Seminar 2

Clinical enzymology:

Enzymatic activity in blood

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Clinical enzymology: enzymatic activity in blood

1. Introduction
2. Measurement of enzymatic activity
3. Factors than can affect the measurement of serum enzymatic activity in clinical biochemistry
4. Interpretation of the results
5. Isoenzymes – what they are, how to test for them

Introduction: clinical enzymology

- Clinical enzymology uses enzymatic reactions to measure the levels of enzymatic activity or of metabolites, mainly in serum, for the **diagnosis, prognosis, monitoring and treatment of diseases**.

- The key idea is that enzyme levels, which we can measure using enzymatic reactions, are altered, i.e. **abnormally high or low**, in blood under **pathological conditions**.

Clinical enzymology

Enzymes in blood

Enzymes released to the blood

Introduction: types of enzymes

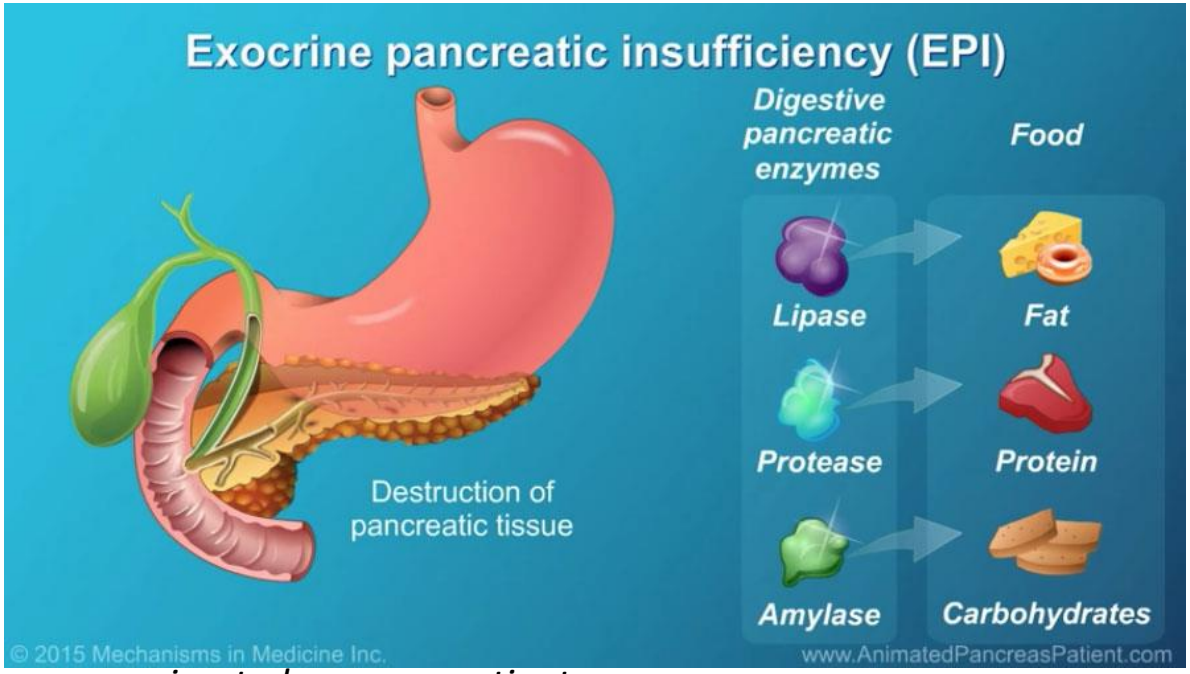
- Enzymes which have their metabolic function **in blood**

e.g., esterases, coagulation enzymes (prothrombin, plasminogen, etc.), lipoprotein lipase, ceruloplasmin

- Enzymes which are released from cells and tissues as a results of cell/tissue turnover BUT under pathological conditions/stress the quantity may be altered:

* Exocrine gland enzymes: e.g. from the exocrine pancreas,

such as amylase, protease, lipase



www.animatedpancreaspatient.com

Introduction: types of enzymes

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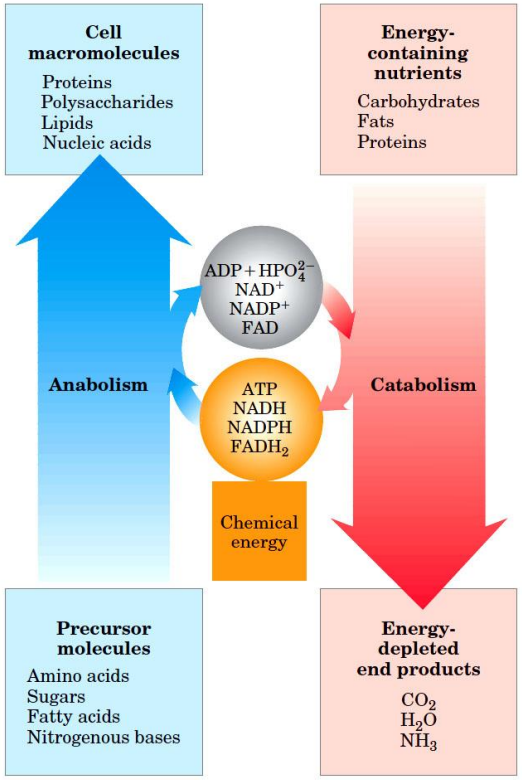
    * Exocrine gland enzymes: e.g., from the exocrine pancreas, such as amylase, protease, lipase

    * intermediary metabolic enzymes

Their levels are low under normal conditions



Introduction: types of enzymes

Exocrine gland enzymes:

They appear in plasma but have no function in it.

Increased production of digestive enzymes from the acinar cells in the pancreas.

Elevated levels of amylase (hyperamylasaemia) or lipase (hyperlipasaemia) in the blood – pancreatitis

Enzymes from the intermediary metabolism:

1. In normal (**healthy**) situations, their release into the blood is due to basal cell turnover, and therefore the **concentrations are low** and lower than the concentrations of the tissue of origin.

2. **In pathological situations**, the release into the blood increases, leading to **high concentrations**.

CAUSES: CELL DAMAGE OR DEATH - Induced by infectious agents, toxic agents (of pharmaceutical or other origin), hypoxia, tumours, etc.

They induce greater cell membrane permeability; and in case of cell death, the release of cell content.

Introduction: types of enzymes

Alteration of blood levels, as levels can increase (induction, cell damage) or decrease (less synthesis, greater degradation)

Objective: the identification of pathological situations

- Changes to the integrity of the cell membrane
- Damage by microbiological, physical or chemical agents (toxins, drugs), hypoxia or tumour-related processes

Introduction: what can affect enzyme values

Increased enzyme activity in blood has high sensitivity and is very useful in clinical diagnosis.

↓

What factors are involved?

Release intensity ⇒

Degree and extent of cell damage
Mass of affected tissue
Type of lesion: inflammation, necrosis

Disappearance in plasma:
Inactivation due to instability
Degradation by proteases
Renal excretion ⇒

Knowledge of the plasma **half-life (t½) of enzymes** enables changes in blood levels to be correctly interpreted

Introduction: half-life of the most common enzymes

The half-life (t½) in plasma of intracellular enzymes is highly variable: knowing this value allows us to correctly interpret changes in blood levels

Enzyma	Half life ($t_{½}$) (hours)
ALT/GPT	50
AST/GOT (cytosolic)	14
AST/GOT (mitochondrial)	6
ALP (placental)	170
LDH-1	107
LDH-5	10
CK-3	20
CK-2	10
CK-1	3

Introduction: interpretation of enzymatic levels

Depending on the amount of change (increase/decrease) in comparison with the baseline reference value and the progression of these changes over time, we can obtain information related to:

- the affected tissue

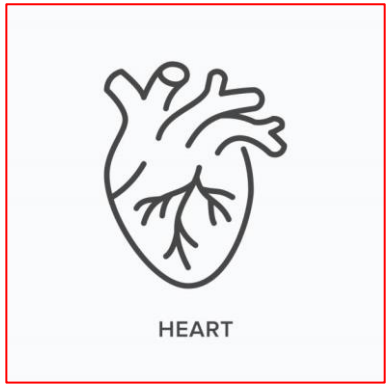
- the severity of the pathological process → increased cytosolic enzymes (greater membrane permeability) or mitochondrial enzymes (cell death)

- the phase of the disease (acute vs chronic)

The release and rate of appearance of enzymes in the blood circulation depends on their intracellular location and the characteristics of the damaged organ or tissue.

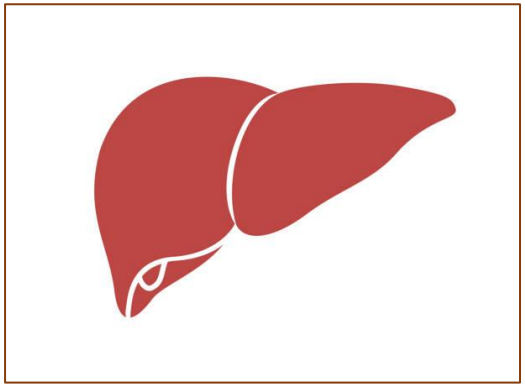
Enzymatic profiles in clinical biochemistry

We measure enzymes in blood as they can act as markers of possible pathologies



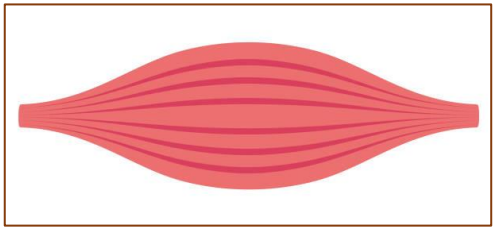
HEART ATTACK

CK-MB
LDH-1
ALT
AST



LIVER DISEASE

ALT
AST
LDH-5
GGT



MUSCULAR DAMAGE

CK-MM

LDH-5

Pics from www.istockphoto.com

Enzymatic profiles in clinical biochemistry

HEART ATTACK

CK-MB
LDH-1
ALT
AST

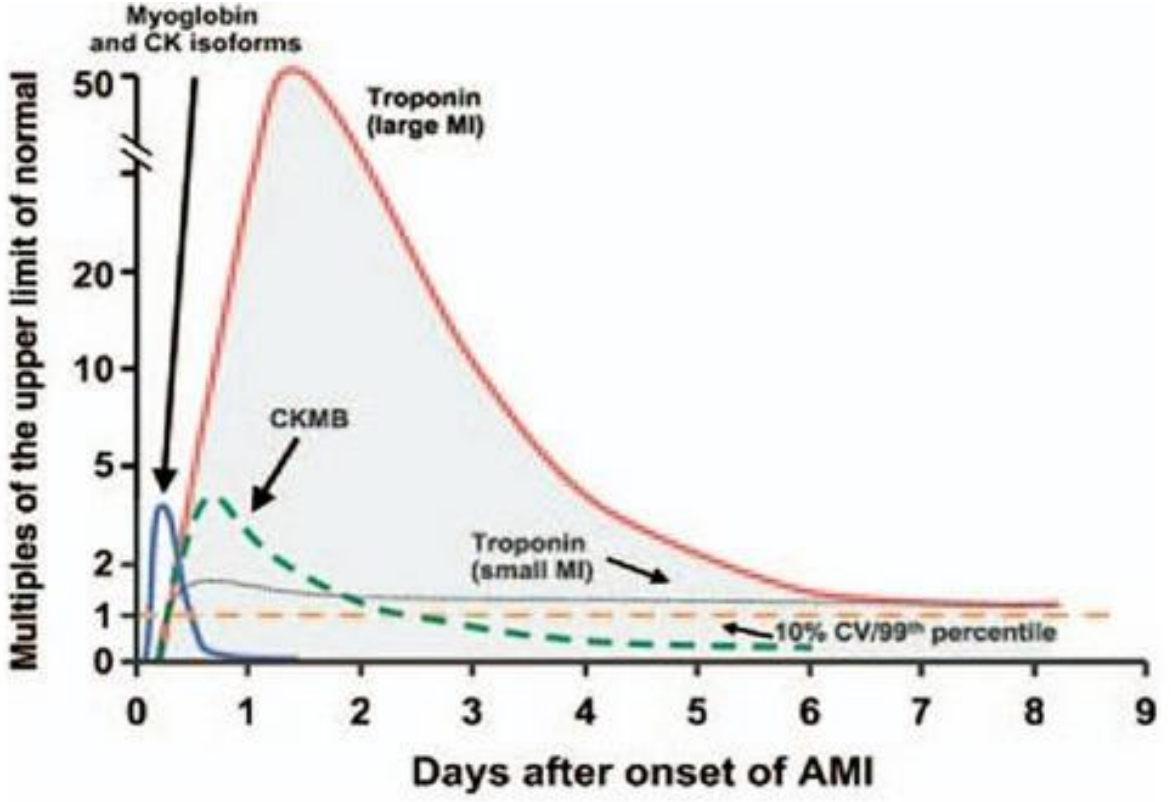


Figure: Timing of the release of various cardiac biomarkers after myocardial injury.
Adapted from Anderson JL, Adams CD, Antman EM, Bridges CR, et al.

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Measurement of enzymatic activity

- Enzymes are very small molecules that are difficult to detect directly, so instead we measure **enzymatic activity (EA)**.

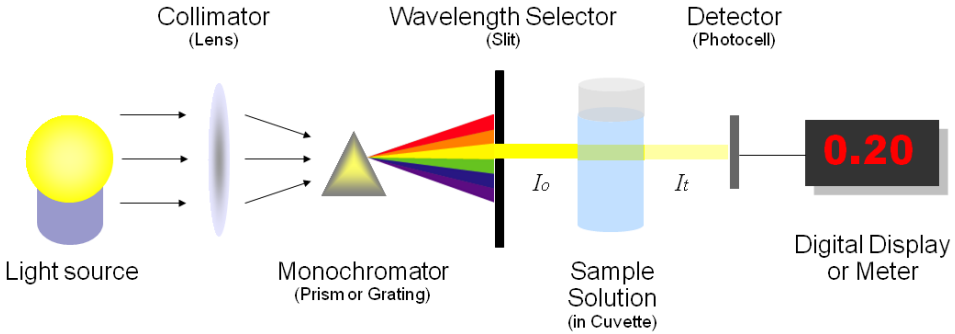
EA= µm generated product/min

Normally, enzymatic activity is measured using **spectrophotometry**

In spectrophotometric tests, we can test for and measure substances which participate in the reaction and which absorb light at a certain wavelength (λ).

Lambert-Beer Law

$$A = \varepsilon\ (mol/L) x b(cm) x C$$

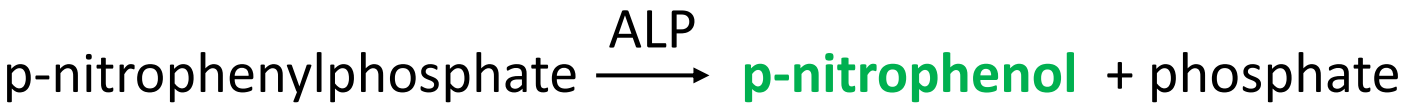


Enzymatic reaction happening here

Quantification using spectrophotometry

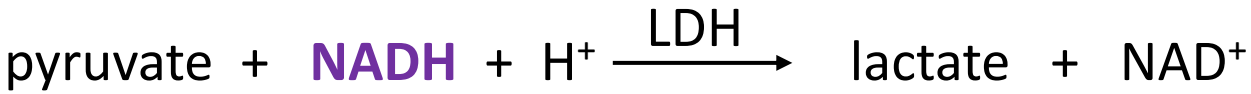
We can test for and measure...

1. The appearance of the reaction product that absorbs light at a certain wavelength.



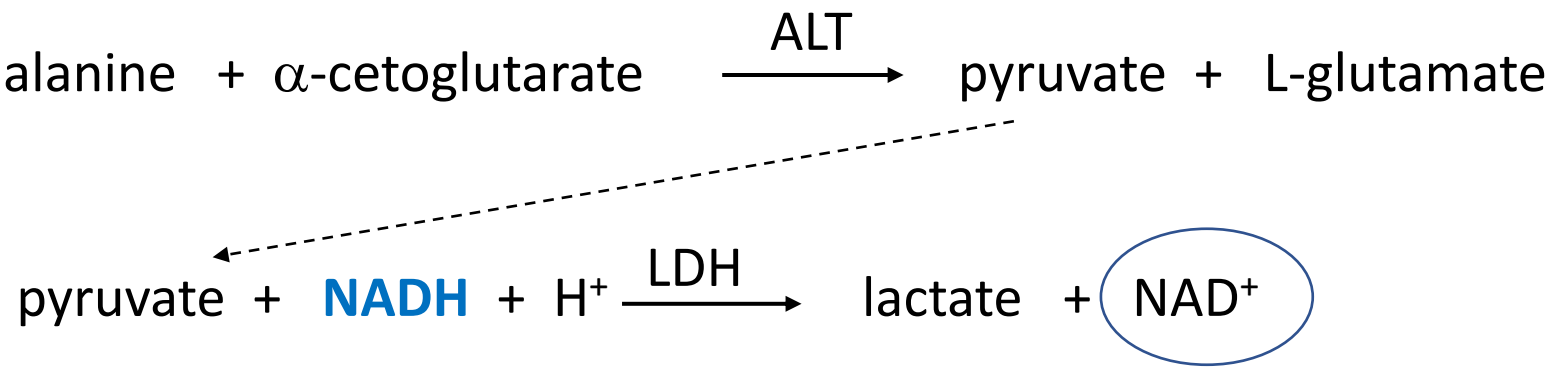
2. The **disappearance** of the substrate if it absorbs light at a given wavelength.

3. A change of a cofactor or other component of the reaction.



Quantification using spectrophotometry

Sometimes none of the elements directly involved in the reaction absorb light. A second reaction is coupled in which, for some of the reactants or products, there is wavelength at which they present an absorption maximum. By analysing the result of this second reaction, we can evaluate the enzymatic activity of the first.



It is very common to use the appearance or disappearance of NADH or NADPH, for which absorbance can be seen to decrease at 340 nm.

Quantification using spectrophotometry

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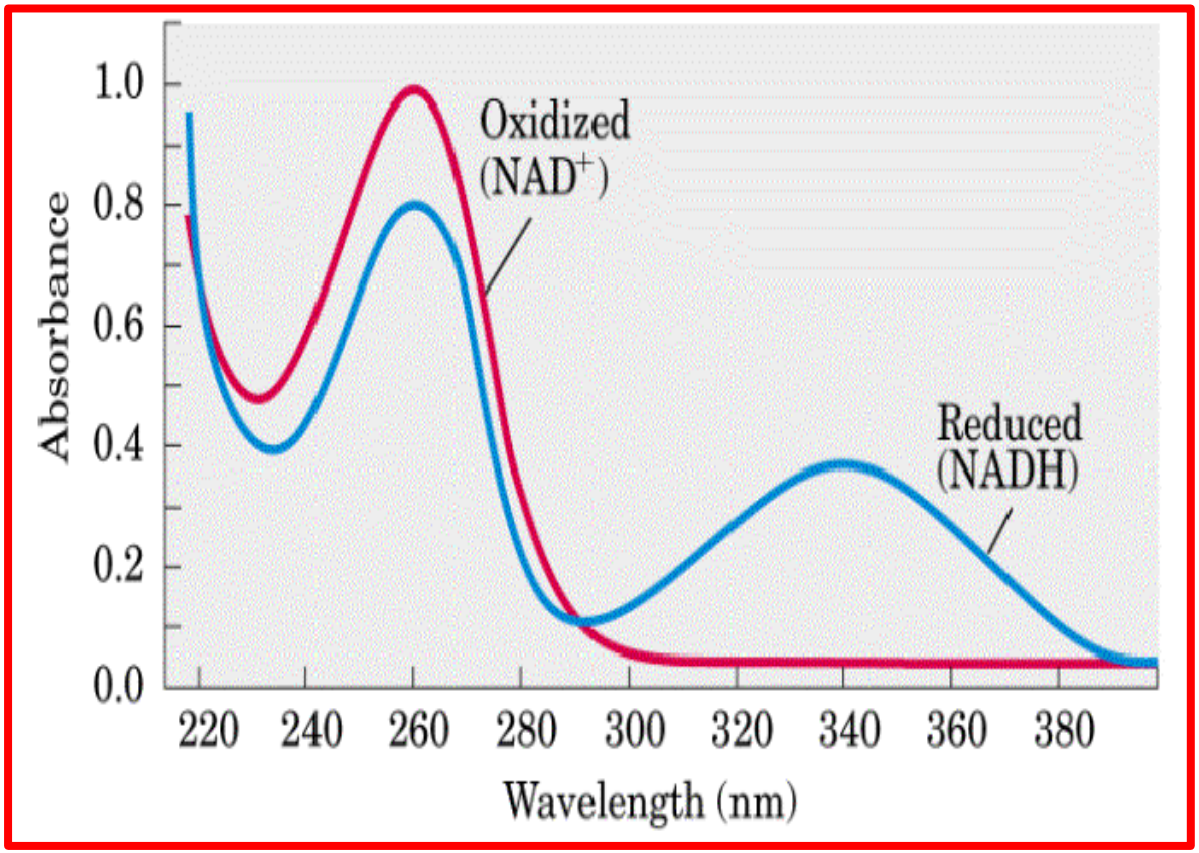
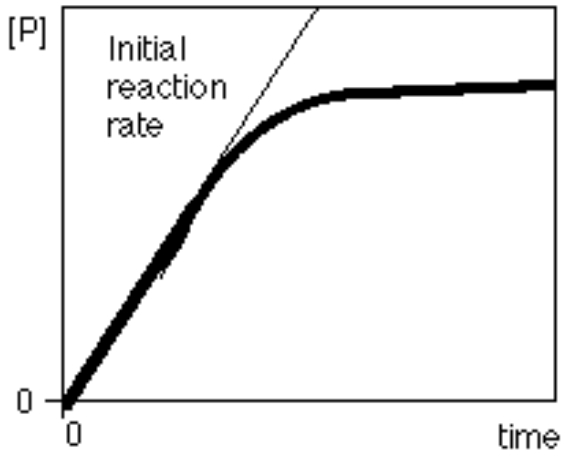
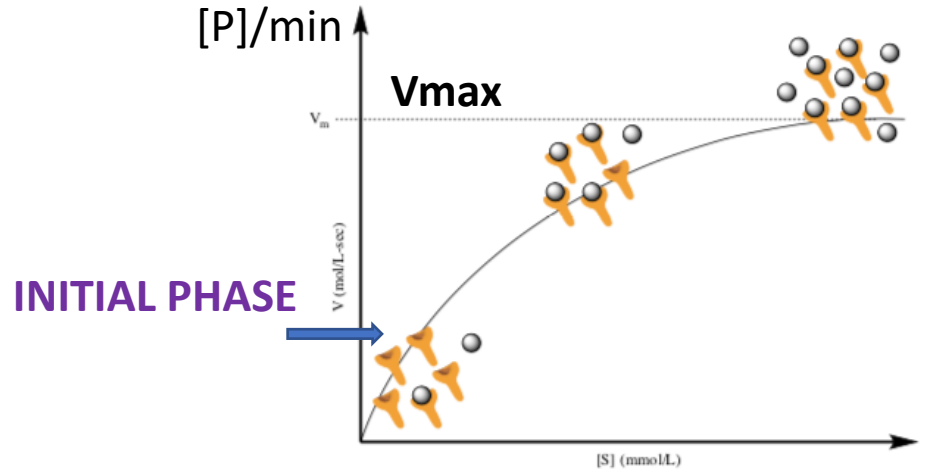


Enzymatic kinetics





INITIAL PHASE: Substrate-enzyme binding. The rate increases with the concentration of the substrate.

SATURATION PHASE: all enzyme molecules are forming the substrate-enzyme complex. The velocity of the reaction is at its maximum (Vmax).

The *in vitro* determination of enzyme activity is carried out during the linear phase since here the appearance of product or disappearance of substrate is **directly proportional** to enzyme activity.

Measurement of enzymatic activity

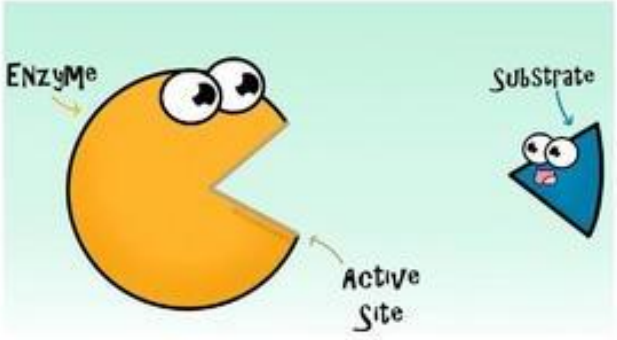
1. The velocity of the enzymatic reaction (product/time) must be directly proportional to the concentration of the enzyme (enzyme limiting factor).
<u>If the activity exceeds a certain value, the test must be repeated with a diluted sample and a dilution factor applied.</u>

2. The substrates and coenzymes must be in excess (so that they are not the limiting factor)

3. Reaction buffer: it will provide the pH and salt conditions

4. Constant temperature and suitable for the enzymatic activity

5. Constant reaction time between different measurements



Measurement of enzymatic activity

The analytical methods that are used to determine the enzymatic activity, and then the difference in ΔA/min can be

- End point methods
- Kinetic methods (multiple points)

Measurement of enzymatic activity

WE DETERMINE

$$\frac{\Delta A}{min}$$

ENZYMATIC ACTIVITY IS CALCULATED AS…

$$\text{AE (UI/L)} = \frac{\Delta A}{min} \times \frac{1000}{\varepsilon \times b} \times \frac{V_F}{V_I}$$

Where:

· ε is the molar extinction coefficient of that compound whose absorbance we are testing in the spectrophotometer

· b is the thickness of the cuvette

· V_F reaction total volume

· V_I sample volume

Ley de Lambert-Beer

$$A = \varepsilon\, b\, C$$

For a given test, all the parameters that appear in colour are constant, therefore the previous formula can be simplified as follows:

$$\text{UI/L} = \Delta A/min \times cte$$

This formula can be applied a to a range of values: if the absorbance is above a certain value, the sample must be diluted and a dilution factor subsequently applied.

Measurement of enzymatic activity

UI/L= ΔA/min x cte

The **enzyme activity unit** (U/IU) is defined as the amount of enzyme that catalyses the conversion of **1 µmol of substrate in one minute**.

Specific activity is the number of enzyme units per milligram of protein (IU/mg prot) or per millilitre of enzyme-containing solution (IU/ml).

The International System of Units (SI) has defined the unit of enzymatic activity as the amount of enzyme that transforms **1 mol of substrate per second: katal (kat).**

1 mole= 106 µmoles and 1 minute = 60 seconds,

1 katal = 60 x 106 U

When the **molecular weight** of the pure enzyme and the **number of active sites** per enzyme molecule are known, the enzyme activity measurements allow the **enzyme turnover number** to be calculated, which is the number of elementary reactions carried out by the enzyme for each active site and per time unit.

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Factors than affect enzymatic values in serum

Inherent to the patient (can be controlled or not): age, sex, pathology (hyperbilirubinemia, lipemia), medication

Sample-related factors:

- Handling: sample taking, handling, transport, conservation, exposure to light, etc.

- Analytical interferences: haemolysis due to inappropriate processing of the sample, inhibitors, drugs, anticoagulants, etc.

- Analytical error: quality control (internal, external)

Factors than affect enzymatic values in serum

Patient-related factors

- Fasting (or not) for 8-12 hours.

The ingestion of food or water can interfere with the results of some tests, especially when evaluating the lipid profile and blood glucose.

Factors than affect enzymatic values in serum

Patient-related factors

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The ingestion of food or water can interfere with the results of some tests, especially when evaluating the lipid profile and blood glucose.

- **Medications that can alter the results**.
More than 150 drugs can interfere with laboratory tests in any of their presentations (increase, decrease, false increase or false decrease)

- Analgaesics and anti-inflammatory drugs can alter liver enzymes or creatinine.

- Glycaemia may appear elevated due to the consumption of oral corticosteroids

Factors than affect enzymatic values in serum

Sample-related factors

- **Transport**: protected from light & from shaking, plasma should be kept cold during transport, but blood stored at room temperature.
- Avoid freezing/thawing multiple times. Separate the clot from the serum as soon as possible (by centrifugation) and avoid working with haemolysed samples.
- Avoid prolonged venous stasis during extraction
- Do not use samples stored for a long time
- **Haemolysis**

The destruction of red blood cells alters the composition of plasma and enzyme determination of LDH, AST, ALT, uric acid, creatinine and cholesterol, etc.

- **Lipaemia** Lipids can alter turbidimetry
- **Jaundice**: Bilirubin can interfere with every analyte except cholesterol.

Factors than affect enzymatic values in serum

Factors during the analysis: pH, T and the presence of inhibitors

Enzymes act at a specific temperature and pH, and their variation can affect the rate of reaction.

Temperature:

At the optimum (critical) temperature, the rate of the reaction catalysed by a given enzyme is maximal.
Below and above that temperature the activity will be lower.

In general, the **critical temperature** for enzymes ranges between **55 and 60 °C**, although the enzymes of some bacteria, which live in hot springs, reach critical **temperatures of 80 to 87 °C.**

Factors than affect enzymatic values in serum

Factors during the analysis: pH, T and the presence of inhibitors

pH:

Each enzyme has certain limit values (maximum and minimum) to be able to perform its activity.
Beyond these values, the enzyme is denatured and ceases its activity.

Within these limits there is a certain pH value, at which the enzyme performs its activity at maximum efficiency: **optimal pH**

Most intracellular enzymes have a pH optimum close to neutral.

Activators/inhibitors

There are multiple metabolites, enzymes, coenzymes, and substrates that can act as activators and inhibitors, affecting enzyme activity.

Take this into account in the experimental design!

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Interpretation of the results

The enzymatic activity is expressed as U/L in serum

U.I.: μmoles of substrate transformed in a minute (or μmoles of product formed in a minute)

For diluted samples, correct the values (*dilution factor*)

Comparison with reference values (basal level, non-pathological serum) – under the same experimental conditions. Use internal controls.

MEET INTERNATIONAL QUALITY REQUIREMENTS

Interpretation of the results

- **Aspartate aminotransferase (AST/GOT)**
  - Normal values: < 40 U/L
  - Important increase: heart attack, hepatitis, trauma
  - Moderate increase: cirrhosis, mononucleosis, jaundice, haemolysis

- **Alanine aminotransferase (ALT/GPT)**
  - Normal values: < 50 U/L
  - Important increase: Shock, hepatitis
  - Moderate increase: cirrhosis, mononucleosis, jaundice

- **Gamma-glutamyl transpeptidase (GGT)**
  - Normal values: < 35 U/L in men y < 25 U/L in women
  - Important increase: Viral hepatitis, liver metastases, biliary obstruction, alcoholic disease
  - Moderate increase: infections that affect the liver (cytomegalovirus, infectious mononucleosis)

Interpretation of the results

- **Alkaline phosphatase (ALP)**
  - Normal values: 85-190 U/L in adults. Up to 500 U/L in children
  - Important increase: obstructive jaundice, cholelithiasis, bile duct neoplasm, cirrhosis, hepatomas
  - Moderate increase: osteogenic bone neoplasms, Paget's disease, hyperparathyroidism

- **Lactate dehydrogenase (LDH)**
  - Normal values: < 120-230 U/L
  - Important increase: myocardial infarction (LDH1), viral hepatitis (LDH5)
  - Moderate increase: haemolysis, cerebrovascular accident, muscular dystrophy

- **Creatine kinase (CK) and isoenzymes**
  - Normal values: < 160 U/L in men and <130 U/L in women
  - Important increase: Heart attack (isoenzyme CK2)
  - Moderate increase: Myopathies, muscular dystrophy, cerebrovascular accident

Other tests

The above assays determine enzyme activity, and are based on the generation/disappearance of a product over time.

Other clinical biochemistry tests:

- Measurement of metabolic parameters: hormones, chemical molecules, ions - they can be associated with different pathologies, and even with stages of these pathologies.

The commonest tests concern the function of the liver, kidneys, or heart, or concern proliferative syndromes (cancer)

The parameters are the substrates or cofactors of the reaction, and techniques similar to those of enzymology are used.

Other tests

METABOLIC PROFILING: THE BIOCHEMISTRY OF THE PATIENT

The study of the set of metabolites helps to form a complete profile of the different functions of the individual.

Carbohydrate metabolism: glucose, insulin, C-peptide

Ions: Na^+, K^+,

Renal function: urea and creatinine

Lipid metabolism: cholesterol (HDL and LDL), triglycerides

Iron metabolism: iron profile (iron, ferritin and transferrin)

Others: vitamin B12, folic acid, thyroid enzymes (T3, T4 and TSH) and cytolysis enzymes (LDH) among others.

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Isoenzymes

Isoenzymes:

- In some cases enzymes that carry out the same chemical reaction are released by different tissues: these are isoenzymes (or isozymes).

- Isoenzymes form a set or family of enzymes that catalyse the same reaction but which differ in structure and physicochemical properties.

- Depending on their tissue of origin, isoenzymes have different characteristics and can be distinguished using different methods.

- The damaged organ or the affected tissue can be determined by the identification of particular isoenzymes and measurement of their activity.

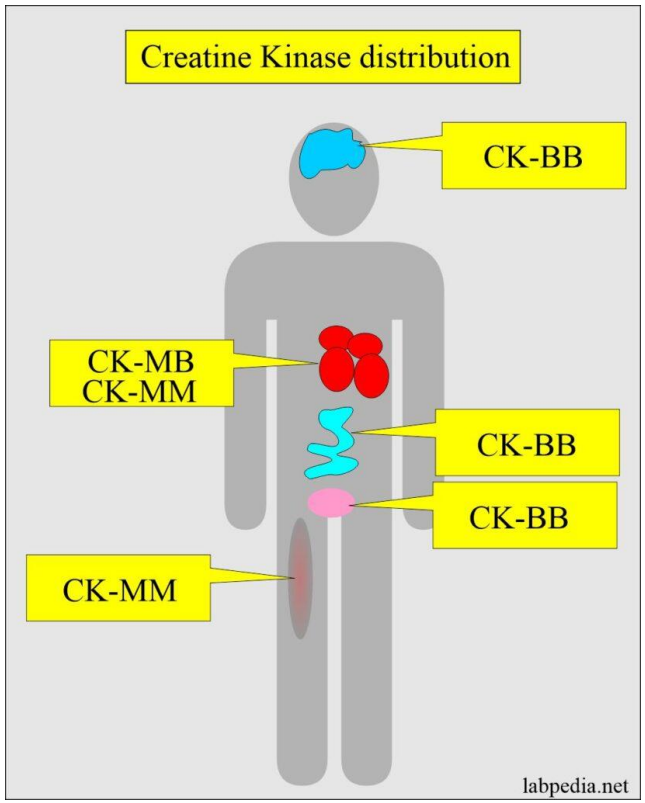
Isoenzymes

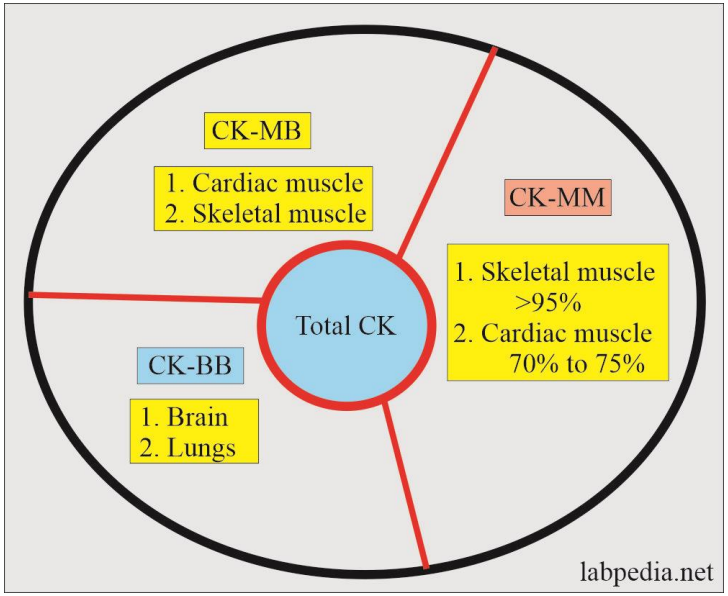
They may differ with respect to:

size, charge, thermostability, K_m, specificity for different substrates, their ability to be recognized by specific agents, or immunoreactivity.

These **differences** can be used to differentiate between the different isoenzymes using different techniques.

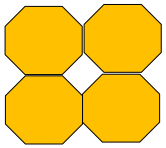
Creatine Kinase

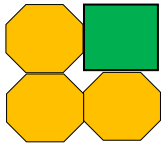


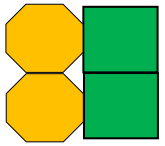


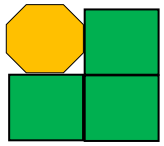
Isoenzymes

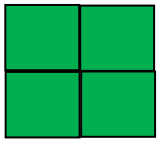
Isoenzymes of lactate dehydrogenase (LDH)

LDH 1  LDH1 = α4

LDH 2  LDH2 = α3β1

LDH 3  LDH3 = α2β2

LDH 4  LDH4 = α1β3

LDH 5  LDH5 = β4

LDH is formed by 4 subunits.

Two subunits  α  β

5 different isoenzymes

They differ with respect to **electrophoretic mobility**, so we can use this to distinguish them.

Each tissue has a different isoenzyme.

Plasma increased values can give us information about:

LDH-1: Myocardial infarction, red blood cell diseases such as haemolytic anaemia, kidney diseases and testicular tumours.

LDH-2: lymphomas.

LDH-3: lung disease

LDH-4: pancreatitis.

LDH-5: liver diseases, muscular dystrophy.

Isoenzymes

- **LDH-1 (α4): in the heart and erythrocytes.**
- **LDH-2 (α3β): in leukocytes. Abundant in the heart, but less so than LDH 1.**
- **LDH-3 (α2β2): in the lungs**
- **LDH-4 (α β 3): in the kidneys, placenta and pancreas.**
- **LDH-5 (β 4): in the liver and skeletal muscle.**

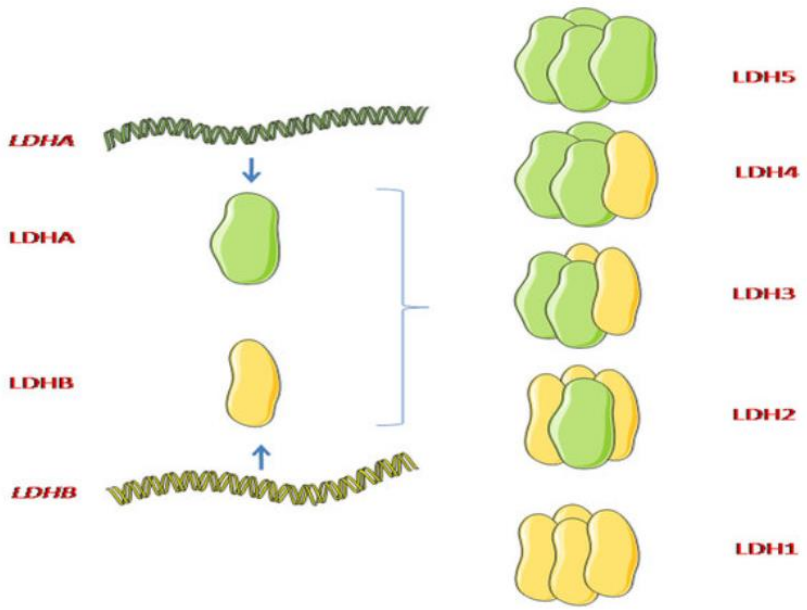


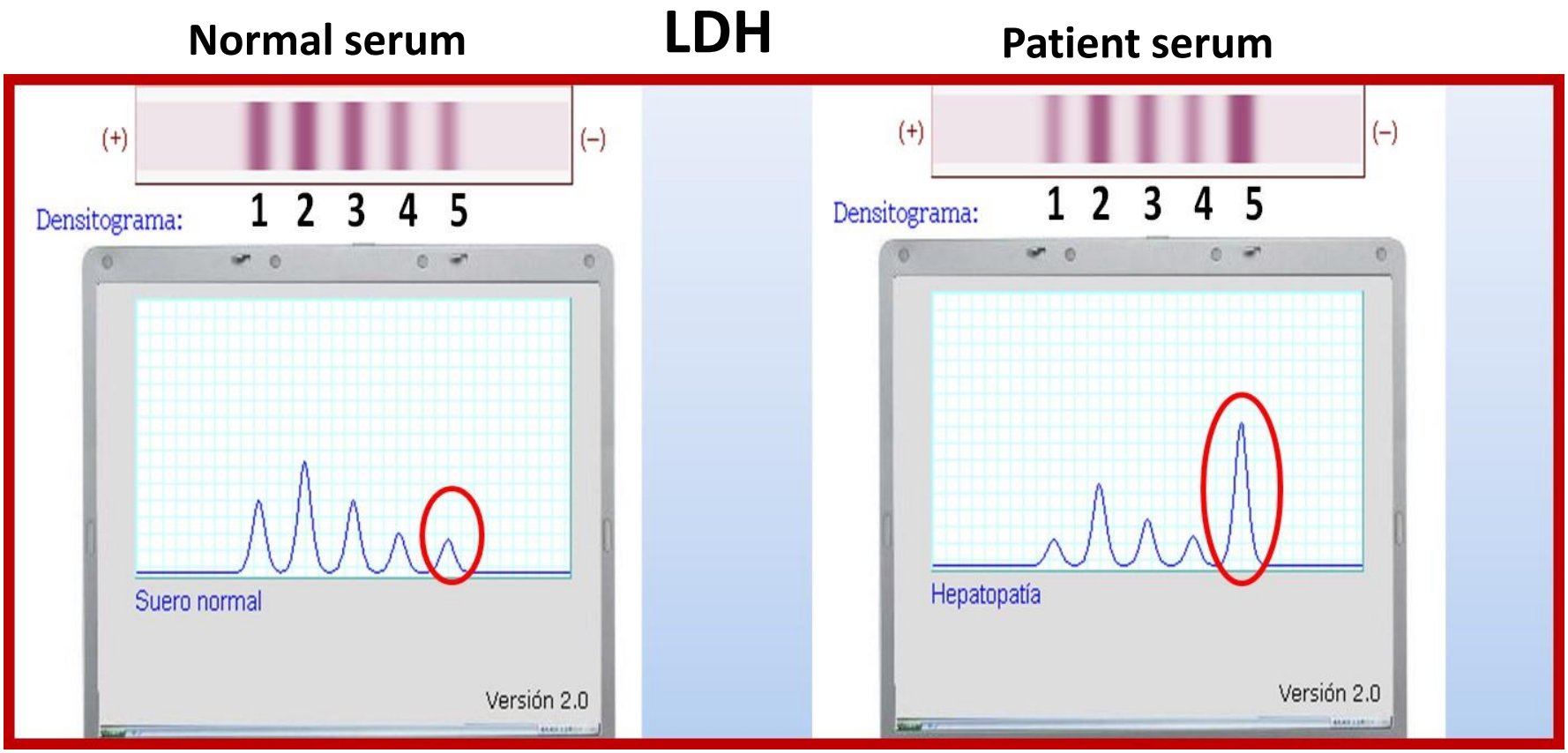
Figure: Lactate dehydrogenase (LDH) subunits and their combinations. Lactate dehydrogenase (LDH) consists of two different subunits LDHA and LDHB. LDHA and LDHB can be assembled into combinations: LDH1 is composed of four LDHB subunits; LDH2 contains three LDHB subunits and one LDHA; LDH3 has two LDHB/LDHA subunits; LDH4 possesses one LDHB subunit and three LDHA subunits; while LDH5 is composed of four LDHA subunits. Figure conception adapted from Doherty et al., (2013). Graphical elements adapted from Servier Medical Art.

Isoenzymes: identification and separation

NORMAL VALUES

- **LDH-1: 17-27%**
- **LDH-2: 27-37%**
- **LDH-3: 18-25%**
- **LDH-4: 8-16%**
- **LDH-5: 6-16%**

Isoenzymes: identification and separation



Isoenzymes: temperature affects activity

LDH isoenzymes: temperature impacts on activity

LDH1 and 3: heat resistant (heart and lung)

LDH 2, 4 and 5: thermosensitive (liver)

NORMAL VALUES

- **LDH-1: 17-27%**
- **LDH-2: 27-37%**
- **LDH-3: 18-25%**
- **LDH-4: 8-16%**
- **LDH-5: 6-16%**

Enzymatic activity is measured in inactivated serum and then a comparison is made with serum activated at 60C for 30 min.

Heat resistant

Normal serum:	**30-60 %**
Liver disease:	**≤ 30%**
Heart attack:	**≥ 60%**

Isoenzymes: activity depends on T

Example: isoenzymes of alkaline phosphatase (ALP)

Their thermosensitivity/thermoresistance differs according to tissue origin

This enables some isoenzymes to be inactivated, indicating the origin of the tissue damage

Table: Inactivation of ALP activity after treatment of serum sample at 56°C for 15 min.

Tissue of origin	Inactivation (%)
Liver	50-70
Bone	90-100
Intestine	50-60
Placenta	0

Testing for isoenzymes

· The different capacity of each isoenzyme **to bind inhibitors** makes it possible to use catalytic inhibition techniques (selective inhibition of isoenzymes).

· Sometimes each isoenzyme has a **specific substrate**, which allows the use of substrate specificity techniques, e.g., LDH-1 can use ß-hydroxybutyrate as a specific substrate.

· Other techniques take advantage of **immunological differences** and use antibodies that react exclusively with certain isoenzymes.

Isoenzymes study

Isoenzyme forms can be tested for or quantified via any differential property:

- **Electrophoretic mobility (charge, molecular weight)**
- **Thermal stability**
- **Use of inhibitors or different substrates**
- **Immunological methods**