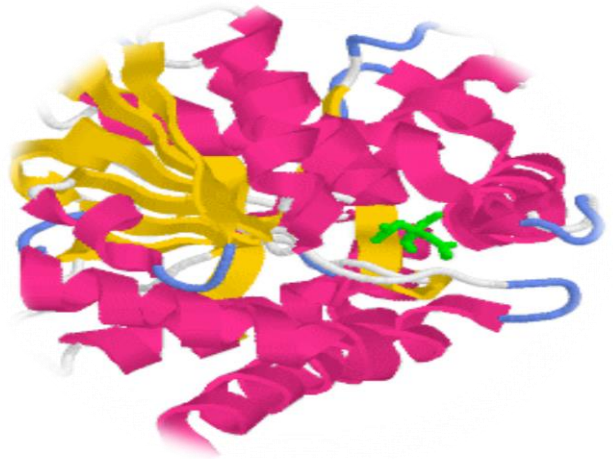
322 BCH

Method of Enzyme Assay



Objective

To study the different methods for determining enzyme activity.

Method of Enzyme Assay

- Enzyme activity is measured in vitro under conditions that often do not closely resemble those in vivo.

- The objective of measuring enzyme activity is normally to determine the amount of enzyme present under defined conditions, so that activity can be compared between one sample and another, and between one laboratory and another.

Method of Enzyme Assay

1- End point assay:

Alanine transaminase (colorimetric\ endpoint assay)

2- Discontinuous assay:

Lactate dehydrogenase (UV/ kinetics)

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.

ENZYME ASSAYS

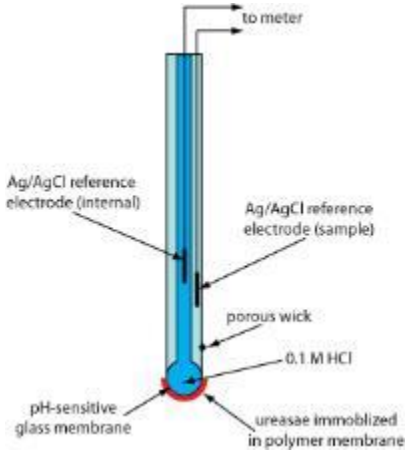
Enzyme assays can be split into two groups:

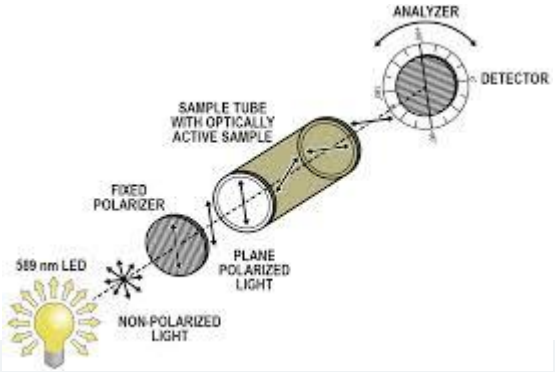
- **Continuous assays**, where the assay gives a continuous reading of activity.

- **Discontinuous (Endpoint) assays**, where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

Methods of quantitatively following enzyme reactions:

1. **Fluorescence methods**
2. **Sampling methods**
3. **Manometric methods**
4. **Electrode Methods**
5. **Polarimetric Method**





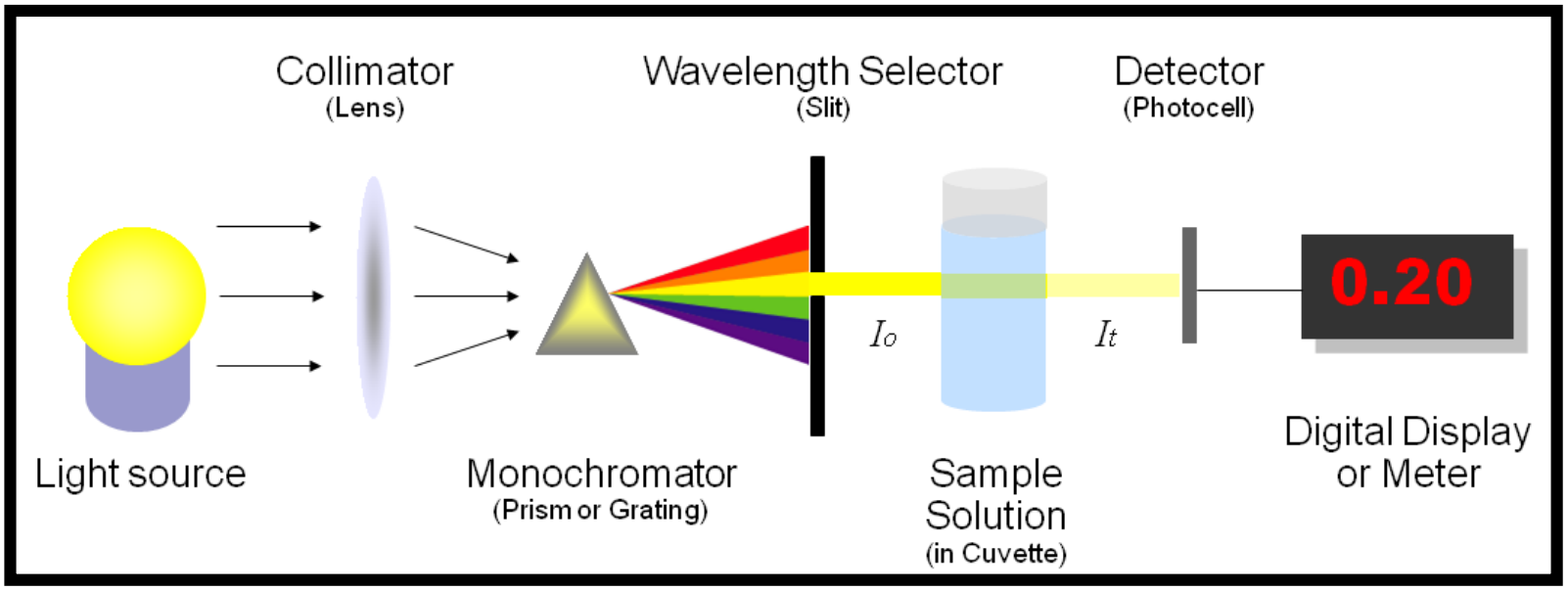
6. Spectrophotometric methods.

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

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

- **Spectrophotometer**

- It is an instrument used to measure the intensity of light at a given wavelength that is transmitted or absorbed by a sample.



*__Wavelength in this instrument divided into__: **Invisible range(ultraviolet) from 100 to 360 nm [Quartz cuvette are used]** -**Visible range (400 -700 nm) [Glass or plastic cuvette are used]**

Blank: contain everything except the compound to be measure.

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 by S

Oxidized form	Reduced form
NAD	NADH
NADP	NADPH
FAD	FADH2

- **have an absorption band at 340 nm in the reduced state**

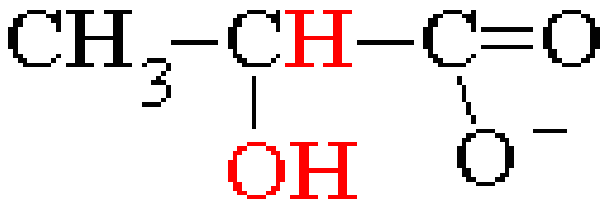
Use of Co-enzyme in Assay

- The common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms.

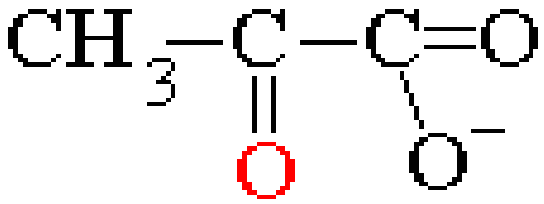
NAD+ does not absorb Ultraviolet at 340 nm.
NADH strongly absorbs Ultraviolet at 340 nm.

- An oxidoreductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at a wavelength of 340 nm as it consumes the coenzyme.

If NADH product: increase the absorbance / min
If NADH substrate: decrease the absorbance / min



NADH strongly absorbs ultraviolet at 340 nm.



NAD+ does not absorb Ultraviolet at 340 nm

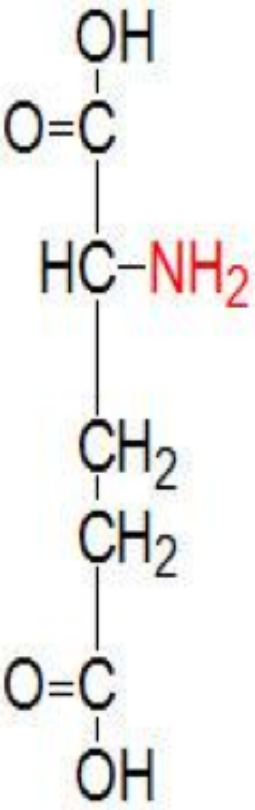
1-Discontinuous assay:

An example that you will study in this lab is Alanine transaminase

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

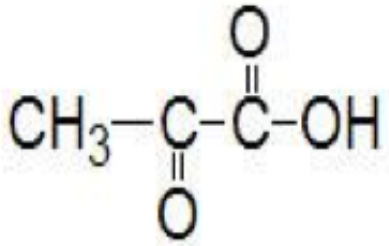
- It is important in the production of various amino acids.

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.

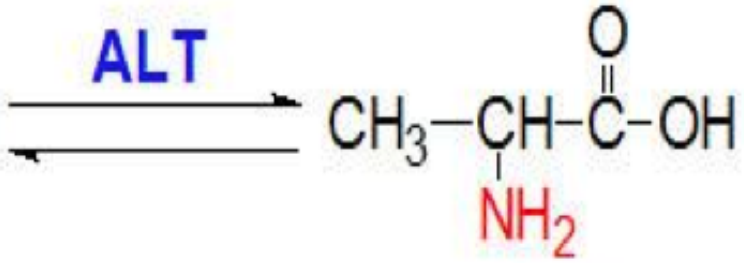


glutamate

+

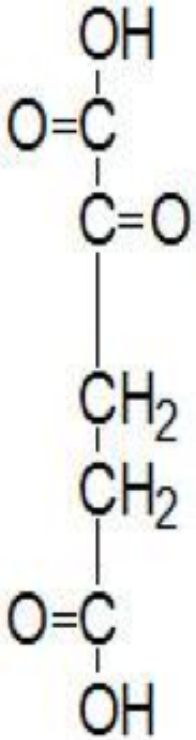


pyruvate



alanine

+



α-ketoglutarate

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 associated with the liver.

- thus , an elevated level ALT is a sensitive index of acute hepatocellular injury.

- Elevated serum ALT (SGPT) level are found in hepatitis, cirrhosis , and obstructive jaundice. Levels of ALT are only slightly elevated in patient following a myocardial infraction.

Principle

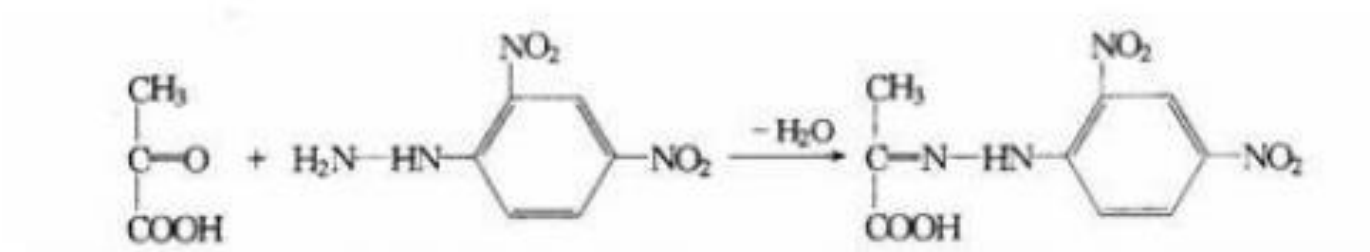
- In this method **ALT** catalyzes the reaction of L-alanine and α- ketoglutaric acid to form pyruvate and glutamate under controlled conditions (37 °C) and pH 7.4 + 0.05.

Alanine + a-ketoglutarate = pyruvate + glutamate

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

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

Reaction



pyruvate 2, 4-dinitrophenylhydrazine (DNPH)

2,4-dinitrophenylhydrazone

- **This assay as an example of colorimetric\ endpoint assay**
- **Why colorimetric? Because it will give a brown color**
- Normal Range: 10-40 units per liter (**U/L**)

PROCEDURE:

	BLANK	SAMPLE	
ALT Substrate	0.5 ml	0.5 ml	
Pre-warm at 37 °C for 5 minutes and add: (using timed intervals)			
Distilled Water	0.1 ml	-	
Sample	-	0.1 ml	
Mix, and incubate at 37 °C for exactly 30 minutes (use same timed intervals), and add:			
Color Reagent	0.5 ml	0.5 ml	
Mix, and return at 37 °C for exactly 10 minutes, then add: (use same timed intervals)			
Color Developer	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**. In case of contact, flush affected area with large amounts of water. Seek medical attention.

- 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:

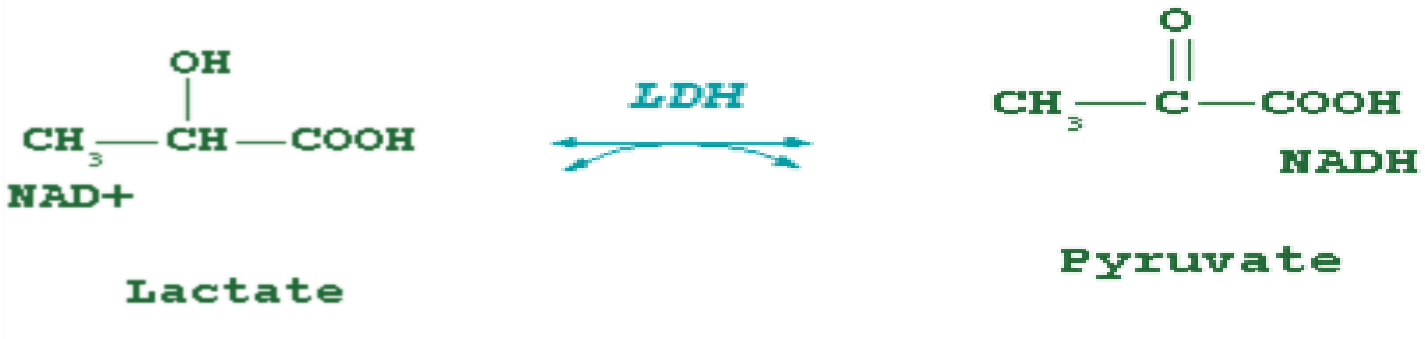
Absorbance at 546 nm	ALT activity
0.025	2.5
0.050	5.5
0.075	9
0.100	12
0.125	17
0.150	21
0.175	25
0.2	30
0.225	35
0.250	41

- 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.

Absorbance at 546 nm = ...
ALT (SGPT) activity of serum sample (from graph)= .....U/L

Continuous assay:
An example in this lab is lactate dehydrogenase

It catalyzes the conversion of lactate to pyruvate.



- This is an important step in energy production in cells.
- Many different types of cells in the body contain this enzyme. Some of the organs relatively rich in LDH are the heart, kidney, liver, and muscle.
- **This reaction will be detected by UV, because NADH will absorb at 340 nm**

LDH diagnostic importance

- LDH is present in almost all of the tissues in the body and becomes **elevated in response to cell damage**.

- **Elevated level** of LDH in serum are found in myocardial infraction, liver diseases, renal diseases, certain forms of anemia, malignant diseases and progressive muscle dystrophy.

- NORMAL RANGE OF LDH:
  - (80-285) U/L male
  - (103- 277) U/L female

NADH is **product : increase** the absorbance /min

NADH is **reactant: decrease** the absorbance /min

Procedure

Pipette into clean and dry test tubes:

LDH Reagent	3 ml
Pre-warm at 37° C for 3 minutes and add	
Sample	0.1 ml
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	

Results

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

Calculations

LDH Activity (U/L) = ΔA/min x 4984
LDH Activity (U/L) =

Discussion

- You should write generally about the first and second experiment, and what you get from each and then compare them to the normal range and also commet about your results (for example if it higher that the normal range what could be the reasons)