fill the **mmol** column

2. Then add to this **Drops** number the next value from the grey column
e.g. **3** + **4** and fill the **Drops** column with the total.
Fill down to the end of the column in the same way.

3. Multiply the number in the **Drops** column with the volume of one drop calculated earlier and fill the **Vol** column



Time (sec)	1. 50 ml ph…	2. 2.5 ml Et…	3. 2 ml of es…			
	Drops per 20sec	Drops		ml)	mmol (n=cv)	
0	0			0	0	
20	3	3				
40	4					
	4					
80	3					
100	2					
120	6					
140	4					
160	6					
180	13					
200	2					
220	6					
240	0					
260	6					
280	3					

Procedure: Step 4 – Part A

Student 2 (no gloves)

1. Calculate the cumulative total drops for **ethyl acetate**. Save.
2. Fill in the axis labels and the title of the graph in the Excel file.

Need help?

Student 1 (gloves on)

- Place **50 ml** of **phosphate buffer AND 2.5 ml** of **ethyl propionate** in the beaker **using the liquid dispensers.**
- Place the beaker on the magnetic stirrer, add in the stir bar and turn it on to stir.
- Rinse and dip the pHmeter electrode carefully in the beaker.
- Using a **plastic pipette, add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**.

Caution: Do not go beyond pH 7.4! If pH >7.45, the enzyme will die – so please start again!

- Stirrer video
- pHmeter video

Procedure: Step 5a – Part A

Student 2 (no gloves)

1. Keep the timer ready. Turn it on when the esterase is added by **Student 1** (*).
2. **Record** the **number of drops** counted out by Student 1 **every 20 s** in the peach column of Table 1 in the '***A. Substrate effect on rate***' worksheet, till 280 s. Save.

Need help?

Student 1 (gloves on)

- Pipette out **2 ml** of **esterase** enzyme (kept in ice) into the beaker (*)
- Using a **plastic pipette, add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**. ***Caution: Do not go beyond 7.4! If pH >7.45, the enzyme will die – so start again!***
- **Count out the number of drops** added **every 20 s** until **280 s**.
- Empty the beaker into the liquid waste, and rinse it out at the sink, saving the stir bar.

➢ Micropipette Video

➢ Waste disposal video

Procedure: Step 5b – Part A

Skip this step if you have already weighed 6 drops of KOH. If the balance is busy, you can do this in Step 7b.

Student 1 (no gloves)

1. Weigh an empty weighing dish and note down its weight in your Prac file.
2. Pass it on to **Student 2**
3. Note the weight of weighting dish with 6 drops KOH from the plastic pipette in your Prac file (#).

Student 2 (gloves on)

- Add **6 drops of KOH** into the pre-weighed weighing dish, with the same plastic pipette and determine the **weight**.
- Place the weighing boat in the disposal tray and report the weight to **Student 1**(#).

Procedure: Step 6 – Part A

Student 1 (no gloves)

1. Calculate the cumulative total drops for **ethyl propionate**. Save.

Student 2 (gloves on)

- Place **50 ml** of **phosphate buffer AND 2.5 ml of ethyl butanoate** in the beaker using the liquid dispensers.
- Place the beaker on the magnetic stirrer, add in the stir bar and turn it on to stir.
- Rinse and dip the pHmeter electrode carefully in the beaker.
- Using a **plastic pipette, add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**.

Caution: Do not go beyond 7.4! If pH >7.45, the enzyme will die – so start again!

Need help?

- Stirrer video
- pHmeter video

Procedure: Step 7a – Part A

Student 2 (no gloves)

Need help?

1. Keep the timer ready. Turn it on when the esterase is added by **Student 1** (*).
2. **Record** the **number of drops** counted out by Student 1 **every 20 s** in the green column of Table 1 in the '***A. Substrate effect on rate***' worksheet, till 280 s. Save.

Student 1 (gloves on)

- Pipette out **2 ml** of **esterase** enzyme (kept in ice) into the beaker (*)
  - ➢ Micropipette Video
- Using a **plastic pipette, add KOH** to the beaker **one drop at a time to adjust the pH to 7.4**. ***Caution: Do not go beyond 7.4! If pH >7.45, the enzyme will die – so start again!***
- **Count out the number of drops** added **every 20 s** until **280 s**.
- Empty the beaker into the liquid waste, and rinse it out at the sink, saving the stir bar.
  - ➢ Waste disposal video

Procedure: Step 7b – Part A

Skip this step if you have already weighed 6 drops of KOH. If not, you need to complete this now!

Student 1 (no gloves)

1. Weigh an empty weighing dish and note down its weight in your Prac file.
2. Pass it on to **Student 2**
3. Note the weight of weighting dish with 6 drops KOH from the plastic pipette in your Prac file (#).

Student 2 (gloves on)

- Add **6 drops of KOH** into the pre-weighed weighing dish, with the same plastic pipette and determine the **weight**.
- Place the weighing boat in the disposal tray and report the weight to **Student 1**(#).

Procedure: Step 8 – Part A

Student 1 **&** **Student 2** (no gloves)

1. In the Part A Excel worksheet, calculate the cumulative total drops for **ethyl butanoate**. Save.
2. Using the volume of 1 drop of KOH, complete the '**Vol**' columns for all the three esters.
3. Using the formula ***n* = *cv***, where [KOH] = 20 mM, complete all the '**mmol**' columns.
4. The graph should refresh itself, with 3 coloured lines – if not, call you tutor to help.
5. Save the Excel file.
6. If all is well, clean up your work bench and return your tray
7. Go to Parts B and C.

Procedure: Step 9a – Packing up

Student 1 and Student 2 (gloves on)

1. **Wipe your bench clean with a Kimwipe.**
2. Take the **stir bar** out using the **magnetic rod**, rinse into the liquid waste container and return it to the labelled tray
3. **Tip the solution** from the beaker into the **liquid waste container.**
4. **Take the tray back to the back bench.**
5. **Tip the liquid waste into** the **liquid waste disposal container**.
6. Empty the **used tips** into their **respective trays/containers**.
7. Put the used Pasteur pipette in the ***Chemical Waste bin.***
8. **Wash** the following **using the water taps** and put them in the drying trays next to the sink:
   a. the beaker
   b. the liquid waste container and
   c. the used tip container.

Procedure: Step 9a - Packing up

Each team to return the following to their respective collection locations

Student 1 (gloves on)

1. **5 mL pipette (green pipette)**
2. **Tips for these pipettes**
3. **a bottle with 20mM KOH**

Student 2 (gloves on)

1. an **RO water wash bottle**
2. a box of **Kimwipes**
3. a **permanent marker**

- Leave the tray on the backbench.

3. Ethyl propionate and ethyl butanoate calculations (similar to the ethyl acetate procedure!

1. Fill the peach and *pale* green cells with the **cumulative total number of drops added** in the column "**Drops**"
2. Using the volume of 1 drop calculated (see Slide 23), fill up the column "**Vol**"
3. Then, calculate the amount of KOH using n = cv, where c is 20mM (use only 20 as n is in mmol). **Save.**
4. **The graphs for ethyl propionate and butanoate will refresh.**
5. **Complete the graph title and axis labels.**

1. 50 ml phosphate buffer;				1. 50 ml phosphate buffer;			
2. 2.5 ml Ethyl propanoate;				2. 2.5 ml Ethyl butanoate;			
3. 2 ml of esterase.				3. 2 ml of esterase.			
Drops per 20sec	Cumulative Total			Drops per 20sec	Cumulative Total		
	Drops	Vol (ml)	mmol (n=cv)		Drops	Vol (ml)	mmol (n=cv)
			0				0

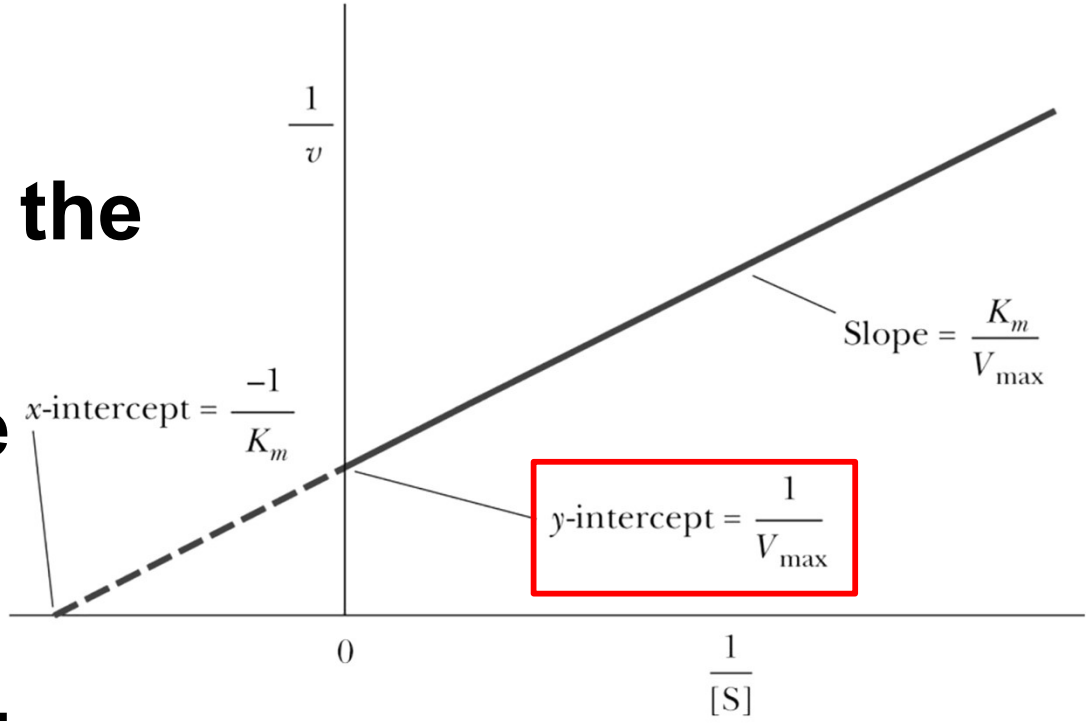
(B) K_m and V_{max} (calculation)

- Open '**B. Km and Vmax (calculation)**' worksheet of Prac 5 Excel file.
- **From the different concentrations of NAD^+ provided in Table 2**, calculate $1/[NAD^+]$
- From the reaction curve details provided, fill in the **ΔA** values for 120s in Table 2.
- From these, calculate **ΔA** values for 1 min.
- Complete the rest of Table 2 as described in the Prac 5 Data Excel file. The plot of 1/v vs. 1/[S] should refresh.
- From the graph, determine the values of V_{max} and K_m

(B) V_{max} calculation

V_{max} is calculated from the graph

- $1/V_{max}$ is where the line cuts the y-axis, called the y-intercept.
- If you see the straight line equation (y = mx + c) in the chart window, $c = 1/V_{max}$
- Just calculate 1/y-intercept (or 1/c) to get the value of V_{max}
- Don't forget the unit for V_{max} in the Prac 5 Quiz.

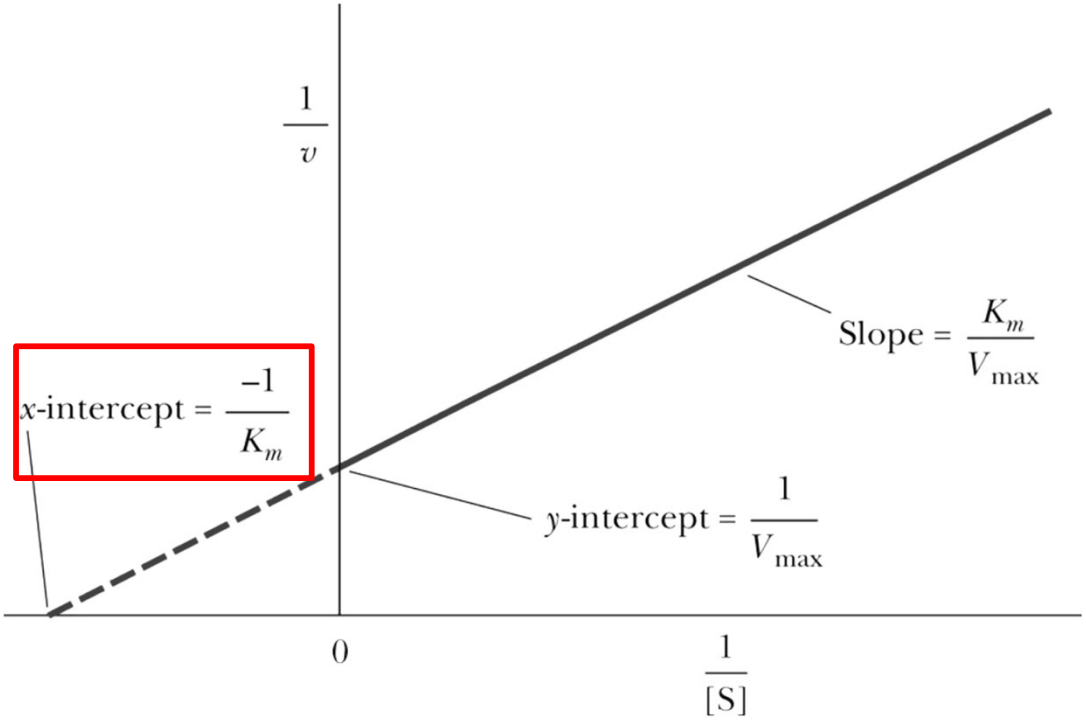


(B) K_m calculation

K_m from the graph

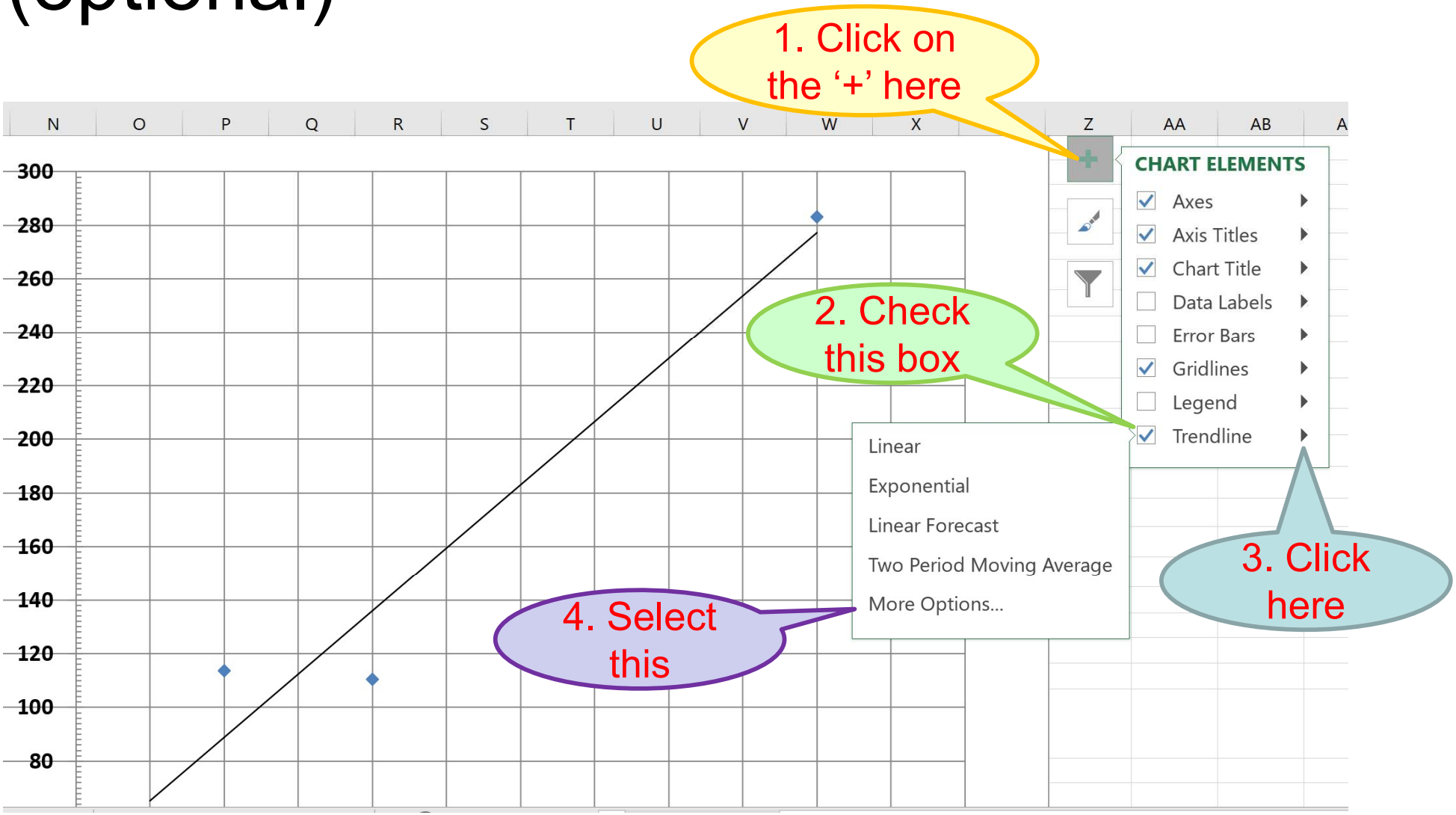
- $1/K_m$ is where the line cuts the negative x-axis, called the x-intercept.
- The line should extend back to the negative x-axis
  - K_m = -1/(x-intercept)
  - Don't forget the unit for K_m in the Prac 5 Quiz.
  - If you see the straight line equation (y = mx + c) in the graph window,

    m/(y-intercept) = K_m



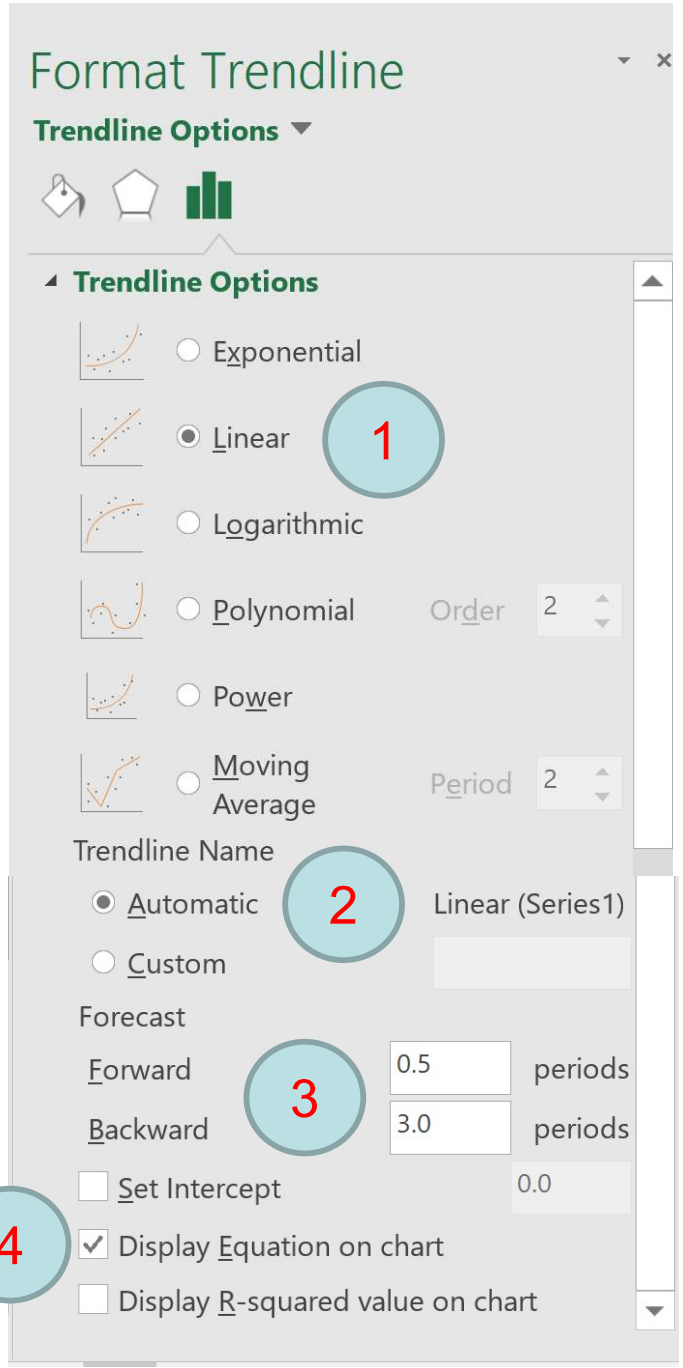
- You can also calculate the slope (m) of the line:
  - Select any two points <u>on the line</u>: (x1,y1) and (x2,y2)
  - m = (y2-y1)/(x2-x1).

To see the equation in Excel (optional)



What to select from Trendline options

- Make sure Linear is checked
- Automatic is fine
- Extend line both ways:
  - ❖Forward 0.5 (only if needed)
  - ❖Backward 3.0 (line should intersect the negative x-axis)
  - ❖You can change these values for your graph….
- Select Display Equation on chart.



(C) Allostery (q&a)

- Study the graph provided and the rate of the GDH reaction on its own and in the presence of effectors, GTP and ADP, in Table 3.
- Answer all the questions.
- Save.

Winding up

All team members

- *Clean up your workspace and return your tray.*
  - *Get your tutor to check your place after returning your trays – thanks*
- Complete all the calculations in the Data file and save the excel file on the desktop.
- Email the file to all team members, just in case.
- Complete the Prac submission individually as described in the next slide.
- You may use your laptops now, if there are no chemicals around.
- Submission due at the end of your practical session.
- *Remember to logout of your account on the computer!*
- *Take your USB drive with you!*

Submitting your data and Results

- Each student to login to iLearn and follow the instructions for completing the prac.
- **Upload your 'Prac 5 data file' individually to ilearn.**
  - ❖ Check your Prac 5 data file carefully to see if it is complete.
  - ❖ When you are 100% happy, upload your file to iLearn.
    - ➢ DO NOT EMAIL US YOUR DATA FILE AS ILEARN DOES NOT ALLOW US TO UPLOAD IT FOR YOU
    - ➢ IF YOU NEED TO RE-SUBMIT CONTACT GURPREET/ABIDALI/SHOBA, TO RESET YOUR SUBMISSION TO DRAFT
- You will then get access to **Prac 5 Quiz**
- **Complete the Prac Quiz individually before you leave the lab!**
- ***We need your data file and completed Quiz for grading!***