Clinical Biochemistry

EVROPSKÁ UNIE
Evropské strukturální a investiční fondy
Operační program Výzkum, vývoj a vzdělávání

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Clinical biochemistry

Assays:
- elements, ions, gases
- small molecules
- proteins
- enzyme activities
- others

Materials:
- serum
- plasma
- urine
- others

Any analytes related to patient's health could be analyzed and used in diagnostics. Many elements, ions, dissolved gases, small molecules, enzyme activities and proteins are routinely assayed in clinical laboratories. They can be assayed in blood (serum or plasma), urine or other samples (stool, liquor and others).

Clinical biochemistry

Sample type may be encoded by the color of a test tube. These colors differ from country to country. Most blood analytes can be assayed in serum. Serum is sampled into red test tubes (in CZ). In order to analyze plasma the blood is sampled into violet test tubes contain disodium EDTA, which binds Ca^{2+} and blocks coagulation. These samples are used for hematological testing (counting of blood elements). Sodium citrate also chelates Ca^{2+}, but reversibly. It is contained in black or blue test tubes for sedimentation rate test or for blood coagulation tests, respectively (concentrations are different). Green test tubes contain NaF. It also binds Ca^{2+}, but also intracellularly. Therefore it blocks glycolysis. It is used to measure glucose and related analytes.

Clinical biochemistry – correlation with clinics

Glucose – diabetes

Electrolytes – circulation

Plasma urea and creatinine – kidney function

ALT, AST, ALP, bilirubin, total protein – liver function

Troponin, CK – cardiac diseases

C-reactive protein – inflammation

Clinical biochemistry

Units:

Concentration	M, mM, µM, g/l
Catalytic activity	katal (kat)
Pressure (dissolved gasses)	mmHg
Osmolarity	osmole

Concentrations are expressed in usual units (molar or mass). Katal is enzymatic activity unit equivalent to the amount of the enzyme that converts one mol of substrate in one second. It is a huge unit, therefore it is rather used as nanokatals. Special units are used for other variables. For example, blood pressure and partial pressures of dissolved gases are expressed in mm of mercury (mmHg). Osmole is equivalent to 1M of osmolytically active particles (molecules and ions). Units may differ from country to country, especially between Europe, UK and US.

Essential ions, dissolved gases, small-molecule compounds

- pO_2 – 10-13 kPa (88-108 mmHg) – electrochemically
- pCO_2 – 4.6-6.0 kPa (35-45 mmHg) – modified pH electrode
- pH – 7.35-7.45 – glass electrode
- Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{2-} – ion-selective electrodes, AAS
- Li^+ – linked to psychiatric disorders
- Cu^{2+} - Wilson's disease (copper accumulation)
- osmolarity – osmometer
- iron, iron binding capacity
- urea – kidney failure

Dissolved oxygen is measured by an amperometric electrode. Dissolved carbon dioxide is measured by a modified pH electrode. The pH electrode itself is immersed into a solution, which is separated from the analyzed solution by a membrane. The membrane is permeable only for carbon dioxide. It passes through the membrane, dissolves and produces carbonic acid, which causes change in pH. The value of pH can be measured by standard pH electrode. Other ions are measured potentiometerically by ion-selective electrodes or by atomic absorption spectrometry (AAS). Urea is measured enzymatically. It is first hydrolyzed by a plant enzyme urease to carbon dioxide and ammonium. Ammonium can be analyzed by ion-selective electrode. Alternatively it can react with 2-oxoglutarate and NADH catalyzed by glutamate dehydrogenase. NADH is converted to NAD^+ which reduces absorbance at 340 nm (near UV). The reaction rate depends (almost) linearly on concentration of ammonium (and urea).

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

Glucose + O_2 → glucono-1-lacton + H_2O_2 *glucose oxidase*

H_2O_2 + peroxidase substrate → color product *peroxidase*

Glucose is important for diabetes diagnosis. It can be assayed by reaction with fungal enzyme glucose oxidase. It oxidases glucose by oxygen and yields peroxide. This reacts with a synthetic chromogenic substrate to form color product. This reaction is catalyzed by enzyme peroxidase (typically from horse radish). Peroxidases destroy peroxides in plants by using it to oxidize some available plant secondary metabolite. Glucose oxidase and peroxidase reactions can be carried out in “one pot”.

Glc → Glc-6-P → NADPH *hexokinase, Glc-6-P dehydrogenase*

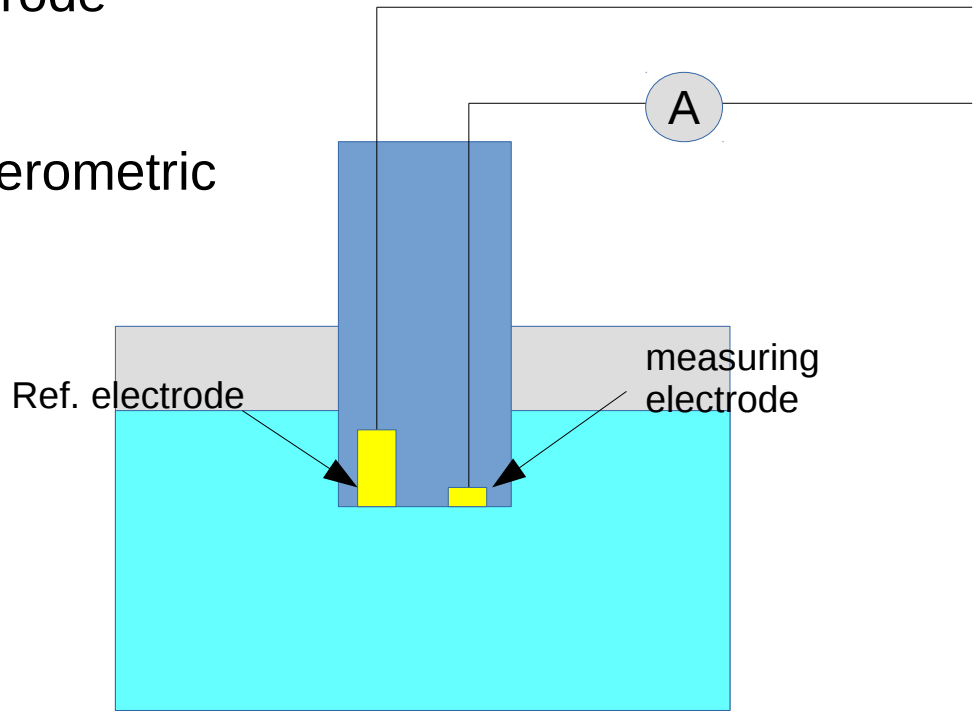
Alternatively, glucose can be assayed by phosphorylation by ATP by hexokinase followed by oxidation by $NADP^+$ by glucose-6-phosphate dehydrogenase.

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

General amperometric sensor



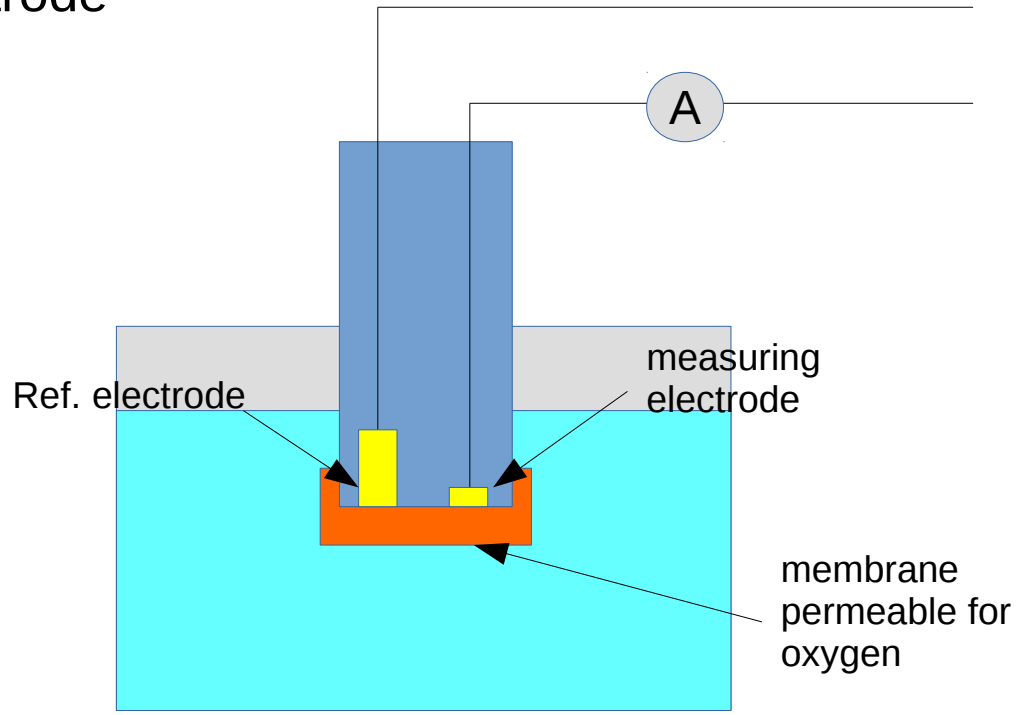
Glucose oxidase reaction can be also used in an amperometric sensor (enzyme electrode). A general amperometric sensor can be composed of measuring and reference electrode. It is immersed into the solution and potential is applied on electrodes. The current is limited by the rate of electrochemical reaction, which is limited by diffusion on the electrode. This process depends linearly on the concentration of the analyte that can react spontaneously on the measuring electrode.

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

Oxygen sensor



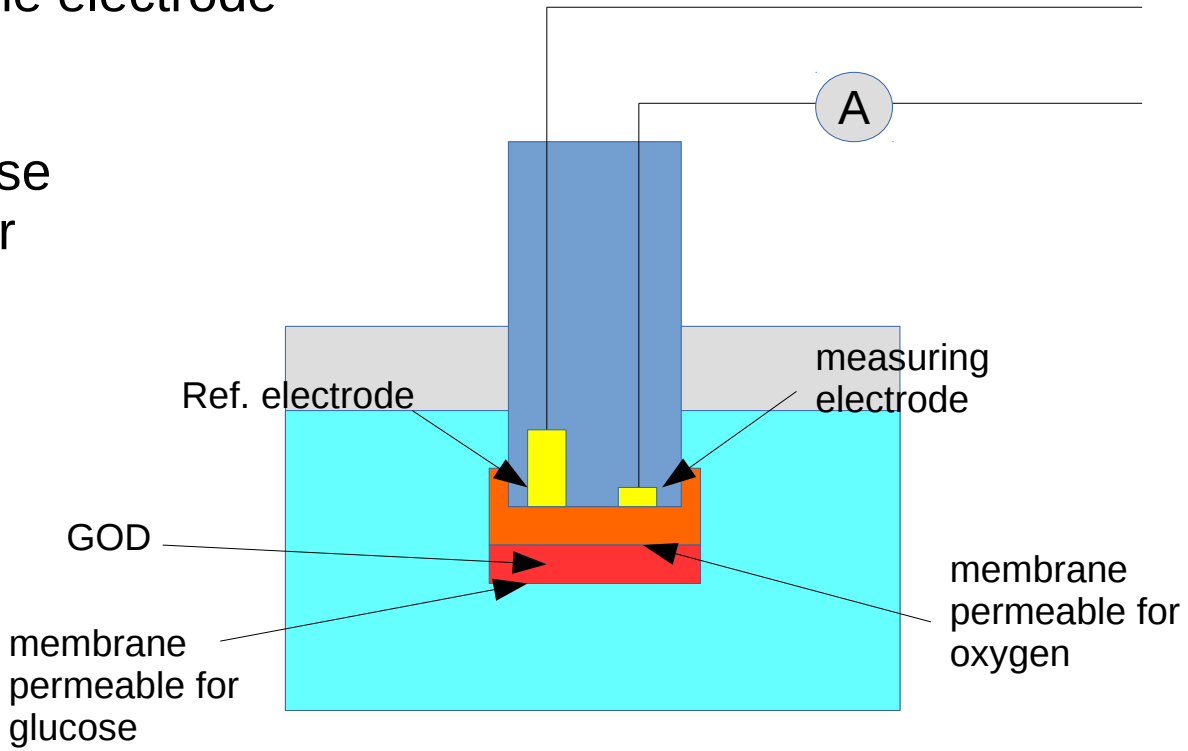
By adding a compartment separated by a membrane permeable only for oxygen it is possible to restrict reactions only to oxygen. Such sensor is used to measure oxygen concentrations in clinical biochemistry, biotechnology, fish production etc.

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

Glucose sensor

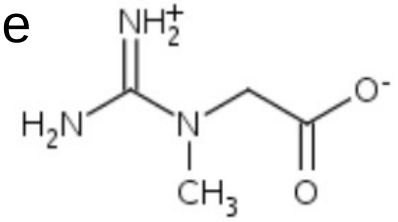


By adding another compartment separated by a membrane permeable only for glucose with glucose oxidase it is possible to measure glucose. These sensors are used in clinical labs or by diabetic patients.

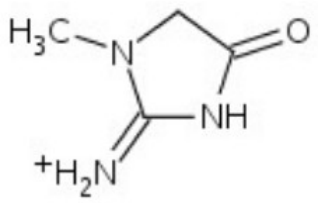
Essential ions, dissolved gases, small-molecule compounds

Creatinine – reaction with picric acid

creatine

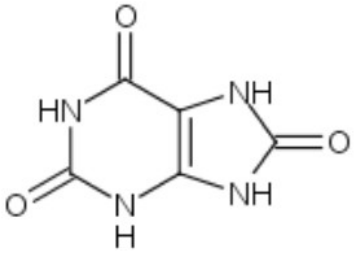


creatinine

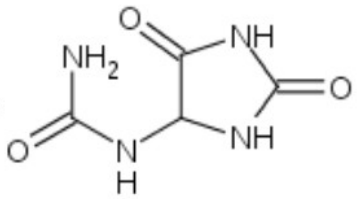


Uric acid – higher in gout

uric acid + O_2



allantoin + H_2O_2



Bilirubin (direct, conjugated) – liver diseases, hemolysis

- diazocoupling with reagent

Creatine spontaneously forms its cyclic form creatinine. It can be assayed by an old school reaction with picric acid (Jaffe reaction from 1886). Uric acid is assayed by urate oxidase (the rest is the same as for glucose assay). Bilirubin is assayed by a diazocoupling reaction with diazonium salt (diazotized sulphanilic acid). It is also a pretty old school reaction (1883).

Essential ions, dissolved gases, small-molecule compounds

- Lactate – lactate dehydrogenase – low oxygen supply

Lactate can be assayed by lactate dehydrogenase and NAD^+.

We must explain reasons behind popularity of NAD(P)H based assays. NADH or NADPH absorb at 340 nm. This is near UV range. Not so many compounds present in biological samples absorb at this wave length, so there are relatively little interferences. Moreover, this wave length makes it possible to use cheep plastic disposable cuvettes. Some analytes can be measured by end-point method (entire analyte is converted and concentration of the product is measured) or kinetically (the rate of reaction, which depends on the concentration of the analyte, is measured).

Proteins

Total protein can be measured in urine or serum by one of established methods. Special proteins are usually measured by enzyme-linked immunosorbent assay (ELISA). There are many formates of ELISA for assays of small molecules, proteins or antibodies. For assays of some protein (e.g. CRP) it is possible to use the following format: an anti-CRP antibody is immobilized on 96-well plate. Next, the sample is added. If CRP is present, it binds to the antibody. After washing away of unbound sample the second anti-CRP antibody labeled by an enzyme is added, incubated and unbound antibody is washed away. If CRP is present in the sample, the second antibody binds to it. Activity of the label enzyme (horseradish peroxidase or alkaline phosphatase) is detected.

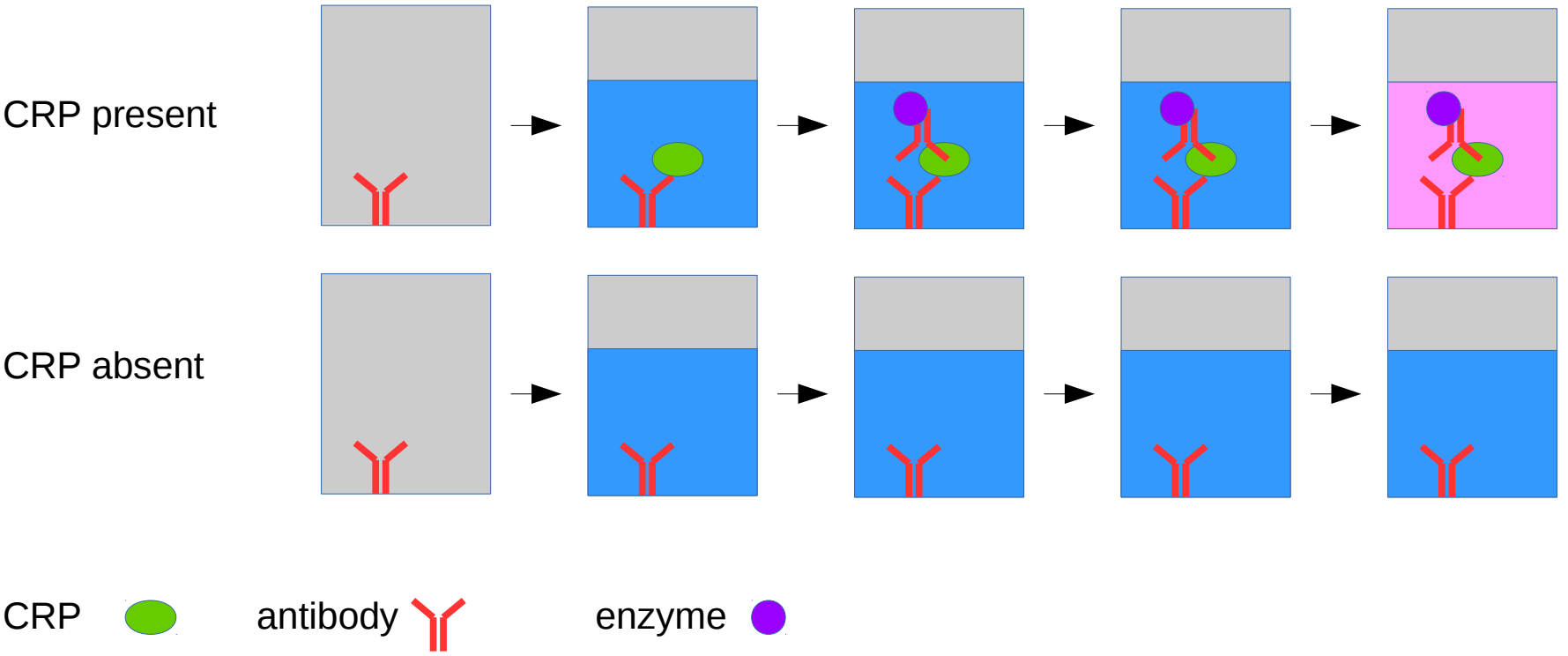
It is also possible to use only one antibody and to add enzyme-labeled CRP. The added enzyme-labeled CRP competes with the natural CRP from the sample and activity of the enzyme is thus inversely related to CRP concentration in the sample.

ELISA can be also used to diagnose infectious diseases. For example, primary HIV test is done by measuring anti-HIV antibodies in patient’s blood. The 96-well plate is coated by HIV antigens. Next, sample is added. If sample contains anti-HIV antibodies they bind onto antigens. After a washing step a labeled anti-human IgG antibody is added to detect presence of antibodies.

Protein assayed: CRP, Orosomucoid, α1-lipoprotein, complement, fibrinogen

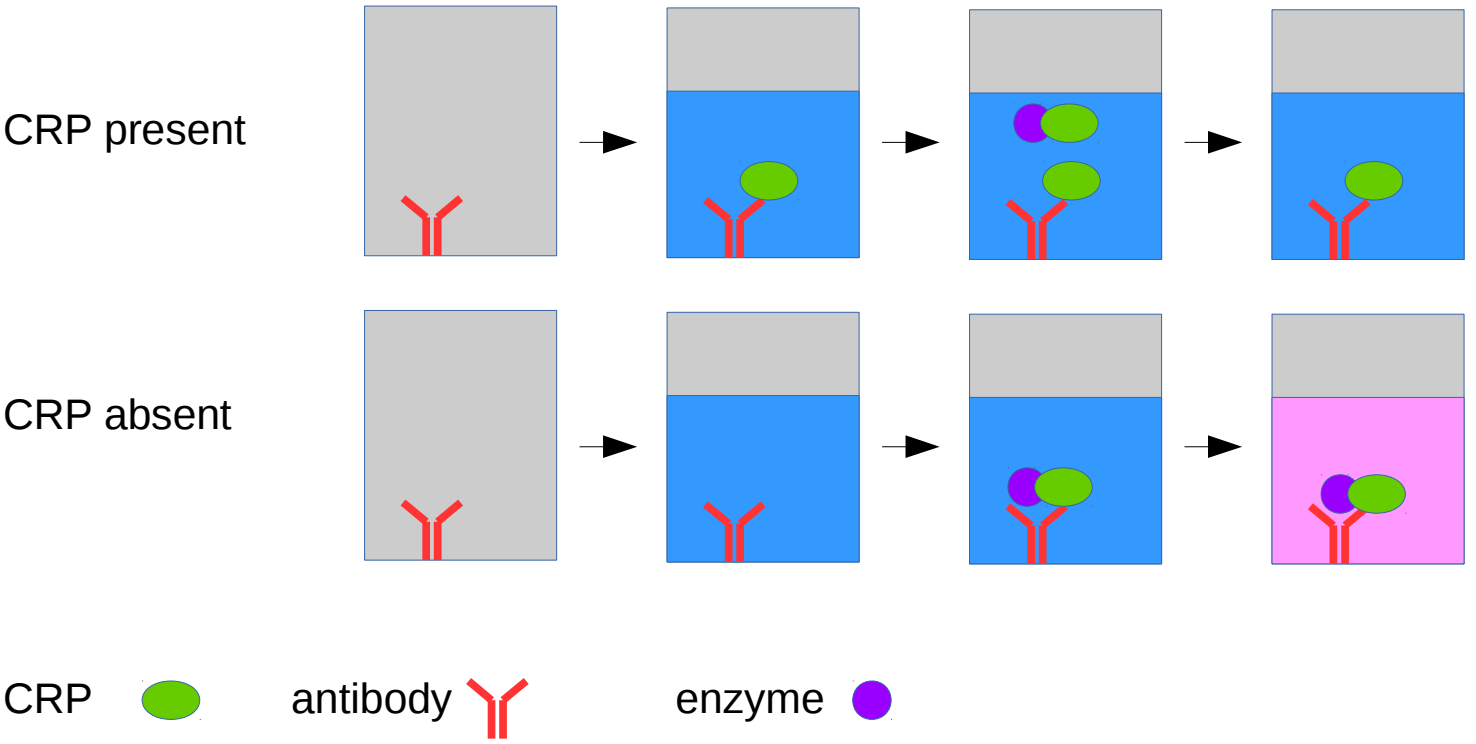
Proteins

- total protein – biuret method, Lowry, UV, Bradford
- C-reactive protein (CRP) – inflammation – ELISA
- Orosomucoid, α1-lipoprotein, complement, fibrinogen ... – ELISA



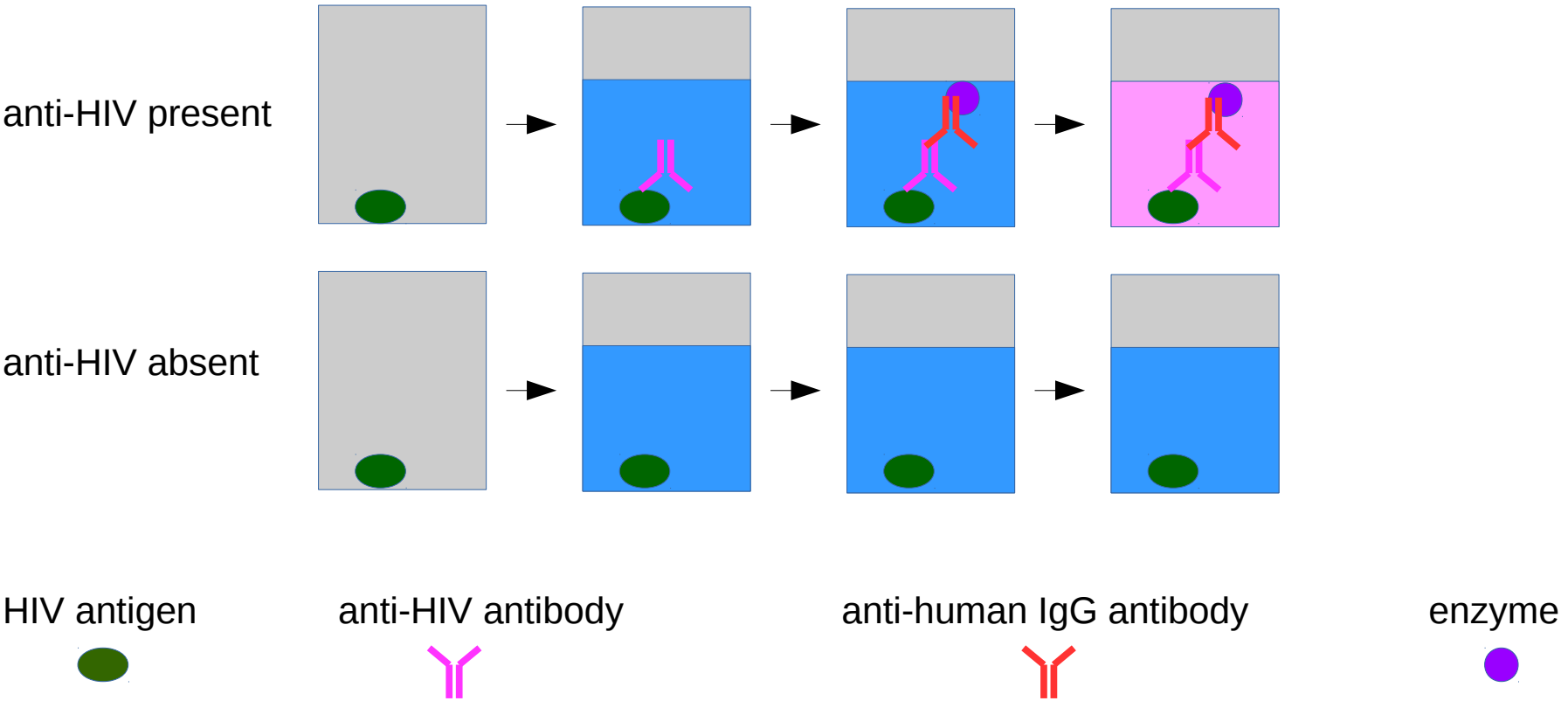
Proteins

- total protein – biuret method, Lowry, UV, Bradford
- C-reactive protein (CRP) – inflammation – ELISA
- Orosomucoid, α1-lipoprotein, complement, fibrinogen ... – ELISA



Proteins

- total protein – biuret method, Lowry, UV, Bradford
- C-reactive protein (CRP) – inflammation – ELISA
- Orosomucoid, α1-lipoprotein, complement, fibrinogen ... – ELISA



Enzyme activities

Enzymes activities are frequently measured in clinical laboratories. The relationships between activity of some enzyme and some disease are rather indirect. For example, activity of alanine transaminase (ALT, alanine aminotransferase) in blood is elevated during liver diseases. This enzyme is intracellular liver enzyme and is activity in blood is very low for healthy individuals. Some liver diseases cause damage of liver cells and leaking of their content, including ALT, to the blood stream. It would be possible to measure concentration of any intracellular liver protein, but ALT assay is the most convenient. Since we are not interested in the ALT activity itself, we in fact want to measure its concentration (which is linearly dependent on the concentration), we can give the enzyme optimal conditions for its activity (excess of substrates, optimal pH, etc). We do not have to follow conditions (pH or substrate concentrations) in blood or liver.

Enzymes can be assayed in continuous or end-point measurements. In the continuous design, buffer, enzyme (sample) and substrates are mixed in the cuvette. Next it is inserted into spectrophotometer. Absorbance as a function of time is measured and rate is obtained by a linear fit. In the end-point design the mixture is incubated for some time (e.g. 10 min) and then the reaction is stopped by addition of strong acid, base or by other change in conditions. After that the absorbance is measured. The advantage of continuous assay is its accuracy. Advantages of end-point assay are high throughput and possibility to use wide range of analytical techniques (continuous assays can be used only together with optical, electrochemical and other non-destructive methods).

Chromogenic substrates are (usually) synthetic colorless substrates that are converted to color products. Similarly it is possible to use fluorogenic or luminogenic substrates.

Enzyme activities

- Alanine transaminase (ALT) – liver diseases

alanine + 2-oxoglutarate → pyruvate + Glu *ALT*

pyruvate + NADH + H^+ → lactate + NAD^+ *lactate dehydrogenase*

- Aspartate transaminase (AST) – heart attack, liver diseases

aspartate + 2-oxoglutarate → oxaloacetate + Glu *AST*

oxaloacetate + NADH + H^+ → malate + NAD^+ *malate dehydrogenase*

ALT and AST assays are examples of coupled assays. These enzymes catalyze reactions with almost no change in spectra or other easily measurable properties. Therefore, they are coupled in the assay with another enzyme. The mixture for ALT assay contains buffer, alanine, 2-oxoglutarate, NADH and decent amount of lactate dehydrogenase. ALT from the sample would form pyruvate, which is immediately converted to lactate while NADH is oxidized. The rate of decrease of absorbance at 340 nm is linearly dependent to ALT activity.

Enzyme activities

- Lactate dehydrogenase – heart attack, liver diseases, hemolysis
  Direct measurement of NADH

- Alkaline phosphatase – bone and some liver diseases
  chromogenic substrate (*o*-nitrophenylphosphate)

- Acid phosphatase – prostate cancer
  chromogenic substrate, also immunochemically

- α-Amylase – pancreas diseases
  coupled with α-glucosidase, maltose phosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase

- γ-Glutamyltransferase – liver and blander diseases
  chromogenic substrate γ-glutamyl-*p*-nitroanilinide + Gly-Gly →
  p-nitroaniline + γ-Glu-Gly-Gly

Enzyme activities

- Creatine kinase (CK) – heart attack, muscle diseases

creatine phosphate + ADP → creatine + ATP *CK*

ATP + glucose → ADP + glucose-6-phosphate *hexokinase*

glucose-6-phosphate + $NADP^+$ → gluconolactone-6-P + NADPH + H^+ *Glc-6P dehydrogenase*

or

creatine + ATP → creatine phosphate + ADP *CK*

ADP + PEP → ATP + pyruvate *pyruvate kinase*

pyruvate + NADH + H^+ → lactate + NAD^+ *LDH*

- CK-MB (muscle & brain) – heart attack (more sensitive)

three dimer forms (MM, MB and BB) – immuno assays

CK is a traditional enzyme in clinical assays. It was found that two isoenzymes exist – muscle (M) and brain (B) form. Interestingly, this dimeric protein can also form mixed dimers (MB). These mixed dimers are typical for myocardium. Several methods were developed to specifically determine CK-MB. It can be also assayed immunochemically (ELISA). However, CK is being replaced by immuno assays of other proteins, such as troponin.

Lipids

- Cholesterol – cardiovascular risk
  cholesterol oxidase + peroxidase
- Total cholesterol – cardiovascular risk
  same with esterase
- HDL & LDL – cardiovascular risk
  selective sorptions and precipitations
- Triacylglycerols
  lipase + glycerol kinase + glycerole-3-phosphate oxidase + peroxidase

Cholesterol can be assayed by cholesterol oxidase and peroxidase. Total cholesterol contains free cholesterol and cholesterol esters. It can be assayed by the same procedure with additional esterase. HDL and LDL are assayed by specific separation of lipoprotein particles using special sorbents followed by cholesterol assay.

Other methods

Flow cytometry
Histology, histochemistry
Cultivation of pathogens
Immunoassays
DNA-based methods
Magnetic Resonance Imaging
Positron Emission Tomography
Single Photon Emission Computer Tomography