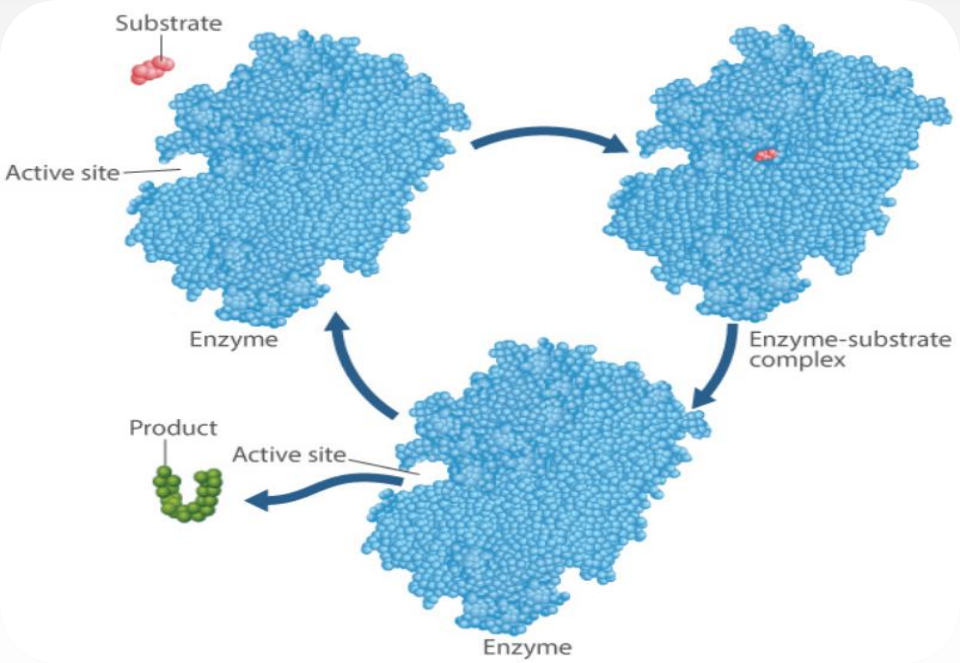
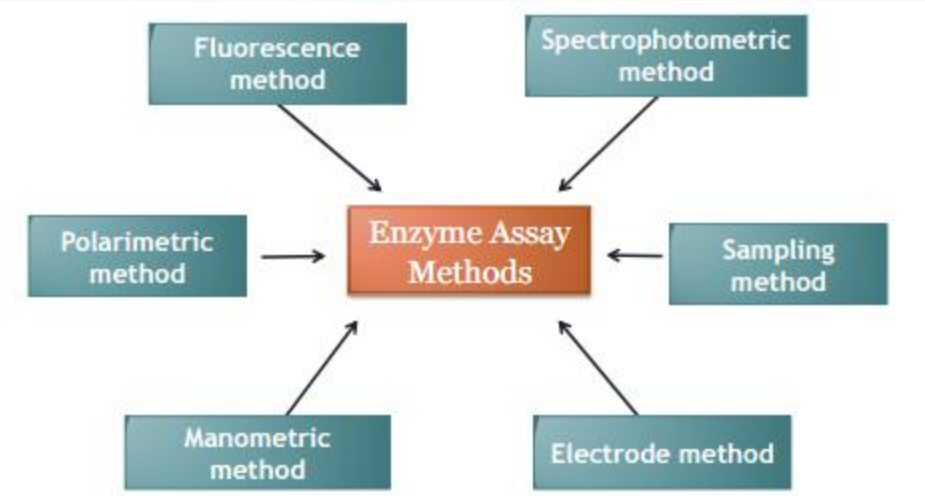
Method of Enzyme Assay



Objective:

•To study the different methods for determining enzyme activity.

•Use these method to diagnose certain diseases.



How to follow a reaction?

Enzyme assays: Are laboratory methods for measuring **enzymatic activity**.

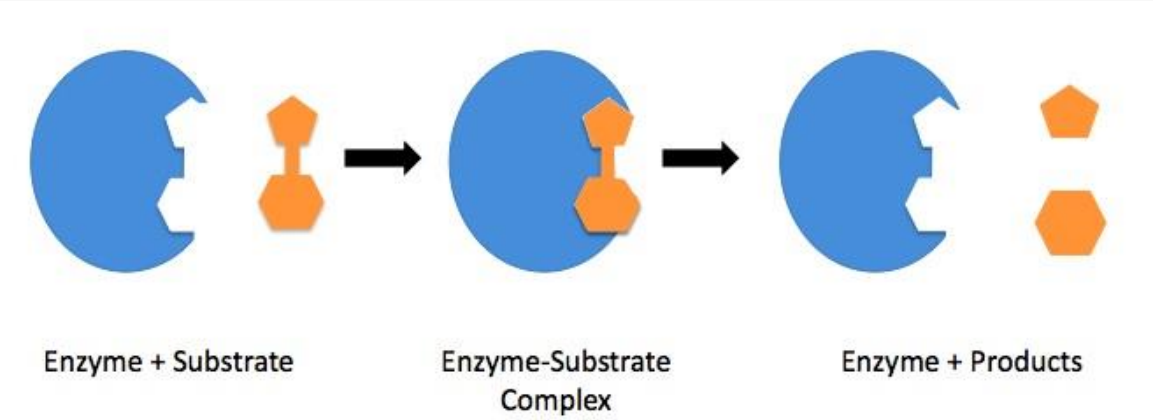
•Enzyme assays measure either the **consumption of substrate** or **production of product** over time.

•**Different enzymes require different estimation methods** depending on the type of reaction catalyzed, the nature of S and P or coenzyme.

Methods of quantitatively following enzyme reactions

How to follow an enzymatic reaction?

- First you must have a complete knowledge about the reaction itself
- Does the substrate or product has the ability to absorb light, fluorescence, any production of gases, production of H+?
- After that you can use this properties to detect the reaction



Methods of quantitatively following enzyme reactions

Fluorescence Methods
Using fluorometer

e.g. NAD+ and NADP+ do not fluoresence in their oxidized forms, but the **reduced form have a blue fluorescence**.

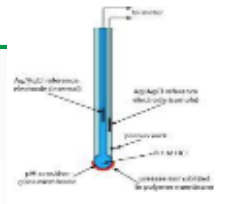
Manometric Methods
Using manometer.

It is suitable for reactions in which one of the component is a gas. e.g.

Oxidases (O2 uptake), **Decarboxylase**(CO2 output)

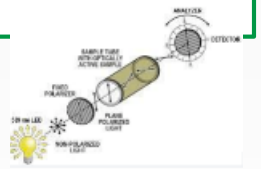
Electrode Methods
Using a pH meter

Reactions which involve the **production of acids** where H+ conc. is measured



Polarimetric Method
Using polarimeter

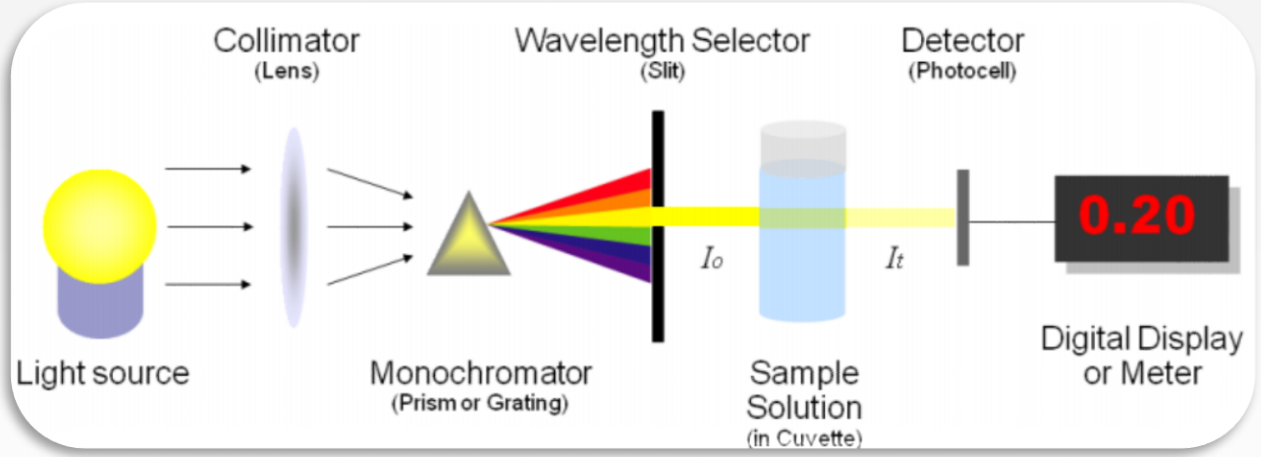
For isomerases that convert one isomer to another.
e.g. D-glucose →L-glucose



Spectrophotometric Methods

Spectrophotometric method

In spectrophotometric assays, you follow the course of the reaction **by measuring a change in how much light the assay solution absorbs.**



What is blank solution?

It is a solution that contains everything except the compound to be measured.

Spectrophotometric methods

Sample absorb Invisible light

(ultraviolet “UV”) 100 to 360 nm

Quartz cuvette

Sample absorb Visible range

Glass or plastic cuvette

If the light is in the **visible region** you can actually see a change in the color of the assay, these are called

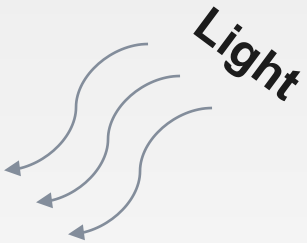
[colorimetric assays]

Examples of using Spectrophotometric methods

1- cases in which <u>product absorb</u> but not the substrate.

e.g.

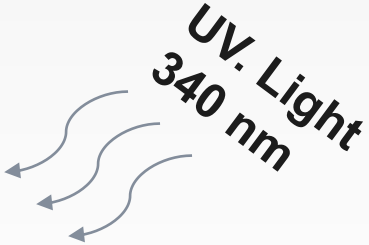
Fumarate → (**Fumarate hydratase**) → **malate**



2- the <u>Co-enzyme</u> undergoes change in absorption upon reduction or oxidation

Oxidized form
- **NAD**
- **NADP**

Reduced form
- **NADH**
- **NADPH**



If **reduced form** was product: increase the absorbance / min

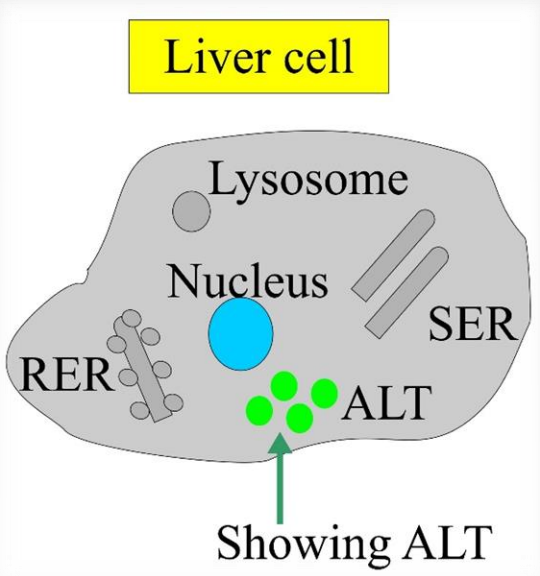
If reduced form was substrate : decrease the absorbance / min

Two types of Enzyme assays:

- **Continuous assays,** where the assay gives a continuous reading of activity.
- **Discontinuous (Endpoint) assays,** Where the reaction is stopped and then the concentration of substrates/products determined.

Alanine transaminase (ALT) in serum

- Continues assay
- Discontinues assay

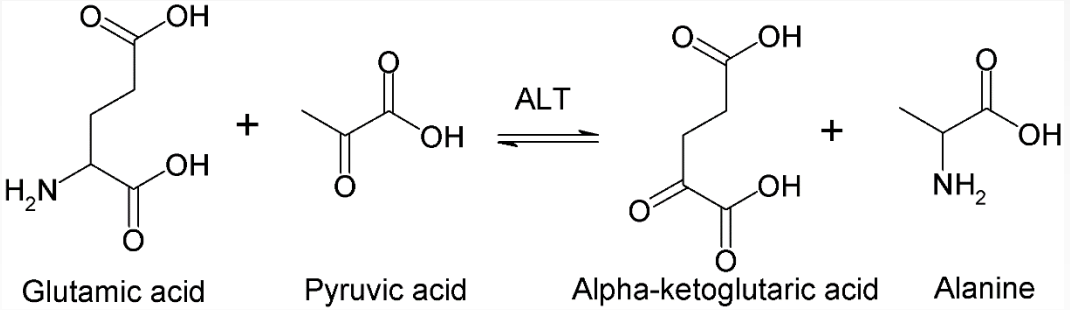


Alanine transaminase (ALT)

•ALT is an enzyme that catalyzes a type of reaction (transamination) between an amino acid and α-keto acid.

•It is important in the production of various amino acids.

- Transfer of amino group of an amino acid to α-keto acid resulting in formation of new amino acid and new keto acid.

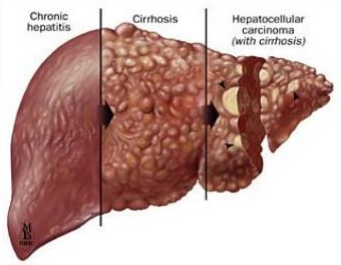


ALT diagnostic importance

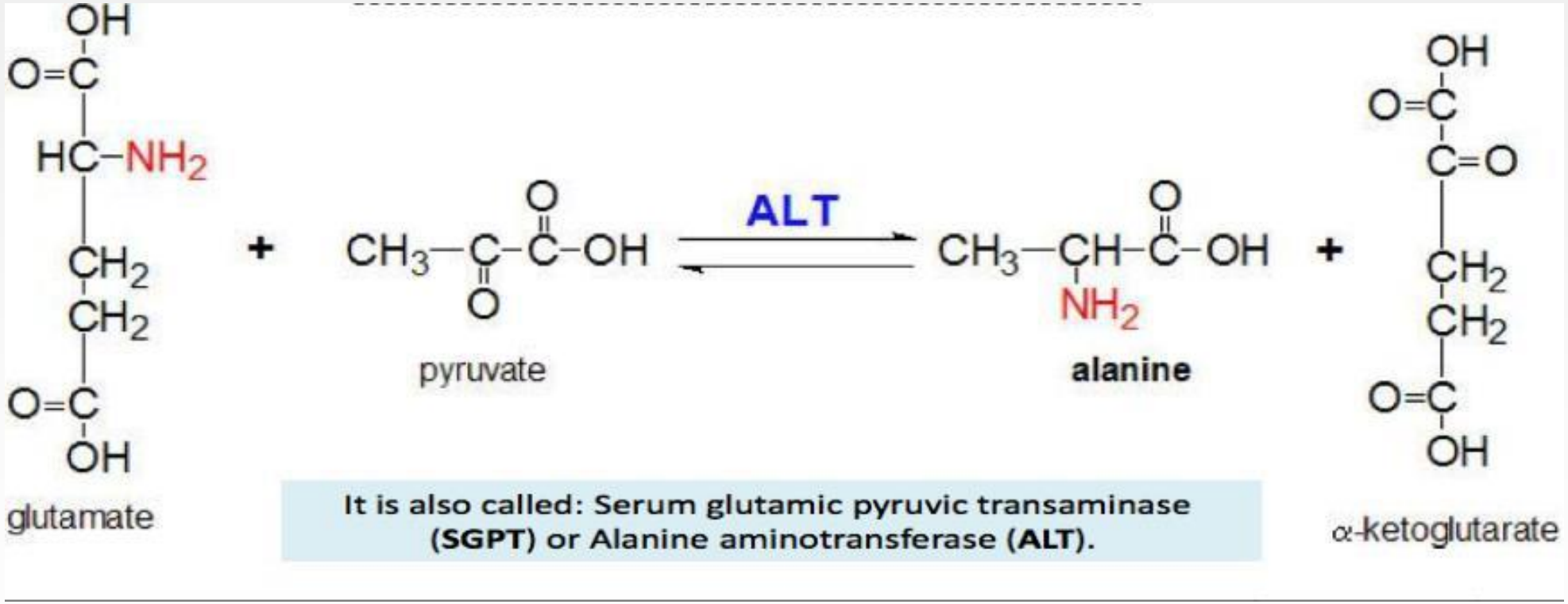
- ALT is found in serum (**at low level**) but is most commonly found in liver.
- Thus , an **elevated** level of ALT is a sensitive index of ***acute hepatocellular injury***.
- Elevated serum ALT levels are found in hepatitis, cirrhosis , and obstructive jaundice.

•**NORMAL RANGE OF ALT:** (up to 42) U/L ➞ males

(up to 32) U/L ➞ females

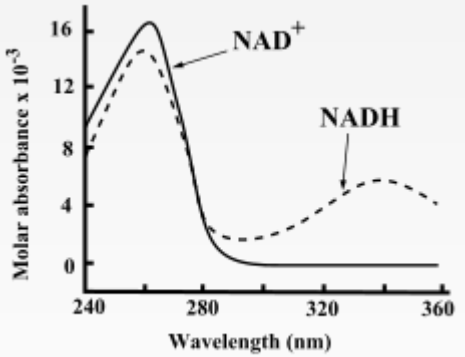


If Substrate and Product can not absorb light? What is the solution?



1-Continuous Assay

- The reading will be continues (1 min for 3 min).
- Because nether the S nor P can absorb light, the following can be done:



First, we will add the enzyme to the Substrate

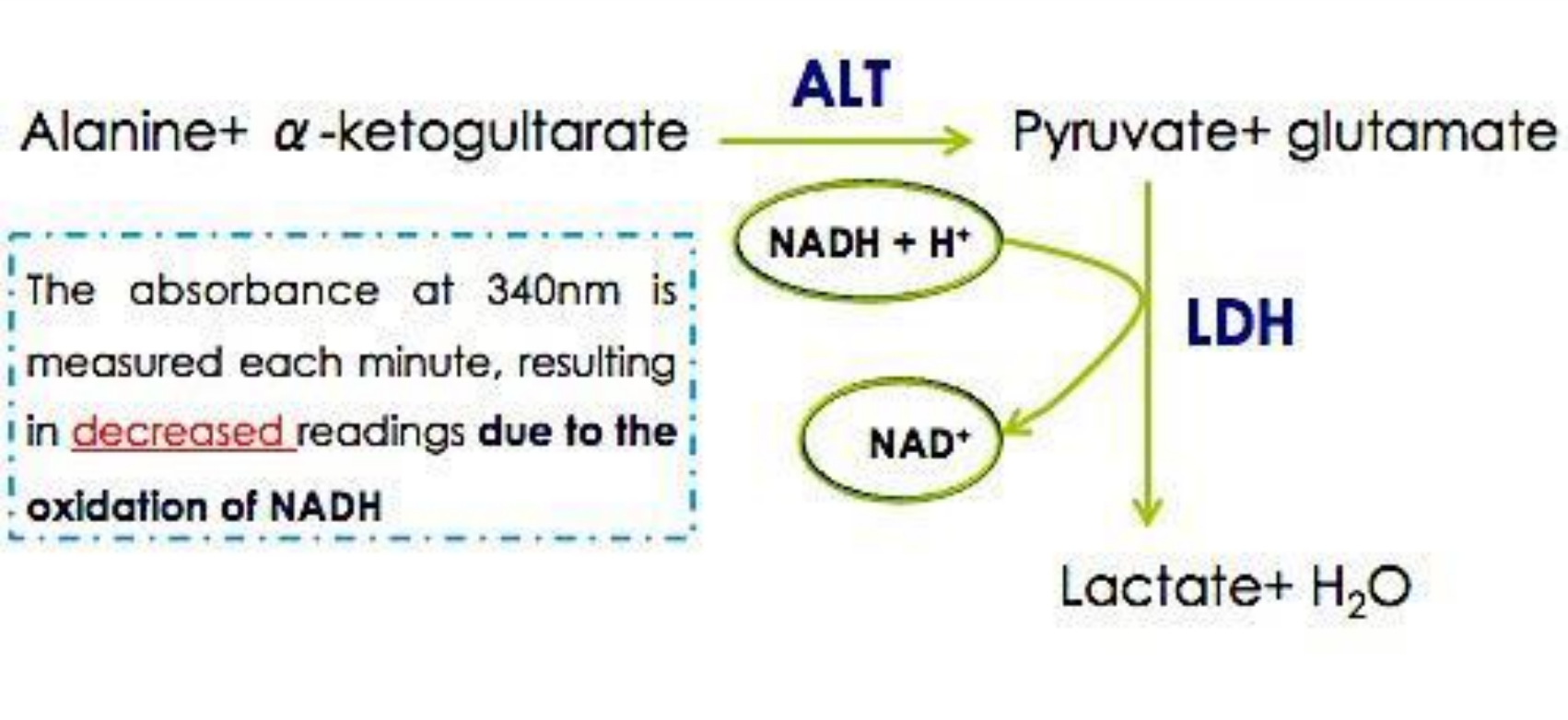
1. **Alanine + α- ketoglutarate → Pyruvate + glutamate**

Then, Another enzyme (**LDH**) and **NADH**+H+ will be added:

2. **Pyruvate + NADH+H+ → L-Lactate+ NAD+ +H2O**

The absorbance at **340nm** is measured each minute without stopping the reaction, resulting in **decreased** readings **due to the oxidation of NADH.**

Principle


Alanine+ α-ketogultarate
ALT
Pyruvate+ glutamate
NADH + H+
LDH
NAD+
Lactate+ H2O
The absorbance at 340nm is measured each minute, resulting in decreased readings due to the oxidation of NADH

Method

Pipette into clean and dry test tubes:

ALT Reagent	3ml
Pre-warm at 37°C for 3 minutes and add	
Serum Sample	0.2 ml = (µl) ??
Mix and incubated at 37 °C for 1 minute, then read absorbance (at 340 nm against distilled water) every minute for 3 minutes) and determine ΔA/min	

Choose the following on the spectrophotometer:

2) Applications → 2) Simple Kinetics → wave length (340 nm) → Seconds → Duration (180 sec = 3 min) → Intervals (60 sec= 1 min) → Print Data Table (off) → Press start (2 times)

Results

Time	Absorbance 340nm		∆A/min=((A1-A2)+(A2-A3))/2
1 min	A1		
2 min	A2		
3 min	A3		

Calculations:

ALT Activity (U/L) = ΔA/min x 1768

2-Discontinuous Assay

• In this method **ALT** catalyzes the following reaction

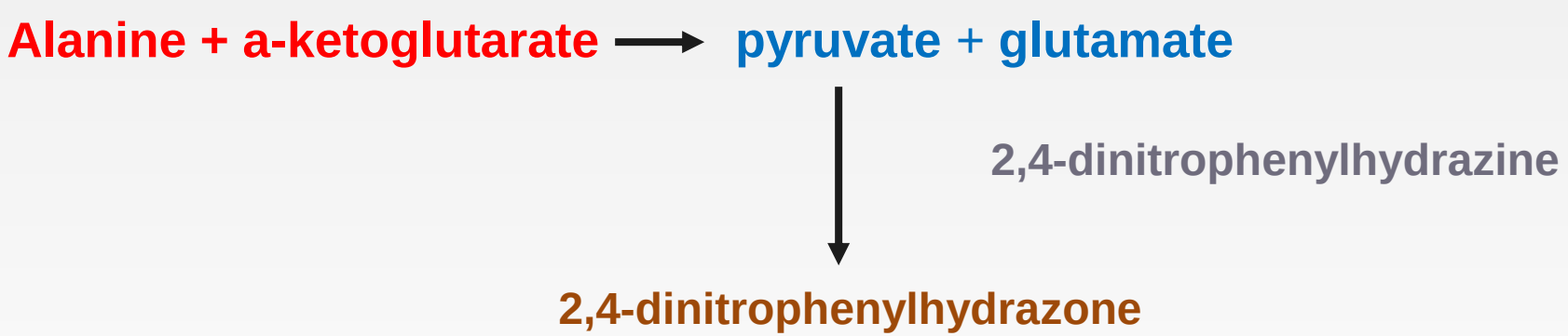
Alanine + a-ketoglutarate ⟶ pyruvate + glutamate

•ALT is assayed by following formation of **pyruvate.**

•Because nether the S nor P can absorb light, the following can be done:

- •The addition of acidic 2,4-dinitrophenylhydrazine (DNPH) lead to the formation of **2,4-dinitrophenylhydrazone (colored product that can absorb light),**
- •Then ,NaOH will be added (to stop the reaction) , so that the **colored product** may be measured at **546nm**.

Reaction-discontinuous



- This assay as an example of **colorimetric\ endpoint assay**

Method:

	BLANK	SAMPLE
ALT Reagent	0.5 ml	0.5 ml
Pre-warm at 37 °C for 5 minutes and add:		
Distilled Water	0.1 ml	-
Serum Sample	-	0.1 ml
Mix, and incubate at 37 °C for exactly 30 minutes, and add:		
Color Reagent (DNPH)	0.5 ml	0.5 ml
Mix, and return at 37 °C for exactly 10 minutes, then add:		
Color Developer (NaOH)	5.0 ml	5.0 ml
Mix, and return to 37 °C for exactly 5 minutes. Read absorbance of all tubes at 546nm against blank.		

Precautions

- COLOR REAGENT contains 1 N Hydrochloric acid which **causes burns**.
- COLOR DEVELOPER contains 0.5 N Sodium hydroxide which is **corrosive**.

In case of contact, flush affected area with large amounts of water. Seek medical attention.

Results:

Absorbance at 546 nm	ALT Activity (U/L)
0.025	2.5
0.050	5.5
0.075	9
0.100	12
0.125	17
0.150	21
0.175	25
0.2	30
0.225	35
0.250	41

- The data shown in the table is used to convert absorbance at 546 nm into enzymatic activity in U/L of serum.
- Draw graph using the data in table with absorbance on the Y- axis and enzymatic activity in U/L on the X-axis.

Note: Don't forget title of the graph "Standard Curve" and the x- axis and y- axis with their units

-Absorbance at 546 nm = ……

-ALT (SGPT) activity (from graph)= ……

Discussion:

- Mention the diagnostic importance of ALT (Introductory paragraph)
- Explain the difference in the principle of each ALT assay.
- Compare your result with ALT normal range [in males], and diagnose the patient's state (what disease could the patient have or not).
- Compare between continuous and discontinuous assays values.