bioanalytics II

analytical methods in clinical praxis

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recommended reading

Tietz fundamentals of clinical chemistry
C.A. Burtis, E.R. Ashwood, D.E. Bruns, Elsevier, 2008

lecture syllabus

laboratory medicine
: samples – character, preparation
: instrumentation, integration and miniaturisation
: quality check and control
: choice of analytical method
: optimisation approach
: analytical set
: analytical result expression

basic methods and principles
: colourness and its analytical use, indicator reactions
: protein determination and enzymatic analysis
: immunoanalysis
: analysis of nucleic acid
: medical microbiology

determination of chosen analytes
: case study: determination of ALP
: albumin, barbiturates, sodium, ethanol...

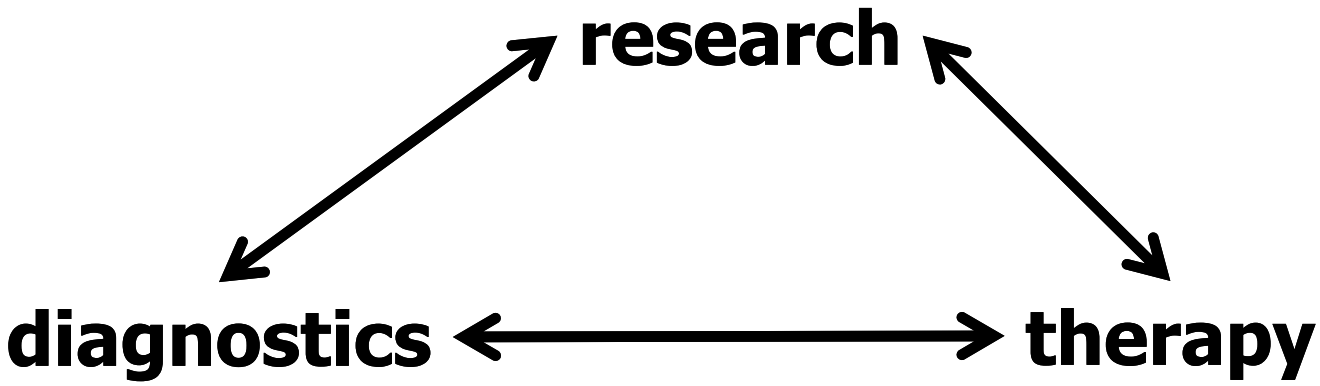
laboratory medicine

: analysis of body fluid component with diagnostical importance

: determination of analytes and metabolites or their groups,
including drug level monitoring

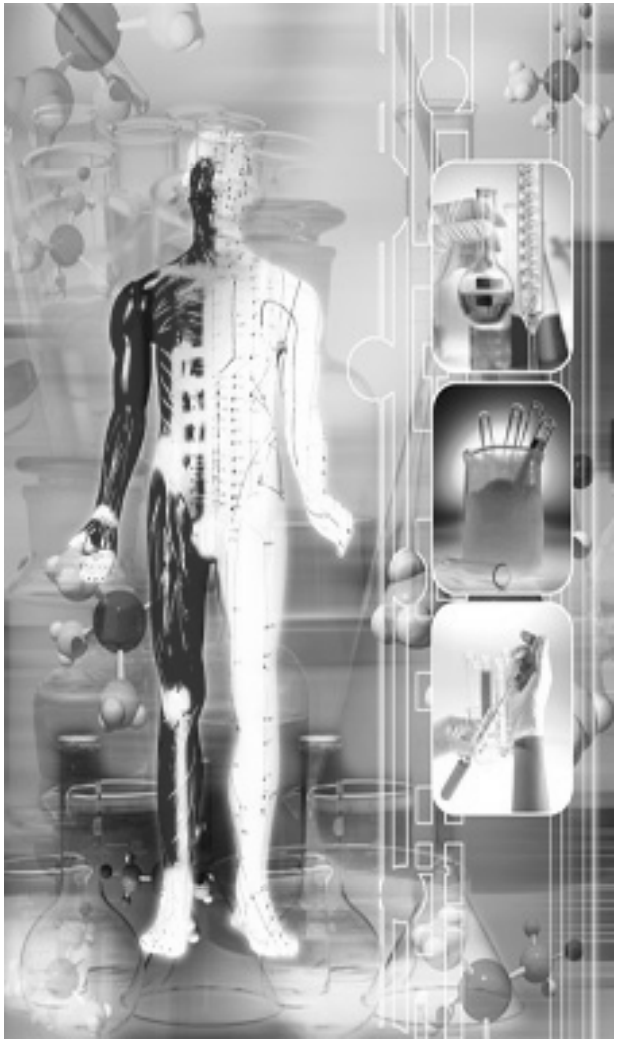
: shelters branches dealing with laboratory diagnostics:
clinical chemistry and biochemistry, haematology, medical microbiology,
immunology

classically traded as separate clinical disciplines



IFCC definition
(*international federation of clinical chemistry*)

„Clinical chemistry is an application of chemical, molecular and cellular principles and technologies with an aim to understand human health and illness, and to allow their classification. Presentation of analyses results in regard to the illness cause and health care is the core of the branch. On the interface of laboratory and clinic, transformation of such data follows into specific and general information related to patient and illness. Deepening of knowledge about health and illness through basic and applied research is the task of the branch."



laboratory medicine : in EU 5 – 10 analyses *per capita per annum*

: the world biggest producer of analytical data
: touches almost all analytical branches

laboratories
: hospital
: private
: consolidate laboratories
: diagnostic centres

huge range of analytical activity
: incomparable to any other science or industry branch

sample
in laboratory medicine

biological

: invasive (blood, cerebrospinal liquid, tissues, gastro-duodenal juice)

: non-invasive (saliva, urine, faeces, sputum, breath)

blood

blood = suspension of cellular particles in liquid (plasma)

cellular particles : blood-cells (erythrocytes and leukocytes)

: blood-platelet (thrombocytes)

blood coagulation (off blood stream) – change of sol. fibrinogen $\Rightarrow$ insol. fibrin

blood *coagulation* > solid blood pie + blood serum

blood serum – similar to plasma, without coagulation agents

plasma and serum

mixture of inorganic and organic compounds in water

native plasma – separated from blood-cells of blood in container with non-polar surface (plastic) without anticoagulants
: as close as possible to what circulates in blood stream

plasma – from blood in container with anticoagulants

serum – separated for coagulated blood in container with polar surface without anticoagulants

: preparation of plasma is *faster*
: 20 % more is gained of plasma than serum
: plasma lowers the risk of unwanted haemolysis (in serum up to 10x higher)
: in serum after centrifugation unwanted secondary coagulation
: serum is an artefact
: no protein ELFO in plasma $\Rightarrow$ fibrinogen co-elutes with the γ-globulines
: anticoagulants introduce ions into plasma, which could not be then analysed
: lots of analytes mask and inhibit some plasma enzymes

urine

light yellow liquid produces by kidneys and excreted through urethra and urocyst

contains: urea, chlorides, ions of sodium, potassium, phosphates, sulphates, creatinine and uric acid

cerebrospinal liquid
(liquor)

clear, sparse liquid circulating between cerebral ventricle, central spinal channel and permeation gate between brain, spinal chord and their meninges

contains: electrolytes and similar organic compounds (~ blood plasma, but different concentrations)

e.g. glucose, proteins, lactate, pyruvate, cholesterol, enzymes, salts and certain amount of lymphocytes

what influences concentration of analytes in biological samples

a) given

influences

b) variable

influences given

race and sex

e.g. different reference values of analytes: creatine kinases, α-amylases, granulocytes

: higher for men than for women

:: women have mostly lower and narrower reference intervals

: increasing in order: Caucasian, Asiatic, African

age

e.g. different reference values of analytes for newborns, children, adolescents, adults and senior

biorhythms – chronobiological influences

linear : changes according to age

cyclic : daily (circadian)
: monthly (lunar)
: seasonal (seasons of year)

biorhythms **cause changes** in *analyte concentrations*

cyclic b. – varying concentrations: day-to-day, during the day

important **change of biorhythms** during *pregnancy*

variable influences

diet

: alimentation, starvation/abrosia ($\Rightarrow$ malnutrition)

Δ concentration of fats, saccharides, proteins $\Rightarrow$ Δ levels of serum ammonium and urea

physical strain

short-term and intense strain
: consumption of ATP, ↓ level of glucose and lactate

long-term strain
: ↑ conc. of ions: sodium, potassium, calcium, phosphorus, ALP, albumin, urea, bilirubin, AST, pyruvate kinase, CK

measure of change is *individual* and *depends on circumstances*

altitude influence (*stress of organism*)
: ↑ concentration of C-reactive protein, β_2-globulin, uric acid, haemoglobin and haematocrit

adaptation on **high altitudes** is *slow* and *takes weeks*

adaptation back to **low altitudes** is *fast*, takes *few days*

common drugs

coffein
: ↑ glucose conc., unesterified fatty acids and catecholamines

nicotine (cigarette)
: acute and chronic change

e.g. ↑ conc. of serum fatty acids, glucose, fibrinogen, cholesterol, free glycerol, aldosteron and cortisol, some hormones and tumour markers and heavy metals (Cd, Cu, Pb)

alcohol
: intensity and length of consumption makes influence

steady influences: ↓ conc. of serum glucose, metabolic acidose
(ethanol⇒acetaldehyde⇒acetate)

long-term influences: ↑ act. of enzymes GMT, GLD, AST and ALT (intoxication of livers)

chronic alcoholism: ↑ conc. of triacylglycerols, cholesterol and some hormones

other common influences

biological fluids – complex of inorg. and org. molecules, protein pseudo-solutions and fat-droplet emulsion

compounds could be bound to proteins ⇒ their content in organism is dynamically changing; changes are related to their individual stability or decomposition processes (metabolism and bacteria)

biological samples – potentially infective material ⇒ safety rules

number of analytes in sample – hundreds + their derivatives with variable content and changeable photo- and thermostability = **biological matrix**

influence of collection and transport – so-called *preanalytical phase*

necessity of **fast transport** and **storage in cold** or **conservation**

influence of medication

patients samples – **drug interferences**

results are *biased* or *disable completely* conduct of analyses

information on **interferences** – estimated **empirically**
: influences of **individual drugs** (common)
: influences of **drug combinations** (almost unknown)

: blood collected on an empty stomach after drug drop for 24 – 72 h
: necessity of knowledge of drugs used by patient

analytical drug interference – observed by IFCC, known analytical interferences in databank

standard (normalised) operational approach **(SOA)** – analysis of composite sera of donors or patients enriched by known amount of respective drug
: analysis result is statistically evaluated

preanalytical phase

collection, transport and storage of sample

processes and operations with analysed material sample untill analysis

biological material collection and transport

analytes have **limited time-stability**
: are metabolised, thermolabile or photolabile

stability – storage period, when under defined conditions the initial analyte content is not changed; concentration or activity

it is expressed as time, during which initial content of analyte is not changed more than 1.5-time more than reference interval with 95% probability

collection rules

influence of patient state

posture – conc. of high molecular compounds are lower when collected in recline and higher in about 15 % when standing

physical strain – Δ concentration of compounds involved in energetic metabolism, it comes to thickening of macromolecular compounds, the activity of AST and CK enzymes is increased, increases level of creatinine, decreases level of thyroxin

blood

: collection in a *sit-down* and at least after *30 min of rest*
: *contraction of arm* by elastic bandage and after *disinfection* of puncture location
: bandage must be *quickly released* – *freely flowing blood* is collected
: before puncture, patient with bandage *should not exercise too long*

slow release of bandage and too intense arm exercise ⇒
important influence on levels of some serum analytes:
↑ conc. Na^+ and Ca(II), haemoglobin, cholesterol, ALP, proteins, bilirubin and some enzymes; ↓ concentration of glucose, creatinine, phosphate...

venous blood

collection morning on empty stomach
last light food at ca 6:00 pm, and then next day morning only a small amount water or tea without sugar

drop medication at least for 24 – 72 hours

: most of analyses (except for haematology) is done using **serum**

: after blood collection and before separation of serum off blood-clot it is necessary to wait at least 30 minutes, what is a period needed for coagulation process

: using coagulations accelerators, only 10 minutes are enough

capillary blood

puncture by lancet into finger, ear lobe or heel (children)
: considerate

: dropping or drain (by micropipette or capillary) 1 – 3 drops of blood

: immediate analysis (determination of glucose on diagnostic strip)

: transport in plastic microtube with anticoagulant and anti-glycolytic agent

anticoagulantia / anticoagulants

compounds able to complexate ions of endogenous calcium in sample and thus prevent process of blood *coagulation*

: sodium or potassium salts of citric or oxalic acid, or EDTA

: heparin, accelerator antithrombin III (inhibitor of blood coagulation)

: hirudin (anticoagulans of leech *Hirudo medicinalis L.*)

in haematologic analyses and tests, it is on the other side important to re-calcify uncoagulable blood and thus re-new coagulation abilities by adding of surplus of calcium salt to saturate anticoagulants

collection containers

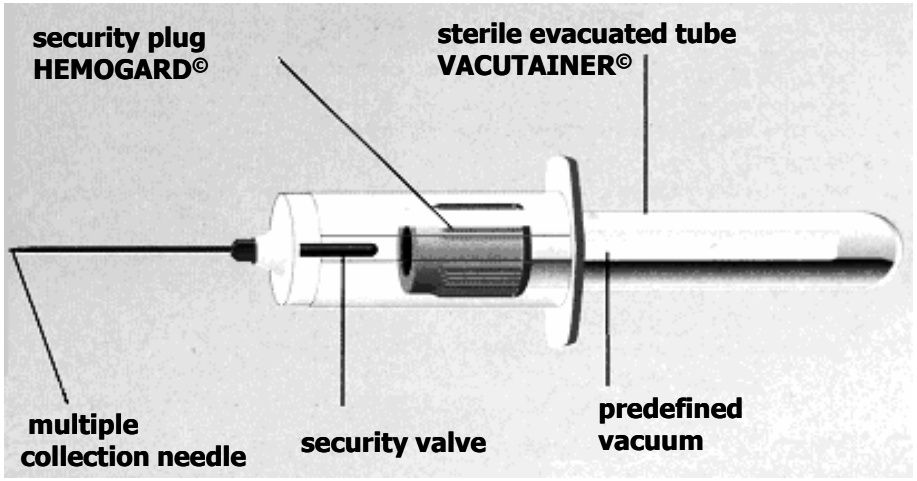
open system – open tube

closed system – evacuated container or test-tube with rubber plug, or special injection syringe serving for sample collection and in parallel also as centrifugation tube

contains – anticoagulantia or compounds speeding-up blood coagulation (gelatine, aprotinin, polystyrene spheres *etc.*)

glucose determination – special doses with agents suppressing glycolysis in combination with anticoagulants

anticoagulants are mostly *salts* and introduce into biological samples certain ions, which ions could not be determined in samples handled this way



special **separation gel** – separates serum or plasma of blood coagulum or blood-cells after centrifugation; there is no need for fast separation of serum/plasma from rest of the blood

containers for blood-samples collection are resolved by colour for easier manipulation; according to respective ISO norm

red – clean; golden – gel for centrifugation
grey – glucose (NaF, K_2(ox)), green – heparin
violet – EDTA, blue – citrate…

disposables – class of tools; closed containers and other plastic one-off tools for collection, transport, centrifugation, dosing and storing of other body fluids in sterile design are in-between them

others: automatic pipettor extension, containers and test-tubes for urine samples, plastic ELISA plates, plates for determination of blood groups *etc.*

haemolysis

degradation of erythrocytes ⇒ changes quality of collected blood
: proceeding of collected blood to serum or plasma optimally *till 30 minutes*, *at latest till 1 hour* after collection

manifestation – ↑ conc. of potassium and chlorides, ↑ activity of enzymes ALT, ↓ glucose

appearance – normal serum and plasma – yellowish and transparent
: haemolysis ⇒ *red* (release of haemoglobin)
: *milky cloud* – emulgated fat droplets – **chylous (lipaemic) serum**

haemolytic and chylous serum or plasma are *for most analyses impropriate*

glycolysis

content of glucose in blood **rapidly decreases** after collection – *blood-cells still live*

serum/plasma must be stored at +4 °C

effective, but *complicates transport*

temperature 15 – 25 °C
⇒ content of glucose goes down in a day to **~30 %**, in 2 days to **6 %**

temperature +4 °C
⇒ content of glucose goes down in a day to **~80 %**, in 2 days to **32 %**

biochemical nature of glycolysis – *catabolism of glucose*

: aerobic glycolysis – **CO_2** and **water** are final products
: anaerobic glycolysis – **lactate** is final product
: pentose cycle – direct oxidation of glucose; hexose ⇒ pentose

suppression of glycolysis

: suppression of function of important catabolic enzymes

glyceraldehyde-3-phosphate dehydrogenase –
dehydrogenation of glyceraldehyde-3-phosphate to 1,3-bis(phospho)glycerate

: **inhibited** by *monoiodacetic acid* (*ca* 0.5 mg per ml of blood sample)

enolase – transformation of 2-phosphoglycerate to phosphoenolpyruvate
metalloprotein – Mg(II) in active centre

: **inhibited** by *fluorides in combination with endogenous phosphates* (*ca* 2 mg/ml of KF or NaF; blood could be stored up to 24 h at room temperature)

hexokinase – phosphorylation of glucose

: **inhibited** by *mannose in surplus* (competitive substrate, 15 mmol mannose, stability of blood up to 12 h at room temperature)
contraindication: no use of hexokinase method for glucose determination

: **inhibited** by *fluorides in combination of anticoagulantium* (EDTA; per 1 ml of blood 1.6 mg Na_2EDTA and 2 mg KF)
contraindication: EDTA, KF interferes or inhibits determination of some analytes (e.g. metalloproteins)

urine

one-shot collection (morning urine) or *collected urine* (12 or 24 hours)
: *catheterisation*

hygiene of collection – bacterial **contamination**

one-shot collection

: basic analysis and urine sedimentation
: analysis at latest till 2 hours after collection

collected urine

: determination of some ions, urea, glucose, microalbuminuria, creatinine and creatinine clearance

: determination of some hormones (17-ketosteroides, 5-hydroxyindolacetic a. and vanillyl mandelic a.) – conservation by diluted HCl

: quantitative analysis of 24 h urine – relation of analyte content to its daily excretion in urine; volume elimination

conservation agents

: 5 ml 10 % thymole in 2-propanol per l of urine – for most of analyses

: sodium azide, 10 mmol/l urine – glucose, urea, uric acid, Na, Ca, oxalates, citrates

: 25 ml HCl solution 6 mol/l for dU – 5-hydroxyindolacetic a., Ca, Mg, P, catecholamines

: sodium carbonate, 2g/l urea – porphyrines, urobilinogen

: benzoic acid, 10 mg na dU – glucose

: adjustment of urine pH over 8 – uric acid

cerebrospinal liquor

lumbal punction

determination of proteins, glucose, chlorides *etc.*

except for neurological investigations, routine analytical methods are used

duodenal juice

probe of duodenum

analyses of digestion enzymes (trypsin), acids, bile acids and colorants

fast changing sample, must be therefore specially collected into containers cooled with ice and immediately analysed

other biological samples

faeces, sputum, fester, saliva, sweat, sperm, mucous membrane smear and samples of organs and tissues

sputum, sweat – not subject of routine analyses in clinical laboratories, usually in microbiology, histochemistry *etc.*

faeces – i.e. occult bleeding (blood in faeces)

saliva – analysis of drugs of abuse (alcohol *etc.*) and some steroids

sample preparation

: whole blood centrifugation, deproteination, mineralisation, preconcentration

centrifugation

separation of **sediment** off *supernatant*

<u>conditions</u> – relative centrifugation force (RCF), time and temperature

RCF – how many times is the centrifugation acceleration higher at bottom then gravitational acceleration (g)

intense centrifugation leads to *unwanted haemolysis* of blood

deproteination

deproteination – necessary for determination of some analytes

analyte
: *in supernatant* – some ions or substrates

: *in sediment* – organic phosphor, total protein in strong lipaemic sera, protein nitrogen by Kjeldahl method *etc.*

deproteination techniques
physical – centrifugation, ultracentrifugation, adsorption and denaturation by heat

time consuming, designed for special cases

chemical – immunoprecipitation, dehydration or salting-out

fast

dehydration

often used
fractionation of proteins or for special analytical cases

strongly depends on pH – proteins have as positive as negative charges
: in acidic media – cations, in alkali media – anions
: isoelectric point (pI) – specific pH at which protein is electroneutral
:: at isoelectric point proteins are labile and do easily precipitate

dehydration: **using organic solvents** or **salting-out**

competition of *protein* with *precipitant* for **water**
$\Rightarrow$ takes some water off protein $\Rightarrow$ protein is precipitated

: methanol, ethanol, acetone

: done at pI

dehydration of proteins is mostly reversible process

salting-out

usual
precipitation of proteins in form of insoluble salts

precipitants:
: anionic (trichloroacetate, perchlorate, picrate, thungstate, molybdenate, sulphosalicylate, metaphosphate)

: cationic (zinc, mercury, cadmium, uranium, thorium, iron, copper and lead)

mineralisation

special cases only

mineralisation in a dry way

for determination of C, H and N by elemental analysis or determination of metal in biological/organic matrix by means of atomic absorption spectrophotometry

mineralisation in a wet way

for determination of organic phosphorus, determination of total protein nitrogen by means Kjeldahl method

kjeldahlisation in clinical chemistry – mixture contains conc. sulphuric acid with different salts, e.g. K_2SO_4 and $CuSO_4$, $HgSO_4$ and SeO_2

preconcentration

preconcentration of trace, otherwise not determinable, amounts of analytes (proteins)

methods
dialysis, ultrafiltration or separation on column

: not carried out in common clinical laboratories

instrumentation
analysers; organisation, integration and analysis miniaturisation

history

important change in last 40 years

manual plant ⇒ **automatic analysers**

collection – blood is using injection needle put into open glass test-tube
: it is either un-closed, or in better case closed with cork or rubber plug

in laboratory – grumous blood mixed by glass stick, centrifuged and serum over precipitate is drained by Pasteur pipette, carry-over to other tube for analysis

: samples and prepared agents are gauged into reaction tubes, and let proceed respective chemical reaction, recast reaction mixture into cuvette of photometer, measure absorbance and carry-on with analyte content calculation

: determination of one analyte – 50 to 500 µl of serum and other 1.5 – 2 ml of agents

comparison: around r. 1930 was volume of reaction mixture for determination of alkalic phosphatase through inorganic phosphate several millilitres and incubation lasted *ca* 48 hours

analysers – liquid agents

flow-through (50's) ⇒ **centrifugal** ⇒ **tracked** (70's) ⇒ **revolver**

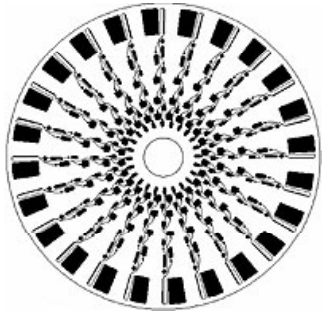
: multi-channel analysis – parallel analyses
: multi-component analysis – series of analyses in one sample

flow-through a.

glass capillaries, samples separated by air bubbles
: multi-channel analysis

high *accuracy* and *consistency* of analyses

centrifugal a.

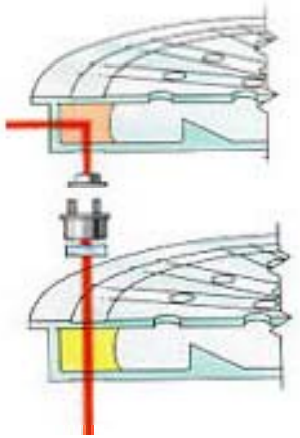


rotor with pits arranged radially from centre

two reaction spots **separated** by elevated **partition**

outer pit – photometric cuvette, transparent window perpendicular to rotor

: dosing of sample and agents by centrifugation; sample fusion with agents
:: recast into measurement cell



rotor – 28 positions for samples, standards and controls, rinsable

: *batch analyses* (one-after-one method measurements)

disadvantages: not selective (no *random access* analyses)

tracked a.

copies reactions in test-tube

: continuous belts placed with lines of tubes in water bath

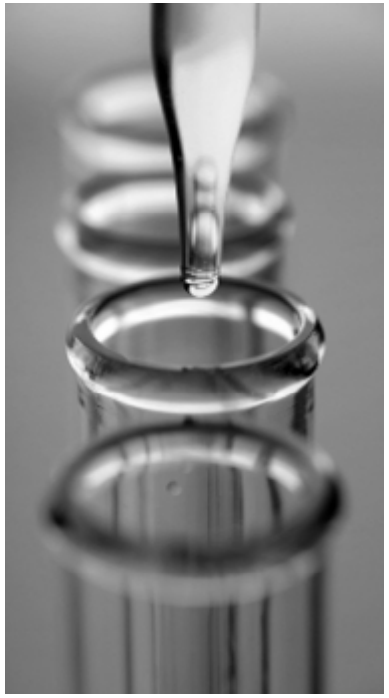
: linear movable doser pipettes serum and fixed doser individual agents

: after reaction completion tube content was taken up into measuring cuvette

: test-tubes are then rinsed, dried and used again for analyses

:: verbatim single file of „marching tubes"

<u>disadvantage</u>: high consumption of sample and agents



revolver a.

wheel of sample holders, agents and reaction cuvettes with **doser system**
: **processor** controlled
: absorbance measurement 340 – 600 nm by commutable filters (5 – 8)

small, middle or big
: average hour performance reached (analyses per hour)
:: performance goes from hundreds up to thousands per hour

method pallet, without re-programming: 15 – 50

special analysers – chosen groups of analytes according to medical demands
: dangerous drug monitoring, urine analysis, hormone determination, oncomarkers determination, coagulation haematologic instruments (blood particles counter *etc.*)

urgent (acute) investigations – special small a.

analysers – dry chemistry

dry chemistry – agents in dry state

plates or **strips** – carriers for reagents

principles of signal measurement:
colourimetry, reflectometry, potentiometry and immunochemical reactions

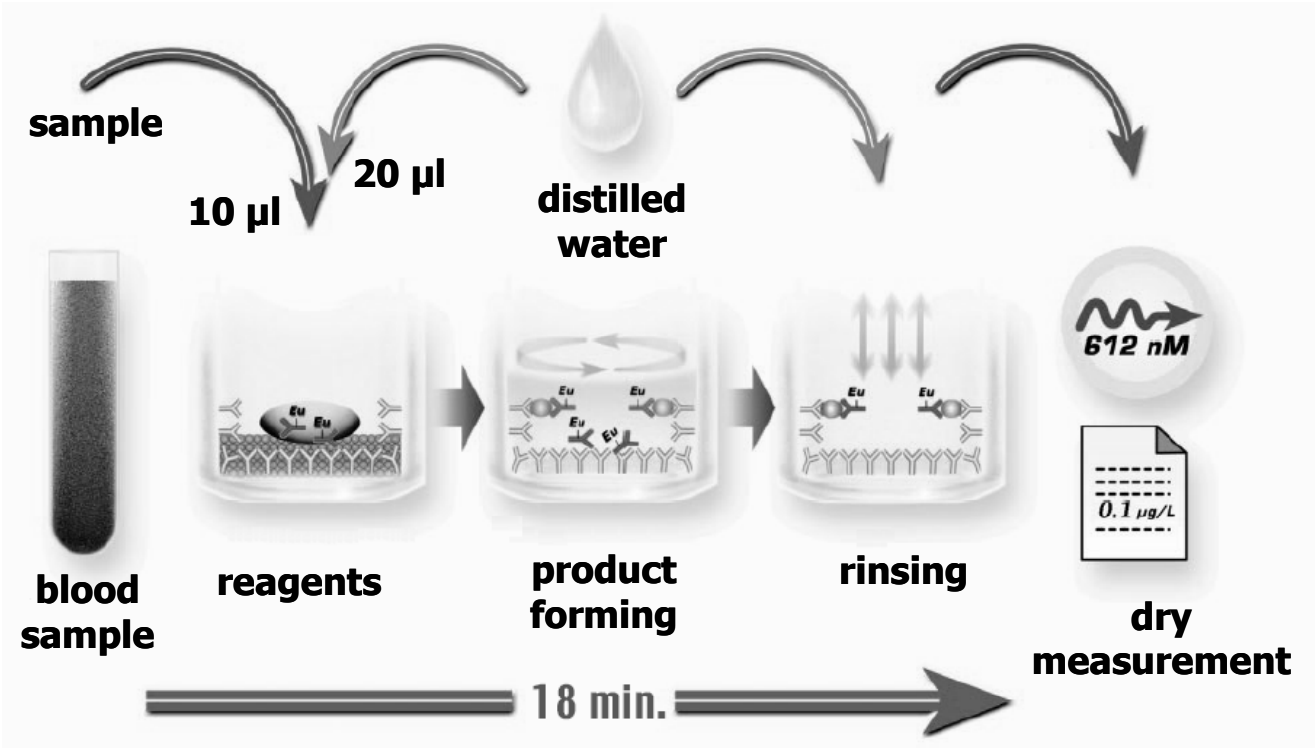
up to 50 different analyses types

analyser with plates: contains holder with plates, stand for more samples and doser

sample into window on one side of plate; soaked into reaction zone ⇒ respective chemical reaction; response is measured in window on the other side of plate

analysers with diagnostic strips: reflectometric measurement of reaction spot colour

allows either individual analyses or analysis of a series of analytes



dry chemistry – impossibility of fast sample transport or results; small analyte volume

: easy operation; not suitable for high-quality screening;
reference determination

other analysers

AAS (atomic absorption spectrometry)**, FAS** (fluorescence absorption spectrometry)**, centrifuges, CZE and PAGE of proteins and lipoproteins, scanners, pH-metres, osmometers, coulometers, fluorimeters, NA analysers, chromatographs, mass spectrometers…**

change of **laboratory diagnostics** to **laboratory medicine**

⇒ **advances in instrumentation!**

laboratory medicine ⇐ pathology and laboratory praxis in the beginning of 19cc (C. Bernard, R. Virchow, J. Hopkins and others)

till 30‘s used to additionally approve diagnosis – **laboratory diagnostics**

development after 1950 – rapid increase in number of laboratory investigations with accession of novel diagnostic method, namely with **immunodiagnostics** (around 1960), speed up by **automation** and **computerisation** of analyses (after 1970); and by **molecular biology** (after 1990)

1970 – 1990 : inter-annual increase of analyses number in *ca* 12 %, costs of laboratory diagnostics reached already almost 10 % of total costs of health care

redeployment

efficiency of work – consolidation of detached sections (haematology, clinical biochemistry, immunology, partially microbiology) ⇒
⇒ **consolidate laboratories**; faster and cheaper complex service

bed-side monitoring
fast analysis at bed of patient by staff of clinic
acute analyses *in situ* – so-called **point-of-care testing** (POCT)
clinical chemistry of acute states
investigation of so-called internal state of patient: blood pH, pO_2, pCO_2, ions of sodium, potassium, chlorides and haemoglobin or haematocrit

patient self-monitoring (home diagnostics) analysis or investigation can patient conduct at **home alone**
check of actual state, medication dosing, or signal to visit doctor

miniaturisation and automation – robotised complexes,
so-called diagnostic centres

centralisation

consolidated laboratories

simultaneous biochemical, haematological, immunochemical analyses, detection of some target NA sequences + microbiological analyses

concentration of analyses originally from isolated laboratories of clinical biochemistry, haematology, microbiology, immunochemistry and so on, into a complex, which is able to provide investigation of biological samples on one place of main laboratory faster and cheaper, across traditional branch division

diagnostic centres

: complex analyses pallet
: high degree of automation
:: localisation of samples with patient bar codes on cart moving on a trajectory around mutually compatible analysers, fully automated sample gauge, analysis conduct and release finding

: modular connection of compatible analysers
: controlled complex
: analyses practically without human touch

samples of patients in disposable collection containers with **bar code**, which includes **requested analyses** + **identification** marks

sample division into tubes as per requested analyses
: samples are automatically moved between analysers
: cumulation of diagnoses and patient database

: **core laboratory**
:: *ca.* 75 % of agency
:: analytical chemistry, haematology, toxicology, immunology, urine analyses

: **microbiology**
:: *ca.* 20 % of agency
:: blood and urine sample cultivation, serology

: **transfusion services**
:: *ca.* 5 % of agency
:: blood-typing and pre-tranfusion cross-matching tests

small, specialised laboratories **acute analyses, analyses for consultation centres** (diabetology, urology, toxicology *etc.*), where is proper or necessary to conduct basic **analyses in situ** and **as fast as possible**

long-term trend
: Δ size of analytical spot from **micro-** to **nanometre**
: Δ volume of sample and agents to **submicrolitres**
:: microchips has analytical spots of size approx. 10 to 100 μm
:: size of human erythrocytes is 7 μm

microelectronics terminology

chip (orig. thin plate of semiconductor): **assembly of analytical spots on matrix** (microwells or microdots containing all necessary reagencies); + other functional elements: channels, micropumps, sensors *etc.*

array (orig. arrangement in rows and columns; system of organised elements): a way how to arrange system of analytical spots and functions on matrix

microanalytical unit – whole analytical system, i.e. complete microanalyser, including dosers, valves and detectors of measured signal

technology of microanalytical devices
$\Rightarrow$ capture of certain target groups in sample by appropriate sensor (probe)

sensor – affinity system (antibody, enzyme, protein, NA or complete biological system)

detection – optical methods, electrochemical or measurements reacting on mass (e.g. acoustic waves)

basic division of microanalytical devices

: microreaction plates with high density of reaction spots
: on-surface reactive spots
: microchips and nanochips
: sensors and biosensors (biochips)

microreaction plates with high density of reaction spots

originally microtitration ELISA plate: transparent polystyrene, 8x12 (96) wells on 9x12 cm, volume of well is *ca* 0.2 ml

contemporary microreaction plates: from 192 to 20000 microwells, volume 125 µl to 50 nl

praxis: chip devices with *ca* 100 analytical spots; compromise between miniaturisation and its price

microdosers (based on *ink-jet* technology, dosing micro- to nanolitres)
microdetectors (connection of microscope with photometer or fluorometer) measures signal (e.g. absorbance) in microwell of plate upside down; well is simultaneously reaction cell and also measuring cuvette

measurement: method „mix and measure"

microreaction plates – polymer material; casted or drilled wells by laser, laser ablation *etc.*

microreaction plates – microchip and microarray

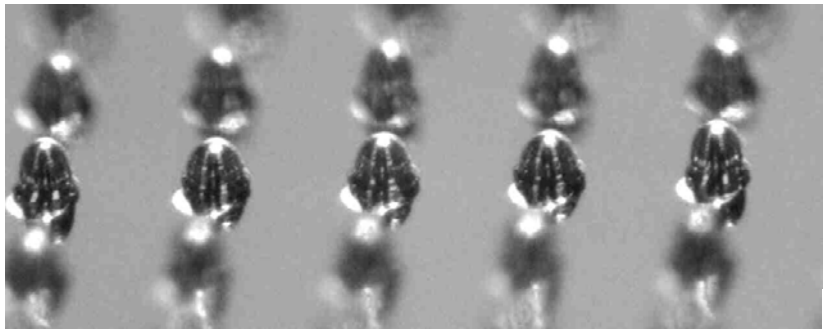
on-surface reaction spots

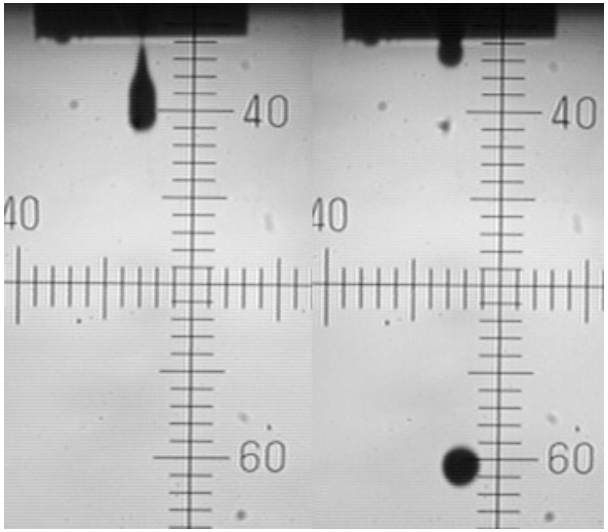
reaction system – directly on a plate, *ca* 1 to 2 cm^2 (not in microwell)

: hundreds of microdots of size 10 – 100 μm

plate material: glass, silicon, plastics (Teflon, polymethylmetacrylate, polycarbonate, polypropylene, polyacrylamide *etc.*)

sample loading: *ink-jet* technique or photolithography

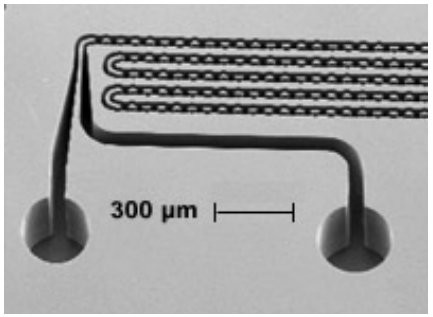




analytical signal measurement: reflectometry

microchips and nanochips

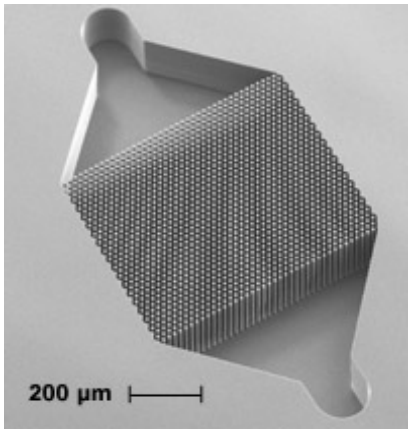
microchips



matrix/holder – *ca* 1.5x1.5 cm; thickness of few millimetres
materials: glass, silicon, hydrophobic plastics

reaction spots: wells or microdots; connected by nanochannels with valves; channels are filled with gel;
: possibility to attach electrodes ⇒ electric voltage between well/sample and sensor

sample flow-rate is monitored by **sensors with laser diode** (excitation by **fluorescence**; detection by photomultiplier; labelling of reactants/samples by fluorophores)



analysis: DNA, RNA, drugs *etc.*

fluids are mobbing on principle of **electrokinesis**

: electroosmosis; uses el. field to move conductive aqueous solutions
: electrophoresis; separation of molecules by el. field according to charge

external micropumps – other solution delivery possibility
: but they are much bigger that microchips; difficult connection to μ-chip

centrifugation analysers – solution for external pumps

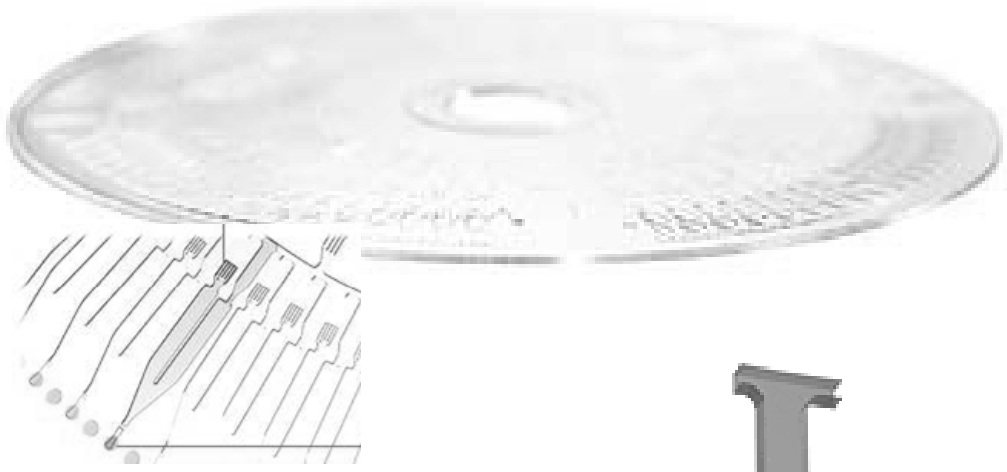
originally for analyses in weightlessness
microchip for centrifugation analysis – **LabCD**

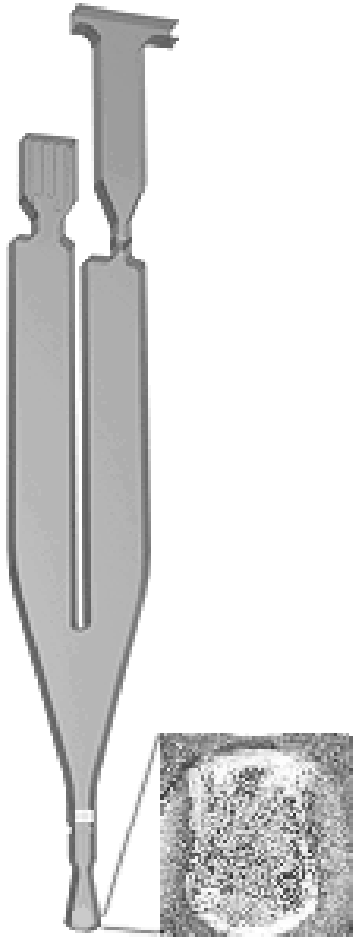
material: three-layer disc, multiplicate determination of one analyte type (*ca* 96 same microcuvettes)
content: controlling software, heating elements and microchannels, reaction wells, valves and microcuvettes
liquid delivery – capillary forces, centrifugation forces

mixing, dilution, washing; heating, filtering, lysation, separation of cells, photometric or fluorimetric detection

modification – system of so-called **surface-directed liquid flow inside microchannels** – microfluid system ⇒ fast laminar flow, molecular diffusion

channels made by **photolithography**





LabCD function

1. sample loading
2. centrifugation preconcentration on column
3. column washing
4. column elution
5. sample in detector

nanochips

further miniaturisation microchips ⇒ **nanometres**
copying frequently function on biomacromolecules (e.g. collagen – cable, DNA – memory unit, protein membrane – pump)

ultramicroanalyses (nanoanalyses) – drugs, biologically active substance, immunoanalysis, mitochondrial DNA *etc.*

limitations – limits of analysis sensitivity and costs (rapidly increasing)
complications – evaporation of sample microvolumes and agents on microchip

sample: volume 1 µl, analyte 2 fmol/l = 6020 molecules of analyte

volume reduction to 1 nl ⇒ only **6** molecules of analyte!!
: mostly under limit of detection of microchip analytical method

: preconcentration procedure: **flow-through** *etc.*

use of microelectrodes on silicon

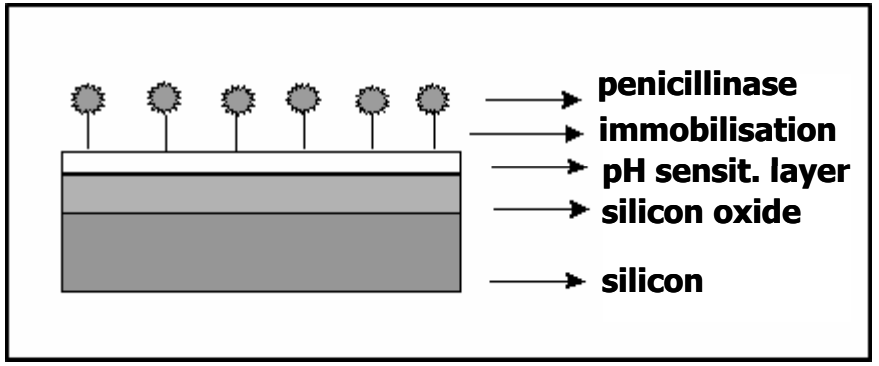
e.g. **resistance** of thin **metal layer** (Au, < 30 nm) is **increasing** with presence of other **atoms and molecules on** Au **surface**

: electrons hitting the metal film (~ mirror) are reflected
: in a place where other atoms or molecules are adsorbed, electrons are not reflected, but scattered
⇒ change of Au film resistance (↑); resistance = *f* (concentration)

use: e.g. determination of heavy metal traces in solutions (ions: Cd, Pb, Ni, Tl, Zn, their organic complexes), limit of detection in *ppb* (parts per billion)
flow-through cells – drinking water quality, corrosive processes, but also in organisms

chip sensor for penicillin

silicon with pH-sensitive structure (Si_3N_4)



penicillin + H_20 $\xrightarrow{penicillinase}$ 6-aminopenicillan acid + H^+
measured with sensitive pH-layer

biosensors (biochips)

biological system (enzyme, receptor, organ) with **analytical chip**

use: medicine, biotechnology, check and monitoring of food and environment

principle: connection of biomolecules with silicon technology **EIS** (capacitive electrolyte insulator semiconductor)
BioFET (biologically sensitive field-effect transistors)

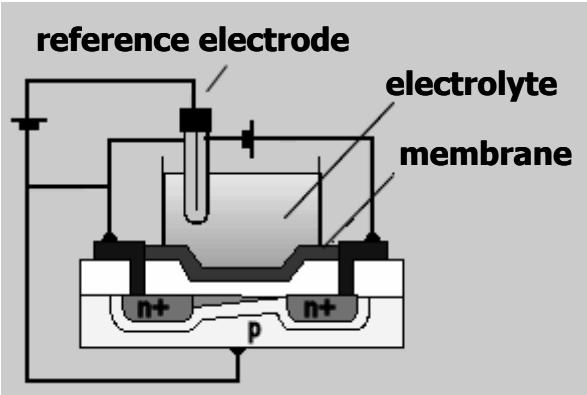
model analytical system

biosystem + analyte $\rightarrow$ product + electrons/protons (H^+)

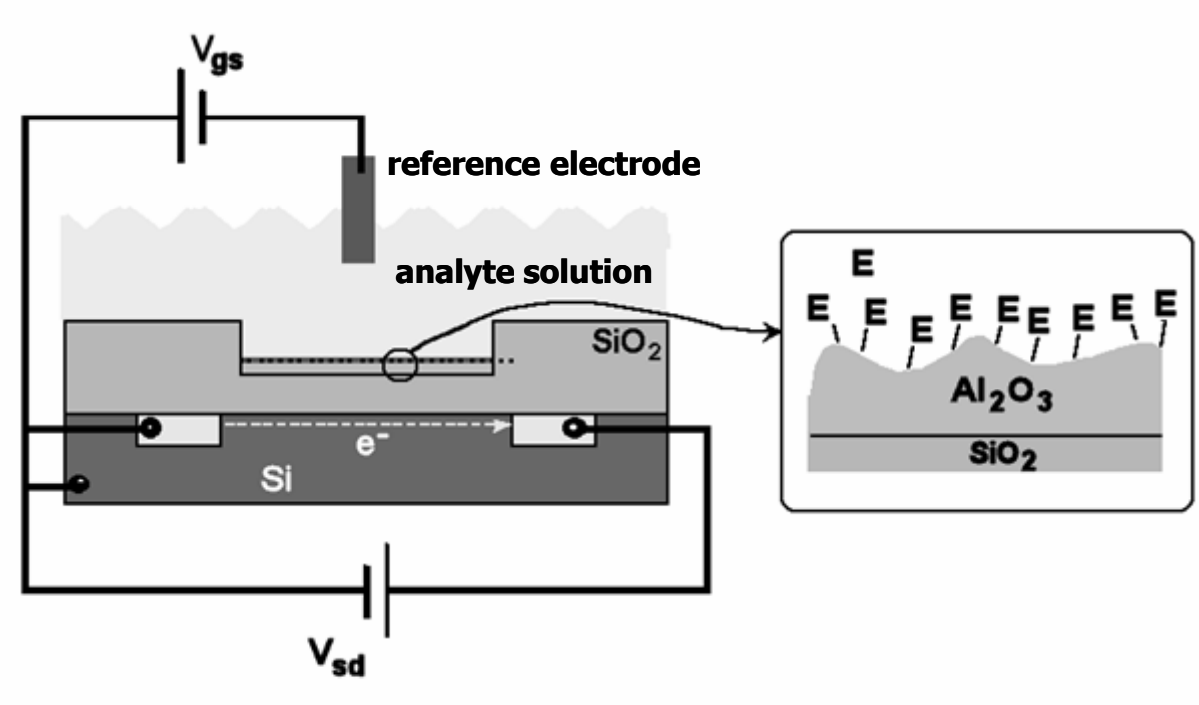
optionally

biosystem1 + analyte $\rightarrow$ product1
biosystem2 + product1 $\rightarrow$ product2 + electrons/protons (H^+)

analysis of metabolites, personal ID (sweat) *etc.*



ISFET
ion-selective field-effect transistor

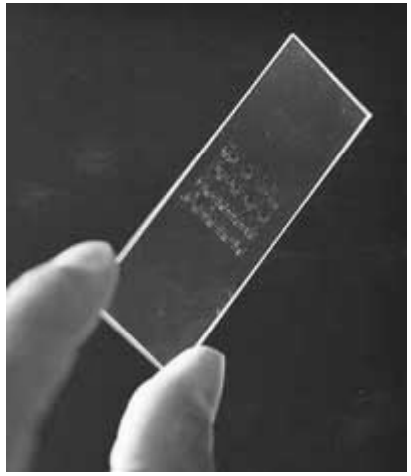


ENFET
enzyme-controlled field-effect transistor

DNA-chips

determination of **target nucleic acid sequences**

detection of pathogens, prenatal diagnostics, forensic diagnostics, Point of Care Testing (POCT)



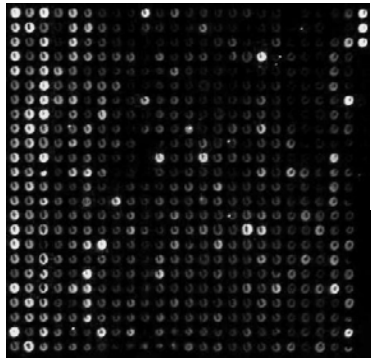
principle: on a chip – **probe with DNA sequence DNA** (Watson-Crick pairing) **complementary to target NA sequence**

only complementary sequences do interact!

sample **TAACGCGATTGTGTGAC**
probe **ATTGCGCTAAGTCACTG**

probe is labelled e.g. **luminophore ⇒ laser induction + fluorimeter**

fully caught **red**
partially caught **yellow**
not caught **green**



immunoassay miniaturisation (competitive, non-competitive)

imunoanalysis on adsorptive support
: dry chemistry method

material: filtration paper, woven fleece
: anchored reaction components; diffusible secondary labelled antibody

sample diffuses through adsorptive material **into reaction zones**
1 to 5 minutes ⇒ **coloured strips** indicate test result

detection: visually or single-purpose reflectometer

analytical system **binary single-purpose tests** – yes/no

analytical cassettes (cartridges)

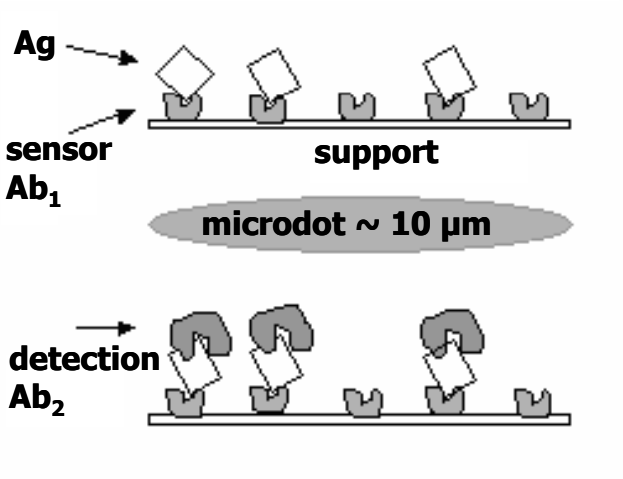
material: plastic cartridge with agents in dry or liquid state

sample is mixed in a system of channels with agents
detection: in place of optical cuvette the analytical signal is read

: analysis of drugs, or acute analysis

microchips for determination of one type of analyte

on microchip – set of same analytical spots – microdots
microdot – immobilised primary antibody (Ab_1), in surplus



sample/antigen (Ag) is added ⇒ **anchored immunocomplex** $[Ab_1{}^b\text{-}Ag]$

rinse; secondary labelled antibody is added (Ab_2*) ⇒
⇒ **sandwich immunocomplex** $[Ab_1{}^b\text{-}Ag]\text{-}Ab_2$*

detection: spectrophotometric

primary antibody – antibody sensoric
secondary antibody – antibody inducing

multifunctional microchip – microdots with different types of antibodies ⇒
⇒ **simultaneous** determination of several **different analytes**

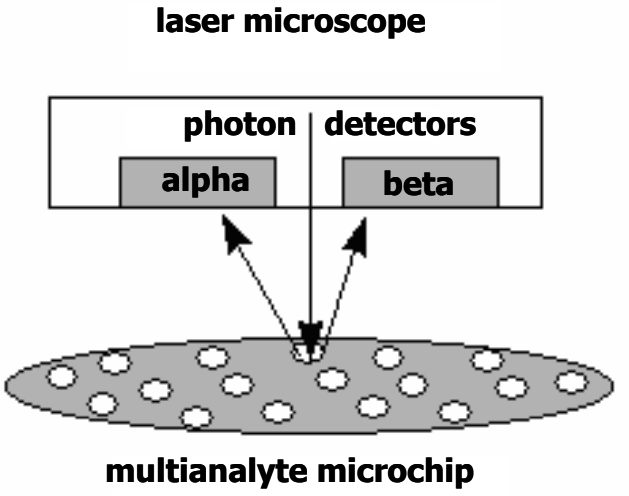
microchips for simultaneous determination of several analytes

analytical spots (microdots) **– different primary antibodies (Ab_1)**

e.g. for antigen Ag_a primary antibody $Ab1_a$, for antigen Ag_b primary antibody $Ab1_b$ *etc.*

rinse; mixture of monoclonal labelled secondary antibodies for determined antigens is added (*$Ab2_a$, *$Ab2_b$, *etc.*)

detection:



multianalytical immunomicrochips – POCT (*ca* 8 to 10 analytes), oncomarkers, allergens, hormones for endocrinology, screening of chosen analytes of blood donors in transfuse stations *etc.*

quality in clinical analysis
its monitoring and management

„fatal" importance of clinical medicine
application of norms ISO 9000

quality

characteristic and desired qualities or features of product or service

quality is indirectly proportional to variability of product or service
: variability – diversion from design/aim

examination of all steps of whole analyte determination
: starting with sampling up to calculations; analyst training, agent preparation, lab-ware cleaning, waste disposition *etc.*

concept for their proper **performance** is **resulting**, along with creation of **requirements** for operations with minimal diversions from prescribed procedure, including **quality assurance mechanism**

definitions according to Czech laws (ČSN ISO 8402)

quality

total sum of properties and attributes of product (e.g. analytical kit) or of service (e.g. analysis), which guarantees ability to satisfy previously given or presumed requisites

quality policy

overall purpose and orientation of company activity in field of quality as defined by top management (e.g. laboratory head)

quality management

part of overall control function, which prescribes and defines quality concept

quality system

structure of organisation and responsibility, procedures, processes and sources needed for realisation of quality management; major requirement – all procedures must be unmistakable, properly documented and fulfilled; quality system is regularly monitored and complemented; all persons included should actively take part in introduction and keeping of the quality system

quality manual

quality management in laboratory – **quality manual**
: present in each certified or accredited laboratory

comprehensive document including all aspects of **laboratory activities**, starting with **top management** down to **laboratory cleaning**

„in-house norm" – transformation of general norms ISO 9000 and EN 45000, respectively, for good laboratory praxis (GLP), safety norms, laws and decrees *etc.* in accordance to particular laboratory conditions

overall quality concept

1) laboratory and its duties – laboratory name, address, fax, e-mail, tel. numbers, name of responsible head

2) laboratory working time – way and period of sample reception

3) list of supplied services – (including analytes)

4) communication between laboratory and users – description of application forms, of result forms, rules for telephonic result forwarding, way of correction or complementation of forwarded analytical results, time period necessary for analysis procedure, including the way of monitoring its keeping

5) sampling (timing) – instructions for patient, sample transport, sample procedure up to preparation of trial sample and storage including expiration date; provision of unambiguous patient identification; way of exclusion of objectionable samples, decrees for forwarding of partial samples transfer to different laboratory sections

6) analysis procedure – written instructions for procedures used; traceability of each analysis starting with application up to laboratory results; calibration including concurrence to metrologically higher etalons

7) result interpretation – information on possible consultations with laboratory personal, assigned contact personal

8) taking part on clinical staff seminars, meetings with external medical practitioners

9) education of laboratory and clinical staff, nurses and students

10) information on procedure changes, accreditation or certification

11) taking part on research and development – concept of laboratory attendance on research and development, monitoring system and names of responsible personal

12) ways of ensuring the mandatory discretion

13) personal numbers in each category

14) information on laboratory equipment – names of instruments, its manufacturers, year of acquisition, prise, guarantee, localisation, service, service manual and its placing; calibration

15) security rules of laboratory and hospital – placing of respective decrees, records on incidents and injuries

quality is not a state, but a dynamic, improving process

calibration, controlling and reference materials

wrong analysis ⇒ wrong decision ⇒ health harming or death

measuring process – respective concentration should be assigned to measured signal using calibration materials (standards)

controlling-regulatory process – analysis result validation and its including into quality management; analyses monitoring in longer time scale

: demands suitable controlling and reference materials

50ies – 70ies of 20th century

manual analyses (photometric, titration and suchs) or semiautomatic (drainable cuvettes)

: sample volume 20 – 500 µl, agent sample 1 – 5 ml

: **aqueous solutions** prepared of fresh analyte

today

automatic analysers

: sample volume in microlitres, agent volume in tens of microlitres

: *viscosity* ⇒ *problems*; calibration solutions w/o biological matrix do not fulfil

:: requirements on similarity with analysed sample

:: instead of aqueous solutions **calibrators with proteinic matrix**

: **certified reference materials**

aqueous calibration solutions and standards

IFCC – whenever possible – calibration using aqueous standard solutions

stabilisations and **protection** (oxidation, bacterial contamination and so)

use: enzyme attestation, verification of analytical method yield, check of photometer wave-lengths and so

preparation: weighing; highly pure chemicals, redistilled water

storage: glass ampoules or tight vials
: under inert atmosphere of N_2 or CO_2

auxiliary substances: albumin, polysaccharides, sugars, glycerol (enzyme stabilisation), cysteine, dithiothreitol, ascorbic acid (anti-oxidation protection), complexing agents, (e.g. EDTA, masking of heavy metals catalysing oxidation), different buffers and products of enzyme hydrolysis of substrates (increasing stability of enzymes) and conservation additives (sodium benzoate, sodium azide and such)

they should not contain a considered standard as a contamination
(and if, only trace and defined amount)

control sera and urines

for operative quality management (for so-called inner quality control)

liquid – frozen (– 80 °C)

: originally animal sera: equine, bovine, porcine
: substituted later with sera of human origin

lyophilised – more stabile

: prepared in at least two concentrations covering not only analyte reference interval, but also the pathologic values

sera w/ attest – determining accuracy in longer time period
sera w/o attest – determining repeatability

parameters: pH 7 to 8, difference in content in one batch < 0.1 %, free glycerol < 0.2 %, should be sterile, residual humidity < 1 %, stability in cold 3 years, difference in content of labile components < 4 %, turbidity after reconstitution and dilution with water 1:9 measured in 1 cm cuvette as absorbance at 700 nm under 0.05 and at 340 nm under 0.2

serum calibrators

similar composition and behaviour as analysed sample

preparation: similar to control sera; addition adjusted analyte concentration

lyophilisates – stability

commutability with defined methods

preparation and attestation: as with control sera

content of individual **analytes** is given by definitive or **reference methods** which are certified by reference materials

preparations for **normal** and **pathologic** analyte values

multi-calibration preparations

certified reference materials (CRM)

calibration of definitive and reference methods
testing/comparison of routine methods (**commutability**)

RM – material or substance, values defined for instrument calibration, method evaluation for measurement or determination of values in materials

CRM is RM documented with certificate
: certified method is accompanied by uncertainty on defined confidence level

analyte concentration and matrix in CRM **same** as in analysed **sample**

suitability of RM for given aim is **examined** (ISO Guide 35)

batch of CRM must be **homogenous**
: difference between representative sample measurement must be always lower than an overall uncertainty of all measurements

CRM must have stated its **expiration period**

CRM – accompanied with attestation

co-ordination by **european committee within european union**, e.g. **institute for reference materials and measurements** in Belgium (IRMM)

validation and good laboratory praxis

confirmation by measurement (testing) and measures of **objective proof**, that individual **demands** for **given aim** were **fulfilled**

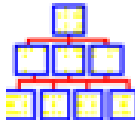
validated analytical method – gives medicinally accurate results and using analytical result within patient treatment leads not to health harming

approval of **overall error** – sum of random and systematic errors

good laboratory praxis (GLP)
: internationally concerted system of assurance and control of quality
: includes organisation of tests, studies and conditions, under which are non-clinical studies planned, proceeded, monitored, recorded and archived

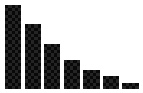
operative quality management

tools of QM so-called „**basic seven**“:



stem-and-leaf display – control data itemised in a way one could quickly judge global data division

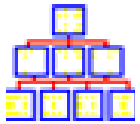
check list – calendar with noticed causes of faults; lines create the calendar, into which when and how many times the given trouble appeared is written



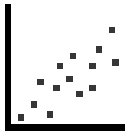
Pareto chart – histogram with columns expressing rate of individual fault types in descending order

cause and effect diagram – graphic assay of faults and its causes

flow chart, defect concentration diagram – graphic presentation of instrument or process with sensitive points marked



scatter diagram – correlation graph serving to estimate mutual dependencies in analysed problem



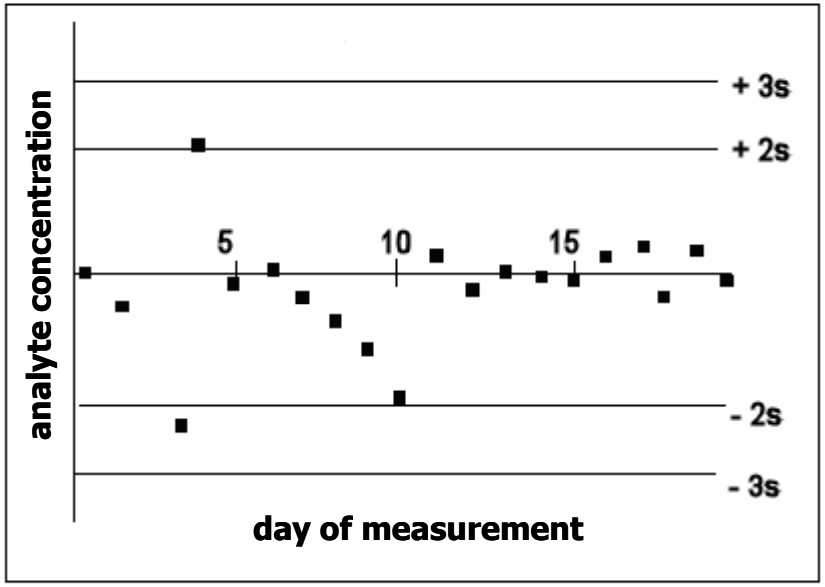
control chart

regulation diagram

: 1931 W.A. Shewhart
: 1950 S. Levey and E.R. Jennings – clinical biochemistry

simple graphic interpretation of Gaussian distribution

set of data with normal distribution ⇒
in interval of standard deviations
–s to **+s** lies **68.3 %** of data
–2s to **+2s** lies **95.5 %** of data (*warning* limit)
–3s to **+3s** lies **99.7 %** of data (*regulation* limit)



x axis – time
y axis – signal measured

: defined portion of data must lies within the respective zones in graph

: half of them must be alternatively above and below the **x** axis

cumulation only towards one side of interval ⇒ **systematic error** in analytical process

: out of warning limit ⇒ **once for a month**
: out of regulation limit ⇒ **once for 18 months**

:: out of warning limit more frequently
⇒ mistakes in analytical process or process gets out of control

deviations: Δ standard deviation (s) ; Δ deviation (B)

operation of regulatory diagram
average series length (ASL)
ø number of points, when inserted point indicates method out of control

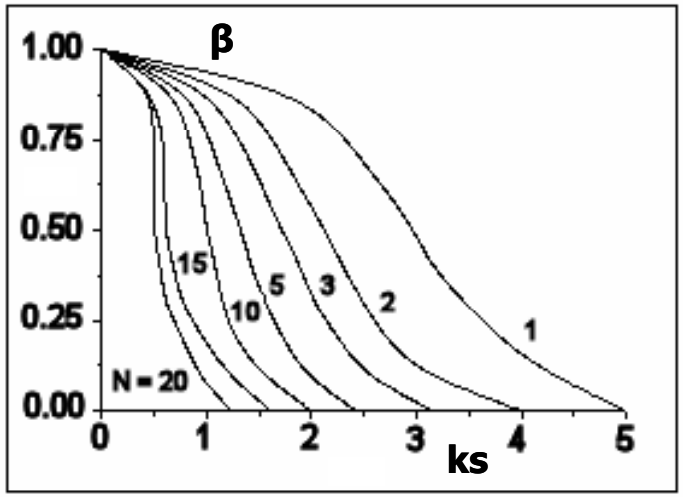
$$\mathrm{ASL} = 1/p,$$

where p is probability, that any point crosses the control limits

operative characteristic curves
β error dependence, i.e. probability of not noticing the shift

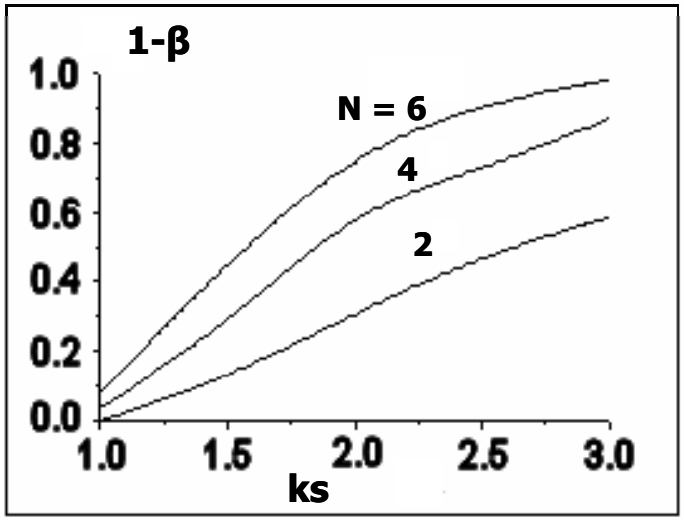
$$\beta = \Phi(L - k\sqrt{N}) - \Phi(-L - k\sqrt{N}),$$

where Φ is Gaussian function, L is multiplicand of s according to chosen limit, k is factor respecting change of x in multiplications of s, N is number of samples



power function

on contrary to operative characteristic curves
y axis – (1–β), so probability of denying



external quality assurance

(EQA)

part of general quality management of each laboratory (*ca* once in 2 months)

: method validation
: laboratory monitoring its precision in comparison to other laboratories or generally valid demands

what is considered?

: deviations in regard to state-of-the-art, in comparison towards data gained by means of reference or definitive methods, respectively

: achieved level of all committed laboratories, according to not only inter-, but also to intra-laboratory variations in recorded data

: relation between data recorded and way of calibration, analytical procedure, commercial analytical kits and instrumental park used

: achieved contemporary level in dependence on analyte concentration in control materials

international harmonised protocol for proficiency testing of (chemical) analytical laboratories

: since 1992
: IUPAC, ISO and AOAC (*association of official analytical chemists*)

IFCC material: elementals of external quality assurance

precise and protocolary test organisation

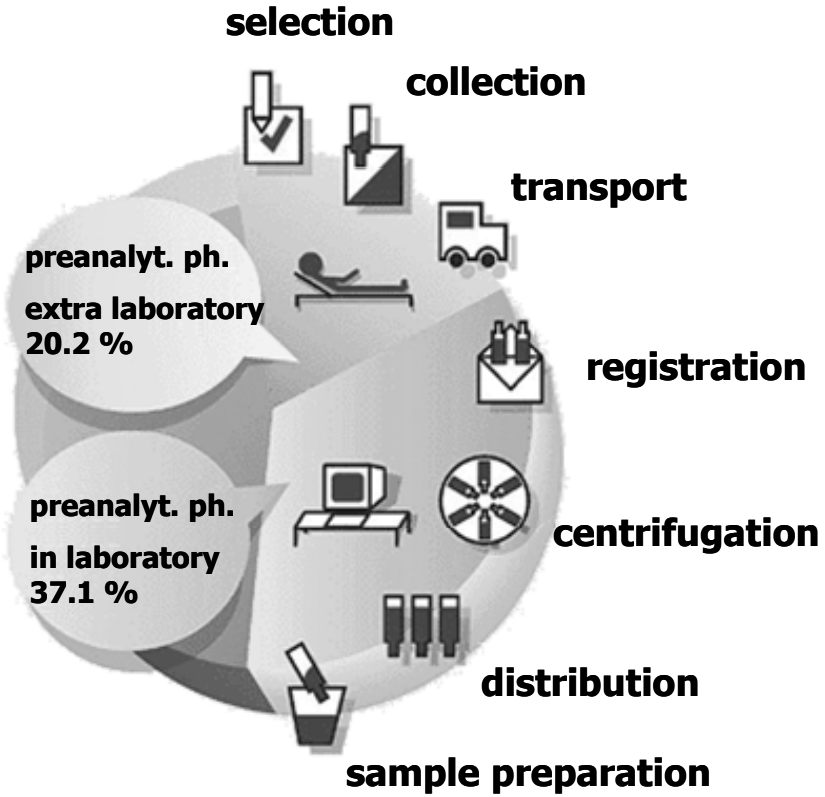
statistical test evaluation

z-score
$z = (x - X_a)/s$,
where s is target standard deviation, x is measured quantity, X_a defined (real) quantity value and z has value of standard normal quantity

z-score interpretation
$|z| \leq 1$ is **good score**
$|z| \leq 2$ score sufficient
$2 \leq |z| \leq 3$ problematic score
score $|z| \geq 3$ insufficient score

importance of laboratory evaluation

success of laboratory in external quality assurance
its **performance** is inside of **tolerance limits** accepted by respective (**inter**)**national authority in clinical chemistry** for given time period



: preanalytical phase

: analytical phase

: postanalytical phase

controlled and monitored – only ***its own analytical procedure***

omitting ***preanalytical phase*** (sample collection, transport and storage)

errors of **preanalytical phase** – up to 50 % **X analytical procedure** ~25 %
the rest is assigned to so-called **postanalytical phase**

one major error within *ca* **1600 analyses**
sample preparation (55 % caused by haemolysis), insufficient sample volume (21 %), sample confusion (12 %) and coagulated sample (5 %)

major error harms life ⇒ more strict control of **preanalytical phase**

Kill as few patients as possible & 56 other essays on how to be the world's best doctor, Arlan Cohn, 2004

postanalytical phase
: sample and result storage
: transmutation of analytical result into well-found info (raison d'être of work)
: communication with practicing medics, feed-back
: meta-analysis of results

analytical methods
choice and optimisation

choice of method/procedure is **given by** analytical chemistry **development**

: qualitative
: quantitative

originally: chemical methods
: number of identifiable analytes was low, only few tens

since 70ies: methods biochemical/enzymatic/molecular biologic
: spectrophotometry
: industrial manufacturing of enzymes, analysers

analytical procedure is of clinical laboratory interest
: demanding requisites – analytical and also clinical needs

result inaccuracy of test: including preanalytical errors, systematic errors, random errors and mistakes
biological influences: intra- and inter-individual variability, errors caused by non-standard sample collection
uncertainty: ability of test to give accurate conclusions (diagnosis, prognosis, or therapeutic decisions)

history of glucose determination:

1) **redox properties** in alkali media, oxidation by picric acid, ferricyanide or by Cu(I) reduction; work demanding, low sensitivity, unspecific

2) by ***o*-toluidine through Schiff base**; sensitive and specific; *o*-toluidine – carcinogenic, agent contains also glacial acetic acid

3) determination using **enzymatic approaches** (GOD)

characteristic attributes of analytical method

defined and described analytical method
: characteristic attributes, standardised in international norms ISO and in derived or transferred national norms

specific clinical requisites

method accuracy in regard to biologic variability

range of calibration function – minimal range of calibration function in range of analyte reference values

analytical method – in principal function of given analyte in organism

ecological and toxicological requisites

analysis of potentially **infectious** biologic material

never use poisons for analyses, carcinogens, corrosive or flammable materials

inevitability: creatinine determination by picric acid, total protein by biuret reaction with NaOH, haemoglobin determination using haemoglobin cyanide *etc.*

analytical method attributes

analytical performance characteristic
: one characteristic out of sum, which are necessary for checking the precision of measuring procedure and its suitability for given aim, and which might be assigned with experimental value

defined by **IFCC** and **IUPAC**

accuracy or **trueness**
: closeness of identity between average value obtained in large test set data and accepted reference value

accepted reference value
: value, which serves as approved reference value and which is derived as
a) theoretic
b) agreed (certified), based on experimental work
c) assigned (certified), based on experimental collaboration
d) if not a), b), c); expected value of measured quantity, i.e. median value of specified basic data set

bias
difference between median value of test set data and accepted reference value

determination: by means of CRM or RM

recovery
relatively expressed difference between values measuring sample with known amount of added analyte and values of sample without addition, related to added amount

precision
closeness of identity between independent test results obtained under given conditions

repeatability
result identity determined under repeatability conditions (same laboratory, same method, same instrumentation, same operator, during short time period)

reproducibility
identity of identification under reproducibility conditions (same method, different laboratory, different operator, different instrumentation, different time)

precise specification – serves as ground for construction of regulation diagrams

uncertainty of measurement
parameter associated to measurement result, characterising variation of values, which might be assigned to measured quantity with good reason

includes many components

type A uncertainty – characterised by standard deviation derived from statistic distribution of results
type B uncertainty – characterised by standard deviations derived from supposed distributions gained in experience

uncertainty as standard deviation – **standard uncertainty $u_{(x)}$**
resulting method uncertainty – **combined standard uncertainty $u_{c(x)}$**
uncertainty *– interval around measurement result* (extended uncertainty **U**)

$U = k*u_{c,}$ where **k** is extension coefficient
normally distributed values and **k = 2** $\Rightarrow$
$\Rightarrow$ result in given interval with probability 95 %

calibration
set of procedures, which under specific conditions set up relation between values of measured quantities and respective calibration values (etalons)

calibration function $S = f(c)$

analytical sensitivity – 1st derivation of the function above on concentration

$$dS/dc = df(c)/dc$$

calculus of calibration function of calibration data by **regression analysis**

advantage – *linear dependence* (limits of confidence, simpler calculations)

limit of detection (L_D, **LOD**) – is related to confidence limits

$$\alpha = \beta = 0.05$$

β – probability of false negative result
α – probability of false positive result

limit of quantification (**LOQ**)
the lowest measurement value, for which the uncertainty could be defined;

IUPAC: for LOQ uncertainty (variation coefficient) = 10 %

measuring interval
closed value interval, which could be obtained by given measuring procedure; it is restricted by upper and lower detection limits; within photometric methods, the linear range of calibration curve is chosen $T = f(\log c)$

linearity
concentration range, in which analyt. signal is linear function of concentration

calibration (linear function):
: 10 concentrations in working interval, 3 – 4 measurements per concentration
: test on homoskedasticity (equal distribution of their standard deviations)
: regression line construction
    :: normal regression (homoskedasticity)
    :: weighted regression (heteroskedasticity)
: test on linearity
: calculation of confidence limits

analytical specificity
ability of measurement procedure to measure only the desired quantity

expression – as unspecificity, i.e. as effect of random sample component different from analyte causing changes in indication of measuring instrument and thus introducing systematic error

interference
systematic error of measurement caused by analytic interferent

analytic interferent – sample component, which is also part which influences quantities; it it-self causes not changes in indication directly, but indirectly

robustness (ruggedness)
method ability to produce acceptable results of measurement also in a case of small deviation in measuring procedure or in sample composition

comparison with other methods

e.g. routinely in laboratory used method and its comparison to reference method (IFCC), or with definitive method, if at disposal

carry-over

is not method characteristic, but says, if there is no cross-contamination within two after one running samples (mutual influence of low signal after strong and vice versa)

problem of filling and flow-through cuvettes

<u>procedure</u>:

15 analyses, 5x with water (absorbance A_1 to A_5)
5x with sample (absorbance A_6 to A_{10})
finally 5x with water (absorbance A_{11} to A_{15})

$A_6 - A_5 = A_{10} - A_5 = A_{10} - A_{11}$ and $A_{15} - A_5 =$ **0**, no carry-over happens

<u>carry-over</u> – numerically in % as a ratio of carry-over $100x(A_{10} - A_6)/(A_{10} - A_5)$

criteria of analytical method choice

analysis in laboratory medicine
: sense only in regard to evaluation of patients health

two main aspects

clinical usefulness – the quality demanded by medical doctors

analytical determination quality – errors and uncertainties, interference and specificity of method

method choice according to analytical attributes

according to state-of-the-art
procedures are chosen according to contemporary demanded clinical needs using analytical methods

according to panel experts
: empirical findings of experts in specialised medical branches
: compromises taking in account state-of-art more than real needs

according to results of quality management
: with increasing quality of analytical methods costs and laboriousness decrease

choice according to clinical requisites

medics experience in confrontation either **state-of-art** in analysis

not possible to design **universal requisites** on quality procedures

disadvantage – it differs a lot and it is not possible to find common criteria

choice based on biologic variability

the most relevant to clinical and analytical requisites
: intra- and inter-individual variability of analytes is almost constant
(even in higher age)
: the geographic and temporal transfer is possible

choice according to clinical importance

each change of health state may lead to concentration change of some analytes composition; for practical reasons it is useful to indicate only some changes – **need to define *useful* change indication**

binary results test

test results: positive/negative (yes/no)

diagnostic importance (contingence table 2x2)
: lines – results found within group *sick* and within group ***non****-sick*

patient	**positive test**	**negative test**	**sum**
sick	correct (cp)	false (fn)	N*cp + N*fn
non*-sick*	false (fp)	correct (cn)	N*fp + N*cn
sum	N*cp + N*fp	N*fn + N*cn	N*(cp + fn + fp + cn)

sensitivity
: probability, that within *sick patient* a positive test result will be found

$$\textbf{\textit{sens}} = N*cp \,/\, (N*cp + N*fn)$$

standard deviation $s_{sens} = \sqrt{[(sens * (1 - sens) / N]}$

specificity
: probability, that within ***non**-sick patient* a negative test result will be found

$$\textbf{\textit{spec}} = N*cn \,/\, (N*fn + N*fp)$$

standard deviation $s_{spec} = \sqrt{[(spec * (1 - spec) / N]}$

non-sensitivity
: probability of expecting false negative test result within sick patient
: non-sensitivity is complementary to sensitivity

$$(1 - sens) = \textbf{\textit{non-sens}} = N*fn \,/\, (N*fn + N*cp)$$

non-specificity
: probability of expecting false positive result within non-sick patient

$$(1 - spec) = \textbf{\textit{non-spec}} = N*fp \,/\, (N*fp + N*cn)$$

prediction

: probability of sickness if test result is positive (or non-sickness test negative)

: described by to conditional probabilities

$$\textbf{\textit{predpos}} = N*cp \,/\, (N*cp + N*fn)$$
$$\textbf{\textit{predneg}} = N*cn \,/\, (N*cn + N*fn)$$

	T	**¬T**	**Σ**
D	94	6	100
¬D	5	95	100
Σ	99	101	200

sens = 94/100 = **0.94**
spec = 95/100 = **0.95**
non-sens = 6/100 = **0.06**
non-spec = 5/100 = **0.05**
predpos = 94/(94 + 5) = **0.95**
predneg = 95/(95 + 6) = **0.94**

prevalence importance

: probability of sickness in defined population in defined time period

prevalence – ratio of sick patients to all tested patients

100 D + 100 ¬D ⇒ 200 tests ⇒ preval = 0.5

sensitivity and *specificity* is **not changed**, if number of tested changes, the *prediction* also **changes**

at any time, compare only comparable testing groups

	T	**¬T**	**Σ**
D	470	30	500
¬D	475	9025	9500
Σ	945	9055	10000

heart attack prediction **D** = 500, **¬D** = 9500 ⇒ ***preval*** = 0.05

population X cardiacs

sens = 470/500 = **0.94**
spec = 9025/9500 = **0.95**
predpos = 470/945 = **0.497**
predneg = 9025/9055 = **0.997**

incidence
: sickness appearance in defined time interval (e.g. year)

e.g. diabetes
: prevalence in USA – 2.00 %, i.e. *ca* 4 millions of citizens have diabetes
: incidence in USA – 1.99 %, i.e. each year 398 000 of new diabetes cases

efficiency
: ratio of all positive results to total number of results

$$\textbf{\textit{efficiency}} = (N*cp + N*cn) / (N*cp + N*cn + N*fp + N*fn)$$

likehood and likehood ratio

likehood
: *probability is measure of phenomenon appearance within* ***given hypothesis***
: *likehood is phenomenon appearance measure within* ***different hypotheses***

likelihood quotient LQ (likelihood ratio)

$$\boldsymbol{LQ = sens \,/\, (1 - spec)}$$

instead of *prevalence* and *prediction* value: **chance** (W)
post *– chance after conducting the test*
ante *– chance before conducting the test*

$$\boldsymbol{W_{post} = LQ * W_{ante}}$$

relation between probabilities and chances

$$\boldsymbol{W = P \,/\, (1 - P);\; P = W \,/\, (1 + W)}$$

$$W_{ante} = P \,/\, (1 - P) = 0.05 \,/\, (1 - 0.05) = 0.0526$$

$$LQ = sens \,/\, (1 - spec) = 0.94 \,/\, (1 - 0.95) = 18.8$$

$$W_{post} = LQ * W_{ante} = 0.0526 * 18.8 = 0.98888$$

$$W_{post} \,/\, (1 + W_{post}) = 0.98888 \,/\, (1 + 0.98888) = 0.497$$

analytical method classification

definitive methods

based on *isotope dilution* (ID) and *mass spectrometry* (ID-MS), or on combination of *ID* with *gas chromatography* (ID-GC)

: mostly not applicable into daily praxis – *complex and laborious*
: serve mainly within **attestation of calibrators and control preparations**

reference methods

elemental, thoroughly studied and defined measuring procedure, which analytical attributes (uncertainty and bias) allow its use to check accuracy of other measuring procedures and to characterise reference material

recommended methods

(according to IFCC) with well described logical steps, which are ordered as they were defined and recommended by relevant authority

routine methods

methods, which fit none of the above groups

: must be **commutable** with reference method

commutable method – gives on representative set of native sera same results and reference method

analytical method optimisation

optimal conditions for analysis

: reaction mixture composition (type, component concentration, pH and reaction mixture temperature *etc.*)

: individual steps and order of agents adding

finding optimal reaction conditions – study of higher number of parameters, their influence is estimated

there is *mostly* only **one particular optimal combination**

single variable approach (SVA)

relaxation method

studies the reaction parameters separately; demands higher number of independent measurements; studies separately even those parameters which might correlate (may lead to incorrect conclusions)

multi variable approach (MVA)

studies parameters in a complex way; parameters are changed in parallel; methodically correct

: demands **experimental design** (ED)

experimental design

way how to conduct experiments to get out of minimal number of points maximum of information and thus the best multivariable function description

factorial design

full factorial experimental design (FED)
: contains all possible combinations of chosen factors

parameters: number of factors and number of levels of each factor

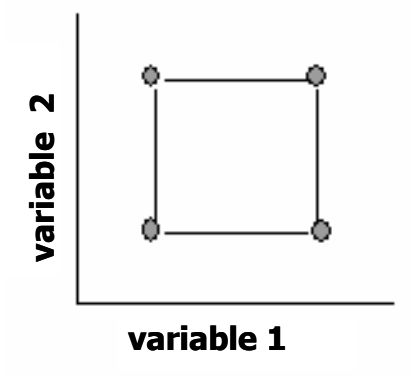
number of factors (f) is related to number of input variables (component number)

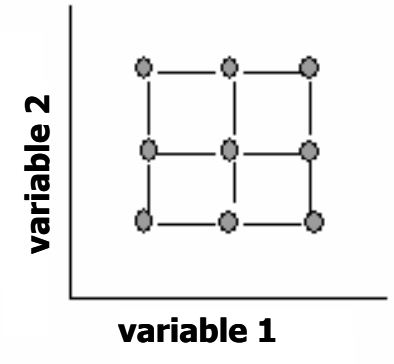
number of levels (L) is number of values of each input variable (*e.g.* number of measured concentrations)

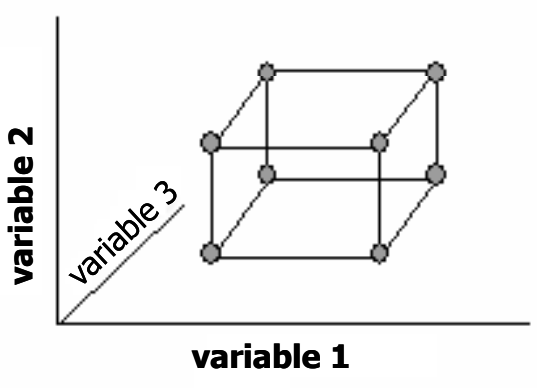
number of points of factorial design (total number of experiments *n*)

$$n = L^{f}$$

three-level two-factor factorial design
(L = 3); 3^2 of experiments







two-level two-factor factorial design
(L = 2) *the simplest* ; 2^2 of experiments

two-level three-factor factorial design
(L = 2) 2^3 of experiments

<u>fractional factorial experimental design</u> (FrED)
decreases number of experiments in contrast to FED (which is sometimes to complex and laborious)

: still describes influence of each parameters and controls possible interactions

: useful in cases of expensive and time-demanding experiments

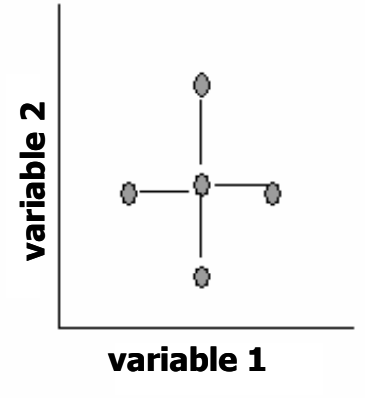
star design

: other variant of experimental design
: might be FrED variant of factorial design
: three-level two-factor factorial design ⇒ two-factor star design

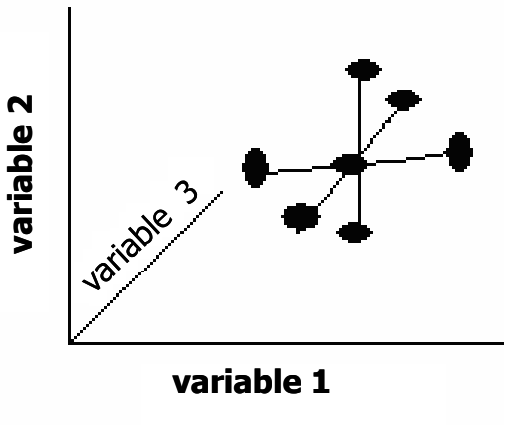
contains (**2xf+1**) of experiments, where **f** is number of dimensions (components)

location of star design points is given *by location of central point*

other points are *located symmetrically around centre*



two-factor star design
2xf+1 of experiments

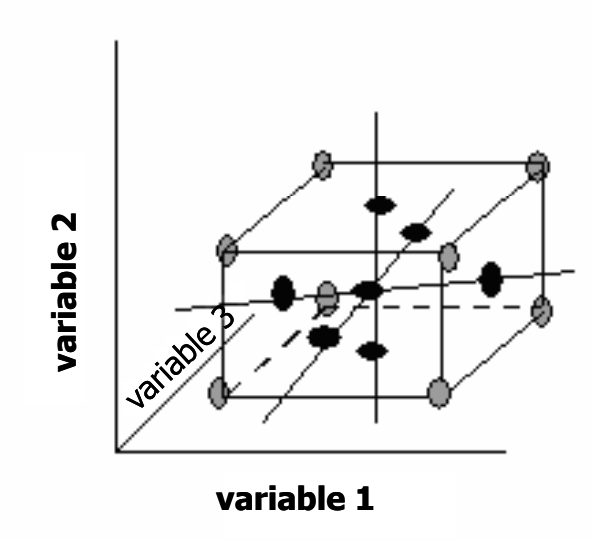


three-factor star design
2xf+1 of experiments

central and non-central composite design

combination of factorial and star experimental design – *complex hyper-flat*

central composite design – centres of both designs are equal
non-central composite design – centres are not located equally



five-level three-factor central composite design
2^f + 2xf+1 of experiments

approximation methods and algorithms

optimisation – intention to „discover" numerically function of output on optimised parameters – ***approximation***

black box : algorithms do not describe physico-chemical properties, but „only" numerically assign relations between variables

partial least squares (PLS)

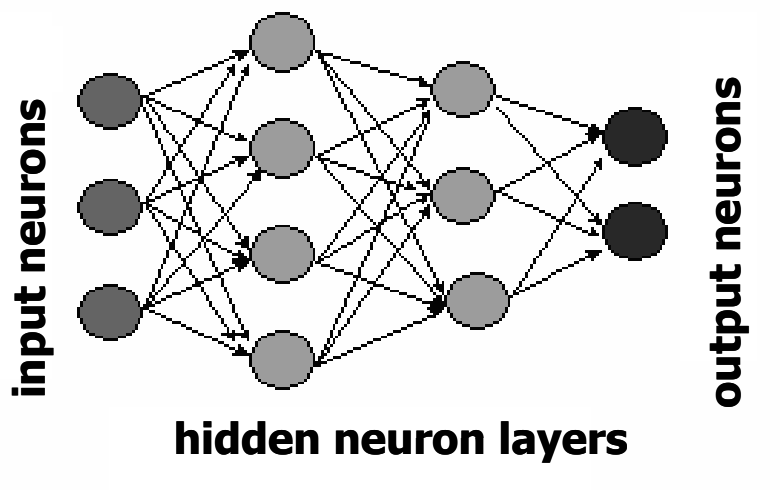
PLS – MVA, values for all analysed mixture components are calculated at once

canonical correlation (CC)

artificial neural networks (ANN)

mimicking biological system of mutually inter-connected neurons

: processors – **neurons**
: way of connection – **network topology**

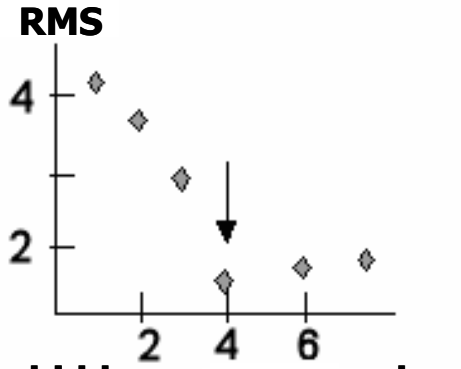


neurons are arranged into **layers**
output of ***n*-th layer** are lead to each neuron in **layer *n* + 1**

first, **input layer** – inserts values for processing
last, **output layer** – response values of whole ANN due to change of input parameter conditions

number of neurons in input and output layers are given by number of input and output variables

inner, **hidden layers –** depends on complexity of approximated function



connection between neurons

represented by rational number – *connection weight* (*w*)

prediction learning of *output values* with *minimal error* of values predicted by ANN *comparing to experimental values* – repeated setting up of numeric inputs of transformation function and monitoring of outputs into functional (real) value

error – ***total sum of squares*** (TSS)
sum of squares of differences of predicted and input values

$$TSS = \sum_{i=1}^{n} (z_i - OUT_i)^2$$

z_i – output variable value z for given triad (x, y, z), OUT_i – its predicted (output) value, **n** – number of elements in training set

each neuron (except for output) sums values from previous layer and multiplies them by connection weight ***w*** :

$$NET_j = \sum_i (INP_i * w_i) + BIAS_i$$

INP_i – input value, w_i – respective weight value and $BIAS_i$ – bias value, which is so-called bias parameter and is necessary for correct set-up of neuron value NET_j and for whole output of network

NET_j – neuron j in neural network

OUT_i – transformation of NET_j (output) sum

$$OUT_i = 1 / (1 + e^{-NET_j})$$

set training/learning – **n** set of parameter given by experimental design
testing – at least 3 sets of parameters inside of plan borders
verification – at least 3 sets of parameters inside set borders
(also borders them-selves)

analytical kits
in laboratory medicine

analytical kits

before 1960 – analytical agents prepared in clinical laboratories

today – analytical agents in forms of microchips designed for special analytical instruments could not be prepared at all in laboratory

requirements for analytical kit

ready-for-use

single-step method – one working solution

two-step method – two working solutions; enzymatic assays

stabile at least **12**, but preferably **24 months**
: individual agent after opening at 2 – 8 °C stabile for a week

fast analysis method – signal, most commonly absorbance, until 5 min, kinetic measurement in interval of 10 s at maximum

method without sample pre-treatment – deproteination, mineralisation, pre-concentration of analyte *etc.*

analytical kit make-up

manufacturing procedure – *technological regulations*
: description of all manufacturing, checking and other operation in a form of standard operation procedure

analytical kits *manufacturing* is organised like pharmaceuticals manufacturing

liquid agents
preparation: weighing (including water; more precise)
vessels: glass or plastic, volume 10 to 200 ml

stabile – non-corrodible, non-underflowing, gas non-permeable (oxidation, outflow of shielding inert gas)
: low-pressure PE, PP, PET (polypropyleneterephtalate) *etc.*

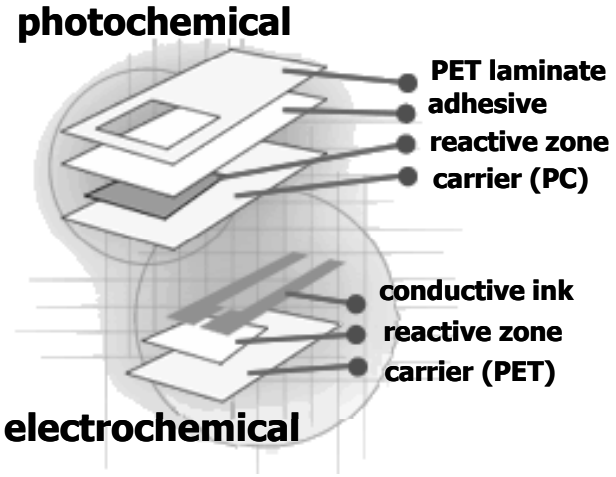
filling: inert gas washing, pump dosing, bacterial filters (microfiltration), labelling (bar code)

solid agents

preparation: weighing the solid into container, solid mixture is tabletted and tablets are sealed (blister)
: mills, mixing and homogenising equipment, tabletting press, granulators;
under low air humidity (< 20 %)

sensitive agents (enzymes, proteins) – **lyophilisates**
: lyophilisation (freeze drying, freeze sublimation) – removal of water by sublimation in deep vacuum from frozen water solution of product; water condensates in cooler, which has much lower temperature comparing to product (higher temperature gradient)

lyophilisation damper – substances allowing more easier process (proteins, cellulose derivatives *etc.*)



kit completion
: semi-automatic, on belt

outer container – final label
storage: at room temperature or in cold-boxes

kits for dry chemistry

diagnostic strip

reaction zone – on plastic strip
impregnation: concentrated, liquid analytical agent soaked in absorptive material; fixation **built-up plastic net**

absorptive support – **defined properties** (grammage, i.e. mass in g/m^2 and absorptivity for liquids) ⇒ soaks any time almost the same amount of sample

storage: drier (silikagel/molecular sieve)

content of analytical kit

name of the kit
: analyte; analysis principle
: storage condition, expiration period, number of analyses
: information on poisons, inflammables and corrosives

manual
: kit principle (with chemical equations), references (lit. and patent.), agent compositions, reaction/incubation mixture composition, procedure of their preparation, storage and stability
: analyte reference values, work-flow scheme, recommended calibration and control materials, notes on sample, security notes

analytical results
how to express them

content of analyte in sample

originally: weight, activity per volume (in biological fluid)
g and mg/dl, also gram percents (g%) and milligram percents (mg%)

1977: **SI** system
weight (g, mg) ⇒ **molar amount** (mol)
concentration – mol/l (analyte with defined molecular weight)

enzymes: concentration of catalytic activity instead of enzyme concentration

catalytic activity **1 katal** – enzyme amount, which proceeds **1 mol** of substrate **in 1 s** under defined reaction conditions

concentration of catalytic activity – catalytic activity related to volume
content of catalytic activity – catalytic activity related to weight

international unit **U** (IU) – enzyme amount, which proceeds in **1 min 1 µmol** of substrate under given conditions

1 U = 1 µmol/min = 16.67 nmol/s = 16.67 nkat

enzyme/protein mass concentration
: analyses in immunodiagnostics and within enzyme CK
:: concentration is expressed in **mg** or **µg** of **protein/l**

number of elements in biological fluids (cells, particles, different objects)
:: numerical concentration, i.e. **particle count in litre**

urinal sediment – **arbitrary numerical concentration** (arbitrary unit)
: simplification of particle (elements) count expression per defined (agreed) volume

after urine centrifugation, individual element counts are read in field of microscope vision in counting cell of defined volume (Bürker cell)

other use: analyses of other tests in urine (e.g. protein test), in cerebrospinal liquor, in faeces (iron content)

IFCC and **IUPAC**: abbreviation system for expression of analytical results for sample types out of SI frame

analysis result interpretation

analyte – part of sample, which we determine (*creatinine in serum*)
: determination with certain precision depending on content, stability, determination method *etc.*

analytical method/approach – analyte determination method (*Jaffé reaction with picric acid in alkali media*)

analysis result – value in regard to diagnosis (*creatinine clearance*)

test on function – tested state in regard to diagnosis (*glomerular filtration*)

index – diagnostic ratios of analyte contents

analytical variability – preanalytical and also analytical phase

biological variability **(BV)** ***of population*** – complex characteristics of variability of biochemical test results; sum of intra- and inter-individual variabilities

expression: coefficient of variation in per cents (*CV*)

estimation: analytical approaches, which have CV < *ca* 1/3 BV interval

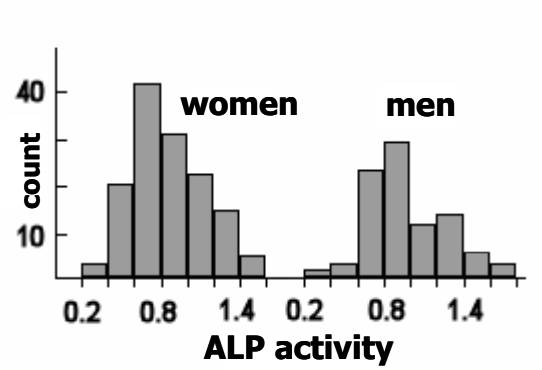
agreed deviation from *analyte target value* (TV) – instead of **BV**

target value : average value of all result in a set after their recording
: average estimated by definitive or reference method, in cases, when definitive neither reference method are not yet established, comparative method may be used

relationship between analytical result and patient state

healthy (normal) or he/she has **analyte** values **deviated** (pathologic)

for common analytes there are so-called **reference values** (formerly normal or physiologic value)



: reference values of **reference individual** – donor
: **reference groups**
: **reference population**

acquiring: *sample analyses* of persons, who are classified as *healthy*
reference (physiologic) interval – laboratory test values, *between which lie majority of the values obtained by reference population measurements*

analytic set

functional sets of individual **analytical methods**

clinico-biochemic queries of medics
: liver set, set for fat metabolism, set for glucose tolerance disruption *etc.*)

other reasons – organisation, security, economy

acute (statim) analysis
determination of one or group of analytes as soon as possible and non-stop

glucose (diabetes), creatinine or urea (kidneys), bilirubin and aminotransferase ALT (liver tests), creatine kinase and troponin (heart), α-amylase or lipase (pancreas), acido-basic blood equilibrium, basic toxicological tests and basic urine tests

basic biochemical test
basic (screening) tests on analytes characterising function of main body organs

total protein, bilirubin, creatinine, urea, glucose, cholesterol, uric acid, ALP, aminotransferases ALT and AST, event. GMT or CK

basic haematologic tests

determination of blood picture

haemoglobin, erythrocytes, haematocrit, leukocytes, thrombocytes, reticulocytes, osmotic resistance of erythrocytes, basophilic granulation of erythrocytes *etc.*

organisation division of analyses into sets

immunochemical tests

immunoelectrophoresis

serum proteins, α1-foetoprotein, immunoglobulins A, G, M, prealbumin, α1-antitrypsin, α2-macroglobulin, transferrin, caeruloplasmin, CRP, prostatic specific antigen *etc.*

radioimmunoanalytical tests

thyroxine, triiodthyronine, thyrotropin, luteinisation hormone, follicle stimulating hormone, prolactine, choriogonadotropin, estradiol, progesteron *etc.*

test of cerebrospinal liquor

chemical determination of analytes + different special tests

basic urine test

pH, nitrites, proteins, glucose, bilirubin, urobilinogen, keto-substances, osmolality, erythrocytes and haemoglobin, leukocytes, urine sedimentation

supplementary analyses

morphologic analysis of urine sediment (epithelial cells; granular, wax, cellular, erythrocytal, leukocytal, bile casts; pseudo-casts, spermatids and microbes (yeasts, bacteria, trichomonades, mould)

drug monitoring

monitoring of those pharmaceutical levels, which in higher doses are toxic or over-dose is meaning, or because patients may have lower tolerance to them

special analysers

the most monitored pharmaceuticals – digoxin, phenytoin, phenobarbital, valproic acid, diazepam, theophylline, gentamicin, tobramicin, methotrexat, ciclosporin *etc.*

drugs of abuse

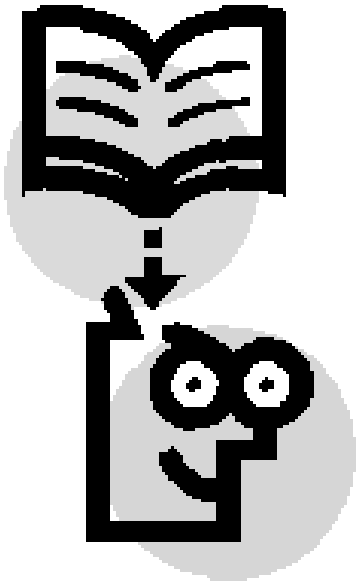
instrumental method (HPLC, GC, MS, CZE *etc.*)

alcohol, amphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine, methadone, opiates, antidepressives, anabolic steroids *etc.*

selected instrumental analytical methods
in laboratory medicine

spectrometric methods

: UV-Vis, fluorimetry
: AAS, AES
: mass spectrometry



electrochemical

: coulometry, potentiometry

separation methods

: chromatography (liquid, gas)
: electrophoresis (gel, capillary)

high performance liquid chromatography (HPLC)

principle

separation based on different distribution constants of analytes in two-phase system (solid-liquid)

distribution ratio D $D = C_S / C_M$

C_S – analyte concentration on solid (stat.) phase, C_M – in liquid (mobile) phase

retention time t_R

: time, which it takes to analyte to go through column

void (dead) time t_M

: time, which it takes to mobile phase itself to go through column, or non-interacting substance (its $t_R = t_M$)

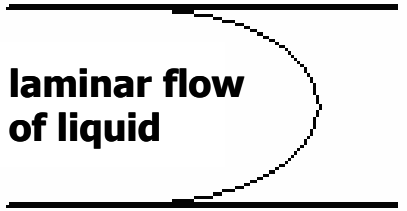
sorption – analyte from mobile phase is adsorbed on stationary phase
desorption – reverse process
chromatography – dynamic equilibrium of analyte sorption and desorption

four main sorption mechanisms: adsorption, distribution (extraction-like), ion exchange on ion-exchanger and steric exclusion (stationary phase with defined porosity, separates substance according to their size and molecular mass)

adsorption – surface effect caused by electrostatic interactions (weak bods; H-bridges, bond dipole-dipole and bond dipole-induced dipole)

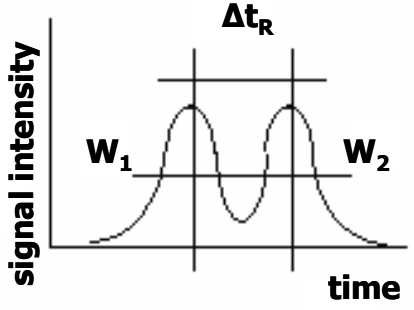
analytes **compete** *with mobile phase* for **limited** number of **binding sites** on adsorbent surface

substance chromatographic separation – symmetric or Gaussian concentration profile in direction of mobile phase motion, i.e. zones or peaks



quality of chromatographic separation
: column efficiency
number of theoretic plates N $N = 5.54*(t_R / W)^2$
W – peak half-width

height equivalent of theoretic plate *H* $H = L / N$
L – column length
high H ⇒ efficient s. of multi-component substance mixtures



resolution R_S $R_S = 2*\Delta t_R / (W_1 + W_2)$
: separation of two neighbouring peaks

$R_S \geq 1.5$ satisfying, $R_S = 1.5$ is so-called baseline separation

stationary phases (SF)

chemically modified silikagel or different copolymers

silikagel Si-OH – polar and weak acid

: modification by alkylchlorosilanes with alifatic carbon chain length 1 to 18; hydrolytically stable siloxanes with typical bonds **Si-O-Si-C**

: most frequently used phase – **octadecylsiloxane** (**ODS** or **C18**); so-called **reversed phase**

mobile phases (MF)

acc. to polarity: *n*-hexane, cyclohexane, methylbenzene, chlorinated carbohydrates, tetrahydrofuran, acetone, acetonitrile, *iso*-propanol, ethanol, methanol, water…

individually or their miscible mixtures

MF flows under pressure up to 40 MPa, flow rates 0.05 – 2.00 ml/min

reversed HPLC – methanol or acetonitrile mixture with water or buffer solution

pH of aqueous part !! – ionised substance forms have lower affinity to C18

HPLC columns

conventional – stainless steel, length 3 to 25 cm, i.d. 3 – 5 mm

microcolumns – length 5 – 50 cm, i.d. 0.5 – 2 mm

capillary HPLC

signal detection

: in MF after elution from column

photometers UV-Vis (PDA/DAD), fluorimeters, refractometers, electrochemical detectors, novel mass spectrometry

HPLC detector sensitivity

: refractometric and conductivity detectors 5.10^{-7} g/cm^3
: photometric detectors 10^{-10} g/cm^3
: fluorimetric and amperometric detectors up to 10^{-12} g/cm^3

HPLC application in laboratory diagnostics

fragile chromosome X syndrome

cause of inherited *mental retardation* (2nd most often after Down syndrome)

manifestation: facial dysmorphy – big ears, big face w/ accent. chin and jaw
dystropies: stereotype, ill communication, hyperactivity and bad spatial orientation

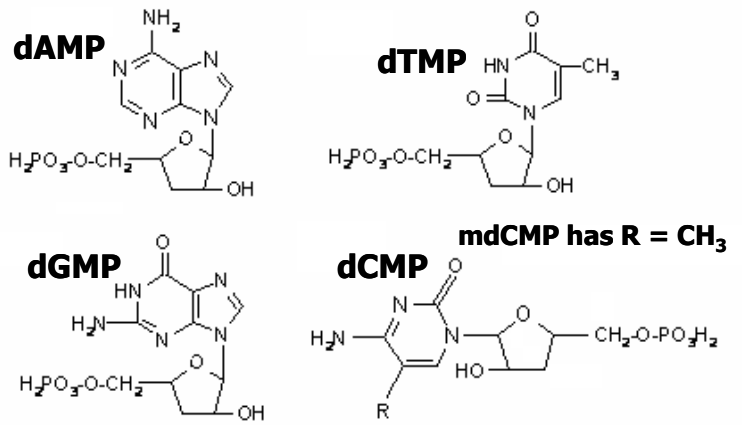
*defect gene **FMR 1*** on chromosome X (fragile **X** mental retardation gene 1)
mutation: trinucleotide repetition multiplication (**SNP**, single nucleotide polymorphism)
: Fra X – CGG repetition multiplication and following **cytosine methylation**
: Cyt methylation of CGG sequence ⇒ gene expression suppression ⇒ Fra X

according to content of **deoxycytidine monophosphate** (dCMP) and its **methylated derivative** (mdCMP) and according to **number of CGG repetitions**, **gene FMR 1** might have **three stadia:**

FRM 1 state	dCMP [%]	mdCMP [%]	CGG number
normal	70 – 100	0 – 30	6 – 53
premutation	50 – 70	30 – 50	< 200
mutation	10 – 50	50 – 90	> 200

estimation of dCMP/mdCMP content ratio

diagnostics of Fra X syndrome



cell ⇒ DNA *endonuclease **Msp** I*>

nucleotide triphosphates *exonuclease III*>

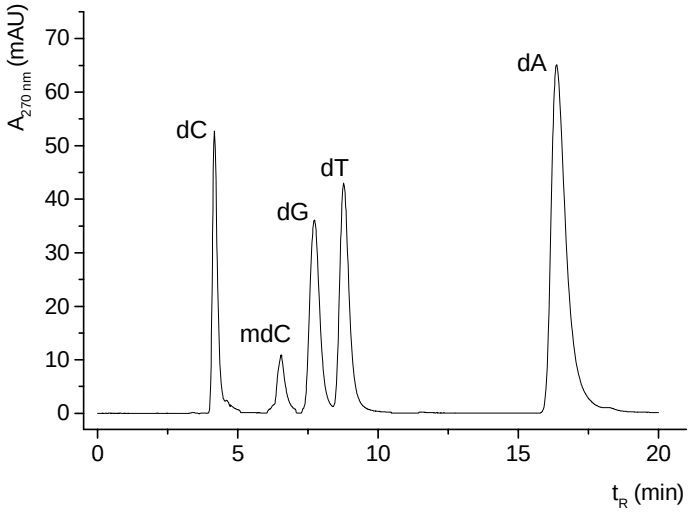
nucleotide monophosphates

$(mdCMP / dCMP)_1$ *vs.* $(mdCMP / dCMP)_2$

%mdCMP = mdCMP / (mdCMP + dCMP)

checking ratios

(mdCMP + dCMP) / dGMP = 1
dAMP / dTMP = 1



improving sensitivity in order of magnitude
UV-Vis ⇒ **fluorescence detection**

derivatisation by **dansyl chloride**

capillary electrophoresis (CE)

principle

separation according to different electrophoretic mobilities of analytes in potential gradient of electric field

d – distance, along which analyte migrates after introduction of potential **E** between two electrodes with distance **S** in time **t**

$$d = \mu * t * (E / S)$$

μ – electrophoretic mobility (it is function of charge, molecular mass and shape; friction forces – slow-down migration in viscose electrolyte)

separation $\Delta d = (\mu_1 - \mu_2) * t * (E / S)$

the most utilised methodics

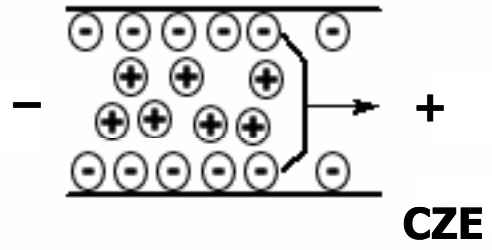
CZE – capillary zone electrophoresis
PAGE – polyacrylamide gel electrophoresis

CZE – capillary zone electrophoresis

: silica capillary of small diameter (50 – 75 μm)

: negatively charged surface (*silanol groups*)

: voltage ~ 10 – 60 kV



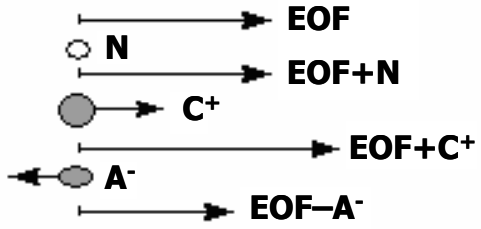
electroosmotic flow (**EOF**)
: basic phenomenon of CE

silanol groups (pK_a *ca* 6.2) ⇒ compensated by buffer cations ⇒ **Stern electric double-layer**

potential gradient ⇒ hydrated cations are in motion ⇒ shift of a whole electrolyte (**EOF**) ⇒ carries towards cathode all present substances (neutral and also ionic)

EOF rate – ↑ w/ ↑ **buffer** pH and voltage; ↓ w/ viscosity

background electrolyte (conductive medium) – buffer (**BE**)



: fundamental influence on constant mobility of separated substances

H_3N^+-CH_2-COOH ⇔ (H_2N-CH_2-COOH ⇔ H_3N^+-CH_2-COO^-) ⇔ H_2N-CH_2-COO^-

separation of neutral species – adding tenside into BE

micelle creation ⇒ encapsulating neutrals ⇒ charged micelles are separated

separation based no their **different solubility in micelles** or based on **differences in distribution coefficients** of analytes between **aqueous** and **micellar phase**

<u>signal detection</u>

: UV-Vis – sensitivity 10^{-13} up to 10^{-16} mol (10^{-5} up to 10^{-8} mol/l)

: amperometric detection – 10^{-11} mol/l

: laser induced fluorescence (**LIF**) – 10^{-14} up to 10^{-16} mol/l
:: derivatisation by fluorophore

PAGE – polyacrylamide gel electrophoresis

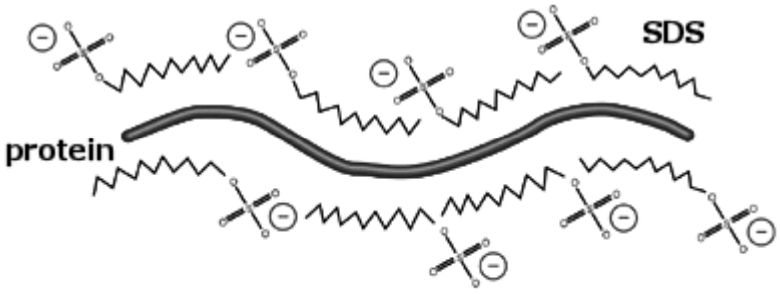
migration in polymeric matrix
(agar, starch, agarose, polyavrylamide)

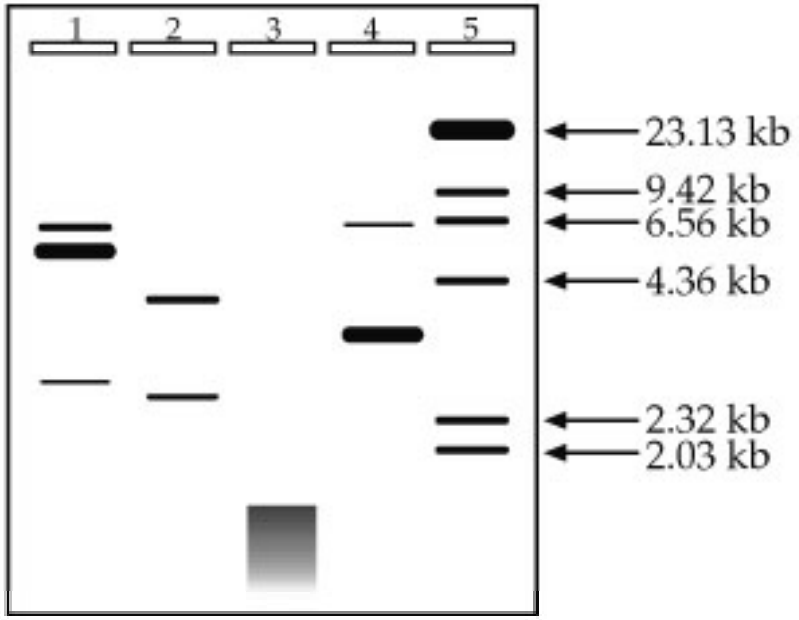
: separation of macromolecules

modes : isocratic, gradient

: denaturing (SDS; *Lämmli*)

: non-denaturing





detection:
staining, densitometry

proteins
Coomassie Blue, silver, SYPRO ruby, Cu(II), Zn(II)
NA
SYBR green, ethidium bromide, Acridine orange

CZE application in laboratory diagnostics

metabolic disorders

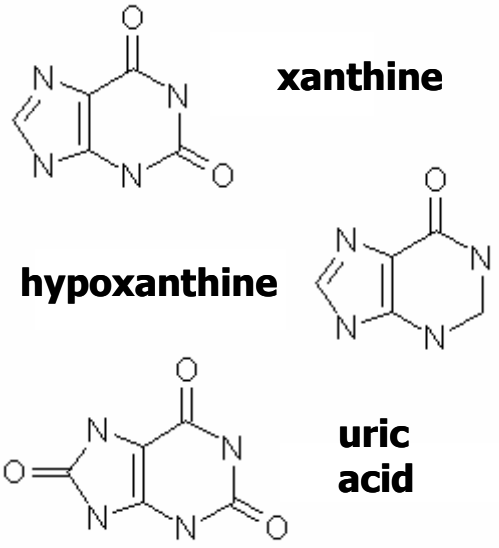
: disrupted metabolic pathways (blocked, absence of enzyme /genetic/)

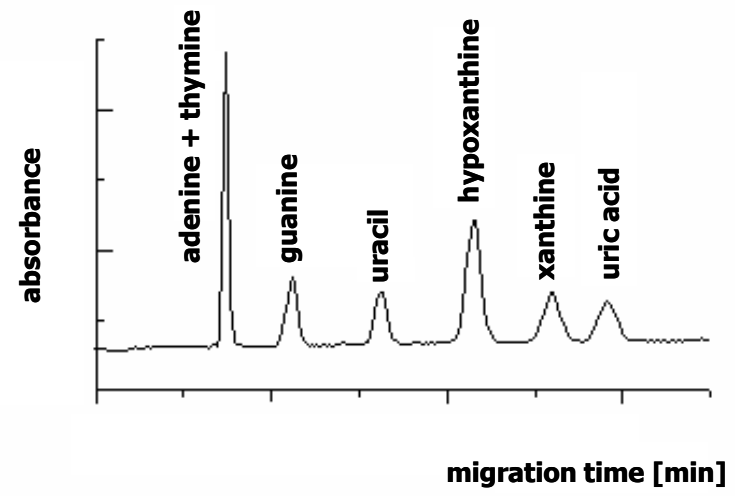
:: *presence of products of defective metabolism* in urine and blood

determination of purines and pyrimidines content

diagnostics of xanthinuria and hypoxanthine phosphoribosyltransferase deficit

BE – borate buffer 30 mmol/l pH 10.2; hydrodynamic injection 10 s, separation voltage 15 kV, temperature 25 °C, UV-detection at 260 nm





mass spectrometry (MS)

: physical method; determination of molecular or atomic mass of analyte

principle

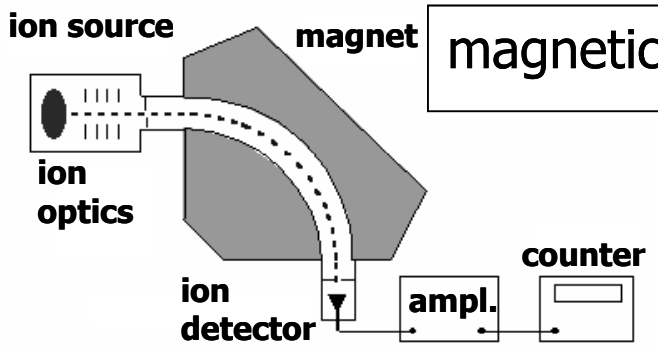
molecules of analyte are **ionised in gaseous phase**; then they are **separated** either in time or space according to mass to charge ratio (*m/z*)

ionisation ⇒ mass analysis ⇒ ion detection

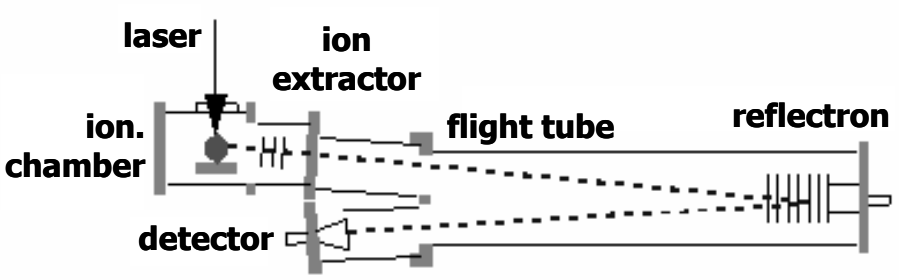
ionisation – by field or high energetic particles (electron, photon, atom)
soft *vs*. hard ionisation

mass analysis

magnetic sector



time-of-flight detector



ion detection – system of dynodes

tandem MS

serially connected *several mass analysers*
+ *secondary ionisation* – **collision cell**

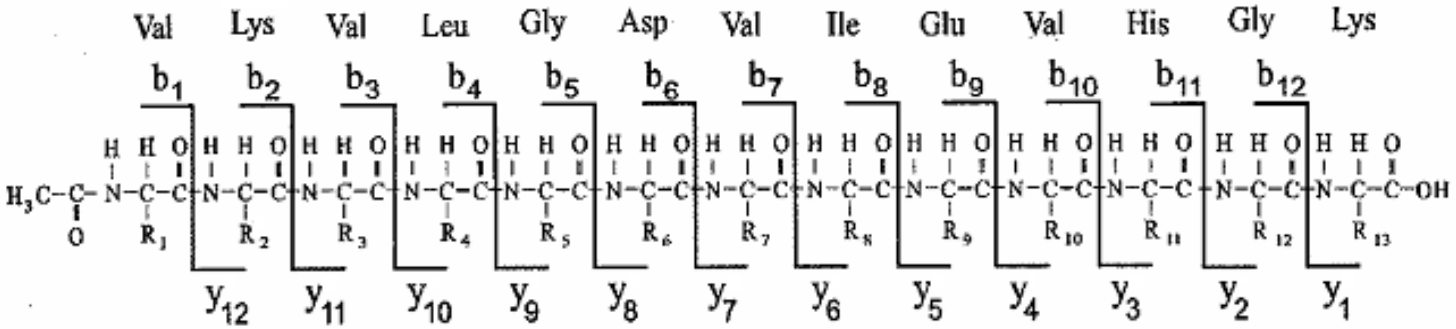
: first analyser separates ions according to *m/z*

: ion selection at certain *m/z*

: secondary ionisation-fragmentation

: fragments separation in second analyser

: fragment ion detection



analysis of proteins and nucleic acids

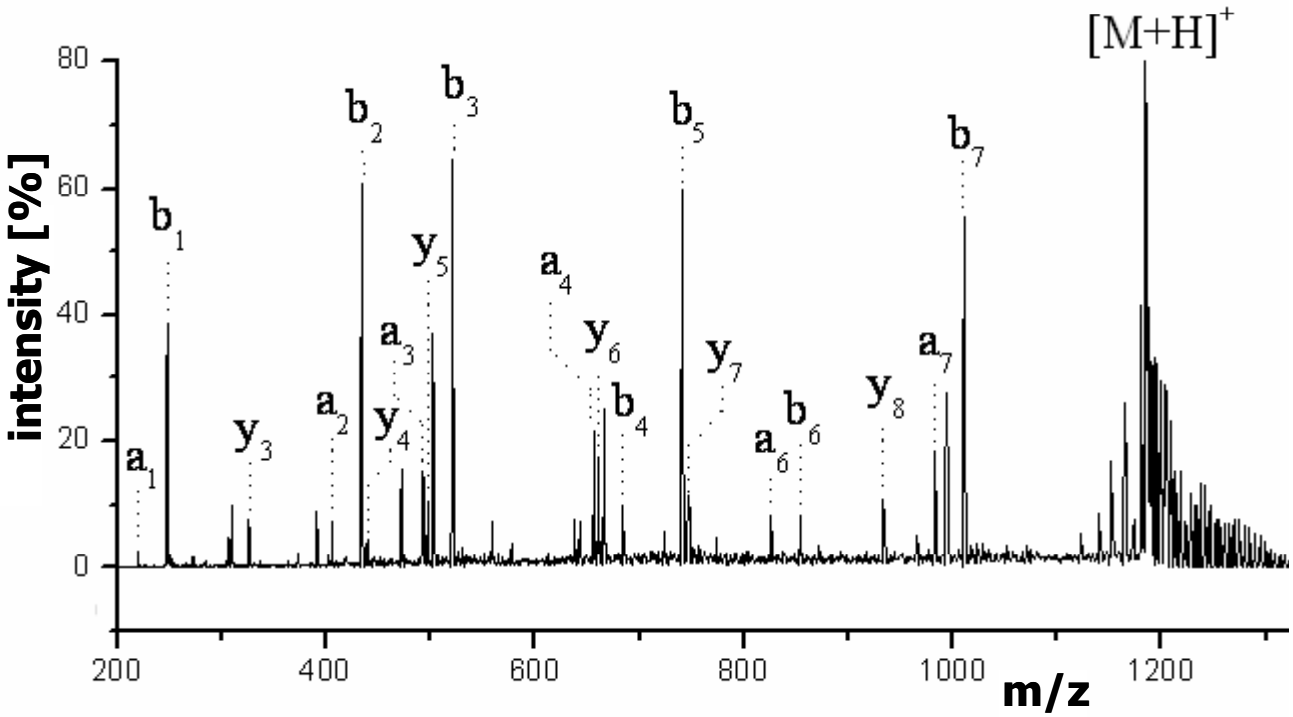
MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight)

MS application in laboratory diagnostics

MALDI PSD TOF

post-source decay

hormone identification by tandem MS



a_n, b_n n = 1 2 3 4 5 6 7 8

pGlu-His—Trp—Ser—Tyr—Gly—Leu—Arg—Pro—Gly–NH_2

8 7 6 5 4 3 2 1 = n y_n

luteinising hormone-releasing hormone (LHRH)

immunochemical analysis
in praxis of clinical diagnostics

immunity system (IS) – information system
: responsible for identity and integrity of individual

components: lymphocytes and antibodies (Ab)
localisation: in blood and lymphatic circulation

function: tolerate intraneous structures, label and remove extraneous

: recognition of intraneous from extraneous

: removal of extraneous: micro-organisms, cells, tissues, proteins and substances with antigenic activity

antigen + **antibody interaction**
: analytical use of immunity system – affinity (specific) interaction
:: non-covalent
:: reversible
:: variable

antigen (*antibody generator*) **(Ag)**

: contains **determinants** (epitopes) ⇒ induces imm. reaction (immunogen)

:: **macromolecule** (> 8 kDa)

:: macromolecular carrier + **hapten**

bacterial toxins, lipopolysaccharides, agglutinins, rheumatoid factor, endotoxins, allergens

antibody (Ab)

: recognise **determinants** ⇒ response of IS to Ag

: specific Ab reacts with particular Ag – **labels it**

: binds to determinants by means of **binding site** (paratope)

:: **immunisation** – exposing IS to new Ag ⇒ creation of new Ab

monoclonal, *polyclonal* Ab – equivalence of epitopes

Ab **cross reactions** – interaction of one paratope with similar epitopes

immunoglobulins

IgA (15 – 20%) – on the surface of exocrine gland mucous membrane

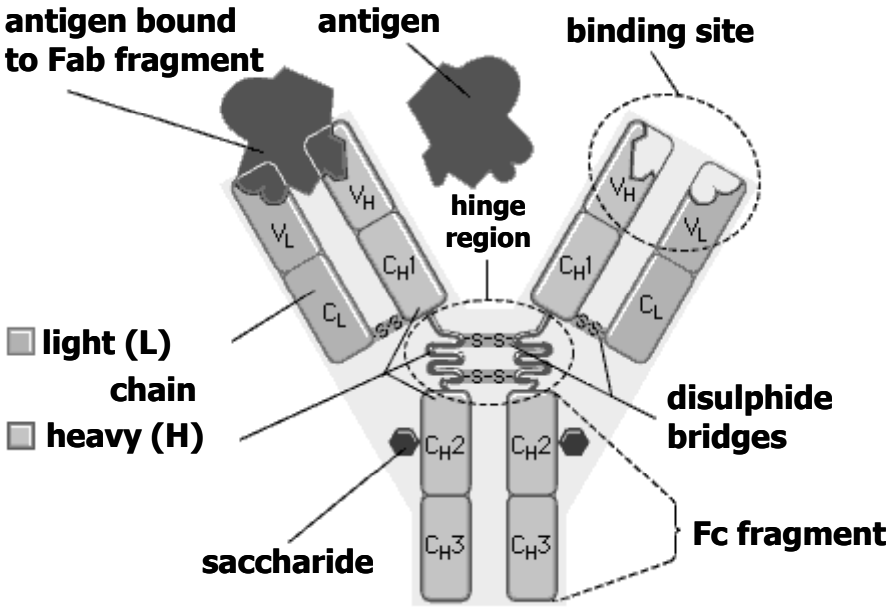
IgD – activation of B-cells

IgE – w/ allergies; double binding: allergen-IgE-glycoprotein

IgG (75%)

IgM (3 – 10%) – intravascular space

antibody – detail of structure



immunoanalysis

interactions

: direct – precipitation, light scattering
: indirect – agglutination, complement bond
: labelling – immunoassay
:: homogeneous
:: heterogeneous
::: direct
::: competitive
::: indirect, sandwich

medium

: solutions
: gel (immunodiffusion)
: immobilisations & their combinations
:: tissues

detection

within direct and indirect immunoanalyses

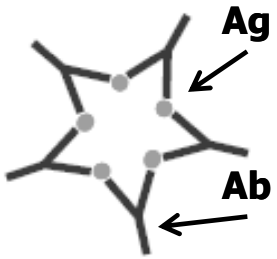
: visual
: optical (nephelometry, turbidimetry)
: mass spectrometric

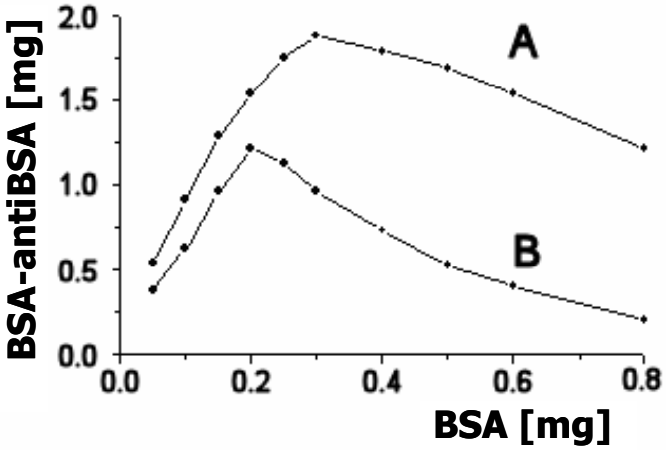
within labelling immunoanalyses

: spectrophotometric, fluorimetric
: radiometric
: electrochemical

direct immunoanalysis – precipitation reactions

bivalent Ab (precipitin), **macromolecular** Ag (precipitinogen), 1:1
precipitate – visible coagulate originated in Ab and Ag molecules only





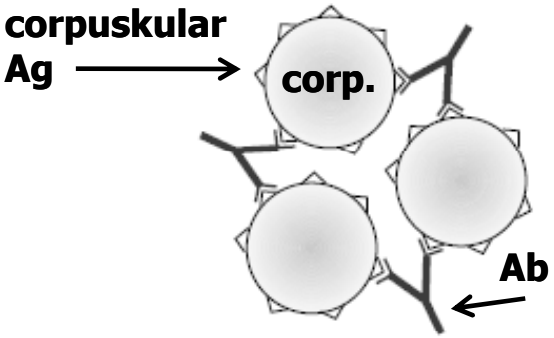
desegregation substances – prevent precipitation

precipitation curve

direct immunoanalysis – agglutination reactions

polyvalent Ab (agglutinin)
corpuscular Ag (agglutinogen)

agglutinate – visible coagulate
originated in macroscopic particles

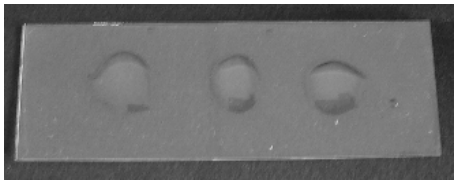


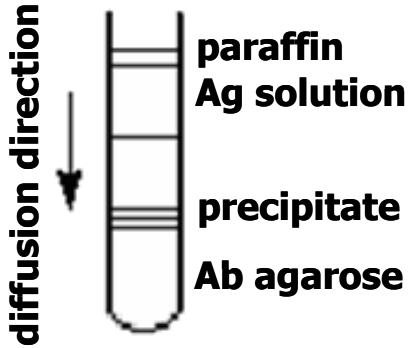
corpusculation Ag: immobilisation on corpuscule (macroscopic particle)
: haemagglutionation, cytoagglutination, latex; complement system

qualitative methods

ring p. – precipitation ring on inter-phase Ag – Ab

plate p. – „plate" immunity reaction





Oudine method – two gel layers, with Ab or Ag

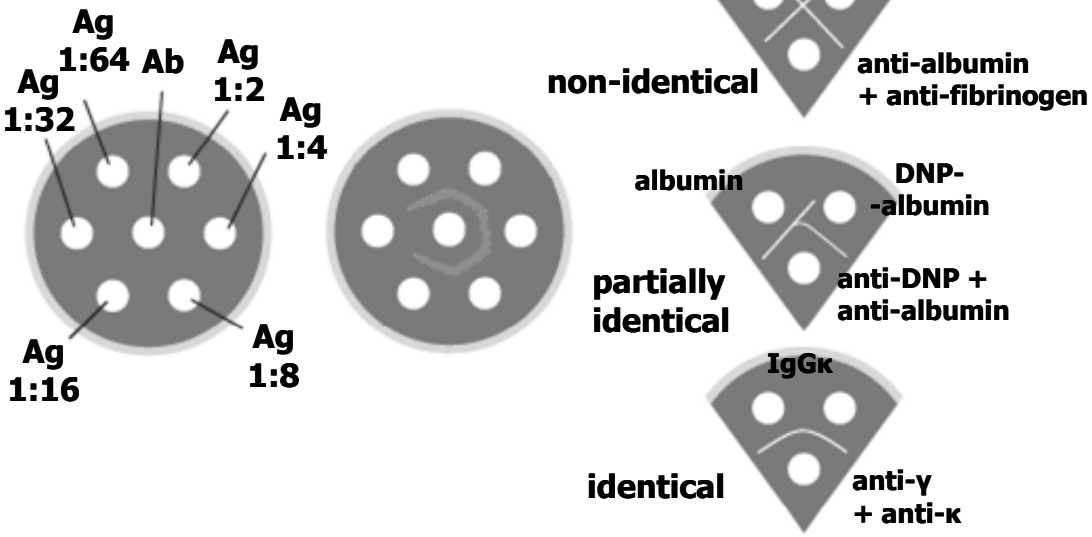
Oakley–Fulthorp method – two gels, with Ab or Ag, detects more [AbAg]

Ouchterlony method

: double radial immunodiffusion

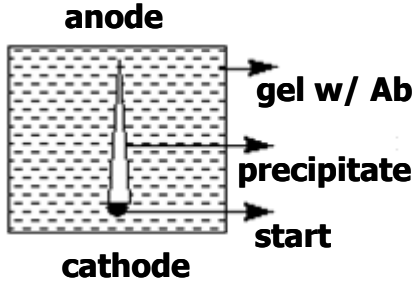
:: concentric system of pits

Ab in the middle
Ag around



Williams & Grabar method

: immunoelectrophoresis

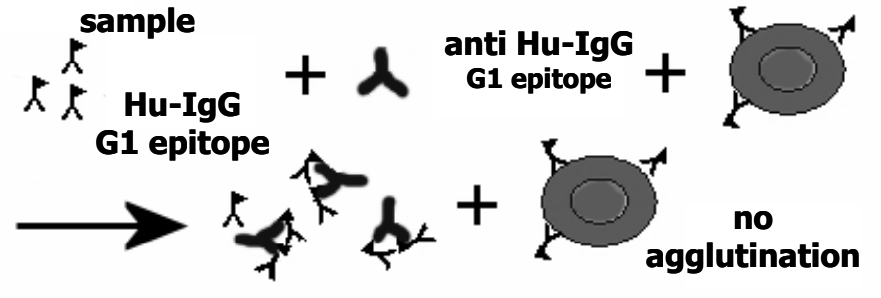


:: starting pits with Ag, lengthwise pit with free diffusing Ab

counter-directive immunoelectrophoresis

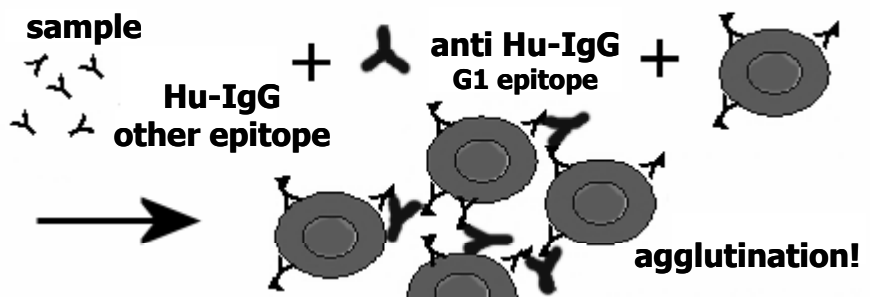
: on opposite ends Ab and Ag, precipitation zones

haemagglutination inhibition test (HIT)



positive – blood sediments

Ab presence ⇒ **no** agglutination

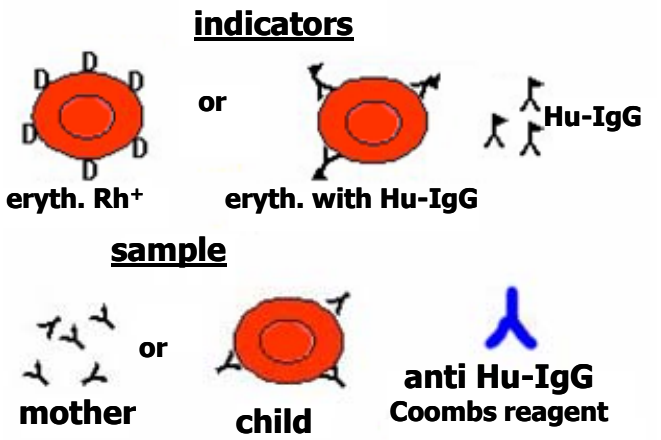


negative – agglutinated erythrocytes

Ab absence ⇒ **agglutination**

Coombs test (CT)

: revelation of antibodies against red-blood cells

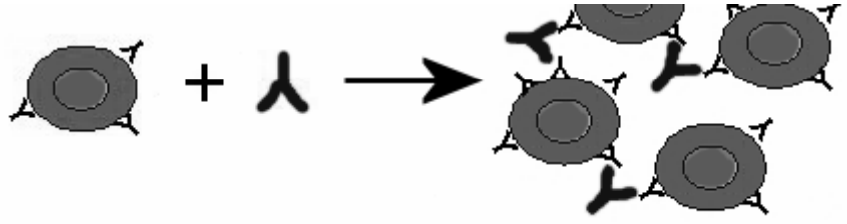


: determination of Ag_1 by un-complete Ab^i

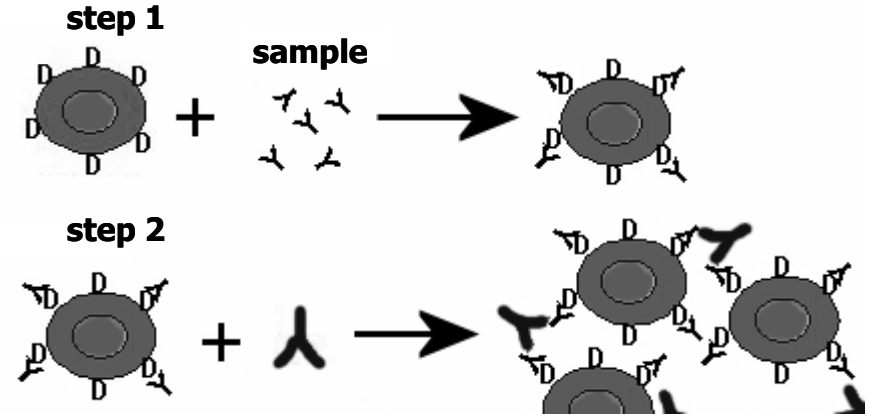
: primary complex: Ag_1Ab^i

: secondary complex: $Ag_1Ab^iAg_2$

direct CT

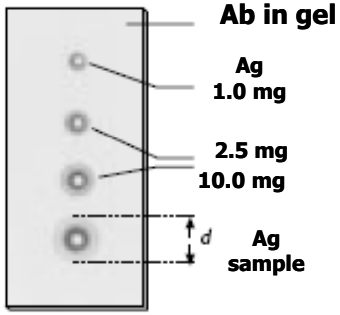


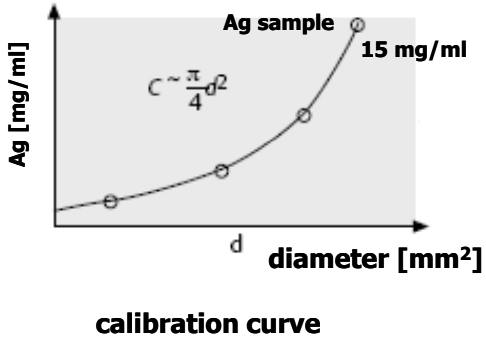
indirect CT



quantitative methods

gravimetric p. – p. weighting, nephelometry, turbidimetry





calibration curve

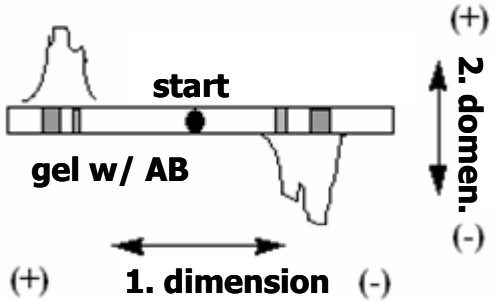
Mancini method
: radial immunodiffusion

:: gel contains Ab
:: line of pits
::: standards (calibration) + sample

Laurel method
: rocket, continuative, cross IELFO; electroimmunodiffusion

:: gel contains Ab, starting pits with Ag; flame shape – semi-quantity

Clarke & Freeman method – 2D IELFO
: 1D separates Ag, 2D interactions with Ab in gel in 90°



tandem IELFO
: similar to 2D IELFO with standard amounts of Ag

immunoanalysis with labelled reagents

immunoassay; **serum neutralisation**

univalent Ab, soluble Ag

: suitable Ab – **monoclonal**

: suitable label (Ab, Ag) – fluoro- or chromophore, radioactive isotope, enzyme

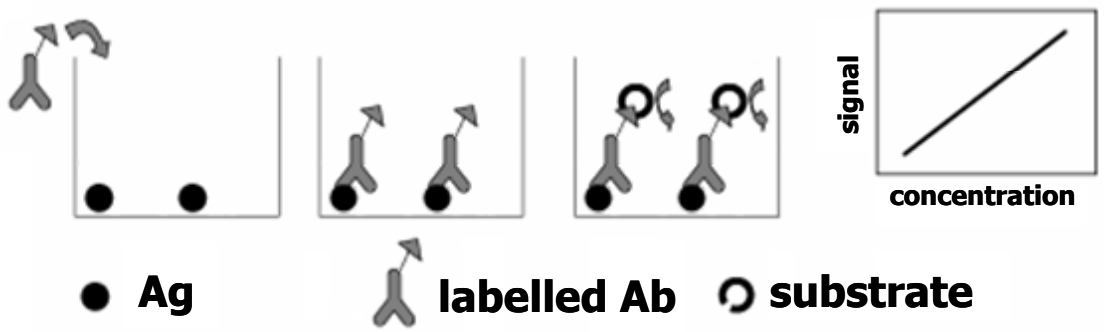
: separation system – immobilisation

: detection

: standard or control measurement

simple immunoassay

: immobilisation of Ag
: known amount of Ab
: bound of Ab to Ag
: Ag contant determination
:: directly – labelled Ab_1
:: indirectly – labelled Ab_2 against Ab_1



$$Ag + Ab^* \leftrightarrow [AgAb^*]$$

advantages: low Ab consumption

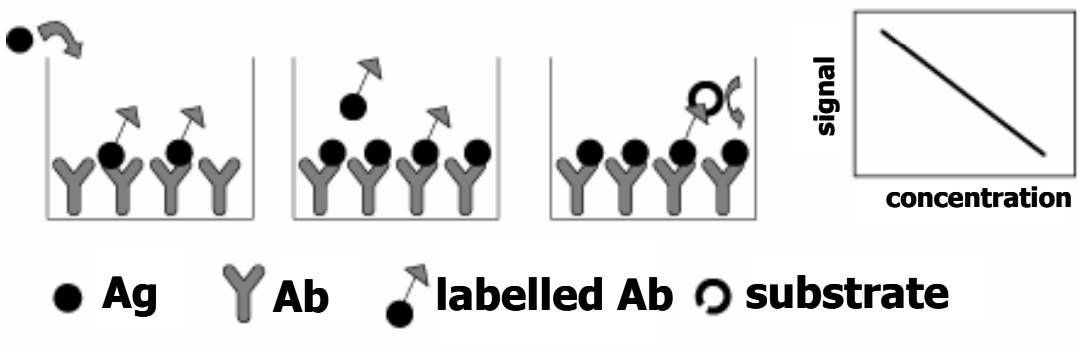
disadvantages: sample immobilisation, labelled Ab,
low specifity, low selectivity (only within direct detection)

competitive immunoassay

limited reagent assay

: limited number of binding sites on plate with Ab
: unknown amount of Ag
: binding site competition with known amount of labelled Ag

:: determination of labelled Ag either free or bound ⇒
unknown amount of Ag



$$2Ab + Ag + Ag^* \leftrightarrow [AbAg] + [AbAg^*]$$

advantages: low Ab consumption

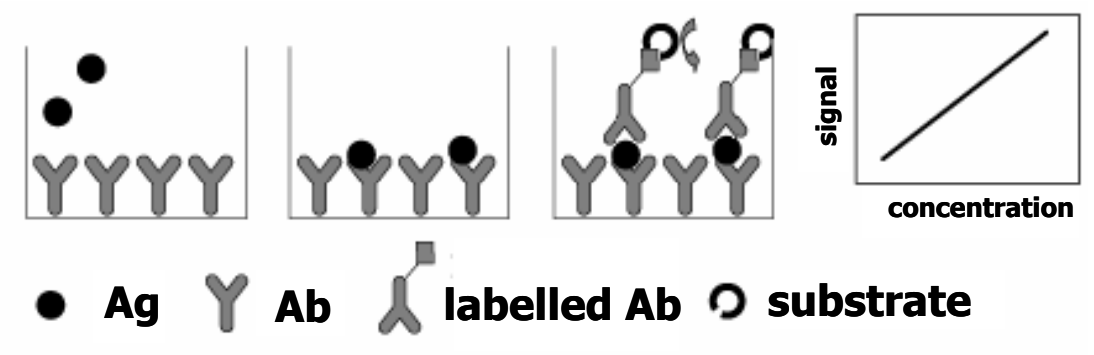
disadvantages: low specificity, low selectivity

sandwich immunoassay, immunometry

reagent surplus assay

: two or more Ab guarantee IA specificity
: theoretical limit of detection is **one analyte molecule**

:: unbound labelled Ab is used to quantify the analyte



$$x\ Ab_1 + y\ Ag \rightarrow y\ [Ab_1Ag] + (x–y)\ Ab_1$$

$$z\ Ab_2^* + y\ [Ab_1Ag] \rightarrow y\ [Ab_1Ag]\text{-}Ab_2^* + (z–y)\ Ab_2^*$$

advantages: elevated sensitivity, elevated specificity

disadvantages: labelled Ab, Ab wasting

FIA – fluorescent immunoassay

fluophore labelled Ab or Ag

design

homogeneous

: Δ fluorescence properties of labelled Ab/Ag when [AbAg] is created

heterogeneous

: fluorescence of bound labelled Ab/Ag after washing-out the unbound one

fluophores used:

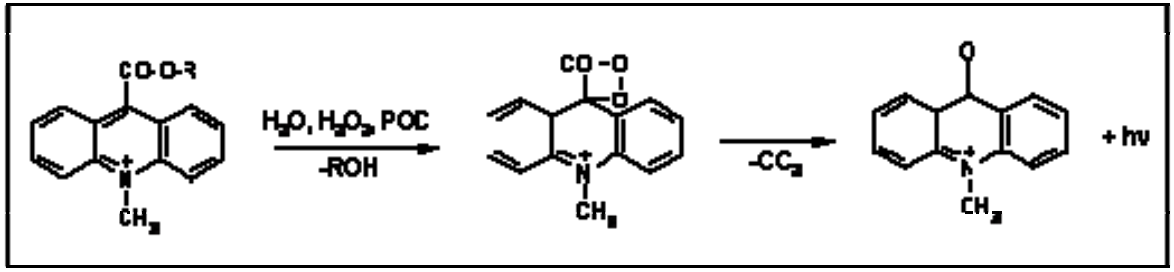
fluorescein, fluorescein isothiocyanate (FITC), rhodamine, umbelliferon, 8-anilin-1-naphthalen sulphate (ANS), lanthanoid chelates (Eu, Tb, Sm)

advantages	disadvantages
: specificity	: background (quenching)
: sensitivity	: instrumentally demanding
: probe size	: limited choice of labels

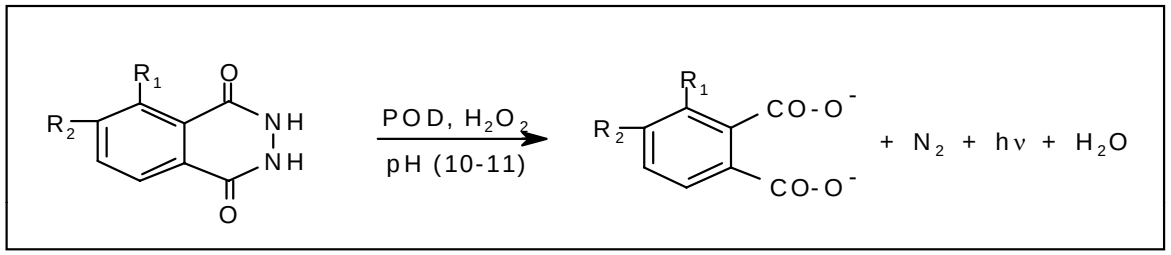
LIA – luminescence immunoassay

chemiluminiscence

acridinium esters – **need no catalysis**

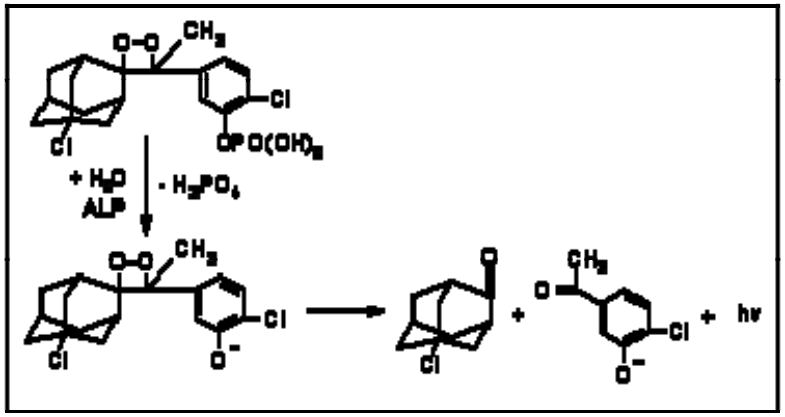


luminol, isoluminol – **needs hydrogen peroxide**; peroxidase



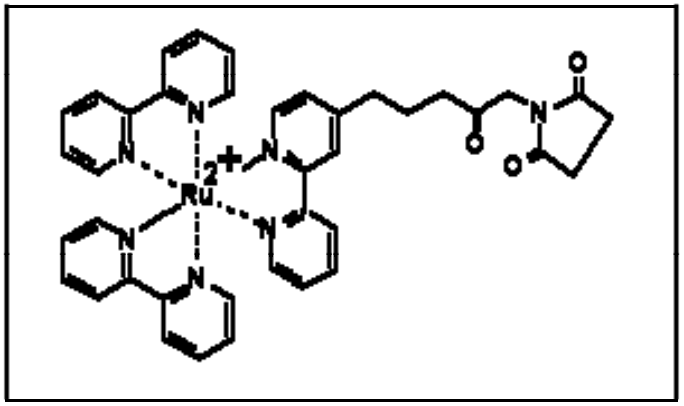
enzymatically induced luminiscence

component: 1,2-dioxoethane



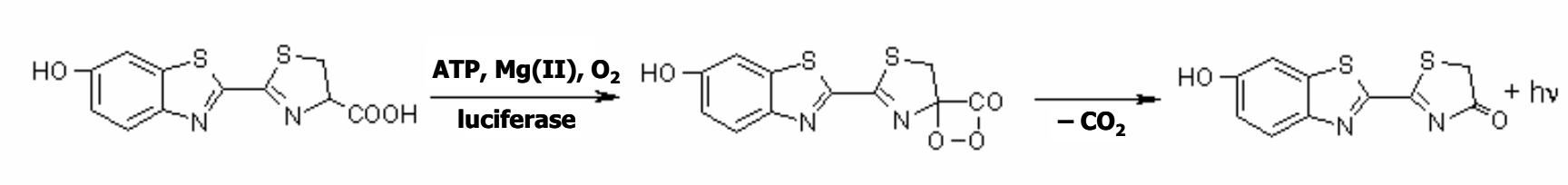
electrochemoluminiscence

NHS ester of $Ru(bpy)_3^{2+}$



anchored on magnetic carrier
label oxidation on Pt or Au electrode at 2 V
+ TPA (tripropylamine) in buffer – *regeneration*

bioluminiscence; luciferin/luciferase



advantages
: S/N ratio
: sensitive
: probe size

disadvantages
: instrumetally demanding

RIA – radioiosotopic immunoassay

radioisotope (unstable / radioctive isotope) labelled Ab or Ag

design:
: heterogeneous, conmpetitive

isotopes used:
^{125}I – protein Ag; 60 days; ^{14}C – haptens; ^{3}H – steroid hormones
other isotopes – ^{75}Se, ^{57}Co

detection: according to radiation – α and β (*scintillator*) or γ (*counter*)

advantages	disadvantages
: flexibility	: toxicity
: sensitivity	: shelf life
: probe size	: waste disposal

EIA – enzyme immunoassay

enzyme labelled Ab or Ag

design

homogeneous

: competitive **Ab + Ag-E + Ag** $\rightarrow$ **[Ab*Ag-E] + Ag-E + Ag**

heterogeneous

: competitive
: direct
: indirect/sandwich

enzymes used:
alkalic phosphatase, peroxidase, galactosidase, glucose oxidase, dehydrogenase, lysozyme and malate dehydrogenase

advantages	disadvantages
: versatility	: unstable
: purposeful	: probe size
: signal amplification	: heterogeneous

immunoanalysers

: fully automated immunoanalyses

: mostly based on heterogeneous sandwich immunoassay (EIA, FIA)
:: every manufacturer has its own design modifications (patents)

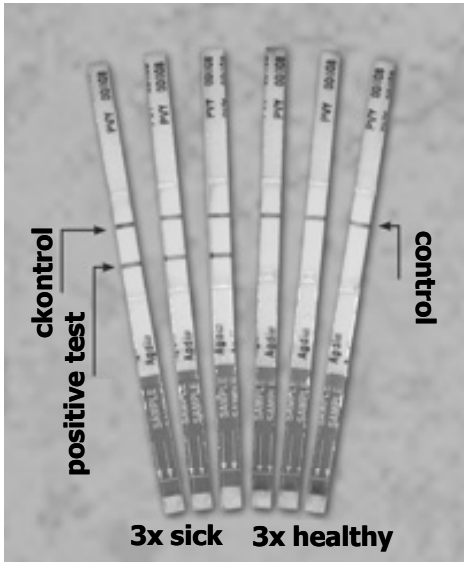
: relatively low costs (mass Ab production)

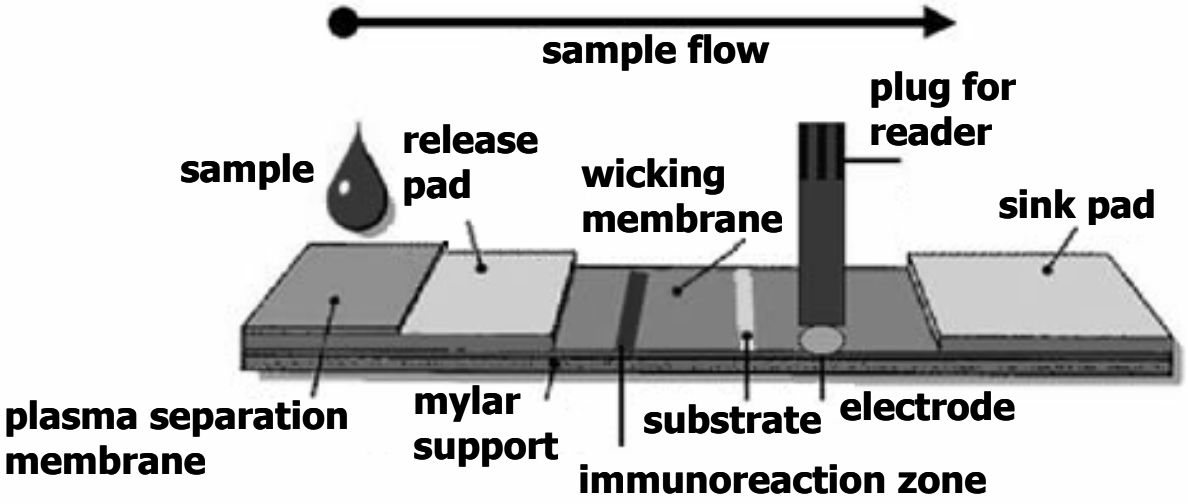
immunostrips

: specialised one-target disposable immunoanalyses

: mostly based on heterogeneous sandwich immunoassay (EIA)

: relatively low costs (mass Ab production)





point-of-care meters based on immunoanalysis

: specialised single- or multi-purpose mini-immunoanalysers

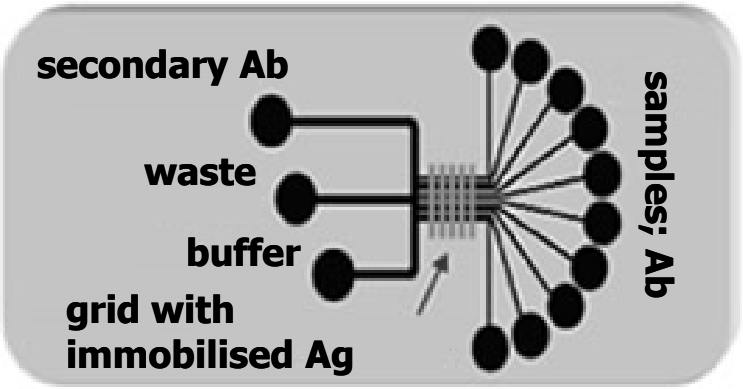
: mostly based on heterogeneous sandwich immunoassay (EIA)

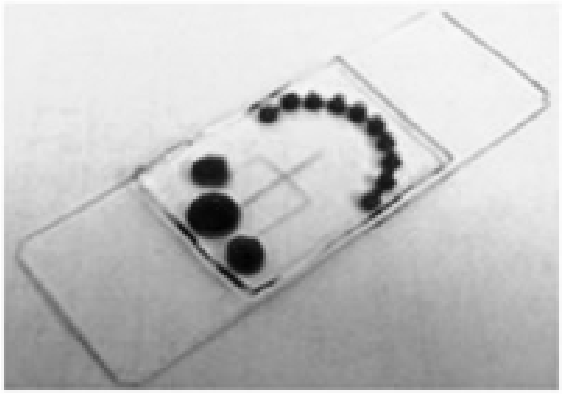
: originated in usage of immunostrips

immunochips

: further miniaturisation

: classical advantages of chip technology



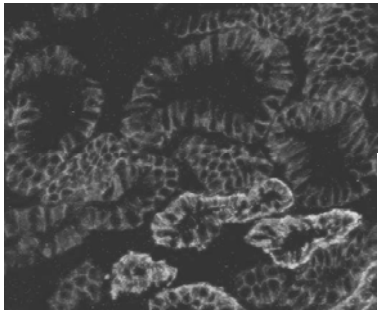


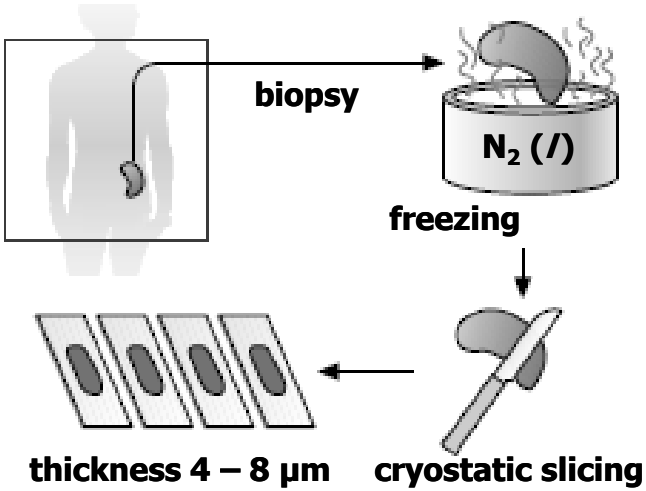
: OptoLabCard+Skinpatch
:: lab-on-foil

⇒ **The SmartBioPhone™**

immunohistochemistry

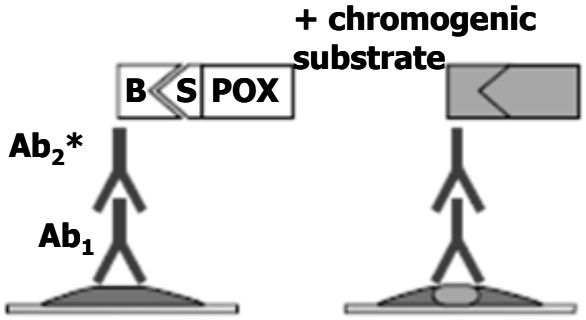
immunocytochemistry



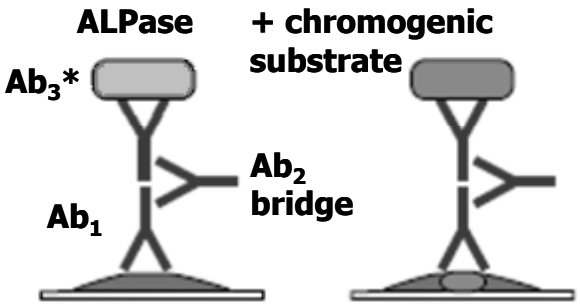


application of immunologic methods to study
tissues and isolated cells
: tumour or pathogenic changes detection

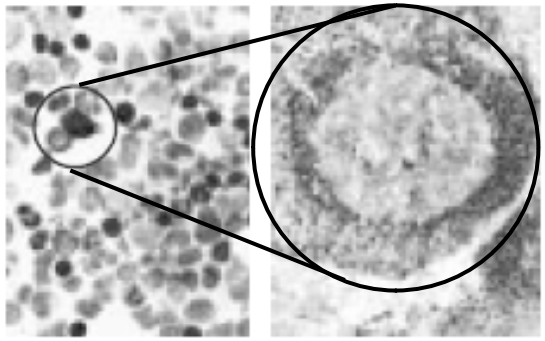
mostly flurophore, metal or enzyme labelling
: direct, indirect, indirect three-stage (Ab-„bridge") – signal amplification



BSPOX method



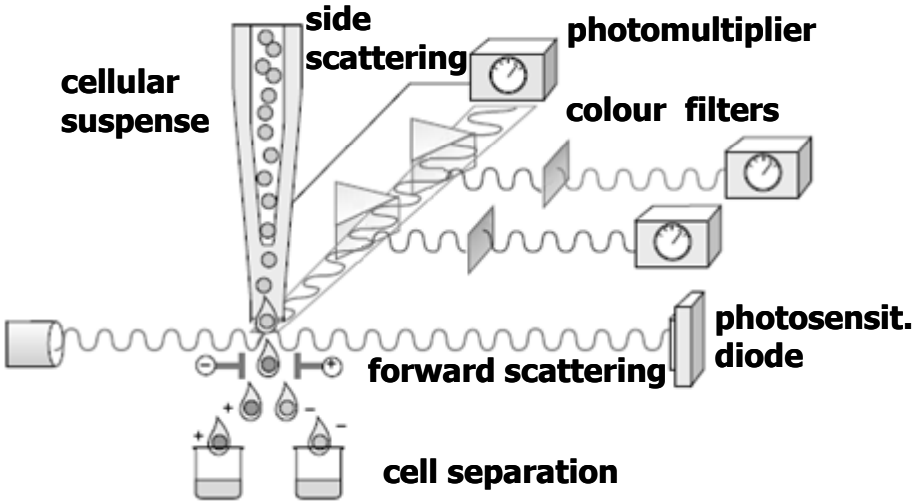
APAAP method

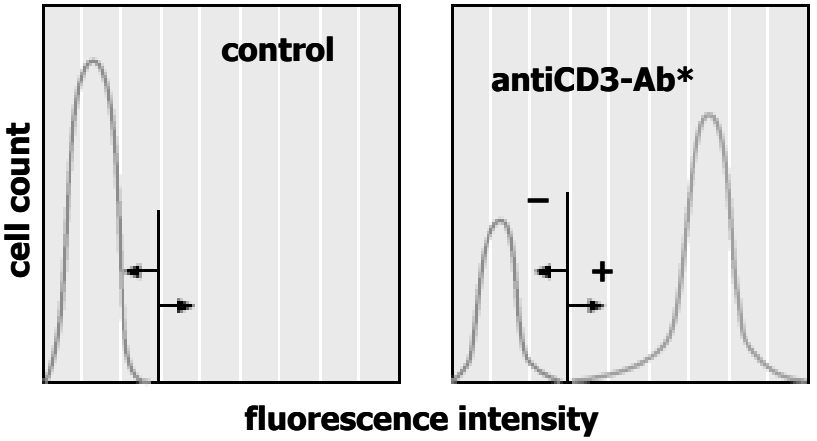


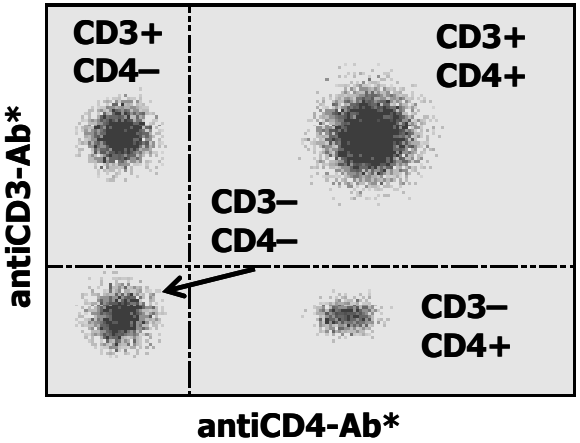
B – biotin, S – streptavidin, POX – peroxidase

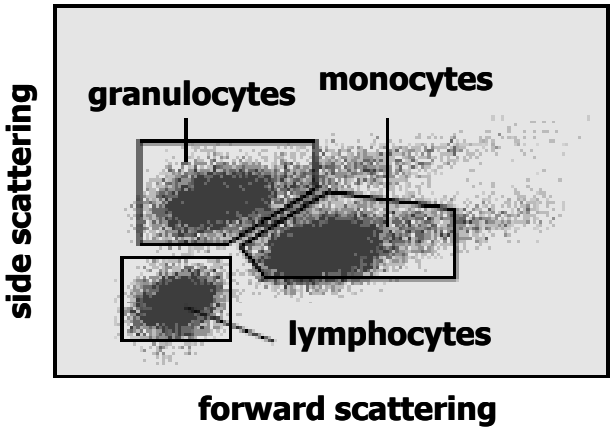
application of immunologic methods to study IS cells
: detection of pathogenic changes in IS

mostly Ab flurophore labelling
: flow-cytometry

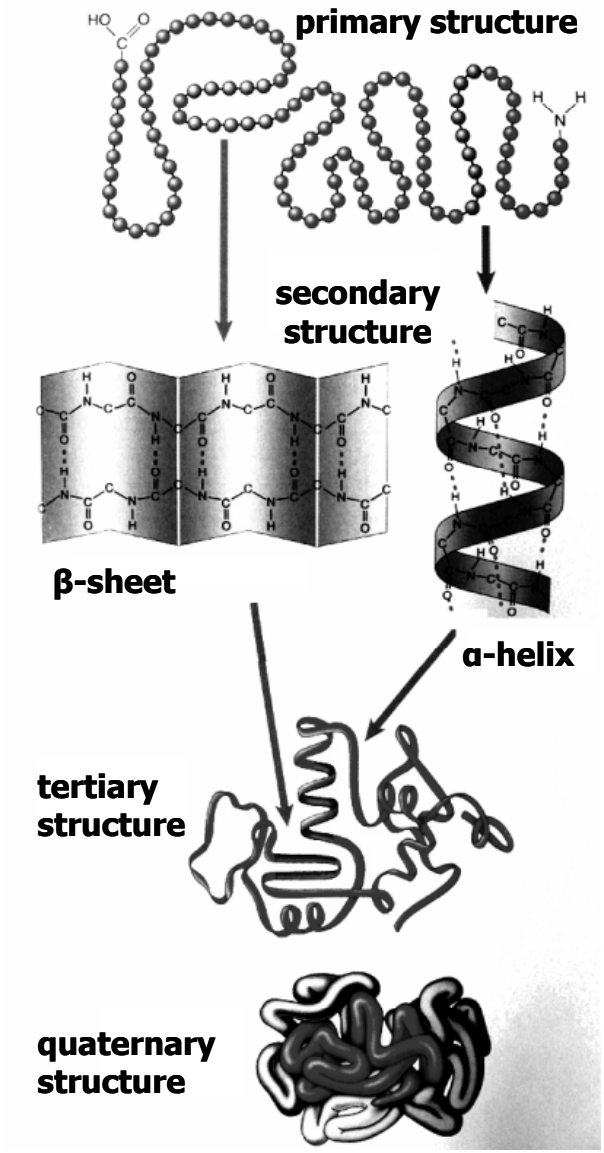








protein analysis
in clinical biochemistry



protein structure

primary structure – amino acid sequence

secondary structure – spontaneous assembly

tertiary structure – 3D (induced by chaperonines)

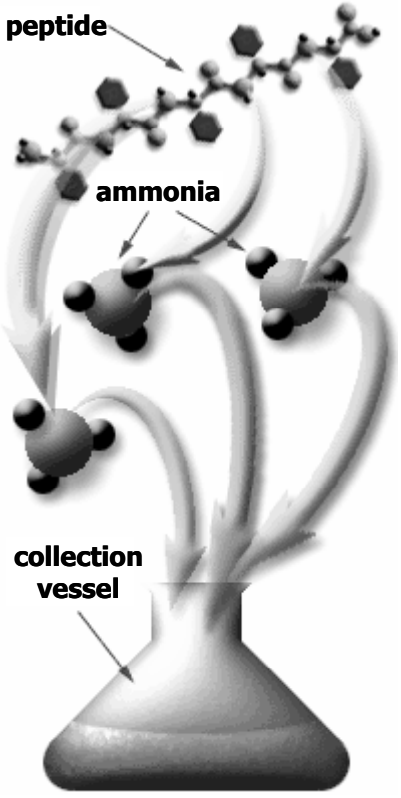
quaternary structure – supracomplexes

specific – determination of particular protein
non-specific – determination of total protein *or* just one protein

non-specific determinations

Kjeldahl method

polypeptide ⇒ **ammonia**, p. content is defined as organic nitrogen



sample is mineralised by concentrated sulphuric acid in presence of catalyst; aminic nitrogen ⇒ **ammonium ions**

ammonium ions are turned into ammonia by heating and are collected by distillation in collection vessel, where it is again turned into ammonium ions

ammonium ions are determined by **neutralisation titration**

spectrophotometric determination (SPEFO)

205; **260** and **280** nm
: peptide bond; aromatic structures

absorbance is influenced by
: secondary, tertiary and quaternary structures
: factors such as pH, ionic strength *etc.*

(un)known sample
: calibration on BSA $\Rightarrow$ absorption coefficient at 205 or 280 nm

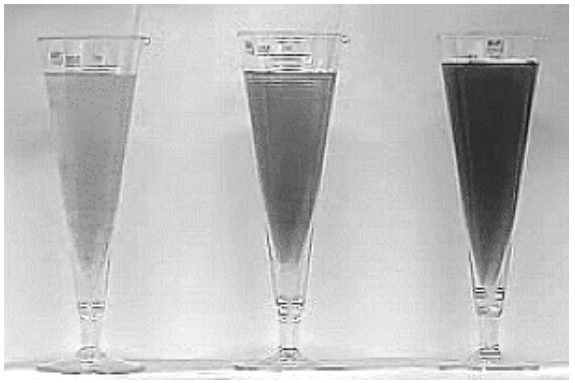
unknown sample with possible DNA contamination
: measurement at 260 and 280 nm
: concentration (mg/ml) = $(1.55 \times A_{280}) - (0.76 \times A_{260})$

non-invasive (consumes no sample)
: imprecise, easy false positive results – contamination

SPEFO – biuret method

: invasive, time consuming, high sample consumption

biuret agent:
25 g potassium sodium tartrate
0.75 g copper sulphate $5H_2O$
1.25 g potassium iodide
:: all in 100 ml 0.2 M NaOH



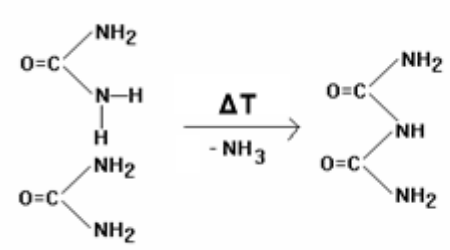
biuret agent | glycine | egg white

: sample of 1 – 10 mg/ml protein

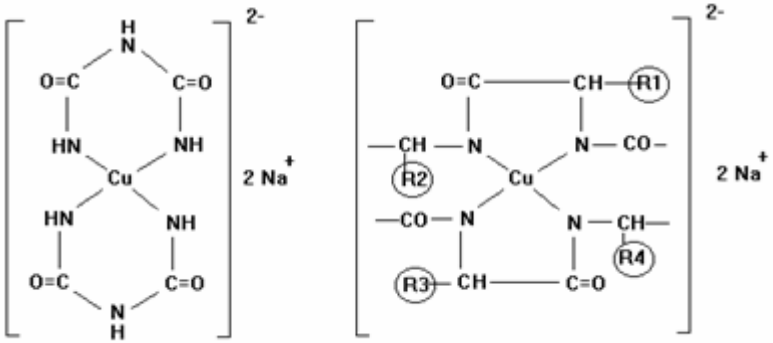
: calibration on BSA

: add biuret agent, stir and let stay for 20 min

: absorbance at 550 nm



tartrate – solubility of Cu(II) in alk. #
iodide – catalysis Cu(II)⇒Cu(I) reduction **?**



lipaemic serum – false positive results

also these groups react
$–CONH_2$, $–C(NH)(NH_2)–$ and $–CSNH_2$

SPEFO – Lowry method

: variation on biuret method

in alkali media with copper ions

: after adding **Folin-Ciocâlteu reagent**, it binds to protein, then is slowly reduced to heteropolymolybdenite blue by means of Cu(II)-catalysed oxidation of aromatic acids; colour changes **yellow** to **blue**

: 100 µl sample + 1.0 ml Lowry reagent (alkali $CuSO_4$)
: incubation 30 min at 25 °C
: + 100 ml of Folin-Ciocâlteu reagent
: incubation 30 min at 25 °C
: absorbance at 595 nm

Folin-Ciocâlteu

750 ml water; 100 g Na_2WO_4; 25 g Na_2MoO_4;50 ml 85% phosphoric acid; 100 ml conc. HCl; 150 g Li_2SO_4 + few drops of Br_2

proteins are different, calibration on BSA is not sufficient

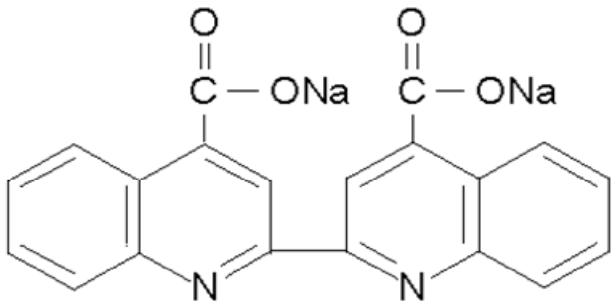
interferents:

barbiturate, CAPS, $CsCl_2$, citrate, cysteine, diethanolamine, dithiothreitol, EDTA, EGTA, HEPES, mercaptoethanol, Nonidet P-40, phenol, polyvinylpyrrolidon, sodium deoxycholate, sodium salicylate, thimerosol, Tricin, TRIS and Triton X-100

SPEFO – modified/Smith-Lowry method

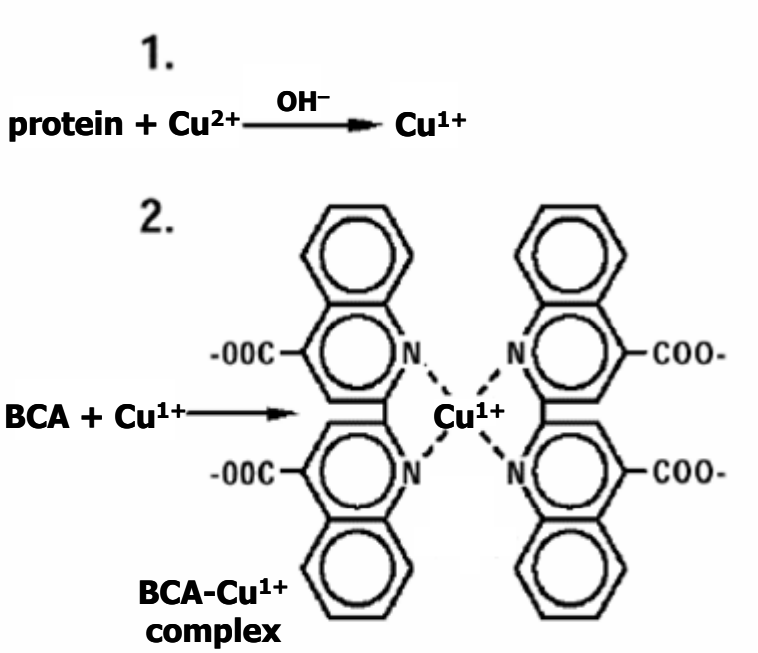
: similar procedure as for Lowry method
: absorbance at 562 nm
: sensitivity not higher, but lowered influence of contaminants

method very sensitive because of precise pipetting



bicinchoninic acid – BCA

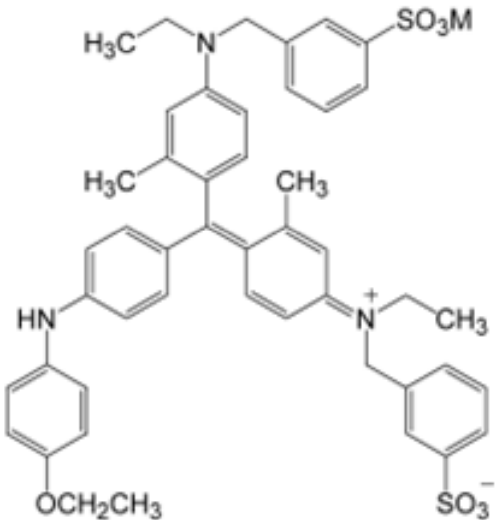
mechanism



SPEFO – Bradford method

Δ absorbance of Coomassie Brilliant Blue G-250 after binding to protein

: calibration on BSA (1 mg/ml)
: absorbance at 595 nm without incubation

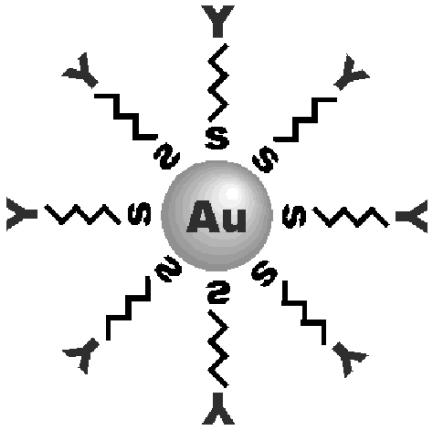


not sensitive to interferents,
except for high conc. of tensides

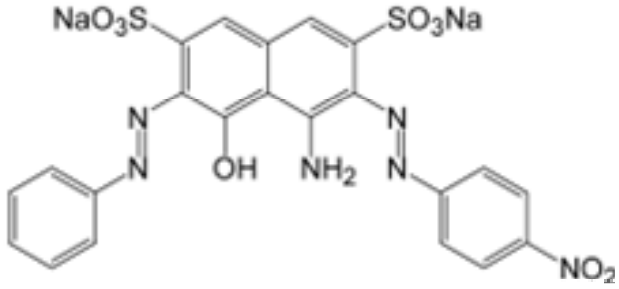
significant difference in protein-to-protein absorption
⇒ **suitable calibration!**

other modifications of SPEFO method

colloid gold



Amidoblack



specific determinations

A. w/o separation
B. w/ separation

A

immunoanalysis

mass spectrometry

peptide mass fingerprinting (PMF)

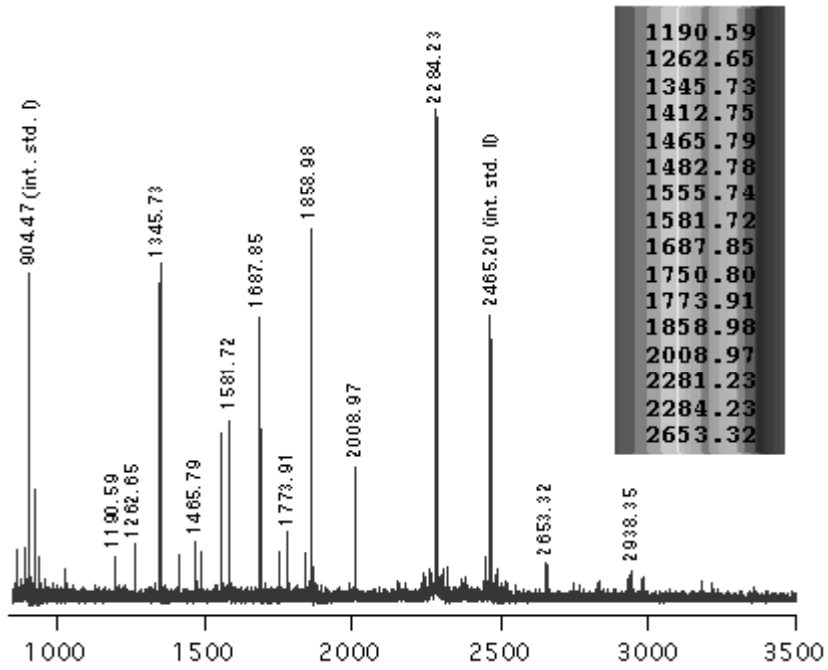
MFLKAVVLTL ALVAVAGARA EVSADQVATV MWDYFSQLSN NAKEAVEHLQ KSELTQQLNA LFQDKLGEVN TYAGDLQKKL
VPFATELHER LAKDSEKLKE EIGKELEELR ARLLPHANEV SQKIGDNLRE LQQRLEPYAD QLRTQVNTQA EQLRRQLTPY
AQRMERVLRE NADSLQASLR PHADELKAKI DQNVEELKGR LTPYADEFKV KIDQTVEELR RSLAPYAQDT QEKLNHQLEG
LTFQMKKNAE ELKARISASA EELRQRLAPL AEDVRGNLKG NTEGLQKSLA ELGGHLDQQV EEFRRRVEPY GENFNKALVQ
QMEQLRQKLG PHAGDVEGHL SFLEKDLRDK VNSFFSTFKE KESQDKTLSL PELEQQQEQQ QEQQQEQVQM LAPLES

: enzymatic cleavage
protease; chemical agents

: specific cleavge
(only at certain AA) – trypsin

: *in silico* cleavage – model cleavage

: comparison of *in silico* & *in vitro* cleavage
statistical evaluation of match
(MOWSE score)



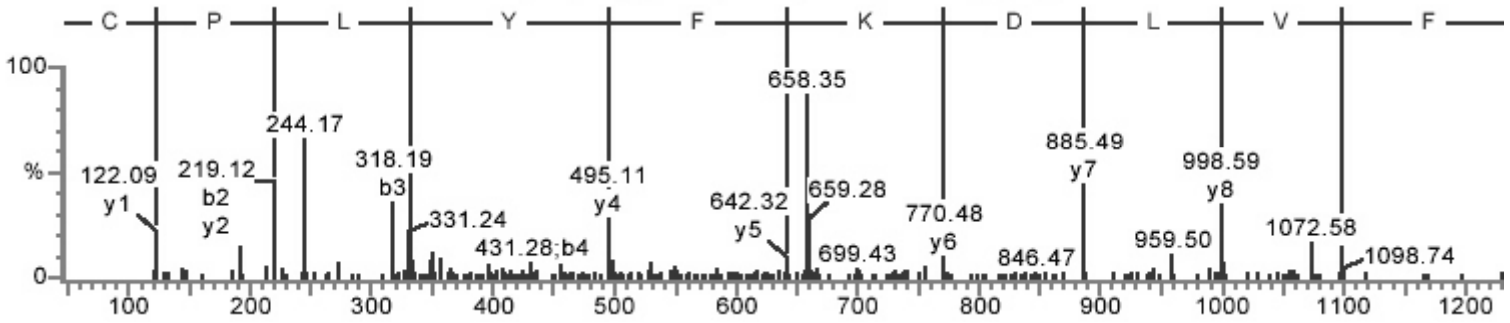
protein sequencing

: enzymatic cleavage – protease

: specific cleavage (only at certain AA) – trypsin

: further cleavage in mass analyser (by field, by collision)

: specific fragmentation of peptide bond



Edman sequencing

separation of phenylthiohydantoine derivatives

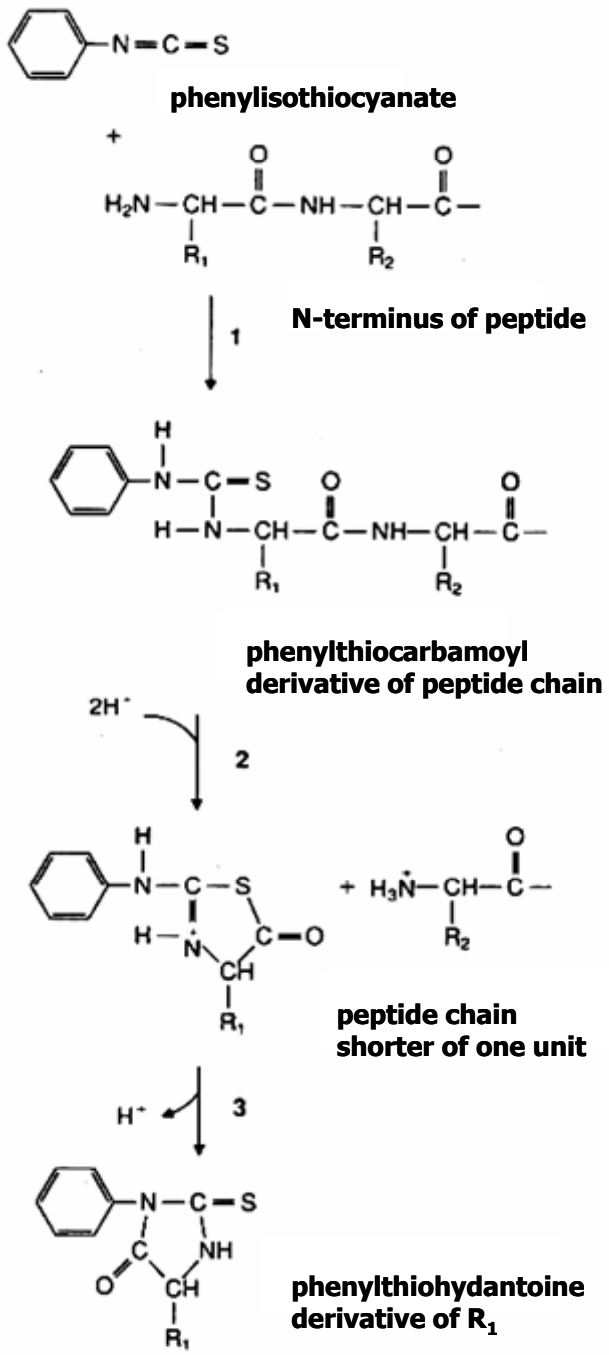
: ion-pairing HPLC
: RP-HPLC
: CZE

other methods of identification – melting point

comparison Edman *vs* MS

MS advantages – low sample consumption
pre-separation on PAGE

ES advantages – automatable
routine method

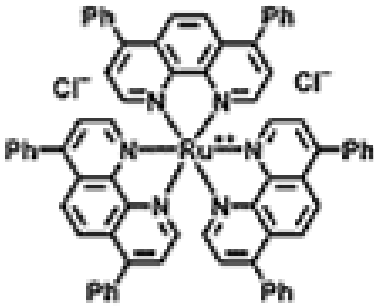


B

<u>gel electrophoresis</u> (PAGE – polyacrylamide gel elfo)
: separation in gel + suitable staining

denaturing PAGE – SDS normalises charge, separation according to M_W
:: densitometry

<u>staining types</u>



: *silver staining* – not quantitative
: *Coomassie Brilliant Blue* – semi-quantitative
: *SYPRO ruby* – quantitative

<u>mass spectrometry</u>
: quantitative using isotopically labelled internal standard

<u>capillary electrophoresis</u>
<u>HPLC</u>
: *UV detection* – unsuitable
: *fluorescence detection* – precolumn derivatisation:
dansyl chloride, *o*-phthaldialdehyde

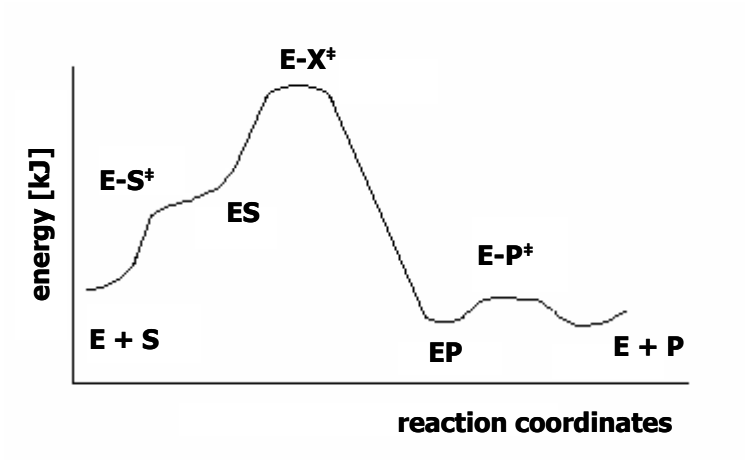
enzyme analysis

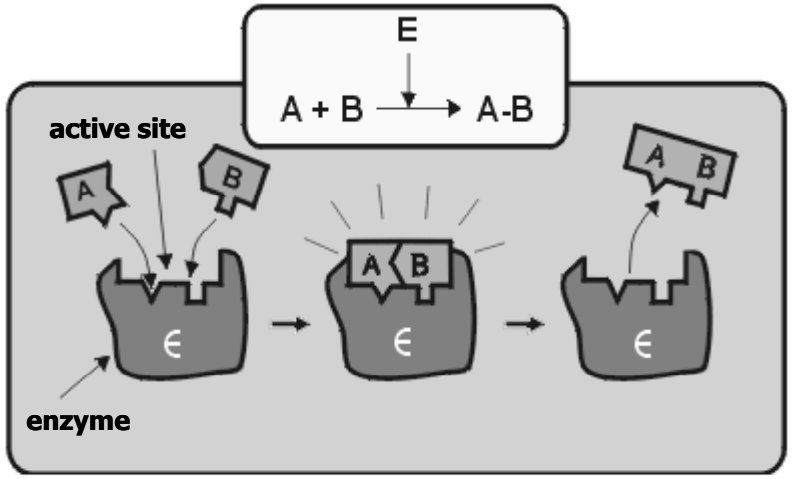
enzyme ~ biocatalyst

holoenzyme = coenzyme (cofactor) + apoenzyme

forms of one enzyme **– isoenzymes**

$$E + S \longleftrightarrow E\text{–}S^{\ddagger} \longleftrightarrow ES \longleftrightarrow E\text{–}X^{\ddagger} \longleftrightarrow EP \longleftrightarrow E\text{–}P^{\ddagger} \longleftrightarrow E + P$$



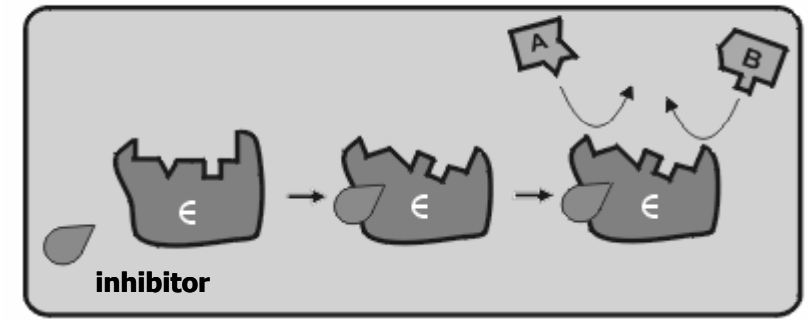


substrate (S) binds into **active site** ⇒ complex enzyme-substrate {ES}
{ES} ⇒ P; **enzyme regenerates**
bond ES – highly specific; depends on AA composition and cofactors

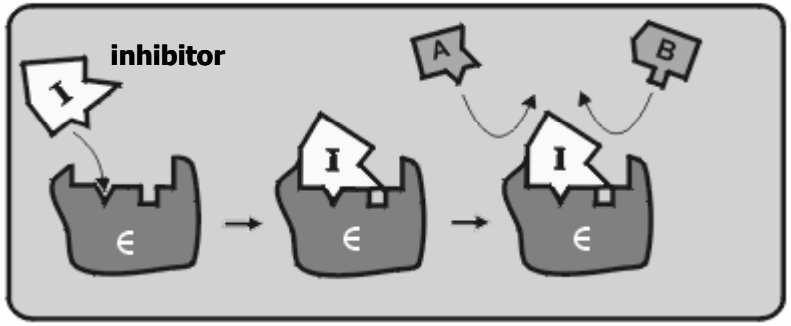
enzyme effect – catalytic activity; concentration of catalytic activity

enzymatic reaction inhibition

allosteric enzymes



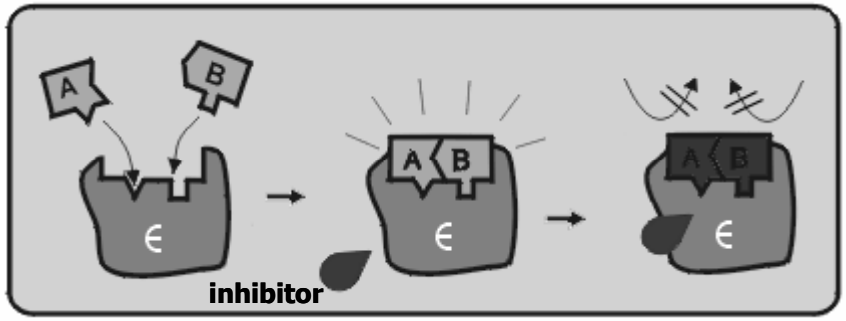
uncompetitive



competitive

inhibitors : specific and unspecific
: reversible and irreversible

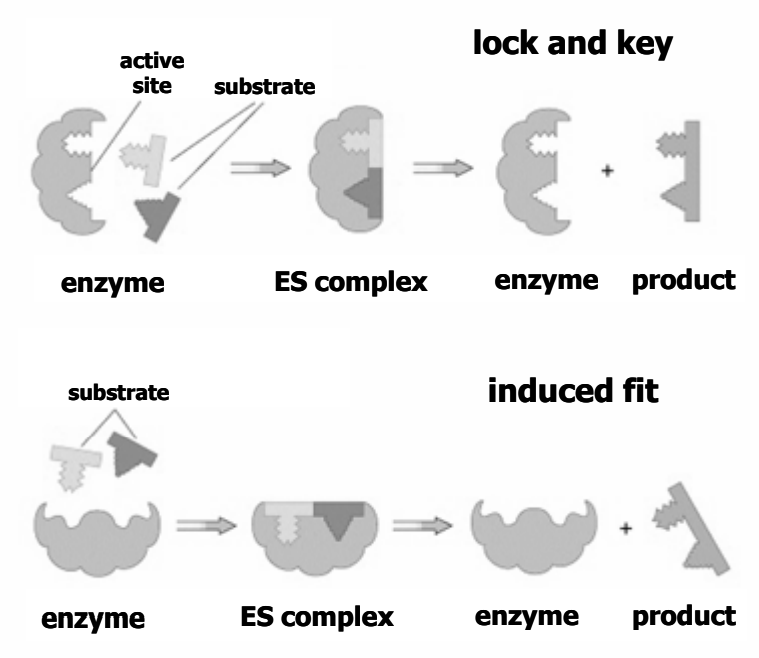
activated enzymatic reactions



acompetitive

: cations: Ca(II), Mg(II), Zn(II)...
: anions: Cl(I)
: organic substances: metabolic intermediates and hormones

mechanism of enzymatic catalysis



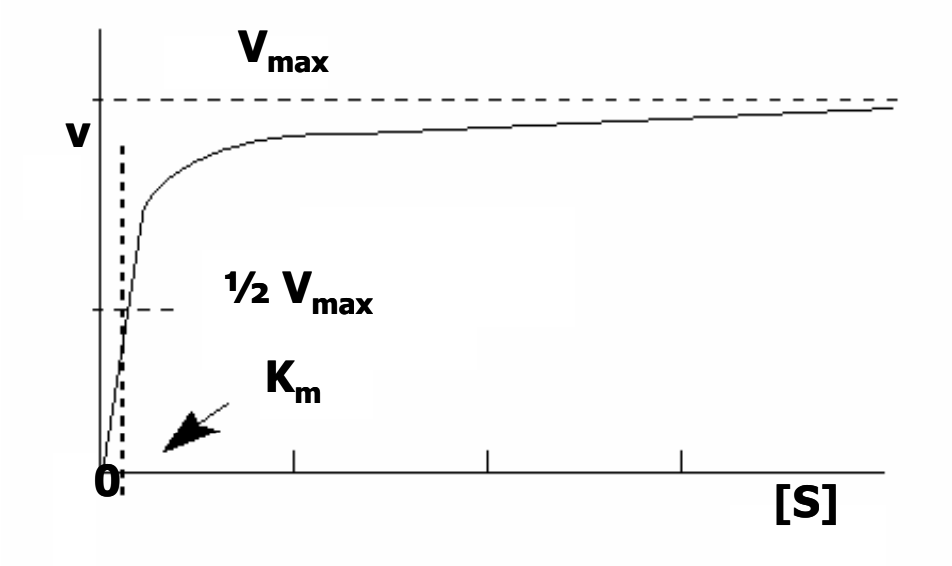
: general acido-basic catalysis

: nucleophilic and electrophilic catalysis

kinetics of enzymatic reaction w/ one S

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES (\rightarrow EP) \overset{k_2}{\rightarrow} E + P$$

$$v = -d[S] / dt = k_1*[E]*[S] - k_{-1}*[ES]$$
$$v = d[P] / dt = k_2*[ES]$$



$$v = V_{max}*[S] / (K_m + [S])$$

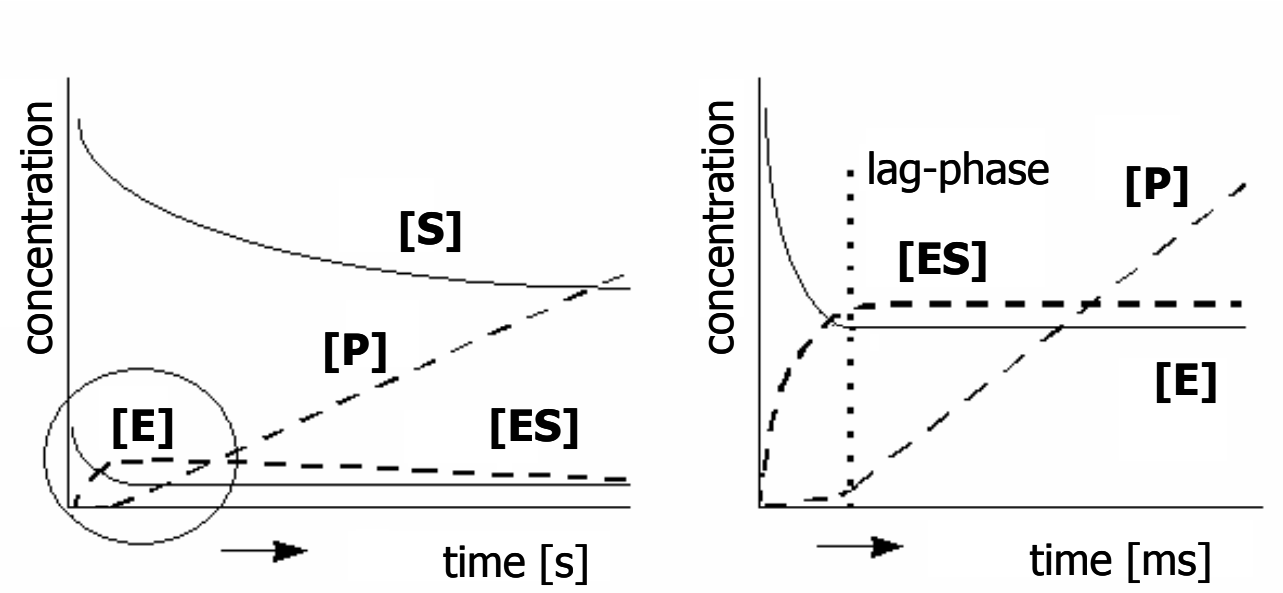
Michaelis-Menten equation

V_{max} – maximum rate

K_m – Michaelis constant;
concentration of S, where $v = ½ V_{max}$

enzyme + substrate ⇒
⇒ short period for ES establishment, so called **lag-phase** (induction period)

[E] of enzyme decreases, [ES] increases up to steady state (ms) ⇒ [ES] = *const.*, [P] = 0



enzyme kinetics *zoom*

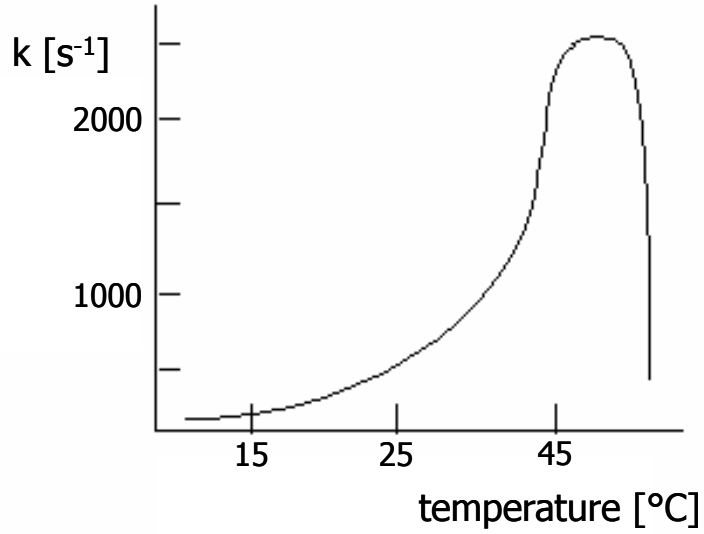
temperature

: range of temperature optima: 0 – 150 °C

:: common range of temperature stability: 20 – 40 °C

to increase temperature stability
:: carrier immobilisation;
:: biotechnological / genetic engineering

enzymatic reaction rate – **Arrhenius law**



pH

given by number and quality of (pK) dissociable groups

: pH optimum of enzyme with synthetic substrates *in vitro*
:: is different from pH optimum for enzyme *in vivo*

buffer

: often important for not only pH optimum set-up
:: **synergic effect** of buffer – effect cummulation

: simple buffers
: ampholytes

buffer as second enzyme substrate

catalytic concentration determination for alkalic phosphatase (ALP)

enzyme activity fundamentally depends on buffer type
: carbonate – ALP works as hydrolase
: amino alcohol – ALP works as phosphate transferase

amino alcohol buffer is simultaneously the second enzyme substrate

catalytic concentration determination for γ-glutamyl transferase (GMT)

amino acid is the second enzyme substrate as glutamyl group acceptor

:: without amino acid – GMT works only as hydrolase
:: optimal second substrate – **glycyl-glycine**
:: simultaneously buffer + synergic influence on GMT; pK_a ~ opt. pH for GMT

buffer as specific moderator with enzyme determination

turbidimetric enzymatic determination of fibrinogen using proteolytic enzyme batroxobine

:: reaction rate specifically increased in presence of glycyl-glycine in *ca* 80 %
:: enzymatic reaction has pH_{opt} = 8, ~ pK_a glycyl-glycine ⇒ simultaneously buffer and activator, synergic effect

buffer as unspecific reaction moderator

enzymatic determination of glucose using glucose oxidase (GOD), peroxidase and oxidative copulation

:: **TRIS**: reaction id slow and slow is also reaching of the equilibrium
:: **phosphate buffer**: reaction rate is dramatically increased and equilibrium is reached quickly

buffer as enzyme activity stabiliser

urea determination by urease

:: –SH group in active site ⇒ buffers combining EDTA (acidic buffer component) with different bases; EDTA shields enzyme before inactivation by heavy metal traces

buffer as problem source in enzyme analysis

determinations using ALP

:: diethanolamine and aminomethyl propanol: deterrent buffer examples
::: honey-like substances, unable to crystallise and difficult to purify
::: contain impurities interfering ALP determinations, which is metalloprotein; complexation of Zn(II) cations in active site of ALP

moderator

activators or inhibitors

inhibitor as an analyte : degree of inhibition points to inhibitor concentration

substrate

practical reasons ⇒ **synthetic substrates**
: structurally defined, industrial manufacturing

: substrate with **auto-indication** properties

4-nitrophenyl phosphate $\xrightarrow{\text{ALP}}$ 4-nitrophenol

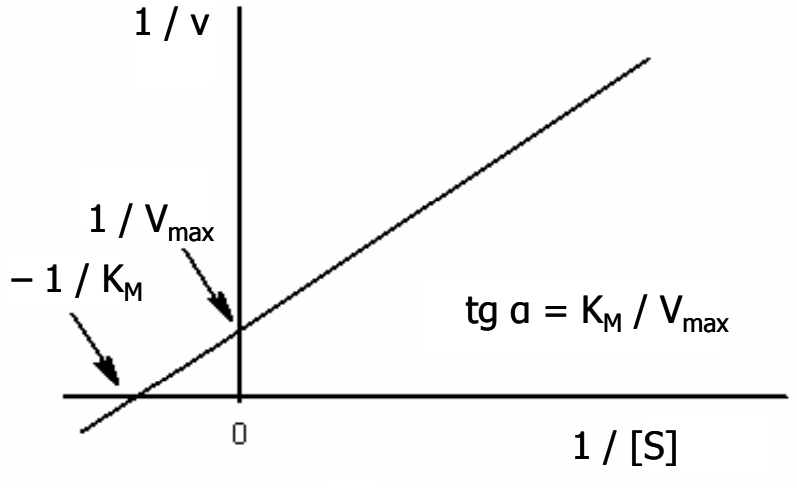
: substrate ⇒ product, which is substrate of **indicator reaction**

1-naphtyl phosphate $\xrightarrow{\text{ACP}}$ **1-naphtyl** + *4-aminoantipyrin*
⇒ quinone monoimine

determination of K_m and V_{max}

linearised Michaelis-Menten equation according to Lineweaver and Burk

$$1/v = 1/V_{max} + K_m/([S]V_{max})$$



alkalic phosphatase (ALP)

absorbance at 400 nm ΔA/min

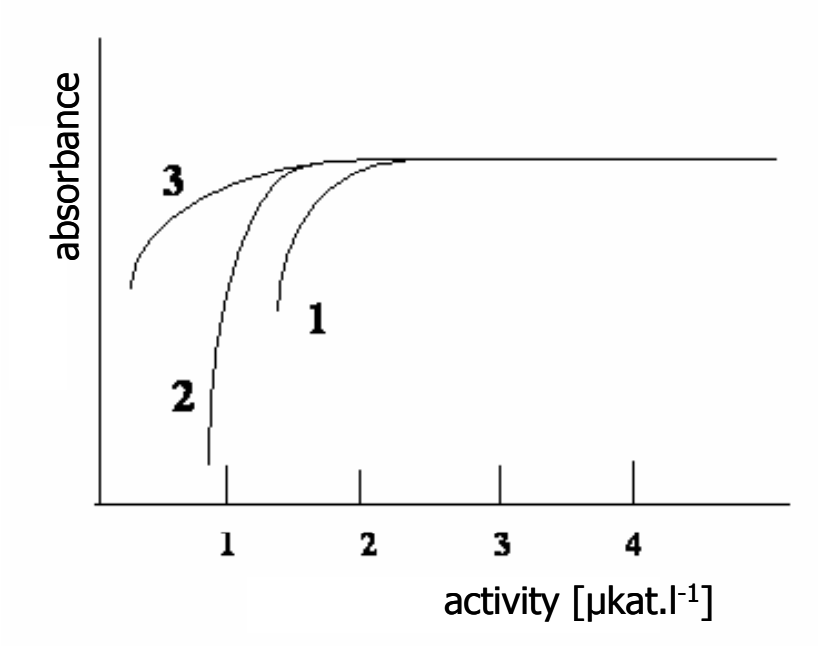
incubation mixture
: ALP + substrate 4-nitrophenyl phosphate
: alkali medium
: buffer N-methyl-D-glucamine, pH 10.1 at 37 ^{O}C

incubation mixture contains always the same amount of enzyme and buffer, it is started by substrate, which concentration is gradually increased

: ΔA measured between 30 and 120 s after start

K_m and V_{max} estimation may be done in graph of linearised equation

enzyme
: fast, elegant, highly specific chemical reaction under mild conditions



uricase
: turns uric acid into allantoin

1 – uricase of bovine liver
2 – uricase of *Aspergillus flavus*
3 – uricase of *Candida utilis*

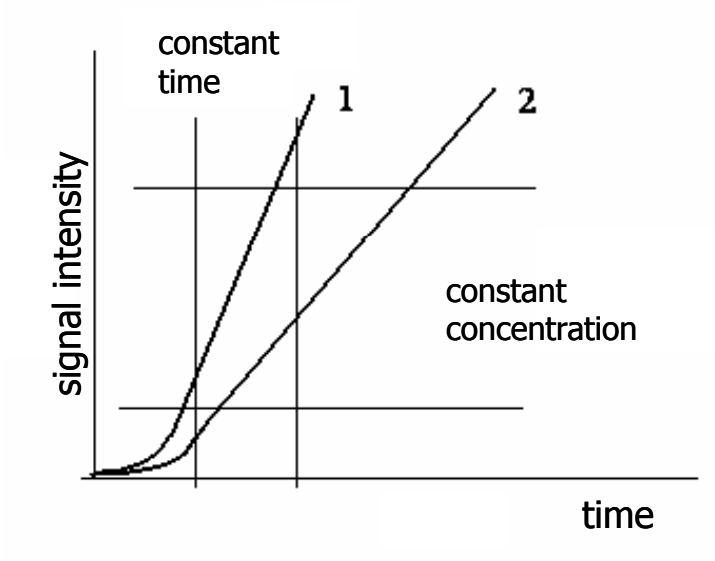
ways of measuring enzymes and substrates including calibration

catalytic activity concentration of enzymes

: analytical approach must be kinetic
: reaction according to zero order kinetics and maximal rate V_{max}

constant concentration method
: out of time necessary to reach specific concentration change

constant time method
: out of signal intensity reached in specific time



sample **1** with higher and **2** with lower activity
vertical lines
: constant time method
horizontal lines
: constant concentration method

calibration

enzyme as analyte – definition of catalytic activity concentration of enzyme by means of product/s/, which is/are created by enzymatic transformation of respective substrate or by measuring its decrease

enzyme as analytical agent – calibration is done using standard solution used instead of sample

calibrants

: *certified reference materials* (CRM)

: *serum calibrators* (lyophilised sera enriched by respective analytes), their content is determined by definitive or reference methods, if available, or using primary standards

brief principles of enzyme analytics

constant temperature of incubation mixture ± 0.1 °C (37 ± 0.1 °C)

enzyme determination: incubation mixture – sample (enzyme), buffer and other needful components of reaction; started by solution of thermostated substrate

: starter volume not >1/20 to 1/10 of total volume
: substrate must be in sufficient surplus > *ca* 20x K_m
: starting also by sample (e.g. serum)

substrate determination: as in 2), starting by sample

catalytic activity concentration of enzyme determination: calibration as within definition of catalytic activity concentration of enzyme by means of product, which is created by enzymatic transformation of respective substrate or by serum calibrators and CRM

substrate enzymatic determination: calibration by standard solution of substrate, or CRM and serum calibrators

optimisation of enzymatic approaches

reaction mixture – multiple components mutually influencing each other, optimisation by so-called relaxation method (SVA)

: gathering data on enzym(s) including K_m (estimation sufficient)

: searching for main parameters of enzyme reaction by MVA optimisation approach

method – **validation** $\Rightarrow$ fulfils for given aim the analytical and clinical demands

DNA analysis
in clinical biochemistry

oncology

cancer diagnostics
presence of mRNA of marker proteins: mRNA tyrosinase (melanoma), GAPDH (lung cancer), E-cadherin (intestines), AIB1 receptor (pancreas), hypermethylation of DNA (prostatic cancer)

prenatal diagnostics

inherited metabolic or neurodegenerative disorders
galactosemia, phenylketonuria, Wilson disease, MCAD (disorder of β-oxidation of fatty acids), Friedreich ataxia, fragile X chromosome syndrome *etc.*

legal medicine

identification by means of so-called genetic fingerprints
kinship identification; paternity, maternity, victim or culprit identification

infectious diseases

proof of etiological agent
: virus (HIV, cytomegalovirus, hepatitis viruses B, C, enterovirus, adenovirus, herpes simplex virus)
: bacteria (*Mycobacteria*, esp. *M. tuberculosis*, *Chlamydia trachomatis*, *Bordetella pertussis*, *Borreliae*, *Neisseria gonorrhoeae*, *Legionella pneumophila*)
: fungi and parasites (*Candidae*, *Aspergillus*, *Leishmania*, *Plasmodium*, *Trichomonas*)

terrorism – anthrax, tularaemia, botulism *etc.*

monitoring of post-transplantation states

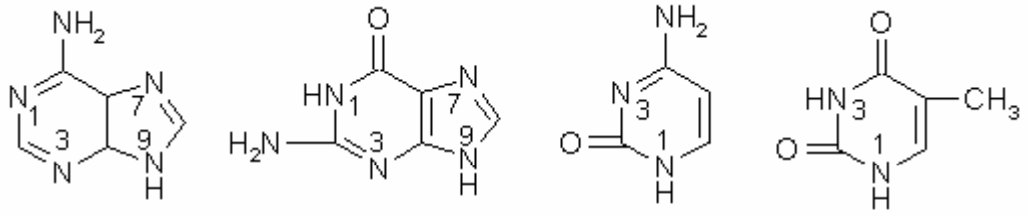
monitoring of patient state after transplantation
level of cytomegalovirus and herpes viruses, DNA-chimerism

food quality monitoring and veterinary medicine

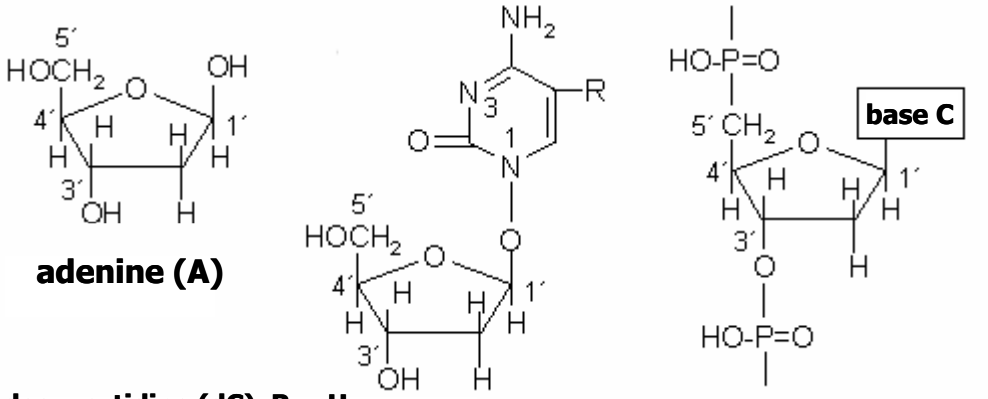
pathogen monitoring
mycobacteria, echinococci, rotaviruses, PRRVS and BVDV viruses within stock animals, helicobacters in milk, *Escherichia coli* O157 mostly in beef or enteroviruses generally in food and drinking water

primary structure – <u>sequence</u>

: **base** = purines, pyrimidines
: **nucleoside** = base + (2‘-deoxy)ribose
: **nucleotide** = nucleoside + 5‘-phosphate
MP – monophosphate, DP – diphosphate, TP – triphosphate



adenine (A) **guanine (G)** **cytosine (A)** **thymine (A)**



adenine (A)

deoxycytidine (dC), R = H

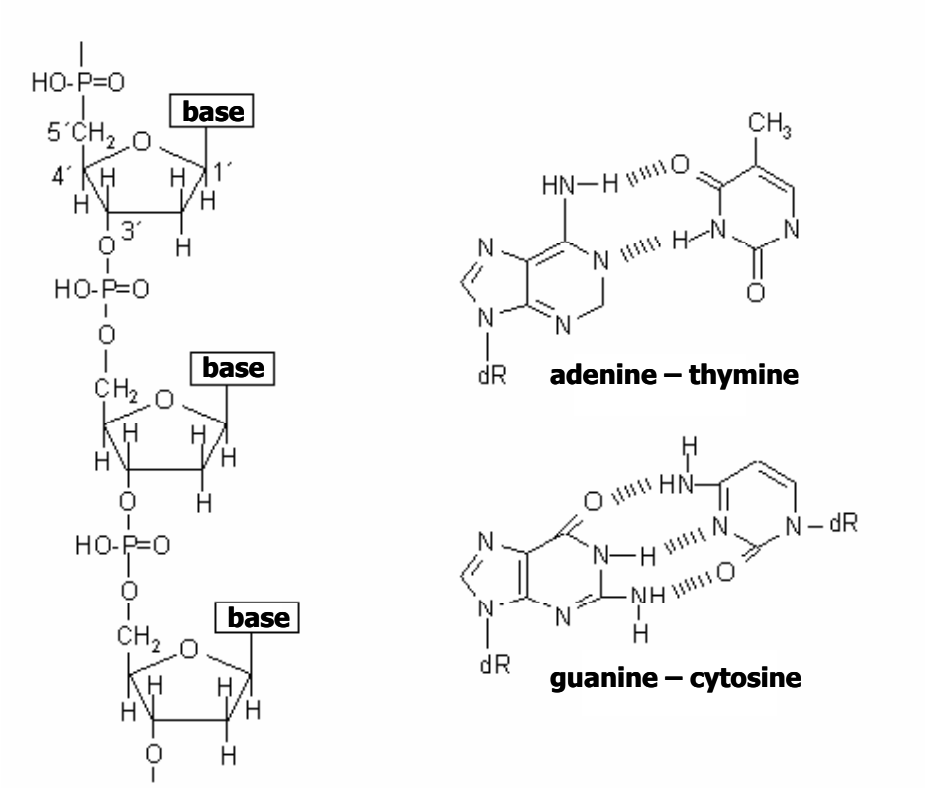
5-methyldeoxycytidine (mdC): R = CH_3

deoxycytidine 3‘-5‘-diphosphate (dCDP)

secondary structure – <u>DNA double-helix</u>

<u>base pairing</u> *(Watson-Crick)*

G – C
A – T



<u>other base pairing</u>

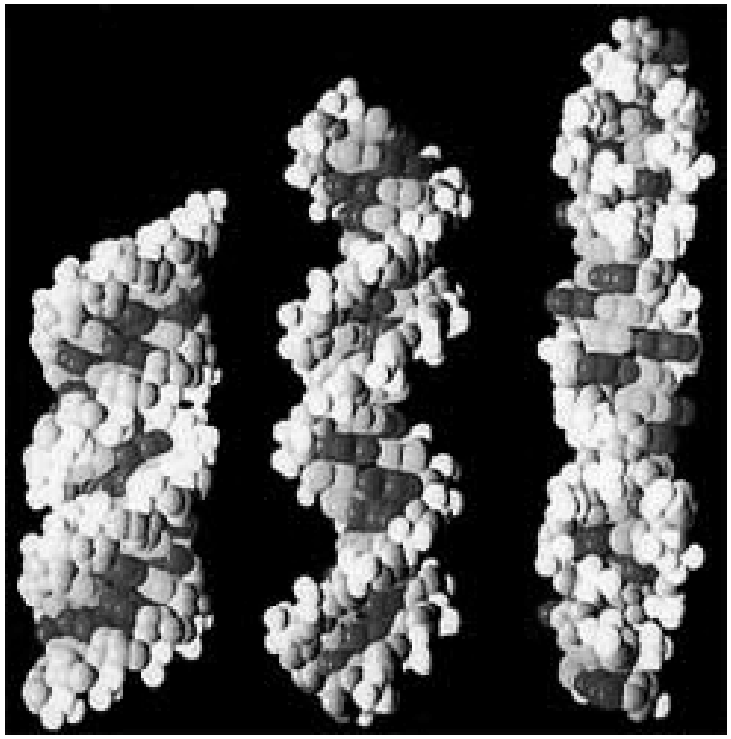
Hoogsteen, Wobble, reversed Watson-Crick

mismatch – sequences not matched

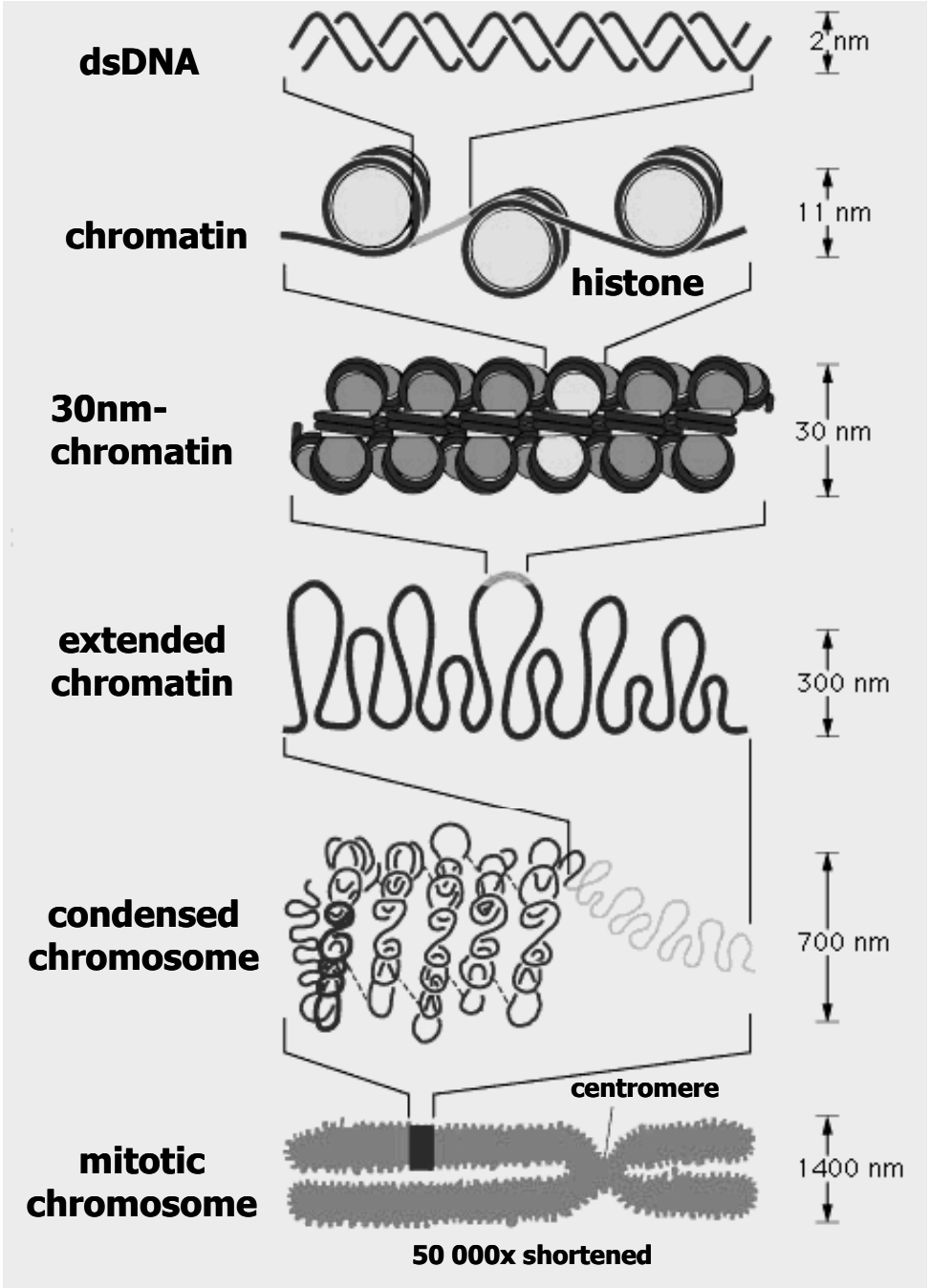
G – A
G – T
A – C

ssDNA – ***single stranded DNA***
dsDNA – ***double stranded DNA***

double-helix – forms



helix **A** **B** **Z**

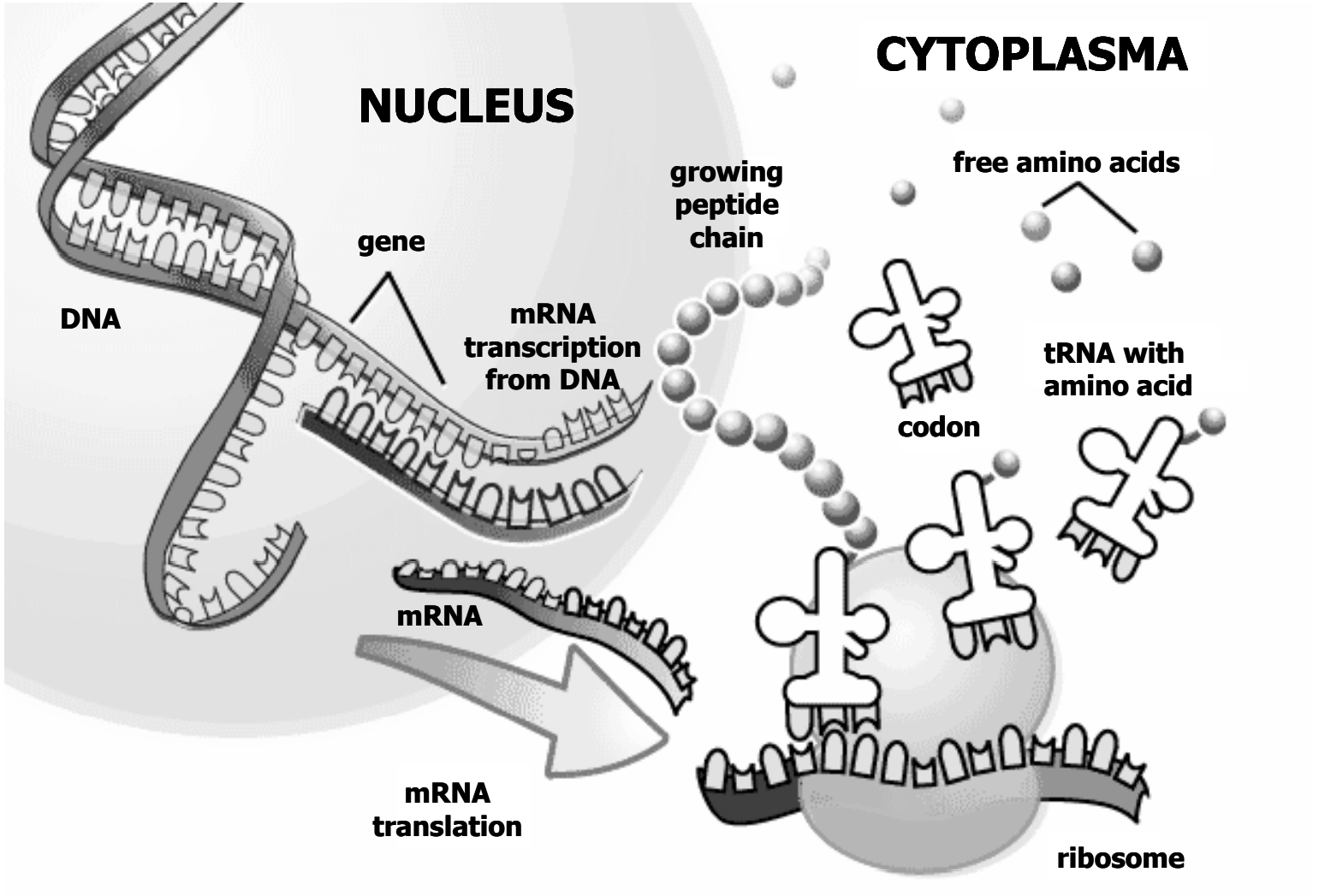


DNA tertiary structure

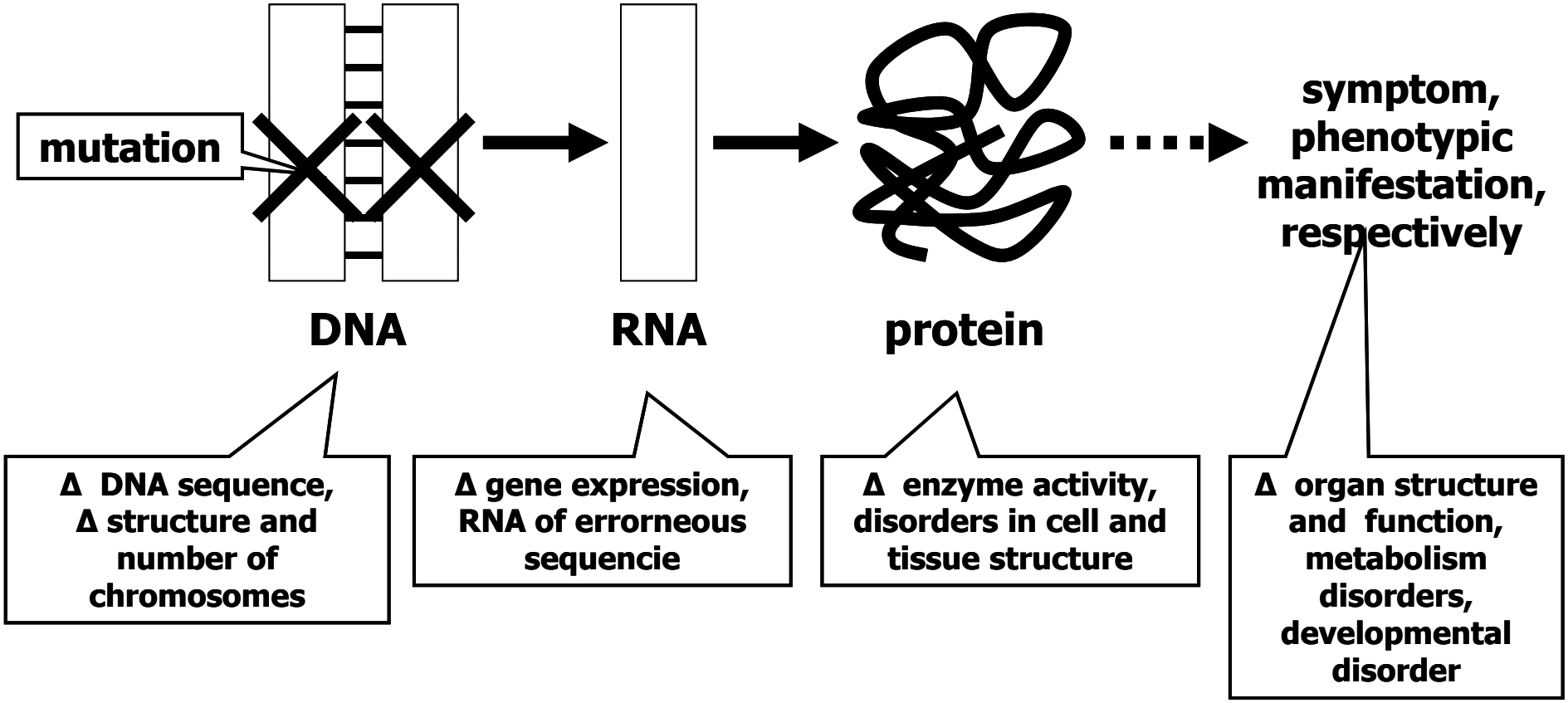
supra-helix
: *topoisomerases*
: histones

<u>transcription</u>; DNA ⇒ mRNA

<u>translation</u>; mRNA ⇒ protein

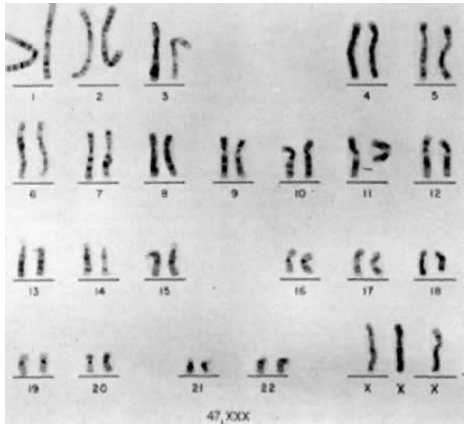


disorders diagnostics



genealogical investigation → **molecular-biological investigation**

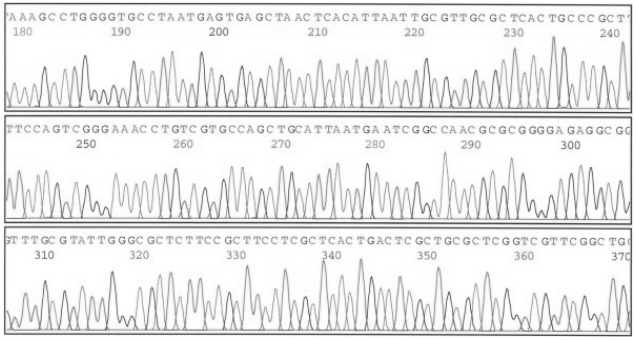
analytical approaches



numerical aberration monitoring
: monosomia (Turner syndrome), polysomia (Down syndrome)

identification of <u>known</u> sequence
: mutations (phenylketonuria)

identification of <u>unknown</u> sequence
: sequencing of new pathogens



DNA <u>state</u> monitoring
: methylation: deoxycytidine (fragile X chromosome syndrome)

basic approaches of NA analyses

: PCR amplification

:: multiplication of original DNA, 25 cycles ~ 2^{24}= 17 x10^6 copies

: cleavage by restriction enzymes

:: fragmentation of amplified DNA (specific)

:(blotting after gel electrophoresis)

:: separation of particular DNA sequence

: hybridisation – on blotting membrane or on chip

:: reaction with complementary labelled ssDNA

PCR: 10 – 500 µg of human DNA
1 – 10 µg of bacterial DNA
0.1 – 1 µg of plasmid DNA

DNA isolation

1) NA extraction + sorption on SiO_2 after cytolysis

2) gradual soft degradation of cells by organic solvents

sample preparation and separation of DNA

affinity purification – based on hybridisation abilities of NA

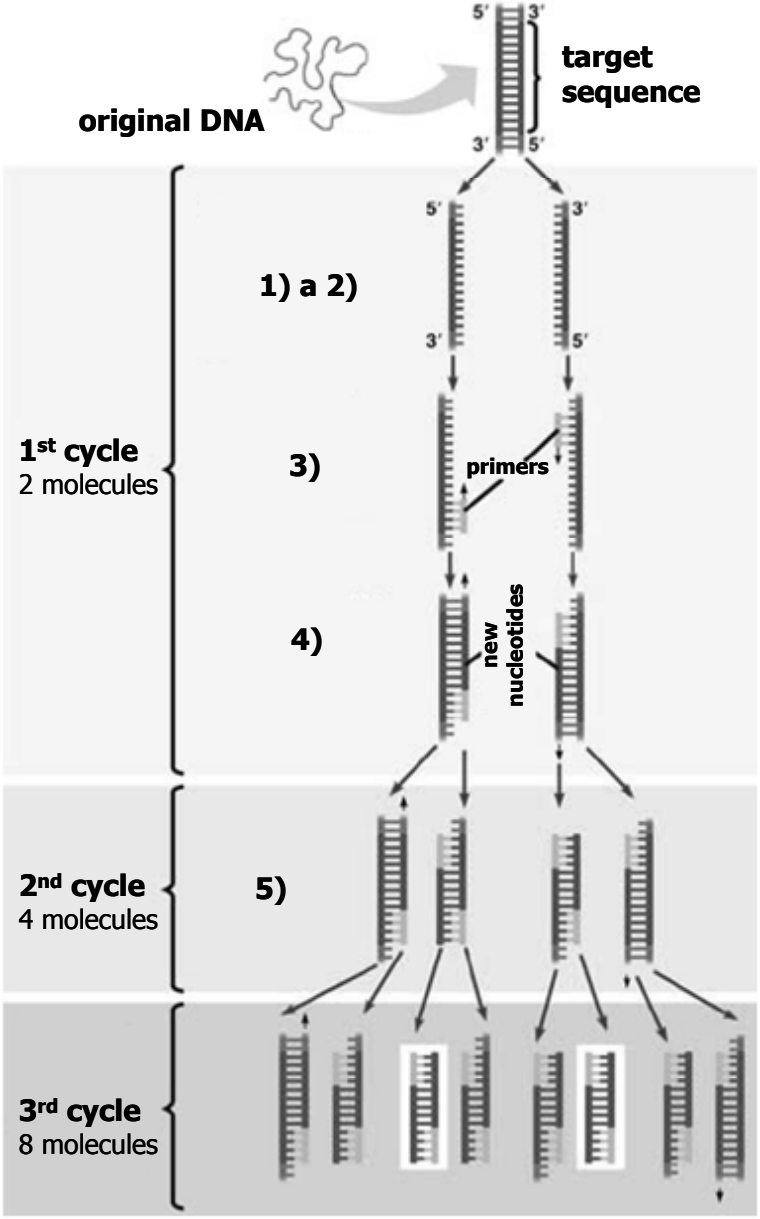
: sample + lysation buffer; + biotinylation buffer; incubation
: after incubation ⇒ microtube with avidin or streptavidin modified surface

adsorption on silikagel
: NA in presence of guanine adsorb on silikagel
: elution by Δ pH and ionic strength

gel filtration
: micro-column filled with gel

: adsorption on silikagel and gel filtration might enhance centrifugation

polymerase chain reaction *(PCR)*



1) **initiation** – heated to 96 °C, 5 min
DNA and primers melt

2) **melting** – heated to 96 °C for 30 s;
add DNA-polymerase

3) **binding** – heated to 68 °C, 30 s

4) **prolongation** – heated to 72 °C for 45 s

5) **repetition** – steps 2-4 repeat
max. 25x

6) **keeping** – final mixture at 7 °C;
preserves DNA from decomposition

NA amplification

PCR	polymerase chain reaction	(DNA)	1985
RT-PCR	reverse transcription PCR	(RNA)	1991
TAS	transcription-amplification system	(RNA, DNA)	1989
3SR	self-sustained sequence replication	(RNA,DNA)	1990
NASBA	nucleic acid sequence based amplification	(RNA, DNA)	
TMA	transcription mediated amplification	(RNA, DNA)	1991
SDA	strain displacement amplification	(DNA)	1992

amplification of hybridisation probe

LAR	ligase amplification reaction	1989
LCR	ligase chain reaction	1991
Q-beta	Q-beta replicase amplification	1988

restriction enzymes

*EcoR*I	*Escherichia coli*	5'G**AATT**C 3'C**TTAA**G
*BamH*I	*Bacillus amyloliquefaciens*	5'G**GATC**C 3'C**CTAG**G
*Hind*III	*Haemophilus influenzae*	5'A**AGCT**T 3'T**TCGA**A
*Mst*II	*Microcoleus* sp.	5'C**CTNAG**G 3'G**GANTC**C
*Taq*I	*Thermus aquaticus*	5'T**CG**A 3'A**GC**T
*Not*I	*Nocardia otitidis*	5'GC**GGCC**GC 3'CG**CCGG**CG
*Alu*I	*Arthrobacter luteus*	5'**AGCT** 3'**TCGA**

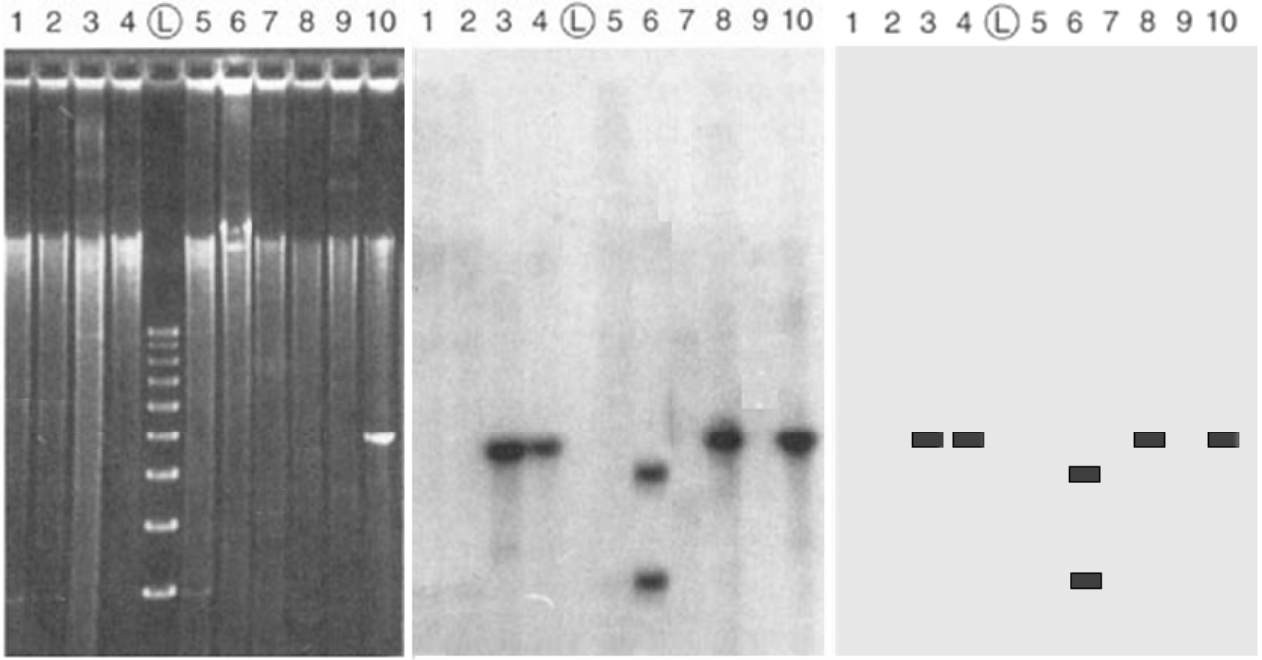
identification of <u>known</u> DNA sequence

blotting

PCR ⇒ DNA cleavage by restriction endonuclease ⇒
⇒ GELFO separation ⇒ **<u>blotting</u>** ⇒ hybridisation ⇒ label visualisation

DNA – Southern blot
RNA – Northern blot

hybridoblot



hybridisation

ssDNA fragment (**analyte**) +
+ labelled complementary ssDNA fragment (**probe**)

: chromophore, fluorophore, radioactive isotope (^{32}P)

cytogenetic diagnostics

FISH – fluorescence *in situ* hybridisation

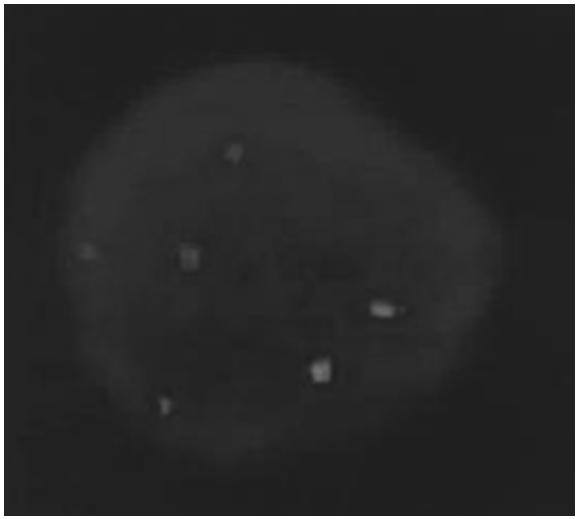
monitoring polysomia, translocation, inversion, deletion

possibility to analyse interphase chromosomes
: does not need to cultivate cells and prepare metaphase chromosomes

complementary pairing of investigated DNA with fluorescence labelled probe

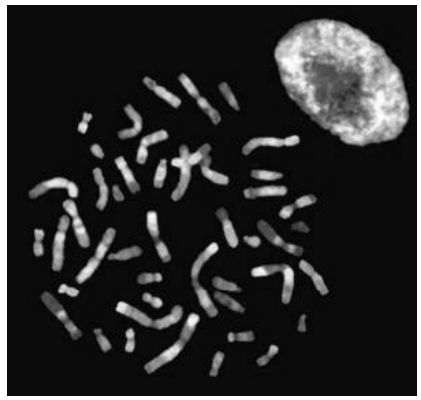
sequence of investigated gene
: individual gene
: whole or part of chromosome
or centromeric or telomeric regions

detection – fluorescence microscopy



mFISH – multicolour FISH high resolution banding

combination of more fluorochromes and of more probes

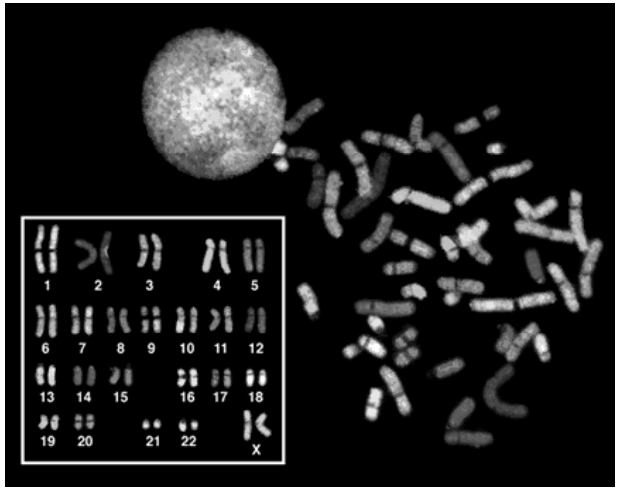


SKY – spectral karyotyping

identification of numeral and structural chromosomal aberrations

complementary pairing – 55 fluorescence labelled probes

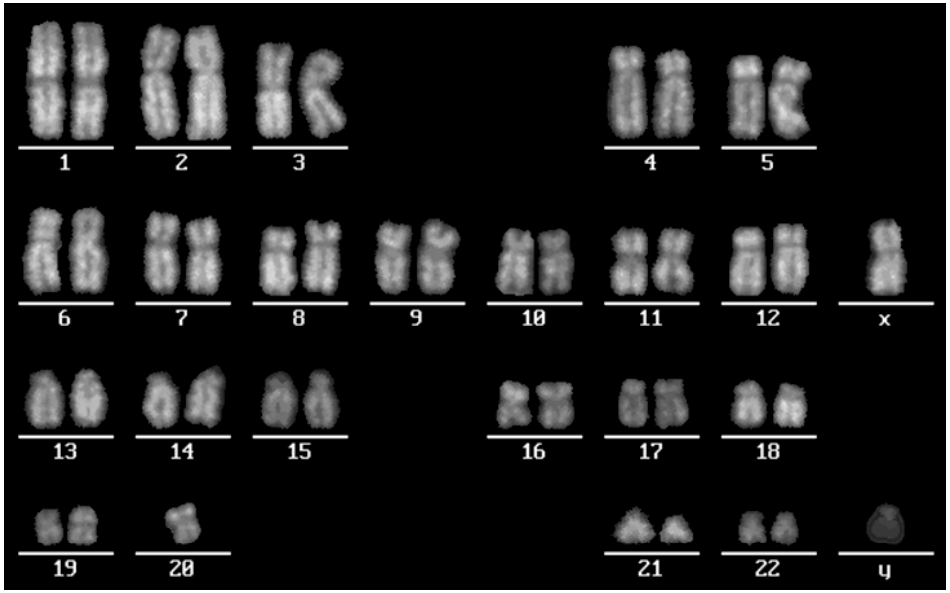
computer imaging and analysis



CGH – comparative genomic hybridization

visualisation of chromosomal disorders

parallel hybridisation of two DNA samples labelled with different fluorochromes
: DNA of patient (tumourous), labelled green
: control DNA (DNA of healthy person), labelled red



gene therapy

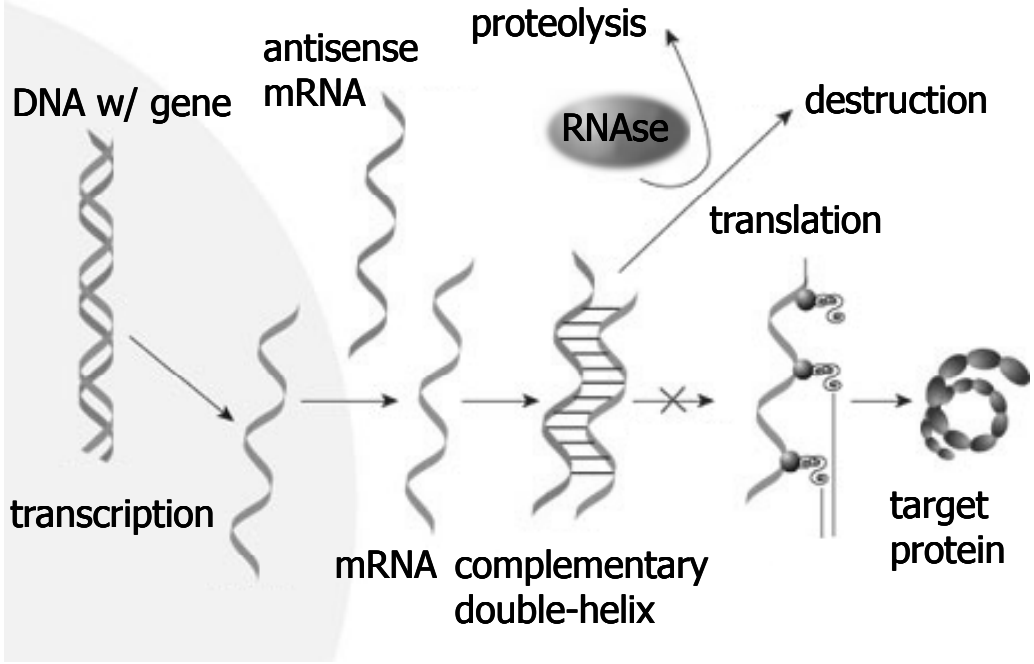
: transfer of genetic material with therapeutic effect (AVV; adenovirus vector)

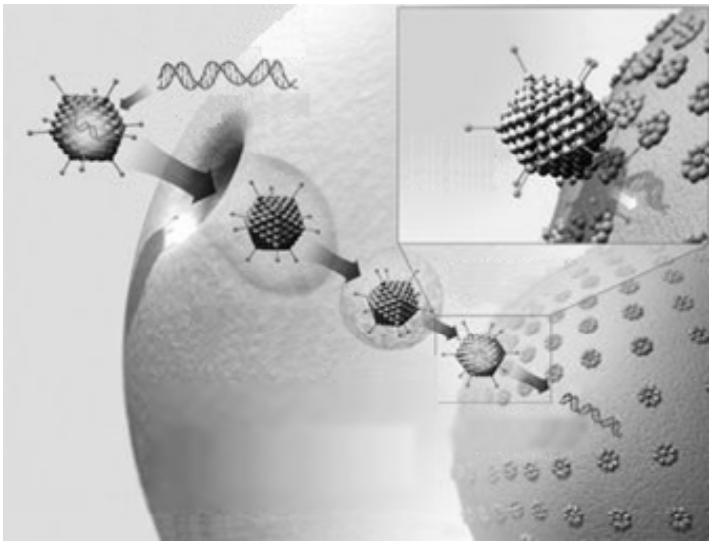
: targeted knock-out of aberrant protein (cancer; antisense therapy)

: renewal of mutated gene expression (inheritable diseases)

: problem of cellular defence mechanisms to foreign DNA (transplantation)

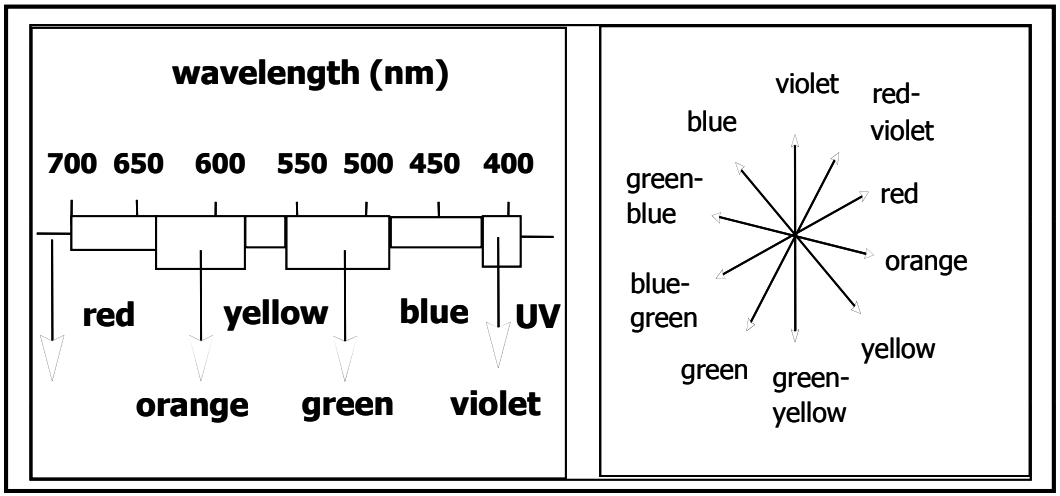
: problem of selective defect cells therapy





colourness
physical phenomenon

indication ⇒ sight ⇒ colour



sunlight – white, colourless

decomposed in prism ⇐ **refractive index is function of wavelength**

: more inclined from straight line of original ray are violet rays, less yellow ones

human eye percepts as colourful rays those with wavelength **400** to **760** nm

if the object absorbs **yellow**, it reflects blue ⇒ appears to be **blue** ***and vice versa***

so-called **complementary colours**

absorbance description

Bouguer-Lambert-Beer law

$$I = I_0 * 10^{-\varepsilon * c * d}$$

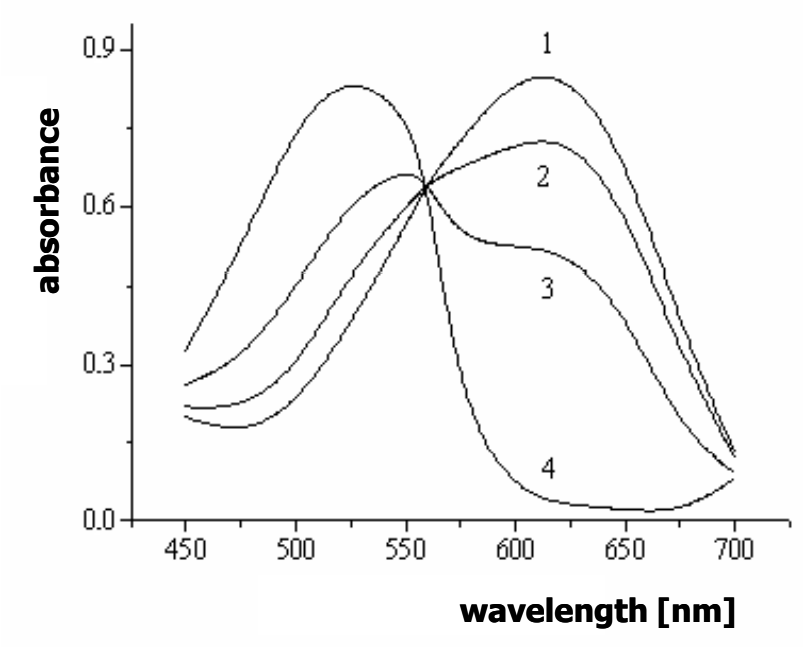
concentration ***c*** [mol/l], molar absorptivity ***ε***, thickness of abs. layer ***d*** [cm]

layer transparency: I / I_0

absorbance A [l/mol.cm] : $A = \log I_0 / I = \varepsilon * c * d$

ε = A / c*d $\Rightarrow$ proportional to **number of molecules** of absorbing substance

spectrophotometric absorption curve (***absorption spectrum***) $A = f(\lambda)$



absorption spectrum:
magon with Mg(II) ions

1 – magon
2 – magon + 2.10^{-6} Mg(II)
3 – magon + 1.10^{-5} Mg(II)
4 – magon + 8.10^{-4} Mg(II)

shifts of absorption maximum:

bathochromic : to higher wavelengths
hypsochromic : to lower wavelengths
hyperchromic : absorbance increase
hypochromic : absorbance decrease

if the spectrum changes indiscreetly ⇒ existence of **isosbestic point**
:*all* colour components of the *same molecule* have in it the *same absorbance*

theory of colourness
absorption of light by molecules

molecule = X atoms (X>2)

absorption of light – valence binding electrons
: the stronger the bond is, the greater the rate of their oscillations is

molecules absorb: light with frequency ≈ to frequency of valence electrons

total inner energy of molecule **E**: $\mathbf{E = E_{rot} + E_{vibr} + E_{electr}}$

σ electrons – higher energy of transition to higher energy level ⇒
⇒ do not take part in light absorption in Vis

π electrons – lower energy of transition to higher energy level ⇒
⇒ may absorb even in Vis

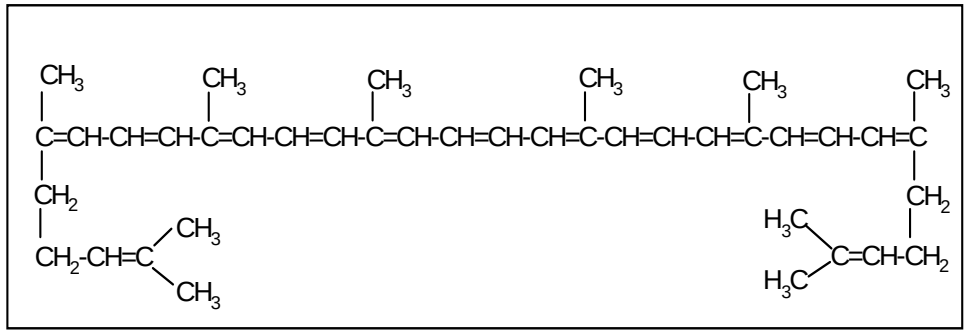
intrinsic cause of colourness:
light energy absorption by **π** electron transition between levels with different **E**

structures causing colourness

chains of conjugated double bonds

excitation energy

ethane	$CH_3–CH_3$	*ca* 180 kJ/mol	λ = 155 nm
ethyne	$CH_2=CH_2$	*ca* 150 kJ/mol	λ = 190 nm
butadiene	$CH_2=CH–CH=CH_2$		λ = 210 nm
lycopene (11 conjugated double bounds)			λ = 506 nm



addition effects

: *substituents* :: nucleophilic, electrophilic
:: charged
: *oxidation | reduction*
: *planarity* of structure
: *complexation* with metal ions
: *detergents*; molecular compounds

substituents – nucleophilic, electrophilic

substituents – charged

influence the electronic distribution

nucleophilic:

: amino group (free electron pair)

: hydroxyl group (two free electron pairs)

benzene λ_{max} = 255 nm > phenol λ_{max} = 275 nm > aniline λ_{max} = 282 nm

electrophilic:

: nitro group, carbonyl group and imino group

nitrobenzene λ_{max} = 268 nm, acetophenone λ_{max} = 279 nm

:: combination of nucleophilic and electrophilic group: bathochromic shift

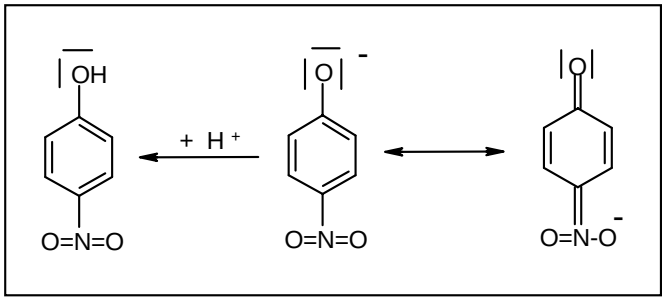
ionisation enhances the effect of substituent presence

nitrophenol pK_a = 7.16

: acidic medium **colourless** λ_{max} = 315 nm (left)

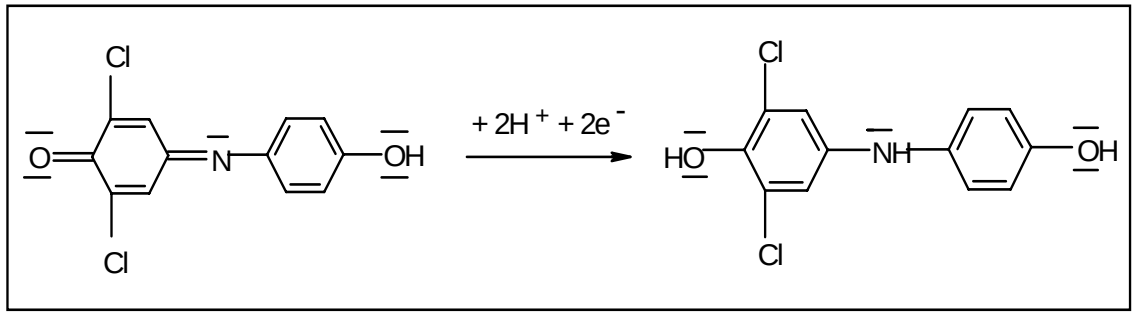
: alkali medium **yellow** (middle)

: quinoid structure (right) λ_{max} = 404 nm



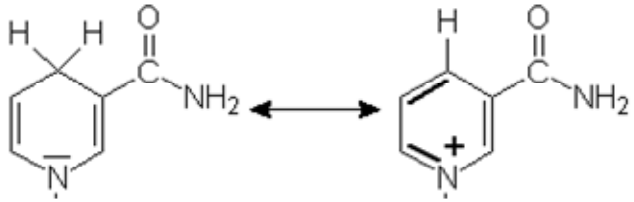
oxidation / reduction

oxidation increases number of conjugated double bonds



2,6-dichlorophenolindophenol – blue
reduction – leucobase (colourless)

reduction rarely enhances absorption



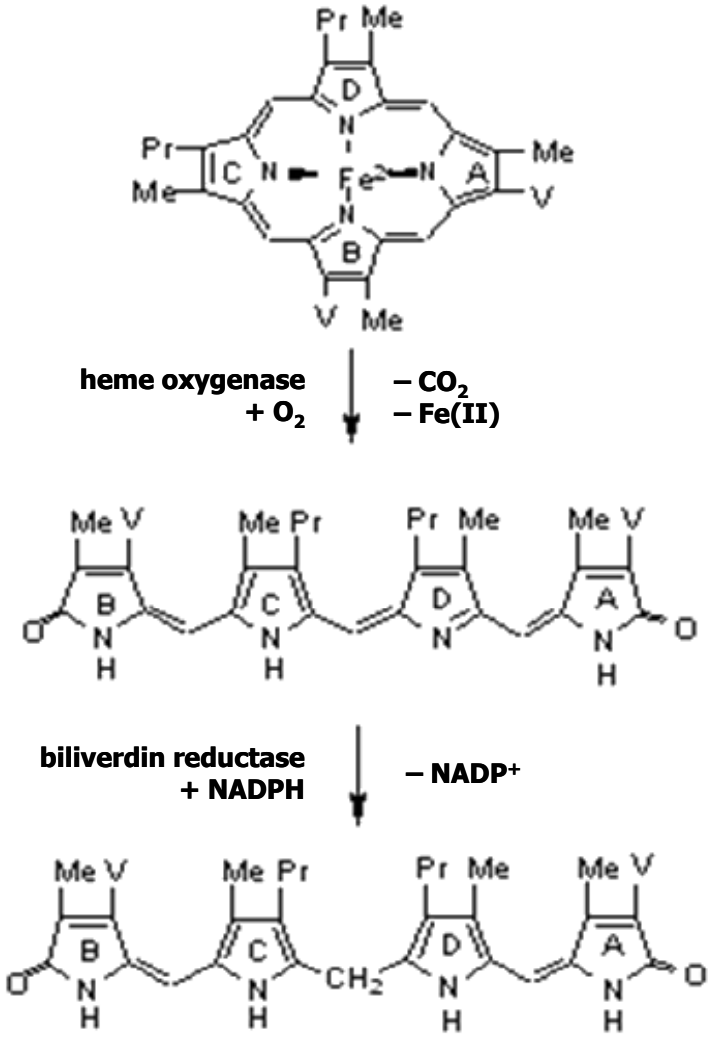
NADH NAD$^+$

coenzymes derives from nicotinamide

$CH_3–CH_2–OH + NAD^+ \rightarrow CH_3–CH=O + NADH + H^+$

structural planarity

free overlapping of **π-***electrons in conjugated system = planar structure*



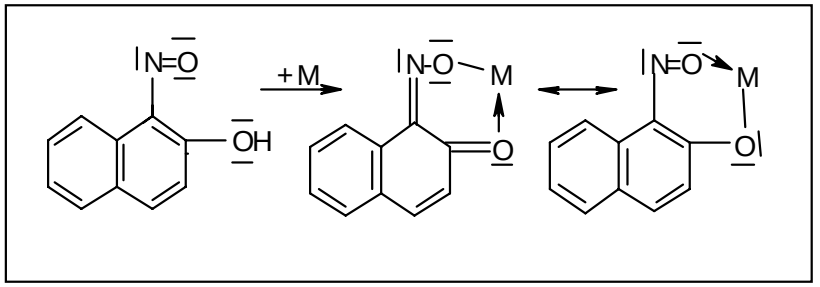
heme – red

biliverdin – green

bilirubin – yellow

metal chelates

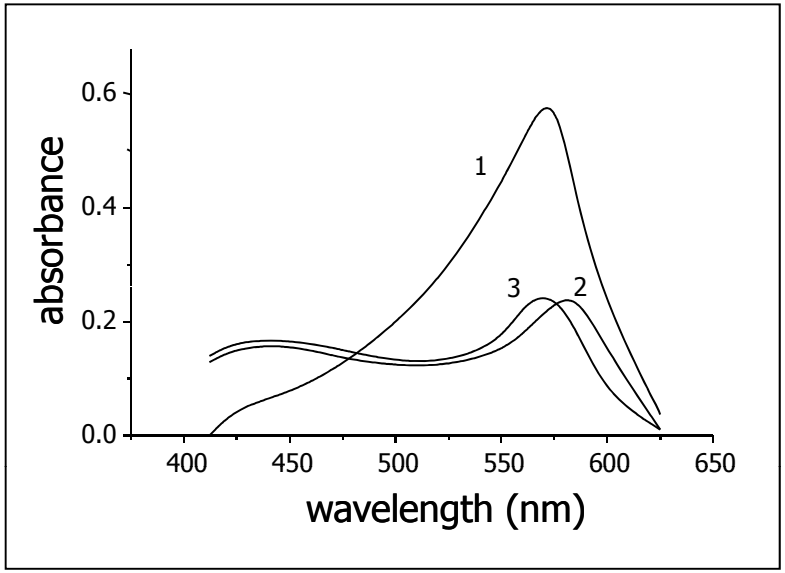
creation of complex {ML} and of co-ordination bond at expense of free electron pair included into system of conjugated double bond is followed by enhancement of colourness



1-nitroso-2-naphtol : yellow-orange
: Fe, Ni or Cr ions
:: green and brown complexes

detergents

alkalic xylenol orange 2.10^{-5} mol/l
1 – XO, pH 10.5
2 – XO and 5.10^{-4} mol/l CPB pH 10.5
3 – XO, pH 6.4



molecular compounds

: aromates, heterocycl. bases & arom. compounds with nucleoph. substituents

: aromatic hydrocarbons with electrophilic substituents **quinhydrone**

use of colourness
in clinical diagnostics

acido-basic indicators

pH determination

: alkalimetric titration ⇒
determination of total protein by Kjeldahl method (NH_3)

: orientational pH determination ⇒
semi-quantitative determination of urinal pH – **mixed indicators**
(continuous transition)

analyte determination

: semi-quantitative determination of serum albumin – protein error

: semi-quantitative urea determination – urease reaction, NH_3 production

: microbial contamination indication – process of sugars into organic acids

redox indicators

determination of reducing/oxidising substances

: determination of ascorbic acid (vitamin C)
: oxidative copulation – glucose determination, uric acid, cholesterol
: ELISA determination with POD (horse-reddish peroxidase)
: AST, ALT enzymes determination (phosphatases)
using secondary enzymatic redox reaction

organo-agents and metallochromic indicators

metal ions determination – Cl (I), Ca(II), Mg(II), Cu(II), Fe(II)

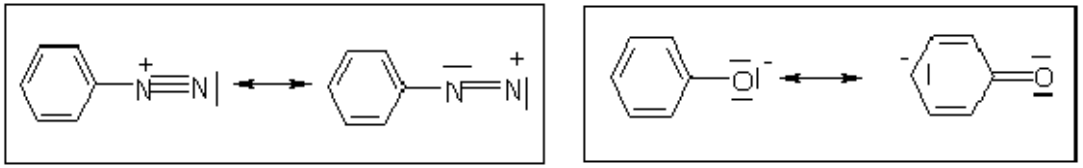
chlorides – titration $Hg(NO_3)_2$; Hg(II) + diphenylcarbazon – blue-violet colour
calcium – arsenaze III, *o*-cresolphthalein complexon
magnesium – magon, calmagit
copper – bathocuproine
iron – bathophenanthroline, ferrozine

copulation agents

determination of reacting substances

azocopulation ⇒

: azo-dyes – conductive connection of conjugation chain of double bonds of original molecules by azo-group ⇒ planary structure of new molecule



: aryl diazonium cation as electrophilic agent

: activation of *p*-position of phenol by dissociation of hydroxyl hydrogen

$$C_6H_5- \bar{N}= \bar{N}^+ + -C_6H_5-R \rightarrow C_6H_5-N=N-C_6H_4-R$$

reaction of bilirubin with diazosulphanilic acid

oxidative copulation ⇒

: molecule with two aromatic rings conjugated through imino-group

: new molecule has **quinoid** structure

: further substituents increase its polarisation ⇒ **highly colourful**

use of created hydrogen peroxide

$C_6H_5–R + 2\ H_2O_2 \xrightarrow{\text{POD}} O=C_6H_4–R + 3\ H_2O$

$R–NH_2 + O=C_6H_4–R \rightarrow R–N=C_6H_4–R + H_2O$

–R is =O, $–NH_2$

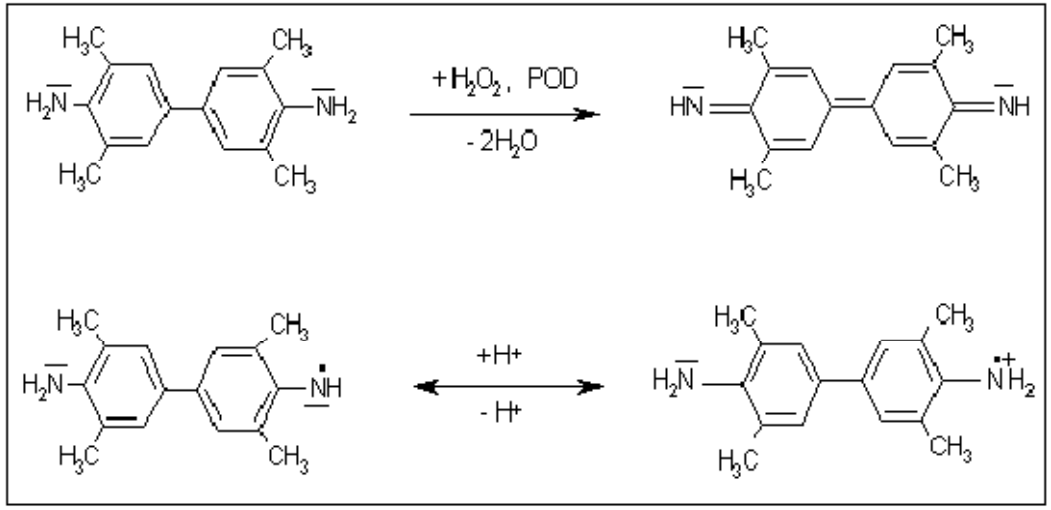
non-chromogenic products are **transformed into colourful**

1-naphthyl phosphate $\xrightarrow{\text{ACP}}$ 1-naphtyl + *copulation ag.* ⇒ colourful product

acidic phosphatase

oxidation ⇒

transformation of reduced **leucoforms** into **colourful**



3,3´,5,5´-tetramethylbenzidine and oxidation to blue

: oxidation intermediate – semi-quinone

chromogenic substrates for enzyme reactions

colourful them-selves, or by enzymatic transformation created colourful product

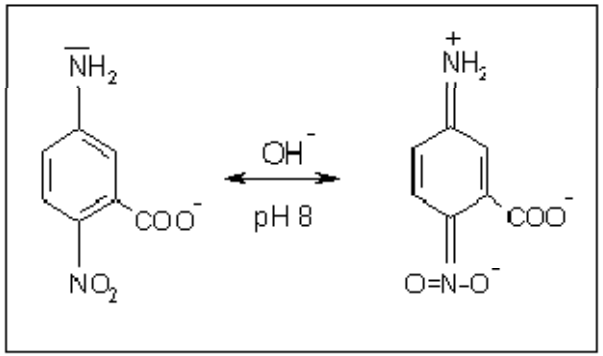
alk. phosphatase

4-nitrophenyl phosphate **ALP>** 4-nitrophenol

312 nm 404 nm

glutamate transferase

L-γ-glutamyl-3-carboxy-4-nitroanilid **GMT>** 5-amino-2-nitrobenzoic acid



biomolecule labelling

: *affinity* – weak interactions
: *covalent*

: peptides/proteins
: NA

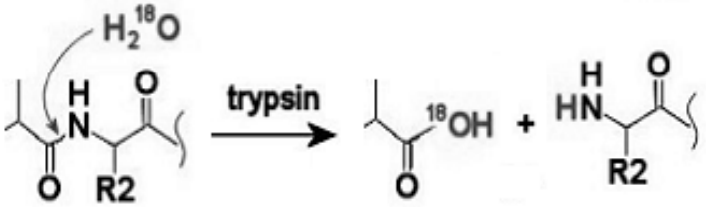
label types

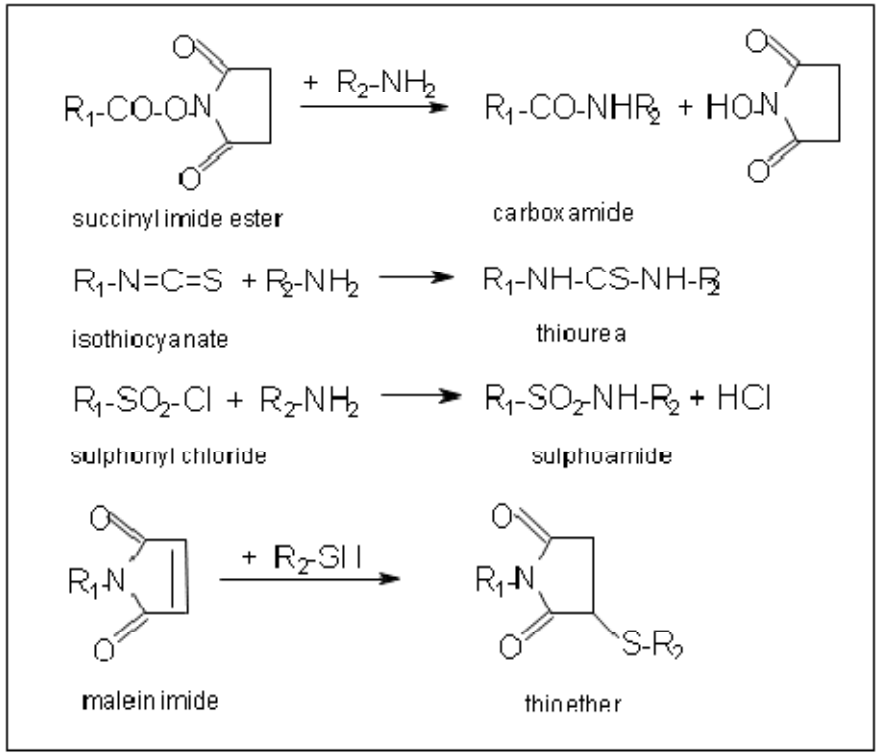
affinity : chromophores, fluorophores

covalent : isotopic (radioactive, heavy)
: chromophores, fluorophores, luminophores
: enzymes

modification

: chemical
: enzymatical





R_1 is label
R_2 is biomolecule

medical microbiology
diagnostic approaches

microorganisms – basic component of biosphere

: pathogenic – health dangerous
: occasional pathogenic – dangerous only sometimes (*Escherichia coli*)

medical microbiology – microbiologic analysis
proof of **etiologic agent** – infection progenitor; its sensitivity to antibiotics and chemotherapeutics

medically important microorganisms

: bacteria
: microscopic fungi
: protozoa
: viruses
: subviral agent (prions)

general approach in microbiologic diagnostics

: clinical investigation of patient

: collection and transport of sample

: evidence and formulation of approach

1st series: microscopy, antigen and DNA/RNA detection

2nd series: isolation of agent, event. indirect proof

3rd series: identification of agent

4th series: determination of sensitivity on antibiotics

: diagnosis and therapy

direct proof: microorganism finding
: microscopically – shape, staining
: immunologically – proof of pathogenic antigens
: genetically – sequencing
: biochemically – proof of specific pathogen metabolites

indirect proof: immunologically – finding of antibodies against pathogens

microscopic proof

: fast and cheap
: less sensitive – from concentration 10^5 / ml

native preparation

: proof of larger microorganisms – some parasites and coetaneous fungi

:: syphilis diagnostics (*Trepomena pallidum,* treponemata)

: mycologic preparations – „native" only in technical sense; stained by Parker ink or fluorescence dye

stained preparation

fixation – disruption and adhesion of microbial cells on microscopic slide
heating of the overlay over flame or denaturation by chemical agents
(regardful; methanol, protein antigens – ethanol, viruses – acetone)

: orientational simple staining – methylene blue
: diagnostic staining (**differential**) – staining according to Gram, Ziehl-Neelsen, Giemsa and fluorescence staining approaches

Gram staining

: presence of microorganisms (significant number)
: size, shape (cocci, bacilli...)
: mutual arrangement (diplo-, staphylo-, strepto- *etc.*)

preparation background – presence and view of macroorganism cells (epithelia or leucocytes) and other structures (mucous fibres *etc.*)

divides microbes into **blue-violet** coloured **gram-positive** microbes
pink to red coloured **gram-negative** microbes

procedure: fixed preparation for 20 s into solution of crystal violet; then for 20 s in iodine solution, alcohol washing (max. 20 s), water washing, immersing into saphranine solution and final water washing

structure of bacterial wall – complex of crystal violet with iodine is within gram-negative bacteria easily washed out by alcohol; they could be then easily stained pink by saphranine or diluted fuchsine

diagnostics of purulent affections (meningitis, gonorrhoea, anaerobic infection, inflammations)

Ziehl-Neelsen staining

bacteria unstainable by Gram procedure (*Mycobacterium tuberculosis*)

procedure: fixed preparation is stained while steam heating in carbolfuchsine, destain by acidic alcohol and re-staining with e.g. methylene blue; acido-resistant bacilli are stained pink, preparation background is blue

fluorescence staining

: more sensitive proof of acido-resistant bacilli

procedure: heat fixed preparation id stained by mixture of fluorescence dyes auramine and rhodamine, differentiates by acidic alcohol, re-staining by fuchsine; on dark crimson preparation background brilliant yellow bacilli

Giemsa-Romanowsky staining

: in haematology serves to stain smear blood; in microbiology to prove protozoa (malaria, trypanosomes, leishmania, trichomonas), poorly Gram stained bacteria (ehrlichia, rickettsia *etc.*) and to picture viral inclusions

procedure: fixed by methanol and stained for 2 h by Giemsa dye (1:10 water diluted). Giemsa dye – azure with eosin; bacteria are stained dark blue, nuclei of protozoa lake, their cytoplasm light blue

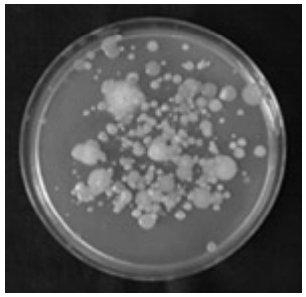
isolation, cultivation of microorganism ⇒ **direct evidence** **cultivation proof**

procedure

: on cultivation media : bacteria, yeasts, mould and protozoa
: in cell cultures (*in vitro*, *in vivo*) : protozoa and intracellular parasites

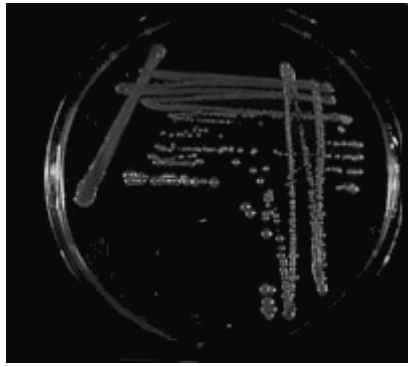
cultivation media

basic liquid medium (bouillon)
: meat extract
:: inoculation by loop, incubation in thermostat



basic solid medium (nutritive agar)
: algae boiled in bouillon – gel (agarose, agaropectin)
:: inoculation by loop – **cross smear**, incubation in thermostat

enriched media
: growth factors (vitamins, AA, nucleotides)
: blood agar, egg media (*M. tuberculosis*)



selective media
: growth inhibitors for unwanted organisms – **selection**

diagnostic media
: growth factors, selection inhibitors, substrate and indicator

End medium (enteric bacilli), MacConkey medium

media for antibiotic sensitivity determination
: growth factors, selection inhibitors, substrate and indicator

Mueller-Hinton agar

transportation media
: without nutrients, wet, metabolism inhibitors
:: 24 h of survival

Amies medium – inorg. salts, sodium thioglycate, active carbon, 0.4% agar

in vitro ***cellular cultures***

: chicken embryos – viruses, ricketsia, chlamydia

tissue cultures, monolayers under nutritive solution (Eagle essential minimal medium)

in vivo

: guinea-pigs, mice

„3 R" rules

: refinement – carefully prepared experiments under best conditions

: reduction – lowest possible number of animals

: replacement – try to evade the vivisection by other approach, e.g. by tissue cultures or genomics

tuberculous mycobacteria – guinea-pigs; rare and unrepeatable samples (liquor, exempt lymph nodes *etc.*)

vivisection is more sensitive than *cultivation* – tularaemia or psittacosis

virology – laboratory mice;
tick-borne encephalitis virus isolation, **coxsackieviruses** (velamentum and hearth muscle inflammation)

proofs for **microbial toxins** – not possible without *vivisection*

diagnostic media

biochemical proof

saccharide media – ability of microorganism to metabolise certain saccharide product ⇒ acid ↓ pH of medium – acido-basic indicator

adonitol, arabinose, cellobiose, galactitol, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannitol, mannose, melesitose, melibiose, rafinose, rhamnose, ribose, sucrose, sorbitol, starch, trehalose and xylose

substrate media – ability to enzymatically proceed the substrate
: deamination – phenylalanine and tryptophan
: decarboxylation – arginine, lysine and ornithine
: hydrolysis – urea, hippurate and tributyrine
: reduction – nitrates to nitrites

metabolic media – take-up of certain substances

citrate, acetate or malate – sources of carbon
: growth factors

auxanogram – determination if yeast grows not better in vicinity of tablet with certain saccharide

used microbial enzymes: N-acetyl-β-D-glucosaminidase (NAG), C8-esterase, α-galactosidase (αGA), β-galactosidase (βGA), β-glucosidase (βGL), β-glucuronidase (βGLR), γ-glutamyl transferase (GGT), leucyl aminopeptidase (LAP), pyrrolidonylarylamidase (PYR), urease and β-xylosidase (βXY)

immunodetection

agglutination on glass-plate
: antigens of corpus and flagellum

Salmonella enteridis, citrobacteria, pseudomonades, *Vibrio cholerae*, bordetella, meningococci

viral identification

virus-neutralising test
: specific antibody inhibits some biological effect of virus

antibiotic sensitivity

antibiotics, synthetic anti-microbial chemotherapeutics

qualitative proof
disc diffusion test

principle: around the disc made of filtration paper saturated with antibiotics, sensitive microbe will not grow – inhibition zone of certain diameter is created

strain sensitive towards given antibiotics – zone is same or larger than within reference strain

tested antibiotics – depends of microbe species, disease characteristics and sample type, of which the microbe was cultivated and on local situation in development of microbial resistance towards antibiotics

quantitative determination

dilution approaches (dilution of antibiotics); minimal inhibition concentration (MIC) of given antibiotics for evaluated microbial strain

: microtitration plate 8x12 with defined concentration of certain antibiotics decreasing in geometric order

: after inoculation and incubation we check, if bouillon remained transparent (total growth inhibition), or if there is precipitate or sediment

MIC – lowest antibiotics concentration able to stop growth; μg/ml, mg/l

minimal germicidal concentration – lowest antibiotics concentration able to kill examined strain
: increasing concentration in series

especially today – **resistance** of microbes towards antimicrobial substances

antibiotics resistance – change of target molecule, worsened penetration into cell, increased excretion of antibiotics from cell or appearance of enzyme, which inactivates antibiotics

proof of microbial component

indirect proof

traces, laid during the infection by **pathogen in organism**

: microbial antigens, toxins, metabolic products and typical NA sequences

in majority of cases it is proof by means of **antibody (Ab) amount determination**

serologic test

immunoassay

syphilis, glandular fever, HIV infection *etc.*
: proof of significant increase of Ab amount; **Ab amount** is called **titre**

case study
method development for clinical diagnostics

determination of alkalic phosphatase

alkalic phosphatase (ALP) – analyte relevant to liver function, growth of bone apparatus, placenta *etc.*

4 main isoenzymes: osseous, hepatic, enteric and placental

case study – typical case for analytical method development

: influence of method, temperature, buffer, modifiers, estimation of optimal substrate concentration, approach for reference interval determination

+ kit manufacture and way of individual agents stabilisation

basic properties of ALP

E.C.3.1.3.1.
ortophosphoric-monoester phosphohydrolase with alkalic optimum

: *catalyses hydrolysis* of phosphate monoesters to inorganic phosphate and respective alcohol

: it may also *hydrolyse* all types of compounds with bonds **P-O-C**, **P-O-P**, **P-S** and **P-N**, with exception of compounds with P-C bond

: in case of phosphate monoesters it may also *transfer phosphate group*

hydrolysis

$$R\text{–}O\text{–}PO\text{–}(OH)_2 + H_2O \xrightarrow{ALP} R\text{–}OH + H_3PO_4$$

transphosphorylation

$$R_1\text{–}O\text{–}PO\text{–}(OH)_2 + R_2\text{–}OH \xrightarrow{ALP} R_1\text{–}OH + R_2\text{–}O\text{–}PO\text{–}(OH)_2$$

efficiency of transphosphorylation depends mainly
on type and concentration of acceptor

ALP : metalloprotein; co-factor Zn(II) (4 atoms)

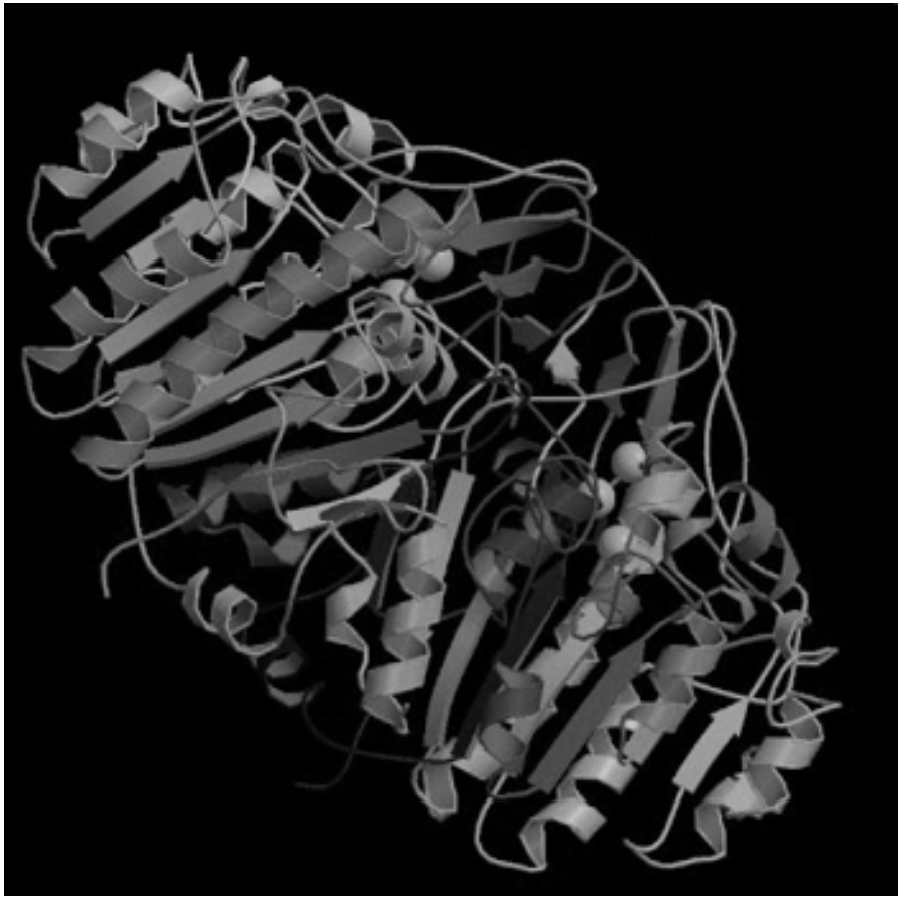
: Zn(II) plays important role within transphosphorylation reactions of ALP
: Co(II) supports hydrolysis, but not transphosphorylation

non-specific inhibitors: EDTA, KCN, cysteine, *o*-phenanthroline, 8-hydroxyquinoline-5-sulphonic acid *etc.*

: removing first 2 Zn atoms, the enzyme activity decreases in about 90%
: remaining 2 atoms are more difficult to remove;
results in completely inactive apoenzyme

specific inhibitor – L-phenylalanine

activators: Co(II), Mg(II) and Mn(II) ions,
whereas Be(II) a Zn(II) act as inhibitors




active site

ALP importance

discovery: 1907 in rice germs

analytical determination and **use** in diagnostics
: 1926 – diagnostics of bone disorders

: 1930 – obstructive jaundice

serum of healthy contains mostly **liver and osseous isoenzymes**

↑ **activity of ALP**
: growth disorders a some osseoms (osseous isoenzyme)

: hepatobiliary system disorders (cholestase and tumour metastases into liver)

↓ **activity of ALP**
: hypothyreosis (cretinism), scurvy, irradiation disease, heavy anaemia and within immunosuppressive medication

season variations: UV-light influence; in winter (low sun radiation) ↑ ALP, in pregnancy ↑ ALP in about 12 to 50 % (placental isoenzyme)

ALP determination methods

catalytic activity: depends on substrate and reaction conditions – type, pH and concentration of buffer, temperature, presence of modifiers

history:

1926 – substrate hexaphosphate

1929 Kay – substrate β-glycerolphosphate, without buffer; determination of inorganic phosphorus (48 h!!!)

later – glycine buffer pH 8.8, phosphorus by phosphomolybdate blue (3 h)

– barbitate buffer pH 10.8

new substrates: phenyl phosphate and 4-nitrophenyl phosphate, phenolphthalein mono- and diphosphates, thymolphthalein monophosphate, 3-O-methylfluorescein phosphate, naphthyl-AS-MX-phosphate, methylumbelliferyl phosphate, indoxyl phosphate *etc.*

increasing sensitivity – phenyl phosphate, determination of released phenol by oxidative copulation with 4-aminoantipyrine and ferricyanide

noval – chromogenic substrates: phenophthalein phosphate
: thymolphtalein phosphat

substrate

: unambiguously defined, sufficient chemical purity, must have hydrolysis product with auto-indicative properties

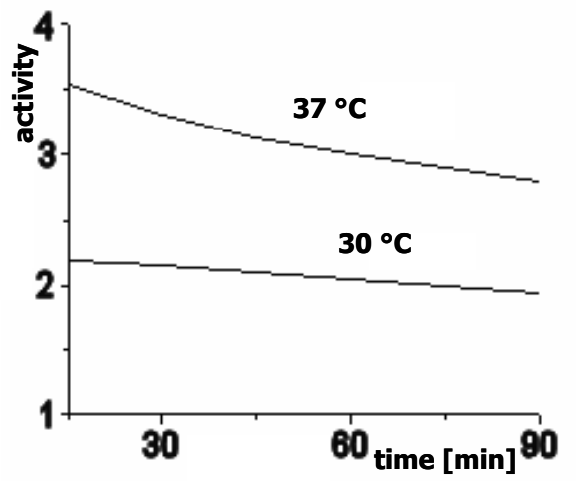
today – 4-nitrophenyl phosphate (NPP **1**), is cleaved into phosphate and 4-nitrophenol (NP **2**)

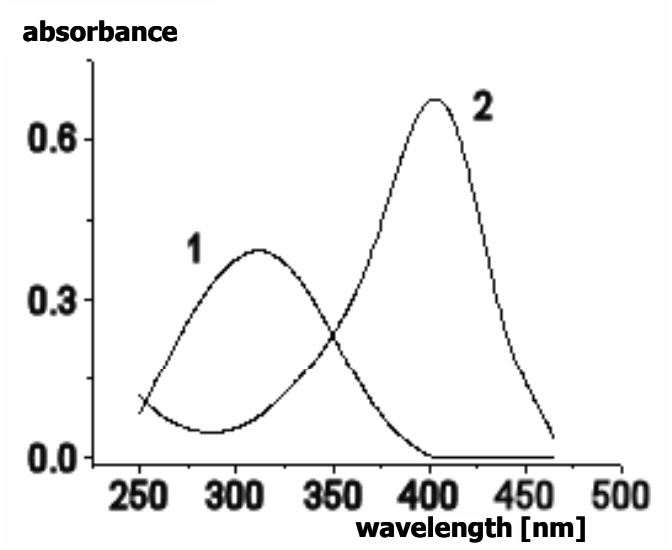
NP is acido-basic indicator with dissociation constant $pK_a = 7.16$

concentration of **NPP**: 5 – 20 mmol/l

temperature

ALP – at 37 °C faster inactivation





pH and buffer

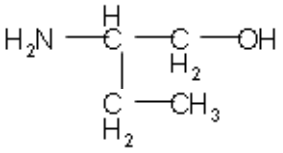
already not used buffers: barbitate, glycine, hydrogen carbonate, 2-amino-2-methyl-1-propionic *etc.*

ALP **activity** is **strongly influenced** by buffer
: buffer is simultaneously also **second substrate** (phosphate acceptor) within transphosphorylation

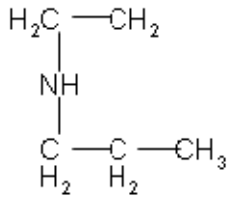
activity influencing by buffer
: neutral / **inert** (carbonate, barbitate *etc.*)
: inhibiting (glycine, propylaminic *etc.*)
: activating (transphosphorylating buffers)

buffer for ALP
: phosphate acceptor
: sufficiently stable
: available in *p.a.* purity
: dissociation constant $pK_a \sim 10$

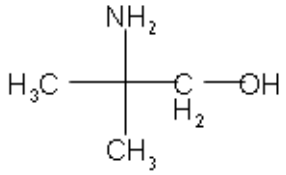
AMP was recommended by **IFCC**



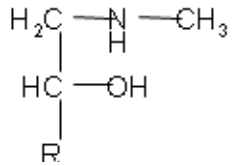
EAE ethylaminoethanol



DEA diethanolamine



AMP 2-amino-2-methyl-1-propanol



MEG N-methyl-D-glucamine

R – glucosed

ways of measurement

before: enzyme activity kinetically, manually and discontinually (two-points)
today: fast kinetic continual approach

modifiers of ALP

inhibitors: compounds of arsenic, phosphates, substances able to bind zinc ions stronger than enzyme

: non-specific chelatogenic inhibitors: EDTA and KCN

specific activator: sodium ion in MEG buffer

ALP determination in MEG buffer

optimisation of ALP determination

: literary research (properties, determination methods *etc.*)

: demarking orientation conditions of analytical approach (buffer type and its pH and concentration, type and concentration of substrate)

: optimisation of incubation mixture composition

exclusively with **NPP substrate**

buffer

: MEG – good transphosphorylation properties, low reactivity, high purity and ready availability

: DEA and AMP excluded for content of inhibiting impurities

reaction conditions and work-flow

temperature	37 °C	wavelength	420 nm
pH (37 °C)	10.1 ± 0.1	opt. path length	1 cm
MEG buffer	0.35 mmol	temperature	(37.0 ± 0.1) °C
NPP substrate	15 mmol/l	signal	ΔA
sodium chloride	70 mmol/l	measurement interval	30 – 120 s
magnesium chloride	0.5 mmol/l	serum/incub. mix (v/v)	1:61 (0.0164)

chemicals purity

NPP: < 0.05 % free 4-nitrophenol, < 0.25 % free inorg. PO_4^{3-}
molar absorbance in 10 mmol/l NaOH λ = 311 nm – 9 867 ± 761 l/mol.cm

MEG: melting point 129 – 131 °C

NP: molar absorbance in 10 mmol/l NaOH λ = 401 nm – 18 380 l/mol.cm

stability of agents

buffer – at least 1 month at +5 °C (without bacterial contamination)

substrate – prepare just before use; in dark and cold at +5 °C stable at least 24 hours

standard solution of NP – in dark and cold stable at least 1 month

calculation of catalytic activity concentration

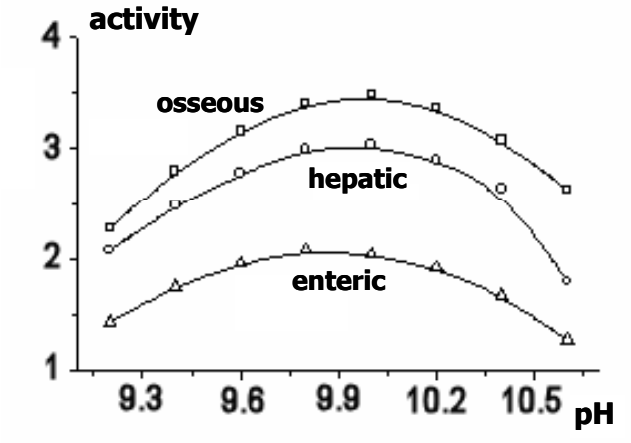
$$\mathbf{fS\text{-}ALP\ [\mu kat/l] = \Delta A_{420} * \mathit{F}}$$

F is factor, $\mathbf{\Delta A_{420} = (\Delta A_1 - \Delta A_2)}$

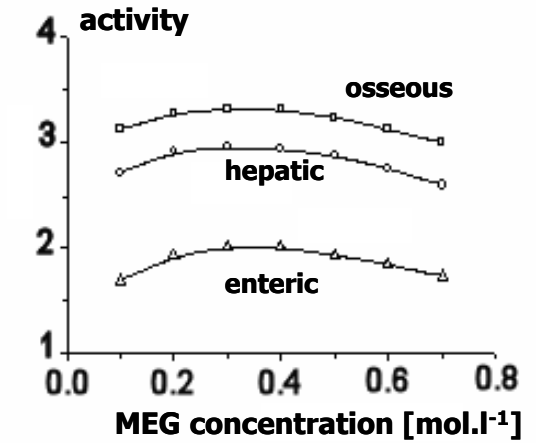
absorbance of reference solution (ΔA_2) – compensates absorbance of NP created by spontaneous, non-enzymatic decomposition of substrate due to alkali medium presence

factor *F* :
1) from calibration solution without ALP
2) using molar absorbance of 4-nitrophenol

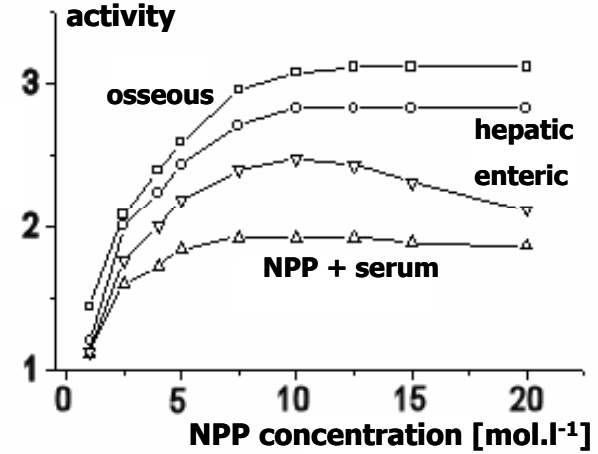
optimal conditions of ALP determination



pH influence



influence of MEG concentration



influence of NPP concentration

reference interval

fS-ALP [µkat/l]: males 0.90 – 2.20, females 0.74 – 2.10, children 1.20 – 6.30
200 adults, 112 boys and 152 girls

repeatability and reproducibility

repeatability: examined in 10 external laboratories, precision in series and day-to-day;
N = 20; ø rel. standard deviation: 3.5 % manual analysis; 2.5 % analyser

reproducibility: in 5 laboratories; 1.4 – 4.2 %

kit for ALP determination

agent 1 (6 vials)
: solid substrate, finally for NPP solution of 1.12 mmol/vial
: substrate solution is prepared by dissolution of one vial content in 5.0 ml of distilled water; in dark and cold at +5 °C, the solution is stable *ca* 1 week

agent 2 (300 ml)
: MEG buffer in liquid state, concentration 0.56 mol/l, which contains as conserving agent 5-bromo-5-nitro-1,3-dioxane 0.3 mg/l and N-methylisothiazolon 1 mg/l

agent 3 (2 ml)
: standard solution of NP in ampoule under N_2 containing NP at 2.4 mmol/l and Na_2EDTA 0.05 g/l

storage: in dark and cold at 2 – 8 °C, stable for 24 months

working procedure: mutual voluminal ratios of serum, buffer and substrate are 1:50:5, so 0.02 + 1.00 + 0.10 ml

importance of buffer in bioanalysis

buffer – reaction medium/environment

: **keeping pH** (optimum)
: **secondary substrate** (enzyme reactions)
: **modifiers** – inhibitors, activators

1900, Fernbach and Hubert: partially neutralised solution of phosphoric acid serves as protection against abrupt changes of solution alkalinity or acidity within research of enzyme amylase

buffer choice

basic characteristics: dissociation constant of acid (part of buffer); pK_a
dilution influence, ionic strength, buffer capacity and pH temperature influence

pH range: $pH = pK_a \pm 1$, while buffer is the most effective at $pH = pK_a$

compatibility and synergic influence of buffer

compatibility

chemical

: good water solubility (bad with barbiturates)
: non complexing for metal i. – Ca(II), Mg(II), Mn(II), Zn(II), Fe(II)/(III) *etc.*
: soluble (so mostly no carbonates, phosphates, citrates)
: non-reactive with other components (proteins, saccharides + borates)
: invulnerable to bacterial contamination (so no phosphates, citrates *etc.*)

biochemical

: influencing activity of some enzymes (phosphates inhibit phosphatases, carboxylases, phosphoglucomutases *etc.*)
: interference in processes of oxidative phosphorylation (barbiturate)
: antisepticity; activity blocking of most of enzymes (phenols)
: ampholytes (zwitterionic) oxidised by flavin mononucleotides (BICIN, TRIS)
: reactive and inhibiting buffers (TRIS)

synergicity

modification – activator (GlyGly; fibrinogen) or secondary substrate (MEG)

main bioanalytic buffers

abbrev	composition	pK_a
MES	2-(N-morpholine)ethane sulphonic acid	6.1
BIS-TRIS	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propandiol	6.5
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane sulphonic acid	7.1
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethane) sulphonic acid	7.5
TRICIN	N-(2-hydroxy)-1,1-bis(hydroxymethyl)ethylglycine	8.1
BICIN	N,N-bis(2-hydroxyeteyl)glycine	8.3
AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane sulphonic a.	9.0
CAPSO	3-(cyclohexylamino)-2-hydroxy-1-propane sulphonic acid	9.6
CABS	4-(cyclohexylamino)-1-butane sulphonic acid	10.7

analysis of selected analytes
inorganic, organic analytes and bioanalytes

ammonium

content: degradation of amino acid in liver; toxic (CNS) ⇒ urea
determination: in plasma; reference interval 11 – 35 μM

chemical methods
procedure: ammonium is alkalised, separated and absorbed into acidic solution (Conway technique)
: titration, conductometry, ISE, Nessler agent (yellow) or Berthelot reaction (reaction with phenol and with alk. hypochlorite into blue quinonimine dye)
laborious and ***non-automatable***

enzymatic methods
enzyme: glutamate dehydrogenase (GLD)

$$\text{2-oxoglutarate} + NH_4^+ + \text{NADPH} \xrightarrow{\text{GLD}} \text{L-glutamate} + NADP^+ + H_2O$$

: absorbance decrease of NADPH at **340** or **365 nm**

procedure: TEA 150 mM, pH 8.6 + 2-oxoglutarate 15 mM, ADP 1.5 mM, GLD *min* 800 U/ml and NADPH 0.12 mM

phosphorus, phosphates

content: inorganic and organic phosphates; bone tissue, nucleic acids, phospholipids, coenzymes, ATP *etc.*
determination: in serum; reference interval 0.7 – 1.6 mM

chemical methods

inorganic phosphates:
: reaction with ammonium molybdate (colourless phospho-molybdate complex)
: with ammonium vanadate-molybdate (yellow complex of ammonium phosphoric vanadomolybdate); hydrolyse partially also organic phosphoric esters and thus makes the determination falsely positive

organic phosphates: after mineralisation of denatured proteins; depends on pH (strong acidic medium)
procedure: serum deproteination (strong acid), supernatant analysis: measured at 340 nm; phosphomolybdenite blue (after reduction tin(II) chloride, aminonaphthalene sulphonic acid, methyl-p-aminophenol sulphate, iron(II)-ammonium sulphate, ascorbic acid *etc.*) at 882 nm; vanado-molybdate phosphoric acid at 420 nm

enzymatic methods

do not catalyse phosphoric ester hydrolysis, no need for sample deproteination
: glycogen phosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase – *NADPH*
: purin nucleoside phosphorylase, xanthine oxidase (peroxidases) – *oxidative copulation*
: sucrose phosphorylase – *NADH*

magnesium

content: 21 – 28 g in *ca* 70 kg (60 % bones, 20 % muscles, 19 % other tissues, *ca* 1 % extra-cellular liquid); cofactor of almost 300 enzymes
determination: in serum/plasma; reference interval 0.65 – 1.05 mM

chemical methods
AAS – optimal; free Mg(II) – ISE
: in analysers – photometric methods with calmagit, magon...

AAS:
procedure: $LaCl_3$ solution (spectral buffer, releases Mg(II) from phosphate complexes and dilutes viscose proteins) concentration 4.3 g/l with 10 ml conc. HCl; sample : buffer 1:50 into flame (acetylene-air), Mg-discharge at 285.2 nm; calibration solution contain interferents Na^+ and K^+

reaction with magon:
procedure: 10 µl serum with 2 ml working solution (20 mM borate buffer pH 9.5 and magon 0.28 mM diluted in mixture DMF and ethanol 5+100), resulting pH *ca* 11; influence of Ca(II) and heavy metals – cyanide masking + physiologic concentrations of Ca(II), Na(I) and K(I) ions; absorbance decrease of blue complex around 500 nm

reaction with calmagit:
procedure: in partially non-aqueous medium with 2-methyl-2-amino-1-propanol; Ca(II) masking by EGTA, measured at 540 nm

hydrogen carbonates

content: blood; diagnosis of acidobasic equilibrium disorders
determination: in whole blood, plasma and serum as CO_2 (after acidification); reference interval depends on instrumentation, usually CO_2 in capillary plasma *ca* 22 – 31 mM

physical methods
gaseous carbon dioxide by *manometry*; laborious and not automatable

chemical methods
continuous measurement – CO_2 diffusion through Si-membrane and sorption as HCO_3^- in alkali buffer, pH 9.2 with phenolphthalein; colouration decrease (acidification) photometry
electrochemical measurement – CO_2 electrode, partial pressure of dissolved gas in blood

enzymatic methods
alkalisation – transformation into HCO_3^-
enzymes: phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH):

$$HCO_3^- + \text{phosphoenolpyruvate} \xrightarrow{\text{PEPC}} \text{oxalacetate} + PO_4^{3-}$$
$$\text{oxalacetate} + NADH + H^+ \xrightarrow{\text{MDH}} \text{malate} + NAD^+$$

procedure: alkali buffer *ca* 70 mM, pH 8 + phosphoenolpyruvate 8 mM, NADH 1.6 mM, microbial PEPC *min* 17 μkat/l and microbial MDH *min* 4 μkat/l
10 μl serum/plasma (tempered cuvette) + 1 ml agent, incubation 5 min at 37 °C, absorbance at 340 nm

chlorides

content: main extra-cellular anion (67 %)
determination: in serum and plasma; reference interval in serum 98 – 107 mM
titration:

$$2\ Cl^- + Hg(NO_3)_2 \rightarrow HgCl_2 + 2\ NO_3^-$$

procedure: in acidic medium, mercury(II) nitrate solution 5 mM, sample is 0.20 ml of serum, indicator diphenylcarbazon 20 mM in ethanol

spectrophotometry:

$$2\ Cl^- + Hg(SCN)_2 \rightarrow HgCl_2 + 2\ SCN^-$$
$$SCN^- + Fe(III) \rightarrow Fe(SCN)^{2+}$$

procedure: measured around 500 nm (red product), range 80 – 125 mM, calibration not linear, linearity by constant amount of $Hg(NO_3)_2$ which binds *ca* 60 mmol Cl^- in 1 L

coulometry:

$$Ag^+ + Cl^- \rightarrow AgCl$$

procedure: generating Ag(I) in a constant rate off anode till equivalence. content of chlorides is then directly proportional to measured time; acidic medium (better conductivity) at presence of gelatine or polyvinyl alcohol (reproducibility)

copper

content: metalloenzymes, in plasma in 95 % on coeruloplasmin
determination: in serum; reference interval (μmol/l) 10.1 – 18.4 (males), 11.3 – 25.2 (females)

AAS:
: only sample dilution necessary

reaction with bathocuproin:
procedure: deproteination + reduction agent (hydroxylamine or pyrosulphite in combination with p-(N-methyl)aminophenol), centrifugation, supernatant is put into other tube and bathocuproin solution is added; in strongly acidic medium orange complex is measured at 480 nm
: depends on glass purity; EDTA, washed by water with ammonium

zinc

content: metalloenzymes
determination: in plasma, serum, saliva, urine; reference interval (serum) 10 – 20 μM

spectrophotometry with 5-Br-PAPS
procedure: 2-(5-bromo-2-pyridylazo)-5-(N-n-propyl-N-3-sulphopropylamino)phenol at pH 8.6 + making agents (other endogenous metals), at 560 nm, not sensitive enough

AAS
procedure: sample is 5x diluted by 5% glycerol, measured at 213.8 nm; urine only from 24 h collection, directly into flame; complications – high salt content

calcium

content: bone tissue (99 %), extra-cellular liquid, free Ca(II) is only active
determination: in serum, only free or also bound; reference interval 2.1 – 2.6 mM

reaction with o-cresolphthalein complexon:
procedure: pH 12 in medium with organic base (2-amino-2-methyl-1-propanol, 2-ethylaminoethanol); measured at 580 nm, 8-hydroxyquinolin suppresses interferences

reaction with arsenazo III:
procedure: imidazole buffer pH 6, blue complex, measured around 650 nm; specific detergents suppress interferences of proteins

AAS:
procedure: similarly to magnesium, measured with Ca-lamp at 422.7 nm, expensive, higher precision and accuracy

ISE:
: Ca(II) activity measured, depends mainly on ionic strength (Na^+ and Cl^-) – calibrators

potassium

content: K^+ in main intra-cellular cation (4.6 mM)
determination: in all body fluids, mostly in plasma and serum, strongly interfered by haemolysis; reference interval in serum 3.8 – 5.2 mM

enzymatic methods
enzymes: tryptophanase (TR), glutamate dehydrogenase (GLD)

$$\text{tryptophan + pyridoxal-5-phosphate} \xrightarrow{TR;K^+} \text{indol + pyruvate + } NH_4^+$$
$$NH_4^+ + \text{2-oxoglutarate + NADPH + } H^+ \xrightarrow{GLD} \text{glutamate + } NADP^+$$

enzymes: pyruvate kinase (PK), lactate dehydrogenase (LDH)

$$\text{phosphoenolpyruvate + ADP} \xrightarrow{PK;K^+} \text{pyruvate + ATP}$$
$$\text{pyruvate + NADH + } H^+ \xrightarrow{LDH} \text{lactate + } NAD^+$$

: Na^+ concentrate lowering by cryptand

chemical methods
AES:
procedure: serum diluted by spectral buffer (lithium or caesium, stabilises effective temperature of flame; anion tensides Brij 35 or Sterox SE for better atomisation); flame propane-air; Na, K, Li and Cs with sharp lines at 589 nm, 768, 671 and 852 nm

ISE:
: electrode with liquid membrane

sodium

content: Na^+ main extra-cellular cation (*ca* 142 mM)
determination: in all body fluids, mostly in plasma and serum, strongly interfered by haemolysis; reference interval in serum 132 – 142 mM

enzymatic methods
enzymes: β-galactosidase (βGD)

2-nitrophenyl-β-D-galactopyranosid $\xrightarrow{\beta GC;Na^+}$ galactose + 2-nitrophenol

2-nitrophenol (chromophore) measrued kinetically at 420 nm, Na^+ content lowered to measurable values by cryptand, e.g. Kryptofix 221

chemical methods
AES:
as potassium

ISE:
electrode with glassy membrane

barbiturates

content: medical rugs (sedative)
determination: in serum (medication monitoring), in urine or stomach (intoxication)

chemical methods
gas chromatography:
procedure: aprobarbital (internal standard) is added to serum, diethylether double extraction, sodium sulphate dehydration and dry evaporation; evaporate dissolved in ethylacetate; GC separation – capillary column (WCOT), flame ionisation detector, carrier gas Ar or He; analysis of different barbiturate types (short, medium and long term affecting)

titration analysis:
procedure: extraction by phosphate buffer pH 7 and chloroform mixture; into organic phase buffer is again added with mercury(II) chloride, extracts and centrifuges; organic phase is titrated by diphenylthiocarbazon (dithizon) solution, change from violet ({Hg(II)-barbiturate} complex) to orange ({Hg(II)-dithizon} complex); hydantoins and cyclic imides of glutaric acid interfere mostly

bilirubin and its esters

content: metabolism of haeme through biliverdin to bilirubin and conjugates (mono- and diglucuronides esters)
determination: in serum and urine; reference interval for total bilirubin in serum (μM): new-borns 68 – 138, children and adults 3.4 – 17.1, out of which conjugated, so called direct bilirubin 0 – 3.4

chemical methods
bilirubin and its conjugates determination (and part of bilirubin covalently bound to albumin) is based of azobilirubin creation (acidobasic indicator): red in weak acidic and neutral pH, in strong acidic and alkali range is blue; **conjugates** (i.e. direct bilirubin) reacts without *accelerators*; **unconjugated bilirubin** (i.e. free) does not react, only in presence of *accelerators* (alcohol, caffeine *etc.*), which release and solubilise bilirubin from its bonds to albumin

determination of **total bilirubin** at presence of *accelerators*; within increased content also the direct bilirubin is determined without accelerators; within new-born jaundice and irradiation of new-borns with UV-light, which serves to decompose and remove of free, unconjugated bilirubin, it was observed creation of so-called photobilirubins, which have in contrary to normal bilirubin different reaction kinetics and may distort results of measurements

spectrophotometry with diazotised sulphanilic acid:
procedure: IFCC method of total bilirubin determination; sample in cuvette + sulphanilic acid solution in HCl (cleaves bilirubin in methylene bridge) with accelerator solution (mixture of caffeine, sodium acetate and benzoate) + $NaNO_2$ solution (diazonium salts); after 10 min in weak acidic medium absorbance is measured of red azobilirubin at 430 – 460 nm; determination through blue form is done by adding alkali buffer solution (NaOH and sodium potassium tartrate), and in pH 12 it is measured at 580 – 620 nm
determination of direct bilirubin is done without accelerator by stopping diazoreaction by ascorbic acid (diazotisation agent decomposition), it is usually added after 5 or 10 min; total bilirubin is determined with cation-active detergent like accelerator (cetyltrimethylamonium bromide)

spectrophotometry after oxidation:
procedure: bilirubin oxidation (yellow) by vanadic acid to biliverdin (green); two-point absorbance measurement before and oxidation (3 min); it is possible also to determine direct bilirubin

enzymatic methods
enzyme: bilirubin oxidase (BOX)

bilirubin (yellow) $\xrightarrow{BOX}$ biliverdin (green)

procedure: pH 4.5, measured in range 424 – 465 nm kinetic in time interval *ca* 5 min; total bilirubin is determined at pH 8.5 at presence of accelerators

ethanol

content: alcoholic intoxication

chemical methods

Widmark method; distillation of alcohol off sample, oxidation by dichromate in sulphuric acid or in glacial acetic acid, surplus of dichromate is then determined by iodometric titration; gas chromatography is used in forensic investigations

: to prove *heavy (chronic) alcoholism*, so called carbohydrate deficient transferrin (CDT) test was developed based on heterogeneous immunoanalysis

enzymatic methods

enzyme: alcohol dehydrogenase (ADH)

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

procedure: sample deproteination by perchloric acid, alcohol is determined from supernatant in alkali buffer pH 8.7 (pyrophosphate, semicarbazide and glycine), absorbance at 37 °C at 340 nm after incubation 25 min

drugs of abuse (DOA)

content: psychofarmaca and their precursors – alcohol, amphetamine, barbiturates, benzodiazepines, cannabinoids, cocaine, methadone, opiates, antidepressives, anabolic steroids *etc.*
determination: belongs to point-of-care testing (POCT)

chemical methods
competitive immunochemistry – fast statim orientation determination of the most usual drugs of abuse, urine screening on diagnostic strip

instrumental techniques – GC-MS or HPLC, serves mostly for consequent quantitative analysis after positive screening test

screening imunoassay
multifunctional (up to 9-zone) strip test, most frequently: amphetamine, barbiturates, benzodiazepines, cocaine, methamphetamine, morphine, phencyclidine and tricyclic antidepressives (or their metabolites)

procedure: *drug from urine* (**mAg**) compete for binding sites with (usually) *murine monoclonal antibody against it* (**Ab_1***), fixed on surface of microparticles, which are sorbed (not immobilised) in lower part of strip; in upper part of strip, there is *immobilised other (second) antibody* **Ab_2** *against murine immunoglobulins* of **Ab_1***

after sinking of multi-strip into urine sample containing some of tested DOAs, the antibody on the start reacts and creates *coloured immunocomplex* [**Ab_1*-mAg**] and rises up

urine without DOA: complex is not created and coloured microparticles with fixed antibody rise up and in the middle part reacts **Ab_1*** with on-there immobilised DOA/metabolite and creates there *coloured immunocomplex* [**Ag-Ab_1***] as **negative control**

urine with DOA: *coloured immunocomplex* [**Ab_1*-mAg**] created with sample rises and is captured at the end of strip, where it reacts with **Ab_2** and creates strongly coloured zone as **positive test** [**Ab_2-Ab_1*-mAg**]; antibody **Ab_1*** is in that case antigen for second fixed antibody **Ab_2**

sensitivity: 10 – 100 ng/ml
colloid gold (Au) microparticles are used, resulting colour of negative control and also positive test is red or blue (if the surface of the microparticle is blue)

glucose

content: represents metabolism of saccharides
determination: in serum, plasma and urine (most commonly determined analyte); reference interval (mM) in serum 3.9 – 6.1, in plasma 3.3 – 5.6 and in 24 h collected urine up to 1.39

chemical methods

reaction with o-aminotoluene
specific (also only galactose and mannose), supernatant of deproteinated sample in medium of glacial acetic acid, when o-aminotoluene (6 % o-toluidine in 80 % acetic acid containing 0.5 % of oxalic acid) condensates with Glu under elevated temperature into glycosylamine, stabilised by Schiff base, green product at *ca* 630 nm
: *agressive chemicals*, *necessity of boiling and sample deproteination*

enzymatic methods

enzymes: hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD), glucose oxidase (GOD), glucose dehydrogenase (GDH), peroxidase (POD)

determination with GDH

$$R\text{-}CH{=}O + H_2O + NAD^+ \xrightarrow{GDH} R\text{-}COOH + NADH + H^+$$

procedure: sample is mixed with working solution (phosphate buffer pH 7.6, GDH *ca* 4.5 kU/l and NAD^+ *ca* 2.2 mM), incubated for 7 min at 37 °C, absorbance increase at 340 nm

determination with HK and G6PD

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{gluconolacton-6-phosphate} + \text{NADPH} + \text{H}^+$$

procedure: sample with working solution (TRIS 50 mM pH 7.5, ATP 1 mM, NAD^+ 2 mM, HK *ca* 3 kU/l and G6PD 2 kU/l) at 37 °C, after 5 min at 340 nm; HK is for glucose unspecific enzyme, specificity lies in consequent reaction

determination by oxidative copulation with GOD and POD

most practical

$$\text{R-CH=O} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{R-COOH} + \text{H}_2\text{O}_2$$

$$\text{R-NH}_2 + \text{C}_6\text{H}_5\text{-OH} + 2\,\text{H}_2\text{O}_2 \xrightarrow{\text{POD}} \text{R-N=C}_6\text{H}_4\text{=O} + 4\,\text{H}_2\text{O}$$

procedure: sample with working solution (phosphate *ca* 0.15 mM pH 8.0, GOD 10 kU/l, POD 1 kU/l, AAP 1 mM and 3-methylphenol 10 mM) at 37 °C, absorbance change at 500 nm in interval 30 – 90 sec or resulting colour after 15 min; second reaction in unspecific, is interfered by reductive compounds (vitamin C)

special analysers

personal glucometers for self-control of diabetics

procedure: immobilised GOD and electrode system in dry state, indication by amperometry (so called Clark electrode):

$$\text{H}_2\text{O}_2 \xrightarrow{\text{Pt}} 2\,\text{H}^+ + \text{O}_2 + 2\,\text{e}^-$$

or: $\text{glucose} + \text{O}_2 + \text{R-Fe(II)} \xrightarrow{\text{POD}} \text{gluconate} + \text{R-Fe(III)}$

pyruvate

content: product of lactate oxidation catalysed by LDH enzyme,
determination: in blood only in small amount approx. 41 – 67 nM

enzymatic methods
enzyme: lactate dehydrogenase (LDH)

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

creatinine

content: cellular product of muscular energetic metabolism of creatine
determination: basic investigation of serum and urine (kidney function – creatinine clearance); amount is proportional to muscle mass size; reference interval for serum creatinine is under 115 μM

chemical methods
Jaffé reaction
red-orange Janovsky complex (adduct of creatinine with picrate 1:1); unspecific, react also non-creatinine chromogens: proteins, glucose, ascorbic acid, guanidine, acetone, acetoacetate and pyruvate

procedure: serum with working solution (picric acid 4.4 mM, NaOH 150 mM and Na_2HPO_4 13 mM), absorbance after 10 s or more precisely after 2 min at 492 nm

enzymatic methods

determination through quinonimine dyes

enzymes: creatininase (KRN), creatinase (KR), sarcosine oxidase (SOX), peroxidase (POD)

$$\text{creatinine} + H_2O \xrightarrow{KRN} \text{creatine}$$
$$\text{creatine} + H_2O \xrightarrow{KR} \text{sarcosine} + \text{urea}$$
$$\text{sarcosine} + H_2O + O_2 \xrightarrow{SOX} \text{glycine} + HCHO + H_2O_2$$
$$2\ H_2O_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{POD} \text{quinonimine dye} + 4\ H_2O$$

procedure: suitable derivatives of phenol – TBHB (2,4,6-tribromo-3-hydroxybenzoic acid) and EHSPT (N-ethyl-N-(2-hydroxy-3-sulphopropyl)-m-toluidine), absorbance at 550 nm

triacylglycerols (TG)

content: 95 % of all lipids stored in tissues are created by saturated fatty acids C12 to C18

determination: in serum (independent factor of ischemia of the heart muscle risk), reference interval lower than 1.7 mM

chemical methods

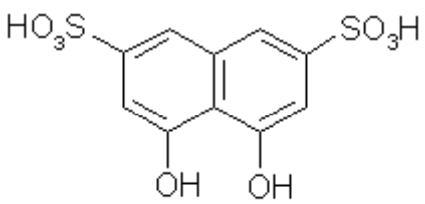
coloured product absorbing at 570 nm, or must be transformed by acetylacetone and ammonium acetate (Hantzch condensation) into yellow 3,5-diacetyl-1,4-dihydrolutidine, which might be determined by photometry or fluorimetry

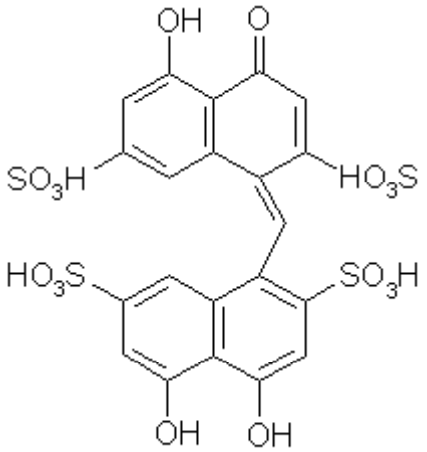
described approaches maybe (after extraction of TG) described by these equations:

$$TG + ROH/OH^- \rightarrow glycerol + 3R\text{-}COOH$$

$$glycerol + IO_4^- \rightarrow HCHO + HCOOH + H_2O + IO_3^-$$

$$HCHO + chromotropic\ acid + H_2SO_4 \rightarrow colour\ product$$





or:

$$4\ HCHO + NH_4OH + CH_3COCH_2COCH_3 \rightarrow 3,5\text{-diacetyl-1,4-dihydrolutidine} + 5\ H_2O$$

enzymatic methods

combination with chemical method, use of created glycerol

<u>enzymes:</u> glycerol kinase (GK), glycerol-3-phosphate dehydrogenase (G3PD), pyruvate kinase (PK), lactate dehydrogenase (LDH), diaphorase (DF), lipoprotein lipase (LPL), glycerolphosphate oxidase (GPO), peroxidase (POD)

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$
$$\text{glycerol-3-phosphate} + \text{NAD+} \xrightarrow{\text{G3PD}} \text{dihydroxyacetone phosphate} + \text{NADH} + \text{H}^+$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$
$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$
$$\text{NADH} + \text{H}^+ + \text{INT} \xrightarrow{\text{DF}} \text{red formazan INT} + \text{NAD}^+$$

INT – iodonitrotetrazolium violet, *ca* 500 nm

combination with chemical methods too complex and not automatable

determination through quinonimine dyes

$$\text{TG} + 3\,\text{H}_2\text{O} \xrightarrow{\text{LPL}} \text{glycerol} + 3\,\text{R-COOH}$$
$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$
$$\text{glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + \text{H}_2\text{O}_2$$
$$2\,\text{H}_2\text{O}_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{POD}} \text{quinonimine dye} + 4\,\text{H}_2\text{O}$$

phenols: 4-chlorophenol, or 3,5-dihydroxybenzene sulphonic acid (DHBS), or N-ethyl-N-(3-sulphopropyl)-m-anisidine (ESPAS)

determination through NADH

$$\text{TG} + 3\,\text{H}_2\text{O} \xrightarrow{\text{LPL}} \text{glycerol} + 3\,\text{R-COOH}$$
$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$
$$\text{glycerol-3-phosphate} + \text{NAD}^+ \xrightarrow{\text{G3PD}} \text{dihydroxyacetone phosphate} + \text{NADH} + \text{H}^+$$

procedure: with only one solution, one-step and automatable

rheumatoid factor

content: pentameric immunoglobulins IgM with specificity for Fc fragment of IgG, similar properties also within monomeric IgM, IgA and IgG
determination: in serum, indication (up to 75 %) of all rheumatic disorders

latex fixation test
procedure: separation according to Cohn (extraction by set of ethanol solutions on rocks), fraction II is mixed with latex particles, on which γ-globulin is passively sorbed, agglutination appears; sensitivity from 2 U/ml

Rose-Waaler test
haemagglutination test (ovine erythrocytes modified by tannin /strengthens cells/ covered by lapine antibodies against ovine erythrocytes); erythrocytes do agglutinate with sample containing human rheumatoid factor based on cross reaction between lapine and human IgG

C-reactive protein (CRP)

content: belongs to so-called proteins of acute phase and to risk factors of ischemia of heart muscle
determination: in serum, its concentration increases *ca* 7 h after the inflammation and culminates during 1 to 3 days, when it may reach thousand of mg/l, reference interval is under *ca* 1.6 mg/l

CRP may be determined by various immunochemical methods, most commonly by latex particle methods

immunoturbidimetric determination
DuREL (dual-radius enhanced latex) method – latex microparticles in two sizes with two types of monoclonal antibodies fixed with different affinity to CRP (large particles with higher affinity); at low analyte concentration larger particles react preferably, at higher concentrations also the smaller ⇒ sensitive, broad and linear measuring range

$$CRP + Ab \rightarrow [CRP\text{-}Ab]$$

: turbidity at 340 nm

trypsin

content: EC 3.4.21.4, serine proteinase, in duodenum
determination: in plasma, in duodenal liquid, in faeces; test of exocrine pancreatic function disorder, reference interval approx. 150 ± 77 µg/l

enzymatic methods
immunochemically (namely RIA)

photometry with chromogenic substrates

L-TAPA + H_2O $\xrightarrow{\text{trypsin}}$ N-α-tosyl-L-arginine + 4-nitroaniline

yellow chromophore 4-nitroaniline; similar chromogenic substrate benzoyl-L-arginine-4-nitroanilid

procedure: sample is diluted with physiological solution + buffer TRIS 200 mM pH 8 + substrate 1 mM, at 37 °C after 10 min absorbance at 405 nm

expectancy test (hCG)

content: human chorionic gonadotropine (hCG), glycoprotein, dimer à 40 kDa; hormone produced by placenta
determination: in maternal blood and urine; since 8th day after conception, hCG level in urine during pregnancy increases dramatically, highest at 8th week, positive test at hCG content above 20 – 50 U/l

latex agglutination test
procedure: on support glass; in urine, hCG reacts with monoclonal antiserum (Ab, murine), and either inhibits following agglutination reaction after addition of latex particles with fixed hCG, or nor (reaction went on); in-parallel so-called positive control is ran

hCG low content (cannot bind all antiserum) $\Rightarrow$ **agglutination happens, negative**:

$$hCG + Ab \rightarrow [hCG\text{-}Ab] + Ab$$
$$Ab + hCG\text{-}latex \rightarrow [Ab\text{-}hCG\text{-}latex]\downarrow$$

hCG high content (all bound) $\Rightarrow$ **agglutination happens not, positive**:

$$hCG + Ab \rightarrow [hCG\text{-}Ab]$$
$$[hCG\text{-}Ab] + hCG\text{-}latex \rightarrow \text{no agglutination}$$

diagnostics strip test
with colloid gold
strip contains in site of sample introduction (down) sorbed, but *not fixed monoclonal antibody against hCG on colloid gold particles* (**Ab_1-Au**), up (in the direction of diffusion) there are two zones with immobilised antibodies; first zone contains *fixed secondary antibody for hCG* (**Ab_2^b**), second zone *contains fixed antibody for Ab_1* (**Ab_3^b**)

hCG high content
hCG reacts with primary antibody into complex, diffuses up and is caught in *the first zone* with fixed **Ab2** as **{Ab2-hCG-AuAb1}** *sandwich*; *red zone* appears (**positive reaction**)

hCG low content
no complex, Ab1 diffuses up, and is caught in *the second zone*; there appears *red zone* of colloid gold (negative reaction), serves as positive control

: instead of colloid gold e.g. organic dye

<u>strip with antibody labelled with enzyme</u>
: similar to colloid gold
: *primary hCG antibody is labelled with suitable enzyme* (**Ab_1***); in a location of *fixed* (**Ab_2^b**) is in reaction zone suitable substrate and auxiliary compounds

hCG high content
complex **[Ab_1*-Ag]** is created, which diffuses and is caught on **Ab_2^b** ⇒ **[Ab_1*-Ag]-Ab_2^b**; enzyme catalyses decomposition of colourless substrate and *zone turns coloured*

hCG low content
no complex, labelled primary antibody is caught in *control zone by third antibody* (**Ab_3^b**) **[Ab_1*-Ab_3^b]**; second zone also contains enzyme substrate – *coloured zone*

substrates:
: for enzyme POD – TMB (in acidic medium) : blue colour
: for enzyme ALP – phenolphthalein- or thymolphthalein phosphate (alkali medium) : red to blue colour

quantification of hCG by ELISA
sandwich technique; second Ab is a conjugate with POD, substrate ABTS

Mycobacterium tuberculosis

content: *Mycobacterium* is acido-resistant bacillus, pathogenic (tuberculosis) *M. tuberculosis* and *M. bovis*, atypical mycobacteria, e.g. *M. Kansasi* and *M. avireni* and etiological agent of leprosy *M. leprae*; less than 10 bacilli are already infectious
determination: sputum, morning sample, *ca* 5 – 10 ml 3 days one after one, sometimes also liquor, purulence, biopsy, urine; not stainable according to Gram

cultivation proof
procedure: decontamination (non-specific microflora) NaOH 1 M in presence of laurylsulphate, HCl neutralisation; medium inoculation (*egg* with salts, asparagine, glycerine, stark and malachite green; or *fluid* containing glutamate, L-alanine, casein hydrolysate, bovine serum *etc.*), incubation at 37 °C, recorded after 1, 3, 6 and 9 weeks (excluding contaminated media); within positive result it proceeds with detection of caught strains and starts with sensitivity test (resistance) towards drugs; for distinguishing *M. tuberculosis* and *M. bovis* the ability to produce niacin is evaluated, by reduction of nitrates to nitrites, sensitivity towards thiophene-2-carbonic acid hydrazide (so called TCH-test) and hydrolysis by Tween 80; *M. tuberculosis* has, in contrast to *M. bovis*, all listed tests positive

PCR proof

HIV (AIDS)

content: body fluids, virus of human immunodeficiency, retrovirus
determination: in serum, immunochemical approaches (ELISA)