Chemistry and Biochemistry

Theory of laboratory exercises

1st year, DENTISTRY

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Department of Medical Chemistry and Biochemistry

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Clinical Biochemistry – Examination of Blood

Examination of blood I (proteins)

When blood is allowed to clot, several plasma proteins contribute in forming the matrix of the clot. The resulting solution, lacking fibrinogen, fibrin, and several clotting factors, is known as ***serum***. Most clinical determinations are made on serum rather than plasma.

Normal plasma contain as much as 63-80 g/L of protein. Of this, 53-65% is ***albumin***, the rest is composed of a mixture of ***globulins***. Most plasma proteins are synthesized in the liver. However, the gamma-globulins are synthesized by a class of lymphocytes known as ***plasma cells***. More than 80% of the total hepatocyte proteosynthesis is directed for export into plasma. Therefore one of the hallmarks of severe liver disease in an abnormality, usually a decrease, in one or more plasma proteins.

The principal function of plasma proteins are the generation of an intravascular colloid osmotic pressure (mainly albumin), specific and nonspecific transport, defence (circulating antibodies), coagulation and fibrinolysis, and availability of certain enzymes and/or their precursors.

Serum proteins are usually separated by zone electrophoresis on media, such as cellulose acetate or agarose gel, into five main arbitrary classes in the order of mobility. Most clinical electrophoretic analysis are carried with a barbital buffer of pH about 8.6. At this pH all normal serum proteins are negatively charged and migrate toward the anode of the electrophoretic cell. The highest negative charge and thus the highest anodal mobility shows albumin (pI = 4.8), the gamma-globulins are the least negatively charged. The quantification of the individual classes of plasma proteins following electrophoresis involves staining and densitometric scanning.

Individual proteins may be measured accurately and specifically by various ***immunotechniques***, such as immunodiffusion, immunoelectrophoresis, immmunofixation, enzyme immunoassays, immunoblots etc. In the Mancini radial immunodiffusion, serum diffuses from a cup cut into agar gel containing a specific antiserum, and the radius of the resulting precipitation zone is proportional to the protein concentrations. In immunoelectrophoresis, after separation by simple electrophoresis, a second electrophoresis causes the separated protein to pass into a sheet of gel containing anti-whole-human serum antiserum. The proteins are precipitated with their specific antibodies, and the area under each protein peak is directly proportional to the concentration of that protein. These (and other) techniques can be used to estimate any of the most abundant proteins as well as other important plasma proteins whose concentration is too low to be detected by zone electrophoresis.

In front of the albumin fraction migrates a small, by zonal electrophoresis not detectable fraction of ***prealbumin***. Prealbumin takes part in the thyroxine transport and its estimation is useful especially in the determination of protein malnutrition. Being rich in amino acid tryptophan and with a short half-life, its concentration rapidly falls in malnutrition and rises when an adequate diet is given.

The most abundant protein is ***albumin***. Mature human albumin consist of one polypeptide chain of 585 amino acids and MW of about 69,000. Albumin is synthesized in the

liver. Therefore, the synthesis of albumin is depressed particularly in diseases of the liver. The plasma of patients with liver disease often shows a decrease in the ratio of albumin to globulin (A/G ratio). The plasma of certain humans lacks albumin (***analbuminemia***). Subject with analbuminemia shows only moderate oedema, due to the compensatory increase in globulins. Because of the relatively low molecular weight, high concentration, and the binding capacity for inorganic ions, albumin is responsible for 75-80% of the ***oncotic pressure*** of human plasma. Many metabolites, such as free fatty acids and bilirubin, are poorly soluble in water. Albumin fulfils the function of a ***carrier*** to enhance the solubility of these substances. Albumin binds also poorly soluble drugs, such as aspirin, digoxin, the coumarin anticoagulants, and barbiturates so that they are efficiently carried through the bloodstream. About 50% of the calcium in the plasma exists as a complex with albumin.

Major plasma proteins			**%**
Albumin			53 - 65
Globulins	Alpha-1	α_1-antitrypsin orosomucoid α_1-lipoprotein transcortin (CBG)	2 - 4
	Alpha-2	α_2-macroglobulin haptoglobins ceruloplasmin	8 - 13
	Beta	transferrin β-lipoprotein C-reactive protein C_3-complement (fibrinogen)	9 - 16
	Gamma	IgG 80% IgA 12% IgM 7% IgD < 1% IgE < 1%	11.5 - 19

Alpha-1 globulins fraction:

α_1-antitrypsin comprises 80-90% of the α_1-globulin. Its function is protective through anti-proteolytic activity. It inhibits trypsin, elastase, and certain other serine proteases by forming complexes with them. Deficiency is inherited as a recessive character and presents either as neonatal hepatitis or as emphysema (degenerative affection of alveoli) in early adult life.

Acid α_1-glycoprotein with a high sugar moiety (38%) is called ***orosomucoid***. Its concentration increases in inflammation.

α₁-Lipoprotein corresponds to the high-density lipoprotein (HDL) of the blood plasma.

The main plasma glucocorticoid binding protein is called ***transcortin*** or ***corticosteroid-binding globulin*** (***CBG***)

In the first globulin fraction may be involved also so called ***alpha-fetoprotein*** (***AFP***). AFP is normally synthesized almost exclusively by the fetal liver. After birth the level falls and raised levels are found with many malignant conditions, especially hepatoma and malignant teratoma, as well as viral hepatitis. In obstetrics, raised levels are found in the amniotic fluid of foetuses with neural tube and other defects.

Alpha-2 globulins fraction:

α₂-Macroglobulin, a high-molecular weight (MW ≈725.000) plasma protein, is another protein that play an important role in the body´s defence against excessive action of proteases. It shows also a certain antithrombin activity. Macroglobulin concentration is increased in nephrotic syndrome (due to its large molecule which poorly passes through the glomerulus).

Haptoglobins are α_2-globulins capable of binding free hemoglobin and may play an important role in the degradation of hemoglobin. Free hemoglobin passes through the glomerulus, whereas the hemoglobin-haptoglobin complex is too large to pass through. The function of haptoglobin thus appears to be to prevent loss of free haemoglobin and its valuable iton. The half-life of hemoglobin-haptoglobin complexes is much shorter than that of haptoglobin alone, and as a consequence, low levels of haptoglobins are found in patients with hemolytic anemias. On the other hand, haptoglobin is an acute phase protein, and its plasma level is elevated in inflammations.

Ceruloplasmin carries 90% of the copper present in plasma, the remaining 10% is accounted for by albumin. It has a blue colour because of its copper content. Ceruloplasmin bears a special relationship to Wilson´s disease, a condition of copper toxicosis.

Beta globulins fraction:

Transferin plays a central role in the body´s metabolism of iron because it transports iron in the circulation to sites where iron is required, e. g. from the gut to the bone marrow and other organs. Free iron is toxic, but association with transferrin diminishes its potential toxicity. The concentration of transferrin in plasma is about 3g/L. This amount can bound approximately 3 mg of iron per litre, so that it represents the ***total iron-binding capacity*** of plasma. However, the protein is normally only one-third saturated with iron. In iron-deficiency anemia, the protein is even less saturated with iron, whereas in conditions of storage of excess iron in body (e.g. hemochromatosis) the saturation with iron is much greater than one-third.

β-lipoprotein corresponds to the plasma low density lipoproteins (LDL). They transport free and esterified cholesterol with a little triglyceride. This fraction contains the majority of the total plasma cholesterol.

C-reactive protein is a serum protein which can react with a somatic C-polysaccharide of the pneumococcus. Its concentration increases early in acute inflammation and reflects activity. Its synthesis is stimulated by interleukin 6 (IL-6)

The ***complement system*** is involved in foreign cell lysis and inflammation. It has nine main factors but C3 and C4, which make up 95% of total, are the two proteins most easily

measured immunochemically. Low levels of complement are of more importance, indicating increased consumption or decreased synthesis. It is acute phase marker.

A rise in ***fibrinogen*** (in the plasma only) is a sensitive indicator of connective tissue disorders such as rheumatoid arthritis.

Gamma globulins fraction:

The γ-globulin fraction contains the ***immunoglobulins*** (***Ig***), which on electrophoresis normally separate as a diffusely stained band. Intensely stained, narrow, localized bands observed on electrophoresis are called ***paraproteins*** and are signs of a ***monoclonal gammopathy***. The immunoglobulins are a mixture of proteins all of which possess antibody activity. There are five main classes of immunoglobulins: IgG, IgM, IgA, IgD and IgE. These all have similar molecular weight, except IgM, which is made up of five subunits and IgA, which is normally a dimer. The Ig monomers possess the common basic structure consisting of two heavy chains and two light chains covalently linked by disulphide bonds. The light chains have a molecular weight of about 22,000, the molecular weight of heavy chains ranges from 50,000 to 75,000. Immunoglobulins are synthesized by plasma cells in the lymphoid tissue and bone marrow. According to the clonal selection theory, a given plasma cell synthesizes Ig of a single molecular type only. Tumours of Ig-synthesizing cells are called ***plasmocytomas*** (***myelomas***). The paraproteins they produce are seen on electrophoresis of plasma proteins. Sometimes, light or heavy chains are produced only. Then they may be detected also in urine, e.g. as Bence-Jones protein consisting of intact light chains. Paraproteins are found in serum on screening, by electrophoresis, the precise biochemical identification is carried out by immunotechniques. The incidence rises markedly with age (1% over 50, 3% over 70 years of age). No malignancy can be found in about 25% of the patients with paraproteinemia (***benign gammopathy***).

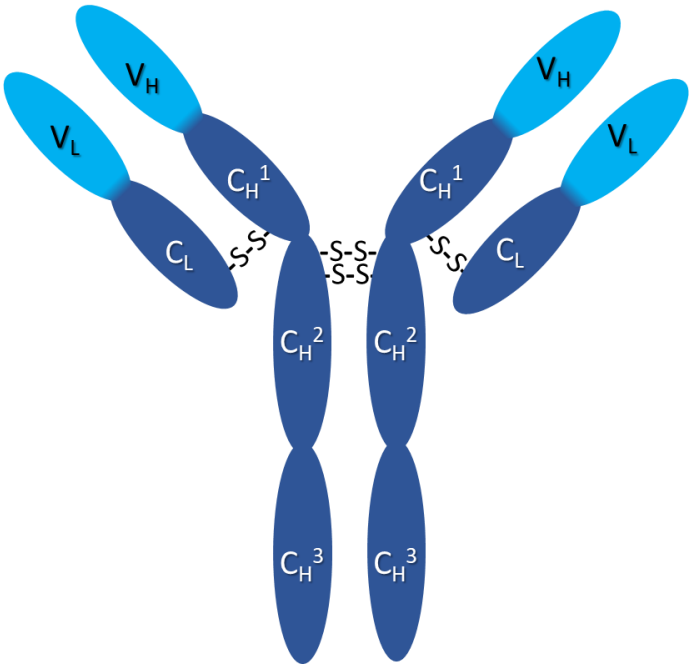


Diagram of IgG molecule made up of two heavy (H) and two light (L) chains. V_L and V_H are N-terminal variable sequence of heavy and light chains, reacting with the antigen, C_L and C_H1-3 are C-terminal constant sequence of heavy and light chains

Assessment of plasma protein examination

The concentration of plasma ***albumin*** varies much more than does that of globulin. Albumin is affected much more readily by the protein intake, whereas globulin is regenerated more quickly after haemorrhage. The generalization may be made that the albumin fraction is rarely increased above normal and the globulin rarely decreased below normal. An increase in the concentration of albumin may occur in dehydration. In such conditions the globulin and albumin fractions will be proportionally increased. Diminution in plasma albumin occurs in the following conditions:

1. Loss of albumin (proteinuria in nephritis and nephrosis, ascites, extensive burns, haemorrhage, etc.)
2. Inadequate supply
3. Impaired synthesis (cirrhosis)
4. Excessive protein catabolism (diabetes mellitus)
5. Plasma dilution

Plasma proteins whose concentration alters following trauma, surgery, tissue necrosis (myocardial infarction) or inflammation are known as ***acute phase reactants***. These are: haptoglobins, orosomucoid, alpha$_1$-antitrypsin, C-reactive protein, complement and fibrinogen. Note that they are present in the fastest globulin fractions and rise in the conditions mentioned above.

Dysproteinemias - changes in the representation of individual proteins characteristic of certain diseases:

DYSPROTEINEMIAS (simplified)

Condition	*Total* P	*A*	*alpha*$_1$	alpha$_2$	beta	gama
hypoproteinemic	↓ ↓	↓↓	N, ↑	N ↑	↓	↓, N
nephrotic	↓ ↓	↓↓		↑↑		
hepatic	↓, N	↓↓	↓	↓	↓	↑
acute inflam.		↓	↑	↑		N
chronic inflam.		↓	↑	↑		↑
hyper-gamma	↑	↓				↑
analbuminemia		↓↓↓				
agammaglobulin.						↓↓↓

X-chromosome-linked ***hypogammaglobulinemia*** is due to lack of B lymphocytes. Without special precautions, affected babies die from infection with pyogenic bacteria. Unlike in Ig-synthesizing tumours, in chronic inflammation the overall increase in IgG fractions involving various types of antibody molecules is found. This condition is called ***polyclonal gammopathy***.

The liver plays an important part in normal protein metabolism. Its chief function in this connection appears to be the deamination of amino acids, urea formation, and the formation of

many important proteins. Fibrinogen is formed entirely in the liver and remarkable variations may be observed in the fibrinogen content of blood plasma in liver disease. In the presence of severe liver damage the fibrinogen may fall to extremely low levels. Prothrombin, a factor essential for normal coagulation, is formed in the live as well. For the maintenance of a normal plasma concentration, adequate hepatocellular function is necessary, along with the adequate vitamin K supply.

The liver plays also a major role in the formation of plasma albumin. The diminution in serum protein in hepatic disease occurs chiefly if not entirely in the albumin fraction. In some instances, particularly in acute forms of liver disease, serum albumin may be only moderately reduced and the serum globulin increased. This increase is observed much more commonly in hepatocellular disorders than in obstructive jaundice, and in some cases, especially in cirrhosis, may be so great as to more than counterbalance the albumin deficit, the total serum protein concentration being actually increased. The estimation of the A/G ration can be used to establish the disproportion between albumin and globulin fraction concentrations

Examination of blood II (glucose, lipids)

Blood glucose and disorders of carbohydrate metabolism

Relative constancy of the ***blood glucose concentration*** (3.6 -5.6 mmol/L) depends upon the precisely adjusted balance of glucose entering the blood and glucose leaving the blood. The input of glucose is derived from dietary carbohydrate, hepatic glycogenolysis and gluconeogenesis from protein. On the other hand, glucose is removed from the body by oxidation to carbon dioxide and water, conversion to fatty acids in adipose tissues, conversion to muscle glycogen or, if blood glucose is high, by renal excretion.

Insulin is the only hormone capable of lowering level of glucose in blood or preventing its excessive rise, because it increases carbohydrate utilization by all the metabolic pathways. Insulin primarily increases cell permeability to glucose and most, if not all, its other metabolic effects are secondary to this. In muscle, insulin enhances glycogen formation, in adipose tissue, insulin promotes the synthesis of fatty acids from glucose. Insulin also increases hepatic glycogen formation, increases protein synthesis especially in muscle. Hence, insulin has been termed the hormone of nutrient storage.

In contrast to insulin, ***glucagon*** causes glycogenolysis, lipolysis, ketogenesis and gluconeogenesis. ***Growth hormone***, ***adrenaline***, ***glucogenic steroids*** and, indirectly ***adrenocorticotropic hormone*** (***ACTH***) all tend to increase blood glucose level or to prevent its excessive fall. Growth hormone causes increased protein synthesis and antagonizes insulin (e. g. depresses the glucose uptake). Adrenaline mobilizes liver glycogen by the activation of phosphorylase and stimulates the secretion of ACTH, which in turn enhances the secretion of glucogenic steroids which increase gluconeogenesis.

Clinical diabetes mellitus is broadly classified into two types:

1. ***Type I, juvenile, insulin-dependent***
   Type I diabetes is a chronic autoimmune disease associated with selective islet beta-cell destruction. More than 95% of individuals express the "diabetogenic" HLA allele DR 3/4.
2. ***Type II, maturity onset, not insulin-dependent***, often controlled by diet but may require oral hypoglycaemic agents. Virtually all patients with type II diabetes have some degree of insulin resistance due to defects at the level of the insulin receptor and at several post-receptor steps involved in insulin action.

Metabolic abnormalities in diabetes include hyperglycaemia, glycosuria, polyuria and dehydration, hyperlipidemia, ketonemia and ketonuria, acidosis, change in plasma electrolytes, and sometimes azotemia. Hyperglycaemia is due to an absolute or relative insufficiency of insulin, causing deficient utilization of carbohydrate, glycogenolysis and gluconeogenesis. ***Glucosuria*** depends on the concentration of glucose in plasma, glucose T_m, GFR, and other factors. Glycosuria causes an osmotic diuresis and the resulting ***polyuria*** results in ***dehydration*** in which the water loss is shared by entire body water. Associated sodium deficiency leads to water loss in the extracellular fluid. The decreased utilization of glucose increases the utilization of triglyceride with formation of glycerol and ***non-esterified fatty acids*** (***NEFA***). The NEFA

are utilized by the tissues for energy production or are utilized in the liver to triglycerides. Those, together with cholesterol synthesized in the liver from acetyl-CoA, results in ***hyperlipoproteinemia***. The fatty acids are degraded into acetyl-CoA which, it not enough oxaloacetic acid is available, is converted into acetoacelyl-CoA or synthesized into cholesterol via beta-hydroxy-beta-methylglutaryl-CoA. The latter may produce acetoacetic acid, which, with the reduction product beta-hydroxybutyric acid and its decarboxylation product acetone, form the ***ketone bodies***. Acetoacetic acid can be utilized by the tissues, especially muscles, for the production of energy, but if it is formed at a rate greater than the muscle can utilize, then ketone bodies accumulate in the blood and appear in the urine. In the initial stages of diabetic ketosis, the associated fall in bicarbonate (***metabolic acidosis***) is compensated by fall in pCO_2, in later stages, however, the fall in plasma bicarbonate becomes so extreme that pCO_2 fails to decrease proportionately and compensate sufficiently acidosis.

Elderly diabetic patients are subject to the ***late complications of diabetes***: macrovascular disease (heart infarction, peripheral vascular disease, stroke), microangiopathy (retinopathy, diabetic glomerulosclerosis), neuropathy. Several disorders may share a common pathogenesis. One of the main pathogenetic mechanisms of late diabetic complications is ***non-enzymatic protein glycation*** as a result of chronic hyperglycaemia. While proteins with a short half-life are gradually degraded, proteins with a longer half-life undergo changes that gradually affect their functions. Oxidative damage by free radicals contributes to the development of these changes, so we speak of ***glycooxidation***. Schwann cells, glomerulus, and possibly retinal capillaries contain aldose reductase which reduces glucose to sorbitol. It was shown that sorbitol accumulation may damage these tissues by causing them to swell.

The estimation of ***blood glucose*** is one of the most important laboratory tests. The most precise determination include enzymatic tests. The most common is the combination of glucose oxidase and peroxidase reactions:

$$\text{glucose} + O_2 \xrightarrow{\textit{glucose oxidase}} \text{gluconolactone} + H_2O_2$$

$$H_2O_2 + \text{chromogens} \xrightarrow{\textit{peroxidase}} \text{coloured product} + H_2O$$

Many patients with diabetes control their treatment by simple devices for measurement of glucose in capillary blood. The most advanced devices, insulin pumps intended for continuous subcutaneous infusion of insulin, allow continuous blood glucose monitoring.

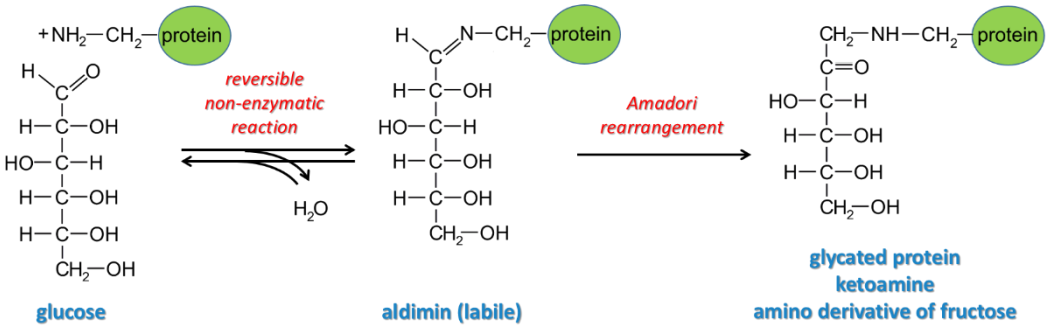
In the diagnosis of diabetes, single determination of blood sugar sometimes give borderline results difficult to interpret. A ***glucose tolerance test*** must then be performed. Because of the impairment of glucose tolerance occurring during fasting the patient must be given a full carbohydrate diet for 3 days before test. After fasting overnight, blood (sometimes also urine) specimens are analysed for glucose before and usually 2 hours after the administration of 75g glucose in 250-350 mL of water. Persons with a normal glucose tolerance have a fasting blood glucose below 6 mmol/L, which does not rise during the test above 10 mmol/L and returns to normal within 2 h. Impaired glucose tolerance is present when the fasting

blood glucose is about 6-7 mmol/L and rises to a level above 10 mmol/L (glycosuria!) and the values between 7.8-11 mmol/L are observed 2 hours after the glucose uptake. In diabetes, both the fasting and final levels are higher. Impaired glucose tolerance is observed not only in diabetes mellitus but also in hyperthyroidism, severe liver disease, Cushing´s syndrome, and many other conditions.

In patients with impaired glucose tolerance or mild diabetes it is sometimes useful to obtain ***serum insulin*** levels. The presence of normal or elevated serum insulin with a peak response to oral glucose indicates islet cells reserve and possible normalization of blood glucose with diet restriction.

In the biosynthesis of insulin, a proinsulin consisting of two subunits of insulin linked by a ***connecting peptide*** (***C-peptide***) is cleaved for secretion. Whereas insulin may be degraded in the liver, C-peptide remains unimpaired and thus provide a measure for the pancreatic insulin production. Insulin level is normally 0.5-0.8 µg/L, C-peptide level is as much as 0.9-3.5 µg/L.

An important tool monitoring diabetic control is the blood concentation of glycosylated hemoglobin HbA_{1c}. HbA glycosylated on the N-terminal valine of the beta-chain by a non-enzymatic reaction is proportional in amount to the mean blood glucose concentration over the preceding 2-3 months (normal value 20-42 mmol/mol). Thus, measurement of HbA_{1c} provide an objective measure of average glycemia during the preceding 6-12 weeks. It was shown that in uncontrolled diabetics high concentrations of glycosylated hemoglobin will diminish delivery of oxygen to the tissue because of the block of conformational changes.



Mechanism of non-enzymatic protein glycation

Hypoglycaemia is the clinical state due to a low plasma glucose, usually less than 2.2 mmol/L, but the severity of symptoms may be related more to the rate of fall then to the actual glucose concentration. Irreversible coma may quickly develop if the condition is not effectively treated caused by the impairment of the normal CNS metabolism.

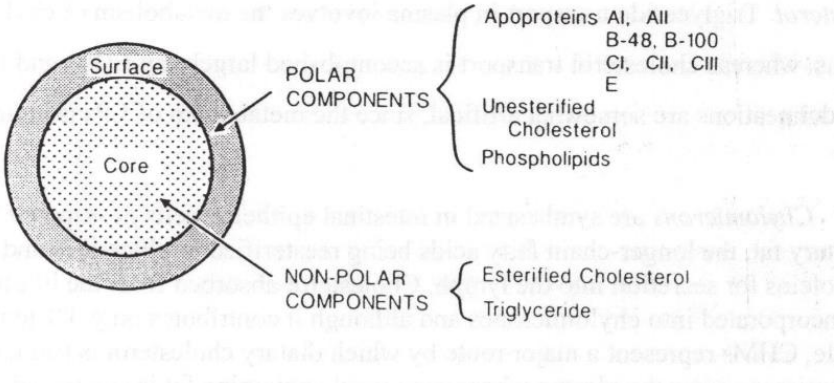
Galactosemia is due to deficiency of the gene directing the synthesis of the enzyme galactose-1-phosphate uridyl transferase concerned with the conversion of galactose to glucose. Children with the disease fail to thrive and the accumulation of galactose and the sugar alcohol galactitol causes mental retardation, liver damage, cataracts, and other defects. Normal growth and development can occur if galactose-free regimen is started early enough. Early diagnosis is therefore essential.

Glycogenosis involves glycogen storage diseases, a number of different clinical forms according to which of enzymes responsible for degrading glycogen is deficient. Gierke´s disease (hepatomegaly, failure to thrive, hypoglycaemia, ketosis) is due to a deficiency of glucose-6-phosphatase. Diagnosis depends upon glycogen and enzyme estimation of various tissues.

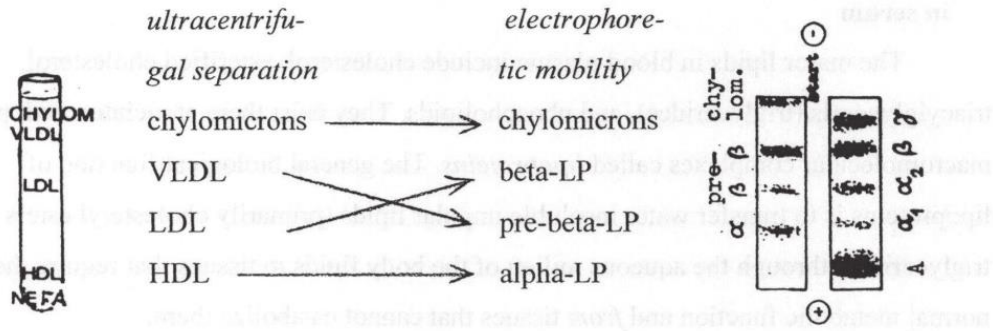
Lipid metabolism and its disorders

The major lipids in blood plasma include cholesterol, esterified cholesterol, triacylglycerols (triglycerides), and phospholipids. They exist there associated with proteins in macromolecular complexes called ***lipoproteins***. The general biological function of lipoproteins is to ***transfer*** water insoluble unpolar lipids (primarily cholesteryl esters and triglycerides) through the aqueous milieu of the body fluids ***to*** tissues that require them for normal metabolic functions and ***from*** tissues that cannot catabolize them.

Lipoproteins are globular or spherical particles that contain polar components (phospholipids, unesterified cholesterol, and proteins) on the surface in contact with body fluids, and a core of nonpolar lipid, including cholestery esters and triglycerides (see the figure). The proteins of lipoproteins (LP) are called ***apolipoproteins*** (***apoproteins***). They are required for several important functions, including the synthesis of LP, the activation of enzymes important in LP metabolism, and the interaction of LP with cell surface receptors that promote the cellular uptake of LP.



The plasma lipoproteins are divided into five major families, including ***chylomicrons, very low density LP (VLDLs), intermediate density LP (IDLs), low density LP (LDLs), and high density LP (HDLs)*** Each family contains a spectrum of particles with similar metabolic functions, and the families are differentiated from one another by certain physicochemical properties, such as their hydrated density range (ultracentrifugation) or their migration during electrophoresis:



The density of a lipoprotein is determined by the ratio of protein to lipid in the particle. The least-dense LPs contain the least amount of protein as a percent of their composition. As the protein content in a LP family increases relative to lipid, the hydrated density also increases. The least-dense LPs are also the largest and contain the greatest amount of triglyceride (chylomicrons, VLDLs). In contrast, HDL particles, the most dense of the LPs, are the smallest in the size and contain more phospholipid per particle than the LPs of lower density.

The constituents of lipoproteins of most clinical interest are ***triglycerides*** and ***cholesterol***. Triglyceride transport in plasma involves the metabolism of chylomicrons and VLDLs, whereas cholesterol transport is accomplished largely by LDLs and HDLs. However, these delineations are somewhat artificial, since the metabolism of LPs is highly interrelated.

Chylomicrons are synthesized in intestinal epithelial cells in response to the ingestion of dietary fat, the longer-chain fatty acids being reesterified to glycerides and combined to apoproteins for secretion into the lymph. Cholesterol absorv´bed from the intestinal lumen is also incorporated into chylomicrons and although it contributes only 5% to the mass of the particle, chylomicrons represents a major route by which dietary cholesterol is taken into the body. Chylomicrons enter the plasma whenever a meal containing fat is consumed, and they are removed from plasma with a half-life of about 30 min. After entering the plasma they aquire additional apoproteins, especially CII, from HDL. Apo CII activates ***lipoprotein lipase*** anchored to capillary endothelial cells to hydrolyze the core triglyceride to free fatty acids (non-esterified fatty acids, NEFA, FFA). NEFAs are taken into tissues for oxidation (e.g. muscle) or storage for future use (adiose tissue). After most of the chylomicron-triglyceride core is hydrolyzed, the particle dissociated from lipoprotein lipase as a ***chylomicron remnant***. The remnant particles are rapidly removed from the circulation in the liver by a receptor that binds with Apo E on the remnant particle surface.

VLDLs are somewhat smaller in size and of higher density than the chylomicrons. They are produced in the liver and secreted as triglyceride-rich particles containing many

apoproteins, among them Apo B and the Apo C, which are partly transferred from HDLs. VLDLs interact with lipoprotein lipase in capillaries, where the triglyceride core is hydrolyzed to yield NEFAs, principally for adipose tissue nad muscle.

LP class	CHYL	VLDL	LDL	HDL
Elfo mobility	start	pre-beta	beta	alpha
Size (nm)	10^2-10^4	30-70	15-70	7.5-10
Composition:	%	%	%	%
PROTEINS	1	10	22	50
PHOSPHOLIPIDS	4	15	23	30
CHOLESTEROL	5	15	45	18
TRIGLYCERIDES	90	60	10	2
Apolipo-proteins	Apo A Apo B Apo C I-III Apo E		Apo B	Apo A I Apo A II Apo D Apo E

When VLDL remnants are released from lipoprotein lipase, they are termed ***intermediate –density LPs*** (***IDLs***). IDLs particles have two major metabolic fates: they may be taken up by the LDL receptor on the liver or may be converted to ***LDLs***, the cholesterol-rich particles that supply cholesterol to cells via LDL receptor pathway or by receptor-independent mechanism (one third of total LDL). LDL receptors are found in most tissued troughout the body, however, more than 60% are located in the liver.

Cells require ***cholesterol*** for normal function and they obtain it from two major sources: biosynthesis from acetyl-CoA and uptake of lipoprotein-cholesterol from the intestinal fluid. Cells must also be able to rid themselves of excess cholesterol, since they lack enzymes to break apart the sterol nucleus. Cells will take up cholesterol from LDL via the LDL receptor. The interaction with the receptor is mediated by ApoB100. Depending on the tissue, cholesterol can be used for new membrane formation, bile acid formation (liver), steroid hormone production (ovary, testis, adrenal). When adequate amounts of cholesterol are entering the cell via LDL receptor pathway, cells supress their endogenous synthesis as reflected by the decreased activity of ***HMG-CoA reductase***, the rate-limiting enzyme in cholesterol biosynthesis. Cells also decrease the number of LDL receptors active on the cell surface and they increase the activity of ***acyl-CoA: cholesterol acyltransferase*** (***ACAT***) to esterify any excess cholesterol for storage as cholesteryl ester droplets. The removal of cholesterol is mediated by the disk-shaped "nascent" HDL particles (produced in the liver, intestine, and perhaps other tissues), which acquire unesterified cholesterol when they come in contact with cell membranes. This activity is enhanced by ***lecithin-cholesterol acyltransferase*** (***LCAT***), an enzyme produced in liver and active in plasma. LCAT exterifies the cholesterol on the surface of the disks, obtaining the fatty acid from number 2 carbon of lecithin (facilitated by ApoAI). The nonpolar chosteryl esters then migrate to the core of the disk, evolving into spheric particles. These are indications that the liver directly takes up HDL through the agency of a specific HDL receptor.

The tissue cholesterol initially acquired by nascent HDL particles may eventually be transferred as cholesteryl ester to triglyceride-rich lipoprotein during their catabolism. If this transfer is to chylomicron remnants, the transferred cholesteryl esters are returned to the liver. If the transfer is to VLDL remnants and IDLs, the cholesteryl esters may return to the liver as IDLs, or they may appear in LDLs. Thus "reverse cholesterol transport" does not often involve a direct route from peripheral tissues to the liver, but depends on the repeated transfer of chlesteryl esters among lipoproteins before final excretion occurs through the liver.

Lipoprotein (a) is another lipoprotein found to a variable extent in human plasma. This LP is important because its presence and concentration are linked to increased risk for coronary heart disease, independent of the other LPs. LP(a) is comparable in size to LDL, but it is somewhat more dense and exhibits pre-beta migration on electrophoresis. It is sometimes called ***sinking pre-beta LP*** to distinguish it from VLDL. The major lipid is cholesteryl ester, but the protein content is unusual in that it consists of Apo B100 linked by disulfide bonds to a plaminogen-like protein. LP(a) is detectable by sensitive immunoassay techniques.

All of the risk factors established thus far, lipid disorders pay a key role in the pathogenesis of atherosclerotic vascular diseases, especially of coronary heart disease. It is widely accepted today that a serum cholesterol level of about 5 mmol/L represents the cutoff value beyond which the risk increases progresively. However, the atherogenic significance of the total cholesterol must be viewed judiciously. These is evidence that LDLs, as the carrier of ca 70% of the total cholesterol, are the most potent atherogenic lipoproteins. Patients with low LDL cholesterol have a high life expectancy. In contrast to LDL, an increase of the second cholesterol-rich class – the HDL – is not associated with risk. It has become apparent that there is an inverse relation between coronary heart disease and the HDL (or HDL cholesterol) concentration. This would seem to indicate that s raised HDL may even be protective against disease.

Several enzymatic methods currently exist for the assay of ***cholestrol***, one of which has become established worldwide. The method is based on three enzyme catylyzed steps:

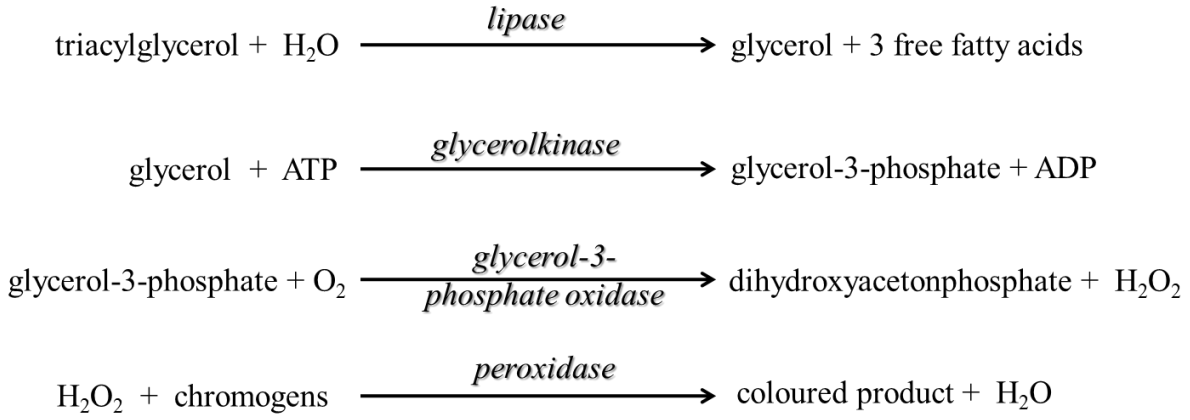
$$\text{cholesteryl esters} + H_2O \xrightarrow{\textit{cholesterolesterase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{cholesterol} + O_2 \xrightarrow{\textit{cholesteroloxidase}} \text{4-cholesten-3-on} + H_2O_2$$

$$H_2O_2 + \text{chromogen} \xrightarrow{\textit{peroxidase}} \text{coloured product} + H_2O$$

In most countries the precipitation technique employing phosphotungstic acid/Mg2+ ions is the most commonly used for determination of ***HDL-cholesterol***. After centrifugation, HDL fraction remains in the supernatant. Here, cholesterol is estimated by the same method as total cholesterol. ***LDL-cholesterol*** may be calculated from the value of total cholesterol, triglycerides and HDL-cholesterol, or may be estimated by a suitable precipitation method.

Determination of the ***triacylglycerol*** (***triglyceride, TG***) concentration has become a basic routine test as many patients with coronary heart disease exhibit significantly elevated TG levels. Determination of the TG concentration is performed mainly by the enzymatic method:



Hyperlipoproteinemias occur as primary, mostly familial inborn defect, or secondary to hypothyroidism, renal or hepatobiliary disease, alcoholism, diabetes, gout, oestrogen or corticosteroid therapy. An international classification was proposed by ***Fredrickson***. Routine typing is based on measurement of fasting serum cholesterol and triglyceride, with inspection of the serum after 18 hours at 4°C.

Type I is a rare disease, chylomicronemia is due to deficiency of extrahepatic LP lipase, or Apo C-II defect.

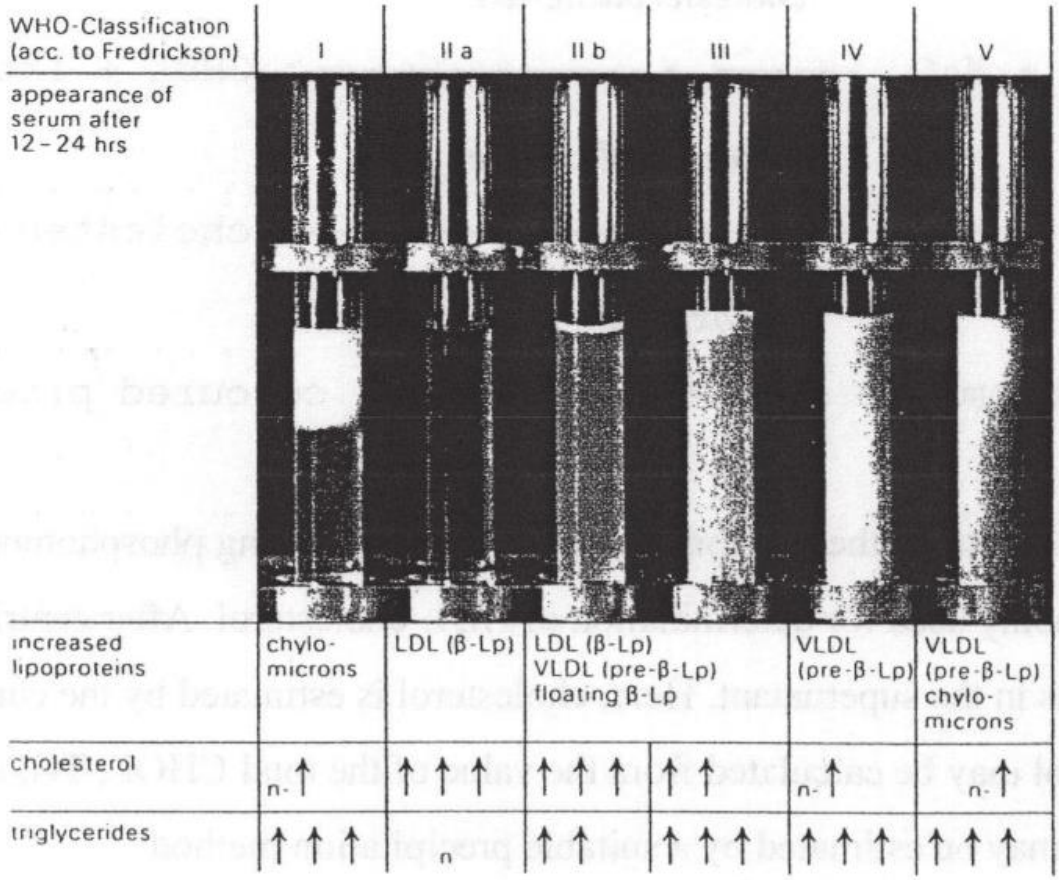
Type IIa represents familial hypercholesterolemia. The serum is clear, electrophoretic analysis show a dense beta-LP band. This disorder commonly presents after a myocardial infarct. The disease may be due to a defect in the cellular uptake of cholesterol from LDL or from failure in regulation of cholesterol biosynthesis. Statins, e.g. Lovastatin, an HMG-CoS reductase inhibitors, are used in the medical treatment.

Type IIb shows the tendency for VLDL to be elevated in addition.

Type III is caused by the deficiency in remnant clearance by the liver due to abnormality in Apo E. IDLs are shown in electrophoresis as a “broad beta”.

Type IV, familial hypertriglyceridemia, is a common condition, associated with overproduction of VLDL, but cholesterol and LDL are normal. It occurs in patients with ischaemic heart disease and/or peripheral vascular disease.

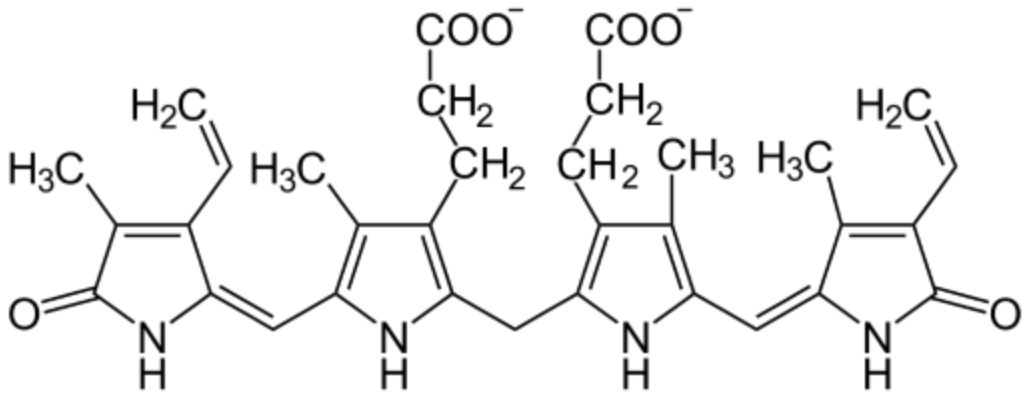
Type V is accompanied by elevated chylomicrons and VLDLs, but this is the least common hyperlipoproteinemia.


WHO-Classification (acc. to Fredrickson)
I
II a
II b
III
IV
V
appearance of serum after 12 – 24 hrs
increased lipoproteins
chylo-microns
LDL (β-Lp)
LDL (β-Lp)
VLDL (pre-β-Lp)
floating β-Lp
VLDL (pre-β-Lp)
VLDL (pre-β-Lp)
chylo-microns
cholesterol
n-
n
n-
n-
triglycerides

Examination of blood III (nitrogen compounds)

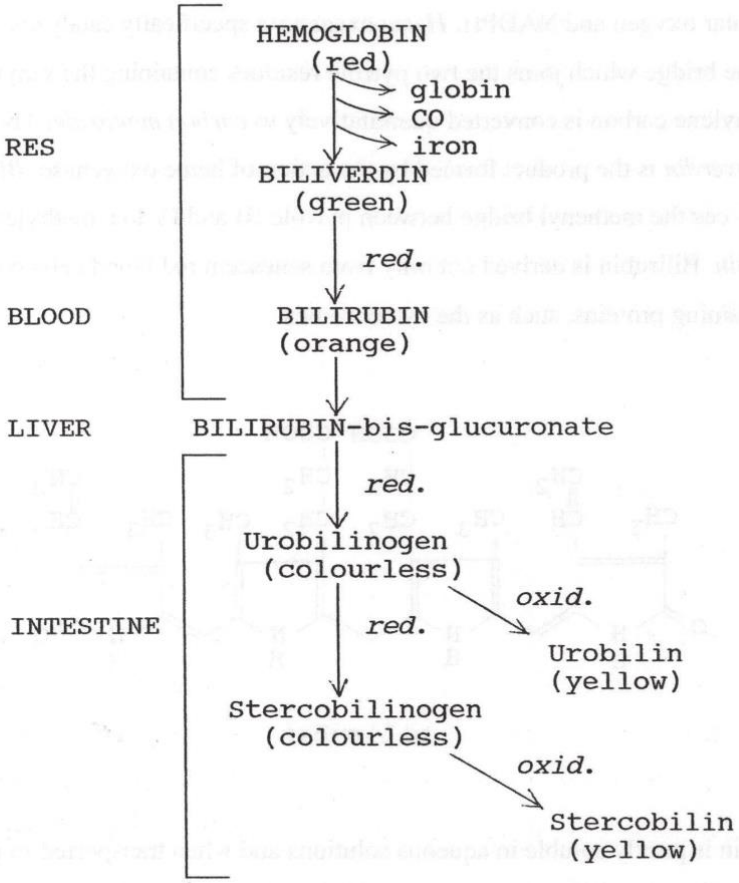
Bilirubin and its catabolism, jaundice

Heme is degraded by a microsomal enzyme system in reticuloendothelial cells that requires molecular oxygen and NADPH. ***Heme oxygenase*** specifically catalyzes the cleavage of the methylene bridge which joins the two pyrrole residues containing the vinyl substituents. The alpha-methylene carbon is converted quantitatively to ***carbon monoxide***. The green linear tetrapyrrole ***biliverdin*** is the product formed by the action of heme oxygenase. ***Biliverdin reductase*** reduces the methenyl bridge between pyrrole III and IV to a methylene group to produce ***bilirubin***. Bilirubin is derived not only from senescent red blood cells but also from other hem containing proteins, such as cytochromes.



Bilirubin

Bilirubin is poorly soluble in aqueous solutions and when transported in plasma, it is bound to serum albumin. Albumin containes one high-affinity binding site and another with lesser affinity. The weak affinity of the second site does not allow it to serve effectively in the transport of bilirubin in extreme concentrations (neonatal hemolysis). A number of drugs compete with bilirubin for binding sites and can displace bilirubin from albumin. Bilirubin on serum albumin is rapidly cleared in the liver by a carrier-mediated system at the sinusoidal surface. This transport system has a large capacity. Once in the hepatocytes, bilirubin is bound to a cytosolic protein, ***ligandin***. The liver increases the water solubility of bilirubin by the conjugation with glucuronic acid to form a ***bilirubin diglucuronide***. Uridine diphosphoglucuronate serves as a glucuronate donor, and the reaction is catalyzed by ***UDP-glucoronyl transferase***. This enzymatic activity can be induced by certain drugs, e.g. by phenobarbital. Secretion of conjugated bilirubin into bile is mediated by an active transport, which is rate-limiting for the entire hepatic bilirubin metabolism.



Bilirubin diglucuronide is poorly absorbed by the intestinal mucosa. The glucuronide residues are released in the terminal ileum and large intestine (colon) by bacterial hydrolases. The released free bilirubin is reduced to colourless linear tetrapyrroles known as ***urobilinogens*** and ***stercobilinogens***. Urobilinogens and stercobilinogens can be oxidized to coloured products known as ***urobilins*** and ***stercobilins***. A small fraction of colourless products can be reabsorbed by the terminal ileum and large intestine to be removed by hepatic cells and reexcreted in bile (enterohepatic cycle). When urobilinogens and other products are reabsorbed in large amounts in certain disease states, they are excreted in urine.

Meconium containes biliverdin, and stools of very young infants generally contain unaltered bilirubin. With development of the bacterial flora bilirubin is more and more reduced to urobilin and stercobilin, unless intestinal contents are hurried through so rapidly that this reduction is incomplete. The reduction is impaired also when the bacterial intestinal flora is reduced by use of antibiotics.

The normal serum bilirubin concentration is up to 25 μmol/L, of it the conjugated form should not exceed 8 μmol/L . This concentration represents a balance between the rate of bilirubin production and the hapatic clearance of bilirubin. Hyperbilirubinemia results when bilirubin formation exceeds hepatic clearance, as in hemolytic states, or when elimination is impaired due to a liver abnormality (i.e. defective uptake, conjugation, or biliary excretion). This is frequently manifest as ***jaundice*** (***icterus***), which is the visible accumulation of excess bilirubin in the skin, mucous membranes, or sclera, imparting a distinct yellow diccoloration to these tissues. Jaundice is usually evident when serum bilirubin level reaches about 35 μmol/L.

Classification of jaundice (hyperbilirubinemia):

Prehepatic (hemolytic) jaundice (corresponds to ***unconjugated hyperbilirubinemia***) may or may not be associated with excessive hemolysis of red blood cells. In either case the excess bilirubin in the blood is unconjugated. In hemolytic jaundice there is an excessive rate of RBC and of their hemoglobin, so unconjugated bilirubin passes into the blood faster than it may be removed by the liver. Jaundice can sometimes occur as a hereditary abnormality (the Crigler-Najjar syndrome or Gilbert syndrome) without evidence of excessive hemolysis. A common feature of both disorders is impaired or absent conjugation of bilirubin by virtue of decreased hepatic bilirubin UDP-glucuronyl transferase activity. Transient unconjugated hyperbilirubinemia of the newborn (***neonatal jaundice***) is relatively common disorder which develop in the first week of life. Delayed functional maturity of glucuronyl transferase, along with a severalfold increase in bilirubin production from the degradation of fetal hemoglobin, appears to the major cause of physiological jaundice. Physiological jaundice must be differentiated from the haemolytic disease due to maternal-fetal blood group incompatibility. Bilirubin encephalopathy ("kernicterus") is the most feared complication of hyperbilirubinemia in the newborn. The laboratory findings are characteristic: unconjugated bilirubin in the blood, urobilinogen in the urine, the stools are very dark (urobilin, stercobilin), in excessive hemolysis urobilin and stercobilin are found also in the urine. However, bilirubin and bile acids are absent in urine.

Hepatic (hepatocellular) jaundice (usually corresponds to ***mixed hyperbilirubinemia***) may be e.g. due to hepatitis or due to drugs and toxins which cause diffuse hepatocellular injury. In hepatic jaundice the blood plasma contains both conjugated and unconjugated bilirubin. The increase in conjugated form is explained by the impaired transport into the bile which is the rate-limiting step in overall transhepatic transport of bilirubin. The laboratory findings are relatively rich: both form of bilirubin are present in the blood, the plasma activities of many enzymes are increased (ALT, AST, LDH, gamma-glutamyl transferase), imbalance in plasma proteins causes the decrease of A/G ratio, excretory liver tests are positive. In urine, bilirubin and increased amounts of urobilinogen and urobilin are fAound. When the jaundice is severe, urobilinogen is found in urine only in early stage of condition and during recovery. The stools may be pale or clay-coloured.

Posthepatic (obstructive) jaundice (usually corresponds to ***conjugated hyperbilirubinemia***) is due to the obstruction of biliary passages from gall stones, tumours or due to intrahepatic cholestasis. The distension of the biliary passages was believed to rupture the biliary canaliculi within the liver with regurgitation of bile in the venous sinusoids. There is more likely an abnormality of transport of conjugated bilirubin to the biliary canaliculi. Conjugated bilirubin, therefore, passes into the blood. In posthepatic jaundice less pigment then normal reaches the gut and feces become less pigmented (clay-coloured). Bilirubin is always present in the urine, along with the bile acids, and urobilin is absent. A high percentage of cases of obstructive jaundice show markedly increased alkaline phosphatase.

Laboratory finding in jaundice:		*prehepatic (hemolytic)*	*(intra)hepatic (hepatocellular)*	*posthepatic (obstructive)*
urine	bilirubin	-	+	+
	urobilinogen	+	+	-
	urobilin	+	+	-
	bile acids	-	+	+
serum	conjugated bilirubin	-	+	+
	unconjugated bilirubin	+	+	-
	↓ A/G ratio	-	+	-
	ALP	-	-	+
	ALT, AST	-	+	-
	functional tests	-	+	-

A quantitative assay for bilirubin was introduced by ***Van den Bergh*** by application of Ehrlich´s test for bilirubin in urine. This reaction is based on the coupling of diazotized sulfanilic acid (Ehrich´s ***diazo*** reagent) and bilirubin to produce a reddish-purple azo compound in acid solution. In alkali, azobilirubin is blue. In the clinical setting conjugated bilirubin is expressed as ***direct bilirubin*** because it can be coupled readily with diazonium salts. Unconjugated bilirubin is bound to albumin and will not react until it is released by the addition of an organic solvent such as ethanol. This reaction is indirect Van den Berg reaction and non-conjugated bilirubin is therefore called ***indirect bilirubin***. Extreme concentrations of bilirubin in some cases of neonatal hyperbilirubinemia are estimated by a direct photometry of serum bilirubin at two wavelengths.

Non-protein nitrogenous compounds of the blood – urea, uric acid

The non-protein nitrogen of the blood (=NPN), that portion of nitrogenous substances not precipitated by the usual protein precipitants, includes especially urea, uric acid, amino acids, creatine, creatinine, and ammonia. From a metabolic standpoint, the non-protein nitrogenous constituents of the blood are usually of grater interest than plasma proteins since they represent products of the intermediary metabolism. The total NPN concentration, although measurable, is of a little diagnostic value, and it is substituted by the measurement of individual component concentrations.

Urea is in man the chief end-product of protein catabolism. It is produced in the liver following transamination of alpha-amino nitrogen, oxidative deamination of glutamate, and reactions of urea cycle. Urea is an extremely diffusible substance and, as such, exists in all body fluids in practically the same concentration, as it is eliminated exclusively by the kidneys, its concentration in the blood is influenced markedly by the renal function,. However, blood urea concentration corresponds also to the ure production, which is influenced by the protein intake, increased or decreased catabolic ratem and a live disease. Blood urea ranges from 3,0 to 8,0 mmol/L.

Creatinine (cratine anhydride) is formed in muscle from creatine phosphate by irreversible, nonenzymatic dehydration and loss of phosphate. Its production is proportional to a patient´s body mass and therefore relatively stabe over time. Creatinine is excreted solely by the kidneys and its excretion is primarily determined by the glomerular filtration rate. Plasma creatinine roughly doubles for every 50% fall in glomerular filtration rate. Normal range is given as 60-100 μmol/L for men and 50-90 μmol/L for women. Generally, in renal failure the rate of excretion of notogenous non-protein components is much less than their rate of formation and there is a rapid increase in their concentration in the blood, i.e. the is ***azotemia***.

Uric acid is the end product of purine metabolism in humans. The average person eliminated approximately 4.4 mmol of uric acid each day. About two thirds of the urate is excreted in the urine, and one third is excreted in the intestinal secretions, where bacteria degrade uric acid to allantoin and carbon dioxide by a process of intestinal uricolysis. An elevated serum urate concentration byond the upper limit (450 μmol/L in men, 360 μmol/L in women) is called ***hyperuricemia***. There are two major causes of hyperuricemia in humans: increased production of uric acid and dreased renal excretion of uric acid. Hyperuricemia needs to be distinquished from ***gout***. Although only a minority of hyperurixemic patients ever become gouty, all patient with gout have hyperuricemia at some stage in their clinical course. Gout is further manifest by recurrent attacks of a characteristic type of acute arthritis, in which crystals of monosodium urate are demonstrable in leukocytes of synovial fluid, by deposits of urate around the joints of the extremities, and often by the renal damage.

One of the final products of protein-amino acids degradation in most organs is ***ammonia***, ***NH_3***. Ammonia is also absorbed from the lumen of the colon, as a product of bacterial deamination of protein from diet, sloughed epithelial cells, and form the action of bacterial urease on lumenal urea. This potentially toxic compound can be detoxicated by two routes. The first is a preliminary step and involves transfer of ammonia to ketoacids (such as 2-oxohlutarate) to form amino acids, or to glutamate to form glutamine.The kidney can excrete ammonia from glutamine to acidify urine. However, the final detocification for most of the NH_3 occurs in hepatocyte through synthesis of urea. In fulminant ***hepatic failure***, blood ammonia increases byond the limit of 50 μmol/L because of hepatocyte death and loss of urea cycle enzymes. In chronic liver disease, shunting of portal-venous NH_3 to the systemic circulation is the major factor for increased blood NH_3, although decreased urea synthetic capacity may be a contributing factor. Relatively rare inherited deficiences of urea cycle, responsible for retarded mental development, are of interest in that they are all associated with hyperammonemia.

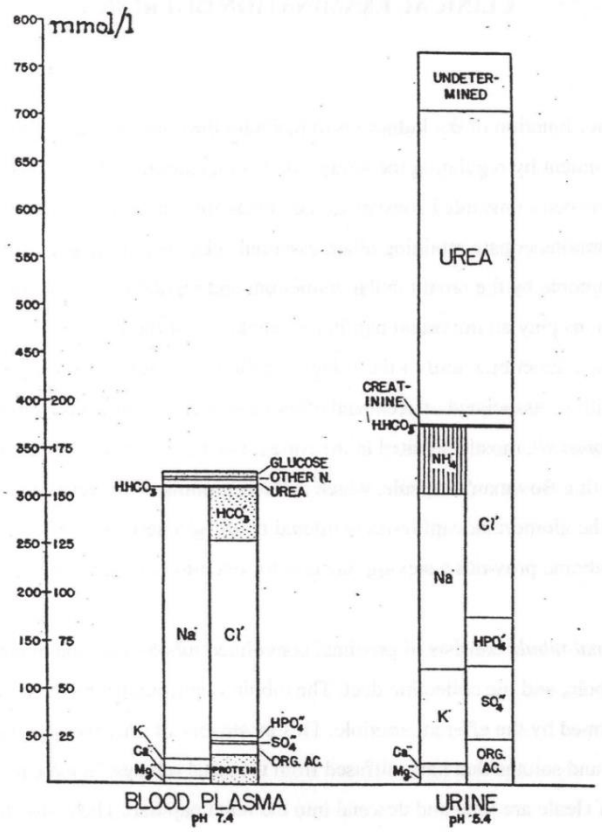
Clinical Biochemistry – Examination of Urine

The chief function of the kidneys is to maintain the constant state of the body´s internal environment by regulating the volume and composition of the cellular fluid. Kidneys remove from the body unwanted substances, i.e. metabolic waste (e.g. urea), drugs and toxic agents while simultaneously retaining other, essential substances (e.g. glucose, amino acids). Formation of ammonia by the renal tubular epithelium and regulation of excretion of certain anions and cations play an important part in the regulation of the acid-base equilibrium.

The basic functional unit of the kidney is called a nephron. It is composed of a glomerulus, with its associated afferent and efferent arterioles, and a renal tubule.

The ***glomeruli***, mostly situated in the cortex, consist of a bundle of 20-40 capillary loops invaginating Bowman´s capsule, which is the beginning of the renal tubule. The plasma that traverses the glomerular capillaries is filtered by the glomerular membrane. Especially the basement membrane provides a sieving function for retention of plasma proteins and blood cells.

The renal tubule consists of proximal convoluted tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct. The tubule is surrounded by the peritubular capillaries, formed by the efferent arteriole. The meshwork of microvessels functions to remove water and solutes that have diffused from the renal tubules. In man, about one-seventh of the loops of Henle are long and descend into the renal papillae. These structures are important in the countercurrent system, by which the kidneys concentrate urine. The proportions of long loops of Henle is much higher and the renal papillae into which they extend are longer in desert animals which have adapted for water conservation.



Physical examination of urine

Naked-eye appearances

Urochrome is the chief pigment and is responsible for amber colour of normal urine. In addition, normal urine contains traces of urobilin and other pigments. Urobilinogen is colourless, contained also in traces. Irrespective of pathological conditions, urine may be pale owing to intake of large volumes of fluid or deeper in colour (more orange) owing to copious sweating or to reduction of fluid intake(=”concentrated”).

Substances which may make urine depart from normal amber:

red – blood, haemoglobin, myoblobin, beets
port-wine – porphyrin
brownish-black – melanin (oxidation of melanogen), alkaptonuria (oxidation of homogentisic acid)
brown – bilirubin, methemoglobin
orange – small amount of bilirubin
greenish – biliverdin (oxidation product of bilirubin)
deep yellow – riboflavin, tetracycline antibiotics, certain chemotherapeutic
white – chyluria

Under naked-eye appearance will be noted also whether urine is clear or cloudy, whether there is any deposit visible (see the examination of urinary sediments). As a rule phosphates are deposited when the urine is alkaline and urates or uric acid when it is acid. Brownish urates may be then dissolved in KOH, phosphates in acetic acid. The deposits mentioned in a cooled specimen are in most cases of no significance. On the other hand turbidity caused by the massive occurrence of white blood cells and microorganisms is always pathological.

Odour

There may be a characteristic odour, as, for example, of ammonia due to decomposition by bacteria (increased pH), or unpleasant odour of phenylacetic acid in phenylketonuria (“mousy smell”).

Volume

An average urine output ranges from 1 to 1.5 L/day. ***Oliguria*** is said to be present when the urine amount is less than mL/day, ***anuria*** less than about 100 mL/day. When the urine output exceeds 2.5 L/day, ***polyuria*** is present.

Density

The specific gravity may be taken with an urinometer. It ranges about 1.003 to 1.035.

pH value

The limits for pH encountered in urine range from 4.5 to 8.0, an average being about 6. Owing to bacterial decomposition an acid urine may become alkaline in a very short time, e.g. in 2-4 hours at room temperature.

Basic chemical examination of urine

Proteinuria

Normal urine contains a small amount of protein insufficient to give a positive reaction to the usual chemical tests. The normal daily amount is about 150 mg consisting of up to 40 mg of albumin, rather less globulin, and Tamm-Horsfall mucoprotein. An increase is important in the diagnosis of renal disease, and especially of the early stages of pyelonephritis.

Proteinuria may be due in part to an increased permeability of the glomerulus, to impaired reabsorption of normal amounts of protein from the glomerular filtrate or to tubular excretion. Distribution of proteins in urine in pathological proteinuria varies with the primary disease. The more glomeruli are seriously affected the larger are the pores in the basement membrane which thereby allow higher molecular weight protein to pass through. If the pores remain small the proteinuria is ***selective***. Using special methods (e.g. immunoassay) permit classification of proteinuria as selective or non-selective. The clearance ration of IgG/transferrin may be used. In minimal change glomerular nephritis in children there is usually selective proteinuria and a good prognosis.

Orthostatic or postural proteinuria, common in adolescents and young adults, is due to venous vasoconstriction caused by a lorditic erect posture (3% of otherwise normal adolescents). A transitory proteinuria can also follow exercise or common pyrexial illnesses.

Overflow (prerenal) proteinuria occurs with low molecular weight proteins, e. g. in Bence-Jones proteinuria of myeloma. Bence-Jones proteins are light chains of the myeloma Ig and are precipitated at 45-55°C and re-dissolve at 95-100°C. Bence-Jones protein is now usually detected by gel electrophoresis of urine. Proteins originating from the renal tract include Tamm-Horsfall mucoprotein which forms hyaline casts in normal urine, IgA associated with renal inflammatory disease such as pyelonephritis, and urinary enzymes.

Determination of sugar in urine

Reabsorption of glucose increases with rising plasma concentration, but there is a maximum rate for this process. Thus if the load of glucose filtered by the glomeruli exceeds the maximum rate of glucose reabsorption (T_mGlc=27-33μmol/s) then ***glycosuria*** occurs. Some subject have individual nephrons with low T_mGlc and excrete glucose in the urine at normal plasma levels and are said to have renal glycosuria. In general T_m values decrease with age. The glucose plasma level corresponding the T_m in healthy young individuals is approximately 9 mmol/L.

In diabetic coma, when concentration of glucose in the plasma is very high, the concentration of glucose in urine will depend on the maximum osmotic work of which the kidneys are capable, as well as on other substances requiring excretion, i.e. there will be no direct relationship to the concentration in the blood.

Mellituria (excretion of other sugars) must be distinguished from glycosuria (excretion of glucose), these are usually congenital metabolic defects:

Fructosuria is rare inborn disorder of fructose metabolism, a deficiency of the enzyme fructokinase. Alimentary fructosuria is occasionally due to ingestion of fructose or fruits.

Galactosuria is one of symptoms of congenital galactosemia, a disease characterized by inability to metabolize galactose to glucose.

Lactosuria is common in nursing mothers and in infants on a milk diet for a long time.

Pentoseuria is a rare inborn error of metabolism, a deficiency of the enzyme L-xylulose reductase, alimentary pentosuria is occasionally encountered in the "fruit season".

Simple detection of glucose in urine is based on its reducing properties (contains an aldehyde group) or on its ability to react with glucose oxidase.

Most sugars are reducing substances and as such may be detected in urine. However, many other substances reduce to some extent the detection reagent used: uric acid, glucuronic acid, creatinine, tetracyclines, ascorbic acid, gentisic acid, etc. Due to the non-specificity of reduction tests, much more specific enzyme-based strip tests are preferred today.

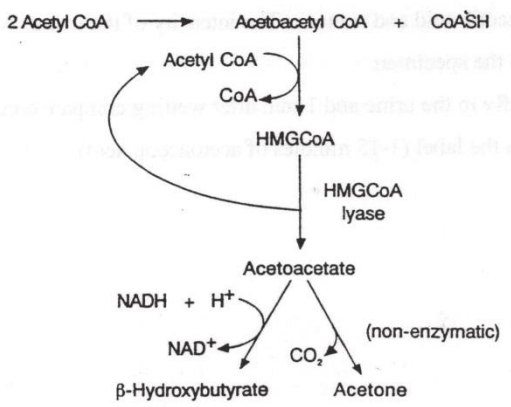
In the past, the quantitative determination of glucose in urine was performed polarimetrically (glucose is optically active). Glycosuria and different melliturias may be distinguished by paper chromatography.

Ketonuria

The fatty acids are degraded in the liver into acetyl coenzyme A (acetyl-CoA) which, if sufficient oxaloacetic acid is available from carbohydrate oxidation, is oxidized in the citric cycle. If not, or if fatty acid degradation is excessive, acetyl-CoA is converted into acetoacetyl-CoA or synthesized into cholesterol via ***beta-hydroxy-beta-methyl-glutaryl-CoA***. The latter may be converted into ***acetoacetic acid***, which, which with the reduction product ***beta-hydroxybutyric acid***, and its decarboxylation product ***acetone***, form the ***ketone bodies***.

Acetoacetatic acid can be utilized by the tissues, especially the muscles, for the production of energy, but if it is formed by the liver at a rate greater than the muscles can utilize, then ketone bodies accumulate in increased quantities in the blood, ketoacidosis develops and keto bodies appear in urine.

The routine tests are for acetoacetic acid and acetone, using nitroprusside. Beta-hydroxybutyric acid, a major part of ketone bodies, is therefore not detected.



Hematuria

The definition of ***haematuria*** encompasses the presence of gross blood in the urine on the one hand to as few as three erythrocytes per microscopic field in a urine sediment. Hematuria may be extrarenal or intrarenal. ***Extrarenal*** injury involves urinary ways (pelvis, ureter, bladder, prostate), ***intrarenal*** causes include e. g. injuries of glomeruli (inflammation) and malignancies (Grawitz´s tumour). ***Myoglobin*** released following muscle injury gives a red-brown colour to the urine. Following intravascular hemolysis, free ***haemoglobin*** may be filtered and excreted, giving a distinctly red colour to the urine. Both pigment cause standard reagents and dip sticks to give a positive reaction for haemoglobin. The colour reactions are catalysed by the hem iron.

Bile pigments in urine

Urine is tested for bile pigments on account of its colour, or of the colour of the patients skin or conjunctiva (icterus, jaundice), or as a part of a routine examination. Normally there is no bilirubin in urine, or possibly the merest traces. In general terms bilirubinuria signifies disease of the liver or obstruction of the bile ducts. Tests for bilirubin depends on oxidation to green (biliverdin) or blue (bilicyanin) pigments, or formation of azobilirubin.

In the intestine bilirubin is reduced to a mixture of colourless chromogens referred as "***faecal urobilinogen***". These chromogens are later dehydrogenated to give orange-red pigments, the so called "***faecal urobilin***".Some faecal urobilinogen is reabsorbed and via portal circulation passes to the liver, where it is normally either metabolized or re-excreted in the bile. In liver disease the reabsorbed urobilinogen is diverted to the blood circulation and, as threshold concentrations for kidney excretion equals zero, urobilinogen is found in urine. Urobilinogen gives Ehrlich´s reaction with *p*-dimethylaminobenzaldehyde in presence of HCl. Urine is examined for urobilinogen during routine examination or because liver damage or excessive hemolysis is diagnosed. Normally urine contain traces of urobilin, and there quantities do not affect its colour. When there is an excess of the pigment urine is more orange than usual. Tests for the pigment depend on the characteristic absorption spectrum on the fluorescence of the zinc-urobilin compound (Schlesinger´s test).

Renal functions

Glomerular and tubular functions, clearance

Glomerular filtration is the initial step in urine formation. The glomeruli filter about 180L of fluid per day although only about 1.5 L is passed as urine, due to tubular reabsorption. The filtrate is an ultrafiltrate, i.e. it has the composition of plasma except that is almost free of protein. Ultrafiltration across the glomerular capillary is determined by imbalance between trans-capillary hydraulic and colloid osmotic pressure gradients. As blood flows along the glomerular capillary, the protein concentration rises because of the protein-free nature of the ultrafiltrate and oncotic pressure increases. On the other hand, net ultrafiltration pressure decreases at the efferent end of the capillary and, as a result of both factors, net filtration ceases. The ***glomerular filtration rate*** (GFR) refers to the volume of glomerular filtrate formed per unit of time and is related to body size, or, more accruable, the surface area and is normally about 2 mL/s (120 mL/min) per 1.73 m^2 body surface area.

As a measure of GFR, a clearance of a substance which is freely filtered and neither secreted nor absorbed by the renal tubules, can be used. Renal clearance of a given substance is the ration of the renal excretion rate of the substance to its concentration in the blood plasma, In other words, it is the volume of plasma which would be completely cleared of the substance.

If volume of urine excreted per sec is equal to ***V mL***, and if concentration of substance in urine is equal to ***U mmol/mL***, then quantity of substance excreted per sec is equal to ***U*×*V mmol*** = ***renal excretion rate***. If concentration of substance in plasma is equal to ***P mmol/mL***, volume of plasma providing U×V mmol of the substance is its ***clearance C***.

$$C = \frac{U \times V}{P}$$

As a measure of GFR, a clearance of inulin, endogenous creatinine, ^{51}Cr-EDTA, radioactive cobalamine or mannitol can be used. Inulin, a fructo-polysaccharide that does not occur naturally in the body, should be administered intravenously. Hence, in clinical practice it is more convenient to determine the clearance of creatinine, although a small amount of creatinine is secreted in humans and, therefore, the clearance might be slightly higher than GFR. Creatinine clearance declines with age, and so does also the GFR. Measurement of ***plasma creatinine*** itself may be also used as a day-to-day indication of changes in GFR, mainly in patients with renal failure. Plasma creatinine roughly doubles for every 50% fall in GFR. However, using serum creatinine concentration alone as a measure of the net GFR can lead to serious errors, especially in elderly individuals.

In current clinical practice, the ***estimated GFR*** (***eGFR***) value, which is calculated from serum creatinine concentration using formulas (such as MDRD or CDK-EPI equations) including factors such as age, gender, weight, and race, can in many cases replace complicated determination of creatinine clearance which need urine collection.

The clearance of many substances are much greater than the GFR because ***tubular excretion*** results in a greater clearance of plasma than can be caused by glomerular filtration alone. *p*-aminohippuric acid (PAH) in low plasma concentrations has an extremely high plasma

clearance. The renal clearance is nearly complete and PAH concentration in renal venous plasma may be taken to be zero. Accordingly, the clearance of *p*-Aminohippuric acid C_{PAH} actually measures the ***effective renal plasma flow*** (ERPF, about 10mL/s or 600mL/min).

From clearance of inulin C_{IN} and C_{PAH}, a ***filtration fraction*** (***FF***) can be calculated:

$$FF = \frac{C_{IN}}{C_{PAH}}$$

FF is a measure of the fraction of the entering plasma volume that is removed through the glomeruli as filtrate (about 20% of the entering plasma volume).

When C_X is less than C_{IN}, excretion is by filtration and reabsorption. In this case, the amount of the substance excreted in the urine is less than the amount of the substance filtered during that time.

APPROXIMATE CLEARANCE VALUES

glucose	0.00 mL/s (unless the threshold concentration is reached)
Na^+	0.01 mL/s
K^+	0.15 mL/s
osmotically active solutes	0.05 mL/s
urea	1.25 mL/s (75 mL/min)

The most clinical useful ***tubular function tests*** include the urinary sodium (Una), fractional excretion of sodium (FENa), urinary concentrating and diluting ability, and urinary acidification ability.

Urinary concentrating and diluting ability:

Normal serum osmolality is about 290 mmol/kg. Urine of this osmolality is called ***isotonic***. With an increased volume of fluids in the body, the excretion of water due to regulatory mechanisms increases and the urine becomes ***hypotonic***. On the other hand, if water intake is restricted, urinary osmolality rises, the urine becomes ***hypertonic***, reflecting to body need to conserve water. If urine osmolality fails to increase, a water regulatory defect should be suspected. Similarly, as serum tonicity falls (dilution), the appropriate renal response is a water diuresis. If the appropriate response does not occur, a defect in water regulation is suggested.

Renal failure, in which the end products of metabolism accumulate in excess in the body fluids, merges with the late stages of ***renal insufficiency*** (in which, under limited intake of protein in diet, the concentrations of end products are maintained within normal limits). The concentrations of urea, creatinine, uric acid and also inorganic phosphate and potassium in plasma are raised and the accumulation of acidic products reduces plasma bicarbonate and leads eventually to metabolic acidosis.

Acute renal failure, associated with both impairment of glomerular filtration and ischaemic damage to the tubules, is a common complication of many primary medical and surgical conditions end even today has a high mortality. In chronic renal failure, as at the number of functional nephrons falls there is an increased amount of urea excreted per nephron, causing ab osmotic diuresis. Patients become salt depleted, the loss of renal parenchyma reduces renal ammonium synthesis and aggravated the metabolic acidosis. A complex of signs, symptoms and biochemical abnormalities in advanced chronic renal failure is referred to as ***uremic syndrome***.

Examination of urinary sediment

This examination remains one of the most useful non-invasive clinical tests. It complements the physical and chemical examination of urine.

Deposits may be classified under three headings, each beginning with "C": ***Cells***, ***Casts***, and ***Crystals*** and amorphous chemical deposits. In the first group are included white blood corpuscles (WBC), red blood corpuscles (RBC), epithelial cells, bacteria, yeast cells, etc. Casts are commonly of three types, i.e. hyaline, granular and cellular.

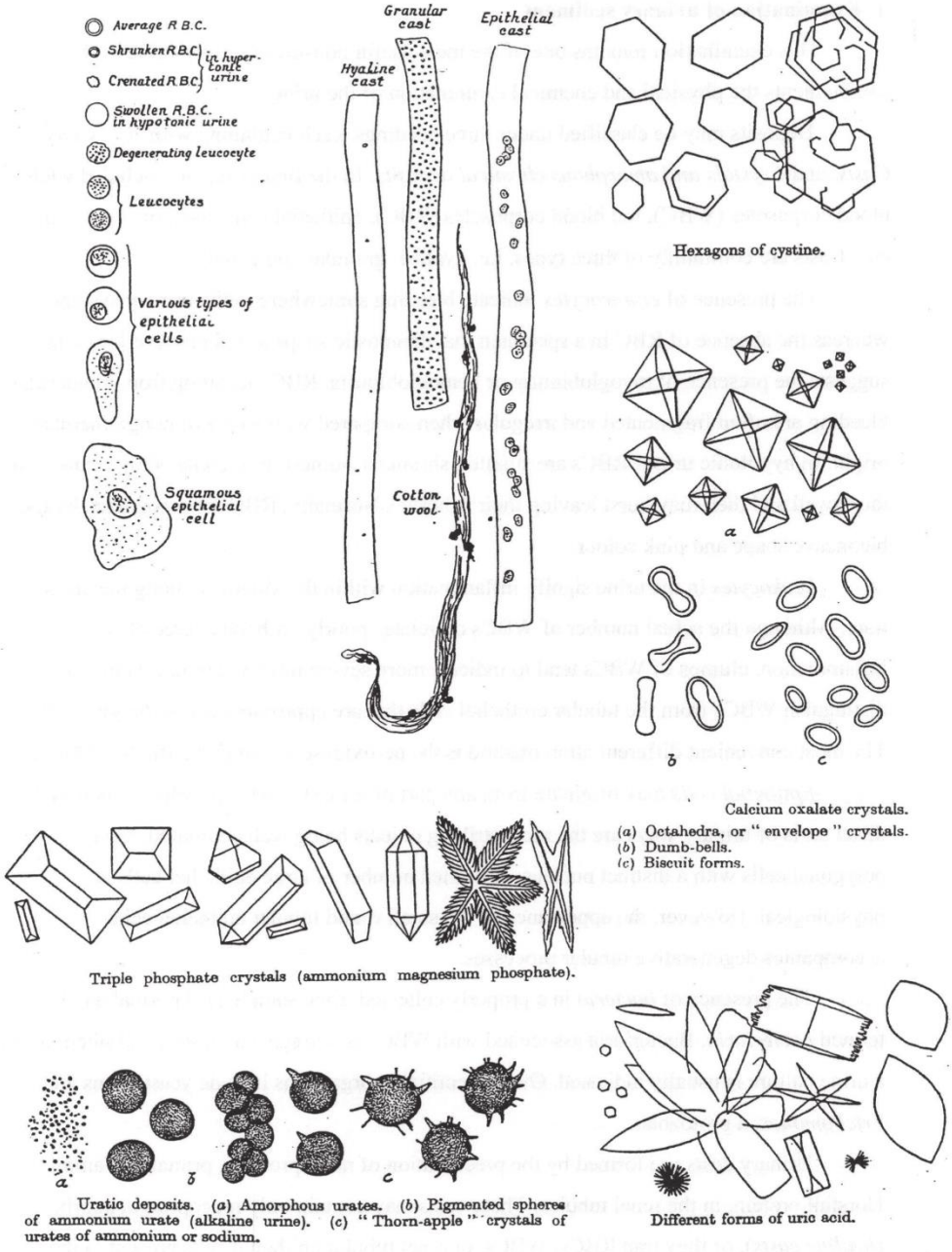
The presence of ***erythrocytes*** indicate bleeding somewhere in urinary system, whereas the absence of RBC in a specimen that diagnostic strips tested positive for blood suggests the presence of myoglobinuria or hemoglobinuria. RBCs resulting from glomerular bleeding are often fragment and irregular when compared with RBCs of non-glomerular origin. In hypotonic urines RBCs are smaller, shrunken sometimes crenated. In dilute urine they swell and they may burst leaving their "ghosts". Normally, RBCs are recognized by the biconcave shape and pink colour.

Leucocytes in the urine signify inflammation within the kidney or along the urinary tract. Although the actual number of WBCs correlates poorly with the degree of inflammation, clumps of WBCs tend to indicate more severe inflammation. It is important to distinguish WBCs from tubular epithelial cells that are approximately of the same size. The most convenient differentiation method is the peroxidase staining specific for WBCs.

Epithelial cells may originate from any part of urinary tract. Epithelial cells from the distal part of urinary ways are the most striking objects being well contoured, large polygonal cells with a distinct nucleus. A limited number of large epithelial cells is physiological. However, the appearance of the small round tubular epithelial cells accompanies degenerative tubular processes.

The presence of ***bacteria*** in a properly collected urine sample is abnormal and is termed ***bacteriuria***. Bacteriuria associated with WBCs is strongly suggestive of infection, and a urine culture is usually indicated. Other identifiable organisms include yeast forms and ***Trichomonas***, a protozoan.

Urinary casts are formed by the precipitation of mucoproteins, primarily Tamm-Horsfall protein, in renal tubules. These casts may contain only mucoprotein matrix (***hyaline casts***), or they trap RBCs, WBCs, or renal tubular epithelial cells present in the lumen (***cellular casts***). Clinically, RBC casts are generally diagnostic of glomerular bleeding and indicate the presence of glomerulonephritis. Large number of cellular casts indicate the presence of inflammation either within the glomerulus or around the tubules (interstitial nephritis). With time, cellular elements within casts degenerate, losing their identifiable characteristics and leaving ***granular casts***. ***Waxy casts*** may represent further degradation of this cellular material to a homogenous appearance. Red cell casts may degenerated into ***haemoglobin casts***. The significance of degenerated casts is the same as that of their parent cast.



Crystals can be a normal finding, although they may occur with increased frequency in renal stoneformers. Only ***cystine crystals*** are always abnormal and indicative of disease. Cystine crystals have a flat, hexagonal shape and indicate the presence of cystinuria. Clinically a deposit of ***uric acid*** or ***urates*** is most often caused by cooling urine in vitro, and only occurs at acid reaction (ammonium urate excepted). Uric acid crystallises in many forms (barrels, plates, prisms, needles, etc.). Urates are often amorphous, but sodium urate and ammonium urate may by crystalline (thorn-apple crystals). Like uric acid, urates are usually pigmented, and re-dissolve on warming, or in excess alkali. Crystals of ammonium magnesium (triple) ***phosphate*** are often seen in urinary deposits. Morphologically the commonest form of ***calcium oxalate*** is the flattened octahedron or "envelope" crystal, though it may appear in the shape of a biscuit or dumb-bell.

Examination of amino acids and their metabolites in urine

Renal aminoaciduria, in contrast to overflow aminoaciduria, occurs in several inborn errors of metabolism of which cystinuria and variants of the Fanconi syndrome are the most common. Cystinuria is a genetically determined deficiency of tubular reabsorption of cystine and the basic amino acids lysine, arginine and ornithine. Of these four, the less soluble cystine crystallizes leading to the formation of cystine calculi. Of the two types of Fanconi syndrome, the most severe syndrome in infants is accompanied with cystine deposits in the body fluids, non-specific aminoaciduria, glycosuria and vitamin-D –resistant rickets.

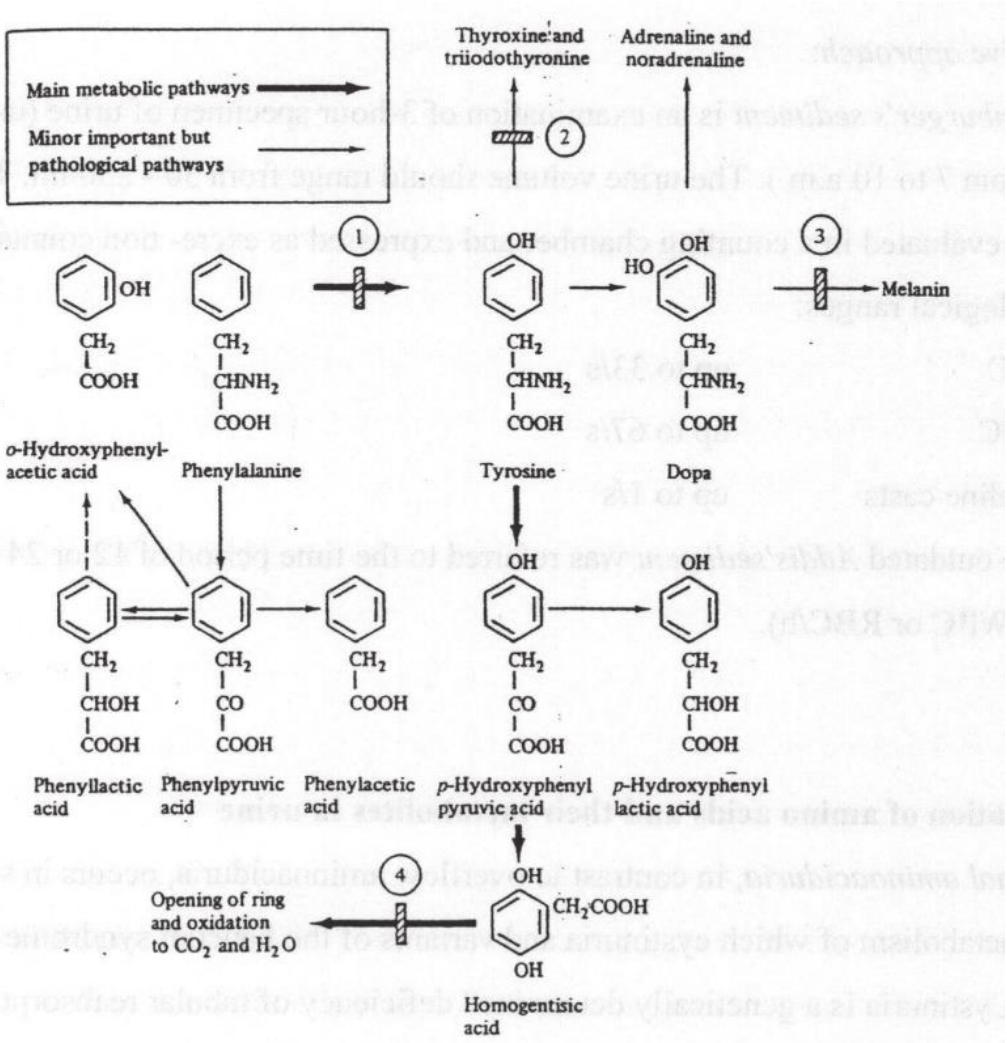
In some inheritable biochemical disorders as well as in liver disease there is a production of amino acids in excess which exceeds the normal reabsorptive capacity of renal tubules, leading to ***overflow aminoaciduria*** (e.g. homocystinuria, cystathionuria),

There are many inborn errors of metabolism of the branched chain amino acids, leucine, valine and isoleucine. ***Maple syrup urine disease*** is due to deficiency or abnormality of the common oxidative decarboxylase, leading to urinary excretion of increased amounts of the three amino acids and their ketoacids.

In ***alkaptonuria*** and ***albinism***, there are blocks in the metabolism of phenylalanine and tyrosine respectively. In former, the metabolism of these amino acids is blocked at the homogentisic acid stage; this acid is therefore excreted. The urine darkens on standing and is strong reducing.

In ***phenylketonuria*** (PKU) the mental disturbance is related to the excretion of phenylpyruvic acid and *o*-hydroxy-phenylacetic acid together with phenylalanine, phenyllactic acid and phenylacetic acid. The patients have an inherited deficiency of the enzyme phenylalanine hydroxylase (or a cofactor) responsible for forming tyrosine from phenylalanine which is present in the plasma in increased concentrations. These patients can be effectively treated with low-phenylananine diets started as early in their lives as possible. Therefore it is carried out the screening of the newborns (48 – 72 h after birth). At present there are 18 diseases tested in the Czech Republic, the phenylketonuria was the first disease tested.

In circumstances in which ***acute porphyria*** (an inborn error of the porphyrine metabolism) is suspected, i.e. in surgical emergences, nervous and psychiatric conditions in which the diagnosis is obscure, a test for s porphobilinogen should be carried out. This is identical with the Ehrlich aldehyde test for urobilinogen. However, the coloured product cannot be extracted with amyl alcohol.

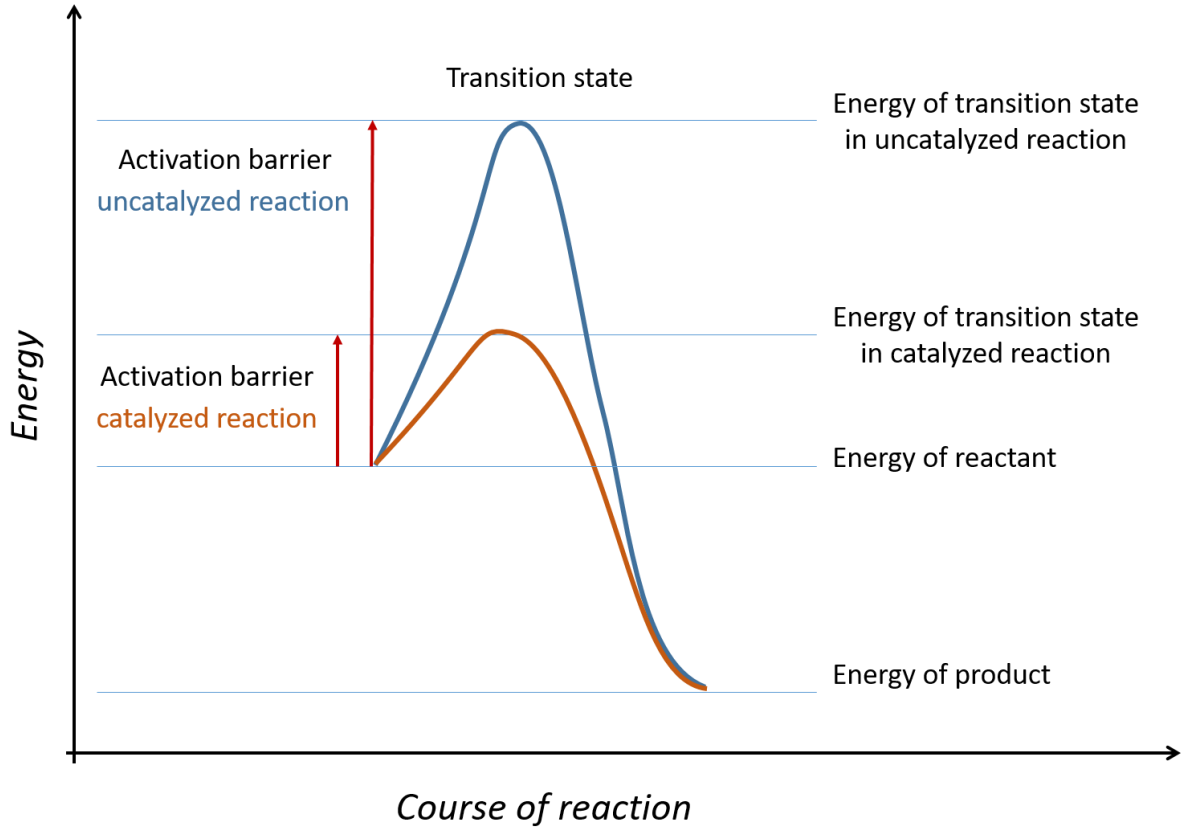


Site of metabolic blocks responsible for phenylketonuria (1), one form of cretenism (2), albinism (3), and alkaptonuria.(4).

Enzymology

Introduction to enzymology

Virtually all the biologically important reactions are catalysed. This is achieved with the help of specific biocatalysts known as ***enzymes***. The enzymes are able to increase the rate of reactions that may occur in the particular cell or tissue. It should be noted that enzymes do not change the chemical equilibrium neither total energy input or output of the reaction. *Enzymes increase reaction rates by decreasing the activation barrier of the reaction.*



Almost all known enzymes are ***proteins***. The molecular weights of enzymes cover a wide range. For example, the enzyme ribonuclease is relatively small, having molecular weight approximately 13,700. In contrast, aldolase, an enzyme of glycolysis, has a molecular weight of approximately 156,600. It is composed of four subunits, each with a molecular weight of about 40,000. Pyruvate dehydrogenase, which catalyses the conversion of pyruvate to acetyl-coenzyme A, is a multi-enzyme complex in which the components are so tightly organised that the entire system can be isolated as a discrete, particulate entity from many tissues. The complex from pig heart has a molecular weight of about 1×10^7. Each complex contains no fewer than 42 individual molecules, including several important and essential cofactors. The entire structure of the pyruvate dehydrogenase complex is required for catalysis.

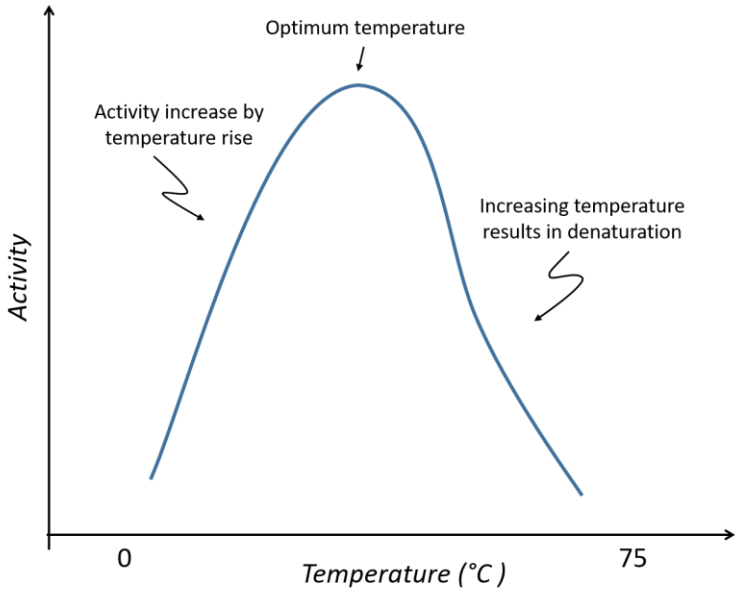
In addition to the protein component, many enzymes require non-protein constituents for their function as catalysts. These accessory moieties are variously termed prosthetic group, cofactor, and coenzyme. The term ***prosthetic group*** applies to any non-amino acid portion of an enzyme that confers on that enzyme some particular property. Prosthetic groups are connected to the protein part either covalently (hem in cytochromes) or non-covalently (hem in haemoglobin). The term ***cofactor*** is also broadly defined. Small organic molecules such as phospholipids are essential to maintain certain enzyme proteins in a conformation suitable for catalysis (β-hydroxybutyrate dehydrogenase), even though they do not directly participate in the catalytic event. Some enzymes require a cation (e.g. magnesium) or less often an anion (e.g. chloride) as a cofactor. Enzymes that require a metallic ion to be present within the protein structure for their function form the family known as ***metalloenzymes*** (e.g. carbonic anhydrase contains a zinc atom in each molecule). The term ***coenzyme*** applies to organic molecules, often but not always derived from a vitamin, which are essential for activity of numerous enzymes. Some coenzymes are tightly bound to the protein portion of a given enzyme; indeed, the enzyme may be denaturated when attempts are made to remove the coenzyme. In other instances the coenzyme is bound so loosely that simple dialysis will separate it from its protein partner. Coenzymes always participate in the catalytic reaction. The complete functional complex of protein plus all required accessory factors of any kind are known as the ***holoenzyme***; the protein part, free of cofactors, is termed an ***apoenzyme***.

Probably the most striking property of enzymes is their specificity, even if it is not always absolute. Urease or catalase are the examples of enzymes with absolute specificity towards their substrates, chymotrypsin on the other hand shows a somewhat lesser specificity, it prefers to cleave peptide bonds in which one participant amino acid has an aromatic ring.

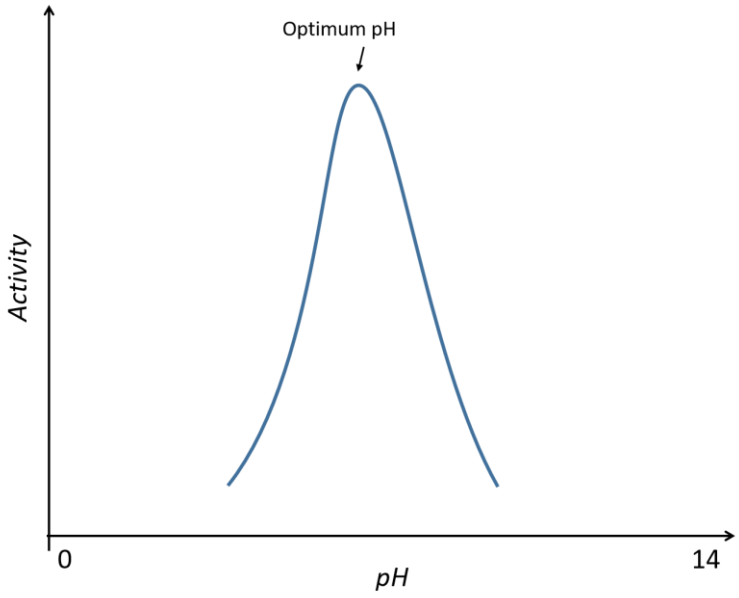
Enzymes isolated from their natural sources can be used in vitro to study in detail the reaction they catalyse. Reaction rates may be altered by varying such parameters as pH or temperature, by changing the ionic composition of the medium, or by changing ligands other than the substrate or coenzymes.

Since protein structure determines enzyme activity, anything that disturbs this structure may lead to change in activity. Denaturation of proteins, which means the spatially random arrangement, can be produced by many agents. These include heat and chemicals that destroy hydrogen bonds in the protein, such as urea at high concentration, detergents such as sodium dodecyl sulfate, and sulfhydryl reagents such as mercaptoethanol. Enzymes frequently show great thermal sensitivity. When heated to temperatures greater than 50°C, most but not all (e.g. enzymes of thermophilic bacteria) enzymes are denaturated. High-temperature denaturation is usually irreversible.

In general, the rate of a chemical reaction increases with increasing temperature (a temperature increase of 10°C will approximately double the rate). In the case of enzyme reactions, however, this only applies to a certain extent - until the denaturation leading to a reduction in the reaction rate prevails. Most enzymes show a certain ***temperature optimum*** at which the activity is maximal. The changes in activity above and below the temperature optimum are not always symmetric.



Enzyme activity is also related to the ionic state of the molecule and especially of the protein part, since the polypeptide chains contain groups that can ionize to a degree that depends on the prevailing pH. As is true of proteins generally, enzymes have an ***isoelectric point*** at which their net free charge is zero. The pH of the isoelectric point (pI) as a rule is not the same as the pH at which maximal activity is demonstrated. The ***pH optima*** shown by enzymes vary widely. Pepsin, which exists in the acid environment of the stomach, has a pH optimum at about 1.5, whereas arginase, an enzyme that cleaves the amino acid arginine, has its optimum at 9.7. However, most enzymes have optima that fall between pH 4 and 8. Some enzymes show a wide tolerance for pH changes, but others work well only in a narrow range. If any enzyme is exposed to extreme values of pH, it is denatured. The sensitivity of enzymes to altered pH is one reason why regulation of body pH is so closely controlled and why changes from normal may involve serious consequences.



Enzymes differ from other proteins in that they possess what has been termed as ***active catalytic site***. The active site can be regarded as being composed of a relatively small number of amino acid residues, not necessarily in immediate sequence in terms of primary structure. However, these amino acids interact in a manner that allows catalysis to occur. Because of the peculiar and highly individualized ways in which peptide chains may be folded, amino acids some distance apart in the primary structure may contribute to the active site. At the same time, if some molecular change occurs, the necessary interaction of amino acids composing the active site probably will be weakened or lost. This accounts for relatively mild treatment possibly causing denaturation.

Some enzymes, namely those with strong irreversible effects (e.g. proteolytic enzymes of digestive tract, enzymes of blood clotting cascade, etc.) are synthesized as inactive precursors also named ***proenzymes*** or ***zymogens***. Typical activation mechanism is the excision of a peptide fragment followed by a change of conformation and a formation of the active site.

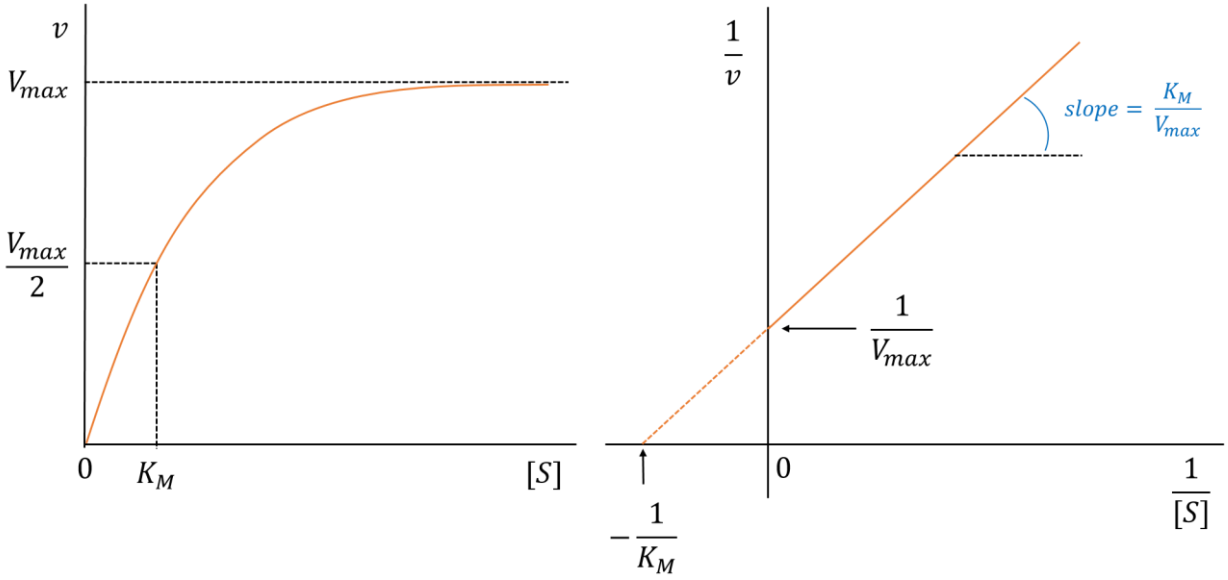
In many species, including human, different molecular forms of certain enzymes may be isolated from the same or different tissues. The different molecular forms have been termed ***isoenzymes*** or ***isozymes***. Lactate dehydrogenase (LDH) and malate dehydrogenase have been thoroughly studied as examples of isoenzymes. LDH is composed of four subunits. The two subunit types, differing in amino acid content and sequence, can be combined into tetramers in five ways. If one subunit type is identified as "M" (the major form found in muscle or liver) and the second as "H" (the major form found in heart), the tetramers could have compositions M_4, M_3H, M_2H_2, MH_3 a H_4. These can be separated by electrophoresis. In humans the content of several isoenzymes differs in heart and liver, and this difference was used in the past in diagnostic differentiation of diseases of the liver and myocardium.

A unit of enzymatic activity is the katal (kat) which is defined as the moles of substrate transformed per second. Enzyme concentrations in the analyzed body fluids (serum, cerebrospinal fluid, urine, etc.) are expressed as kat/L (mkat/L, μkat/L, etc.)

When determining the activity, we usually measure the decrease of the substrate or the increase of the product. It is also possible to measure coenzyme changes, i.e. anything that is convenient from a laboratory point of view. Various synthetic substrates yielding coloured products, or even subsequent reactions based on the properties of by-products of the enzyme reaction, have proven themselves.

Enzyme kinetics

It was mentioned above that enzymes increase the reaction rate by their catalytic action. Let us explain some quantitative aspects of enzyme reaction kinetics. The quantitative analysis of enzyme action depends largely on measured reaction times. If the initial reaction rate, defined as the rate observed for a given amount of enzyme when the concentration of product formed is nearly zero, is plotted as a function of the substrate concentration, the results appear similar to those shown below:



The curve connecting the observed points would be a hyperbola and would asymptotically approach a maximum value, as shown by V_{max}. This is the maximum initial velocity that can be obtained without increasing the amount of enzyme.

The hyperbola described by a plot of reaction velocities as a function of substrate concentrations is difficult to use. If reciprocals of the velocities are plotted as a function of the reciprocal substrate concentrations, the hyperbola is converted to a straight line. The double-reciprocal plots are frequently called Lineweaver-Burk plot.

If the usual convention is followed, representing concentrations by means of brackets, that is, by letting [S] stand for the molar concentration of the substrate, and if a few assumptions are made regarding the experimental situation, one can obtain a useful mathematical equation that describes the enzyme kinetics.

Assume for the present that:

1. The system involves only a single substrate S.
2. The system is at steady state, that is, [ES] is a constant and the free enzyme E is in equilibrium with complex enzyme-substrate ES.
3. The system is established so that [E] < [S] on a molar basis.
4. Since the analysis deal with initial reaction rates (i.e. almost no product P is formed yet), [S] >> [P] and [P] is negligible under these conditions.

The reaction mechanism of such a single-substrate reaction may be formulated as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

where k_1, k_2, k_3, and k_4 are the respective rate constants.

At the steady state the concentration of ES is constant; that is, the rate at which it is being formed is the same as the rate at which it is being broken down. Under these conditions the rate equation can derived:

$$\underset{\textit{rate of ES formation}}{k_1[E][S] + k_4[E][P]} = \underset{\textit{rate of ES dissociation}}{k_2[ES] + k_3[ES]}$$

Since the analysis is restricted to initial reaction rates, [P] is negligible and [S] is virtually constant. Thus the term involving [P] can be dropped and those involving [ES] can be collected to give the following:

$$k_1[E][S] = (k_2 + k_3)[ES] \qquad \text{or}$$

$$\frac{[E][S]}{[ES]} = \frac{k_2 + k_3}{k_1} = K_M$$

The ratio of rate constants can be replaced by a single constant, $\boldsymbol{K_M}$, known as ***Michaelis constant***.

If in the reaction mechanism, which consists of a sequence of several reactions, one is significantly slower, then the reaction rate of this reaction determines the resulting reaction rate of the entire reaction mechanism. Here, the formation of the product is a decisive step. The observed initial velocity will be the following:

$$v = k_3\,[ES]$$

The enzyme present is either free or bound in a complex with substrate. Only the total enzyme concentration $[E]_0$ is accessible for measurement.

$$[E]_0 = [E] + [ES]$$

Combining the above mentioned equations yields:

$$\frac{([E]_0 - [ES])[S]}{[ES]} = \frac{k_2 + k_3}{k_1} = K_M$$

$$[ES] = \frac{[E]_0[S]}{\frac{k_2 + k_3}{k_1} + [S]}$$

The observed initial velocity is then the following function:

$$v = k_3 \frac{[E]_0[S]}{\frac{k_2 + k_3}{k_1} + [S]}$$

The maximum initial velocity is achieved only when all the enzyme is in the form of the active complex ES, from which it follows that:

$$V_{max} = k_3 [E]_0$$

For a given enzyme concentration, the maximum initial velocity is constant.

Using the above constants, the final version of ***Michaelis-Menten equation*** can be derived:

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

A significance of K_M is seen from this equation. When it is equal to concentration of substrate ($K_M = [S]$), than $v = \frac{1}{2} V_{max}$. This relation is actually the definition of K_M:

The Michaelis constant is the substrate concentration at which the initial reaction velocity is equal to half maximal. Both, Km and [S] are expressed in the same units, moles per litre - ***mol/L***.

Also, when [S] is significantly higher than K_M, K_M can be dropped to yield:

$$v = V_{max} = k_3 [E]_0$$

On the other hand, if [S] is negligibly low compared to K_M, then:

$$v = \frac{V_{max}[S]}{K_M}$$

These relations should be followed in any laboratory reaction. In designing any assay to measure the amount of an enzyme in blood or other material, it is important to ensure that sufficient substrate is present to saturate the enzyme completely, that is, to convert it entirely to the enzyme-substrate complex (the reaction rate in this case depends only on the concentration of the enzyme, it does not depend on the concentration of the substrate). On the other hand, when substrate is measured in enzymatic reaction, the relative excess of enzyme is necessary to have the reaction rate to be a function of substrate concentration.

The significance of K_M in metabolism centres its operational definition as the concentration of substrate at which the initial velocity is half its maximum. From this, some important points can be made:

1. The concentration of substrate in vivo will play a role in the rate of the conversion to product only if its concentration approximates the K_M.
2. If an enzyme has two or more substrates that can be converted to their respective products, each substrate having its K_M and V_{max}, the rates of conversion of each substrate can be calculated from the Michaelis-Menten equation. This calculation requires that the *in vivo* concentration of each substrate is known. If the *in vivo* concentration is much less than the K_M, that substrate will not be significantly converted to product. An example is the alcohol dehydrogenase which “prefers” ethanol to other alcohols.

3. If a substrate can be converted to a product by either two enzymes, the enzyme with the lower K_M will convert the majority of the substrate to its specific product. The physiological importance of the reaction can be predicted, using K_M, V_{max} and the *in vivo* concentration of the substrate.

Inhibition and regulation of enzyme activity

Enzymes can be inhibited by specific molecules or ions. In irreversible inhibition, the inhibitor is bound covalently to the enzyme or is otherwise attached so tightly that dissociation of the inhibitor from the enzyme is very low. Reversible inhibition, on the other hand, is characterized by a true equilibrium between the free enzyme and the inhibitor and the enzyme-inhibitor complex. Competitive inhibitors prevent the substrate from binding to the active site. They decrease the reaction rate by reducing the number of enzyme molecules that bind the substrate. Non-competitive inhibitors, on the other hand, reduce the turnover number. Distinguishing between the two types of inhibition is possible by determining whether the inhibition can be suppressed by increased substrate concentration, which is typical for competitive inhibition.

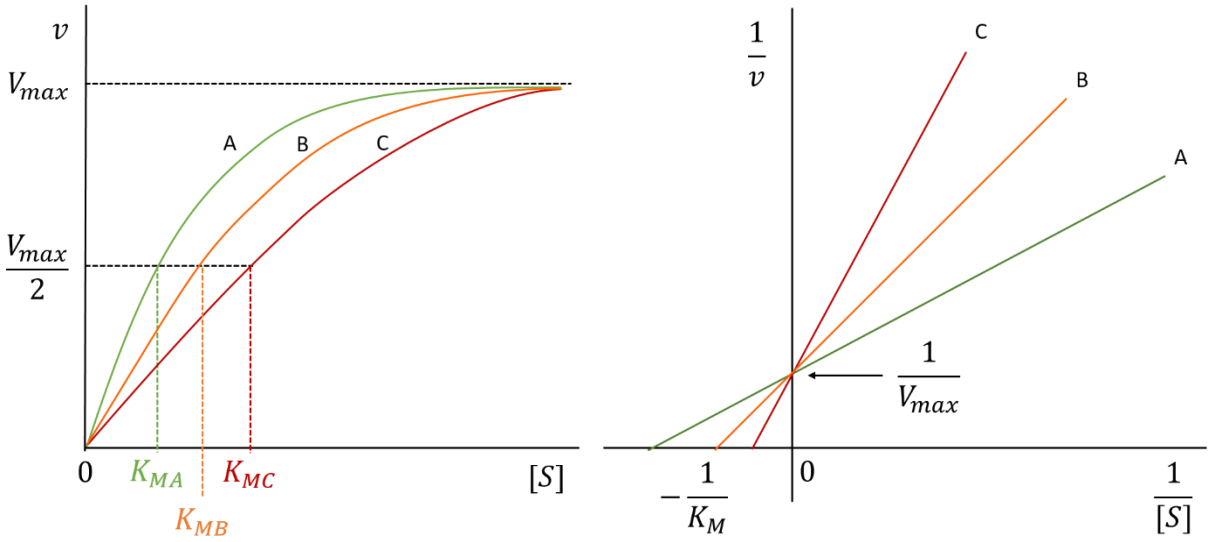
The activity of many enzymes is tightly regulated *in vivo*. In this regard, so-called allosteric interactions, which are interactions between spatially different sites of enzymes, are important. In enzyme regulation, we very often encounter a phenomenon in which the end product of a biosynthetic pathway inhibits the enzyme catalysing the reaction near the beginning of this pathway (= ***feedback inhibition***). Enzymes are also often controlled by regulatory proteins of the calmodulin type, whose conformation reflects the level of calcium ions. Covalent modification through phosphorylation of serine, tyrosine or threonine residues in the enzyme structure is also an important tool for enzyme regulation. The most powerful regulatory mechanism is probably associated with the formation of inactive precursors, which are transformed into active enzymes by proteolytic cleavage - the so-called ***proteolytic activation***.

The principal of enzyme catalysis is usually the selective stabilization of an activated intermediate, which is bound by the enzyme more tightly than the original substrate. That is why structural analogues of activated intermediates are the most effective enzyme inhibitors. This also applies to immunogens, since the interaction of antigen with antibody strongly resembles an enzyme-substrate reaction, and the best producers of antibodies are immunogens (antigens) that mimic an activated intermediate.

Lineweaver-Burk plots can be used to assess the nature of enzyme inhibition. If catalysis is to occur, a certain structural correlation must exist between the substrate on the one hand and the active site of the enzyme and the surroundings on the other. Anything that alters or interferes with this “fit” will inhibit or prevent catalysis. Metabolites, drugs, or toxic substances may inhibit enzymes so that normal catalysed reaction occurs at a lower rate, if at all. The inhibitors may be classified according to how they react with the enzyme:

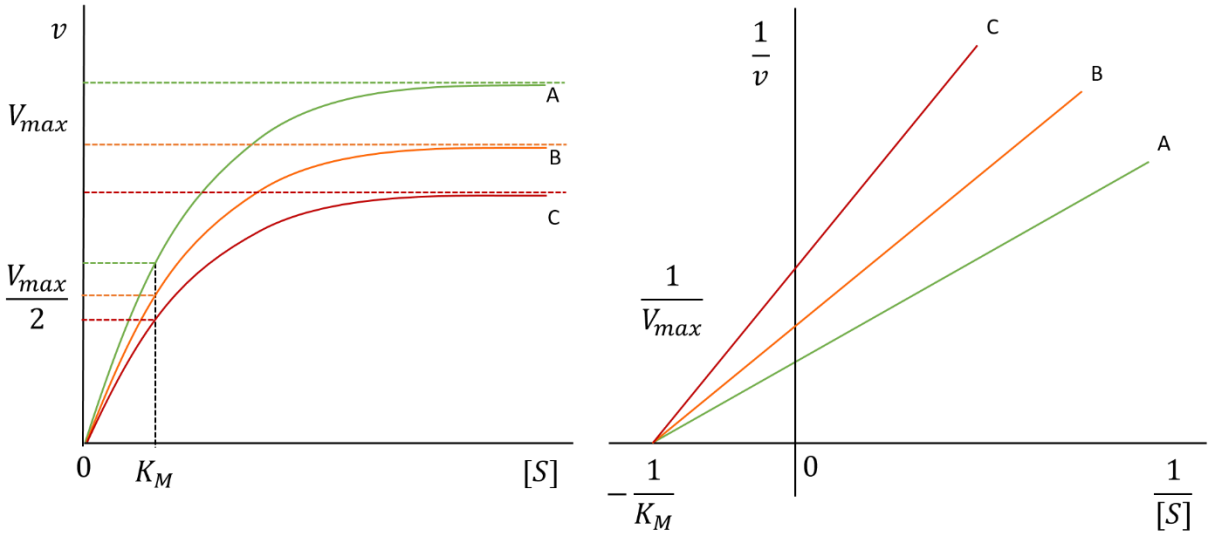
Competitive inhibitors bind reversibly with the enzyme in competitions with the substrate. When the inhibitor is bound to the enzyme, the normal substrate cannot form the ES active complex, and thus less enzyme is available for catalysis. Since the competition for the enzyme is proportionate to the concentrations of the substrate and the inhibitor, a sufficient concentration of the substrate will overwhelm the inhibition, and the V_{max} will be the same as with no inhibitor present. At concentrations in which substrate and inhibitor are more comparable, the K_M for the substrate will be increased.

Competitive inhibition depicted by Lineweaver-Burk plots. A, Normal uninhibited reaction. B and C, Two different inhibitor concentrations (B < C).



Noncompetitive inhibitors bind either to the enzyme or the enzyme-substrate complex. In this case the V_{max} is decreased without the change in the K_M for the substrate. Even an extremely high substrate concentration cannot completely eliminate the inhibitory effect.

Noncompetitive inhibition depicted by Lineweaver-Burk plots. A, Normal uninhibited reaction. B and C, Two different inhibitor concentrations (B < C).

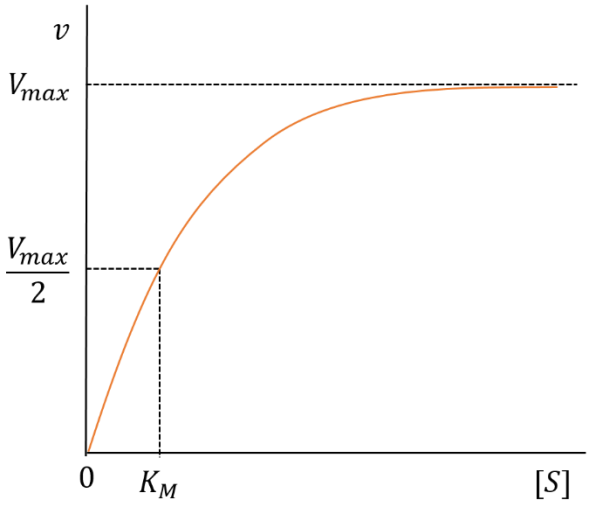


Some enzymes do not follow the kinetics of the Michaelis-Menten model. A significant group of such enzymes are subject to control by molecules that bind to sites on the enzyme other than the catalytic site. Such molecules, called ***effectors***, influence the binding of the substrate to the catalytic site. These enzymes are known as ***allosteric enzymes***. Some allosteric enzymes are composed of subunits of identical or closely related peptide chains. The quaternary conformation is modified by the appropriate allosteric effectors. One or more of functional sites on these enzymes may be catalytic, whereas one or more other sites may be regulatory and not identical with the catalytic or active sites. In some instances regulatory and catalytic sites are on different subunits; in other instances regulatory and catalytic sites are locates on the same subunit. When the reaction velocity of an allosteric enzyme is plotted as a function of substrate concentration, a sigmoid rather than hyperbolic curve is obtained. One can see that the shapes of the allosteric curves are changed considerably by altering the concentration of either positive or negative effectors. In effect, decreasing the amount of negative effector or increasing the amount of positive effector produces a response equivalent lo lowering the K_M of the substrate. In the most general case allosteric kinetics can be represented by the following equation:

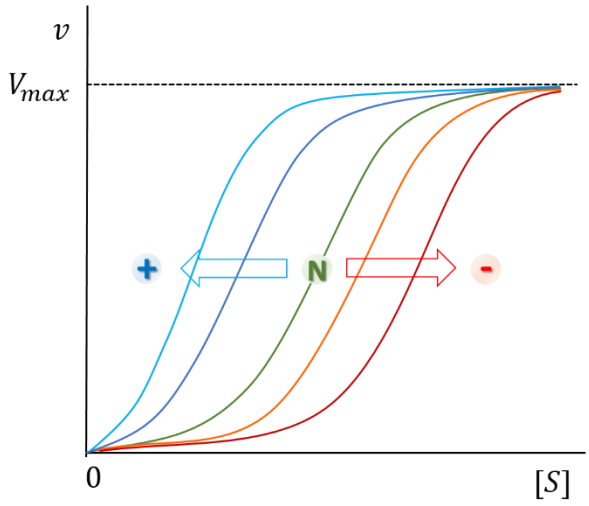
$$v = \frac{V_{max}[S]^n}{K + [S]^n}$$

where n is a coefficient that represents the interaction of the binding site, K represents a measure of the affinity of substrate for enzyme (other symbols have their previously stated meaning).

Allosteric enzymes composed of multiple subunits often show a ***cooperative effect***. The binding of the first substrate molecule to the enzyme will affect the conformation of the other active sites and change the affinity of the enzyme for other substrate molecules. Therefore, in a certain range of substrate concentrations, a minimal change in substrate concentration will cause a significant change in the reaction rate, much larger compared to a classical enzyme. This effect guarantees the maintenance of the substrate level within a certain range.



Michaelis-Menten kinetics



Allosteric kinetics

Clinical applications of enzymes

Measurement of enzyme activity is a useful monitor of overt disease, of genetic tendencies toward a disease state, and of a patient´s response to a particular type of therapy.

A growing number of purified enzymes are becoming commercially available. These can be employed as reagents for accurate determination of small amounts of such blood constituents as glucose, urea, uric acid, cholesterol and triacylglycerols. Frequently these methods are more specific and faster than the chemical determinations used previously.

Enzymes may be employed as diagnostic aids in yet another way, which takes advantage of their turnover numbers. Different antibodies are often used to determine specifically many antigens as molecules of interest. Before use, the generated antibody is modified by coupling it to some indicating enzyme with a fairly high turnover number, such as horseradish peroxidase or alkaline phosphatase. If sample containing specific antigen is added to the modified antibody, the antibody reacts with the antigen to form complex antigen –antibody. The resulting complex is washed to get rid of the excess of unbound antibodies. Then a suitable substrate is added to the antigen-antibody complex, which is converted into a coloured product by the effect of the enzyme present. The intensity of the colour produced is directly proportional to the amount of antigen present. Recently, a large number of different commercial kits are available for similar determinations, known as EIA (enzyme-immunoassay) or ELISA (Enzyme Linked Immuno Sorbent Assay).

Our knowledge of enzyme reactions and the sources of enzymes in tissue allows us to detect changes in body function and assess the presence or absence of specific tissue damage. Knowing where certain enzymes are concentrated in the body and how their levels in body fluids change during the course of disease are powerful diagnostic tool which reveal important information about our health. Most enzymes detected in body fluids are not formed there, but rather released from cells. The amount of given enzyme within a cell varies from one moment to the next as the metabolic demands on the cell change. Some enzymes are formed specifically for release into the circulation, since this is where they carry out their metabolic functions. The various coagulation factors are enzymes and can be found in high levels in circulating blood. Enzymes responsible for the metabolism of proteins within the circulation are found in higher levels in blood than in most tissues.

On the other hand, the levels of activity of most enzymes in blood or other body fluids are quite low. The body fluid concentration of a given enzyme reflects a variety of processes. One major factor is the amount of ***cellular turnover*** – how rapidly the old cells are dying and breaking down. As a cell reaches the end of its life span, it disintegrates and releases its contents into the surrounding tissues. The enzymes within the cell escape, and a certain amount of this material enters the bloodstream, urine, CSF. Since most cells turn over fairly slowly, only low levels of the enzyme are observed in the body fluid under normal circumstances. Once the enzyme enters the body fluid, further changes in concentration take place. In blood (and, to a lesser extent, other fluids) there are a number of proteases, that attack the various enzymes and metabolize them at different rates. The circulating levels of some enzymes are also affected through excretion by the kidney. Proteins with a molecular weight of less than approximately 60,000 are filtered by kidney and excreted in the urine. One such enzyme is pancreatic amylase

which is readily excreted by way of the kidneys and its level in urine is higher than that in blood.

The routine turnover of cells and the accompanying steady level of an enzyme in a body fluid is altered in a striking fashion in many disease states. Instead of a fairly constant concentration of the enzyme, the level may rise markedly over a short time and then subside fairly rapidly to the normal amount for that enzyme. These drastic alterations of the normal enzyme level provide valuable diagnostic clues as to the underlying pathological state in the body. In many disease states, one of the consequences is the rapid destruction of the tissue. Let us the case of ***myocardial infarction*** as an example. As a result of loss of oxygen, changes in heartbeat, decreased blood flow, or other factors, heart tissue begins to break down very quickly. The rate of cell breakdown rises, and greatly increased quantities of certain enzymes and released into the circulation. Later, when the cell turnover returns to normal, metabolism (and excretion) of the excess enzyme occurred, and the usual level of enzyme in circulation is restored.

Each enzyme has some tissue specificity. By knowing the tissue specificity of any enzyme, we can better ascertain where the damage occurred in the body. Enzymes of high specificity (amylase, acid phosphatase) are found predominantly in one type of tissue. Those enzymes which have moderate specificity are more widely distributed in the body. Some enzymes (alkaline phosphatase and lactate dehydrogenase) are ubiquitous – they are found everywhere. Even with this very wide distribution, these enzymes can provide valuable diagnostic information if we study the proper parameters. In case of lactate dehydrogenase the estimation of isoenzymes will increase the tissue specificity. Another enzyme, where the isoenzymes are a useful tool, is creatine kinase.

Molecular biology

Leiden mutation detection

This laboratory exercise uses elements of the problem-based learning (PBL) approach.

problem: "Leiden mutation" - from all points of view (molecular basis, hemostasis physiology, pathophysiology, clinical significance and diagnostics)

Leiden mutation is an autosomal intermediate (incomplete dominance) hereditary single-point mutation in the hemocoagulation factor V gene. It is the most common genetic cause of thrombophilia (increased blood clotting) in the European population. This mutation was discovered in 1993 (published in 1994) and named after the site of discovery - the city of Leiden in the Netherlands.

Molecular basis

At the molecular level, it is a *Single Nucleotide Polymorphism* (SNP) referred to as rs6025 in the databases. It is a G1691A substitution, *i.e.* the replacement of G (guanine) by A (adenine) at position 1691 in the coding region of the Factor V gene. The Factor V gene is located on the long arm of the first chromosome (locus q23). . The gene contains a total of 25 exons, with the Leiden mutation (also referred to as Factor V Leiden) located at exon number 10.

ATATTAATTGGTTCCAGCGAAAGCTTATTTATTTATTTATTATCATGAAATAACTTTGCA

AATGAAAACAATTTTGAATATATTTTCTTTCAGGCAGGAACAACACCATGATCAGAGCAG

TTCAACCAGGGGAAACCTATACTTATAAGTGGAACATCTTAGAGTTTGATGAACCCACAG

AAAATGATGCCCAGTGCTTAACAAGACCATACTACAGTGACGTGGACATCATGAGAGACA

TCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCGAG

GAATACAGGTATTTTGTCCTTGAAGTAACCTTTCAGAAATTCTGAGAATTTCTTCTGGCT

Part of the nucleotide sequence of the Factor V gene. The exon 10 is highlighted.

As a consequence of this mutation / substitution / polymorphism / change in the gene, the primary structure of the produced hemocoagulation factor V protein (a cofactor, without proteolytic function) is altered. At position 506, the amino acid arginine is replaced by glutamine. According to the change in genetic information, this is a non-synonymous missense SNP. That is, the base-exchange codon encodes another amino acid, and thus the function of the protein may be altered.

FV wild type

CTG GAC AGG C**G**A GGA ATA CAG AGG GCA

Leu-Asp-Arg-**Arg**-Gly-Ile-Gln-Arg-Ala

FV Leiden

CTG GAC AGG C**A**A GGA ATA CAG AGG GCA

Leu-Asp-Arg-**Gln**-Gly-Ile-Gln-Arg-Ala

Arg 506 Gln G 1691 A

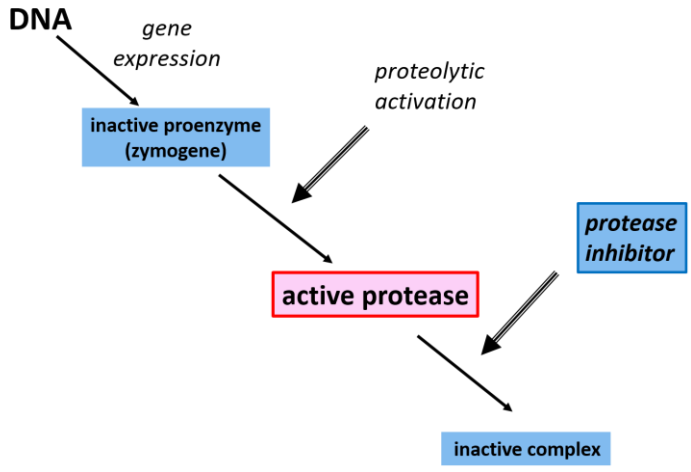
Part of the nucleotide and amino acid sequence of the wild type and mutated gene. The nucleotide and amino acid substitution are highlighted.

Hemostasis

Hemostasis is a vital process protecting the body against excessive blood loss in violation of vascular integrity (violation of bloodstream continuity). The following mechanisms are involved in the haemostasis: vascular reactions (vasoconstriction), platelet activity (activation and accumulation at the site of injury) and blood clotting, called hemocoagulation. These processes lead to the formation of a blood clot (thrombus).

Distinguish thrombus and coagulum: thrombus is a blood clot intravitally and intravasally, whereas a coagulum is a blood clot formed extravasally or postmortem.

Hemocoagulation itself is a cascade of enzyme reactions leading to the conversion of fibrinogen to insoluble fibrin. This process requires the interplay of many coagulation factors, phospholipids and calcium ions. Coagulation factors are referred to by name and Roman numeral, which is of historical character only, *i.e.* it does not indicate a sequence of reactions. Coagulation factors usually have the character of proteolytic enzymes (FII, VII, IX, X, XI, XII, precalikrein), according to the mechanism of action they belong to serine proteases. They are synthesized in the liver in the form of inactive proenzymes (zymogens), which are sequentially activated. Activation of individual proenzymes to active enzymes consists in their proteolytic cleavage by the enzyme activated in the previous reaction.



Simplified scheme of expression, activation and inhibition of proteolytic enzyme

There are also additional factors that accelerate the activation of zymogens (FIII, V, VIII…). Proteolysis is not enough for the successful involvement of coagulation factors II, VII, IX, X and anticoagulant factors - proteins C and S. They undergo posttranslational treatment first (carboxylation of 10-12 glutamic acid residues on γ carbon), which unconditionally requires vitamin K. The purpose of this posttranslational treatment is to increase the ability to react with Ca^{2+} ions and to bind the coagulation factor to the phospholipid membrane. In the absence of carboxylation, the affinity of the factor for the membrane is low, resulting in a clotting disorder.

Coagulation events are divided into two systems: internal and external / external, but which are not independent of each other and together aim to activate factor X. In the internal system (all procoagulation factors are in the blood) factor XII (the so-called Hageman factor) is activated by contact with a negatively charged surface (collagen, platelet phospholipids) of the damaged vessel. Factors IX (the so-called Christmas factor), VIII (the so-called anti-haemophilic factor) and X (the so-called Stuart-Prower factor) are gradually activated with calcium ions. The external system begins with the release of factor III - tissue thromboplastin. Factor III, along with released membrane phospholipids from cells, factor VII (called proconvertin) and calcium ions, leads to activation of factor X. A common pathway then continues for both systems. Activated factor Xa, calcium ions, platelet phospholipids, activated factor Va (so-called proaccelerin) and inactive prothrombin (factor II) form a prothrombinase complex. Prothrombin to thrombin may then be activated. Thrombin is a serine protease that cleaves two fibrinopeptides from fibrinogen. This allows the spontaneous polymerization of fibrin monomers by non-covalent bonds. The fibrin stabilizing factor (Factor XIIIa), which stabilizes the newly formed fibrin polymer, is then affected. Insoluble fibrin together with the platelets forms a clot, a plug closing the wound.

Haemocoagulation is a multistep regulated process because spontaneous blood coagulation in the circulation can have fatal consequences. Therefore, several coagulation factor inhibitors circulate in the blood along with coagulation factors. These inhibitors are among the serpins (serine protease inhibitors). These include mainly antithrombin, protein C (PC), protein S, and endothelium-bound thrombomodulin.

However, haemocoagulation also occurs in the undisturbed venous system if there is a stasis in the blood. This is a pathological process leading to thromboembolism (explained in more detail in the Clinical Significance chapter). This is also due to a malfunction or lack of natural coagulation inhibitors, often as a result of a genetic disorder (e.g. FV Leiden).

Another, more distant mechanism following bleeding arrest is **fibrinolysis** (removal of thrombus) and activation of fibroblasts and smooth muscle cells. The result is healing of the injured tissue.

Primary fibrinolysis is a natural physiological process whose goal is to remove the unnecessary thrombus from the healed vessel. Fibrin (to a lesser extent fibrinogen) is cleaved by plasmin into soluble fibrin grafts. Plasmin (formerly called fibrinolysin) is an active form of plasminogen protein. It is a serine protease. Activation of plasminogen to plasmin is caused by the active enzyme, tissue plasminogen activator (t-pA). On the other hand, rapid degradation of plasmin is caused by α2-antiplasmin, a plasma protein that forms an irreversible inactive complex with it.

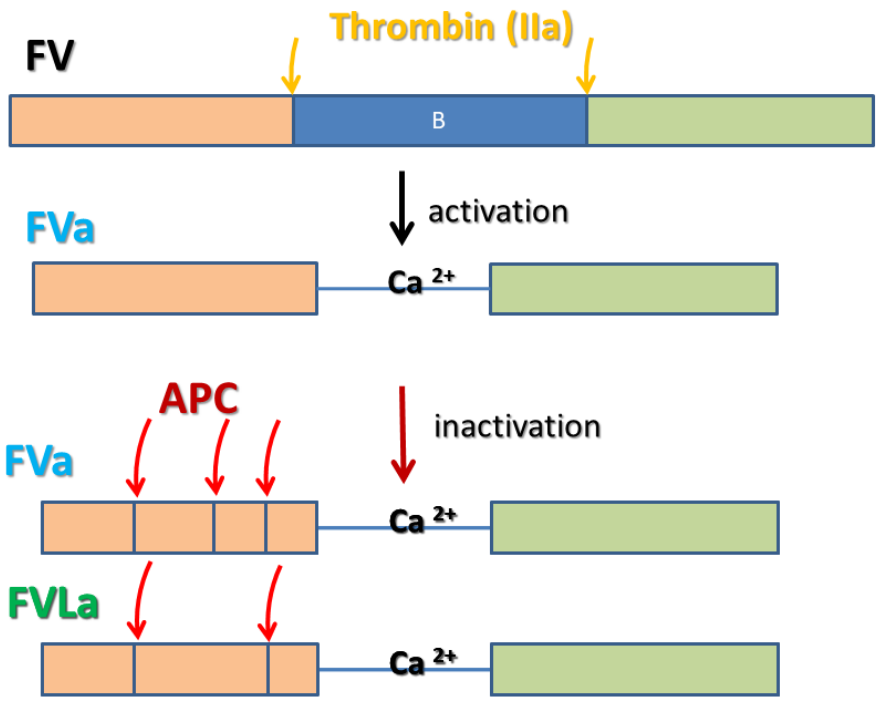
The breakdown of fibrin releases products of this degradation into the blood. These are two linked D fragments of the fibrin protein, called **D-dimers**. Their determination has found application in clinical practice in the diagnosis of thromboses and embolisms. Due to the fact that both the formation of a clot and its degradation are physiological events, we cannot simply associate elevated D-dimer levels with a disease. However, if the D-dimers are negative, we can exclude the presence of any clot in the bloodstream (thus, the diagnosis of thrombosis or embolism can be excluded). It is a relatively inexpensive, fast and, above all, patient-free test, and is therefore used in clinical practice to categorize patients suspected of having, for example, pulmonary embolism. Using the test, the physician can divide the patients into healthy patients, no longer needing further investigation, and those who need to be further proven or eliminated by other methods.

Secondary fibrinolysis (more commonly referred to as thrombolysis) is a relatively dangerous but often life-saving medical procedure, used today mainly in massive pulmonary embolism. Fibrinolytic activity preparations (alteplase, streptokinase, etc.) are administered to the patient's bloodstream in order to dissolve the clot closure of the artery and restore blood circulation thereafter. The major risk may be severe bleeding, making it impossible to resolve the situation that required thrombolysis.

The flawless formation of a coagulum limited in a particular place and time is conditioned by the correct interaction of many specific substances. Some work to strengthen and accelerate blood coagulation, while others are designed to inhibit the blood coagulation mechanism. The task of this complex system is to ensure a dynamic balance that not only stops bleeding in the event of injury, but also prevents uncontrolled blood clotting that ultimately closes the vascular bed.

Pathophysiology of Leiden mutation

Factor V (FV) is a glycoprotein, a coagulation factor, also called proaccelerin or labile factor. Unlike most coagulation factors, it does not have enzymatic activity, it acts as a cofactor. FV is activated by thrombin on FVa. Upon activation, it cleaves into two chains, which are linked by calcium ions, see in the next figure. FVa binds to specific receptors on the platelet membrane and together with factor Xa and prothrombin forms a prothrombinase complex. The prothrombin is then cleaved by activated factor Xa to thrombin. Thus, the role of FVa is on the side of procoagulant factors. It might appear that the mutation of factor V, which has procoagulant effects, should result in a decrease in the efficiency of the coagulation system. Surprisingly, the substitution of 1 amino acid in the structure of the FV Leiden protein compared to the FV wild type has no effect on its pro-coagulation activity. The thrombophilia associated with the Leiden mutation is conditioned by a change in its degradation.

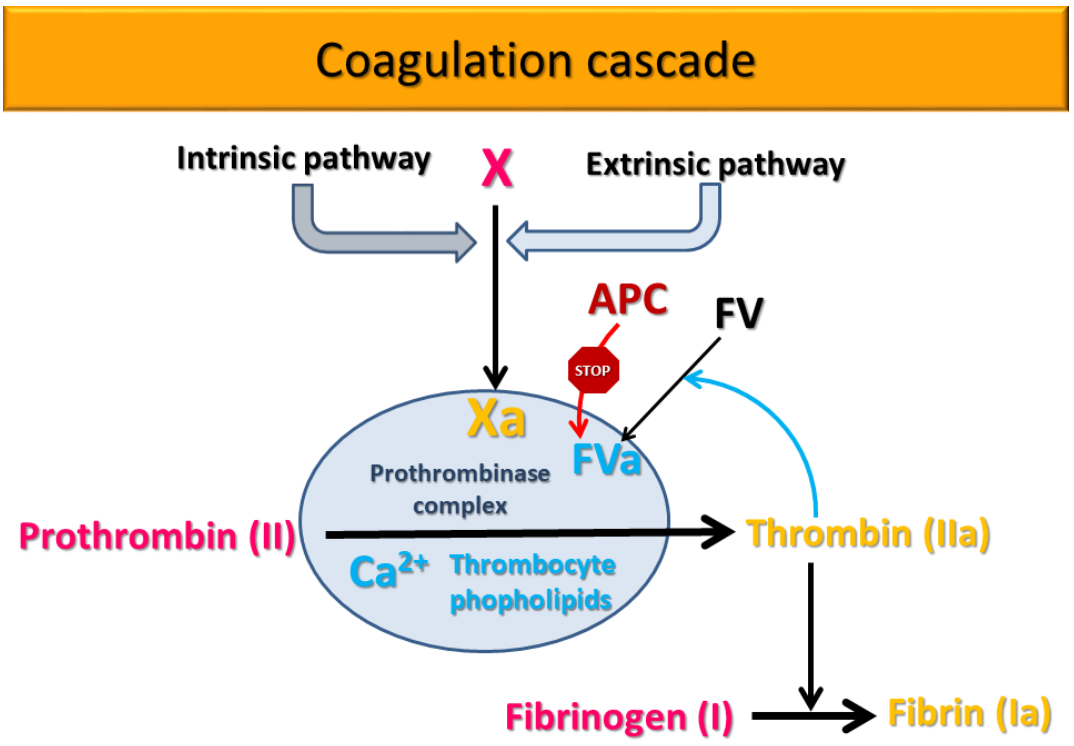


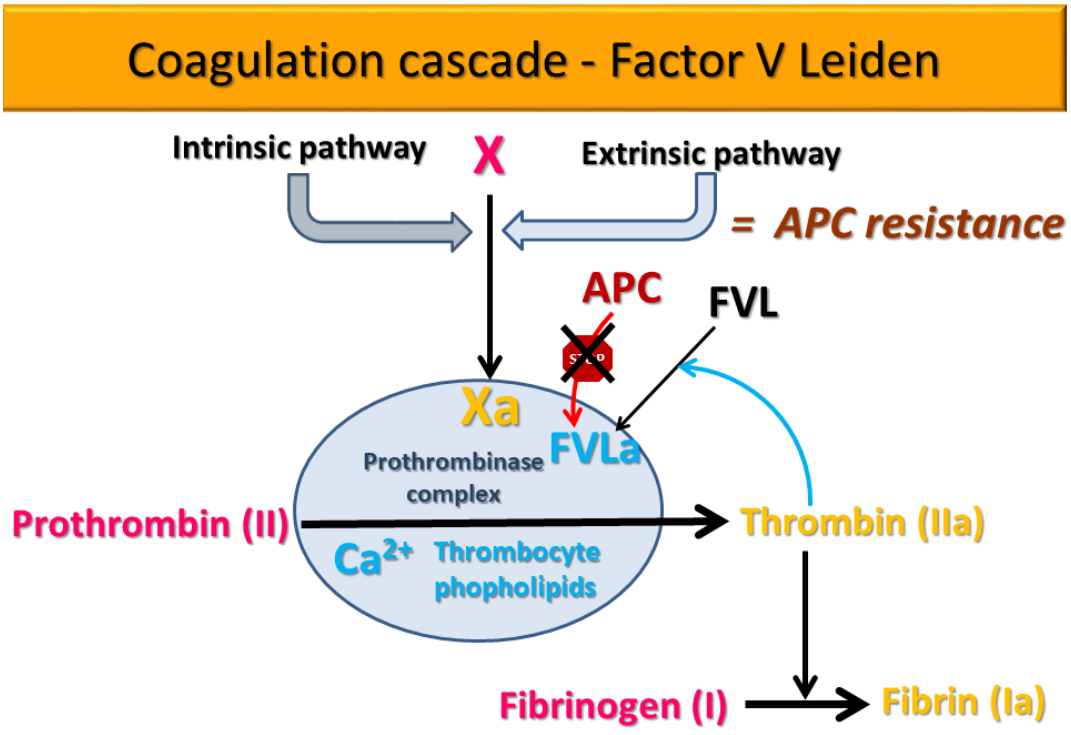
Factor V (FV)

Activated factor Va is inactivated by activated protein C (APC), which limits its action in coagulation. APC binds to FVa at the site of three different arginine residues and causes inactivation by proteolytic cleavage.

The Leiden mutation of factor V causes the amino acid substitution arginine for glutamine at the 506 position of FV, where there is one APC binding site. This is more difficult to bind to PV. The result is the persistence (extension of duration) of FVa activity. This thrombophilin state is the result of prolonged exposure to FVa.

The Leiden mutation is also sometimes described as APC resistance - resistance of activated factor Va to the anticoagulant activity of activated protein C (APC). APC is required for the inactivation of factor Va and VIIIa, and is one of the principal physiological inhibitors of coagulation. These inhibitors are among the serpins (serine protease inhibitors). These include mainly antithrombin, protein C (PC), protein S, and endothelium-bound thrombomodulin.



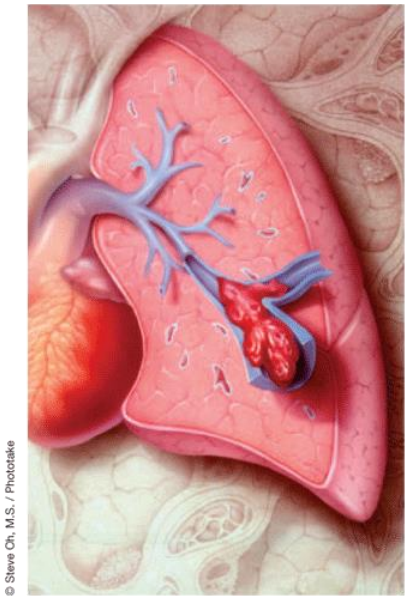


Simplified scheme of hemocoagulation cascade with emphasis on APC relationship

Clinical significance

The Leiden mutation shows an interesting dependence on race and geographical location. In the Czech Republic, homozygotes for factor V Leiden occur in 1 per 5,000 inhabitants, heterozygotes account for approximately 5% of the population. The highest occurrence of the FV Leiden mutation was detected in Sweden, while it is a very rare mutation in Asian or African populations. This is explained by the fact that the mutation originated in the Caucasian population about 20 to 34 thousand years ago. Factor V Leiden mutations (and other thrombophilic mutations) were retained in the population for a long time, so they had to be evolutionary advantageous. Thrombophilic mutations lead to quicker haemostasis, for example during combat, hunting or after delivery, which increases the likelihood of the wearer's survival. But what was once advantageous today is becoming a burden. Factor V homozygotes for Leiden are about 80 times higher risk of developing deep vein thrombosis. In heterozygotes, the risk is about 8 times higher without any other risk factor and 30 times higher when combined with combined hormonal contraception or hormone replacement.

Deep vein thrombosis is the formation of a blood clot (thrombus) in the deep venous system. The clot leads to obstruction, i.e. a restriction of the blood flow through the vein. Deep vein thrombosis mainly affects deep distal veins of the lower leg, except popliteal veins or femoral veins up to the iliac vein. Tearing the thrombus and passing it through the right atrium and ventricle into the pulmonary artery creates the most serious complication - **pulmonary embolism**. It is a life-threatening condition caused by obstruction of the a. Pulmonalis or its branches. Superficial vein thrombosis, which is usually accompanied by inflammation (thrombophlebitis), does not lead to pulmonary embolism.



Pulmonary embolism

Normally, there is a balance in the body between the formation and dissolution of thrombus. In the pathogenesis of thrombosis, risk factors known as the **Virchow Triad** are involved. These are: **1. changes in hemodynamics** - slowed blood flow called venostasis. This can be caused by long-term bed restraint, limb immobilization, long travel with limited movement. **2. damage to the vessel wall**, e.g. during surgery, injury, inflammation, arteriosclerosis. 3**. thrombophilic conditions**, e.g., mutations of coagulation factor genes (Leiden factor V mutation), or conditions acquired during life, for example by tumour growth. Even in patients with an increased risk of thrombus development, it is necessary to create a trigger such as infection, trauma, dehydration, smoking, hormonal changes, cancer.

Prevention and treatment monitoring

In thrombophilic conditions, there is a real risk of recurrence of deep vein thrombosis, in women there is a higher risk of obstetric complications leading to abortion or premature labour. Women with thrombophilia should also not use hormonal contraceptives, as estrogens increase the tendency for blood to clot even in healthy women. In the case of thrombophilic conditions, prevention by a specialist hematologist should be ensured, especially before planned surgical interventions, long-term flights, during pregnancy, the period around childbirth and the puerperium. In some cases prevention throughout life is necessary.

Prevention consists of administering anticoagulants, medicines that reduce blood clotting. There are risks to treatment with these agents: overdose can lead to severe bleeding, while underdosing is ineffective. Therefore, it is usually necessary to monitor the condition of the coagulation system by special tests during treatment.

For short-term prevention, heparin or more modern low-molecular-weight heparin (Fraxiparin, Clexan) has the best properties. Both have a rapid onset of action and their predictable (predictable course) properties allow for fixed dosing. A disadvantage is the need for administration by the parenteral route (parenteral = extracorporeal), e.g., Fraxiparin s.c. - subcutaneously. The efficacy of heparin therapy can be quantified using **activated partial thromboplastin time (aPTT)**. This examination reflects the state of the internal and common pathways of the coagulation system. Low-molecular-weight heparin therapy is usually not required to be controlled, but can be assessed by examining **anti Xa activity** if necessary.

The group of oral anticoagulants is represented by modern xabans or gatranes and, most importantly, still the most widely used, **warfarin**. Warfarin is a coumarin derivative, originally used as a rat poison, which has the ability to inhibit the *vitamin K reductase* enzyme. This enzyme restores a reduced form of vitamin K, which is necessary for the carboxylation of several coagulation factors. Insufficient supply of reduced vitamin K, liver cells are unable to synthesize vitamin K-dependent coagulation factors (II, VII, IX and X). The result is a lower amount of vitamin K-dependent factors circulating in the blood and thus less readiness to coagulate.

The effects of warfarin do not begin to appear until several days after initiation of therapy and are highly variable both interindividually and over time (for example, depending on the vitamin K content in the diet). Therefore, warfarin therapy should be continuously monitored by determining **prothrombin time (PT)**. Tissue factor (F III) along with sufficient Ca^{2+} ions is added to citrate-anticoagulated plasma and the time to coagulation is measured. The measured time in seconds well reflects the functionality of the coagulation system's external path. However, in order to compare values from different laboratories, it has proved more practical to express the results as an **International Normalized Ratio (INR)**. Basically, it is the ratio of patient's prothrombin time to control (healthy) prothrombin time.

The INR of a healthy patient is between 0.8 -1.2. The patient is effectively coagulated at values between 2.0 and 3.5.

$$INR = PT_{patient} / PT_{norm}$$

Methodical part

Isolation of nucleic acids

Nucleic acids can be isolated from any biological material that contains cells with conserved nuclei. A common source of DNA is blood coagulation leukocytes. Typically, 0.5-10 ml of venous blood is collected through a preferably sealed collection system into sterile K_2-EDTA tubes. In prenatal diagnosis, amniotic cells and chorionic villi are a common source. If it is necessary to obtain material for DNA isolation in a non-invasive manner, DNA is taken from the buccal mucosa. DNA can be obtained even from stains of dried blood. The source of RNA is most often tissue obtained by biopsy or non-clotting venous blood.

The quality of the starting material significantly affects the yield, quality and integrity of the isolated nucleic acid. The best results are obtained with fresh material. The specimen should be immediately processed or immediately frozen (in the case of DNA within three hours after collection) and further stored at -80 ° C to avoid DNA fragmentation into shorter fragments or RNA degradation (especially mRNA). The material must be kept in a suitable container free of the nucleases. This is especially important in the case of RNA which is far less resistant, moreover ribonucleases are ubiquitous and very resistant enzymes.

More recently, formalin-fixed and paraffin embedded (FFPE) tissue samples originally obtained for histological examination may be used to isolate both nucleic acids. Although these samples do not provide ideal results (nucleic acid fragmentation), they are very valuable because they are often the only source of biological material, especially in retrospective studies in deceased patients.

Samples for determining gene expression must be handled with particular care so that the measured values correspond to the actual levels present *in vivo* and not to reflect changes that occurred during sample processing. Therefore, samples must be frozen immediately in liquid nitrogen and collected at -80 ° C immediately after collection. In cases where this is not possible, stabilizers are commercially available.

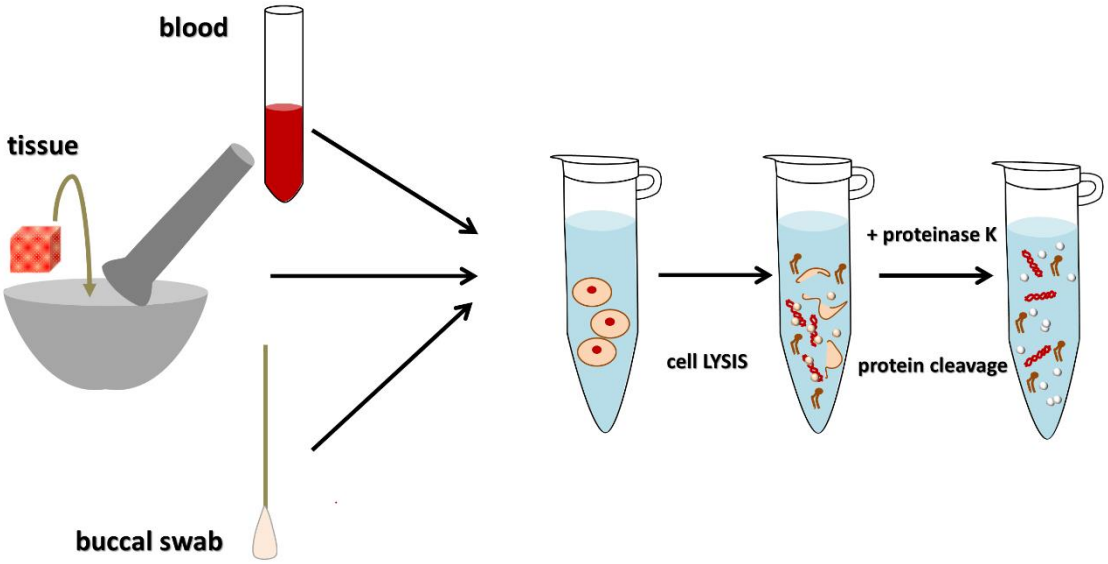
DNA was first isolated in 1869 by Friedrich Miescher, and is now a routine technique of molecular biology.

***Friedrich Miescher** (1844-1895)*

*was a Swiss physician who in 1869 isolated an unknown substance from the cell nucleus of leukocytes, which he called "**nuclein**". He concluded that it was a new substance because its characteristics did not correspond to known proteins or lipids at that time. Nuclein was protease resistant, did not contain sulphur, but contained a large amount of phosphorus. Miescher's name was retained in today's DNA name - deoxyribo**nucleic** acid.*

The isolation method depends both on the nature of the biological material from which the nucleic acid is to be derived and on the method of subsequent analysis of the obtained molecule. In all cases, the first step is lysis of the cells from which the nucleic acids are to be

obtained. In blood cells, a detergent is usually sufficient to disrupt biomembranes. Mechanical disruption must be used to disrupt solid tissues, such as crushing tissue frozen with liquid nitrogen in a mortar, shaking with balls of different materials, special homogenizers. A chelating agent - ethylenediaminotetraacetic acid (EDTA) is also added to the lysis solution to form non-dissociable complexes with calcium ions. This prevents the cleavage of freshly released DNA nucleases (DNases), which are also released during cell lysis. Calcium ions serve as the cofactors necessary for their function. Proteinase K (a bacterial enzyme with a temperature optimum of about 60 °C, which does not require calcium or magnesium ions and which does not inhibit concentrated surfactants, is also added to ensure cleavage of proteins, including DNA-bound histones). To isolate RNA, guanidine thiocyanate and β-mercaptoethanol are added to the lysis solution as ribonuclease inhibitors located on organelle membranes.



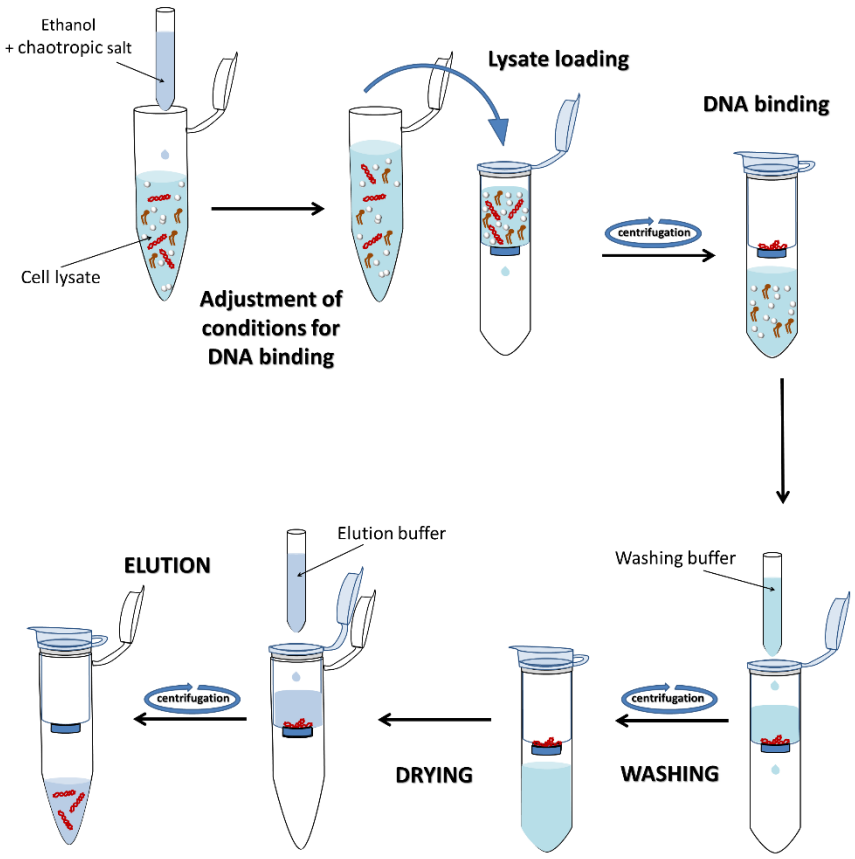
Preparation of cell lysate for DNA isolation

Ballast substances must be removed from the lysate obtained. The obtained pure nucleic acid is diluted to an optimal concentration for further use in a suitable solvent, most often in water or buffer.

Currently, two methods are most often used to remove ballast substances:

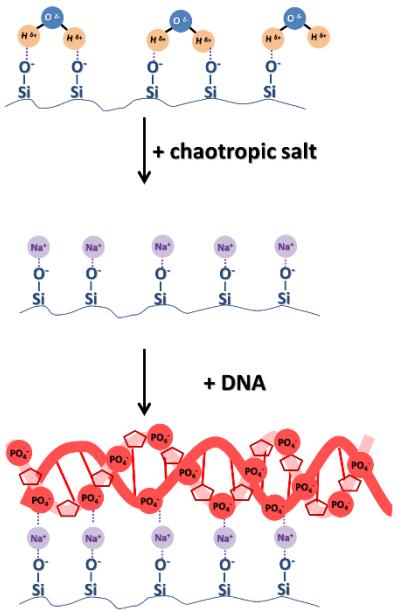
- **Column method**

The column method generally works on the principle of ion exchange chromatography. DNA and RNA molecules carry a negative charge. In the presence of a high concentration of so-called chaotropic salts, nucleic acids bind to the silicate while most contaminants flow through the column. Typically, ethanol or isopropanol is added to the lysate to improve nucleic acid binding to the silicate column. The column is then washed successively with various buffers to remove bound contaminants. Finally, the pure nucleic acid is washed with dilute buffer or distilled water.



Extraction of DNA from cell lysate by column method

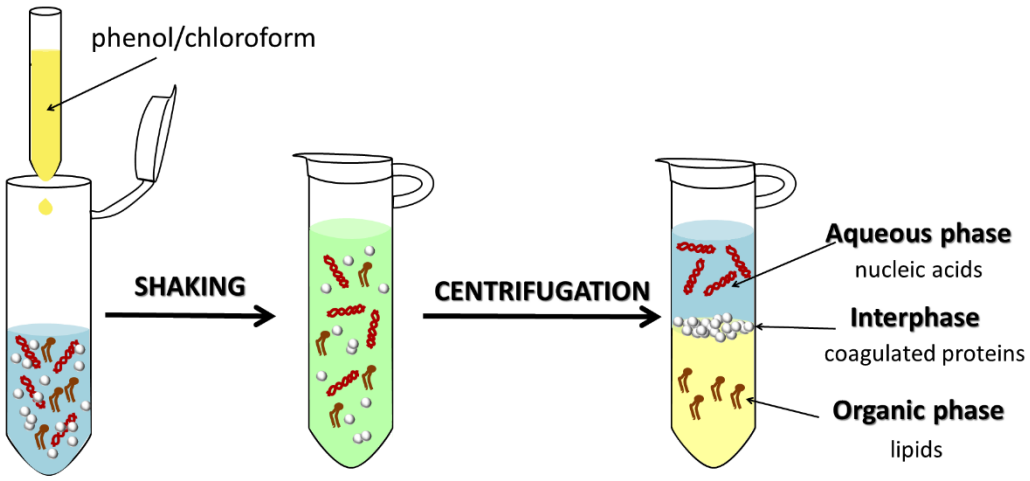
***Chaotropic salts** = ionic compounds, which disrupt the regular structure of hydrogen bonds in water in liquid form. Sodium iodide, guanidine hydrochloride or guanidine thiocyanate are most commonly used in nucleic acid isolation.*



Principle of DNA binding to silicate membrane in presence of chaotropic salts

- **Phenol-chloroform method**

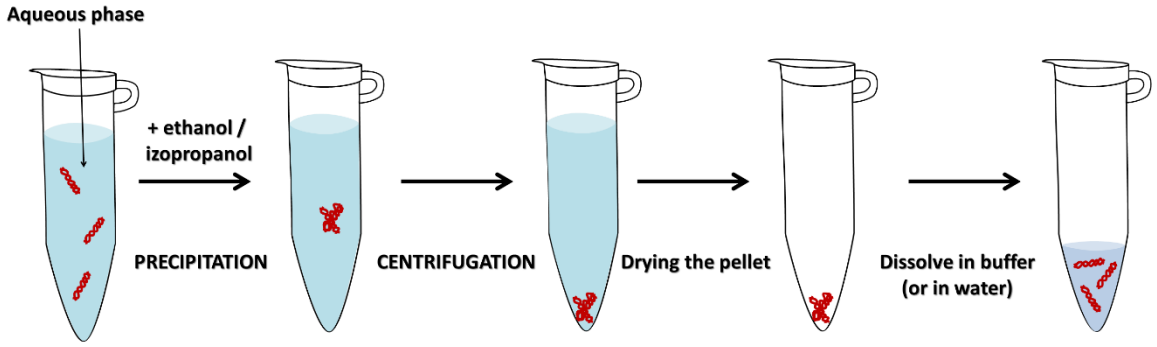
Traditional reliable, inexpensive method that provides very pure nucleic acids. Its disadvantages are laboriousness and lengthy procedure (the entire isolation procedure takes about 3 days), work with toxic, corrosive, flammable and odorous substances. A water-immiscible mixture of phenol and chloroform is added to the lysate to denature the proteins present. By denaturation, proteins become less soluble in water and pass into the organic phase or remain at the interface of the phases, while nucleic acids remain in the aqueous phase. Lipids dissolve in chloroform. Isoamyl alcohol is sometimes added to prevent foaming, especially in protein-rich samples. The resulting mixture was shaken vigorously and then centrifuged to separate the upper aqueous and lower phenol-chloroform phases. At the interface of these phases, an interphase consisting of a white ring of precipitated proteins usually arises. The aqueous phase containing the nucleic acids is carefully transferred to a new clean tube. For perfect protein removal, it is usually necessary to repeat the extraction several times (no white precipitate of proteins will appear at the interface). Even traces of phenol can influence subsequent analysis of isolated nucleic acid, eg phenol inhibits PCR. Therefore, for the last extraction, chloroform alone or a mixture of chloroform with isoamyl alcohol is used to facilitate the removal of phenol from the aqueous phase by increasing its solubility in chloroform.



DNA extraction from cell lysate by phenol-chloroform method

It is necessary to precipitate the nucleic acid from the pure aqueous phase, most often with absolute ethanol or isopropanol. Increasing the precipitation efficiency can be achieved by lowering the temperature and adding salts (e.g. sodium acetate, sodium chloride, lithium chloride or ammonium acetate). Salts facilitate precipitation by removing the hydration coating from the nucleic acid, neutralizing the charge on the sugar-phosphate backbone and thereby reducing its solubility in water. Ethanol is a less polar solvent than water. Thus, the nucleic acid is even less soluble in ethanol and falls out of solution. The presence of salts turns the pellet white.

After removal of the supernatant and washing with 70% ethanol (dissolves the salts present), the obtained pure nucleic acid is dissolved in a suitable solvent, most often in water or buffer.



Precipitation of DNA from the aqueous phase obtained by the phenol-chloroform method

Depending on which nucleic acid we need to obtain, we choose the extraction buffer. The pH of the extraction buffer used greatly influences whether DNA or RNA will predominate in the aqueous phase. In a neutral or slightly alkaline environment (pH ≈ 7-8), DNA predominates. The acidic environment, on the other hand, is more suitable for RNA isolation. The reason is the different charge that these molecules carry in an acidic environment. The sugar-phosphate backbone of nucleic acids carries a negative charge on its surface in a neutral environment. However, if the DNA molecule is in a low pH environment and thus a high concentration of H + ions, the phosphate groups are neutralized. This leads to the loss of the polar nature of DNA and its transition to the non-polar organic phase. On the other hand, single-stranded RNA has exposed nitrogenous bases that retain its polar character and keep it in the aqueous phase.

In any case, but we get the desired nucleic acid contaminated by the other. If it is necessary to work with pure DNA, the contaminating RNA is removed by RNase treatment and the DNA is purified again by phenol-chloroform extraction followed by ethanol precipitation. If we need to obtain pure RNA for further work, remove the DNA present by DNase treatment. RNA is reprecipitated with isopropanol and washed with ethanol.
As described above, total RNA is obtained. Sometimes it is preferable to isolate only mRNA because total RNA contains a high proportion of tRNA and rRNA. After lysis and homogenization of the sample and denaturation of total RNA, selective capture of poly (A) RNA = mRNA on immobilized oligo (dT) 20 affinity is performed, unwanted components are removed, bound mRNA is washed and then released from the affinant into solution.

Currently, there are a number of commercially available kits for isolating genomic DNA, plasmid DNA, total RNA, and individual RNA types (mRNA, rRNA, miRNA, snRNA, etc.) from various biological materials. Automatic nucleic acid isolators are even available for routine clinical laboratories. Because centrifugation is difficult for automatic lines, magnetic beads are used to separate the components. These beads have a silicate surface to which the nucleic acid specifically binds. During washing, the beads are held by a magnet. Finally, the pure nucleic acid is eluted from the surface of the separated beads.

Storage of isolated nucleic acid

DNA is a relatively stable molecule, yet it must be protected from nuclease degradation. Being a very long molecule, it is prone to fracture. Therefore, coarse pipetting (excessive suction and discharge of pipetted fluid) and excessive vortexing should be avoided during isolation and further handling of the DNA sample. The DNA is stored dissolved in a buffer such as TE (Tris / EDTA) buffer (10 mM Tris-HCl (tris-hydroxymethylaminomethane hydrochloride) and 1 mM EDTA (chelaton 3), pH 8.5) because it hydrolyses in water. The Tris component maintains a stable pH, EDTA chelates calcium and magnesium ions, thereby blocking the unwanted activity of DNases and to some extent RNases in the sample. The isolated DNA is stored for short periods (days) at 4 ° C and long term (weeks-months) at -20 °C or -80 ° C. Repeated freezing and thawing, which also damages DNA, should be avoided. RNA is usually stored in nuclease-free water (RNase-free water) at -20 °C or -80 °C.

Quantification and control of nucleic acid purity

Several methods can be used to determine the concentration of nucleic acids and to check their purity - spectrophotometry, electrophoretic evaluation or fluorescence DNA-binding dyes.

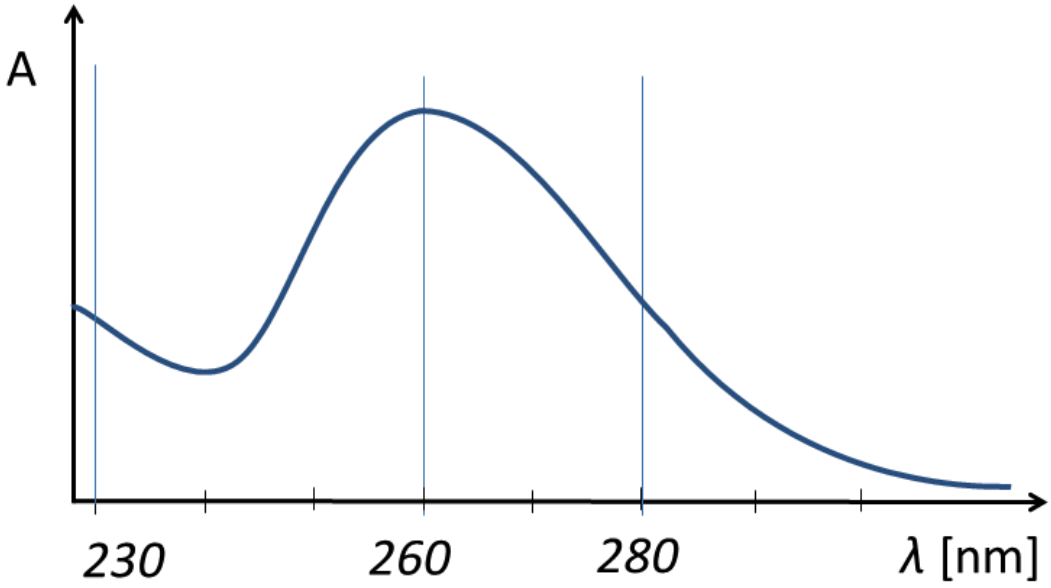
In practice we will use spectrophotometric determination. A pure nucleic acid solution (DNA and RNA) has an absorption maximum at 260 nm, proteins absorb at maximum 280 nm, at 230 nm the absorption maximum has low molecular weight substances (e.g. phenol, chloroform, EDTA, polysaccharides…). Absorbance at 320 nm means the presence of undissolved solid particles or a contaminated cuvette.

The nucleic acid concentration is calculated from the measured absorbance at 260 nm. It is based on the following relationships:

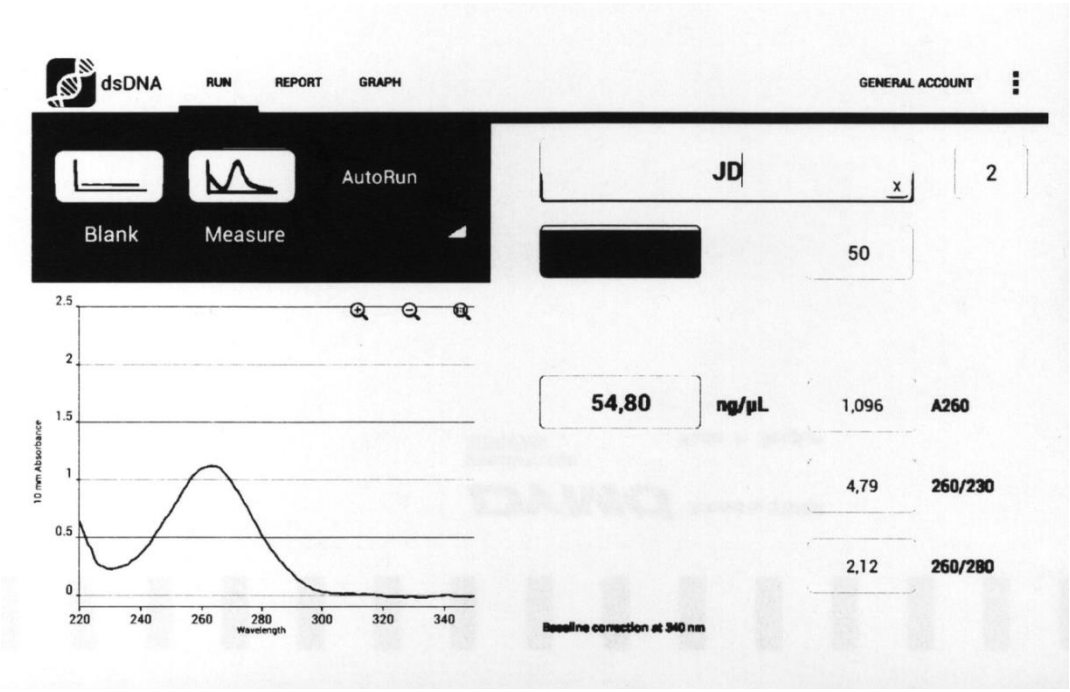
A_{260} = 1 if in the measured solution:

- double stranded DNA (dsDNA) at a concentration of 50 µg / mL
- single-stranded DNA at 37 µg / mL
- RNA of 40 µg / mL

The sample purity is evaluated according to the absorbance ratios A_{260} / A_{280} and A_{260} / A_{230}. The ratio A_{260} / A_{280} for pure DNA should be about 1.8, for RNA about 2. The ratio A_{260} / A_{230} for pure DNA should be higher than 2.0. If the ratio is significantly lower, the solution is contaminated with proteins or phenol. If the required purity of the samples is not met, it is necessary to reprecipitate the sample, which leads to a significant reduction in the impurity content.



DNA absorption spectrum



Sample recording from DeNovix DS-11

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is an enzymatic method allowing in vitro multiplication (amplification) of a selected segment of DNA flanked by short oligonucleotides, called primers. The method works on the principle of nucleic acid replication. It allows very quickly to obtain millions of exact copies from very small amounts of input material, even from DNA from a single cell, i.e. from a single DNA molecule. The reaction itself is based on cyclic temperature changes. Since this is a chaining of these cycles, we call this methodology a chain reaction.

Composition of the reaction mixture

In the polymerase chain reaction it is necessary to insert:

1. **DNA template** - dsDNA as template (template) for synthesis
2. **dNTP** = dATP, dGTP, dCTP, dTTP - a mixture of deoxynucleoside triphosphates representing the building blocks from which a new DNA strand is synthesized.
3. **primers** - two synthetic oligonucleotides that define the amplified region
4. **DNA polymerase** - an enzyme that performs DNA synthesis
5. Buffer = medium suitable for polymerase activity, the composition can influence the yield and specificity of the reaction.

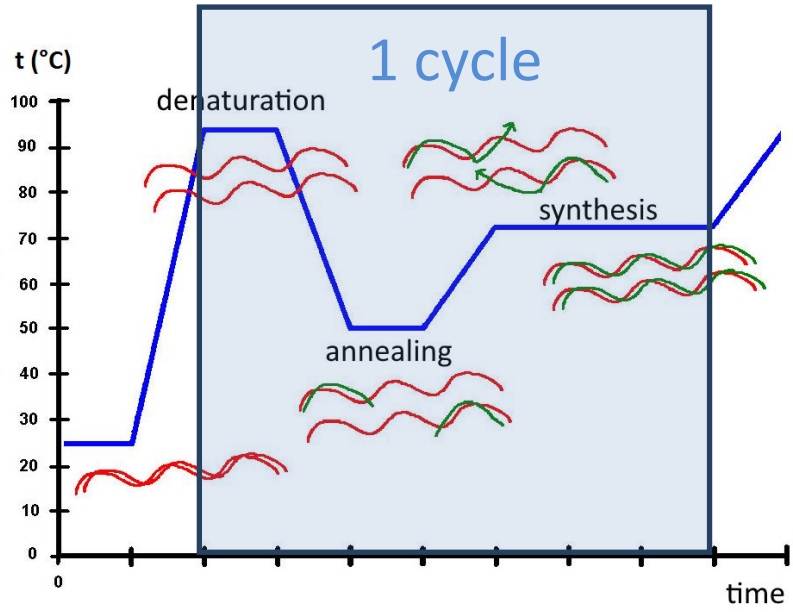
The total reaction volume is selected as needed, most commonly 15-100 μL.

Principle and course of reaction

The reaction, based on cyclic temperature alternation, consists of three periodically repeating steps:
1. Denaturation - by increasing the temperature of the reaction mixture to approx. 95 ° C breaks the hydrogen bridges between the bases in the double stranded DNA. Thus, two single-stranded DNA molecules are formed.

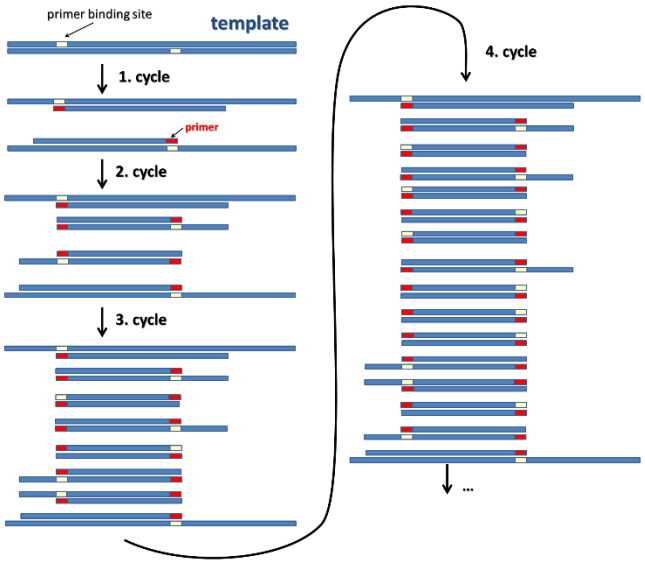
2. Annealing - cooling of the reaction mixture to a temperature at which primers can specifically bind to the complementary sequence of the template DNA strand. The temperature depends on the length and nucleotide composition of the primers. Most often this temperature is between 50 and 60 ° C.

3. Synthesis of new strands - in this step the DNA polymerase primers extend (elongation) - heating the mixture to the temperature optimum of DNA polymerase (usually 72 °C) will allow efficient synthesis of the desired length fragment at a given time. The duration of each cycle depends on the length of the replicated fragment and the type of polymerase.



Temperature variation during PCR

The cycle is repeated periodically 15 - 40 times, resulting in exponential multiplication of the selected fragment. How is it ensured that the currently selected fragment is created? A new strand of DNA is synthesized by a DNA polymerase that does not sit at the primer site and lengthens it. In the first cycle it could theoretically synthesize to the end of the pattern chain. Practically this does not occur, because earlier denaturation occurs in the next, ie the second cycle. In the 2nd cycle, double-stranded DNA is released into individual strands. The 4 strands thus formed are primed again and everything is repeated, except that if the template is a strand formed in cycle 1, the strand ends at the first primer, so that the new strand is of the appropriate length. These short specific products increase exponentially, while longer products (according to the original long template) multiply linearly.



PCR process

In order to avoid the need to add a new enzyme after each denaturation, a thermostable DNA polymerase is used which does not lose its activity even after heating to 95°C. Most commonly, DNA polymerase derived from the thermophilic bacterium *Thermus aquaticus*, called Taq polymerase, is used. In order to prevent polymerase from working at low temperature, which may lead to non-specific amplification, a so-called hot-start polymerase is used. These enzymes typically utilize monoclonal antibodies that bind to the catalytic site of the enzyme, thereby reversibly blocking its activity. The first denaturation cycle results in irreversible temperature denaturation of the antibody, thereby unblocking enzymatic activity.

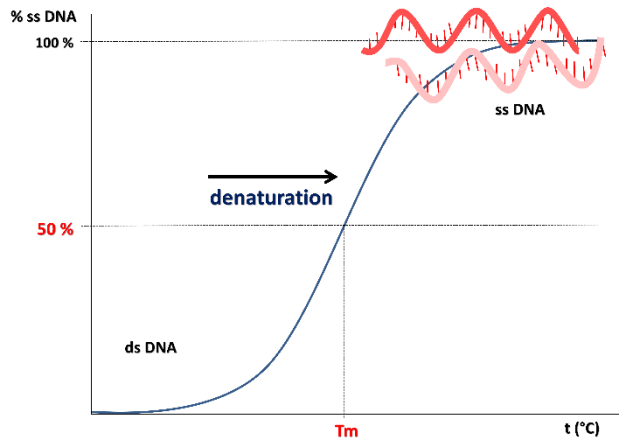
A key condition for success is choosing the right primers. These oligonucleotides, typically 17-25 nucleotides in length, hybridize to the complementary sequence of opposite strands of the template DNA, thus flanking the amplified sequence. Thus, it must be complementary to the target DNA but not complementary to one another or within one of the primers. These oligonucleotides, after hybridization at the 3´-end, are extended by DNA polymerase, which cannot begin synthesis of the complementary DNA strand de novo. Their sequence is given in the 5'-3´ direction of the copied thread, the first one is called forward (upstream) and the second one is called reverse (downstream). The selected sequences should be unique, specific to the amplified sequence, so as not to expand any region other than the desired one. They should also have the same or at least similar annealing temperature. The annealing temperature can be approximately calculated by various methods, but it must always be empirically optimized for amplification to be specific at sufficient yield. At too low a temperature, the primers can anneal to sequences that are not completely complementary. This creates a non-specific product. At too high a temperature, the primers do not hybridize sufficiently and the product is formed in very small amounts.

Last but not least, amplification efficiency is influenced by the quality and purity of the template DNA. The impurities contained in the sample may act as polymerase inhibitors or slow the polymerase reaction considerably by binding to template DNA and making it unavailable for polymerase binding.

Since well denatured DNA is essential for a successful PCR run, initial denaturation (usually 95°C for 2 - 3 min) is preceded by a block of cycles that ensures the separation of dsDNA into two ssDNAs. After completion of the selected number of cycles, a step for synthesizing the fragments of -72°C, 5-7 min, is then inserted as standard, then the reaction mixture is cooled to 4 ° C.

Denaturation and renaturation of DNA

Rising ambient temperatures (as well as strongly alkaline pH) lead to breakdown of hydrogen bridges between the fibres of the double helix. We are talking about DNA denaturation. This change is reversible. Returning to the original double-stranded state, the so-called renaturation (= hybridization), can be achieved by slow cooling. It does not occur with rapid cooling. Under appropriate conditions, fibres of different origins can thus form a double helix only on the basis of base complementarity.



Denaturation of DNA

The stability characteristic of a dsDNA fragment is its melting temperature (Tm). It is the temperature at which double stranded DNA is half denatured. This temperature depends on the length of the fragment, its nucleotide composition (a higher proportion of GC pairs increases the melting point) and other factors such as pH or ionic strength of the solution.

For oligonucleotides shorter than 50 bp, it can be calculated as follows:

Tm = 2× (number of AT pairs) + 4× (number of GC pairs)

The hybridization temperature is 5 °C lower than Tm.

Amplification of RNA

In the case of RNA amplification, reverse transcription is performed followed by polymerase chain reaction (RT - PCR). In reverse transcription, the reaction mixture consists of the following components:

- template RNA
- reverse transcriptase, an enzyme that performs reverse transcription of RNA to single stranded DNA
- a synthetic oligonucleotide
    - for reverse transcription of mRNA, an oligonucleotide oligo $(dT)_{12-24}$ is used which attaches to the polyadenyl chain, or a mixture of oligonucleotides (usually hexamers) with different sequences that attach to random complementary RNA sequences
    - a gene-specific primer is used to reverse transcribe a particular selected RNA with a known portion of the nucleotide sequence
- ribonuclease inhibitor

Common reverse transcription is as follows:

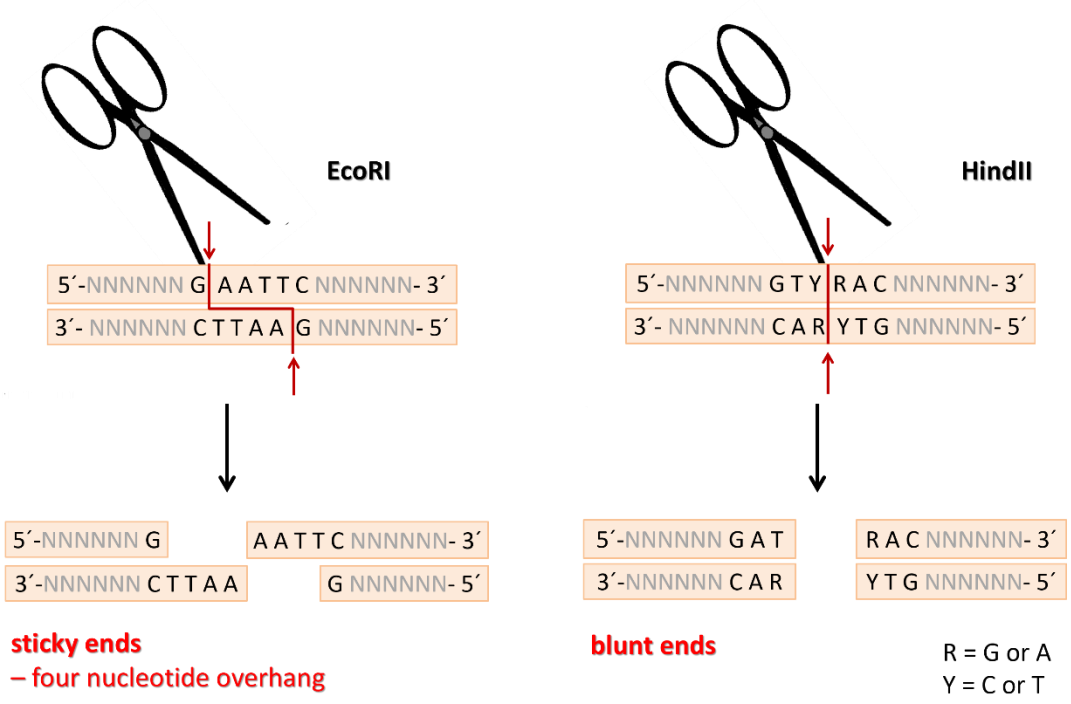
1. Denaturation of RNA prior to reaction mixture preparation
2. Preparation of the reaction mixture
3. Primer attachment at appropriate temperature (for oligo (dT) 15 25 ° C 10 min)
4. Synthetic reaction (approx. 42 °C 60 min)
5. Thermal inactivation of the enzyme (at 99 °C for 5 min)
6. Cooling the mixture to 4 °C

Subsequent PCR, in which reverse transcriptase synthesized 1st strand cDNA is amplified to conventional double stranded DNA, is no longer substantially different from the above PCR.

Restriction Endonucleases (Restrictases)

Restriction endonucleases are bacterial enzymes that break down foreign dsDNA into shorter regions, the so-called restriction fragments. Bacteria serve as a kind of "immune system" that protects them from foreign DNA. The bacterial DNA itself is protected against degradation by methylation at the sites recognized by the sequences. These bacterial enzymes can be isolated and used in a molecular biological laboratory to fragment DNA.

Restriction endonucleases are classified according to their properties into 4 types. Endonucleases of type II have practical application in DNA analysis. These restrictases do not randomly cleave DNA, but recognize specific sequences (restriction sites) and cleave DNA directly in, or in close proximity to, them. These sites are usually about 4-8 nucleotides in length and often have the character of palindromes (inverted repeats). Phosphodiester bond cleavage occurs simultaneously on both chains. If cleavage occurs exactly in the middle of the restriction site, so-called blunt ends are formed. If cleavage occurs at a different location, ends with different lengths of overhang occur, the so-called sticky ends.



Cleavage patterns

Currently, more than 4000 restrictionases are known to recognize more than 300 different sequences. There are databases, eg REBASE® (rebase.neb.com), in which it is possible to search for restrictases, for example according to the recognition sequence.

The nomenclature of restriction endonucleases is quite specific. The first three letters of the name are derived from the genus (first letter) and species names (second and third letters) of the organism from which the restrictase originates, *eg Eco* for *Escherichia coli* or *Hin* for *Haemophilus influenzae*. This is followed by the symbol to denote the strain, *Hind* for *H. influenzae* strain d. The last part of the name is the Roman numeral denoting the individual restrictases produced by the same strain, *HindII* and *HindIII*. Cleavage of DNA by restriction endonucleases is widely used *e.g.* restriction DNA analysis, molecular cloning, cDNA libraries production…

Determination of SNP

A method called "restriction fragment length polymorphism" (RFLP) or its modification by PCR-RFLP = Amplified Fragment Length Polymorphism (AFLP) can be used to determine single nucleotide polymorphisms. In practice we will use a modified method.

In a first step, the DNA to be analyzed is isolated. The region with suspected polymorphism is amplified by PCR. The PCR product obtained is subjected to restriction digestion. The original method uses directly isolated genomic DNA for restriction digestion. If two alleles differ by a nucleotide sequence that is part of a restriction endonuclease site on one of them, that restriction site is disrupted on the other, i.e. the DNA is not cleaved at that site by the restriction site. After digestion with a given restriction enzyme, the fragments of the same length differ initially in length. The fragments obtained are analyzed by gel electrophoresis.

Since genomic DNA is cleaved by a given number of other restriction sites in addition to the target sequence, the cleavage results in a huge number of fragments that form a continuous "smudge" on electrophoresis. Southern blots are therefore required to determine the position of the fragments of the sequence of interest. In the PCR -RFLP method, only a few fragments result from cleavage, and therefore we see the result directly in electrophoresis.

Southern blot/blotting

Southern blotting is a hybridization technique named after E. Southern who introduced the procedure. DNA was separated according to fragment size by agarose gel electrophoresis and converted to a single-stranded structure by denaturation in NaOH solution. Single stranded DNA (ssDNA) is transferred to a nylon or nitrocellulose membrane. The transfer of DNA from the gel to the membrane (blotting) is carried out either by capillary action or by means of electrical forces (electroblotting). DNA is anchored to the membrane (most often by UV radiation). The membrane is then incubated in a solution containing labelled defined DNA probe fragments. These probes hybridize (forming double stranded regions) with complementary strands of the anchored DNA. After hybridization, unbound probes are washed off and the position of the bound probes is detected. If the probes are radiolabelled, autoradiography is performed - the membrane is placed on the X-ray film and exposed. Once the film is developed, a signal will be in place of the hybridized radioactive probe. However, there are also commercially available kits for non-radioactive labelling, such as fluorescent or chemiluminescent labels.

There is a similar hybridization technique for RNA analysis called Northern blot.

Electrophoresis

The basic technique for nucleic acid separation, identification and purification is gel electrophoresis. Nucleic acids behave as polyanions in a slightly alkaline environment (pH ≈8.5). As the number of nucleotides in the molecule increases, its charge increases proportionally. Therefore, they move in the electric field towards the anode. To achieve their size distribution, we use a suitable carrier, most commonly an agarose or polyacrylamide gel, which serves as a molecular sieve. Larger molecules move much worse than smaller molecules.

Agarose gels are used for longer fragments (500 bp - 25 kbp), polyacrylamide gels for shorter fragments. The agarose gel separating ability can be influenced by the agarose concentration.

agarose concentration in the gel (%)	lengths effectively separated DNA fragments (kbp)
0.5	30 – 1
0.7	12 – 0.8
1.0	10 – 0.5
1.2	7 – 0.4
1.5	3 – 0.2

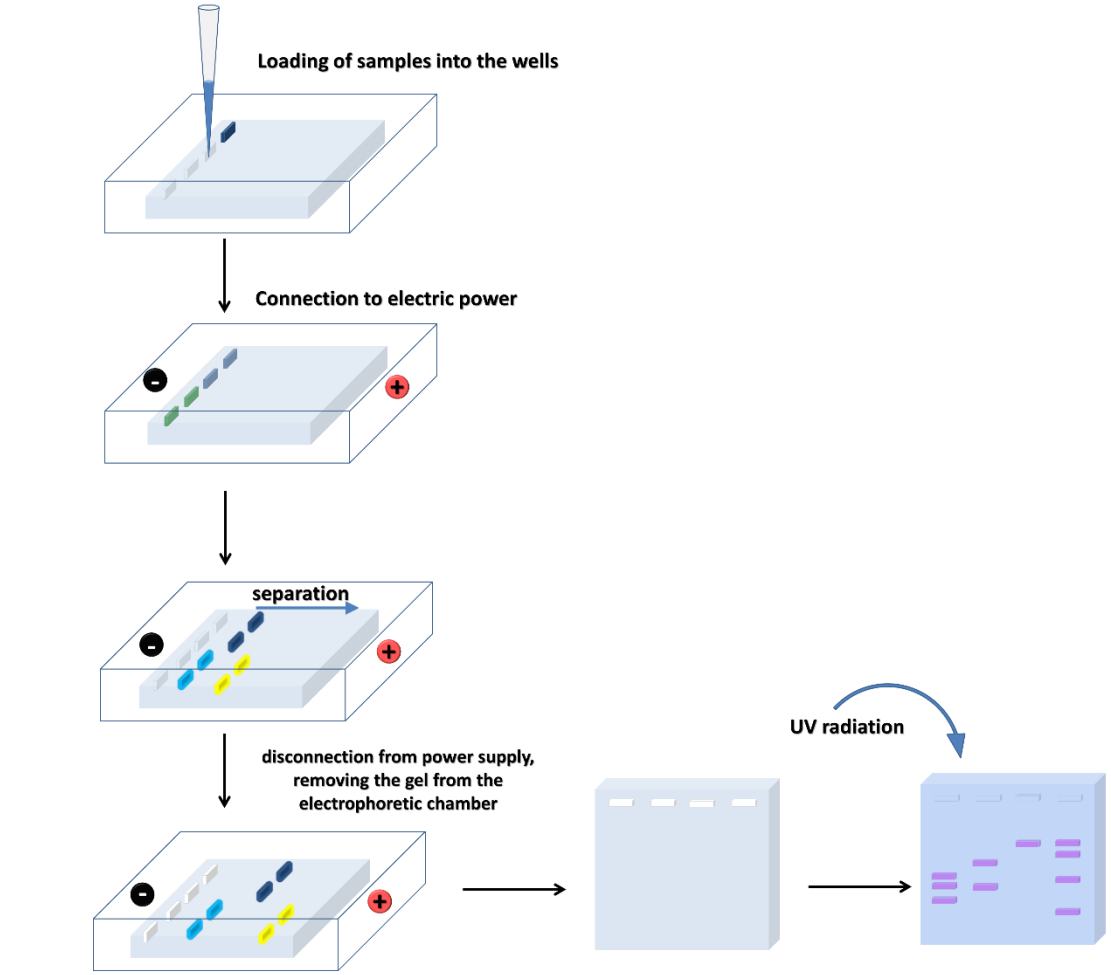
Agarose gel electrophoresis is performed in a horizontal design. Prepare a gel with a given concentration of agarose in electrophoretic buffer (TAE or TBE) having a thickness of 0.5 - 1 cm and at the start of the sample well. The gel is placed in an electrophoretic vessel and poured into the electrophoresis buffer so that a 1 mm buffer layer is above the gel.

Before loading on the gel, the sample is mixed with loading buffer, which increases the density of the mixture and thus facilitates application to the well. The coating buffer also contains a dye which migrates in the same direction as the nucleic acids under the influence of an electric field, thus providing an opportunity to estimate the position of the fragmented fragments. Most commonly, bromophenol blue is used, which proceeds at the same rate as a 500 bp DNA fragment.

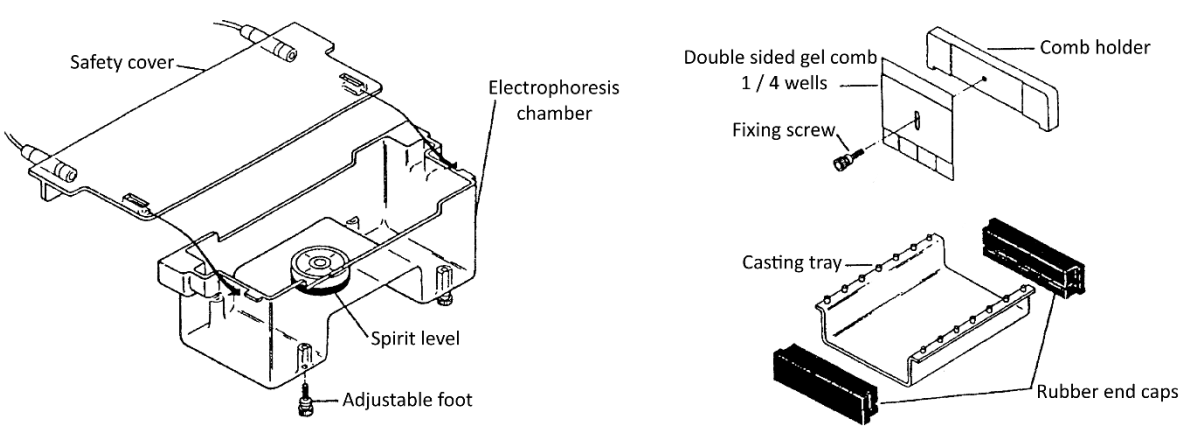
To determine the lengths of the fragments to be analysed, it is suitable to apply both samples and gel to the samples, i.e. a mixture of DNA fragments of appropriate lengths of known size.

After application of the samples, electrophoresis is connected to an electrical source, the voltage is 1-10 V / cm of gel length, and the run time is selected according to the length of the fragmented fragments.

The separated fragments can be visualized by staining the gel with ethidium bromide, a dye that intercalates into DNA and glows orange fluorescent orange under ultraviolet radiation. The gel can be stained after electrophoresis by bathing in ethidium bromide solution (0.5 μg / mL) or ethidium bromide can be added to the gel during its preparation (final concentration of ethidium bromide in the gel - 0.5 μg / mL). Ethidium bromide is a potential carcinogen.

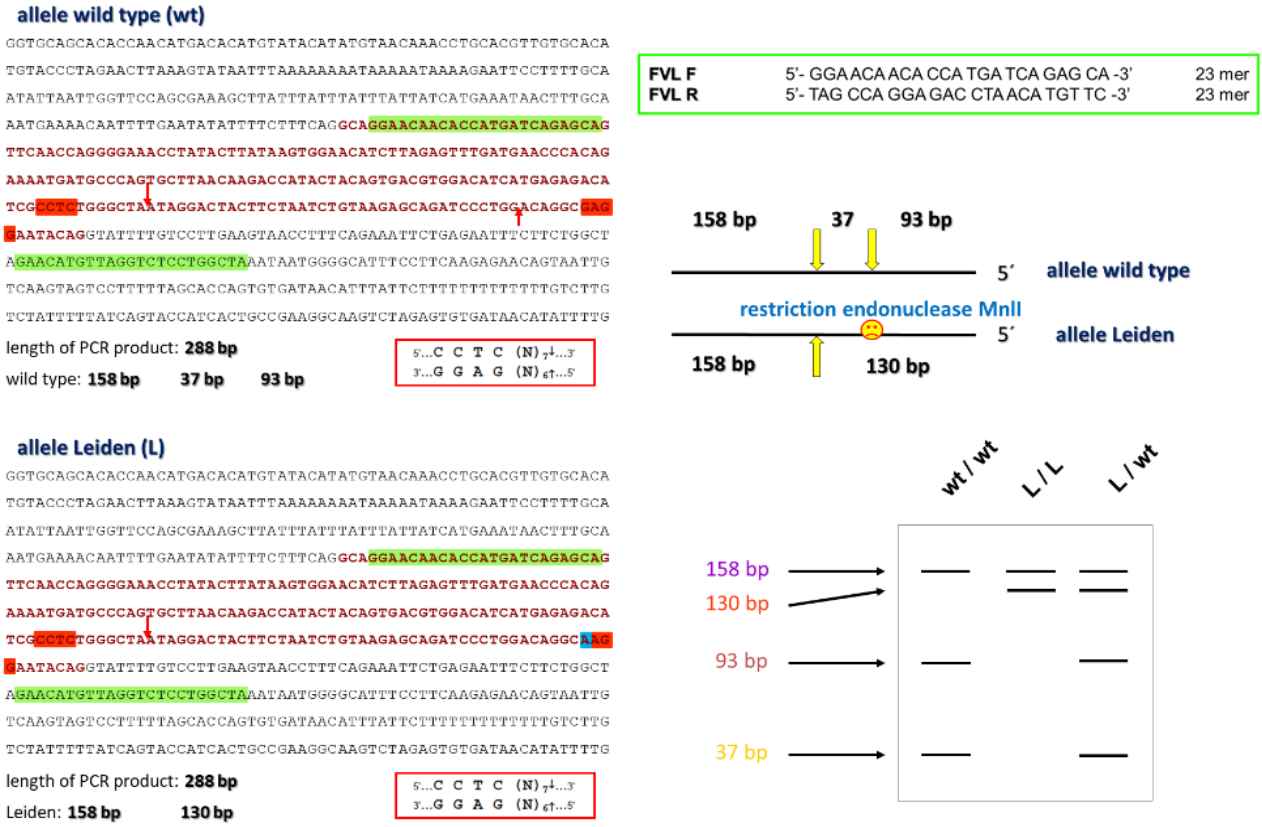


Electrophoresis - principle



Horizontal gel electrophoresis apparatus

Interpretation of results



Cleavage of the PCR product with MnII restriction endonuclease

Why do we see strips of given lengths (158, 130, 93 and 37bp)?

A restriction endonuclease MnII recognizes a particular sequence. The recognition sequence also includes a site where, if present, the Leiden mutation.

For wt homozygotes, 2 restriction sites are present in the PCR product. The product is split into 3 parts. Thus, there will be 3 strips (158, 93 and 37 bp) on electrophoresis.

By changing the nucleotide in the case of a mutation, one of the restriction sites terminates and does not cleave at that site. The product is cleaved at only one site. Thus, only 2 parts are produced. Electrophoresis shows 2 bands (158 and 130 bp). This is the case of the homozygote Leiden.

In heterozygote, which includes both variants, we will see 4 bands (1. is common to both variants - 158bp, 2. uncleaved (L allele) - 130bp, 3. and 4. two splice segments (normal allele) 93 and 37bp).

Tools and slang terms

Eppendorf tube (Eppendorf microtube)

Small plastic tube (usually 1.5 mL) with a lid and conical bottom. It is perhaps the most versatile and most commonly used "container" in biochemistry and molecular biology laboratories. It can be used wherever small volumes are used (in the order of tens to hundreds of microliters). It is used not only for carrying out reactions, but also for preparing and storing solutions. It is also suitable for storing deep-frozen specimens (-80 °C). It can withstand temperatures of 100 ° Cduring reactions. The tube is autoclavable.

Autoclaving is a common and very efficient method of sterilization with humid air at high pressure and temperature (steam sterilization), typically 120 ° C for 15 minutes.

The attached cap easily allows a tight closure that prevents contamination during work and makes the eppendorf container suitable for long-term storage.

The conical bottom allows centrifugation, and this tube shape with a narrow bottom space makes it easy to work with small volumes, i.e. even if there is only a minimal volume in the tube, it is still relatively convenient to pipet from there.

The slang name of eppendorf comes from the name of the company that first launched this type of test tube in 1963 (the German company Eppendorf, founded in 1945 in Hamburg in the Eppendorf district, which gave the company its name). The tubes are made of polypropylene, which is a very chemically resistant material that can withstand extreme temperatures (-90 °C to +121 °C). Over the last 50 years, tube design has evolved into many minor enhancements that can make work more enjoyable, and some are pointing to one particular type of use. Eppendorfs are available with a "safe-lock" lid lock to prevent easy opening, a tube with a completely clear wall that allows their direct use as cuvettes for optical methods. The tube wall may have a coarse volume scale or an "indelible" label area.

Vortex (vortex mixer)

Apparatus designed to thoroughly mix the contents of small tubes and containers. When the tube is pushed into the rubber part at the top of the instrument, the motor starts and the rubber part oscillates rapidly in a circular motion, the motion is transmitted to the fluid inside the container and is vortexed very efficiently. Most instruments have adjustable speed and choice of whether the engine is running continuously or only when the rubber part is pushed on the tube.

Multiple ways are possible to place the vortex tube (press in the center of the rubber part or just lean against the edge). Usually only a short application (5-10 seconds) is sufficient for normal mixing of the solution, but in some procedures intensive stirring for a longer time is required.

When mixing the contents of the eppendorf tube it is necessary that one finger of the hand in which we hold the eppendorf tube secure the lid against possible opening! Otherwise, there is a risk of splashing. For this reason, it is not recommended to vortex corrosives.

vortex = *m*ix on vortex

Microcentrifuge

Microcentrifuge is a centrifuge designed to centrifuge small tubes (eppendorfs).