Historical Background

1815 Kirchoff first indicated the presence of enzymes in living systems

1833 A. Payen & Persoz reported a re-usable factor from malt extract and called it DIASTASE

1837 Berzilius recognised the catalytic nature of biological diastase

1860 L. Pasteur reported fermentation of food stuffs by living cells

1878 Kühne - term 'enzyme': Greek "in yeast"

1894 Emil Fisher studied enzyme specificity for substrate and proposed Lock & Key theory

1897 Hans & Eduard Buchner – filtrates of yeast extracts could catalyse fermentation! No need to living cells

Also prepared pure extracts of "Zymase" from yeast

1903 Henri – first successful mathematical model

1913 Michaelis and Menten – Kinetic theory of enz action.

1958 Koshland – "Induced fit" model

1926 James B. Sumner isolated as well as crystallised "Urease" from jack beans. He was the first one to postulate the proteinaceous nature of enz thou the idea remained unnoticed. Was awarded NOBLE PRIZE

1930 John Northrop and Stanley prepared pure crystals of PEPSIN & TRYPSIN

1930s JBS Haldane wrote the treaty " ENZYMES" and suggested weak interactions b/w E and S

1953 Sanger determined aa seq of INSULIN

1960s AA seq of RNAse

1965 3-dim str of LYSOZYME by X-ray Crystallo.

1965 Monod, Wyman and Changeux – allosteric regulation

1969 Ist enz chemically synthesised "RIBONUCLEASE"

How to define enzyme activity?

Physical properties of an enzyme most often is measured by relative rate by which the substrate is converted to ---> product

- 1 unit ACTIVITY= International unit (IU)

  amount enzyme which converts 1 µmole substrate to product per min at 25°C

  – e.g. **IU= 10 µmole/min**

- 1 unit SPECIFIC ACTIVITY

  # IU of enzymatic activity per mg of total protein present

  – e.g. 10 µmole/min/mg protein or 10 IU/mg protein

Enzyme assays

- **Enzyme assays are laboratory methods for measuring enzymatic activity. They are vital for the study of enzyme kinetics and enzyme inhibition.**
- The assay is the act of measuring how fast a given (unknown) amount of enzyme will convert substrate to product (the act of measuring a velocity).
- Enzyme assays measure either the disappearance of substrate over time or the appearance of product over time.
- An assay requires to determine the concentration of a product or substrate at a given time after starting the reaction.

Measuring enzyme activity

- Enzymes are usually present in very small quantities in biological fluids
- Therefore, Enzymes are **not** directly measured
- They are commonly measured in terms of their catalytic activity
- We don't measure the molecule …
- But we measure how much "work" it performs (catalytic activity)
- That means the rate at which it catalyzes the conversion of substrate to product
- The enzymatic activity is a reflection of its concentration
- Activity is proportional to concentration

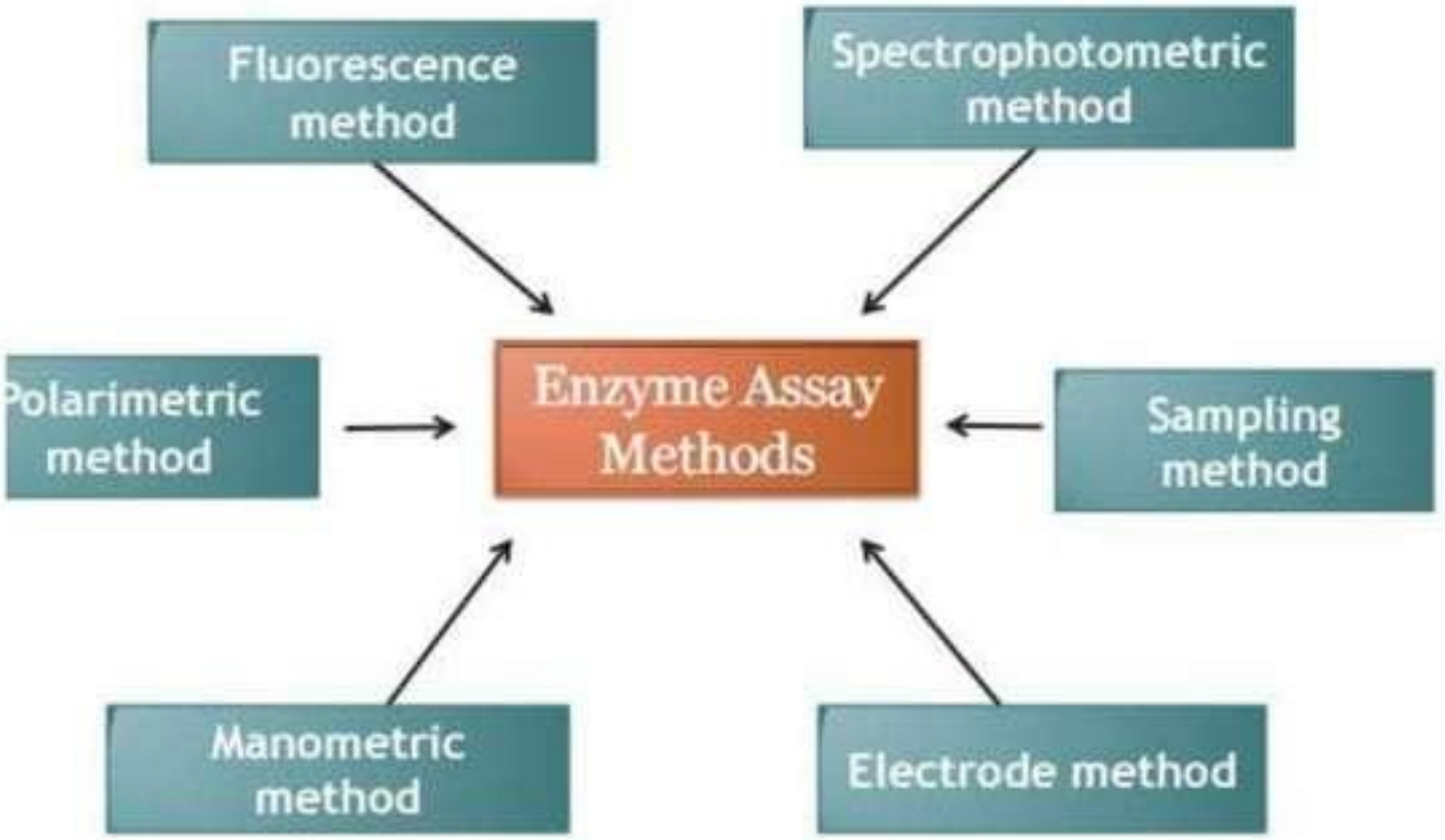
Types of Enzyme assay

Enzyme assays can be split into two groups according to their sampling method:

- Continuous assays, where the assay gives a continuous reading of activity,
  - multiple measurements, usually of absorbance change, are made during the reaction,
  - either at specific time intervals (usually every 30 or 60 seconds)
  - or continuously by a continuous-recording spectrophotometer.
  - These assays are advantageous over fixed-time methods because the linearity of the reaction may be more adequately verified.

Discontinuous assays, where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

- the reaction proceeds for a designated time,
- the reaction is stopped (usually by inactivating the enzyme with a weak acid),
- a measurement is made of the amount of reaction that has occurred.


Fluorescence method
Spectrophotometric method
Polarimetric method
Enzyme Assay Methods
Sampling method
Manometric method
Electrode method

Features of a good E.A.

- 1. Simple and Specific
- 2. Rapid (one doesn't need to wait for hrs or weeks for the results to appear)
- 3. Sensitive (v little sample)
- 4. Easy to use
- 5. Economical

Continuous assays

1. **Spectrophotometric:**

- The spectrophotometric assay is the most common method of detection in enzyme assays.
- It uses a spectrophotometer, a machine used to measure the amount of light a substance's absorbs, to combine kinetic measurements and Beer's law by calculating the appearance of product or disappearance of substrate concentrations
- If this light is in the visible region we can actually see a change in the color of the assay, these are called colorimetric assays.
- UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms.

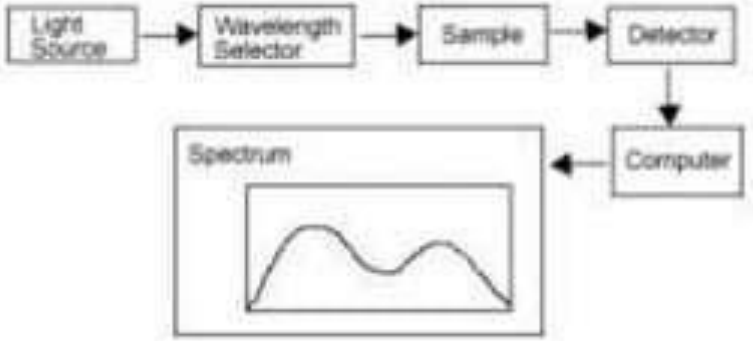
- Many substrates and products of enzyme reactions absorb light either in the **visible** region or in the **U.V.** region.

- Mostly the spectra of S and P are not the same.
  - The conversion of one into another is followed by a considerable change of absorption and by measuring this change the progress of the reaction can be followed quantitatively.

- The enzyme is allowed to react with substrate and the decrease in the conc. of substrate or the increase of product produced will be followed spectrophotometrically

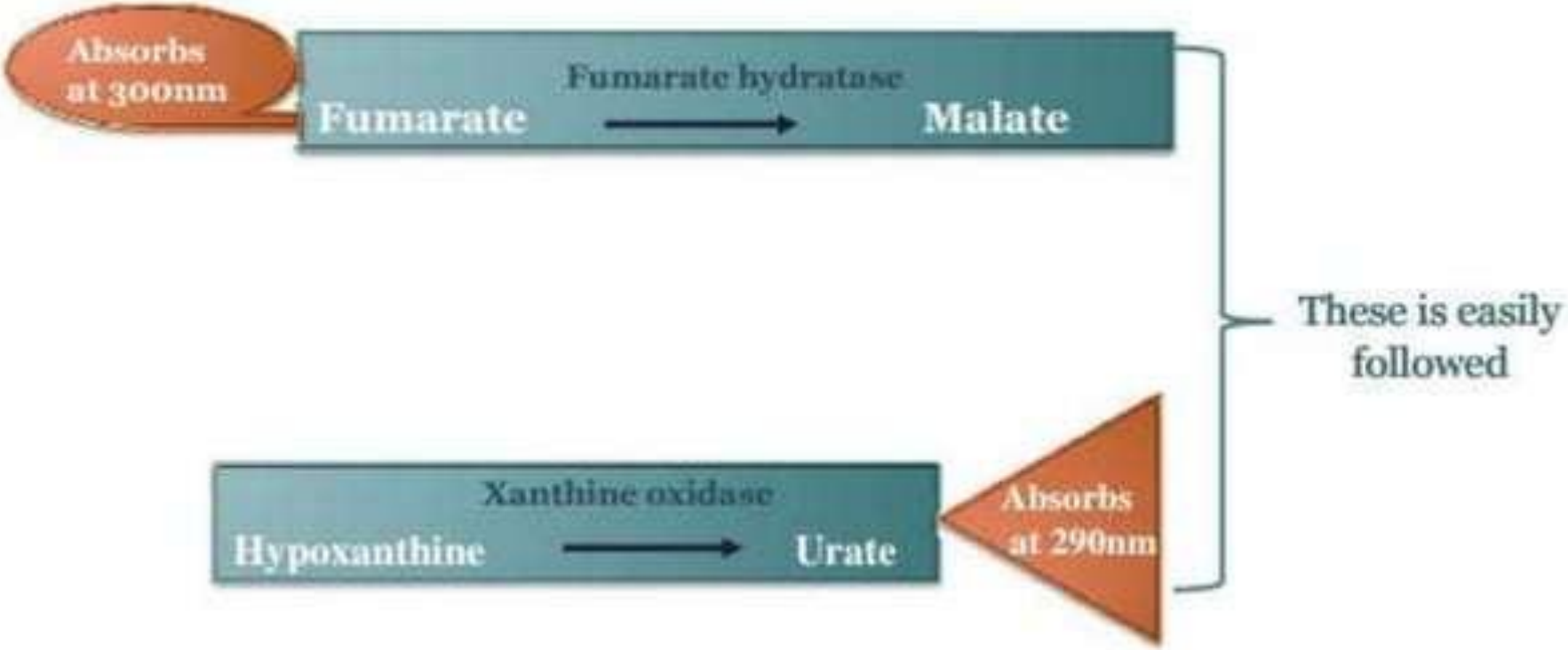
Advantages

- Easy
- Simple
- Sensitive
- Require small sample
- Whole progress curve can be followed quantitatively



Cases in which product absorb but not the substrate

- This will include enzymes catalyze the addition of groups to double bonds or the reverse reaction.



Decrease in absorption at 300nm indicates that the rxn is moving in forward direction in Ist case and increase in abs at 290nm in the IInd case

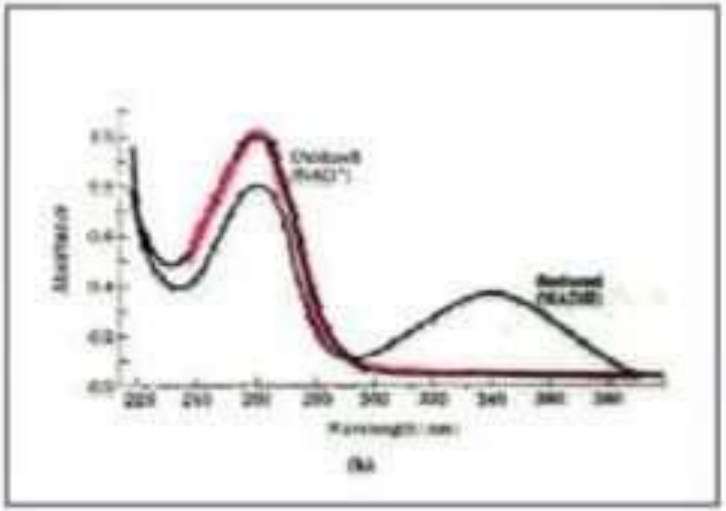
The coenzyme undergoes change in absorption on reduction or oxidation by S (oxidizing enzymes)

$NAD^+ \rightarrow NADH$

Oxidized form Reduced form

$NADP^+ \rightarrow NADPH$

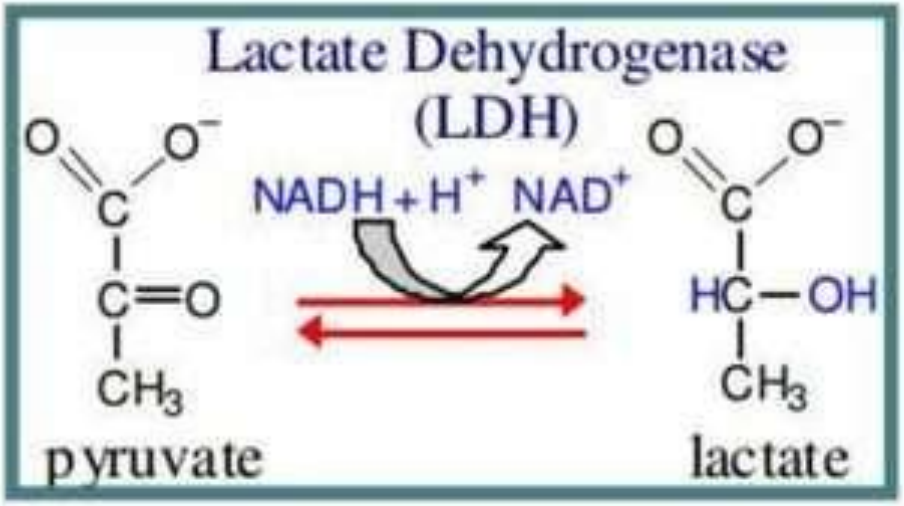
Absorbs at 340nm



$FAD^+ \rightarrow FADH_2$

Much less absorption at 450nm

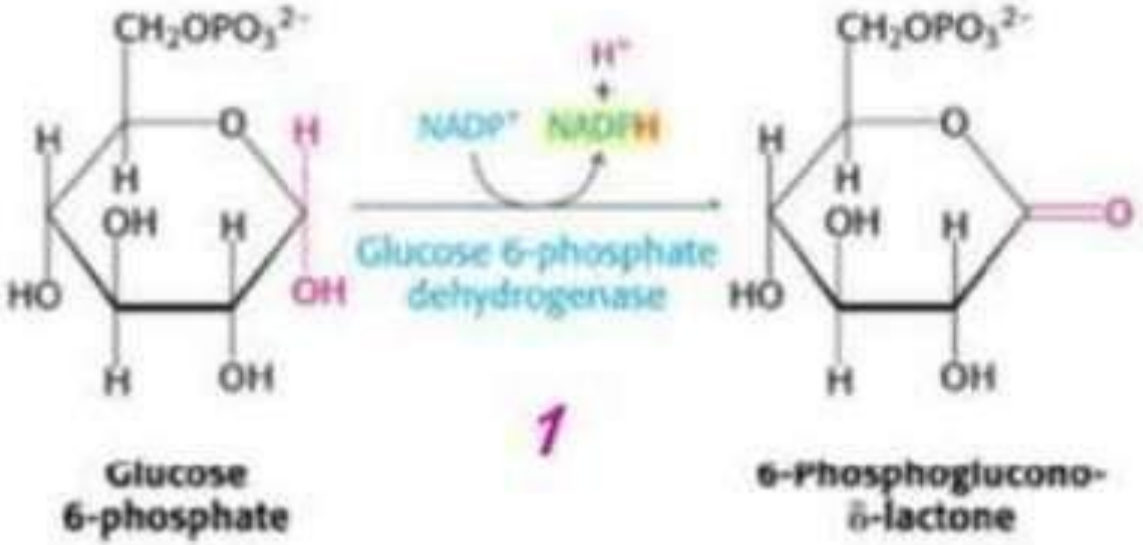
High absorption at 450nm



NADH: Absorbs at 340nm

NAD^+ : No absorption at 340nm

In decrease in abs/min can be used to measure rate of LDH activity



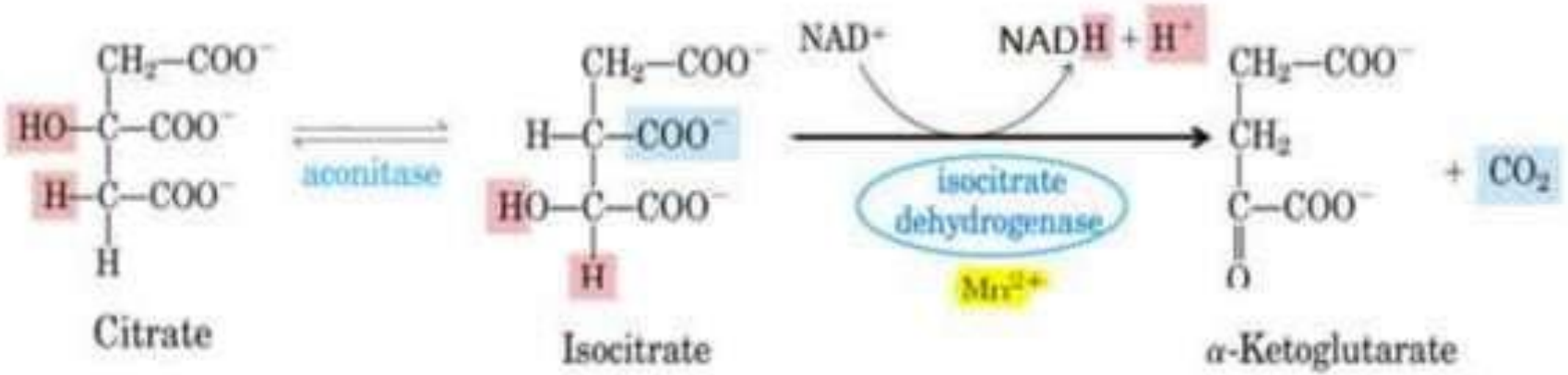
NADPH: Absorbs at 340nm

$NADP^+$: No absorption at 340nm

COUPLED REACTIONS:

- In many reactions, changes in substrates or products are not observable by spectrophotometric methods because they do not absorb light.
- Even when such enzyme reaction does not result in a change in the absorbance of light, it can still be possible to use a spectrophotometric assay for the enzyme by using a coupled assay.
- These reactions can be measured by coupling them to enzymes that can be detected via a spectrophotometer.
- Here, the product of one reaction is used as the substrate of another, easily-detectable reaction.
- This help to follow the first enzymatic reaction.

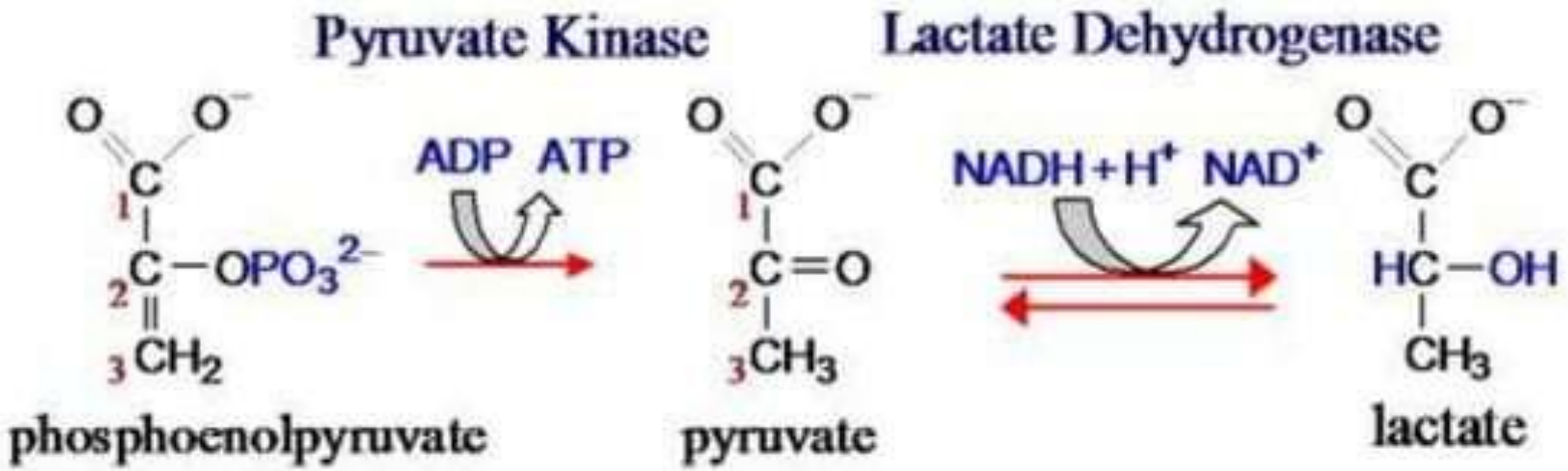
Coupled



NADH: Absorbs at 340nm

NAD^+ : No absorption at 340nm

The assay mixture wud contain– Citrate, aconitase, isocitrate DH and NAD+ with Mn2+



Both ATP and ADP absorb at 260nm

In reaction can be followed by measuring the increase in absorbance at 340 nm (specific for NADH+ H^+ but not NAD^+)

2. Flurescence method/Fluorimetric:

- Fluorescence is when a molecule emits light of one wavelength after absorbing light of a different wavelength.
- Uses a Fluorometer
- Fluorometric assays use a difference in the fluorescence of substrate from product to measure the enzyme reaction.
- FLAVIN COMPOUNDS: fluorescence in reduced form and loose their flouescence in oxidised form

- An example of these assays is again the use of the nucleotide coenzymes NADH and NADPH.
- Here, the reduced forms are fluorescent and the oxidised forms non-fluorescent.
- Oxidation reactions can therefore be followed by a decrease in fluorescence and reduction reactions by an increase.
- More sensitive than spectrophotometric assays, but can suffer from interference caused by impurities and the instability of many fluorescent compounds when exposed to light.
- Detection in small quantities
- Non dangerous

3. Calorimetric: is the measurement of the heat released or absorbed by chemical reactions.

- These assays are very general, since many reactions involve some change in heat and with use of a micro-calorimeter, not much enzyme or substrate is required.
- These assays can be used to measure reactions that are impossible to assay in any other way.

4. Chemiluminescent: is the emission of light by a chemical reaction.

- Some enzyme reactions produce light and this can be measured to detect product formation.
- These types of assay can be extremely sensitive, since the light produced can be captured by photographic film over days or weeks,
- but can be hard to quantify, because not all the light released by a reaction will be detected.

5. MANOMETRIC METHOD

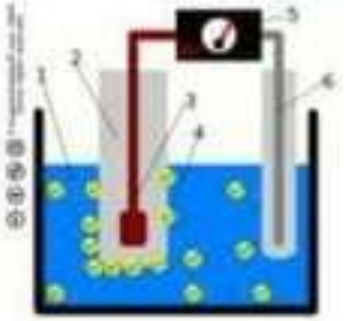
- Use manometer
- These are convenient and accurate methods for following reactions in which one of the component is a gas.
- For the study of:
  - Oxidases (O2 uptake)
  - Decarboxylase (CO2 output)

Emission/ absorption of gas is measured by WARBURG Manometer

6. ELECTRODE METHOD

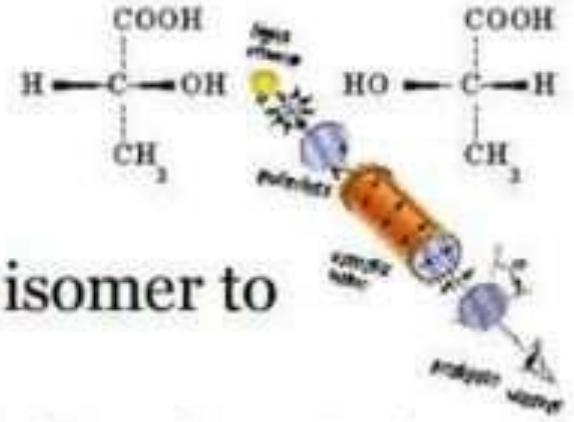
Electrode Method

- To follow reactions which involve the production of acids.
  - Use glass or platinum electrode.
  - In this method pH meter is used to measure change in H+ conc. During enzyme reactions. (i.e. measure change in pH as the reaction proceeds).

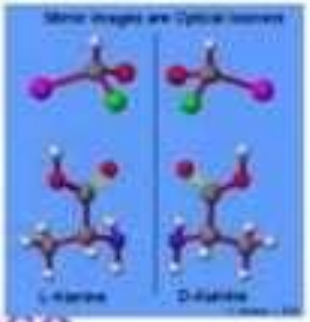


7. POLARIMETRIC METHOD

Polarimetric method



- Use polarimeter
- For isomerases that convert one isomer to another
- Or that convert optically active to inactive or vice versa
- It can be used if both S and P are optically active but different in specific rotation



D-glucose ⟷ L-glucose

Sucrose ⟶ D-glucose + D-fructose

Discontinuous assays

Discontinuous assays are when samples are taken from an enzyme reaction at intervals and the amount of product production or substrate consumption is measured in these samples by different chemical methods.

Radiometric: Radiometric assays measure the incorporation of radioactivity into substrates or its release from substrates.

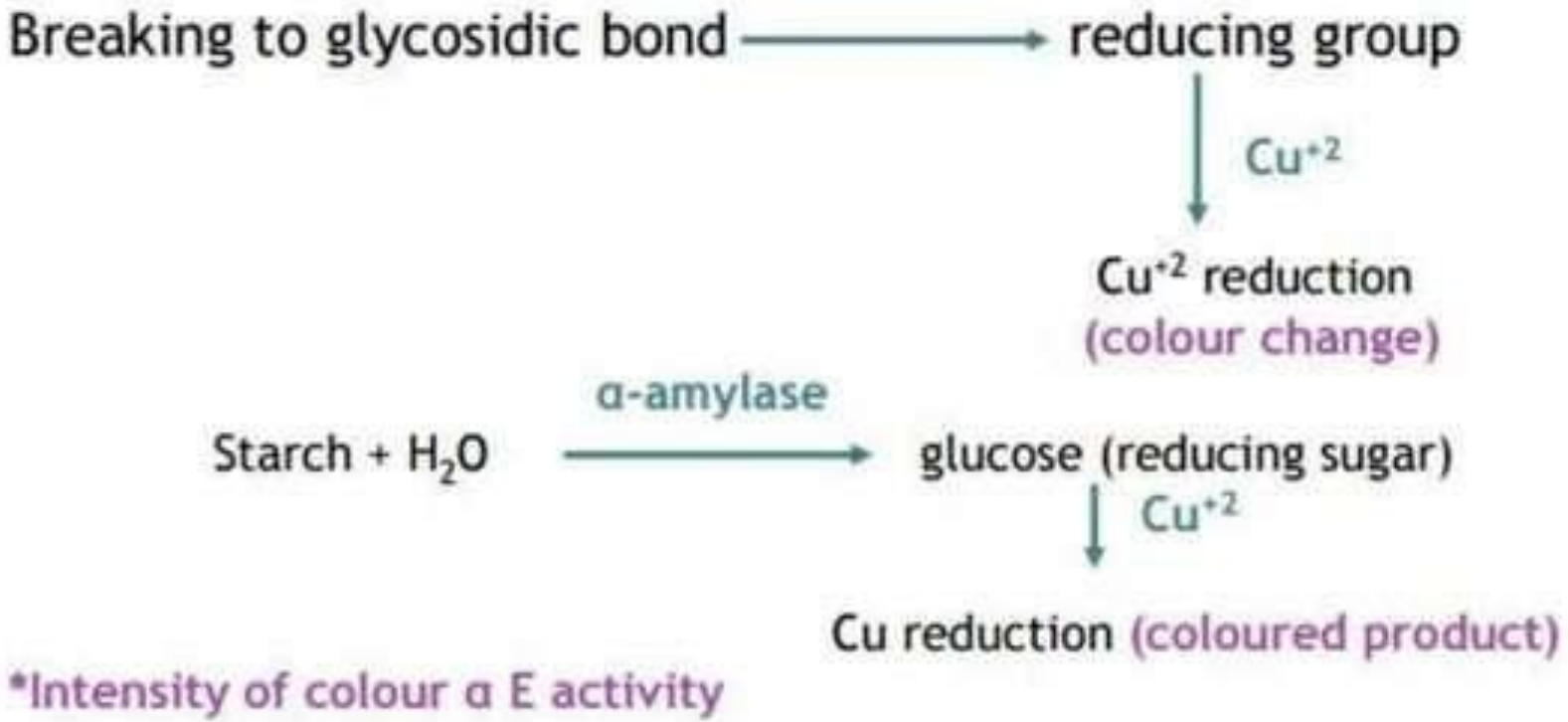
- The radioactive isotopes most frequently used in these assays are 14C, 32P, 35S and 125I.
- Since radioactive isotopes can allow the specific labelling of a single atom of a substrate, these assays are both extremely sensitive and specific.
- They are frequently used in biochemistry and are often the only way of measuring a specific reaction in crude extracts (the complex mixtures of enzymes produced when you lyse cells).
- Radioactivity is usually measured in these procedures using a scintillation counter., which measures the ionizing radiation.
- V sensitive but hazardous

Chromatographic: Chromatographic assays measure product formation by separating the reaction mixture into its components by chromatography.

- This is usually done by high-performance liquid chromatography (HPLC), but can also use the simpler technique of thin layer chromatography.
- Although this approach can need a lot of material, its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag.

For Carbohydrates: Reducing Sugars

- Used for study of enzymes acting on carbohydrates (since the breaking of a glycosidic link produces a reducing group).



*Intensity of colour α E activity