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Use of enzyme (and other) markers in diagnostics of selected pathophysiological states

Evaluation of LDH isoenzymes

Ústav patologické fyziologie LF MU

Aim and importance of laboratory examination

- biochemical investigations
  - used in medicine for a variety of purposes
  - measurement of a substance in a body fluid
  - reflect pathological processes
- 70 – 80 % of medical decisions depend on tests
  - low costs (3 – 5 % of total)
  - last 5 years increase of
    - glucose measurements (10 – 15 % increase)
    - molecular biology (25 – 35 % increase)
    - point-of-care testing
- aims
  - confirming a clinical suspicion (glucose – diabetes)
  - assessment of severity (creatinine)
  - monitoring disease/treatment (HbA_{1c})
  - providing of prognosis
  - screening
  - research of the disease

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Classification of laboratory examinations

- by availability
  - basic
    - quickly accessible
  - specialized
  - highly specialized
    - centralized
- by demands of processing
  - routine
  - statim
    - by 60 minutes since delivery
  - vital indication
    - by 30 minutes since delivery
  - point-of-care testing
    - on-site examination
      - acid-base, oxygen metabolism, diagnostic strips – blood and urine

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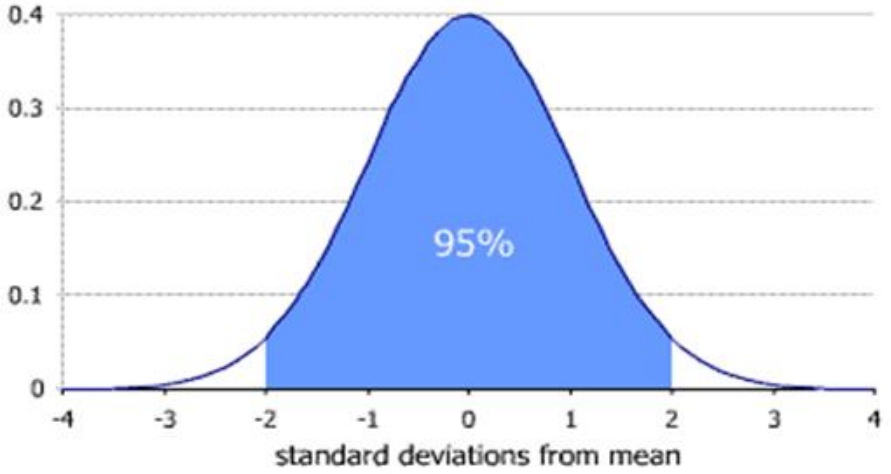
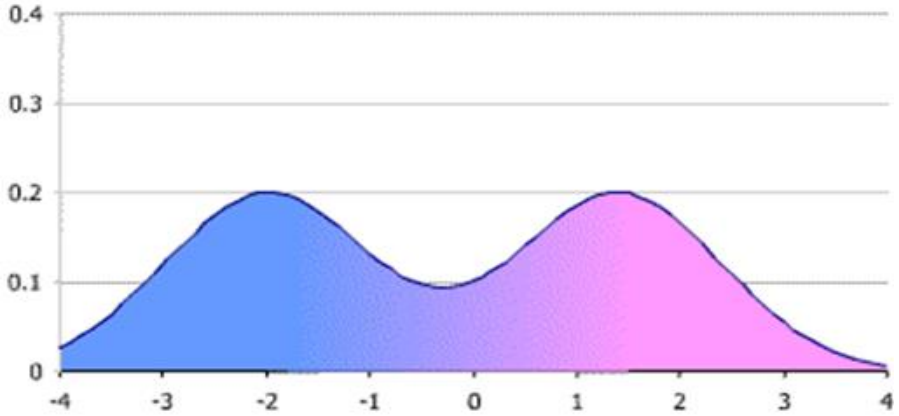
Reference intervals

- rather than normal interval (what is normal?) reference interval of values is used in clearly defined reference group of subjects
  - what is reference group?
    - healthy or better people without state of health which directly affects/interferes with measured variable
- determination
  - historical: x ± 2 SD
    - i.e. 95% of values with normal distribution
- distribution might be influenced by
  - age, gender, race, diet, …
- values outside reference interval
  - statistical/methodological variability
  - biological variability
  - 5% of healthy population outside





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Sample collection and analysis

- preanalytic phase
  - sample collection, storage and transport
  - as many as 60% of errors
- analytic phase
  - follow the good laboratory practice conditions
  - internal and external quality control – elimination of errors
- postanalytic phase
  - results interpretation

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Factors affecting preanalytic phase

- biological
  - influenceable
    - weight – correlation of cholesterol, TAG, cortisol, uric acid with obesity
    - eating habits
      - high-protein diet – increase in urea, cholesterol, phosphates
    - smoking – cholesterol, TAG, cortisol, vitamins B_{12} and C
    - alcohol
      - chronic abuse – increase in ALT, AST, cortisol
      - mild doses – temporary increase of HDL
    - pharmaceuticals and drugs
      - impact on biological processes (induction of enzymes, cytotoxicity), interference
    - physical load
      - depends on the duration and intensity
    - environment – altitude, temperature, travel across time zones
    - mechanic effects
      - muscle trauma – increase in ALT, AST and CK, myoglobin

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Factors affecting preanalytic phase

- biological
  - uninfluenceable
    - race – different enzymatic activities
    - gender
      - minimal differences in childhood
      - in adulthood values ofter higher in men
    - age
      - ALP – high activity in childhood, then decrease, ferritin
    - pregnancy
    - biological rhythms – circadian – hormones, iron, urea
- sample management
  - labelling – all stages of handling, request forms
  - sampling material
  - technique of sampling – nurses vs. doctors
- sample transport
- storage of samples

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Examples of biased results

analyte	result	reason
Glucose	↑	non-fasting
TAG	↑	non-fasting
Creatinine	↑	↑ acetoacetate in plasma
Bilirubin	↓	longterm exposition to sunlight
K^+	↑	hemolysis
Total calcium	↓	blood taken in EDTA
Phosphate	↑	longterm contact with erythrocytes
Cortisol	↑	stress

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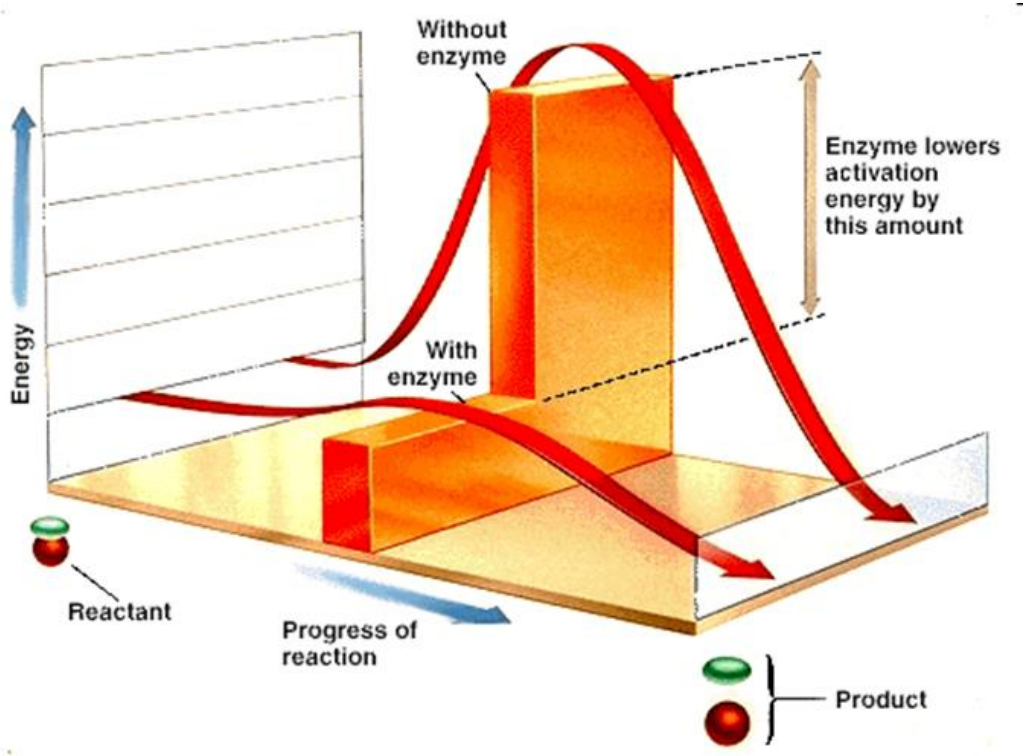
Enzymes

- proteins with catalytic properties
  - virtually all reactions in the cell depend on enzymes
  - decrease activation energy
  - not being consumed
  - change only the rate at which equilibrium is established
  - enzyme molecules are larger than their substrates
    - exception - proteases



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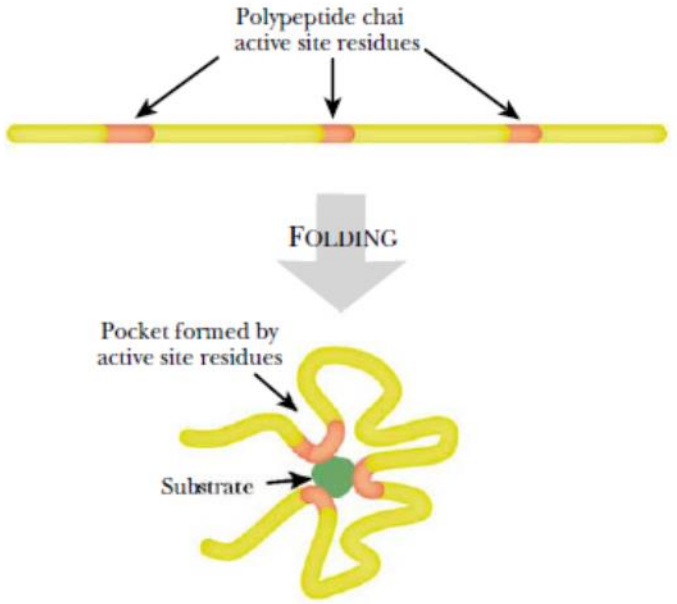
Enzymes

- structure
  - primary
  - secondary
    - conformation of limited sequences of polypetide chain
  - tertiary
  - quaternary
- holoenzyme
  - apoenzyme + cofactor
- cofactor
  - prosthetic group
  - coenzyme
- active site
  - relatively small
  - 3D structure formed as a result of the tertiary structure



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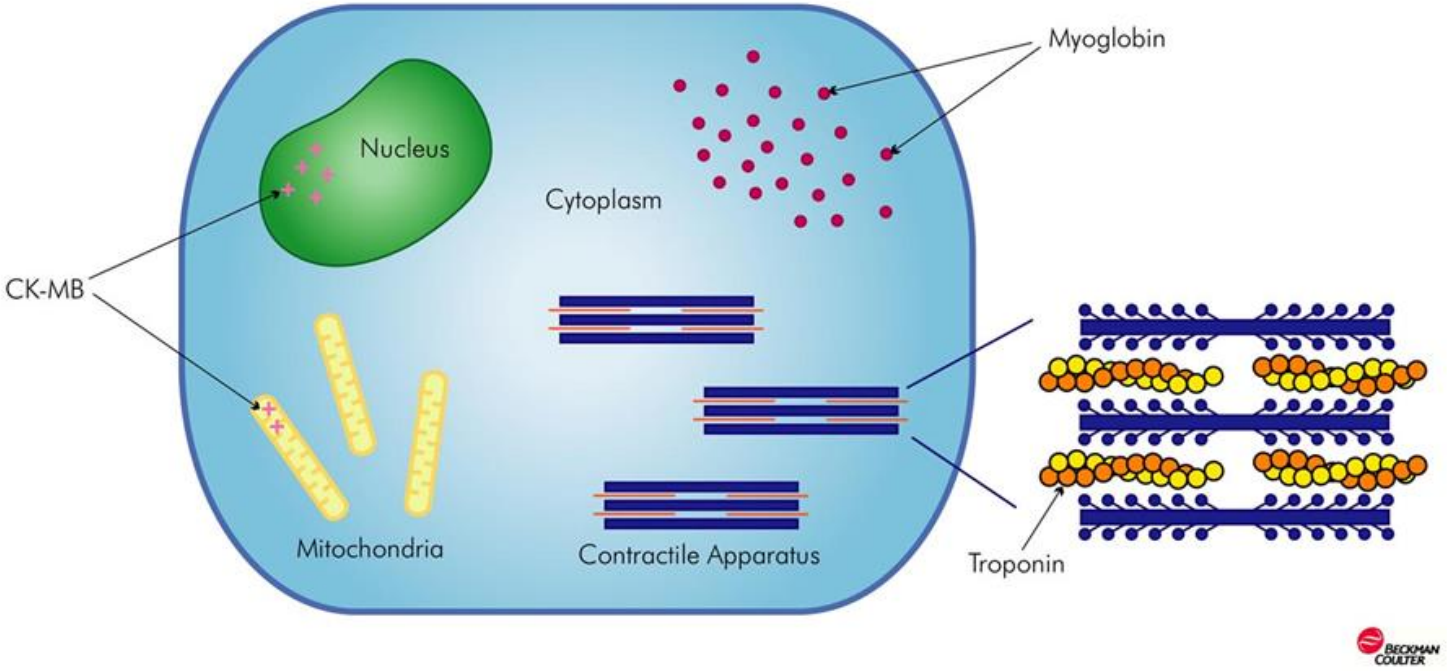
Cellular localization of enzymes/markers

- extracellular
- intracellular
  - membrane-bound
  - cytosolic
  - in organelles



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Plasma enzymes

- specific
  - blood clotting enzymes, ceruloplasmin, lipoprotein lipase
- nonspecific
  - secreted
    - amylase, lipase
  - celullular enzymes
    - enzymes of main metabolic pathways

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Factors affecting plasma enzyme concentration

- intracellular enzyme activity
- intracellular localization
- permeability of plasma membrane
- the extent of cell damage
- the mass of the damaged cell
- the rate of enzyme elimination

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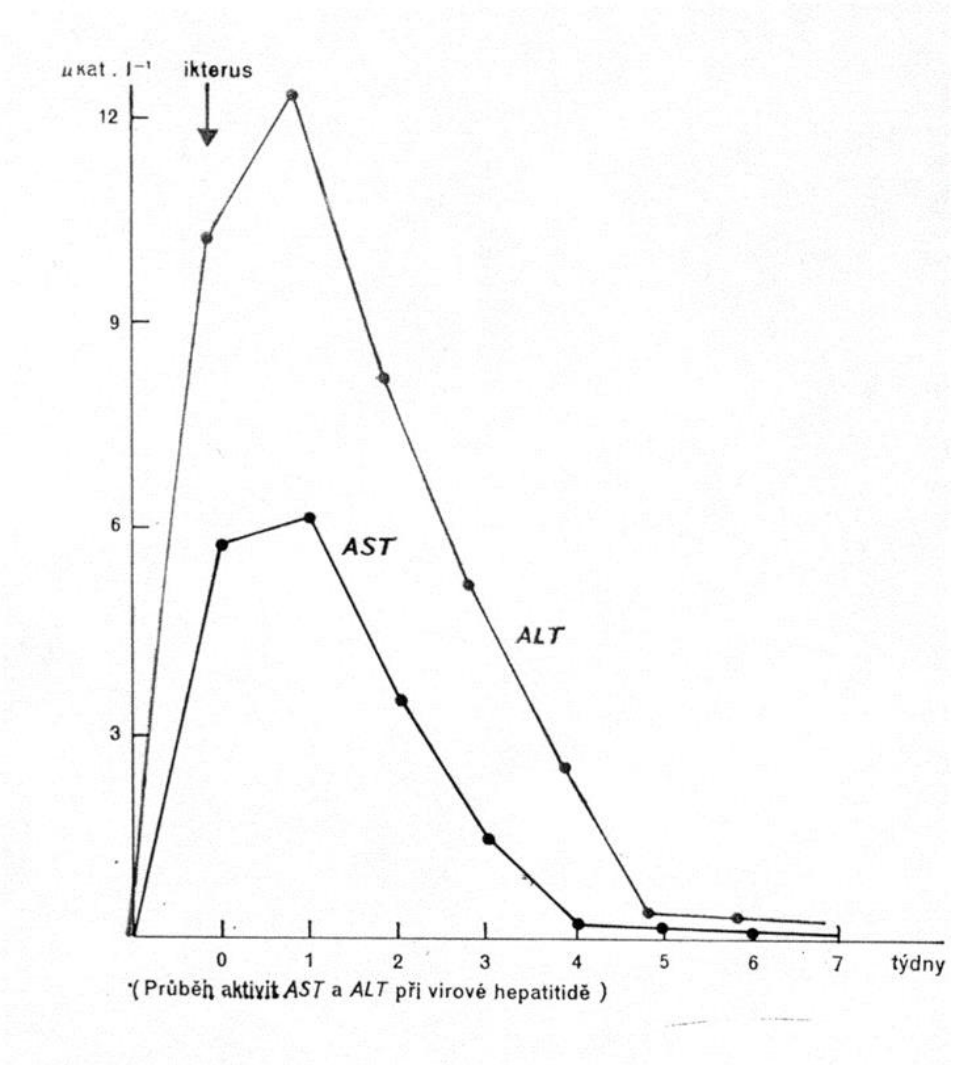
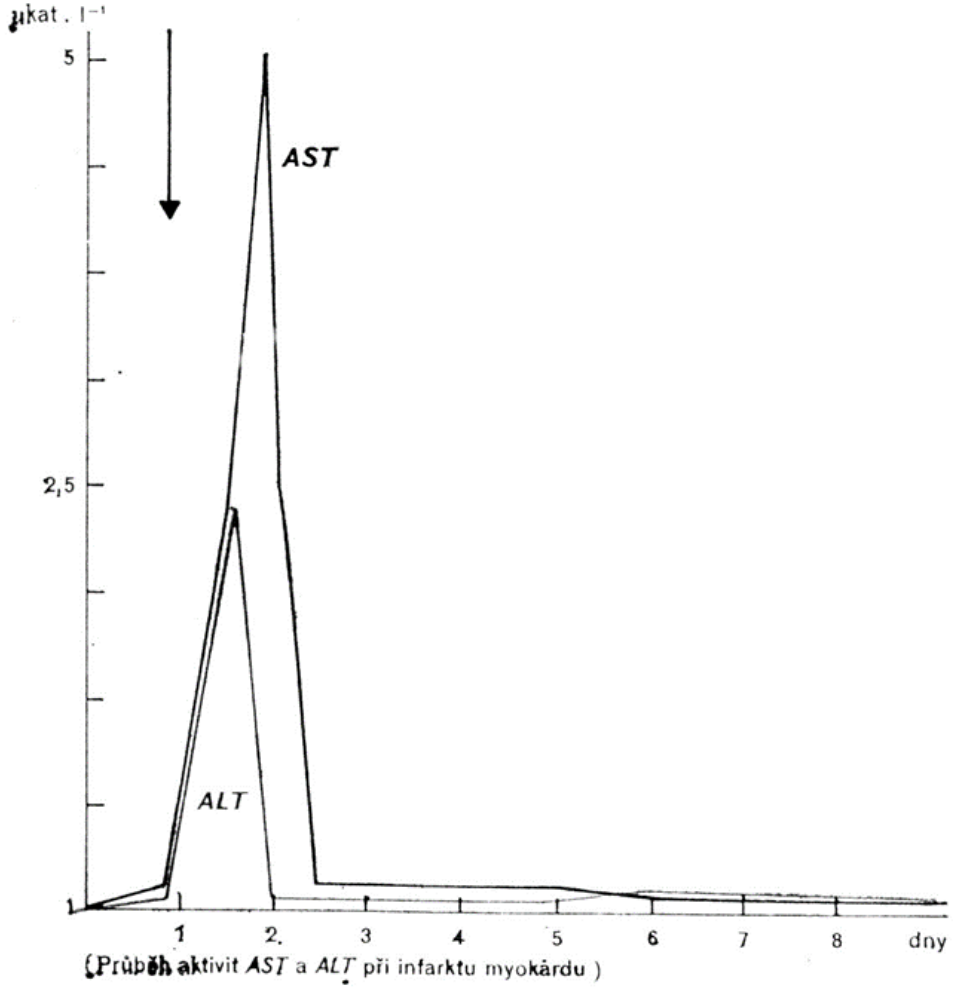
Example



(Průběh aktivit *AST* a *ALT* při virové hepatitidě)



(Průběh aktivit *AST* a *ALT* při infarktu myokardu)

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Different forms of enzymes

- proenzyme (zymogen)
  - inactive enzyme precursor
  - requires a biochemical change
  - angiotensinogen, pepsinogen
- isoenzymes
  - multiple forms of an enzyme that catalyze the enzyme‘s characteristic reaction but that differ in structure
  - primary
    - more than one gene locus coding for the structure
  - secondary (=isoforms)
    - modification of polypeptide chains
- examples
  - glucokinase, LD, CK, PKC, cytochrome P450

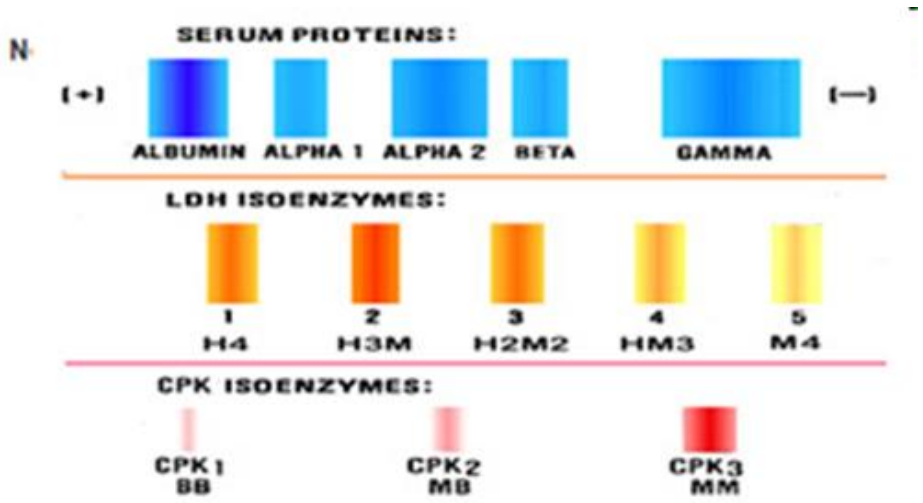

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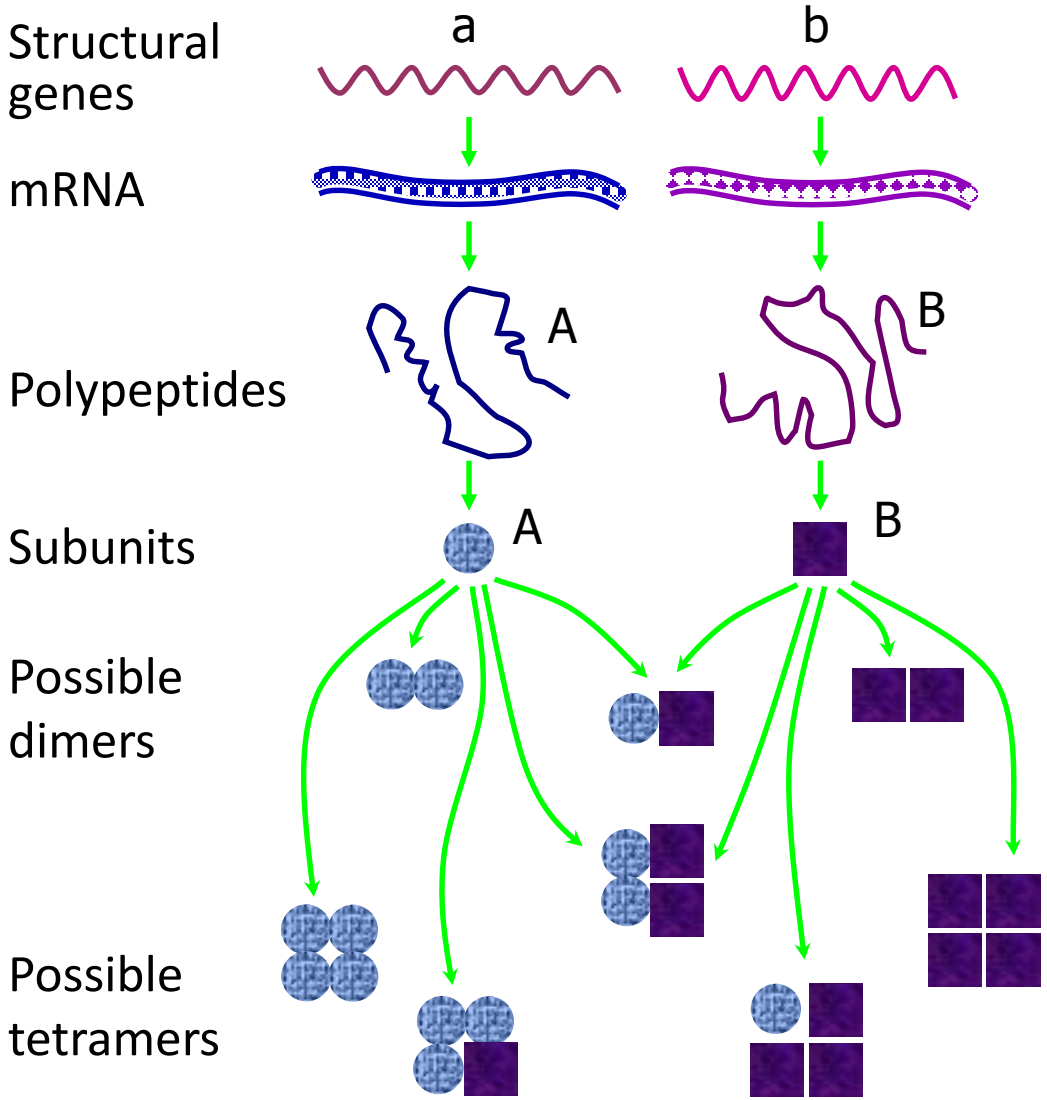
Diagram of the origin of isoenzymes



Distribution of isoenzymes

- distribution of isoenzymes is not uniform
  - variation in the activity at the organ, cellular and subcellular levels
- the basis for organ-specific diagnosis
  - through isoenzyme measurement
- certain loci may be expressed exclusively in a single tissue
  - lactate dehydrogenase – 2 loci
  - third locus active only in mature testes
    - third type of subunit X or C – isoenzyme LD-X or LD-C
- adaptation of metabolic patterns to the changing needs of different organs and tissues
  - pathological conditions may be associated with alterations in the activity of specific isoenzymes

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Changes in isoenzyme distribution during development and disease

- several sets of isoenzymes change during normal development
  - changes result from changes in the relative activities of gene loci
    - skeletal muscle – LD, CK
    - liver
      - 3 aldolase isoenzymes A, B and C are present during embryogenesis
      - isoenzyme B is predominant in the adults
  - changes in the number of cells containing respective isoenzyme
    - increased number and activity of osteoblast
      - elevation of total serum ALP in young people
  - malignant tumors
    - LD – shift in the balance of isoenzymes

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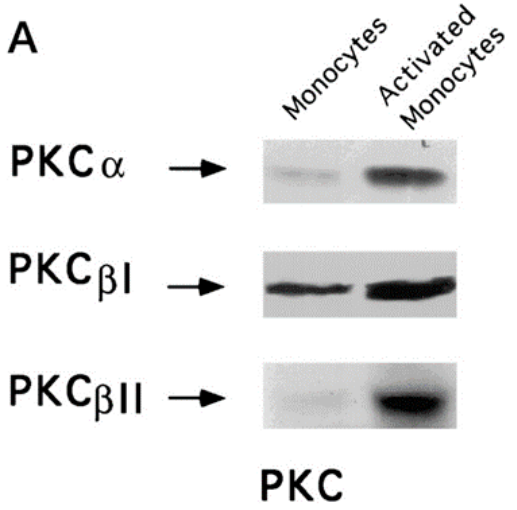
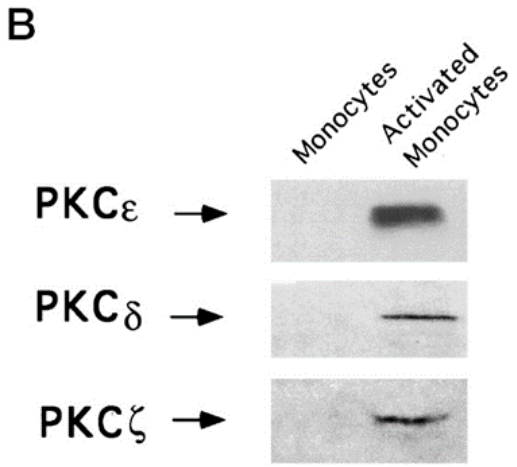
Detection of isoenzymes

- isoenzymes can be distinguished
  - difference in various physical properties
    - electrophoretic mobility, resistance to chemical or thermal inactivation
- physical-chemistry
  - electrophoresis
  - chromatography
- immunohistochemistry
- chemical
  - determination of reaction rate in different settings (pH, t, substrate concentration)





Li Q et al. J. Biol. Chem. 1999;274:3764-3771

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Diagnostic enzymology

- changes in the activity in the serum of enzymes that are predominantly intracellular and that are normally present in the serum at low activities
  - changes in activities of these enzymes in disease – location and nature of pathological changes in the tissues
- the measured levels of an enzyme in blood – result of the balance between
  - the rate at which it is entering the circulation from the cells of origin
  - the rate at which it is inactivated or removed
- existence of multiple forms of enzymes
  - increase in diagnostic specificity and sensitivity

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Leakage of enzymes from cells

- plasma membrane retains enzymes within the cell
  - its integrity depends on the availability of ATP
  - any process impairing ATP production promotes deterioration of the cell membrane
- very high concentration of enzymes within cells
  - ICF/ECF ratio
  - small amount of enzyme can be detected
  - an increase of enzyme activity in plasma is sensitive indicator of cellular damage

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Causes of cell damage or death

Category	Examples
Hypoxia	Loss of blood supply
Chemicals and drugs	Environmental pollutants, drugs, alcohol
Physical agents	Trauma, radiation, electrical energy
Microbiological agents	Bacteria, viruses, fungi
Immune mechanisms	Anaphylaxis, cytotoxicity

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Clearance of enzymes

- urinary excretion
  - few enzyme molecules are small enough to pass through the healthy glomerulus (α-amylase)
- receptor-mediated endocytosis
  - many enzymes are removed by the reticuloendothelial system (spleen, liver, bone marrow)
  - in lesser extent by all cells in the body
  - Kupffer‘s cell
    - LD5, CK-MM, AST
- half-life of enzymes
  - few hours to several days
  - average 6 – 48 hours

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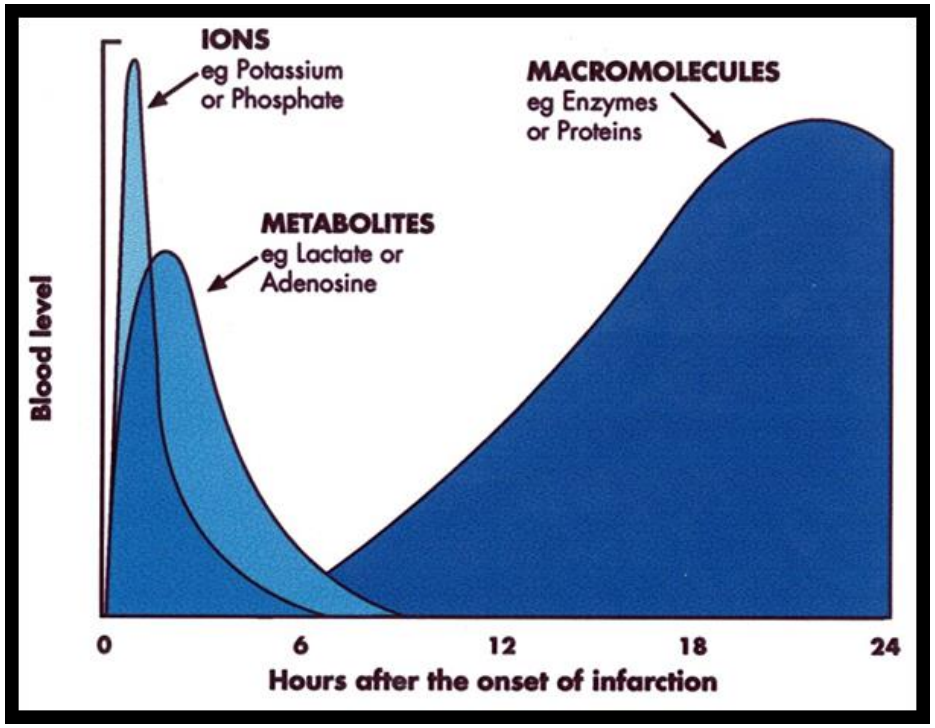
Cardiac markers

- the ideal cardiac marker
  - high sensitivity
    - high concentration in myocardium
      - rapid release for early diagnosis
      - long half-life in blood for late diagnosis
  - high specificity
    - absent in non-myocardial tissue
  - analytical characteristics
    - measurable by cost-effective and simple method
  - clinical characteristics
    - ability to influence therapy and to improve patients outcome
- **the ideal cardiac marker does not yet exist**



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Cardiac markers

- **Creatine kinase (CK)**
- cytoplasmic and mitochondrial enzyme
- catalyzes reversible transfer of phosphate from ATP onto creatine
- ATP + creatine → ADP + creatine phosphate
- dimeric – M (muscle) and B (brain)
- 3 isoform
  - **CK-BB – smooth muscle, brain, prostate**
  - **CK-MB – myocardium (also in skeletal muscle)**
  - **CK-MM – skeletal muscle, myocardium**
- CK-MB – diagnosis of acute myocardial infarction and monitoring of reperfusion in the course of trombolytic treatment of AMI

- **Myoglobin**
- intracellular protein found in cardiac and skeletal muscle cells concerned in aerobic metabolism
- released quickly from damaged cells into circulation (small size, 0,5 – 2 hours)
- the smallest cardiac marker – quick propagation and degradation
- non-specific marker (present also in skeletal muscle)

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Cardiac markers

- **troponins**
- troponin complex – part of the structural proteins, which participates on muscle contraction
  - heterotrimer consisting of troponins I, T and C
- tightly connected with contractile apparatus – low levels of cardiac troponins in the circulation
  - TnI level is undetectable if the heart is not injured (even in the presence of skeletal muscle damage)
- cardiac isoform troponin I (TnI) differs from skeletal muscle isoform - specific determination

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Troponin I

- benefits
  - absolute cardiospecifity
  - long period of liberation – monitoring of course
  - sensitivity – detection of smaller injury
  - not affected by chronic renal insufficiency
- limitation
  - slower onset than myoglobin (nonspecific)

	Myoglobin	TnI	CK-MB
increased after	0,5 - 2 h	3 - 6 h	3 – 8h
peaks between	5 - 12 h	14 - 20 h	9-30 h
remains elevated	18 – 30 h	5 - 7 days	48-72 h

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Cardiac markers - comparison

enzyme	beginning of rise	maximum	normalization	fold in maximum
AST	4-8 hours	16-48	3-6 days	up to 25
CK	3-6 h	16-36	3-5 days	up to 25
LD	6-12 h	24-60	7-15 days	up to 8
myoglobin	0,5-2 h	6-12	0,5-1 days	up to 20
troponin I	3,5-10 h	12-18	7-20 days	Up to 300

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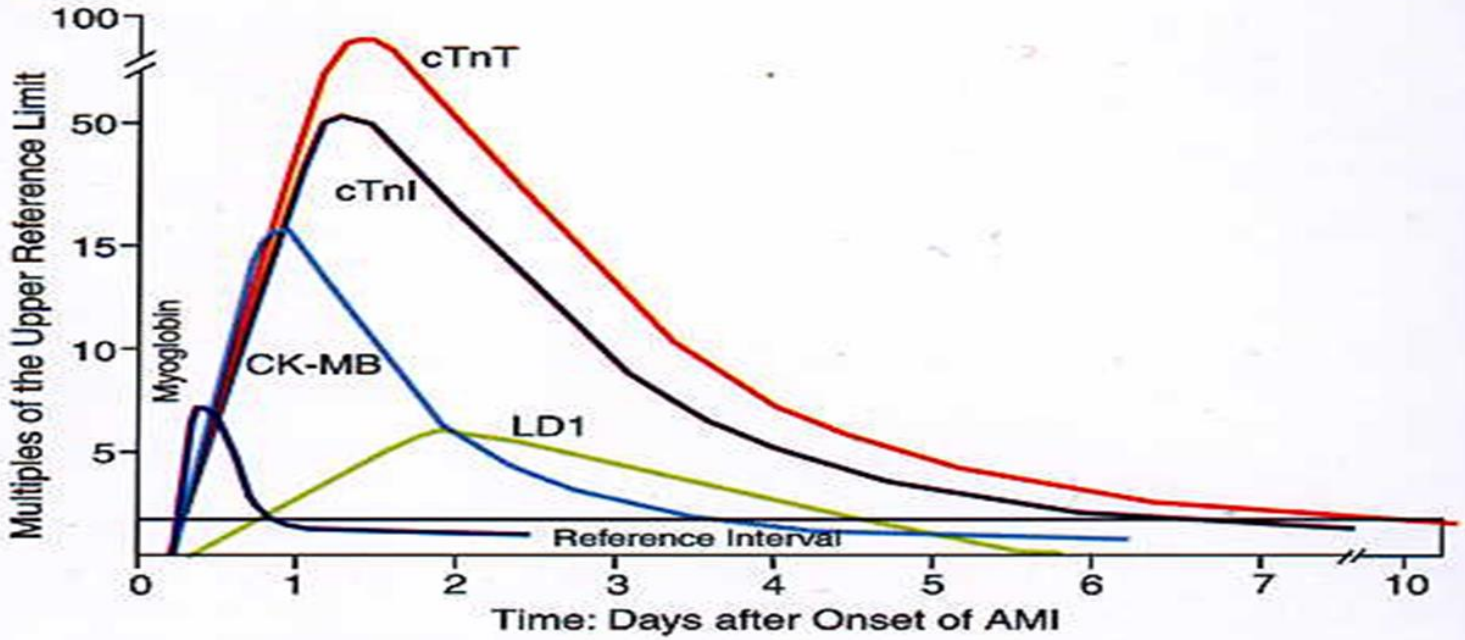

Figure 1. Release of cardiac biomarkers into blood following AMI. Time zero is defined as the moment of onset of symptoms. Marker concentrations are expressed in a common scale—as multiples of the upper reference limit for that marker.[11]

cTnT = cardiac troponin T

cTnI = cardiac troponin I

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Biochemical markers of liver function

- indicators of hepatocyte damage
  - ALT, AST, LDH
- indicator of bile ducts obstruction
  - ALP, GMT
- indicators of synthetic liver function
  - albumin, CHE, LCAT, PT
- tests of conjugation and liver transport of organic anionts
  - bilirubin, urobilinogen

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Markers of hepatocyte damage

- **Alanin aminotransferase (ALT)**
- L-alanin+2-oxoglutarate ↔ pyruvate+L-glutamate
  - reaction is reversible, it proceeds in the syntesis, degradation and transformation of aminoacids
- cytoplasmatic enzyme
- the most abundant in hepatocytes, plasmatic level elevated as early as in the disorder of membrane permeability

- **Aspartate aminotransferase (AST)**
- L-aspartate+2-oxoglutarate ↔ oxalacetate+L-glutamate
  - reaction is reversible, it proceeds in the syntesis, degradation and transformation of aminoacids
- cytoplasmic and mitochondrial isoenzymes
- occurs in liver, myocard, skeletal muscle, kidney and pancreas
- plasmatic level of cytoplasmic isoenzyme elevated as early as in the disorder of membrane permeability, releasing of mitochondrial isoenzyme accompanies hepatocellular necrosis

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Interpretation of ALT/AST elevation

- increased activity of both ALT and AST in many liver diseases
  - extremely high values (10-100x) in toxic and acute viral hepatitis and shock conditions
- plasmatic aminotransferase activity does not tell us anything about excretoric or metabolic function of hepatocytes
- correlation between level of amino transferases and the extent of liver lesions is not the rule
- De Rittis index = AST/ALT
  - less than 0,7…good prognosis
  - 1 and more…bad prognosis (necrosis)
- physiologically and in majority of liver diseases ALT > AST
- exception - AST/ALT >2
  - alcoholic damage
  - postnecrotic cirrhosis

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Markers of bile ducts obstruction

- **Alcaline phosphatese (ALP)**
- membrane bound enzyme catalyzes hydrolysis of phosphate esters at alkalic pH
  - tetramer, into the circulation released as dimer
- widespread - occurs primarily in liver, gut and bones (different isoenzymes)
- plasmatic ALP level – diagnosis of bone and hepatobiliar disorders
- considerable part of liver ALP is localized membranes of cells covering bile ducts
  - membranes are disturbed in cholestasis and ALP is released
- elevated also in other conditions (liver tumors, cirrhosis)

- **γ-glutamyl transferase (GMT)**
- membrane bound enzyme found in liver, kidney, pancreas, gut and prostate
- catalyzes transfer of γ -glutamyl from glutathione on aminoacid and enables the aminoacid transport through membrane
- serum GMT activity determination is used for evaluation of hepatobiliar diseases

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Markers of synthetic liver function

- **albumin**
- synthesized in liver, plasmatic level determination
- long half-life – does not fall in acute disorders
- exclusion of another causes of decline (malabsorption, reduced intake of proteins, kidney disease) → liver disease
- significant decline in alcoholic cirrhosis

- **cholinesterase**
- enzyme generated in hepatocytes and released into blood (secretory enzyme)
- catalyzes hydrolysis of cholin esters in plasma
- enzyme production (thereby plasmatic activity) is decreased when liver parenchyme is damaged or in malnutrition
- irreversibly inhibited by organophosphates

Synthetic liver function

- **coagulation factors**
- produced in liver, short half-life – quick changes
- Quick test – extrinsic coagulation system
- values are changed in disorders of liver parenchyma accompanied by proteosynthesis failure or in obstructive icterus with disorder of lipid and lipid soluble vitamins uptake

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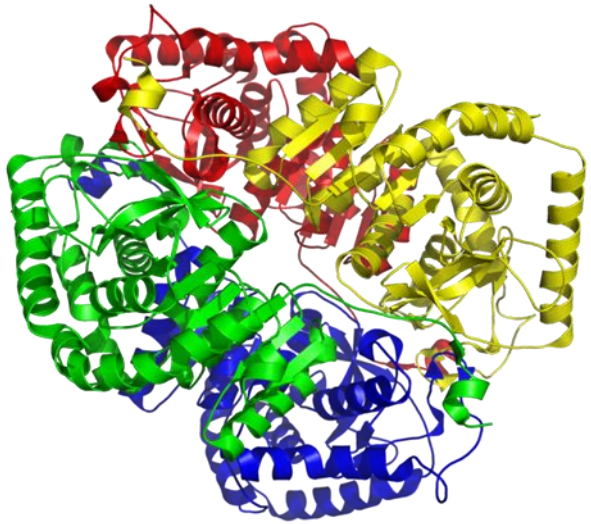
Lactate dehydrogenase (LDH)

- tetramer
  - M (gene LDHA, ch.11)
  - H (gene LDHB, ch.12)
- LDH_1 (HHHH) **31-49%**
  - heart, liver, erythrocytes
- LDH_2 (HHHM) **38-58%**
  - reticuloendothelial system
- LDH_3 (HHMM) **5.5-16.5%**
  - lungs
- LDH_4 (HMMM) **0-0.7%**
  - kidney
- LDH_5 (MMMM) **0-1.5%**
  - skeletal muscle, liver



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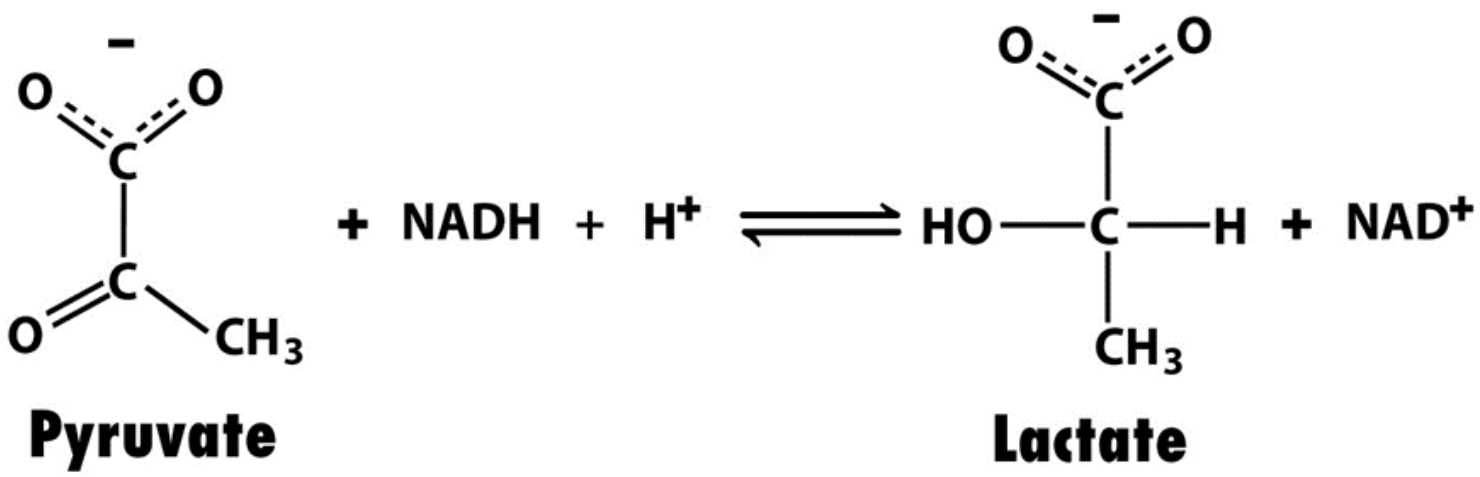
Lactate dehydrogenase

- LDH 1 and LDH 2
  - converts lactate into pyruvate in tissues with aerobic metabolism
- LDH 4 and LDH 5
  - converts pyruvate into lactate in tissues with anaerobic glycolysis



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Biochemistry, Sixth Edition

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Changes in plasma LDH levels

- myocardial injury
  - elevated LDH1 and LDH2
  - ratio LDH1/LDH1>1 (in healthy <1)
  - myocardial infarction (peak 3-4 after MI)
- liver injury
  - elevated LDH4 and LDH5
  - hepatitis, cirrhosis, organic solvent intoxication
- hemolysis
  - elevated LDH2
  - hemolytic anemia, incompatible blood transfusion

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Electrophoretic separation of LDH isoenzymes

- agarose gel, TBE buffer
- staining solution
  - lithium lactate
  - NAD^+
  - stain nitroblue tetrazolium
  - phenazine methosulphate – carrier of electrons between NADH and the dye
- 5 % acetic acid

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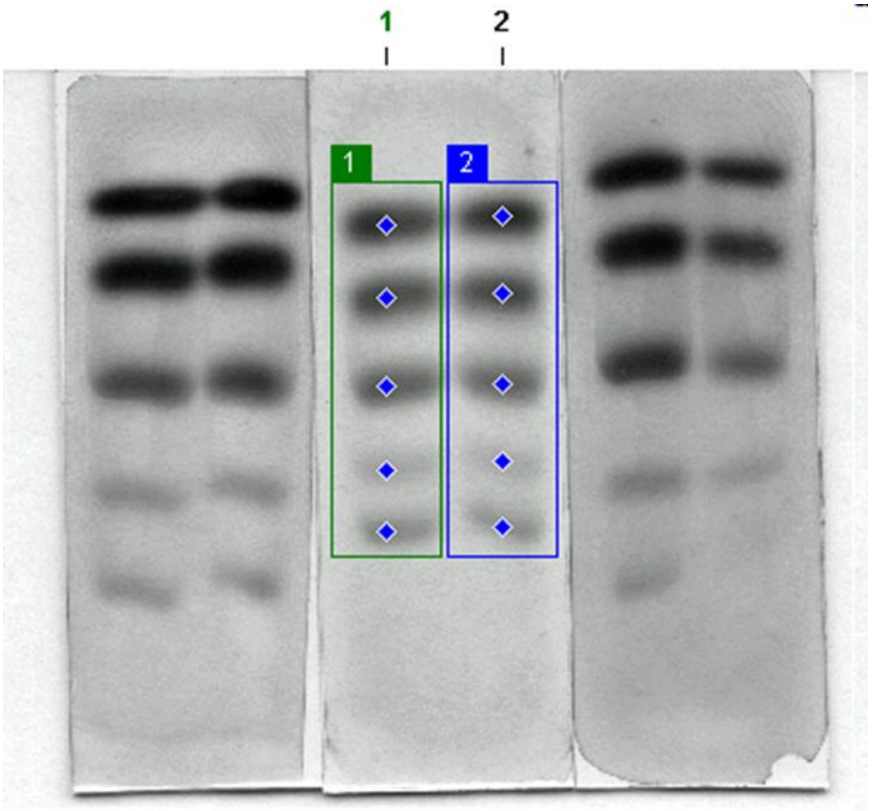
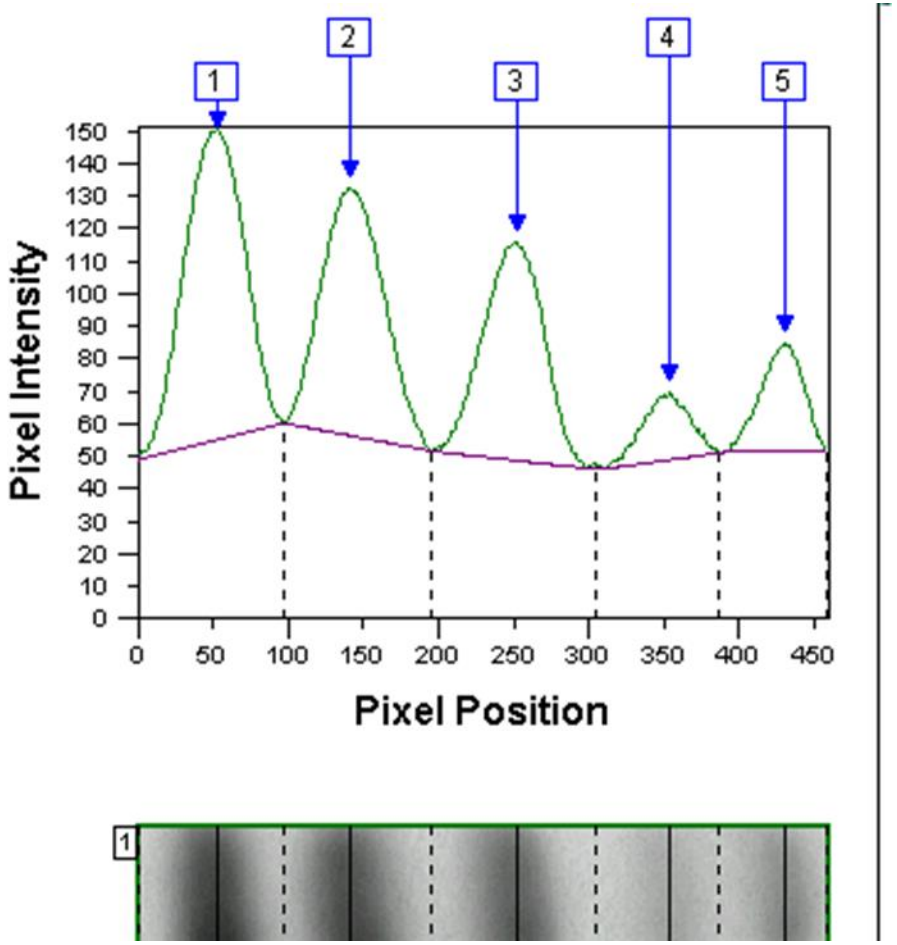
Isoenzymes detection

- lactate + NAD^+ → pyruvate + NADH + H^+
- NADH + H^+ + NBT → NAD^+ + formazan





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