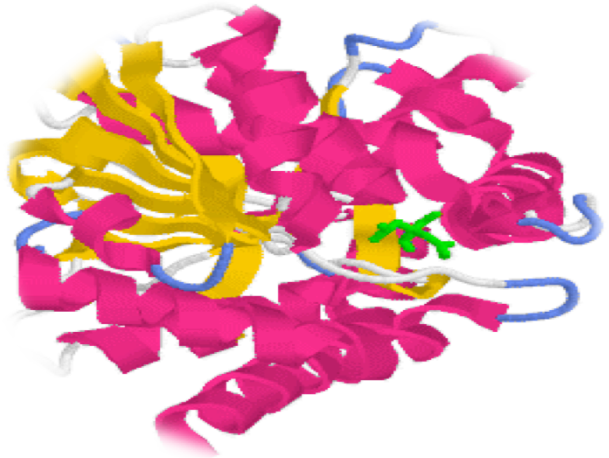
322 BCH

Method of Enzyme Assay



Objective

To study the different methods for determining enzyme activity.

Enzyme assays: Are laboratory methods for measuring enzymatic activity.

- All 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

1.Fluorescence methods: Using a fluorometer .

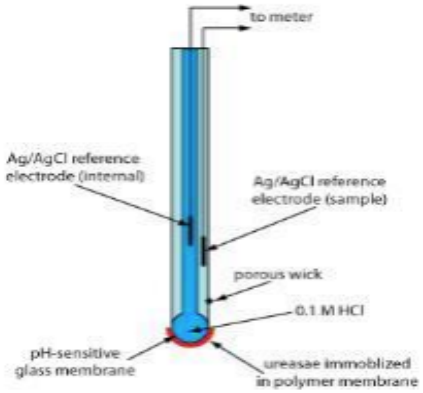
** e.g. NAD^+ and $NADP^+$ do not fluoresence in their oxidized forms, but the reduced form have a blue fluorescence reduction reaction.

2.Manometric methods: Using a manometer.

** It is suitable for reactions in which one of the component is a gas. e.g. Oxidases (O_2 uptake), Decarboxylase(CO_2 output)

3. Eletrode Methods: Using a pH meter.

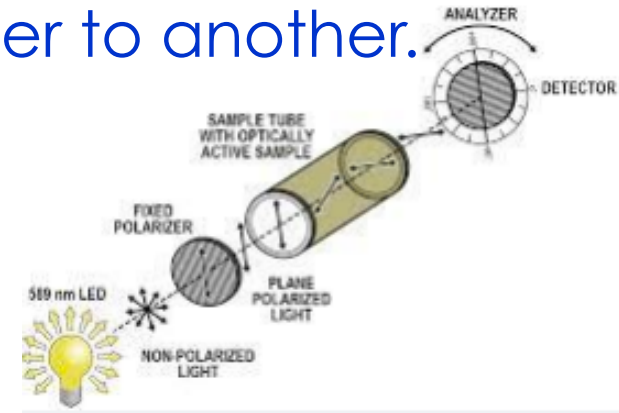
** Reactions which involve the production of acids where H^+ conc. is measured.



4. Polarimetric Method: use polarimeter.

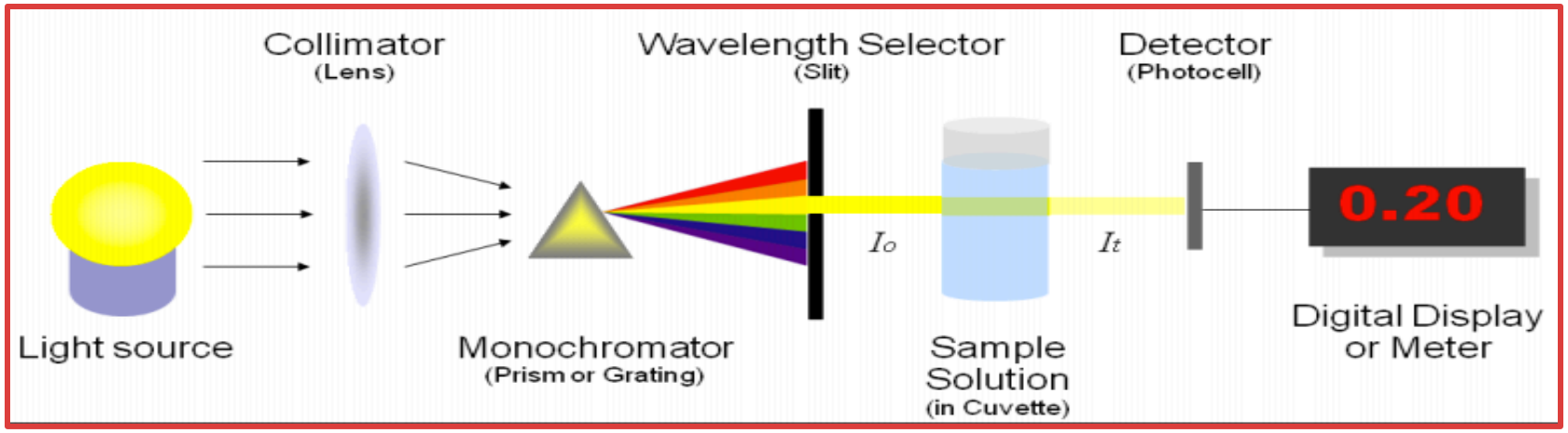
** For isomerases that convert one isomer to another.

e.g. D-glucose → L-glucose



5. Spectrophotometric methods.

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



Wavelength in this instrument is divided into:

-Invisible range(ultraviolet "UV") from 100 to 360 nm

[Quartz cuvette are used]

-Visible range (400 -700 nm)

[Glass or plastic cuvette are used]

[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**]

What is blank solution?

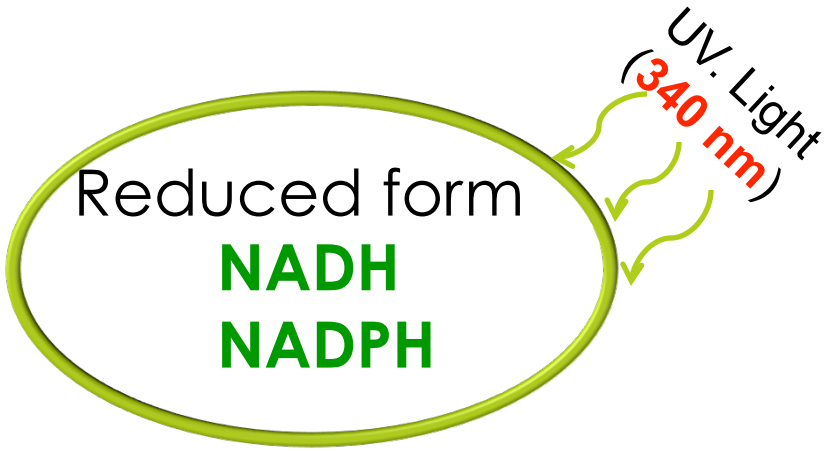
It is a solution that contains everything except the compound to be measured.

When the Spectrophotometric methods can be used?

1- cases in which product absorb but not the substrate.
e.g.

2- the Co-enzyme undergoes change in absorption upon reduction or oxidation

Oxidized form
NAD
NADP

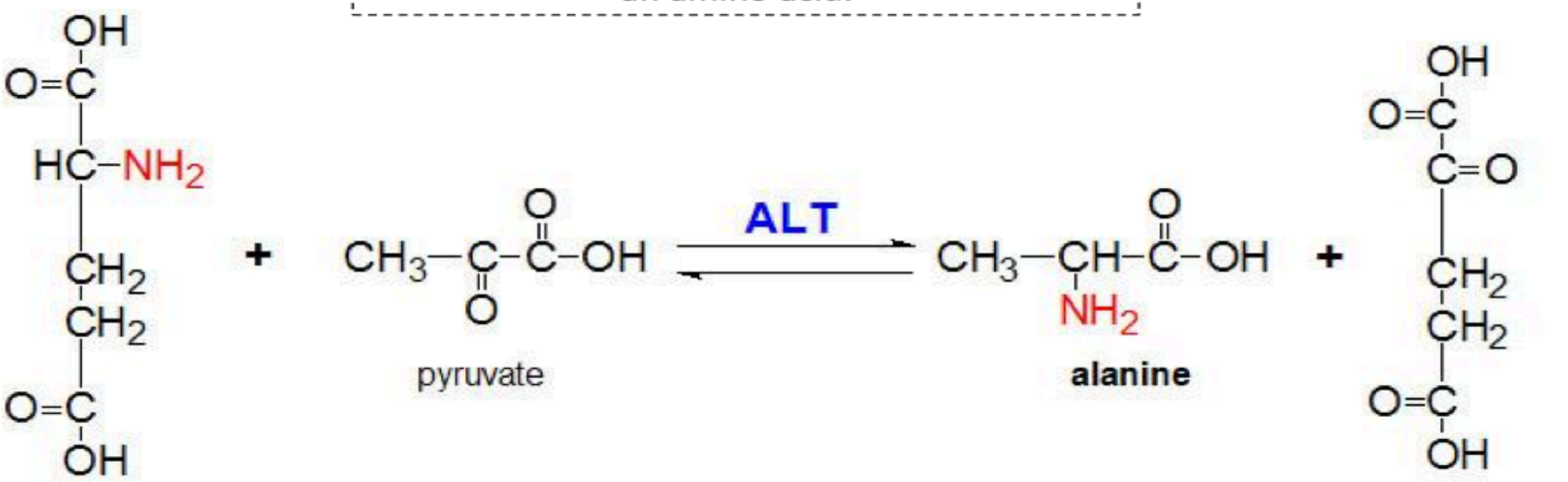


If reduced form was product: increase the absorbance / min
If reduced form was substrate : decrease the absorbance / min

Alanine transaminase (ALT)

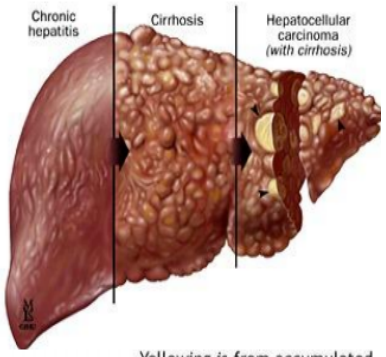
- ALT is an enzyme that catalyzes a type of reaction (transamination) between an amino acid and α-keto acid.
- It is important in the production of various amino acids.

transamination reaction involves removing the amino group from the amino acid, leaving behind an α-keto acid, and transferring it to the reactant α-keto acid and converting it into an amino acid.

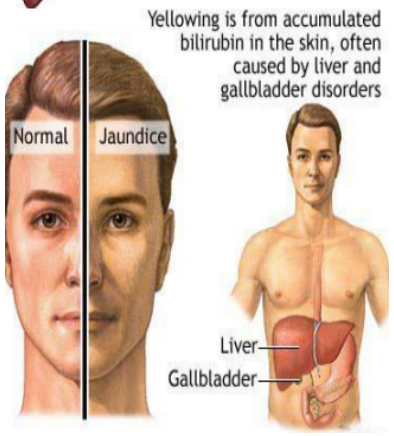


It is also called: Serum glutamic pyruvic transaminase (**SGPT**) or Alanine aminotransferase (**ALT**).

ALT diagnostic importance



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



- **NORMAL RANGE OF ALT:**

(up to 42) U/L → males (up to 32) U/L → females

Enzyme assays can be split into two types:

- **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.

Both types of enzyme assays will be applied in this lab on ALT

Principle of Continuous Assay

1- ALT "*present in serum sample*" catalyzes the transfer of an amino group from alanine to α-ketoglutarate in the following reaction:

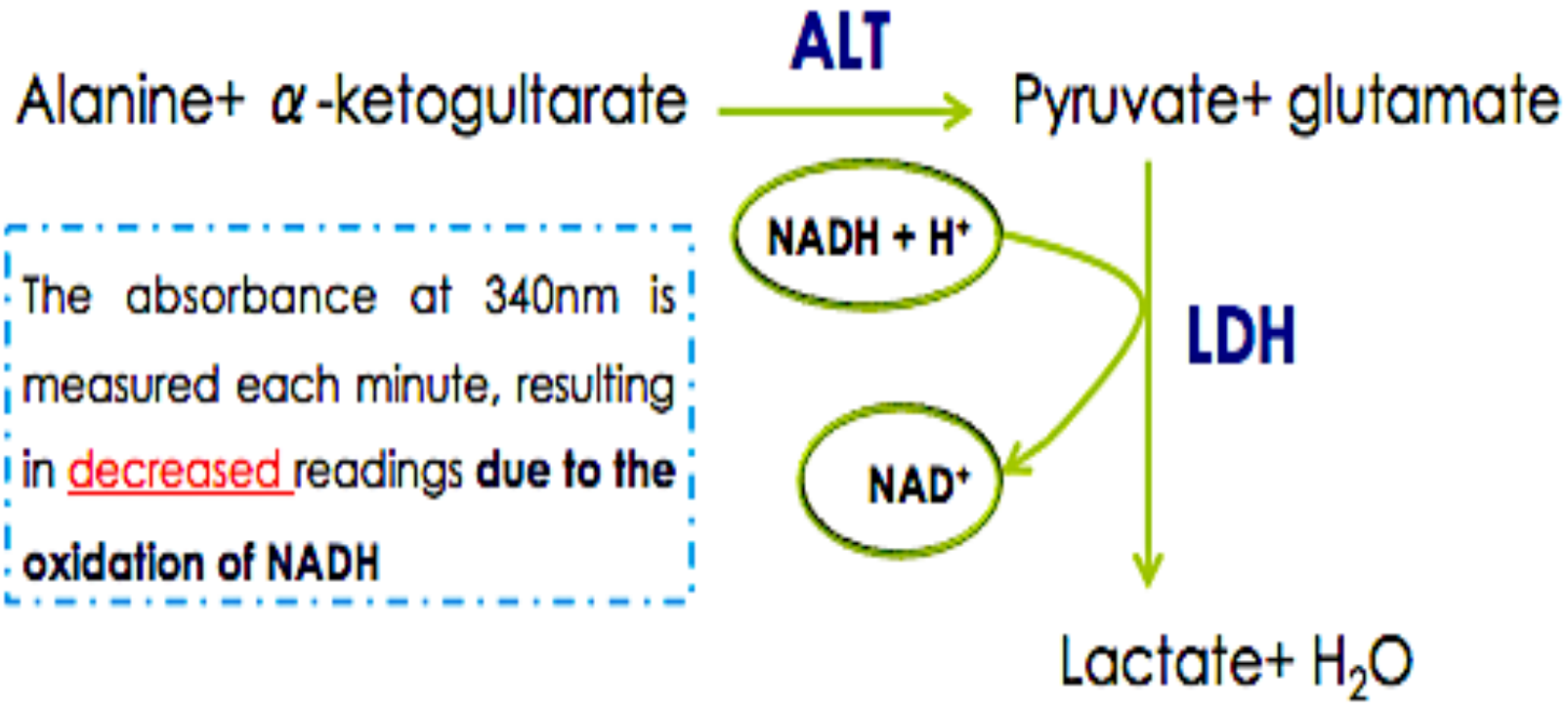
Alanine + α - ketoglutarate → Pyruvate + glutamate

2- Then, the pyruvate formed in the reaction is reduced to L-Lactate by Lactate dehydrogenase (LDH) "*found in ALT reagent*".

Pyruvate + $NADH+H^+$ → L-Lactate+ NAD^+ +H_2O

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

Principle


ALT
Alanine+ α-ketogultarate
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	3 ml	
Pre-warm at 37°C for 3 minutes and add		
Serum Sample	0.2 ml	ml→ µl (x 1000)
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) → 1) Seconds → Duration (180 sec = 3 min) → Intervals (60 sec= 1 min) → Print Data Table (off) → Press start (2 times)

Results

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

Calculations

ALT Activity (U/L) = Δ A/min x 1768

ALT Activity (U/L) =

Principle of Discontinuous Assay

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

Alanine + a–ketoglutarate → pyruvate + glutamate

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

- The addition of acidic 2,4-dinitrophenylhydrazine (DNPH) stops the reaction and forms the 2,4-dinitrophenylhydrazone. So that it may be measured at 546nm.

Method:

Total time= 50 min

	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)= .....U/L

Discussion

- Mention the diagnostic importance of ALT

- 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).