Physico-Chemical Bases of Drug Action

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PREFACE

Development of safe active pharmaceutical substances, reduction of their non desired adverse effects, makes knowledge of mechanism of action of the compounds at the molecular level. The area is one of the most intensively developing field of pharmacology, biochemistry and medicinal chemistry. Due to the complex nature of development of biological effects, aims and methods of investigations conducting on different fields can be different. It is common experimental observation, however, that the biological effects of foreign substances (xenobiotics) is essentially determined by a few basic physical-chemical properties, including structure, acid-base properties and lipophilicity.

The present electronic educational material was compiled based on the main chapters of the course of the authors announced in the PhD School of Faculty of Medicine of University of Pécs. During the course cellular molecular targets, structure-activity relationships, basic physico-chemical properties and nature of interactions of pharmacologically active substances are discussed. Although the course is primarily targeting interest of PhD students the present educational material can help better understanding of Pharmaceutical Chemistry studies of graduate students as well.

Due to complexity of the title field, such an educational material may not necessarily be complete It can provide sufficient basis, however, to those who are working on elucidation of mechanism of action of medicinal products or on development of new active substances or dosage forms.

The editors are expressing their grateful thanks to professor Gábor Halmos (University of Debrecen) for his conscientious revision and constructive comments of the Hungarian version of this educational material. The present material is a translation of the most important chapters of the Hungarian version.

The modular structure of the material allows incorporation of new chapters in the future., which discuss further topics and results of new developments of the field. In this connection, the editor welcomes proposals forwarded to him. Any other comments concerning improvement of the educational material is thankfully welcomed.

Pécs, October 2015.

The editors

I Molecular bases of biological actions - Structural characterstics of drug targets

Studying effects of exogenous substances (xenobiotics) on living organisms is one of the oldest field of research of natural sciences. The bases of these investigations are those ancient observations, which – among others - distinguished edible and toxic plants, recognized painkiller and anti-inflammatory effects of some plant materials, reduced fever and helped healing of wounds. In the early times, recognition of effects of exogenous substances of different origin was the most important information. Although the first written documents are from China, one of earliest well known documents in Europe was found in Egypt by *G. Ebers* in 1874. This 21 m papyrus roll lists 876 medicinal products and more than 500 plants. About one third of these plants (for example: caraway, thyme, linseed, garlic) is still in use nowadays. Around 500 AC the Egyptian herbalists were considered the best. They were the doctors of monarchs from Rome to Babylon.

The Greek and the Roman doctors systematized the knowledge of identification and use of medicinal plants. *Hippocrates* (460-377 BC), the "father" medical sciences listed more than 200 medicinal plants in his book titled "Corpus Hippocratium". He considered the various diseases as a natural issue of human existence, which can be cured by means of appropriate medicinal preparations. Aristotle (384-322 BC) also compiled a book discussing medical importance of herbs. After summary and analysis of knowledge the Greek-roman *Dioscorides* (40-90 AC) compiled the first list of medicinal plants in his book titled *De Materia Medica* appeared in 78 BC. The book lists about 600 medicinal plants. It was the reference book of herbalists over 1500 years. *C. Galenus* (129-199), the acknowledged medical doctor of emperor Marcus Aurelis, compiled a more detailed herbal book. He prepared different pharmaceutical formulations (decoctions, tinctures, creams, patches, etc.), which are called galenicums nowadays.

The basis of modern herbal medicine was set by *Paracelsus* in the beginning of the 16th century. In his book, titled Herbarius, several medicinal plants are described in details. Paracelsus considered the various diseases as a consequence of disturbance of mineral balance of the body, which can be cured by medication of chemistry of it. It was Paracelsus who could use the otherwise toxic compounds of mercury, sulfur and iron. He stated "the *dose makes* the poison". (In its original terms: *„Alle Ding' sind Gift und nichts ohn' Gift; allein die Dosis macht, das ein Ding kein Gift ist."; Each substance is poison, even if it is not alone; it is only the dose, that makes a substance not poison."*)

Foundation of scientific basis of medical treatment is linked to the name of a *C. Bernard* (1813-1878). He performed the exact pharmacological analysis of biological action of curare and laid the foundation-stone of pharmacology. The first idependent pharmavology department was founded *R. Buchheim* (1820-1879) in University Dorpat (Russia) (today Tartu, Estonia) in 1849. His successor, *O. Schmiedeberg*et (1838-1921), is considered as a founder of education of modern pharmacology.

Development of experimental pharmacology has evolved and developed closely with organic chemistry, which helped development of pharmacology by isolation and structure determination of isolated plant materials, and by development of new synthetic methods used to synthesize new derivatives. Today medicinal chemistry is an

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independent discipline, which is organizing the physico-chemical basis of biological actions of drugs at the molecular level.

The first molecular interpretation of biological effects appeared in works of *P. Erlich* (1854–1915) and *J.N. Langley* (1852-1925). According to Erlich's conception, the basic requirement of pharmacological action is linking of the affector molecule to a cellular unit, he called "receptor". („*Corpora non agunt nisi fixata*"). He assumed that the protoplasm of microscopic living organisms contains several sidechains, so called receptors. The physiological function of these receptors is uptake of oxygen and nutriments that are needed for metabolic activity of the cells. In case of advantageous effect, the drug molecules are linked to these receptors through their specific functional groups (pharmacophore groups) and inhibit the physiological functions of the microorganisms. Linkage of the drug molecules and the receptors is realized by means of (typically secondary) chemical bonds. For example, the basic dye molecules are linked to the acidic functional groups of the receptors.

Erlich's above hypothesis has been proved to be right, although the theory has been further developed over the years. Regardless, Erlich's work – foundation of chemotherapy and development of the receptor theory – can be considered as a landmark in drug discovery.

I.1 Molecular bases of drug actions

The original conceptions have been developed based on the actual level knowledge. Thus, based on the molecular basis of development of biological actions of drugs (xenobiotics) they can be divided into two main groups:

1.) Drugs, of which biological actions is developing without interaction with cellular (macro)molecules (target molecules), and
2.) Drugs, of which biological actions is developing through interaction with cellular (macro)molecules (target molecules). These compounds can be further classified based on the nature of interactions, which can be
   a.) covalent, or
   b.) noncovalent

I.1.1 Drugs without interactions with cellular (macro)molecules

Drugs belonging to this group exert their effects as aresult of their physico-chemical prperties. This group includes such as

lubricant laxatives (e.g., paraffin oil)
saline laxatives (sodium sulfate decahydrate)
osmotic laxatives (eg., lactulose)
osmotic diuretics (e.g, mannitol, glycerol)
inhalational general anesthetics (e.g., halothane, enflurane, isoflurane)

I.1.2 Drugs developing interactions with cellular (macro)molecules

I.1.2.1 Drugs developing covalent interactions

Drugs (xenobiotics) belonging to this group can be further classified whether they

a) react with endogenous small molecular weight compounds, or
b) react with cellular macromolecules.

Antacids (e.g., sodium hydrogen carbonate, calcium carbonate, basic magnesium carbonate, aluminum oxide) are frequently used examples of drugs exerting their effect as a result of their neutralization reaction with the hydrochloric acid content of the gastric juice.

The other group is represented by those compounds that interact with cellular macromolecules (target molecules) and react with them to form covalent bonds. The most well-known examples of these compounds are (a) the alkylating antineoplastic agents, and (b) the irreversible enzyme inhibitors.

The most important groups of alkylating agents are

the nitrogen mustards
the nitrosoureas
the alkylsulfonates

These compounds typically undergo metabolic transformation to form reactive electrophilic metabolites, which can react with the nucleophilic functionalities of the target molecules (nucleic acids, proteins, polysaccharides. Because of the high reactivity of the forming electrophilic species, selectivity of the compounds is mainly the result of difference in their distribution and location of primary formation of their reactive metabolites.

Drugs displaying selective inhibitory effects represent an important class among the drugs developing covalent interactions with their cellular target molecules. Most of these compounds are known as selective inhibitor of particular enzymes. The most well-known examples of these compounds are

acetylsalicylic acid (selective COX-1 inhibitor)
tranylcypromine (non-selective MAO inhibitor)
selegylin (selective MAO-B inhibitor
diisopropyl fluorophosphates (cholinesterase inhibitors)
allopurinol (xanthine oxidase inhibitor)
penicillins, cephalosporins (DD-transpeptidase inhibitors)

I.1.2.2 Drugs developing noncovalent interactions

Noncovalent interactions with cellular macromolecules represent the molecular basis of most of the drug molecules. Although noncovalent interactions do not result in formation of new molecules, they are playing an important role in interactions between ions and molecules. These interactions are weaker than the covalent bindings, reversible, and sensitive to fluctuation of the molecular surroundings. The most important noncovalent interactions and their characteristics are summarized in Tale I-1.

Table I-1. The most important noncovalent interactions and their characteristics.

Type of interaction	Characterization	Example	Distance dependence
Ion-Ion	Longest-range, non-directional	ammonium ion – carboxylate ion	$1/r$
Ion-Permanent dipole	Strength depends on orientation of the dipole	ammonium ion - water molecule	$1/r^2$
Permanent dipole-Permanent dipole	Strength depends on relative orientation of the	water molecule – water molecule	$1/r^3$

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Type of interaction	Characterization	Example	Distance dependence
	two dipoles		
Ion-Induced dipole	Strength depends on polarizability of the inducible molecule	ammonium ion – phenyl group	$1/r^4$
Permanent dipole–Induced dipole	Strength depends on polarizability of the inducible molecule	water molecule – phenyl group	$1/r^6$
London dispersion forces	Mutual synchronization of fluctuating instantaneous dipoles	phenyl group-phenyl group	$1/r^6$
Short term repulsion	Interactions of electron orbitals, very short-term	electron orbital – electron orbital	$1/r^{12}$
Hydrogen bond	Electrostatic, partially covalent	secondary amino group – carbonyl group	bond length

The cellular target molecules are typically macromolecules such as,

a.) proteins,
b.) nucleic acids,
c.) lipids (cell membranes) and
d.) carbohydrates

These macromolecules fulfill a variety of functions in the living organisms. In respect of studying molecular basis of drug actions proteins, nucleic acids and the lipid bilayer of the cellular membranes are the most important targets. In the latter, several cellular organelles of protein origin with different physiological functions can be found, among which several can be target of drug molecules. The possibility of modification of functions of the most important cellular targets are introduced in Chapters I-IV.

Naming of the part of the macromolecule which is involved in the interactions – depending on the cellular function of the macromolecule – can be *target molecule*, *binding site*, *active center*, etc. In this chapter the most important structural characteristics of proteins and lipids are discussed.

I.2 Structural characteristics of drug target molecules

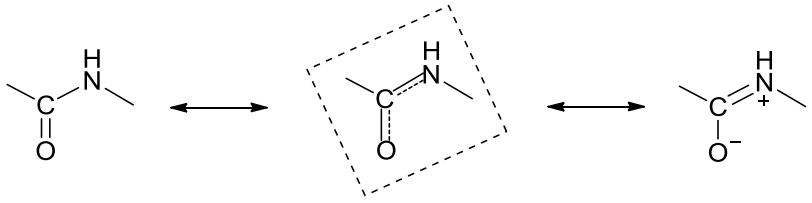
I.2.1 Structure of proteins

Proteins are polymers of amino acids. Their chemical and enzymatic hydrolysis results in formation of amino acids. The *primary structure* of proteins is the sequence of the building amino acids that are linked together by peptide bonds. Peptide bonds are carboxamide bonds that link together the consecutive amino acids involved their

carboxyl and amino functional groups (Figure I-1). The lone pair of electrons of the nitrogen atom and the pi and n electrons of the carboxyl group form a delocalized system. As a consequence of it

1. Rotation around the C-N bond becomes hindered because the bond order of the bond is increased. Isomerization of the more stable trans arrangement into the respective cis isomer needs relatively large energy. In the more preferred trans form the H-N-C=O atoms are located in the same plane (Figure i-1).
2. Due to the delocalization, the nitrogen atom does not show basic character

Figure I-1. Structure of the peptide bond



The sequence of the amino acids is listed starting from the N-terminal using the two or three letter symbols of the amino acid units.

Straight-chain structure of proteins is energetically not favorable, therefore sections of the chain develop interactions to form *secondary*, *tertiary*, and - in the case of oligomeric peptides – *quaternary structural units*. The three dimensional structures are stabilized by means of

hydrogen bonds,
ionic interactions,
hydrophobic interactions, and
disulphide bonds

Accordingly, the three dimensional structure (conformation) of proteins is mainly determined by interactions that can be easily modified by changing the molecular surroundings. (The only exceptions are the covalent disulphide bonds.) The interactions show cooperativity; development of some interactions helps building of other ones.

One of the basic properties of proteins is that their conformations is closely associated with their biological function; the function is linked to a specific conformation. Therefore, modification of the conformation (e.g., as consequence of interaction with xenobiotics) can change biological activity of the protein.

The secondary structure of proteins is stabilized by hydrogen bonds developing between the carbonyl and the amid groups of peptide bonds of the chain. The higher the number the hydrogen bonds the more stable the structure. Structure analysis of proteins revealed that there are three distinguished periodically repeating secondary structural units (motifs). the *alpha helix*, the *beta sheet* and the *beta turn*.

The *alpha helix* is a common secondary structure of proteins and is a right-hand-coiled or spiral conformation (helix) in which every backbone N-H group donates a hydrogen bond to the backbone C=O group of the amino acid four residues earlier (Figure I-2). Most frequently Ala, Cys, Leu, Met, Glu, Gln, His és Lys amino acid residues are participating in formation of alfa helix motifs.

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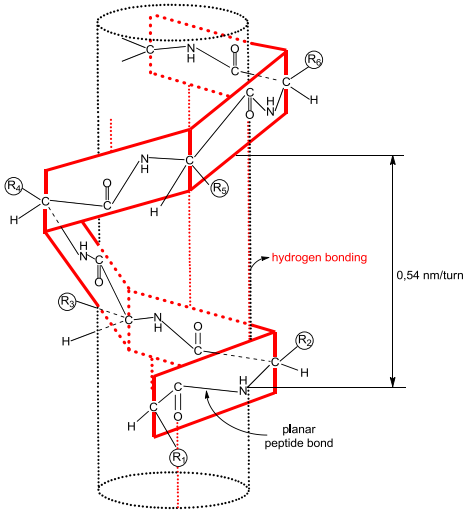
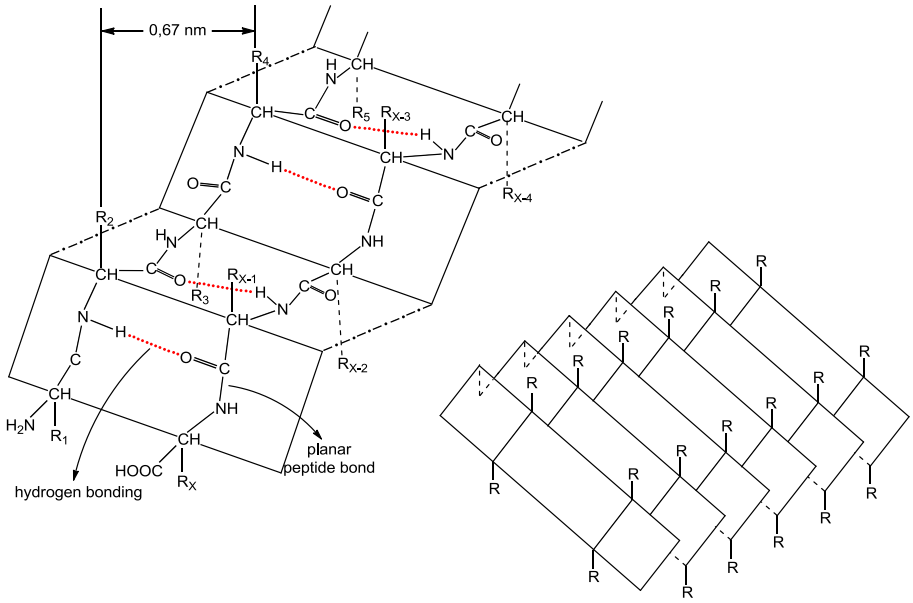
Figure I-2. Structure of the alfa-helix motif of proteins



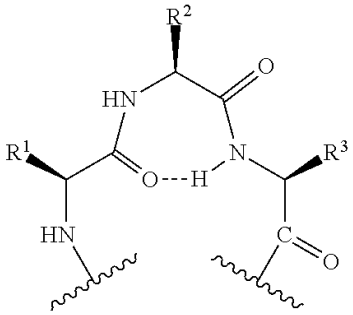
The *beta sheet* (also beta-pleated sheet) is the second form of regular secondary structure in proteins. Beta sheets consist of *beta strands* connected laterally by at least two or three backbone hydrogen bonds, forming a generally twisted, pleated sheet. Occurrence of Val, Ile, Phe, Tyr, Trp, Thr amino acid residues is frequent in beta sheet mitifs. Because peptide chains have a directionality conferred by their N-terminus and C-terminus, beta strands too can be said to be directional. Adjacent beta strands can form hydrogen bonds in *antiparallel*, *parallel*, or *mixed* arrangements.

Figure I-3. Structure of beta-sheet motif of proteins



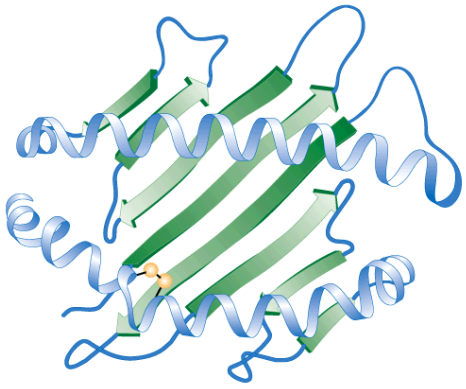
Beta turns are very common motifs in proteins and polypeptides. Each consists of four amino acid residues, which link two beta sheets or alfa helices by means of hydrogen bonds (Figure I-4). The Gly, Ser, Asp, Asn and Pro amino acid residues prefer development of beta turn motifs.

Figure I-4. Structure of beta turn motif of proteins



The term *tertiary structure* refers to a protein's geometric shape. The tertiary structure will have a single polypeptide chain "backbone" with one or more protein secondary structures, the protein domains. Amino acid side chains may interact and bond in a number of ways. The interactions and bonds of side chains within a particular protein determine its tertiary structure (Figure I-5). Native tertiary function is essential for proper functioning of biologically active proteins.

Figure I-5. Tertiary structure of proteins



There are some proteins of which functional form is composed of two or mole polypeptide chains. These proteins are called oligomers, while the combining polypeptide chains are called subunits. Quaternary structure is the combination of two or more chains, to form a complete unit. The interactions between the chains are not different from those in tertiary structure, but are distinguished only by being interchain rather than intrachain.

I.2.2 Structure of lipids

Lipids form a structurally divers group of naturally occurring molecules. Lipids – contrary to proteins and nucleic acids - can be broadly defined as hydrophobic or amphiphilic small molecules. They have limited water solubility; consequently, non-polar solvents (e.g., petroleum ether, chloroform, ether, benzene), dissolve well.

The most important functions of lipids in the living organisms as follows.

1.) Efficient energy sources
2.) Good thermal and electrical insulating and mechanical protection materials
3.) Lipid-protein complexes (lipoproteins) are important cellular units

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4.) Regulatory substances (hormones, vitamins, etc.) of the body metabolic processes.

Classification of lipids, whereas being chemically different substances, is rather spontaneous. Among others, lipids can be classified based on the structure of their lipophilic moieties. Accordingly, it can be distinguished

a.) Fatty acid derivatives (*saponificable* lipids) and
b.) Polyprenyl derivatives (*non-saponificable* lipids).

I.2.2.1 Saponificable lipids

Saponificable lipids have two permanent components: fatty acids and glycerol. Definition of further subclasses is based on the other components of the molecules. Based on this, saponificable lipids often have can be categorized into the following groups:

1. Simple lipids (triglycerides)
   - Neutral fats (fats, vegetable oils)
   - Waxes
2. Complex Lipids
   - 2a. Phospholipids
     - Glycerophospholipids
     - Phosphosphingolipids
   - 2b. Glycolipids
     - Glyceroglycolipids
     - Glycosphingolipids

Simple lipids

Neutral fats

The neutral fats are reserve nutrients of the organisms. The animal (and human) neutral fats are esters of glycerol and three even (12-22) carbon number carboxylic acids (fatty acids). In humans the 16-carbon and 18-carbon fatty acids occur most. Of these palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1 n-9) are the most common ones. (The notation 18:1 n-9 is marking that there is one carcon-carbon double bond in a 18-carbon fatty acid, which is located between the C9-C10 atoms.) In natural fats the configuration of the carbon-carbon double bonds is (*Z*) in most cases. Solid triglycerides are called fats; the liquid triglycerides are called oils. (Oils have high number of unsaturated fatty acids with (*E*) carbon-carbon double bond configuration.

Waxes

Waxes are mixtures of long chain aliphatic hydrocarbons and esters of long chain aliphatic alcohols and saturated fatty acids. Waxes are synthesized by many plants and animals. Waxes form protecting external layer of fruits and hydrophobic coating of feather of birds.

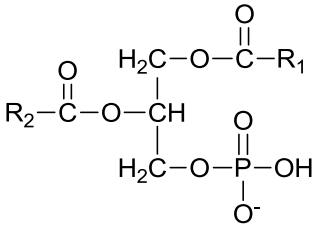
Complex lipids
Phospholipids

Glycerophospholipids

Glycerophospholipids are involved in construction of biological membranes, in particular. Structurally, phospholipid molecules generally consist of two hydrophobic

fatty acids and a phosphoric acid, which are linked to a glycerol molecule via ester bonds. The basic structure of all glycerophospholipids is phosphatidic acid. In cellular glycerophospholipids exclusively *L-phosphatidic acids* occur (Figure I-6).

Figure I-6. Structure of L-α-phosphatidic acid



Glycerophospholipids are *amphipathic* molecules: they are composed of two hydrophobic hydrocarbon chain of fatty acids and a hydrophilic phosphoric acid unit. The phosphoric acid unit is being modified with simple, polar organic molecules, which are bound to it via ester bond. The structure of the alcohols as follows.

Glycerophospholipid	**Alcohol component**	**Structure/Formula**
Phosphatidyl aminoethanol (cephaline)	aminoethano	$HOCH_2CH_2NH_2$
Phosphatidylcholine (lecitine)	choline	$HOCH_2CH_2N^+(CH_3)_3$
Phosphatidylserine	serine	$HOCH_2CHNH_2COOH$
Phosphatidylinositol	*mio*inositol	$C_6H_{12}O_6$
Bis-phosphatidyl-glycerol (cardiolipin)	glycerol	$HOCH_2CHOHCH_2OH$

Plasmalogens are a type of *ether phospholipid*s characterized by the presence of an (*E*)- vinyl ether (enol ether) linkage at the C1 position and an ester linkage at the C2 position of glycerol. In mammals, the C1 position is typically derived from C16:0, C18:0, or C18:1 fatty alcohols, while the C2 position is esterified most commonly by polyunsaturated fatty acids (PUFAs). In mammals the most common alcohols esterifying the phosphoric acid moiety ethanolamine or choline. The compounds mainly occur in muscles and nervous cell membranes.

Glycolipids
Glycosphingolipids

Glycolipids are lipids with a carbohydrate attached by a glycosidic bond. The lipid complex is most often composed of either a glycerol or sphingosine backbone, which gives rise to the two main categories of glycolipids, *glyceroglycolipids* and *sphingolipids*.

Sphingolipids are type of glycolipids in which the lipid part backbone is not glycerol but sphingosine, a 20-carbon amino alcohol (4-sphinganine). Sphingosine (4-sphyngenine) contains two asymmetric carbon atoms. In nature only one of the enantiomers (D-*eritro*-4-sphinganine) occurs (Figure I-7).

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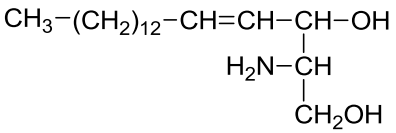
Figure I-7. Structure of sphingosine (4-sphinganine)



In the overall architecture of sphingolipids, stearic acid is the most common fatty acid which is linked to the C2 amino group via acid amide bond. The C1 alcoholic hydroxyl group can be linked to a sugar moiety via glycosidic bond or to phosphoric acid via ester linkage. The phosphoric acid moiety can be esterified by further small molecule polar compounds (e.g., choline). Based on the structure of these latter groups, sphingolipids can be classified into the (a) *glycolipids* or the (b) *phospholpids* groups.

In *sphingomyelin*, most common *phosphosphingolipid*, the amino group is acylated by stearic acid and it is choline that is linked to the phosphoric acid moiety.

In the largest group of sphingolipids (glycosphingolipids) the primary alcoholic functional group is linked to a sugar moiety via a glycosidic bond. Based on the structure of the sugar moiety glycosphingolipids shall be divided into three groups:

1.) *Neutral glycosphingolipids* (cerebrosides),
2.) *Negatively charged glycosphingolipids, containing sialic acid* (gangliosides), and
3.) *Sulfate esters of galactocerebrosides* (sulfated *glycosphingolipid*).

I.2.2.2 Non-saponificable lipids

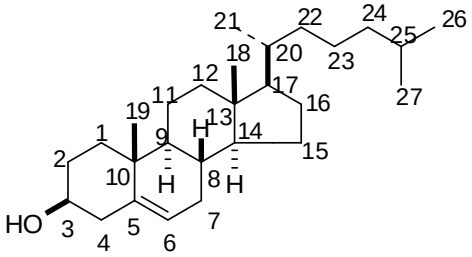
The non-saponificable lipids ca be classified as follows.

- terpenes,
- steroids,
- fat-soluble vitamins (A, D, E, K), and
- prostaglandins

In respect to understanding of molecular basis of drug actions, steroids represent the most important group of non-saponificable lipids. Therefore, only steroids are discussed in this educational material.

Steroids are structurally complex compounds with diverse biological functions. Each of them is a derivative of their common structural unit, the steroid skeleton. The steroid, also known as gonane, skeleton is a four ring condensed ring system. The common precursor of all steroids synthesized in the body is *cholesterol*. (Figure I-8).

Figure I-8. Structure of cholesterol



In cholesterol there are two methyl groups (C10 and C13), an isooctyl group (C17) and a hydroxyl group (C3) linked to the steroid skeleton. In the B ring a ΔC5 carbon-carbon double is located. The stereochemistry of condensation of both the B/C and C/D rings is *trans*. Therefore, the molecule has a relatively planar, lengthwise structure (Figure I-8). Cholesterol can be found in all cells in a free form or as an ester derivative of fatty acids. It is also involved in construction of cell membranes.

Natural steroids can be classified as follows.

1. *Sterines*. Cholesterol derivatives with a C3 hydroxyl and a C17 hydrocarbon chain.
2. *Vitamins D*. Derivatives with vitaminic effects. In fact, the compounds are precursors of the active hydroxylated derivatives formed in the liver.
3. *Bile acids*. Derivatives of cholic acid with detergent activity. They are amphipathic molecules with hydrophobic and hydrophilic regions. Bile acids/salts form micelles and solubilize lipids in the small intestine.
4. *Steroid hormones*. They represent two main classes of hormones: sex hormones and corticosteroid hormones.
5. *Steroid glycosides*. Steroids of plant origin. Some representatives are used in medicine as cardiotonics (e.g., digitoxin).
6. *Steroid alcohols*. Generally toxic steroids of plant origin. Some derivatives are important starting materials for industrial synthesis of steroid drugs.

I.2.3 Structure of the plasma membrane

The functional unit of all living organisms is the cell. The interior and external space of cells is separated and connected at the same time by the plasma membrane. Like all other cellular membranes, the plasma membrane consists of both lipids and proteins. The fundamental structure of the membrane is the *phospholipid bilayer*, which forms a stable barrier between the two aqueous compartments. The fundamental structure of the cell membrane is a thin layer of amphipathic phospholipids (*phospholipid bilayer*), into which cholesterol and glycolipids (e.g., sphingomyelin) are also incorporated. The amphipathic phospholipids are spontaneously arranging so that the hydrophobic regions are isolated from the surrounding water, while the hydrophilic regions interact with the intracellular (cytosolic) and extracellular polar molecular surroundings (Figure I-9). Membrane phospholipids (and proteins) are unable to move back and forth between the inner and outer leaflets of the membrane at an appreciable rate. However, they are able to *diffuse laterally* through the membrane.

While lipids are the fundamental structural elements of membranes, proteins are responsible for carrying out specific membrane functions. In 1972, *J. Singer* and *G. Nicolson* proposed the fluid mosaic model of membrane structure, which is now generally accepted as the basic paradigm for the organization of all biological membranes. In this model, membranes are viewed as two-dimensional fluids in which proteins are inserted into lipid bilayers (Figure I-9). *Singer* and *Nicolson* distinguished two classes of membrane-associated proteins, which they called (a) *peripheral* and (b) *integral membrane proteins*.

Peripheral membrane proteins were operationally defined as proteins that dissociate from the membrane following treatments with polar reagents, such as solutions of extreme pH or high salt concentration, that do not disrupt the phospholipid bilayer. In contrast to the peripheral membrane proteins, *integral membrane proteins* can be released only by treatments that disrupt the phospholipid bilayer

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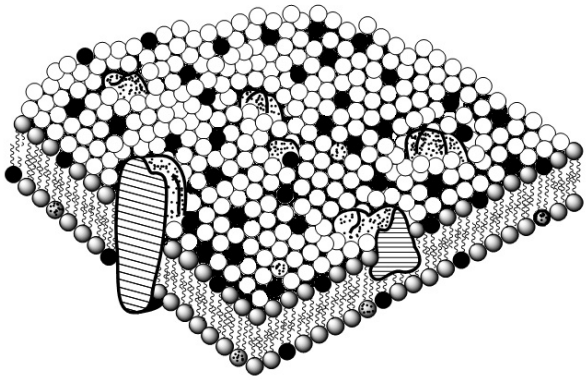
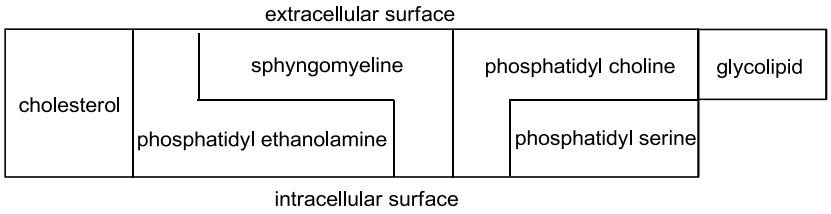
Figure I-9. Fluid mosaic model of the plasma membrane



The plasma membranes of animal cells contain four major phospholipids (*phosphatidylcholine*, *phosphatidylethanolamine*, *phosphatidylserine*, and *sphingomyelin*), which together account for more than half of the lipid in most membranes. It was found that these phospholipids are asymmetrically distributed between the two halves of the membrane bilayer The outer leaflet of the plasma membrane consists mainly of phosphatidylcholine and sphingomyelin, whereas phosphatidylethanolamine and phosphatidylserine are the predominant phospholipids of the inner leaflet. A fifth phospholipid, phosphatidylinositol, is also localized to the inner half of the plasma membrane. Distribution of cholesterol is symmetric. The glycolipids are found exclusively in the outer leaflet of the plasma membrane, with their carbohydrate portions exposed on the cell surface (Figure I-10).

Figure I-10. Distribution of phospholipids in the membrane of human red blood cells



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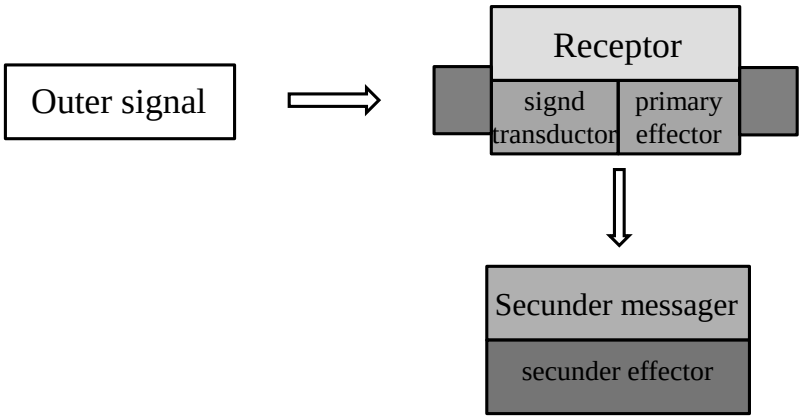
II Receptors as drug targets

Receptors are macromolecules of living organisms specialized in signal recognition and transduction. Large portions of receptors are so called *sensory receptor*, receptors which are involved in recognition and transduction of vision, smell, hearing, mechanical, chemical or other stimuli. In biochemical sense, a receptor is a protein (glycoprotein), which can develop interaction with certain (usually small) molecules and the resulted changes in its three-dimensional structure causes further changes in its molecular environment.

Functions of receptors is manifested through the operation cycle of it. In the first phase of it an extracellular signal, or signal-inducer molecule is bound to the receptor. As a result of the interaction – change in structure and/or conformation - the receptor forwards the information to the (*primary) effector* or a s*ignal transducer molecul*e. The signal transducer molecules activate intracellularly localized, so-called *secondary messenger*s (ions, molecules). Interaction of secondary messengers with the so called *secondary effectors* results in development of the biological result (Figure II-1).

Most receptor are embedded in the cell's plasma membranes; A molecule that binds to a receptor is called *ligand*, and can be a peptide or another small molecule such as a neurotransmitter, hormone, pharmaceutical drug, toxin, or parts of the outside of a virus or microbe. The endogenously designated molecule for a particular receptor is referred to as its *endogenous ligand*. An exogenous ligand (xenobiotic) interacting with the receptor can induce the same biological results as the endogenous ligand or can prevent (inhibit) development of it. Activating xenobiotics are called *agonists*, inhibitory xenobiotics are called *antagonists.*

Figure II-1. Mechanistic function of receptor, seconder messenger and effector



In the original "*receptor theories*" chemical nature of the “receptors” was not subject of detailed studies. With better and deeper understanding of cellular biochemistry, however, it turned out that the original "*receptor*" concept could mean several cellular macromolecules (*target molecule*) and diverse cellular functional units. Accordingly, the drug-“receptor” interaction can be classified as follows.

1. Interaction with receptors
2. Interaction with metabolic, regulatory enzymes
3. Interaction with transport proteins, transporters
4. Interaction with structural proteins
5. Interaction with nucleic acids

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In this chapter interactions with the above defined receptors is discussed.

Biological effect of medicinal products (xenobiotics) is often the result of interaction of the compounds with receptors of endogenous compounds (e.g., acetylcholine, norepinephrine, serotonin, gamma amino butyric acid, etc.) So one of the main targets in research for new drug candidates are receptors, of which biological function can be selectively modified by the new compounds.
Interaction of a foreign molecule (xenobiotic) with a protein can be considered as xenobiotic-receptor interaction (in other words, the protein can be regarded as a receptor) if the following main criteria are met:

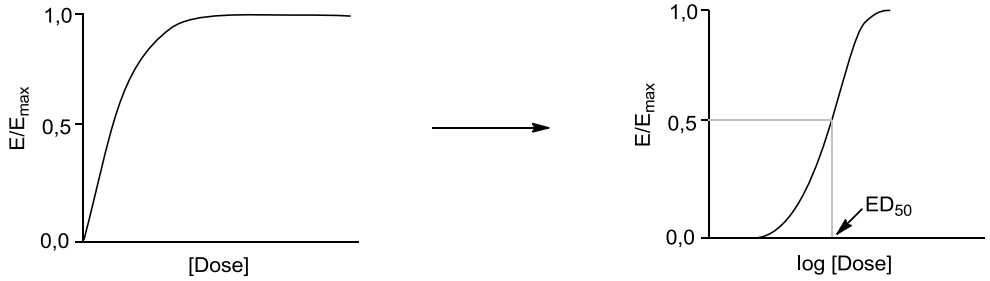
1. The receptor should show high affinity to the ligand,
2. The receptor should show high degree of stereoselectivity against the ligand,
3. The receptor should become saturated at low concentration of the ligand,
4. The ligand-receptor interaction should be reversible,
5. Expression of the receptor should show appropriate balance and tissue specificity,
6. The ligand should be released from the ligand-receptor complex by appropriate agonists and antagonists,
7. The in vitro receptor affinity and in vivo pharmacological activity of the ligand should show positive correlation.

Study of functioning of receptors and that of efficacy of xenobiotics (drug candidates) can be quantitatively characterized by determination of the so-called dose-effect relationship. During these studies the ligand is gradually added in increasing dose (concentration) to the receptor and the biological response is recorded.

A fundamental principle of pharmacology is that the intensity of effect produced by a drug is a function of the quantity of drug administered (or the concentration of the drug at the target site). The principle was concluded from the experimental finding that the effect of a drug was found to be directly proportional to the fraction of receptors occupied by drug and the maximal effect results when all receptors are occupied. The dose-effect relationship can be explained based on the laws governing chemical equilibrium or mass action.

The mathematical relationship between dose and effect based on the drug-receptor interaction can be graphically displayed. The dose-effect curve is shown in Figure II-2. In the graph on the left, the free drug concentration along the X-axis is a linear scale and the shape of the curve is a rectangular hyperbola, in which drug effect asymptotically approaches the maximal effect as the free drug concentration increases. It has become more customary to use semilog dose-effect curves to evaluate the quantitative aspects of the action of a single drug or to compare the actions of several drugs, such as in the plot on the right. When the dose is transformed to a log scale, the response-log dose curve shows a characteristic sigmoid shape. By means of analysis of the curve the ligand efficiency (*potency*) (ED_{50}) (i.e. the dose (or concentration) causing 50% of the maximum effect) or by its strength (*efficacy*) (E_{max}) (i.e., the maximum possible effect of the agonist) can be determined (Figure II-2).

Figure II-2. Different forms of the dose-response curve



The shape of the dose-response curve depends on both *affinity* and *intrinsic activity* of the ligand. *Affinity* is determined by the position of the dose-response curve in relation to the X axis, while intrinsic activity is determined by the height of the curve.

Affinity is a measure of propensity of a drug to bind receptor; the attractiveness of drug and receptor. Affinity can be measured numerically by using the dissociation constant K_D of the drug-receptor complex. The higher the K_D the lower the affinity of the drug.

Efficacy (or *intrinsic activity*) is ability of a bound drug to change the receptor in a way that produces an effect; some drugs possess affinity but no efficacy. Efficacy is numerically characterized by the (E_{max}).value of the agonist.

Potency (*efficiency*) is a measure of drug activity expressed in terms of the amount required to produce an effect of given intensity. Potency is most frequently characterized by the ED_{50} (or ED_{90}) value of the drug.

Xenobiotics of different lipophilic character bind to extra or intracellular receptors based on their actual lipophilicity. Lipophilic molecules (e.g., steroid hormones, thyroid hormones, vitamin D_3, etc.) can easily diffuse through the plasma membrane and linked to the intracellular receptors. Hydrophilic (water soluble) compounds (e.g., peptide hormones, neurotransmitters), however, are not able to penetrate the lipid-reach interior of plasma membranes. Therefore, they can exert biological effects by binding to the extracellular (hydrophilic) surface of the membrane embedded receptors. Based on their localization and function receptors can be classified as follows.

1.) Cell-surface (extracellular) receptors

a.) Ligand-activated ion channels (Ion channel-linked receptors)

b.) Receptors without own enzyme activity
- G-protein-coupled receptors
- Tyrosine kinase-coupled receptors
- Proteolysis-governed receptors

c.) Receptors with own enzyme activity
- Receptor tyrosine kinases
- Tyrosine-kinase-associated receptors
- Receptorlike tyrosine phosphatases
- Receptor guanylyl cyclases
- Receptor serine/threonine-kinases
- Histidine-kinase-associated receptors

2.) Intracellular receptors

d.) Intracellular nuclear receptors

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Homodimer receptors
- Estrogen receptors
- Androgen receptors
- Progesterone receptors
- Glucocorticoid receptors
- Mineralocorticoid receptors

Heterodimer receptors
- Retinoic acid receptors
- Vitamin D3 receptors
- Thyroid receptors

II.1 Cell-surface (extracellular) receptors

Cell surface receptor complexes are integrating into the cell membrane, most commonly their ligand-binding sites are in the extracellular side. Operation of the ligand-activated ion channels and that of receptors without enzymatic activity is based on conformational change of the inactive receptor due to binding of their ligand. Ion channel receptors to regulate the flow of selected ion into and out of the cells. Binding of the ligand causes channel opening.

The G-protein-coupled receptors initiate formation of secondary messengers (cAMP, IP_3 and DAG), which will cause changes in activity of enzymes and genes. Some of the receptors (e.g. cytokine receptors, erythropoietin, interferons, receptors of interleukins, Fc receptors) are operating in combination of tyrosine kinase enzymes (tyrosine kinase-coupled receptors). Activation of proteolysis-governed receptors (e.g., tumor necrosis factor receptors) initiate proteolytic process.

II.1.1 Ligand-activated ion channels (Ion channel-linked receptors)

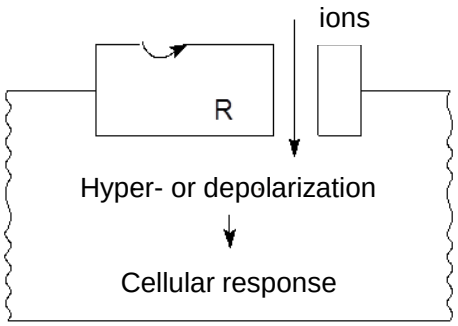
Intracellular and extracellular concentration of ions is critical determinant of several basic cellular functions. Therefore, physiological functioning of ion channels is essential. Both blocking and activation of operation of ion channels is possible. On the basis of nature of the primary stimulus

- voltage-gated ion channels, and
- ligand-activated ion channels

can be distinguished.

Ligand-activated ion channels - also known as ion-channel-linked (ionotropic) receptors or receptor ion channels – expressed in high density in the nervous system and in contractile cells (smooth/striated/heart muscle). As a result of interaction with the ligands ligand-activated ion channels open and sodium, potassium, calcium or chloride ions flow through the channel in the direction of their concentration gradients (Figure II-3.). As a result of the fast ion flow ion channel-linked receptors result fast information/signal transmission. Voltage-gated ion channels will be discussed in Chapter IV.

Figure II-3. Simplified structure of ligand-activated ion channels



According to their structure and functions ligand-activated ion channels can be can be classified as follows.

I.) Cys-loop receptors

Cys-loop receptors are named after a characteristic loop formed by a disulfide bond between two cysteine residues in the N terminal extracellular domain. They are usually pentameric with each subunit containing 4 transmembrane helices. Cys-loop ion channel receptors can be classified based on the nature of the transmitted ions and the chemical structure of the activating endogenous ligands as follows.

1. Anion type cys-loop-type receptor ion channels
   a.) GABAA (chloride ion) receptors,
   b.) glycine (chloride ion) receptors.

They function as inhibitory neurotransmitters in the central nervous system.

3. Cation type cys-loop-type receptor ion channels
   a.) nicotinic acetylcholine nicotinic (nAChR) (sodium, potassium, calcium ions) receptor
   b.) serotonin (5-HT_3) (sodium, potassium, calcium ions) receptors.

They function as activating neurotransmitters in the central nervous system.

II.) Ionotropic glutamate receptors (iGluR)

They form tetramers with each subunit consisting of an extracellular amino terminal domain, (which is involved tetramer assembly), an extracellular ligand binding domain (which binds glutamate), and a transmembrane domain which forms the ion channel). Each receptor is activated by glutamate, but there are several other compounds that bind only to glutamate receptors of specific structure. Based on this latter three receptor subfamilies can be distinguished.

1. AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)-activated (GluA) receptors
2. Cainate (CF) -activated (GluK) receptors
3 NMDA (N-methyl-D-aspartate)-activated (GluN) receptors.

The receptors are permeable to Na^+-, K^+- and Ca^{2+} ions. They function as activating neurotransmitters in the central nervous system

III.) ATP-gated channels

ATP-gated channels open in response to binding the nucleotide ATP. They form trimers with two transmembrane helices per subunit and both the C and N termini on the intracellular side. P_{2X} subfamily of the purinergic. receptors are belonging to this type of ion channels. The receptors are permeable to Na^+-, K^+- and Ca^{2+} ions.

The most important ligand-activated ion channels are shown in Table II-1.

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Table II-1. Some important ion channel receptors

Receptor	**Physiological agonists**	**Ion channel**	**Antagonist***
Nicotine	Acetylcholine	Na^+, K^+, Ca^{2+}	Tubocurarine
$GABA_A$	gamma-Amino butyric acid	Cl^-, HCO_3^-	Bicuculline
Glycine	Glycine	Cl^-, HCO_3^-	Strichnine
Serotonine (5-HT_3)	Serotonine	Na^+, K^+, Ca^{2+}	Granisetron
AMPA, Kainate	Glutamic acid	Na^+, K^+, Ca^{2+}	CNQX, DNQX
NMDA	Glutamic acid	Na^+, K^+, Ca^{2+}	AP5, AP7
P_{2X}	ATP	Na^+, Mg^{2+}, Ca^{2+}	Suramine

*Non-complete list

Another group of the ligand-activated ion channels are represented by the intracellular ion channels that are activated on linkage with secondary messengers. These include the

a) cyclic nucleotide-gated receptor ion channels (non-selective cation channels),
b) inositol triphosphate (IP_3) receptor ion channel (calcium ion channel), and
c) ryanodine receptor ion channel (calcium ion channel).

There are several CNS-active drug molecules, the most important of which are the barbiturates and benzodiazepines., which exert their biological effects through binding to $GABA_A$ receptor.

Barbiturates are binding to the barbiturate specific binding site of the $GABA_A$ receptors. As a result, the of gamma-aminobutyric acid (GABA) mediated chloride ion current is increased due to the increased open status time of the channel. Thus, the membrane gets permanently hyperpolarized and excitability of the neuron is reduced. At higher concentrations they also inhibit the AMPA-activated glutamate receptors as well.

Benzodiazepines exert their effects through allosteric modulation: as a result of their binding to their specific binding site, sensitivity of the receptor for GABA is increased. The result is an increase in the frequency of chloride ion channel opening. (Different mechanism of action from barbiturates.)

II.1.2 **Receptors without own enzyme activity**

Whereas significant proportion of drugs exert their biological effects on G protein-coupled receptor, in the framework of this educational material only the only G protein-coupled receptors of the title class is discussed. Regulation via tyrosine kinase-coupled receptors and proteolysis-governed receptors is discussed in several number of good reviews.

II.1.2.1 G-protein-coupled receptors

Superfamily of G-protein coupled receptors (GFKR) includes a large number of receptors, which constitute one of the largest in the human genome (approx. 3% the encoded protein). These receptors involved in mediating information both of the internal environment (hormones, neurotransmitters) and of the external space (light, smells, tastes). This receptor family includes more than 300 receptors, and nearly 50%

of the present-day drugs exert their effect, through GFKRs. The receptors have no intracellular effector function, binding of their ligands results in activation of specific intracellular signal transduction pathways, which trigger the cell response.

G-Protein coupled receptors (GPCRs) are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices. The extracellular parts of the receptor can be glycosylated. These extracellular loops also contain two highly conserved cysteine residues that form disulfide bonds to stabilize the receptor structure. Some seven-transmembrane helix proteins (channel rhodopsin) that resemble GPCRs may contain ion channels, within their protein.

G-proteins are also embedded into the membranes but unlike the receptors they are mobile and able to communicate with the receptor and effector molecules. The latter may include (a) the intracellular ion channels (e.g., potassium ion channel, voltage-calcium ion channels), or (b) intracellular signal enzymes (e.g., adenylate cyclase, guanylate cyclase or phospholipase-C). This second level of the signal transduction is regulated by secondary messengers released by the effector molecules. The most important G-protein-activated messenger molecules (ions) and their important properties are summarized in Table II-2.

Table II-2. The most important G-protein-activated messenger molecules (ions).

Messenger molecule (ion)	**Source**	**Intracellular target molecules/cell organelles**	**Elimination**
Calcium ion	I. Plasma membrane: a.) voltage-gated calcium channels b.) ligand-activated calcium channels II. Endoplasmic reticulum: IP_3 receptors Ryanodine receptors	Calmoduline Protein-kinases Protein phosphatases Ion channels Further calcium binding proteins	I. Plasma membrane: 1. Na^+/Ca^{2+}-exchanger 2. Ca^{2+}-ATPase II. Endoplasmic reticulum: Ca^{2+}-ATPase III. Mitochondrion inner membrane: Ca^{2+}-uniporter
Cyclic AMP (cAMP)	Adenylate cyclase-ATP reaction	Protein kinase A Cyclic nucleotide-activated intracellular receptors	cAMP-phosphodiesterase
Cyclic GMP (cGMP)	Guanylate cyclase - ATP reaction	Protein kinase G Cyclic nucleotide-activated intracellular receptors	cAMP-phosphodiesterase
Inositol-triphosphate (IP_3)	Phospholipase C-PIP_2 reaction	Inositol-triphosphate (IP_3) receptors (endoplasmic reticulum)	Phosphatases
Diacyl glycerol (DAG)	Phospholipase C-PIP_2 reaction	Protein kinase C	Several enzymes

Note: PIP_2: Phosphatidylinositol bisphosphate

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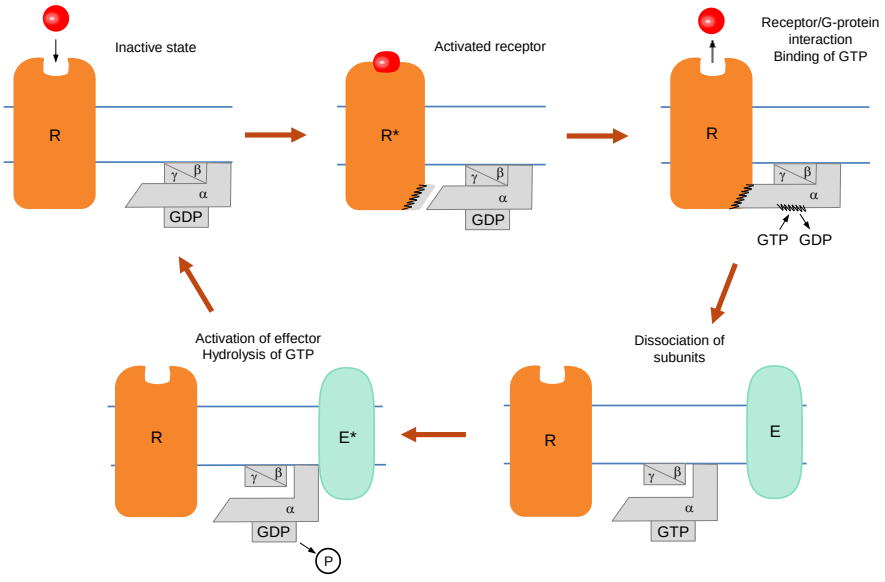
Besides the molecules mentioned in the Table, nitric oxide (NO), (such as an intracellular signal transduction molecule) should also be mentioned. It is synthesized from L-arginine by nitric oxide synthase in smooth muscle cells NO activates guanylate cyclase, which converts GTP into cGMP. CGMP activates protein kinase G - a serine/threonine kinase. The kinase is involved in relaxation of smooth muscle, regulation of platelet function, metabolism of the sperm cells, cell division and synthesis of nucleic acids.

In terms of structure, GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane (7-TM) α-helices (TM-1 to TM-7) connected by three intracellular (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3), and finally an intracellular C-terminus. The G protein-coupled receptor is activated by an external signal in the form of a ligand or other signal mediator. When the receptor is inactive, the IL2 and IL3 domains may be bound to an also inactive α-subunit of a heterotrimeric G-protein. These "G-proteins" are a trimer of α, β, and γ subunits (known as Gα, Gβ, and Gγ, respectively). G-proteins are inactive when they bind GDP, but active when bound to GTP. Upon activation, the receptor in turn, allosterically activates the G-protein by facilitating the exchange of a molecule of GDP for GTP at the G-protein's α-subunit. At this point, the subunits of the G-protein dissociate from the receptor, as well as each other, to yield a Gα-GTP monomer and a tightly interacting Gβγ dimer, which are now free to modulate the activity of other intracellular proteins. The extent to which they may diffuse, however, is limited due to the palmitoylation of Gα and the presence of an isoprenoid moiety that has been covalently added to the C-termini of Gγ. Because Gα also has slow GTP→GDP hydrolysis capability, the inactive form of the α-subunit (Gα-GDP) is eventually regenerated, thus allowing reassociation with a Gβγ dimer to form the "resting" G-protein, which can again bind to a GPCR and await activation. If the signal transmission takes place through the Gβγ subunit, modulation of ion-channel signaling (calcium and potassium ion channels) plays a role.

Figure II-4. Activation model of G- protein coupled receptors



R: receptor, E: enzyme, GDP: guanosine diphosphate, GTP: guanosine triphosphate, α, β , γ: subunits of G protein

On basis of their structure and function seven different Gα subunits can be distinguished (Table II-3).

Table II-3. Classification of G proteins on basis of their α-subunits

G-protein subunit α	Incidence	Function
G_s	all tissues	Adenylate cyclase activation Calcium ion channel activation
$G_{s(olf)}$	olfactory nerve	Adenylate cyclase activation
G_i	almost all tissues	Adenylate cyclase activation Calcium ion channel activation
G_t	retina	cGMP phosphodiesterase (transducin) activation
G_o	brain	Inhibition of calcium ion channel
G_q	almost all tissues	Phospholipase C activation
$G_{12/13}$	all tissues	Activation of monomer ("small"), G-proteins

The most important transmitter-activated G-protein-coupled receptors, as well as their agonists and/or antagonists used in therapy are. summarized in Table II-4.

Table II-4. The most important transmitter-activated G-protein-coupled receptors.

Receptor	Physiological agonists	Non-physiological agonist *	Antagonist *
Opioid (mu, kappa, delta)	enkephalins	Morphine	Naloxone, Naltrexone
Dopamine (D1-D5)	dopamine	Apomorphine (D2-D5) Bromocriptine (D2) Pergolide (D2-D4)	Chlorpromazine (D2) Levopromazin (D2) Sulpiride (D2 / D3) Tiapride (D2 / D3)
Serotonin (5-HT1-5-HT7	serotonin (5-HT)	Ergotamine (5-HT1B), Mescaline (5-HT2A), Trazodone (5-HT2C), 5-Methoxytryptamine (5-HT7)	Clozapine, Risperidone, Mianserin (5-HT2A), Amitriptyline, Clozapine, fluoxetine (5-HT2C), Amitriptyline, Clozapine, imipramine (5-HT7)
muscarinic	acetylcholine	Pilocarpine	Trihexyphenidyl, Metixene, Atropine, Homatropine,

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Receptor	Physiological agonists	Non-physiological agonist *	Antagonist *
			cyclopentolate, Procyclidine
Adrenergic (alpha)	noradrenaline, adrenaline	Phenylephrine, Clonidine, Guanfacine	Phentolamine, Ergotamine, Prazosin, Terazosin, Doxazosin
Adrenergic (alpha + beta)	noradrenaline, adrenaline	Isoprenaline, Dobutamine, Ephedrine	
Adrenergic (beta)	noradrenaline adrenaline	Salbutamol, Terbutaline, Fenoterol,	Oxprenolol, Propranolol, Pindolol, Atenolol, Betaxolol
$GABA_B$	GABA	Baclofen	Saclofen
Glutamate (NMDA)	glutamic acid	N-methyl-D-aspartate (NMDA)	Amantadine
Histamine (H1)	histamine	2-Pyridyl ethylamine	Diphenhydramine, Dimethindene, Chloropyramine, Cetirizine, Promethazine, Loratadine, Terfenadine, Astemizole
Histamine (H2)	histamine	5-Methylhistamine	Cimetidine, Ranitidine, Famotidine
Angiotensin-I	Angiotensin-II	Novoquinidine	Losartan, Valsartan, Irbesartan

* Not a complete list.

II.1.3 **Receptors with own (intrinsic) enzymatic activity**

Receptors with own enzymatic activity are multi-subunit transmembrane proteins, of which intracellular domain possess intrinsic enzymatic activity. As a result of ligand binding, a conformational change takes place in the transmembrane helices, which leads triggering the enzymatic activity and initiating the signaling cascade. Receptors with own enzymatic activity can be classified as follows.

1. Receptor tyrosine kinases (pl. insulin, EGF, VEGF, PDGF and FGF receptors) phosphorylate specific tyrosine units on a small set of intracellular signaling proteins.

2. *Tyrosine-kinase-associated receptors* associate with intracellular proteins that have tyrosine kinase activity. *Cytokines* are the main ligands that signal through tyrosine kinase-associated receptors.

3. *Receptorlike tyrosine phosphatases* remove phosphate groups from tyrosine units of specific intracellular signaling proteins. (They are called "receptorlike" because the presumptive ligands have not yet been identified, and so their receptor function has not been directly demonstrated.)

4. *Receptor serine/threonine kinases* phosphorylate specific serine or threonine units on associated latent gene regulatory proteins.

5. *Receptor guanylyl cyclases* directly catalyze the production of cyclic GMP in the cytosol.

6. *Histidine-kinase-associated receptors* activate a "two-component" signaling pathway in which the kinase phosphorylates itself on histidine and then immediately transfers the phosphate to a second intracellular signaling protein.

Receptors with tyrosine kinase activity have paramount pharmacological importance among receptors with intrinsic enzyme activity. . The extracellular signal proteins that act through *receptor tyrosine kinases* consist of hormones (e.g., insulin) and a large variety of secreted growth factors. Notable examples include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulinlike growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and all the neurotrophins, including nerve growth factor (NGF).

Targeting this latter receptor family resulted in explosive growth in some areas of drug development. So it is to be mentioned

1. *imatinib*, which can inhibit activity of epidermal growth factor (EGF), which is frequently over-expressed in tumor cells, as well as
2. *rituximab*, *cetuximab* and *bevacizumab* which can inhibit activity of epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), respectively. These latter are also overexpressed in some sorts of tumor cells.

The above drugs influencing signal transduction pathways related to tyrosine kinase receptors can effectively prevent pathologically increased proinflammatory cytokine production in autoimmune diseases or growth of certain malignant tumors.

II.2 Intracellular receptors

Intracellular receptors are complex molecules of receptor properties localized in the cell membrane. Intracellular receptors can be classified into two main categories:

(a) nuclear receptors (nuclear receptors), and

(b) receptors that are integrated into membranes of intracellular organelles (e.g., endoplasmic (sarcoplasmic) reticulum, mitochondria) (The latter sort of receptors are not discussed in the present educational material.)

Nuclear receptors primarily mediate effects of lipophilic hormones (e.g., steroid hormones, thyroid hormones, retinoids), vitamins D, lipid sensors as well as that of fatty acids and arachidonic acid derivatives. Nuclear receptors are located in the cytoplasm (type I receptors) or in the nucleus (type. II receptors) linked to co-repressor proteins (e.g. HSP90) or DNA.

Based on sequence homology nuclear receptors are classified into

I. Thyroid Hormone Receptor-like:

- Thyroid hormone receptor (TR)
- Vitamin D-receptor-like (VDR)
  - Vitamin D receptor
  - Pregnane X receptor (PXR)
  - Constitutive androsteron receptor (CAR)
- Retinoic acid receptor (RAR)
- Liver X receptor-like
  - Liver X receptor (LXR)

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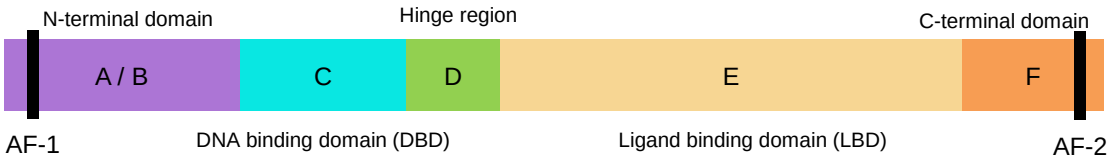
Farnesoid X receptor (FXR)
Peroxisome proliferator-activated receptor (PPAR)
RAR-related orphan receptor
II. Retinoid X Receptor-like
Retinoid X receptor (RXR)
III. Estrogen Receptor-like
Mineralocorticoid receptor (MR)
Glucocorticoid receptor (GR)
Estrogen receptor (ER)
Progesterone receptor (PR)
Androgen receptor (AR)
IV. Nerve Growth Factor IB-like
Nerve Growth factor IB
V. Steroidogenic Factor-like
Steroidogenic factor 1 (SF1)
VI. Germ Cell Nuclear Factor-like
Germ cell nuclear factor

Nuclear receptors are ligand-dependent transcription factors. Structurally they are highly conserved, single polypeptide chains, which contain in the central region a cysteine-rich, Zn(II) ion-binding, so-called. 'zinc finger' regions (LBD), which allows the molecule to bind to the corresponding region of the nuclear DNA strand. The C-terminal region of the receptor can bind its specific steroid ligand (Figure II-5).

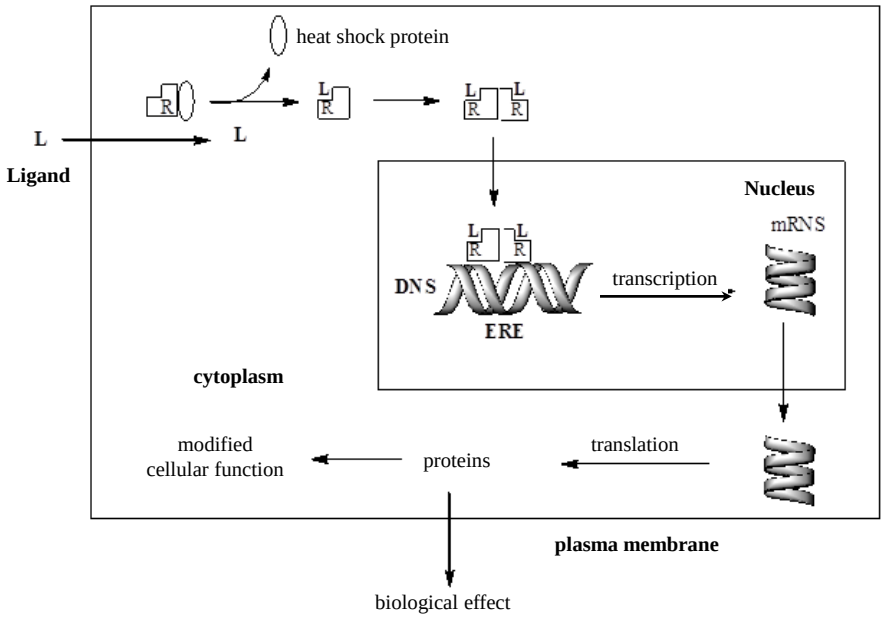
Figure II-5. Simplified structure of nuclear receptors



AF-1: activation function 1
AF-2: activation function 2

Molecular description of operation of nuclear receptors in example of the steroid hormones can be described as follows. Small lipophilic substances such as natural hormones diffuse through the cell membrane and bind to nuclear receptors located in the cytosol (type I NR) of the cell. Hormone binding to the NR triggers dissociation of heat shock proteins (HSP), dimerization, and translocation to the nucleus, where the NR binds to a specific sequence of DNA known as an estrogen response element (ERE). The nuclear receptor DNA complex in turn binds to other proteins that are responsible for transcription of downstream DNA into mRNA, which is eventually translated into protein, which results in a change in cell function (Figure II-6).

Figure II-6. Mechanism of action of estrogen receptors



Type II receptors, in contrast to type I, are retained in the nucleus regardless of the ligand binding status and in addition bind as hetero-dimers (usually with RXR) to DNA. In the absence of ligand, type II nuclear receptors are often complexed with corepressor proteins. Ligand binding to the nuclear receptor causes dissociation of corepressor and recruitment of coactivator proteins. Additional proteins including RNA polymerase are then recruited to the NR/DNA complex that transcribe DNA into messenger RNA.

Several candidate drug molecules developing interactions with nuclear receptors has been underwent clinical trials and has been used to treat various diseases. For example, sexual steroids are used in treatment of hormone substitution therapies (endocrine diseases), breast tumors and used as contraceptives. Synthetic glucocorticoid analogues are used as anti-inflammatory and immunosuppressive agents (e.g. Autoimmune diseases, transplantation, some leukemia). Thyroxine is used in substitution therapy after thyroidectomy, Vitamins A and D are applied in vitamin deficiency. The most important nuclear receptors as well as their agonists and/or antagonists used as a medicine are.summarized in Table. II-5.

Table II-5. Nuclear receptors and their clinically useful agonists and antagonists

Receptor	Physiological agonists	Non-physiological agonist *	Antagonist*
Thyroid hormone receptor (TR)	triiodothyronine, teraiodothyronine,	dextro thyroxine	
Mineralocorticoid receptor (MR)	corticosterone, cortisol, aldosterone	dexamethasone	spironolactone, canrenone, eplerenone
Glucocorticoid receptor (GR)	cortisol, corticosterone	prednisone, prednisolone, betamethasone, triamcinolone	mifepristone (mixed)
Estrogen receptor (ER)	estrone, estradiol, estriol	ethinyl estradiol, mestranol,	clomiphene, tamoxifen,

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Receptor	Physiological agonists	Non-physiological agonist *	Antagonist*
		diethylstilbestrol, dienestrol	raloxifene (a mixed agonist / antagonist) fulvestrant
Progesterone receptor (PR)	progesterone	ethisterone, noretisterone, levonorgestrel, medroxyprogesterone,	mifepristone (mixed), onapristone
Androgen receptor (AR)	dihydrotestosterone, testosterone	methyltestosterone, fluoxymesterone, nandrolone, danazol	cyproterone, flutamide, bicalutamide

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III Enzymes as drug targets

Enzymes are biocatalysts of the body. Operation of enzymes – similar to other catalysts – is characterized by significantly reducing activation energy of the catalyzed reactions. Change of free energy change and numerical value of equilibrium constant expressions, however, are not effected in the catalyzed reactions. As a result, velocity of reactions at a set temperature is increasing. The time needed for completion of reactions or reaching the state of equilibrium is reduced.

The beginning of the modern age of enzyme research is dated to the time of *J.B. Sumner* works, who was able to purify and crystallize urease (1926). Sumner showed that enzymes are proteins in nature.

Since that time it has proved that, most of the isolated enzymes are simple or complex proteins (Although a few are catalytic RNA molecules, such molecules are out of the scope of this educational material.) Accordingly, each physico-chemical characteristics of proteins (see Chapter 1) are characterizing enzymes as well. Thus, structural and three-dimensional structure, characteristic physical-chemical properties of macromolecules, environment-dependent conformation, all play an important role in catalytic function of enzymes.

III.1 Mechanism of enzyme-catalyzed reactions

Enzymes are catalysts (generally proteins) that help to convert other molecules called substrates, into products, but they themselves are not changed by the reaction. Their most important features are catalytic power, specificity and regulation. Enzymes accelerate the conversion of *substrates* into *products* by lowering the free energy of activation of the reaction. Enzymes are particularly efficient at speeding up biological reactions, giving increase in speed up to 10 million times or more. They are also highly specific, usually catalyzing the reaction of only one particular substrate or closely related substrates. Finally, they are typically regulated by various positive and negative feedback systems, thus allowing precise control over the rate of reaction

An enzyme works by binding to a given substrate in such a geometrical fashion that the substrate is able to undergo its inherent reaction at a more rapid rate. This type of reaction is commonly referred to as the *lock and key mode* for enzyme action. It implies that there is a particular part of the enzyme structure, the active site, which specifically binds sterically to a substrate. The enzyme does not actually react with the substrate but merely brings the substrate into the proper alignment or configuration for it to react spontaneously or in conjunction with another substance. Since a reaction proceeds normally by a random kinetic action of molecules bumping into each other, any time molecules are aligned, they will react faster. Thus, for any given enzyme there will be a best fit configuration to the protein in order to align the substrate and to facilitate the reaction. When the enzyme is in its ideal configuration, the reaction will proceed at its maximum rate, and the overall rate of activity will be dependent upon substrate concentration.

Enzymes act as catalysts because of their three dimensional protein structure. This structure is controlled by many factors, but is particularly sensitive to changes in pH, salts and temperature. Small changes in the *temperature* of a reaction can significantly alter the reaction rate, and extremely high temperatures can irreversibly alter both the three dimensional structure of the enzyme and its activity. It may even render the enzyme non-functional; that is, to denature the enzyme. *Salts* can also cause

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denaturation, but the effects of ammonium sulfate are usually reversible. *Heavy metal salts*, by contrast, usually irreversibly alter the structure of the protein, and thus their routine use as fixatives in histological work.

An important point about enzymes is that they are very specific about what they can catalyze. Even small changes in the reactant molecule can stop the enzyme from catalyzing its reaction. The reason for this lies in the *active site* present in the enzyme. All enzymes have an active site, where the reaction is catalyzed Active sites are cracks or hollows on the surface of the enzyme caused by the way the protein folds itself up into its tertiary structure. Molecules of just the right shape (called the *substrate*) and with just the right arrangement of attractive groups can fit into these active sites. Other molecules will not fit or will not have the right groups to bind to the surface of the active site. The usual analogy for this is *a key fitting into a lock*. For the key to work properly it has to fit exactly into the lock. This simplified mechanism was proposed by E. Fischer in 1890.

Some of the enzymes are functioning as simple protein molecules; there are some others, however, which need further, non-protein molecules as well for their proper operation. These molecules are called *cofactors*. Cofactors can be (a) prosthetic groups, (b) coenzymes and (c) metal ions. Prosthetic groups are metal ion or organic molecules, which are covalently bound to the enzyme protein. Coenzymes are also relatively small molecular weight organic molecules, which are linked to the enzyme via non-covalent interactions. Most of the vitamins have coenzyme function. Coenzymes transport chemical groups from one enzyme to another and are released from the enzyme's active site during the reaction. Enzymes with coordinatively bound metal ions are called *metalloenzymes*. Enzymes that require a cofactor but do not have one bound are called *apoenzymes* or *apoproteins*. An enzyme together with the cofactor(s) required for activity is called a *holoenzyme* (or *haloenzyme*).

Metal ions play roles in approximately one-third of the known enzymes. Metal ions may be a co-factor or they may be incorporated into the molecule, and these are known as *metalloenzymes*. Amino acids in peptide linkage possess groups that can form coordinate-covalent bonds with the metal atom. The free amino and carboxyl group bind to the metal affecting the enzymes structure resulting in its active conformation. Metal ions main function is to serve in electron transfer. Many enzymes can serve as electrophiles and some can serve as nucleophilic groups. This versatility explains metals frequent occurrence in enzymes. Some metalloenzymes include *hemoglobins, cytochromes,* phosphotransferases, alcohol dehydrogenase, arginase, *ferredoxin,* and *cytochrome oxidase.*

III.2 Classification and nomenclature of enzymes

Based on catalyzed reactions, the nomenclature committee (Enzyme Commission, E.C.) of the International Union of Biochemistry and Molecular Biology (IUBMB) recommended the following classification.

E.C.1. Oxidoreductases

To this class belong all enzymes catalyzing oxidoreduction reactions. The substrate that is oxidized is regarded as hydrogen donor. The systematic name is based on *donor:acceptor oxidoreductase*. The common name will be *dehydrogenase,* wherever this is possible; as an alternative, *reductase* can be used. *Oxidase* is only used in cases where O_2 is the acceptor. Cytochrome P-450 (CYP) and flavin monooxidase

(FMO) enzymes are playing important role in oxidative metabolism of drugs and other xenobiotics. Coenzymes participating in the reactions of the catalyzed reactions as follows.

Nicotinic acid adenine dinucleotide (phosphate) (NAD^+ and $NADP^+$)
Vitamin B_3 (niacin) (precursor of NAD^+ and $NADP^+$)
Flavin mononucleotide (FMN)
Flavin adenine dinucleotide (FAD)
Vitamin B_2 *vitamin* (riboflavin) (precursor of FMN and FAD)
Lipoic acid

E.C.2. Transferases

Transferases are enzymes transferring a group, *e.g.* a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor). The systematic names are formed according to the scheme *donor:acceptor grouptransferase*. The common names are normally formed according to *acceptor grouptransferase* or *donor grouptransferase*. In many cases, the donor is a cofactor (coenzyme) charged with the group to be transferred. Coenzymes participating in the reactions of the catalyzed reactions as follows.

Adenosine triphosphate (ATP)
Cyclic adenosine monophosphate (cAMP)
Cytidin monophosphate (CMP)
Uridin diphosphate (UDP)
Thiamine pyrophosphate
Vitamin B_1 (thiamine) (precursor of thiamine pyrophosphate)
Pyridoxal phosphate
Vitamin B_6 (pyridoxine) (precursor of pyridoxal phosphate)
Tetrahydro folic acid (THF)
Folic acid (precursor of THF)
Biotin (Vitamin H)
Coenzyme-A (CoA)
Pantothenic acid (precursor of CoA)
S-Adenosyl methionine (SAM)
3'-Phosphoadenosine-5'-phosphosulfate (PAPS)

E.C.3. Hydrolases

These enzymes catalyze the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Although the systematic name always includes *hydrolase*, the common name is, in many cases, formed by the name of the substrate with the suffix *-ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

A number of hydrolases acting on ester, glycosyl, peptide, amide or other bonds are known to catalyze not only hydrolytic removal of a particular group from their substrates, but likewise the transfer of this group to suitable acceptor molecules. In principle, all hydrolytic enzymes might be classified as transferases, since hydrolysis itself can be regarded as transfer of a specific group to water as the acceptor.

E.C.4. Lyases

Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. The

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systematic name is formed according to the pattern *substrate group-lyase*. In the common names, expressions like *decarboxylase, aldolase, dehydratase* (in case of elimination of CO_2, aldehyde, or water) are used. In cases where the reverse reaction is much more important, or the only one demonstrated, *synthase* (not synthetase) may be used in the name. Coenzymes participating in the reactions of the catalyzed reactions as follows.

Thiamine pyrophosphate (*Vitamin B_1*)
Pyridoxal phosphate (*Vitamin B_6*)
Vitamins K

E.C.5. Isomerases

These enzymes catalyze geometric or structural changes within one molecule. According to the type of isomerism, they may be called *racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases* or *cycloisomerases*. Coenzymes participating in the reactions of the catalyzed reactions as follows.

Glucose-1,6-diphosphate
Glyceric acid-2,3-diphosphate
5-Deoxyadenosylcobalamin (coenzyme form of *Vitamin B_{12}*)

E.C.6. Ligases

Ligases are enzymes catalyzing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The systematic names are formed on the system *X:Y ligase (ADP-forming).* In earlier editions of the list the term *synthetase* has been used for the common names. Many authors have been confused by the use of the terms *synthetase* (used only for Group 6) and *synthase* (used throughout the list when it is desired to emphasis the synthetic nature of the reaction). Consequently, NC-IUB decided in 1983 to abandon the use of synthetase for common names. Coenzymes participating in the reactions of the catalyzed reactions as follows.

Adenosine-triphosphate (ATP)
Nicotinic acid adenine dinucleotide (phosphate) (NAD^+ and $NADP^+$)
Vitamin B_3 (niacin) (precursor of NAD^+ and $NADP^+$)

III.3 Kinetics of enzyme-catalyzed reactions

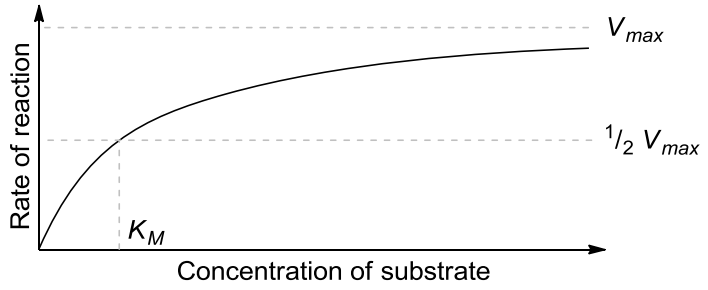
Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or an agonist might inhibit the enzyme. The two most important kinetic properties of an enzyme are how quickly the enzyme becomes saturated with a particular substrate, and the maximum rate it can achieve. Knowing these properties suggests what an enzyme might do in the cell and can show how the enzyme will respond to changes in these conditions.

One of the first things to realize about enzymes reaction is that they do not follow the law of mass action directly. As the concentration of substrate is increased, the rate of the reaction increases only to a certain extent, reaching a maximal reaction velocity at high substrate concentration. This is in contrast with the mass action law, which, when

applied directly to the reaction with the enzyme predicts that the reaction velocity increase linearly as the substrate increases.

The rates of enzyme-catalyzed reactions show a characteristic dependence on substrate concentration (Figure III-1). At low substrate concentration, the initial rate is proportional to both the concentration of the enzyme and the substrate (a second-order reaction). At the substrate concentration increases, it becomes easier for the enzyme to find the substrate, until when all of the enzyme active sites are occupied with bound substrate (or product). At this point the enzyme is said to be *saturated* (i.e., all of the active sites have substrate or product bound), and further increases in the substrate concentration will not increase the rate of the enzyme reaction. This is the maximal rate or $V_{\max}$, which is attained really only at infinite substrate concentration, so it is never fully achieved. At this point, the rate is zeroth order with respect to concentration of the substrate and is dependent only on the enzyme concentration.

Figure III-1. Saturation curve for an enzyme reaction showing the relation between the substrate concentration [S] and reaction rate (V_{max}: maxima rate, K_M: Michaelis-Menten constant)



According to the theory of *L. Michaelis* and *M. Menten*, kinetics of the enzyme-catalyzed reactions can be described by the following simple form:

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{\text{cat}}} E + P \qquad (1)$$

where E = enzyme
S = substrate
ES = enzyme-substrate complex
P = product
$k_{1=}$ rate constant of formation of the enzyme –substrate complex
k_2 = rate constant of dissociation of the enzyme –substrate complex
k_3 = rate constant of formation of the product

Accordingly, the rate of the enzyme-catalyzed reaction (v):

$$v = k_3 \text{ [ES]} \qquad (2)$$

and the rate of formation and dissociation of the ES complex:

$$v_k = k_1 \text{ [[E] [S]} \qquad (3)$$

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$v_d = k_2$ [ES] (4)

can be described by the (2)-(4) rate equations.

At a given [E_t] concentration of the enzyme, part of the enzyme molecules can be found in free from [E], and the rest of the molecules form enzyme-substrate complex [ES] :

[E_t] = [E] +[ES] (5)

The rate of the reaction reaches its maximal value (v_{max}), when all the enzyme molecules [E_t] form enzyme-substrate complex. Then [ES] = [E_t] and

$v_{max} = k_3$ [E_t] (6)

From the above rate equations it can be concluded

$$\frac{v}{v_{max}} = \frac{[ES]}{[E_t]}$$

Based on equation (1)

rate of formation of (ES) = k_1 [E] [S] (8)

and

rate of dissociation of (ES) = k_2 [ES] + k_3 [ES] = ˙(k_2+k_3) [ES] (9)

After starting the reactions, an equilibrium („steady state") concentration of the (ES) complex is set, which is the result of the same rate of formation and rate of dissociation of it. Then:

k_1 [E] [S] = (k_2+k_3) [ES] (10)

Rearranging equation (10):

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

The (k_2+k_3)/(k_1) expression can be unified in a new constant, which is called *Michaelis-Menten constant* (K_M):

$$K_M = \frac{k_2 + k_3}{k_1}$$

Introducing K_M :

$$[ES] = \frac{[E][S]}{K_M}$$

can be obtained.

Taking equation (5) into consideration:

$$[E_t] = \frac{[E][S]}{K_M} + [E]$$

Incorporation of above values of [ES] and [E_t] into equation a (7)

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

can be obtained. Rearrangement of the equation results the most common form of the Michaelis-Menten equation:

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

This equation is used to describe the empirical relationship between the enzyme-catalyzed reaction rate the (v) and substrate concentration ([S]) shown in Figure I-1. At low substrate concentration, where [S] is much smaller than the K_M, then v=[S] v_{max}/K_m, that is, the speed is directly proportional to the substrate concentration. At high substrate concentration, where [S] is much greater than the K_M, $v = v_{max}$, i.e. the maximum speed is independent of the substrate concentration.

If the reaction rate is half the maximum speed,
that is, v = 0.5 v_{max}:

$$0{,}5\ v_{max} = \frac{v_{max}\,[S]}{[S] + K_M}$$

namely

$$[S] + K_M = 2\,[S]$$

and

$$K_M = [S]$$

Thus, the Michaelis-Menten constant is numerically equal to the substrate concentration at which the reaction rate half of the maximum (v_{max}). High K_M value indicates weak, small K_M indicates strong interaction between the enzyme and the substrate.

Note that this description of enzyme kinetics applies only to the simplest reactions in which the enzyme reacts with a single substrate and catalyzes the chemical transformation. Realistically, reactions occur more complex in the body, but in principle the same way.

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III.4 Inhibition of enzyme reactions

In drug discovery, several drug analogues are chosen and/or designed to inhibit specific enzymes. However, detoxification or reduced toxic effect of many antitoxins is also accomplished mainly due to their enzyme inhibitory action. The enzyme inhibitors are low molecular weight chemical compounds. They can reduce or completely inhibit the enzyme catalytic activity either *reversibly* or *permanently (irreversibly*). Inhibitory action of *reversible inhibitors* is reversible because they make reversible association with the enzyme. *Irreversible inhibitors* make inactivating irreversible covalent modification of an essential residue of the enzyme. Studying enzyme kinetics and structure-function relationship is one of the frequently used method to understand the nature and kinetics of enzyme inhibition that, in turn, is fundamental to the modern design of pharmaceuticals in industries

Conceptually, enzyme inhibitors are classified into two types: *non-specific* inhibitors and *specific* inhibitors.

Non-specific irreversible inhibitors include all protein denaturating factors (physical and chemical denaturation factors).

Specific inhibitors attack a specific component of the holoenzyme system. The action depends on increased amount of substrate or by other means of physiological conditions.Specific inhibitors can be described in several forms including;

1) coenzyme inhibitors: e.g., cyanide, hydrazine and hydroxylamine that inhibit pyridoxal phosphate, and, dicumarol that is a competitive antagonist for vitamin K;

2) inhibitors of specific ion cofactor: e.g., fluoride that chelates Mg^{2+} of enolase enzyme;

3) prosthetic group inhibitors: e.g., cyanide that inhibits the heme prosthetic group of cytochrome oxidase; and,

4) apoenzyme inhibitors that attack the apoenzyme component of the holoenzyme;

5) *physiological modulators* of reaction pH and temperature that denature the enzyme catalytic site.

Specific inhibitors can be categorized into three groups. Inhibition can be reversible or irreversible in each group.

1. Competitive (or substrate anaologue) inhibitors.
2. Enzyme-substrate complex (ES) inhibitors. (Uncompetitive inhibitors.)
3. Non-competitive inhibitors. (Mixed type inhibitors.)

1. The *competitive inhibitor* is structurally related to the substrate and binds reversibly at the active site of enzyme and occupies it in a mutually exclusive manner with the substrate. Therefore, the competitive inhibitor competes with the substrate for the active site. The binding is mutually exclusive because of their free competition. According to the law of mass action, relatively higher inhibitor concentration prevents the substrate binding. Since the reaction rate is directly proportional to [ES], reduction in ES formation for EI formation lowers the rate. Increasing substrate towards a saturating concentration alleviates competitive inhibition.

2. *Uncompetitive inhibitor* has no structural similarity to the substrate. It may bind the free enzyme or enzyme substrate complex that exposes the inhibitor binding site (ESI). Its binding, although away from the active site, causes structural distortion of the active

and allosteric sites of the complexed enzyme that inactivates the catalysis. Uncompetitive reversible inhibition is rare, but may occur in multimeric enzymes.
3. The mixed type (non-competitive) inhibitor does not have structural similarity to the substrate but it binds both of the free enzyme and the enzyme-substrate complex. Thus, its binding manner is not mutually exclusive with the substrate and the presence of a substrate has no influence on the ability of a non-competitive inhibitor to bind an enzyme and *vice versa*. However, its binding - although away from the active site - alters the conformation of the enzyme and reduces its catalytic activity due to changes in the nature of the catalytic groups at the active site. EI and ESI complexes are nonproductive and increasing substrate to a saturating concentration does not reverse the inhibition. Reversal of the inhibition requires a special treatment, e.g., dialysis or pH adjustment.
As it was mentioned before, several drug molecules exert ther biological effects thrugh inhibition of enzymes. Some characteristic enzyme inhibitors are summarized in Table III-1.

Table III-1. Drugs with enzyme inhibitor effect

Enzyme	Substrate	Anti-detergent *	Therapeutic significance
GABA-transzamináz	gamma-aminobutyric acid (GABA)	vigabatrin (irreversible)	antiepileptic drug
DOPA decarboxylase	3,4-dihydroxy-phenylalanine (DOPA)	carbidopa, benserazide	drugs to reduce side effects of Parkinson's disease
Monoamine oxidase B (MAO-B)	dopamine	selegiline, rasagiline	Anti-Parkinson drugs
Acetylcholinesterase	acetylcholine	physostigmine, neostigmine, diisopropyl fluorophosphate phosphate (irreversible)	antiglaucoma drugs
Phosphodiesterase 3 (PDE3)	cAMP, cGMP	amrinone, milrinone, enoximone	cardiotonics
Angiotensin-converting (ACE)	angiotensin II	captopril, enalapril, lisinopril, spirapril	antihypertensive agents
HMG-CoA reductase	hydroxymethyl-glutaryl-coenzyme-A	clofibrate, fenofibrate, gemfibrozil, lovastatin, simvastatin, pravastatin, atorvastatin	antihyperlipidemic drugs
carbonic anhydrase	carbon dioxide	acetazolamide,	antiglaucoma and

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Enzyme	Substrate	Anti-detergent *	Therapeutic significance
		methazolamide, dorzolamide	antidiuretic drugs
Thyroid peroxidase (TPO)	tyrosine	propylthiouracil, carbimazol methiamazole,	anti-hyperthyroidism drugs
cyclooxygenase	arachidonic acid	acetylsalicylic acid (irreversible) indomethacin, diclofenac, celecoxib	antipyretic, analgesic/non-steroidal anti-inflammatory drugs
Xanthine oxidase	xanthine	allopurinol (irreversible)	antigout drugs
DD-transpeptidase	D-alanine	penicillin and cephalosporin antibiotics (irreversible)	antibacterial agents

*Incomplete list

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IV Transporters and ion channels as drug targets

Permeation of polar compounds through the double lipid layers of cell membranes is very limited. The only exception is water, which is freely permeates if there is osmotic difference between the two sides of the membrane. Water and the other small, uncharged polar molecules (e.g., urea) get through the holes, which are formed as result of continuous movement of the fatty acid side chains of phospholipids. On the contrary, the lipid bilayer of cell membranes is virtually impermeable for molecules carrying either positive or negative charge. However, small hydrophobic (non-polar) molecules (e.g., molecular oxygen, carbon dioxide, nitrogen) can get through the lipid bilayers. Such processes are called *simple diffusion*. Simple diffusion of drugs across a biological membrane is a natural tendency of molecules to move from the higher concentration to one of lower concentration. Such movement of molecules occurs without interaction with proteins or other membrane-forming components. This movement of molecules is caused by the kinetic energy of the molecules.

Unlike simple lipid bilayers, polar molecules can permeate the biological membranes. This is due to the proteins embedded into the lipid bilayer of the biological membranes, which provide selective absorption of polar molecules. Among these protein complexes transporters and ion channels play important role in absorption of exogenous molecules.

IV.1 Facilitated (carrier mediated) diffusion

After oral ingestion, medicinal products (xenobiotics) are absorbed through the epithelial cells of the oral cavity and the gastrointestinal system. Although most of the drugs (xenobiotics) are absorbed by passive diffusion, several of them are absorbed by so-called facilitated (carrier mediated) diffusion. In this type of transport, particles are moving down their concentration gradient by involvement of specific *proteins embedded* membrane (Figure IV-1). Being passive, facilitated diffusion does not directly require chemical energy from ATP hydrolysis.

All proteins studied so far are transmembrane proteins, which cross the membrane several times. This way, they are providing a continuous transmembrane pathway to the hydrophilic molecules preventing them from interaction with the lipophilic lipid bilayer of the membrane. These proteins can be categorized into two groups:

1. *Carrier proteins*, also known as *transporters*.

These the proteins are developing non-covalent interactions with the transported molecules at the extracellular or intracellular side of the membrane. Interaction of the proteins with the transported molecules (e.g., glucose, amino acids) results in change of conformation of the proteins, which allows dissociation of the transported molecule at the intracellular or extracellular side, respectively.

2. *Channel proteins.*

This type of proteins are not developing interactions with the transporting particles. They constitute a hole (pore) with hydrophilic interior crossing the membrane, through which the ions, or small molecules are transported. Transportation through the channels is much faster (about 1000 times) faster than the transporter-mediated processes.

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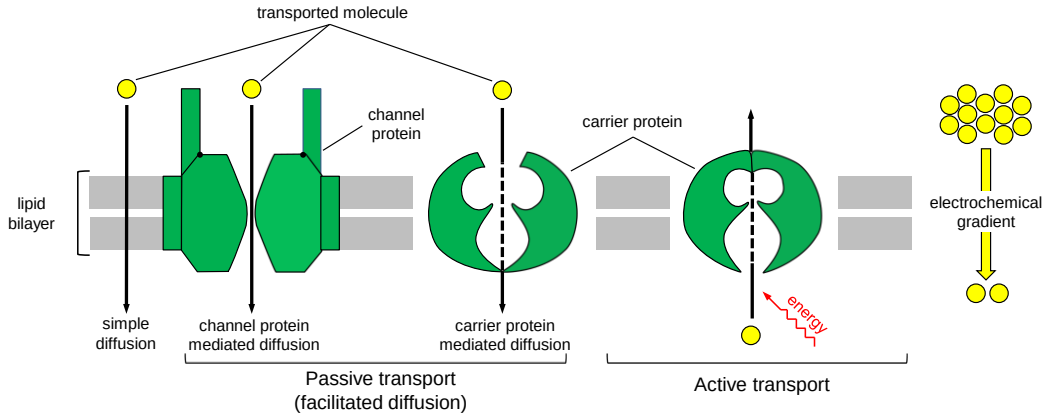
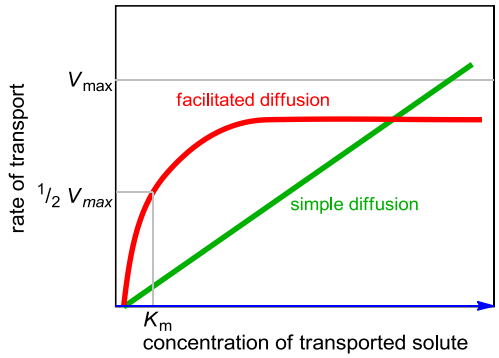
Figure IV-1. Simplified mechanism of simple diffusion, facilitated (career-mediated) diffusion and active transport.



Facilitated diffusion in many ways is similar to that of the enzyme-catalyzed reactions - but in this case new product is not formed. The transporting molecules develop non-covalent interactions with the proteins embedded into the membrane, and translocation of the molecules become possible as a consequence of the binding-induced conformational change of the proteins. The interactions ("molecular recognition") are structure and stereo specific. The process can be inhibited in competitive and non-competitive manner.

If the speed of transport is studied as a function of concentration of the transporting substance, a saturation curve similar to that of the enzyme-catalyzed reactions is obtained. (Figure IV-2). Based on the curve the maximum speed (in analogy to the Michaelis-Menten constant) and the K_t value of the transport can be determined. In case of facilitated diffusion substances are always mowing down their concentration gradient until they reach their equilibrium concentrations. On the contrary, similar investigation of simple diffusions shows the speed of transport process to be directly proportional to the concentration gradient of the transported compound. (Figure IV-2).

Figure IV-2. Concentration dependence of simple diffusion and facilitated diffusion.

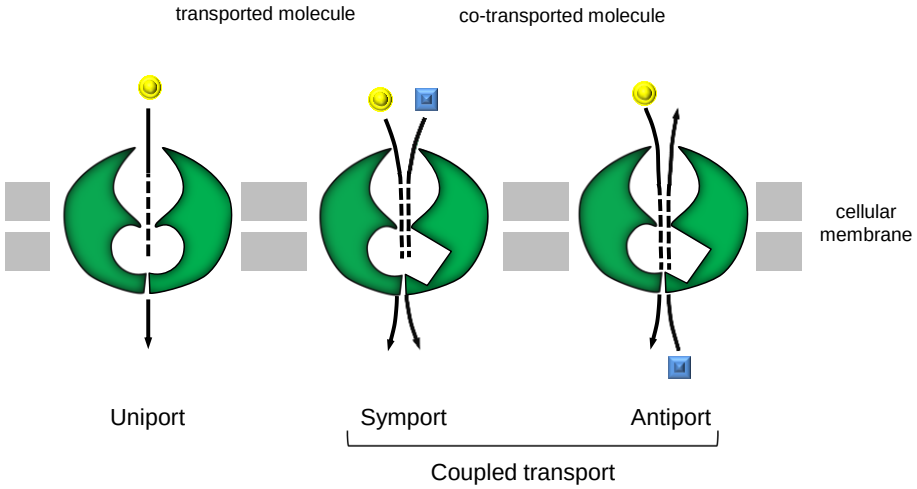


Uniporter carrier proteins work by binding to one molecule of at a time and transporting it with its concentration gradient. One example of such transporters is *glucose transporter* (GLUT-1) embedded into the erythrocyte membrane. Glucose enters the erythrocyte via this specific glucose transporter, at a rate about 50,000 times greater than uncatalyzed transmembrane diffusion.

Other carrier proteins, however, transport two or more different molecules or ions across the cell membrane simultaneously. These type of transporters are called *cotransporters* (or *coupled transporters*), which can be (a) *symporters*, or (b) *antiporters*.

Symporters are integral membrane proteins that are involved in the transport of two or more different types of molecules or ions across the cell membrane in the same direction in relation to each other. *Antiporters (*also called *exchanger* or *counter-transporter*) are integral membrane proteins that involved in transport of two or more different molecules or ions across the cell membrane in opposite directions (Figure IV-3).

Figure IV-3. Principle of operation of *uniport*, *symport* and *antiport* transporters



One of the important antiporters – also found in the erythrocyte membrane - is the *chloride (Cl^-)-bicarbonate (HCO_3^-) exchanger*. It should be noted that during operation of cotransporters (*coupled transporters)* one species of solute moves along its electrochemical gradient, allowing a different species to move against its own electrochemical gradient. These transport processes can be categorized into the so called *secondary active transport* processes (see later).

IV.2 Energetics of transport processes

Energetics of transport processes is characterized by change in free energy accompanied the movement of particles across the membrane. Change in free energy of the transporting material across membrane depends on concentration gradient across membrane. In case of neutral particles, the change can be expressed by the formula as follows.

$$\Delta G = 2{,}3\ RT\ log\frac{[c_2]}{[c_1]}$$

where

ΔG = change in free energy
R = universal gas constant
T = temperature (K)
$[c_2]$ and $[c_1]$ = concentration ($mol(dm^3)^{-1}$) of solute (molecule or ion) in the opposite side of the membrane

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If solute is transported from a solution of higher concentration (c_1) to the solution of lower concentration ($c_1>c_2$), the sign of ΔG is negative; i.e. the transport takes place spontaneously (*passive transport*). For the opposite transport process ΔG is positive, i.e. in the process is nonspontaneous, it takes place only with investment of energy (*active transport*).

In case of transport of ions, the change of free energy depends not only on the chemical concentration gradient but on the difference of the total charge on the two sides of the membrane (membrane potential) as well. In these cases, the above formula is modified as follows.

$$\Delta G = 2{,}3\ RT\ log\frac{[c_2]}{[c_1]} + ZF\Psi$$

where

Z = the charge of transporting ion
F = the Faraday constant (96480 J/ (Vmol)
Ψ = the membrane potential (V)

Therefore, energetics of transport of an ion is determined by the *electrochemical potential* – the sum of the concentration and the electrochemical gradients.

IV.3 Active transport

There are several transport processes in which molecules (ions) are transported against the concentration (and/or electrochemical) gradient. Such transports are always carrier-mediated, nonspontaneous, energy demanding processes. They are always coupled to energy releasing processes. Basically, energy demand of the active transport processes can be covered by three different sources:

1. Hydrolysis of ATP,
2. Light (photon) energy, or
3. Redox energy

IV.3.1 Primary active transport

In most cases, these transport processes are directly coupled to hydrolysis of terminal phosphate unit of ATP. These are the so-called *primary active transport processes*. Most of the enzymes that perform this type of transport are *transmembrane ATPases*. Other sources of energy for primary active transport are *redox energy* and photon energy (light). An example of primary active transport using redox energy is the *mitochondrial electron transport chain* that uses the reduction energy of NADH to move protons across the inner mitochondrial membrane against their concentration gradient. An example of primary active transport using *light energy* are the proteins involved in photosynthesis that use the energy of photons to create a proton gradient across the thylakoid membrane.

One of the most important family of the ATP-using primary active processes are the P-type transmembrane ATPases. Most members of this transporter family are specific for the pumping of a large array of cations. Prominent examples of P-type ATPases are the *sodium-potassium pump* (Na^+,K^+-ATPase), the plasma membrane

proton pump (H^+-ATPase), the *proton-potassium pump* (H^+,K^+-ATPase), and the *calcium pump* (Ca^{2+}-ATPase). A primary ATPase universal to all animal life is the *sodium-potassium pump*, which helps to maintain the cell potential. During operation, sodium ions are transported into the intracellular while potassium ions into the extracellular space. Cardiac glycosides are selective inhibitors of *sodium-potassium pump* (*Na^+,K^+-ATPase*) (Table IV-1). C*alcium pump* (*Ca^{2+}-ATPase*)., which are taking place in maintaining very low (10^{-7} M) intracellular calcium ion concentration, are located in plasma membranes of the cells and in the endoplasmic reticulum of the skeletal muscle cells (referred to as sarcoplasmic reticulum). To maintain low concentrations of free Ca^{2+} in the cytosol, cells use membrane pumps like calcium ATPase. These pumps are needed to provide the steep electrochemical gradient that allows Ca^{2+} to rush into the cytosol when a stimulus signal opens the Ca^{2+} channels in the membrane. The *H+/K+-ATPase pump* (*proton pump*) causes exchange of a proton against a potassium ion through a membrane. This pump is present in the colon, the kidney, but especially the stomach where it is particularly active. In the stomach, this pump causes the secretion of protons into the gastric fluid which becomes acid. *Na^+K -ATPase* and *K^+H^+ -ATPase* transporters ("*pumps*") are molecular targets of several therapeutically useful drugs (Table IV-1).

ABC transporters are transmembrane proteins that utilize the energy of ATP binding and hydrolysis (*primary active transport*) to carry out certain biological processes including translocation of a wide variety of substrates including sugars, amino acids, metal ions, peptides, and proteins, and a large number of hydrophobic compounds and metabolites across extra- and intracellular membranes. Among the latter there can be found lipids, polypeptides, steroids as well as toxins, antibiotics and antitumor drugs. They are named after the structure of their intracellular ATP binding site („ATP binding cassette"). There are several subfamily of human ABC transporters, among which one of the most important one is the ABCB subfamily. The ABCB subfamily is composed of four full transporters and seven half transporters. The *ABCB1* (*MDR1*) gene was discovered as a protein overexpressed in certain drug-resistant tumor cell lines. Cells that overexpress the *MDR1* protein (*P-glycoprotein*) are resistant to or transport a wide variety of hydrophobic compounds including colchicine, doxorubicin, adriamycin, vinblastine, and paclitaxel out of the cells. The *multidrug resistance protein* 1 (*MRP1*, product of the *ABCC1 gene*) is expressed in most tissues throughout the body with relatively high levels found in the lung, testis, kidneys, while relatively low levels are found in liver. Transport specificity of MRP1 proteins is confined to hydrophobic molecules. Possibility of inhibition of both transporters is an intensive field of present-day drug development.

IV.3.2 **Secondary active transport**

There are active transport processes in which the required energy of transportation of one solute is provided by simultaneous transmembrane movement of another solute along its electrochemical gradient. Most of such transporters utilize sodium ion gradient, and transportation of the solute is accompanied by movement of sodium ions into the cell. Since maintenance of the asymmetric sodium ion concentration (sodium ion gradient) is an ATP-dependent process, such transports are called *secondary active transport processes*. Among the *secondary active transport processes* both symport and antiport processes can be found.

The most important representative of *symporters* as follows.

1. the Na^+-glucose cotransporters,

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2. the $Na^+(Cl^-)$-amino acid cotransporters,
3. the $Na^+(Cl^-)$-neurotransmitter cotransporters,
4 the Na^+Cl^- cotransporter,
5. the K^+Cl^- cotransporter,
6. the Na^+K+2Cl^- cotransporter, and
7. the Na^+I^- cotransporter.

The most important representative of *antiporters (exchangers)* as follows.

1. the Na^+Ca^{2+} *exchanger*,
2.the Na^+H^+ *exchanger*, and
3.the $K^+NH_4^+$ *exchanger*.

The $Na^+(Cl^-)$-neurotransmitter (noradrenalin (NA), dopamine (DA), serotonin (5-HT)) and amino acid (pl. GABA) cotransporters are representing a group of transporters of high importance from pharmacological point of views. Among the compounds acting on these transporters there can be found several antiepileptic (GABA), antidepressant (5-HT) and antiparkinson (DA) drugs.

There are several clinically effective drugs of which biological effects are associated with their modulatory action on activity of different transporters. These drugs are summarized in Table IV-1.

Table IV-1. Drugs targeting different transporters

Transporter	**Anti-detergent ***	**Therapeutic significance**
Na^+K^+-ATPase	stofantin, digoxin, digitoxin	cardiotonics
K^+H^+-ATPase	omeprazole, lansoprazole, pantoprazole, rabeprazole, esomeprazole	drugs reducing gastric acid secretion
Na^+Cl^- cotransporter	chlorothiazide, hydrochlorothiazide, metolazone, chlorthalidone, indapamide, clopamid	salt-wiasting diuretics
$Na^+K^+2Cl^-$ cotransporter	ethacrynic acid, furosemide, bumetanide, piretanide, torasemid,	high ceiling/loop diuretics
Na^+I^- cotransporter	potassium perchlorate	anti-hyperthyroidism drugs
Na^+- glucose cotransporter	dapagliflozin	antidiabetic agent
Na^+Cl^--GABA cotransporter	tiagabin	antiepileptic drugs
$Na+Cl^-$-5HT cotransporter	clomipramine, citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, zimelidine	antidepressants
$Na+Cl^-$-NA cotransporter	desipramine, talopram, reboxetine	antidepressants
Na^+Cl^--DA cotransporter	rimcazol	antiparkinson drugs

*Incomplete list

IV.4 Channel proteins

Operation of channel forming proteins shows different mechanism from that of transporters. *Channel proteins* transport water or specific types of ions down their concentration or electric potential gradients (passive transport). They form a protein-lined passageway across the membrane through which water molecules or ions move simultaneously, at a very rapid rate.
Membrane-bound ion channels can be categorized by several different ways. One of the possible classification is based on selectivity of the channels towards different ions. According to this aspect the most important ion channels

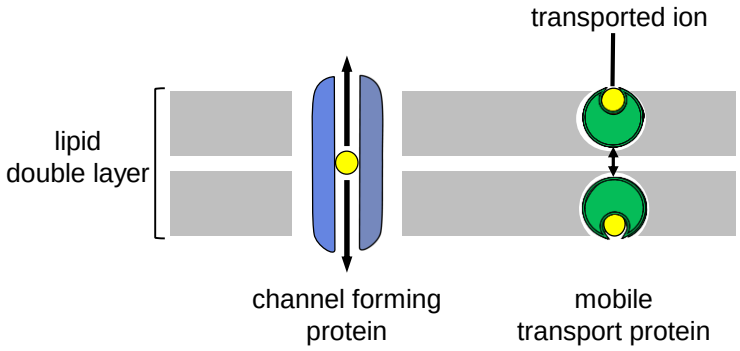
1. Chloride ion channels
2. Proton (hydrogen ion) channels
3. Sodium ion channels
4. Potassium ion channels
5. Calcium ion channels
6. Aquaporins
7. Nonselective cation channels

IV.4.1 Ionophores

Besides the above natural channels, there are some relatively small hydrophobic molecules that can facilitates the transport of ions across lipid membranes. These compounds are called ionophores. Ionophores can be integrated into the membranes and increase its permeability towards certain inorganic ions.

There are two types of ionophores: (a) *channel formers*, which combine to form a channel in the membrane through which ions can flow; and (b) *mobile ion carriers*, which transport ions across a membrane by forming a complex with the ion (Figure IV-4).

Figure IV-4. Principle of operation of channel formers and mobile ion carriers



One of the most known example of mobile ion carriers is *valinomycin*. Valinomycin is a cyclic polypeptide wit hydrophobic surface, which can form chelate complex with potassium ions. The membrane embedded polypeptide can form complex with extracellular potassium ions, which is translocated into the intracellular surface of the membrane, from where it is liberated.

One example of the channel forming ionophores is *gramicidin*. Gramicidin is a hydrophobic protein of 15 amino acids. Gramicidin's bactericidal activity is a result of increasing the permeability of the bacterial cell membrane, allowing inorganic

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monovalent cations (Na^+, K^+, H^+) to travel through unrestricted and thereby destroying the ion gradient between the cytoplasm and the extracellular environment. Channel formation is the molecular basis of antifungal effects of polyene antibiotics (e.g., *neomycin*, *nystatin*, *B-amphotericin*).

Ion channels can be categorized into two structurally different groups. One of them is the group of so called "leaking channels", which allows a slow but continuous flow of ions though the pores. The second type of ion channels are the so called "gated ion channels", of which permeability towards selected ions depends on the different state of a "gate". The "gate" can be "open" or "closed" as a response to chemical or electronic signals, temperature change or mechanical force. "Gated ion channels" can be categorized into the following subtypes:

1. Voltage-gated ion channels
2. Extracellular ligand-activated ion channels (also known as ionotrop receptors)
3. Intracellular ligand-gated ion channels
4. Mechanosensitive ion channels
5. Volume-regulate ion channels
6. Thermosensitive ion channels

In the present electronic educational material, the voltage-gated ion channels and the ligand-activated ion channels are discussed. (Discussion of ligand-activated ion channels is part of Chapter II.)

IV.4.2 **Voltage-gated ion channels**

Voltage-gated ion channels are a class of transmembrane ion channels that are activated by changes in electrical membrane potential near the channel; these types of ion channels are especially critical in neurons, but are common in many types of cells. They have a crucial role in excitable neuronal and muscle tissues, allowing a rapid and coordinated depolarization in response to triggering voltage change. Found along the axon and at the synapse, voltage-gated ion channels directionally propagate electrical signals.

Voltage-gated ion channels are classified on the bases of nature of ions that can move through the channel pore. Although the channels are ion-specific, ions of similar size can get through. Among the voltage-gated ion channels the following ones are of pharmacological interest:

1. sodium voltage-gated ion channels
2. potassium voltage-gated ion channels
3. calcium voltage-gated ion channels, and
4. voltage-gated proton ion channels

Voltage-dependent conductivity of the channels can be explained by the function of the gating particles. Voltage-dependent channels as made of three basic parts: the voltage sensor, the pore and the gate. Their function is demonstrated though operation of the voltage-activated ion channel (Figure IV-5).

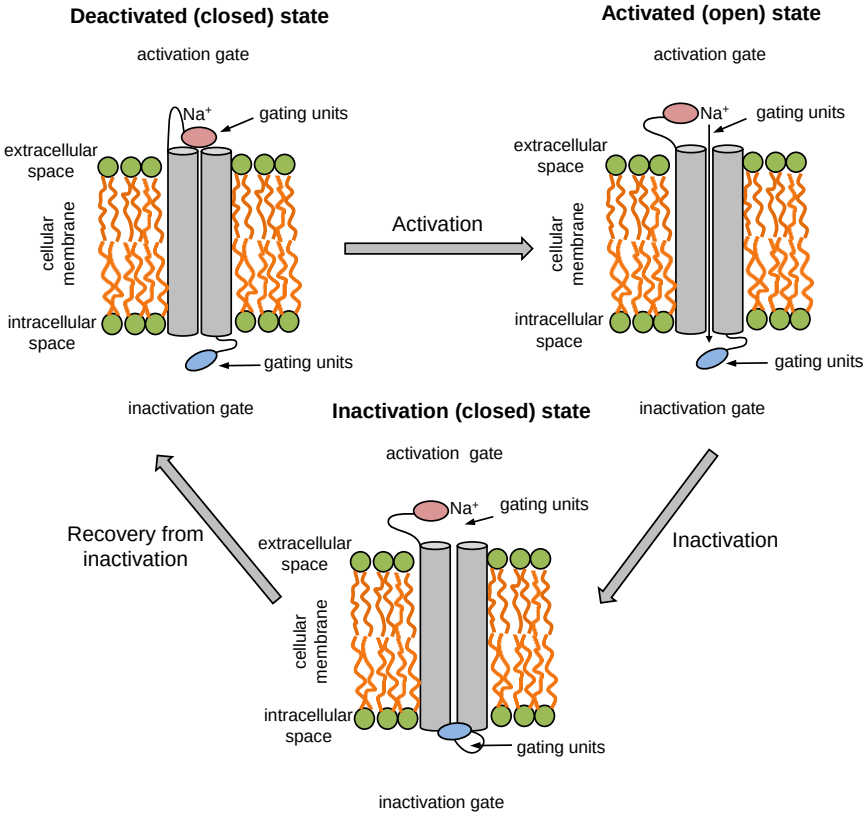
Voltage-gated Na^+ channels have three main conformational states: closed, open and inactivated. Forward/back transitions between these states are correspondingly referred to as activation/deactivation (between closed and open), inactivation/reactivation (between open and inactivated), and recovery from

inactivation/closed-state inactivation (between inactivated and closed). Closed and inactivated states are ion impermeable.

Before an action potential occurs, the axonal membrane is at its normal resting potential, and Na^+ channels are in their deactivated state, blocked on the extracellular side by their *activation gates*. In response to an electric current (in this case, an action potential), the activation gates open, allowing positively charged Na^+ ions to flow into the neuron through the channels, and causing the voltage across the neuronal membrane to increase. This increase in voltage constitutes the rising phase of an action potential.

At the peak of the action potential, when enough Na^+ has entered the neuron and the membrane's potential has become high enough, the Na^+ channels inactivate themselves by closing their *inactivation gates*. With its inactivation gate closed, the channel is said to be inactivated. With the Na^+ channel no longer contributing to the membrane potential, the potential decreases back to its resting potential as the neuron repolarizes and subsequently hyperpolarizes itself.

Figure IV-5. Activated, inactivated and deactivated forms of voltage-gated sodium ion channels



When the membrane's voltage becomes low enough, the inactivation gate reopens and the activation gate closes in a process called deinactivation, or *removal of inactivation*. With the activation gate closed and the inactivation gate open, the Na^+ channel is once again in its deactivated state, and is ready to participate in another action potential.

Local anesthetics exert their biological effect by inhibiting the voltage-gated sodium ion channels. Most of the clinically useful local anesthetics (e.g., procaine, tetracaine, lidocaine, bupivacaine) are tertiary amines with pKa value of 7.0-9.0. Thus, under physiological conditions both the protonated (ionized) and the nonprotonated

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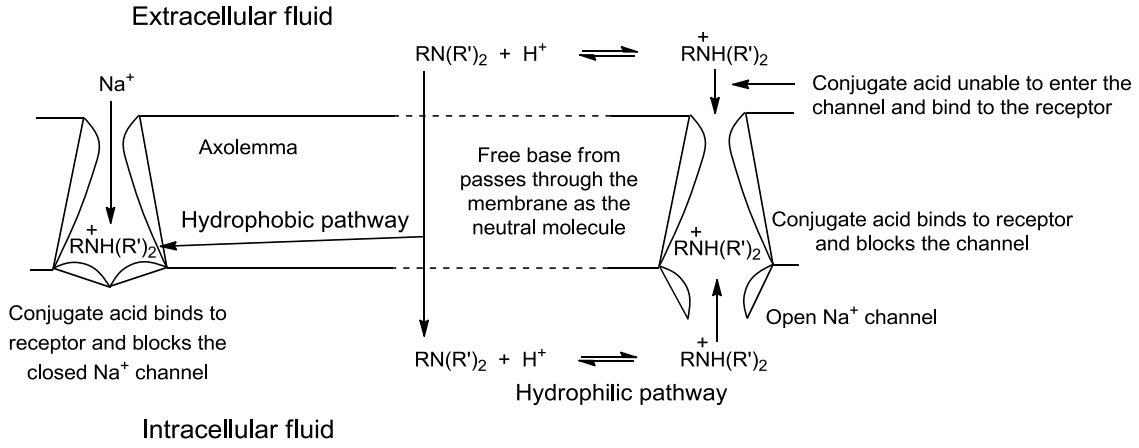
(neutral) forms of the compounds can interact with the channel proteins. In 1984, *B. Hille* proposed a theory of action of local anesthetics on the voltage-gated sodium ion channels. According to Hille"s proposal, protonated anesthetic molecules (similar to the quaternary ammonium compounds) reach their target sites via the hydrophilic pathway externally, which is available only during channel activation. The lipid soluble anesthetic molecules, on the other hand, diffuse across the neuronal membrane in their unionized form. They can interact with the same receptors from either the hydrophilic or the hydrophobic pathway. The compounds can bind by higher affinity to the activated channels, so they can effectively block the flow of sodium ions.

Figure IV-6. Model of effect of local anesthetics on voltage-gated sodium ion channel, as suggested by *Hille*



IV.4.3 **Ligand-activated ion channels**

Discussion of ligand-activated ion channels is part of Chapter II.

IV.5 References

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V Physico-chemical characteristics of drugs - Acid-base properties

Ionic interactions are important factors of the interactions between active pharmaceutical ingredients and the molecules in the organism. That interactions depend on the protonated/deprotonated state of the substance, therefore, characterization of acid-base properties of a compound is essential to understand the biological effect of it. On the other hand, ionization plays important role in in vivo absorption, distribution and elimination of medicinal substances.

V.1 Strength of acids and bases. Definition of the pKa value

According to the *Brönsted-Lowry* definition, an **acid** (generally HA) is a substance that is able to donate proton(s), while a **base** (generally B) is able to accept proton(s). In an acid-base reaction conjugate acid-base pairs take part. The acid becomes to the conjugate base by donating a proton, while the base becomes to the conjugate acid by accepting the proton.

$$\underset{\text{acid}_1}{HA} + \underset{\text{base}_2}{B} \rightleftharpoons \underset{\text{base}_1}{A^-} + \underset{\text{acid}_2}{BH^+}$$

In aqueous solutions, where HA is the **acid**, water accepts the proton and acts as a base:

$$HA\ (aq) + H_2O\ (l) \rightleftharpoons A^-\ (aq) + H_3O^+\ (aq)$$

The equilibrium constant for this reaction is the acidity constant (K_a; „a" refers to *acid*) and is expressed mathematically as:

$$K_a = \frac{[A^-][H_3O^+]}{[HA]}$$

taking $[H_2O]$ to be effectively constant for dilute solutions. K_a is virtually equal to the dissociation equilibrium constant of the acid:

$$HA\ (aq) \rightleftharpoons A^-\ (aq) + H^+\ (aq)$$

It can be seen from the equation, since K_a is a simple ratio, the higher the numerical value of K_a the stronger will be the acid. Dissociation of *strong acids* (e.g. HCl, HNO_3, $HClO_4$, H_2SO_4) is practically 100%, they dissociate completely. Therefore the K_a value is virtually infinite.
In the case of *weak acids* the rate of dissociation is lower, it can be calculated using the equilibrium proton-concentration and the total acid-concentration:

$$\text{dissociation } \% = \frac{[H^+]}{[HA]_{all}} \times 100$$

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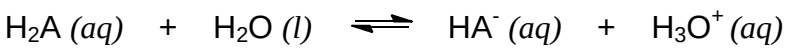
Strength of acids is generally expressed by the term pK_a, which is the negative logarithm of K_a:

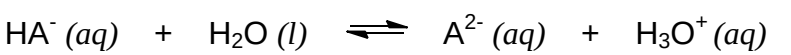
$$pK_a = -\log K_a$$

In case of acids, the lower the value of pK_a the stronger will be the acid and that on a log scale a difference of one unit in pK_a reflects a tenfold difference in acid strength.

Polyvalent acids dissociate in more steps. Separate acid dissociation constants can be defined, depending on the number of acidic functional groups. In case of bivalent acids, usually the second dissociation constant is by several orders of magnitude smaller than the first one. It is much more difficult to remove the proton from the negatively charged HA^- anion, than that from the neutral H_2A molecule.

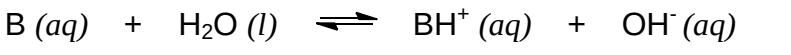


$$K_{a1} = \frac{[HA^-][H_3O^+]}{[H_2A]}$$



$$K_{a2} = \frac{[A^{2-}][H_3O^+]}{[HA^-]}$$

Bases can be characterized with the following equilibrium in aqueous medium:



$$K_b = \frac{[BH^+][OH^-]}{[B]}$$

The higher the numerical value of K_b the stronger will be the base. *Strong bases* are practically totally protonated in aqueous solution, while *weak bases* are only partially protonated.

Anions of weak acids, that means the conjugate base pairs are relatively strong bases. Conversely, anions of strong acids are relatively weak bases.

Multiplication of the dissociation constants of conjugate acid-base pairs is equal to the water ionization constant:

$$K_a \times K_b = K_w$$

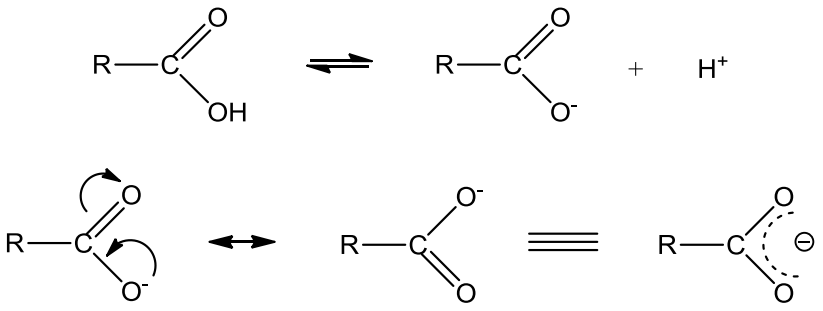
Generally, to characterize the strength of bases, instead of K_b, the K_a or the pK_a values (dissociation constant of the conjugate acid pair) are used. According to the equations it is apparent, that in case of bases, the higher the pK_a the stronger will be the base.

V.2 Acidic and basic functional groups

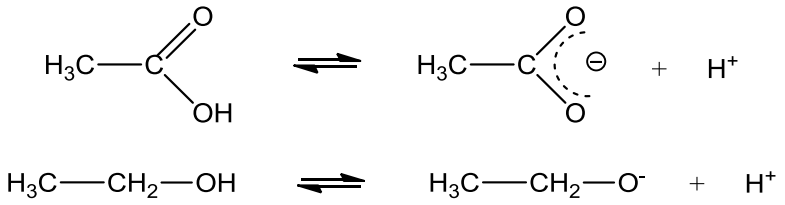
Most of the drugs used in the medicine possess basic (75%) or/and acidic (20%) functional groups, and only about 5% of the drugs is non-ionizable.

V.2.1 Acidic functional groups

The most commonly oocuring functional group conferring acidity on drug molecules is the **carboxyl group**. The pK_a value of carboxylic acids may move in a wide range (pK_a = 2-7) The anion formed by ionization of the acid, called carboxylate ion, is stabilised by the process of *resonance*. The two oxygen atoms in the ion are coequal.



The stabilizing effect of resonance may be seen when the acidity of a simple carboxylic acid such as acetic acid is compared with the acidity of an alcohol such as ethanol. Both compounds can ionize to liberate a proton, but while the anion formed on ionization of acetic acid is resonance-stabilised, the ethoxide anion formed on ionization of ethanol is not stabilised and the negative charge resides wholly on the oxygenatom.



The pK_a of acetic acid is 4.7 while the pK_a of ethanol is approximately 16. This means that acetic acid is almost 10^{11} times more acidic than ethanol. Alcohols are much weaker acids than water and in biological systems are considered to be neutral. The ionization of an alcohol requires the use of a very strong base such as metallic sodium.

A number of commonly used drugs are carboxylic acid derivatives, including the analgesic and anti-inflammatory *aspirin* (pK_a = 3.5), the diuretic *furosemide* (pK_a = 3.9) and the anticancer compound *methotrexate* (pK_a = 3.8 és 4.8).

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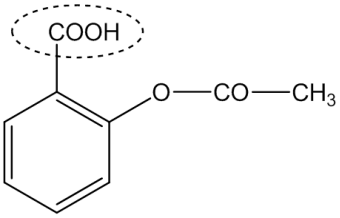
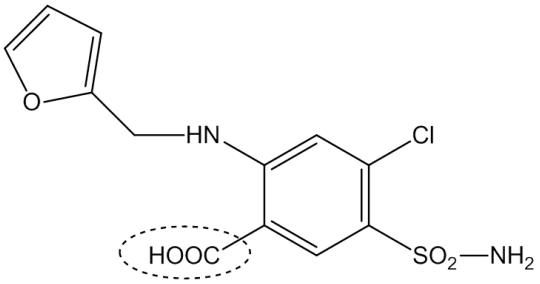
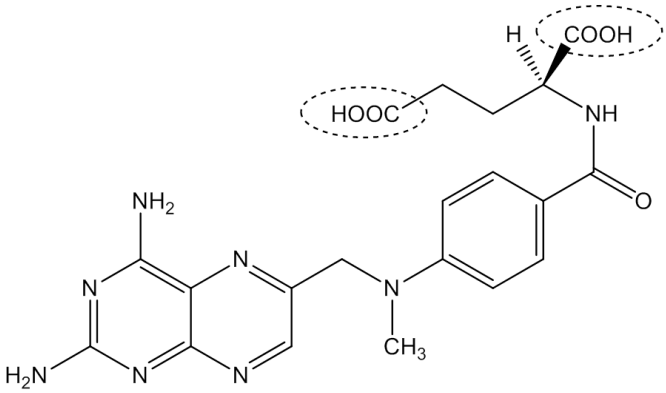


Acetylsalicylic acid

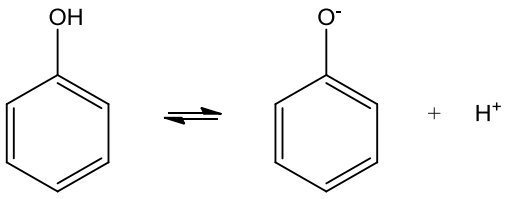


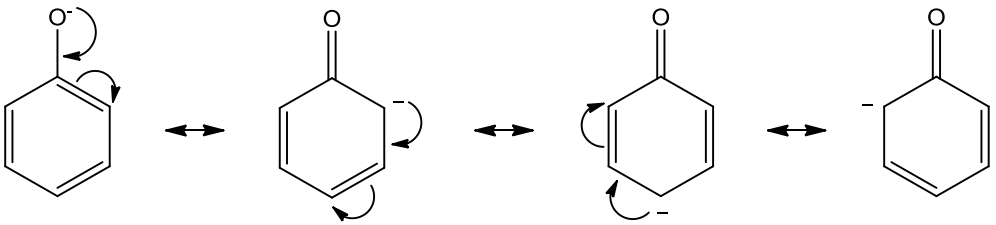
Furosemide



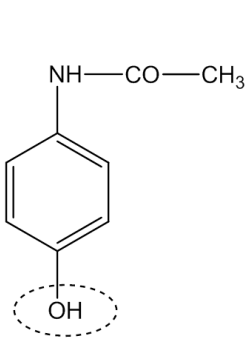
Methotrexate

Another common acidic functional group found in drug compounds is **phenolic hydroxyl group**. Phenols are much stronger acids than alcohols, the pK_a value is about 9-11. They liberate protons to give phenoxide anion, which is resonance-stabilised.

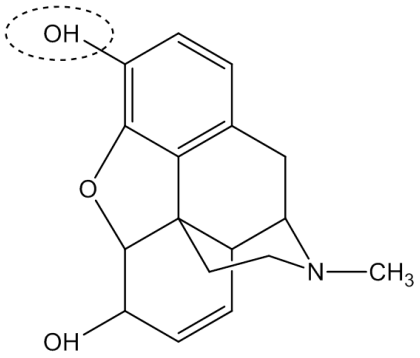




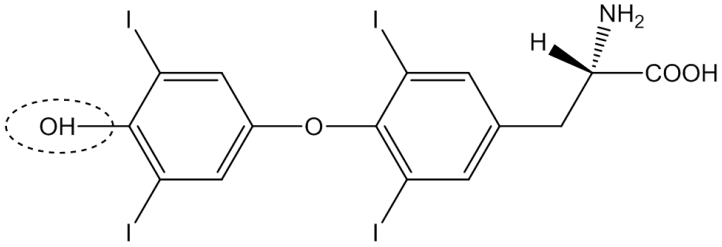
Phenols have pK_a values of approximately 10, which means they are about 10^6 times less acidic than carboxylic acids but are about 10^6 times more acidic than alcohols. A number of common drugs contain the phenol functional groul, including *paracetamol* (pK_a = 9.5), *morphine* (pK_a = 9.9) and *levothyroxine* (pK_a = 10).



Paracetamol

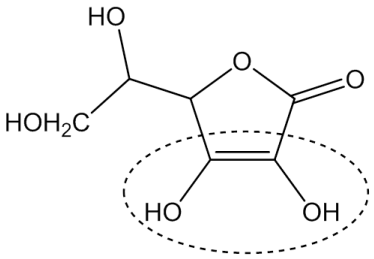


Morphine

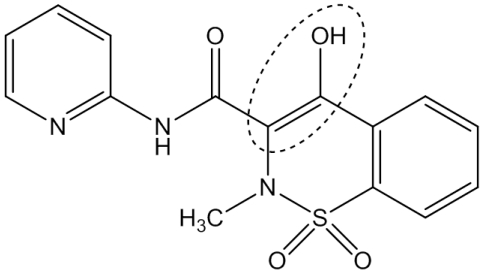


Levothyroxine

Enolic hydroxyl groups are also acidic, especially in the presence of electron withdrawing substituents. *Ascorbic acid* (pK_a = 4.2) and *piroxicam* (pK_a =2.3) contain enolic hydroxyl group.

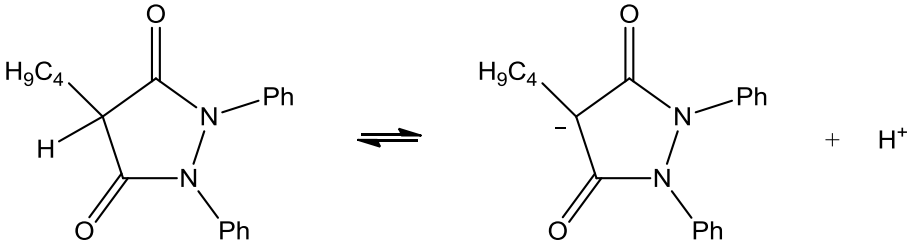


Ascorbic acid



Piroxicam

A typical example of **C-H acidity** is the non-steroidal anti-inflammatory drug *phenylbutazone* (pK_a = 4.4). Phenylbutazone, despite containing nitrogen, is a weak acid, the acidic hydrogen is on the 4-position of the pyrazolidinedione ring. Upon ionization the negative charge is delocalised.



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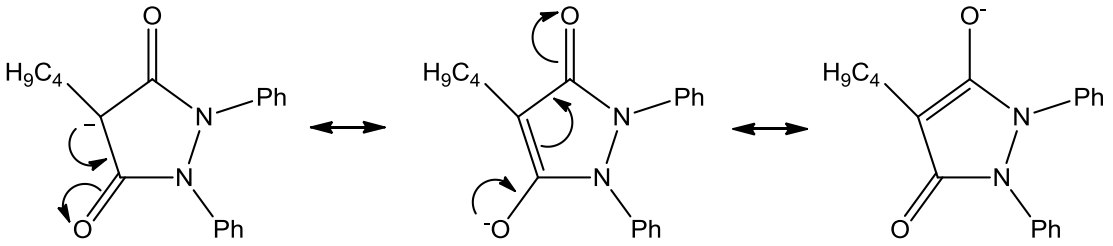
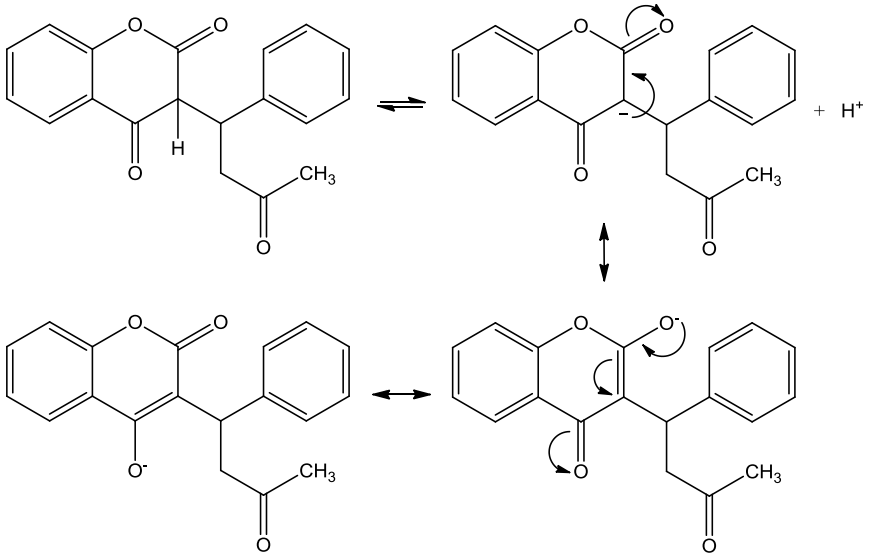
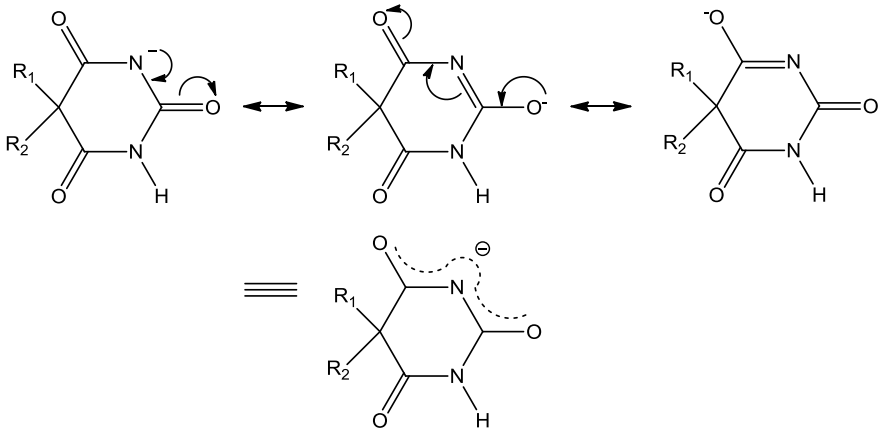


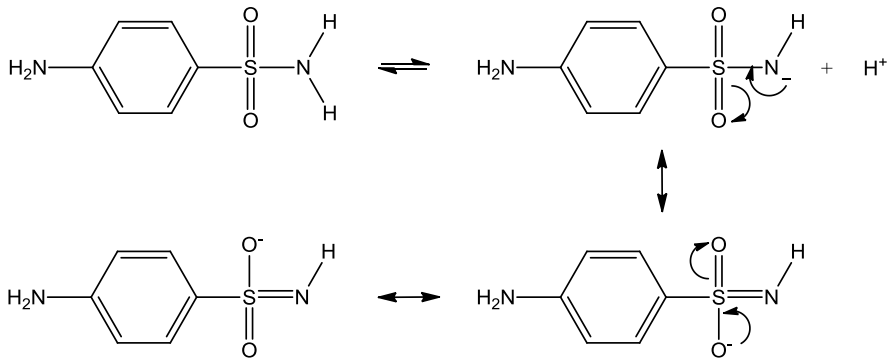
In case of the anticoagulant *warfarin* (pK_a = 5.0) the acidic hydrogen is located between two electron withdrawing carbonyl groups. Upon ionization, the negative charge can be delocalised, which increases the stability of the anion.



Imides are **N-H acids**, an acyl group is connected to the nitrogen atom in the carboxylamide. Barbiturates are cyclic imides. The negative charge formed on ionization delocalizes around the two adjacent carbonyl groups. The pK_a values for barbiturates are typically 7-8 for the first ionization and approximately 11-12 for the second. The drugs are usually administered in the form of the sodium salt to increase the water solubility.

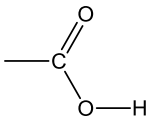
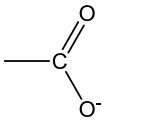
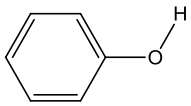
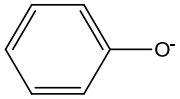
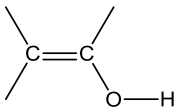
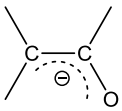
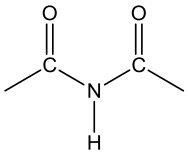
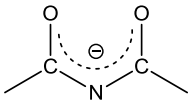
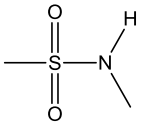
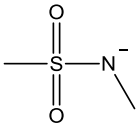


Sulfonamides contain sulfonamido group, and are weakly acidic (pK_a approximately 5-8) due to the powerful electron withdrawing effect of the $–SO_2$- substituent and stabilisation of the resulting anion by resonance.



The most common acidic functional groups and their acidic dissociation constants are collected in the next table.

Table V-1: Acidic functional groups and their dissociation constants

Acid		Conjugated base		pK_a	Example
	carboxylic acid		carboxylate	2-7	aspirin (3,4)
	phenol		phenoxide	9-11	paracetamol (9,6)
	enol		enolate	2-6	ascorbic acid (4,2)
	thiol		thiolate	8-11	captopril (9,8)
	imide		imidate	9-10	phenytoin (8,3) theobromine (10,0)
	sulfonamide		sulfonamidate	9-10	hydrochloro-thiazide (9,7 és 8,7)

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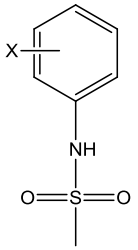
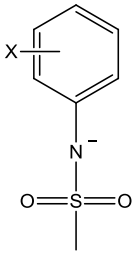
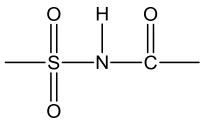
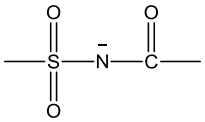
Acid		Conjugated base		pK_a	Example
	N-aryl-sulfonamide		N-aryl-sulfonamidate	9-10	sulfadimidine (7,5)
	sulfonimide		sulfonimidate	5-6	sulfacetamide (5,4)

V.2.2 **Basic functional groups**

Basic functional groups are very common in drug molecules. Basic character is connected to nitrogen atom(s) in the molecule, that means drugs behave as bases if they contain a nitrogen atom with a lone pair of electrons available for reaction with protons. If the lone pair of electrons on the nitrogen is not avalilable for reaction with protons, such as in amides, where the electrons take part in the conjugation, the nitrogen does not show basic character.

Amines will react with water in aqueous solution to release hydroxide ions. They are proton donors, that means Brönsted-bases. However, they are electron-pair donors as well, therefore they are able to form coordinative bonds with protons, electron-pair acceptor compounds and ions. As a consequence they function as bases according to the Lewis theory also.

In the gaseous phase the basicity of amines is only influenced by the substituents. Electron-donating substituents increase the basicity, since they increase the electron density of the nitrogen atom. In gas phase the order of basicity is: ammonia < primary amine < secondary amine < tertiary amine.

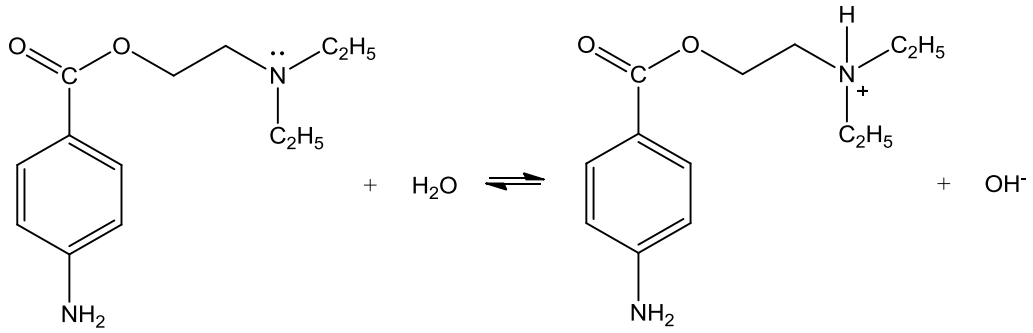
In aqueous solutions the basicity of amines is influenced by dipole-dipole interactions and hydrogen bonding as well, but the alkyl substituents have a dual effect on the stability of the formed ammonium ions too. The alkyl groups encumber the admittance of the water molecules to the amine, hence the solvation. The absence of solvation is destabilizing. Therefore, primary amines could be the strongest bases. However, the alkyl group promotes the delocalization of the charge through its electron-donating property, and stabilizes the ion. From this point of view the tertiary amines could be the strongest bases. The opposite effects deteriorate the basicity of primary and tertiary amines, and the secondary amines come out on the top, they are in fact the strongest bases.

Aromatic amines are much weaker bases than their aliphatic analogues. The reason for this is that the nonbonding electron pair of the nitrogen atom conjugates with the π electrons of the aromatic ring, hence the protonation of the nitrogen atom is more difficult.

The order of basicity in aqueous solutions is the following:

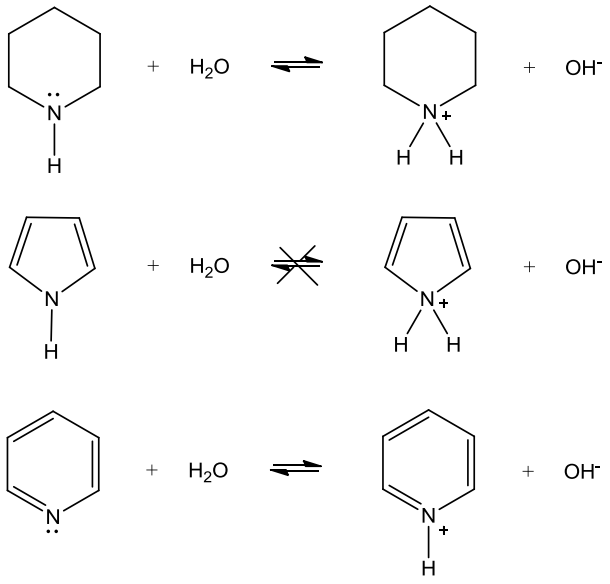
$$Ar\text{-}NH_2 \ll NH_3 < R_3\text{-}N < R\text{-}NH_2 < R_2\text{-}NH$$

This can be illustrated by investigation of basicity of *procaine*, a local anaesthetic drug, which contains an aromatic primery amino group and an aliphatic tertiary amino group as well. The tertiary amino group is a relatively strong base (pK_a = 9.0), the lone pair of electrons is concentrated on the nitrogen atom and is available to accept a proton. Conversely, the lone pair of electrons on the amino group attached to the benzene ring is less available for reaction with protons due to delocalisation into the ring. The aromatic primary amino group is a weak base (pK_a = 2.5).



Many drugs contain nitrogen in a heterocyclic ring. In aliphatic heterocyclic compounds (e.g. *piperidine*), the nitrogen atom is a part of a saturated heterocyclic ring and the long pair of electrons is available for reaction with protons. Compounds of this type are similar in base strength to their open-chain aliphatic counterparts, with typical pK_a values of 8-9.

In aromatic heterocyclic compounds lone pairs on the nitrogen atoms are involved in interaction with electrons of the aromatic ring. In *pyrrol*, the lone pair contributes to the aromatic sextet and is not available for reaction with protons. As a result, pyrrole is considered practically not to be basic (pK_a = -0.27). However, the six-membered nitrogen heterocycle *pyridine* is a weak base (pK_a = 5.2), it can accept a proton.



The most common basic functional groups and the acidic dissociation constants of their conjugate acid pairs are collected in the next table.

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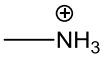
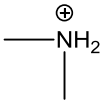
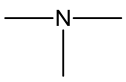
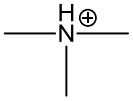
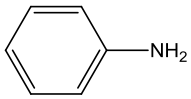
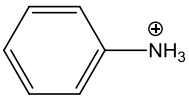
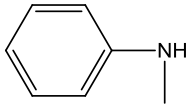
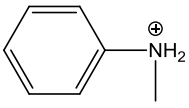
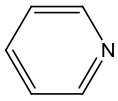
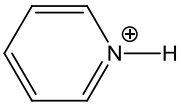
Table V-2: Basic functional groups in drugs and the acidic dissociation constants of their conjugate acid pairs

Base		Conjugated acid		pK_a	Example
	aliphatic primary amine		-ammonium	8-10	amphetamine (9,8)
	aliphatic secondary amine		-ammonium	8-10	ephedrine (9,6)
	aliphatic tertiary amine		-ammonium	8-10	lidocaine (7,9) procaine (9,0)
	aromatic primary amine		-ammonium	2-5	procaine (2,5)
	alkyl-aryl secondary amine		-ammonium	2-5	tetracaine (2,4)
	pyridine		pyridinium	3-6	omeprazole (4,1)

V.3 Relevance of the pK_a value

Knowledge of the pK_a of a substance is widely useful. It is essential to understand the formation of biological effect, but it is necessary to solve several analytical problems, such as determination of the log*P* value or the solubility. However, we have also to know the pK_a of a compound to solve some technological questions, such as preparation of an injection.

The protonated state of a molecule basically influences the fate of the drug in the body, that means the solubility in biological medium, transfer through the membranes, binding to plasma proteins and receptors, and metabolism, respectively.

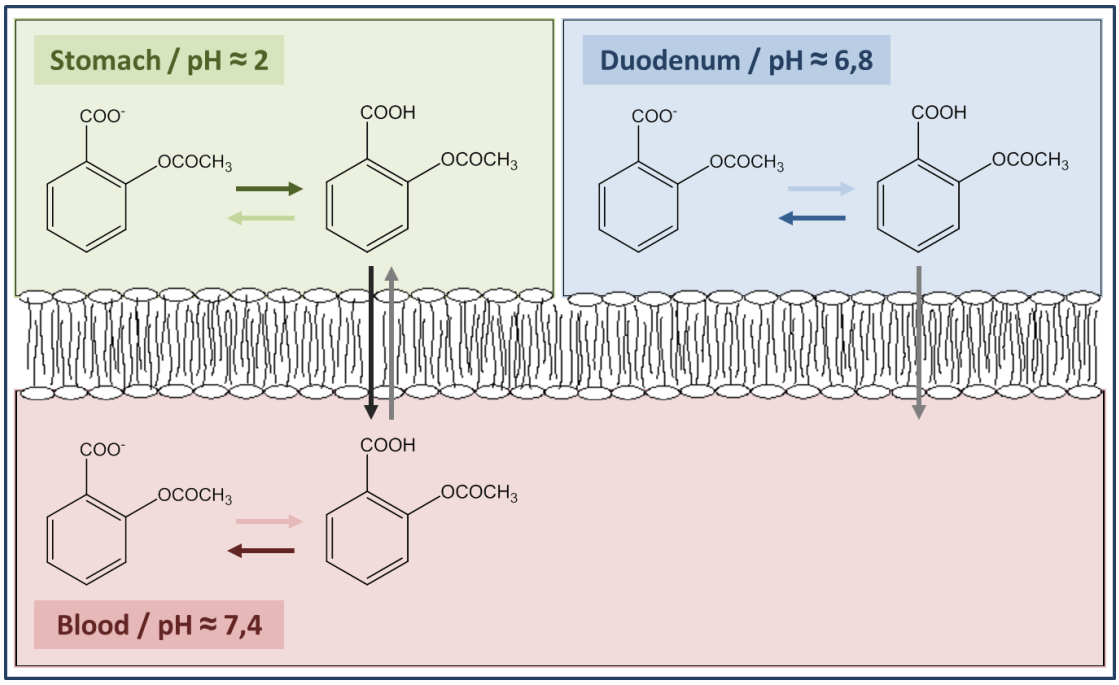
In general, the uncharged, nonionized form (AH and B form) is the so-called „*transport-form*", which is able to pass through the biological membranes. On the other hand, the charged, ionic form (A^- and BH^+ form) is able to bind to transportproteins and receptors, this is the so-called „*receptor-form*".

The ionization state of the molecule depends - besides on the pK_a value of the drug – on the pH of each compartments in the body.

Table V-3: Average pH values in healthy humans at various sites in the gastrointestinal tract and of blood

Compartment in the body	Average pH values
Stomach	1,4-2,1 (fasted state)
	3,7-7,0 (fed state)
Duodenum	4,4-6,6 (fasted state)
	5,2-6,2 (fed state)
Jejunum	6,6
Ileum	7,4
Colon	5,0-8,0
Blood	7,4

Figure V-1. Absorption of acetylsalicylic acid



In case of acidic or basic drugs, the rate of protonated and deprotonated molecules at each pH can be calculated using the ***Henderson-Hasselbach equation*** and the pK_a values of the compound.

In case of acids: $$pK_a = pH + \log \frac{[HA]}{[A^-]}$$

In case of bases: $$pK_a = pH + \log \frac{[BH^+]}{[B]}$$

If the pK_a value of the drug is equal to the pH of the medium, the ionization is 50%, that means the number of ionized and nonionized molecules is the same.

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Example I. *Ionization of acetylsalicylic acid (weak acid) in the stomach*

$$\mathrm{p}K_\mathrm{a} = 3{,}4$$
$$\mathrm{pH}_\mathrm{stomach} = 1{,}4$$
$$\mathrm{p}K_\mathrm{a} = \mathrm{pH} + \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]}$$
$$\mathrm{p}K_\mathrm{a} - \mathrm{pH} = \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]}$$
$$3{,}4 - 1{,}4 = 2 = \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]} = \log 100$$
$$\frac{[\mathrm{HA}]}{[\mathrm{A}^-]} = 100$$

which means, the amount of HA is 100 fold greater than the amount of A^-. Acetylsalicylic acid (HA) moves from the stomach into the blood (good absorption), since it is mainly non ionized on that pH.

Example II. *Ionization of acetylsalicylic acid (weak acid) in the blood*

$$\mathrm{p}K_\mathrm{a} = 3{,}4$$
$$\mathrm{pH}_\mathrm{blood} = 7{,}4$$
$$\mathrm{p}K_\mathrm{a} = \mathrm{pH} + \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]}$$
$$\mathrm{p}K_\mathrm{a} - \mathrm{pH} = \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]}$$
$$3{,}4 - 7{,}4 = -4 = \log\frac{[\mathrm{HA}]}{[\mathrm{A}^-]} = \log 0{,}0001$$
$$\frac{[\mathrm{HA}]}{[\mathrm{A}^-]} = 0{,}0001$$

which means, the amount of A^- is 10.000 fold greater than the amount of HA in the blood. Acetylsalicylic acid is readily absorbed from the stomach into blood. It is practically completely non ionized in the blood.

Example III. *Ionization of lidocaine (weak base) in the stomach*

$$\mathrm{p}K_\mathrm{a} = 7{,}9$$
$$\mathrm{pH}_\mathrm{stomach} = 1{,}4$$
$$\mathrm{p}K_\mathrm{a} = \mathrm{pH} + \log\frac{[\mathrm{BH}^+]}{[\mathrm{B}]}$$
$$\mathrm{p}K_\mathrm{a} - \mathrm{pH} = \log\frac{[\mathrm{BH}^+]}{[\mathrm{B}]}$$
$$7{,}9 - 1{,}9 = 6 = \log\frac{[\mathrm{BH}^+]}{[\mathrm{B}]} = \log 1000000$$
$$\frac{[\mathrm{BH}^+]}{[\mathrm{B}]} = 10^6$$

which means, the amount of BH^+ in the stomach is 10^6 fold greater than the amount of B. So the lidocaine molecules are not absorbed (poor absorption) from the stomach,

since they are almost totally ionized. Absorption from the duodenum (higher pH) is preferable.

In the next table there are some examples illustrating the absorption of acidic and basic drugs.

Table V-4 Absorption of acidic and basic drugs in the body

Drugs	pK_a	State of ionization in the body	Site of absoption
Very weak acids e.g. hexobarbital	>8	unionized at all pH values	absorbed along the entire length of GIT
Moderately weak acids e.g. aspirin, ibuprofen	2,5 - 7,5	unionized at gastric pH; ionized at intestinal pH	better absorption from stomach
Stronger acids e.g. disodium cromogylate	<2,0	ionized at all pH values	poorly absorbed from GIT
Very weak bases e.g. theophylline, caffeine	<5	unionized at all pH values	absorbed along the entire length of GIT
Moderately weak bases e.g. codeine	5 - 11	ionized at gastric pH; unionized at intestinal pH	better absorption from intestine
Stronger bases e.g. guanethidine	>11	ionized at all pH values	poorly absorbed from GIT

V.4 Determination of the pK_a value

Several methods are applicable to determine the pK_a. All methods are based on the experience, that the measured analytical signal depends on the pH, therefore it is related to the protonation or deprotonation of the investigated molecule.

Table V-5: Methods to determinate the pK_a

Method	Substance needed (mg)	Solubility (M)	Type	Capacity (compound/day)
Potentiometry	3-5	5×10^{-4}	not HT	30-40
UV/pH titration				
traditional	1-2	10^{-5}	not HT	1
automated	1-2	10^{-5}	not HT	30-40
SGA	1-2	10^{-2} (DMSO)	HT	200
Capillary electrophoresis				
normal mode	0,01	10^{-5}-10^{-6}	not HT	20
multiplex CE	0,01	10^{-5}-10^{-6}	HT	100

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V.4.1 Potentiometric titration

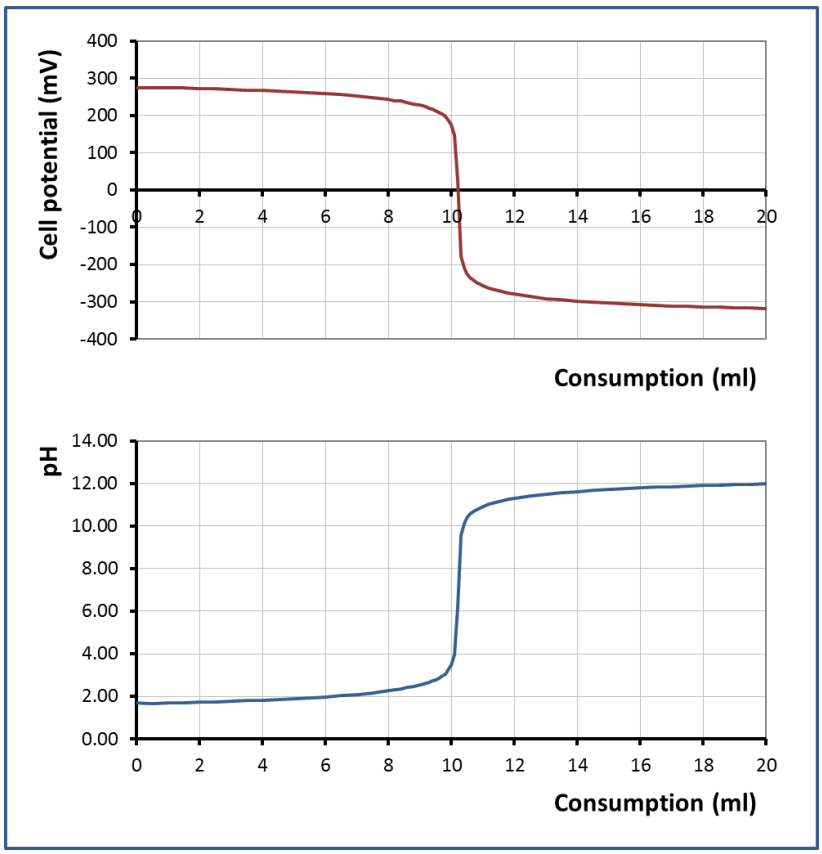
pH-potentiometry is the most widespread, principal method for the determination of protonation constants. There are two ways to carry out pH-potentiometry: the *direct method* and the *difference titration method*.

Direct potentiometric titration

The aqueous and vigorously-stirred solution of the investigated compound is titrated directly with the standardized volumetric solution, which is either a solution of a strong acid (usually hydrochloric acid) or a strong base (usually sodium or potassium hydroxide).

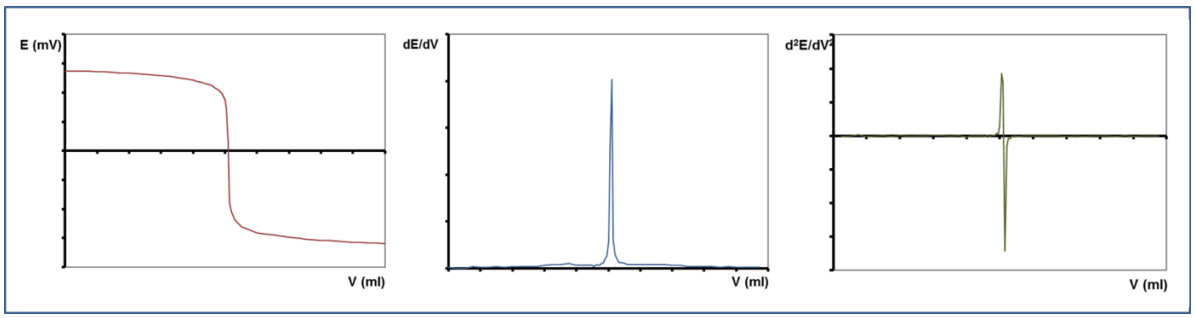
During the titration the cell potential (mV) is continuously measured with a precision combination glass electrode. Since the electrode hase to be calibrated before administration using several buffer solutions, pH values can be assigned to the cell potential values. The plot of cell potential or pH against titrant volume added is called potentiometric titration curve.

Figure V-2.: Potentiometric titration curves



The end-point consumption represents the 100% degree of titration and corresponds to the inflexion point of the titration curve. It is equivalent to the maximum of the first derivative or the zero value of the second derivative of the titration curve.

Figure V-3.: Potentiometric titration curve; the first and the second derivative of the titration curve



The end-point consumption (ml) can be calculated by an appropriate mathematical method (e.g. the difference quotient method). This volume represents the 100% degree of titration. At this point the investigated compound is totally deprotonated or protonated. Using the end-point volume, the degree of titration and the ratio of deprotonated and protonated molecules at different volume values can be calculated. Using the mV – pH calibration curve of the electrode the actual pH values corresponding to these points can be determined. At each pH values the pK_a can be calculated using the Henderson-Hasselbach equation:

$$\mathrm{p}K_\mathrm{a} = \mathrm{pH} + \log\frac{[\text{protonated form}]}{[\text{deprotonated form}]}$$

The pH value corresponding to the 50% degree of titration is equal to the pK_a, since at this point the concentration of the ionized and nonionized form is equal. The most accurate points should be between 30 – 70% degree of titration.

The method is fast and simple, only one titration is enough to calculate the protonation constant. If the compound has more protonation constants, but they differ by at least 4 pK_a units, the method can be applied.

Difference titration method

Two consecutive titrations have to be carried out. First a known amount strong acid with definite concentration is titrated with a strong alkali (known concentration). The second titration is almost the same, except that the investigated compound is added to the strong acid before the beginning of the titration. Both titration curves are illustrated on the same plot.

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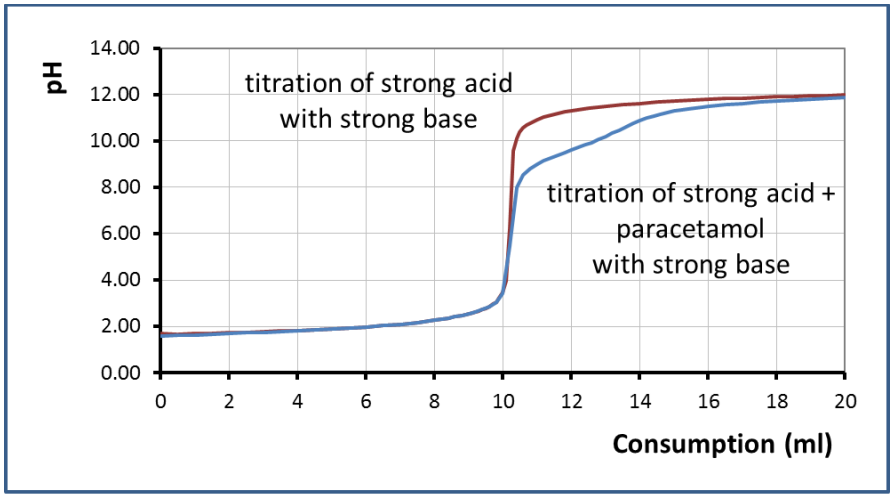
Figure V-4. Potentiometric titration curves – Difference titration of paracetamol



The deviations of the two curves depend on the number of protons dissociated from the investigated compound, therefore the protonation constant can be determined from the difference of the two titration curves using the „H average" ($\bar{H}$) function. $\bar{H}$ is the average number of protons dissociated from the molecule on a definite pH value.

The difference of the two titration curves is constant towards the end of the titration, above a high pH value. It means the compound has become completely deprotonated. Dividing this constant difference by the number of dissociable protons of the molecule we get the alkali consumption for a single proton of the substance investigated under the actual experimental circumstances (weight, concentration of volumetric solution etc.).

The difference in volumetric solution moles can be determined from the distance of the two curves. Divide this by the moles of the substance, we get the $\bar{H}$ value.

$$\bar{H} = \frac{\mathrm{n(difference\ in\ volumetric\ solution\ moles)}}{\mathrm{n(substance)}}$$

$$\mathrm{n(volumetric\ sol.)} = \frac{\Delta V(\mathrm{volumetric\ sol.,ml}) \times c(\mathrm{volumetric\ sol.,mol/dm^3}) \times f}{1000}$$

$$\mathrm{n(substance)} = \frac{\mathrm{m(measured,g)}}{M}$$

$\bar{H}$ can be defined using the protonation constant as well. The calculation schema depends on the number of protonable groups in the molecule.

In case of molecules with only *one protonable group*:

$$\bar{H} = \frac{[A^-]}{[A^-]+[HA]}$$

introducing the protonation constant: $K = \frac{[HA]}{[A^-][H^+]}$

and insetring it into the above equation: $\bar{H} = \frac{[A^-]}{[A^-]+K[H^+][A^-]} = \frac{1}{1+K[H^+]}$

After rearrangement: $K = \frac{1-\bar{H}}{\bar{H}[H^+]}$

Using the $\bar{H}$ and the corresponding $[H^+]$ data pairs the K protonation constant can be calculated at several pH values. The final result will be the average of the K values.

In case of molecules with *two protonable group*:

$$\bar{H} = \frac{2[A^{2-}] + [HA^-]}{[A^{2-}] + [HA^-] + [H_2A]}$$

introducing the protonation constants:

$K_1 = \frac{[HA^-]}{[A^{2-}][H^+]}$ and $K_2 = \frac{[H_2A]}{[HA^-][H^+]}$

and insetring them into the above equation:

$$\bar{H} = \frac{2 + K_1[H^+]}{1 + K_1[H^+] + K_1K_2[H^+]^2}$$

Using two $\bar{H}$ and corresponding $[H^+]$ data pairs the protonation constants K_1 and K_2 can be calculated at several pH values. The calculated values will be more precise if the datapairs are taken from the $\bar{H}_1$ = 0.3 – 0.7 and the $\bar{H}_2$ = 1.3 – 1.7 intervals. Several K_1 and K_2 values should be determined and the average of the them should be the more accurate final result.

Using this method the errors of the direct method can be eliminated. An additional advantage is that this method requires a lower concentration of the compound to achieve reliable results compared to the direct method. Compounds with more protonation constants, even with overlapping pK_a values, can be easiliy investigated.

Advantage of the potentiometric methods is that they are automated and the protonation constants can be determinated very precisely. To optimize the investigations good soluble inert salts (e.g. 0.15 M NaCl) can be applied to maintain the constant ionic-strength. Since the protonation constant depends on the temperature, it is recommended to perform the measurments under termostated conditions.

Disadvantage of potentiometric methods is, that impurities (decomposition products, other ionizable compounds) in the solutions and their carbon dioxide content can cause errors in the calculation. The latter can be eliminated by using of freshly boiled distilled water, or by a blanket of a heavy inert gas (argon, but not helium) on the solution surface.

However, the pH-potentiometry can only be recommended if the solubility of the compound exceeds 0.5 mM in the whole pH range of the titration. The determination will be precise in the pH range between 2 to 12, because the glass

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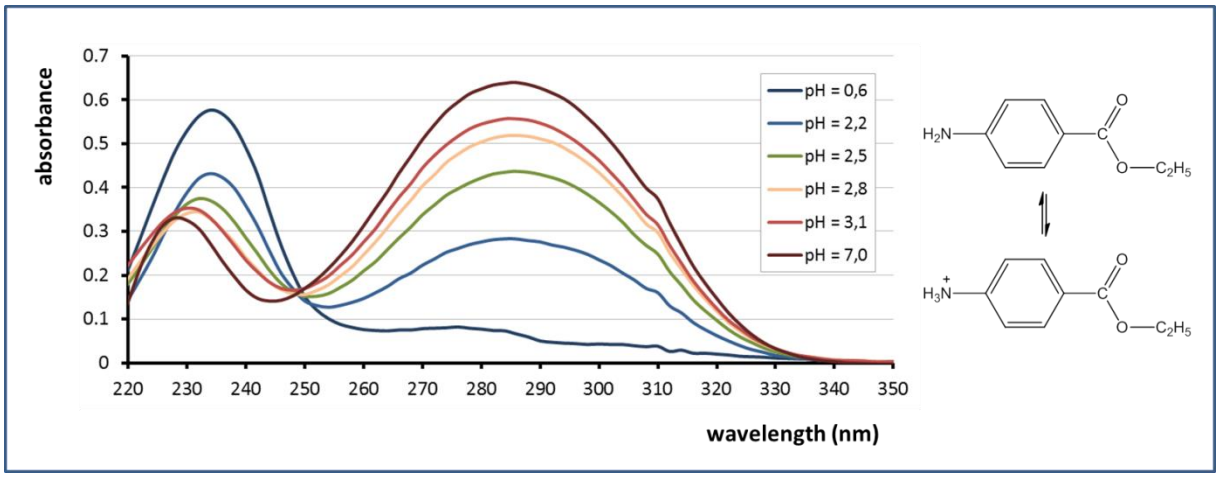
electrode is in that range the most accurate, so the error of determination increases by investigation of strong acids ($pK_a < 2$) or strong bases ($pK_a > 12$).

V.4.2 UV-Vis spectrofotometric method

UV spectroscopy can be a powerful tool of protonation constant determinaton if the compound possesses pH-dependent UV absorption due to a chromophore in the proximity of the protonating group (e.g. phenolate, carboxilate or amino group beside the aromatic ring, nitrogen of the heteroaromatic ring etc.). The protonated state and as a consequence the spectrum alters according to the change of the pH. That means, protonation or deprotonation results in hyper-, hypo-, bato- or hypsochromic shift in the spectrum, as a function of the alteration in conjugation.

Figure V-5.: pH dependence of UV-Vis spectrum of benzocaine



During UV – pH titration the absorbance of the solution of the investigated compound is recorded on constant wavelength as a function of the pH. A series of solutions with same concentration and ionic strength, but with different pH are prepared, and the UV spectra are registered. Evaluation has to be done on that wavelength, where the largest difference between the spectra of the totally protonated and of the totally deprotonated compounds can be seen.

In case of a compound with only one ionizable group, the molecule is totally protonated in the solution with the lowest pH and totally deprotonated at the highest pH. A_{XH} is the absorbance of the protonated molecule, A_X is the absorbance of the deprotonated form and A_{pH} is the absorbance measured at intermediate pH value, respectively. At intermediate pH, the amount of the protonated molecules is expressed as A_X - A_{pH}, while the amount of the deprotonated molecules is A_{pH} - A_{XH}. pK_a can be calculated using the *Henderson-Hasselbach* equation:

$$pK_a = pH + \log\frac{[\text{protonated form}]}{[\text{deprotonated form}]} = pH + \log\frac{A_X - A_{pH}}{A_{pH} - A_{XH}}$$

UV – pH titration can be applied in case of compounds with low solubility, when the potentiometric method is unworkable. Molecules of strong chromophore moiety can be characterized even with solubilities of 50 μM.

The method can be automated, using diode-array UV spectrophotometry coupled to an automated pH titrator. Time of determination becomes significantly shorter.

V.4.3 **Other methods**

SGA method

The so-called *spectral gradient analysis* (SGA) is a 96-well microtitre plate high-throughput method. It is based on a pH-gradient flow technique with diode-array UV detection. A universal buffer was developed in an acidified and an alkaline form. Mixture of the two forms in a flowing stream produce a pH gradient very linear in time. pK_a of one compound can be determined in 4 minutes with satisfactory reliability.

Capillary electrophoresis determination

Capillary electrophoresis determination of pK_as is new, compared to the other techniques. A fused-silica capillary is filled with a dilute aqueous buffer solution. The sample solution is gathered at one end of the capillary, and potential is applied between the ends of the capillary dipped into each of two beakers. Sample species migrate according to their charge and fluid drag. Apparent electrophoretic mobility is determined, which is related to the migration time, the length of the capillary and the applied voltage. The mobility of ionizable compounds is dependent on the fraction of the compound in the charged form. This in turn depends on the pK_a. The plot of the apparent mobility versus pH has a sigmoidal shape, the inflection point is equal to the pK_a.

The method has the advantage of being a rather universal method since different detection systems can be coupled to CE. Because it is a separation technique, sample impurities are not generally a problem. The method is very selecive and the sample consumption is very low.

Other techniques

Further techniques to determine the protonation constant are NMR – pH titration and CD – pH titration methods, respectively. This methods are not rutine techniques already, they are usually applied to solve special problems.

In case of NMR – pH titration the chemical shift of the NMR active atoms in the near of the protonable group(s) is determined as a function of the pH. Advantages of the method are, that impurities do not disturb the determination and it is not required to know the accurate concentration of the compound investigated.

pK_a determination of water-insoluble compounds

Since many substances are very poorly soluble in water, the determination of the pK_a in aqueous solution can be difficult and problematic. Potentiometry can be a good technique for such determination, provided the solubility of the substance is at least 100

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μM. If the substance is only soluble to 1 – 10 μM and possesses a pH-sensitive UV chromophore, then spectrophotometry can be applied. CE methods may also be useful since very small sample quantities are required, and detection methods are generally quite sensitive.

If the compound is virtually insoluble (< 1 μM), then a pH-metric mixed solvent approach (*co-solvent methods*) can be tried. The most explored solvent systems are based on water-alcohol (mostly water-methanol) mixtures. Mixed-solvent solutions of various cosolvent-water proportions are titrated and p_sK_a (the *apparent* pK_a) is determined in each mixture. The aqueous pK_a is deduced by extrapolation of the p_sK_a values to zero cosolvent.

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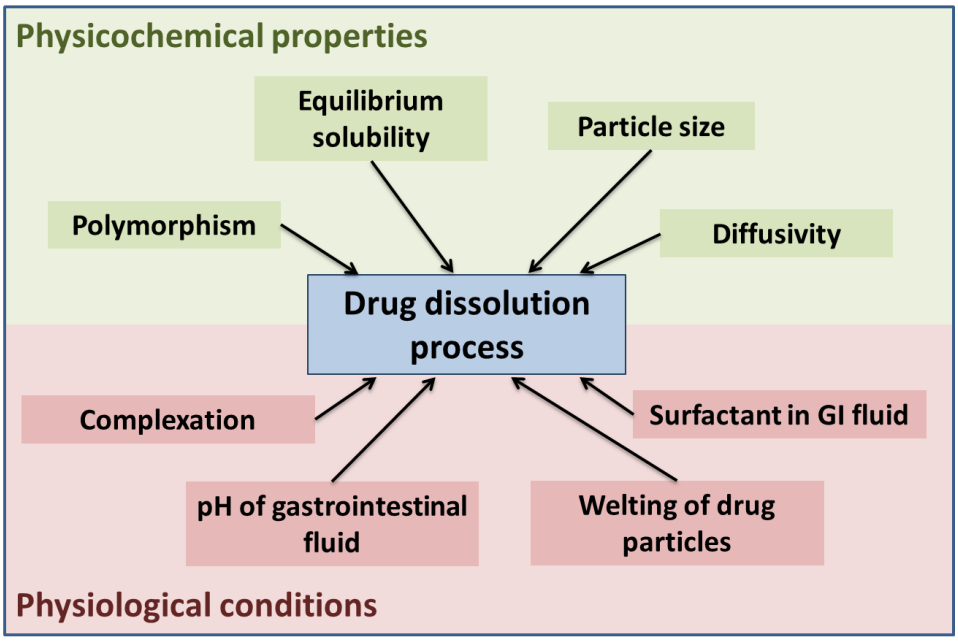
VI Physicochemical properties of drugs - Solubility

Bioavailability of a drug is determinated by a number of properties, including drug dissolution rate as well. Dissolution of a drug plays a special role in oral administartion of a medicinal preparation (tablets, dragees, capsules etc.). Frequently, the rate limiting step in drug absorption from the gastrointestinal tract is drug release and drug dissolution from the dosage form.

Effect of a drug molecule depends on the appropriate water solubility, since the biological and biochemical processes occur in an aqueous medium in the body. The aqueous compartments provide a good solvent for polar compounds, that means hydrophilic character is necessary for good absorption and elimination. However, transport through biological membranes depends on the lipophilic character of the molecule. An optimal drug molecule disposes both lipophilic and hydrophilic properties, that is, it is amphiphil.

All factors – physicochemical and physiological parameters – that influence drug dissolution will probably influence drug absorption and, therefore, bioavailability.

Figure VI-1.: Physicochemical and physiological properties that play an importent role in drug dissolution process



Dissolution in water influences, in addition to the pharmacokinetic parameters, the stability and the optimatization of the formulation process of the medicinal preparation.

Solubility, as a quality requirement, also appears in the pharmacopoeia. The Hungarian Pharmacopoeia VIII. prescribes investigation of dissolution in case of almost all chemical substances.

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VI.1 Definition of solutions

In general, a solution is a homogeneous mixture of two or more components. Solutions may exist in any of the three states of matter, that is, they may be gases, liquids or solids. The terms solute and solvent refer to the components of the solutions; usually the solute is the component is smaller amount and the solvent is the component in greater amount. In pharmaceutical point of view the liquid solutions with solide solutes are the most important.

VI.2 Process of dissolution

Dissolution may occur by different processes.

In case of a *chemical dissolution* profound change occurs, new compounds are formed as a result of chemical reactions and the starting materials are not retrievable using physical methods.

However, generally dissultion is a result of interactions (mostly non-covalent) between the molecules of the solute and solvent. During the process the interactions between the particles in the solute and in the solvent cease, and new interactions will be formed between the molecules of the solute and solvent. This latest process is called **solvation**. If the solvent is water, **hydration** occurs.

During the solvation several intermolecular interactions can be formed, including hydrogen bonding, ion-dipole force, dipole-dipole force, dipole-apolar force and London-type interaction.

Solubility of a compound in a solute at a given temperature and pressure depends on the free enthalpy of the system (*Gibbs-equation*):

$$\Delta G = \Delta H - T \cdot \Delta S$$

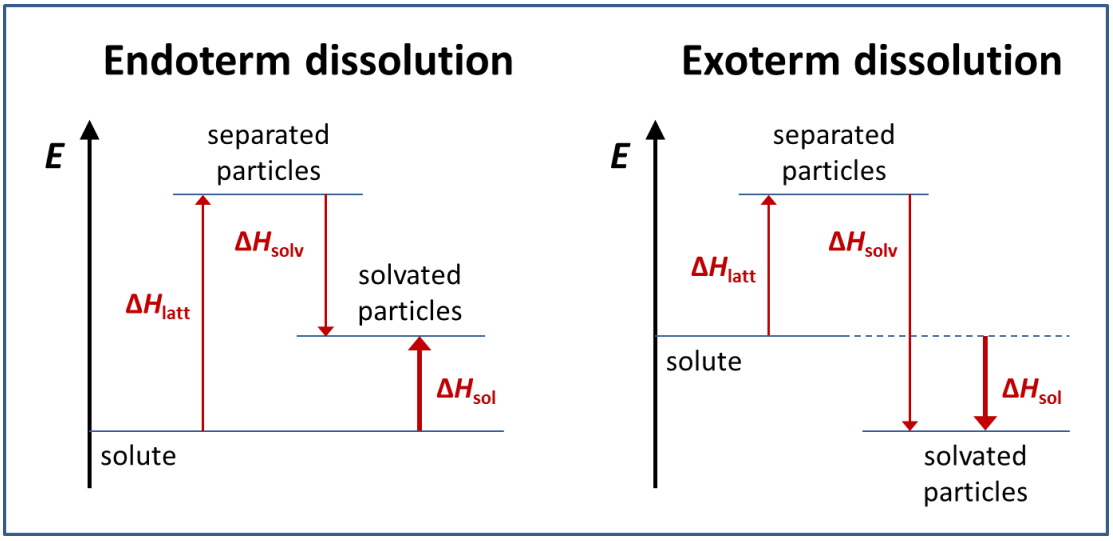
If the ΔG for a reaction at a given temperature and pressure is negative, the reaction will be spontaneous. ΔG depends on the value of ΔH and $T \cdot \Delta S$.

Hydration of ions favors the dissolution of an ionic solid in water. Ions on the surface of the crystal become first hydrated and move into the body of the solution as hydrated ions. Hydration of an ion is accompanied with the change of energy, which is called entalphy of hydration/solvation ($\Delta H_{hydration}/\Delta H_{solvation}$). The ions in a crystal, however, are very strongly attracted to one another. Therefore, the solubility of an ionic solid depends not only on the energy of hydration of ions, but also on lattice energy ($\Delta H_{lattice}$), the energy holding ions together in the crystal lattice.

The heat/enthalpy of dissolution is the enthalpy change associated with the dissolution of a substance (1 mol) in a solvent at constant pressure resulting in infinite dilution. The value of the enthalpy of solution is the sum of the enthalpy of the individual steps, that is, the sum of the lattice energy and the enthalpy of solvation. The process is exothermic when heat is evolved, or exothermic when heat is absorbed.

$$\Delta H_{dissolution} = \Delta H_{lattice} + \Delta H_{solvation}$$

Figure VI-2.: Heat of dissolution



VI.3 Definition and types of solubility

The amount of substance that will dissolve in a solvent depends on both the substance and the solvent. Increasing the amount of the solute, at one point dynamic equilibrium is reached in which the rate at which particles leaves the crystals equals the rate at which particles return to the crystals. At equilibrium, no more solute appears to dissolve. That is the **saturated solution**, a solution that is in equilibrium with respect to a given dissolved substance.

Solubility is the amount of solute that dissolves in a given quantity of solvent at a given temperature to give a saturated solution. There are two approaches to describe the solubility, the so-called *equilibrium solubility* and the so-called *kinetic solubility*.

Thermodynamic (equilibrium) solubility, *S*

The equilibrium solubility of a compound is defined as the maximum quantity of that substance which can be completely dissolved at a given temperature and pressure in a given amount of solvent, and is thermodynamically valid as long as a solid phase exists which is in equilibrium with the solution phase. It also depends on the crystal structure of the substance.

In case of ionisable compounds the solubility depends on the pH. ***Intrinsic solubility*** (S_0) is the equilibrium solubility of the free acid or free base form of an ionisable compound at a pH where it is totally unionised.

Kinetic solubility, S^{APP}

Kinetic solubility is the concentration of a compound in solution when an induced precipitate first appears. It is the instantaneous solubility at the time of sampling, since there is not enough time for developing of equilibrium. The kinetic solubility is always higher than the thermodynamic solubility.

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Kinetic solubility measures a precipitation rate rather than solubility. Typically, the compound is dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of known concentration. This stock is added gradually to the aqueous solvent of interest until the anti-solvent properties of the water drive the compound out of solution.

Solubility results obtained from kinetic measurements might not match the thermodynamic solubility results perfectly, because kinetic solubility is determined for compounds that have not been purified to a high degree or crystallized, the impurities and amorphous content in the material lead to a higher solubility than the thermodynamic solubility. On the other hand kinetic solubility experiments begin with the drug in solution, it is not needed to vanquish the intramolecular forces in the crystal

Kinetic solubility methods are designed to facilitate high throughput measurements which is very usefull in the early phase of drug discovery. In that phase very precise solubility data are not required, knowledge of the approximate values are adequate, since they are appropriate to predict the oral administration of the drug.

Solubility can be expressed in different concentration units, e.g. g/100ml, g/ml, g/liter, mol/liter etc. Solubility is marked with *S*. It can also be expressed in log*S* (p*S*), the negative logarithm of *S*. The correct concentration unit always has to be signed.

Solubility is one of the importent physicochemical characteristics of pharmaceutically active compounds. In the Hungarian Pharmacopoeia (Ph.Hg.VIII.) solubility of substances is characterized by the following terms.

Table VI-1: Descriptive terms used in the Pharmacopoeia (Ph.Hg.VIII.) to characterize solubility of substances (Terms are valid between 15-25°C.)

Descriptive term	Approximate volume of solvent (ml) per gram of solute
Very soluble	< 1
Freely soluble	1 - 10
Soluble	10 - 30
Sparingly soluble	30 - 100
Slightly soluble	100 - 1000
Very slightly soluble	1000 - 10000
Practically insoluble	> 10000

Solubility product constant, *L or* K_{sp}

In general, the solubility product constant is the equilibrium of a slightly soluble (or nearly insoluble) ionic compound. Solubility and solubility constant are related but not identical concepts. Solubility is the equilibrium concentration of the saturated solution at given temperature. Solubility depends on other ingredients present in the solution. However, solubility product constant is an *equilibrium constant*, its value depends only on the temperature and the quality of the substance.

Example:

Molar solubility of AgCl is *S*.

$$AgCl\ (s) \leftrightarrow Ag^+ + Cl^-$$

It can be seen from the equation of dissolution, that $[Ag^+] = [Cl^-] = S$, that is, the ionconcentrations are equal to the molar solubility. The solubility product constant (as equilibrium constant) can be calculated:

$$L = [Ag^+] \cdot [Cl^-] = S \cdot S = S^2$$

Molar solubility of $Al(OH)_3$ is *S*.

$$Al(OH)_3\ (s) \leftrightarrow Al^{3+} + 3\ OH^-$$

In the equilibrium of dissolution the concentration of $[OH^-]$ is three times higher than the concentration of $[Al^{3+}]$. Based on these the solubility product constant can be calculated:

$$L = [Al^{3+}] \cdot [OH^-]^3 = S \cdot (3S)^3 = 27S^4$$

VI.4 Factors influencing the solubility

Solubility is primarily specified by the solute and the solvent, but many other factors may influence it, such as the temperature, pressure, nature of the solute (crystal structure, particle size, polymorphism), presence of other compounds, presence of surfactans, aggregation, formation of micelles etc.

VI.4.1 Chemical structure of solute and solvent

Type and strength of interactions between the particles of the solute and the solvent are primarily defined by the molecular structure of them.

The statement „like dissolves like" describes the process of dissolution. That is, substances with similar intermolecular attractions are usually soluble in one another. Polar solvents dissolve rather polar compounds, apolar solvents dissolve rather apolar compounds, respectively.

Water is a polar solvent. Apolar gases, liquids and solid compounds dissolve are very slightly soluble in water. Molecules, which also contain besides the apolar part a polar moiety dissolve better in water. Naturally, ionic compounds and polar substances are very soluble in water.

VI.4.2 Temperature

Dissolution is always accompanied by alteration of the temperature, consequently, the solubility depends on the temperature. Dissolution can be an exothermic process, in that case solubility decreases with rising temperature. However,

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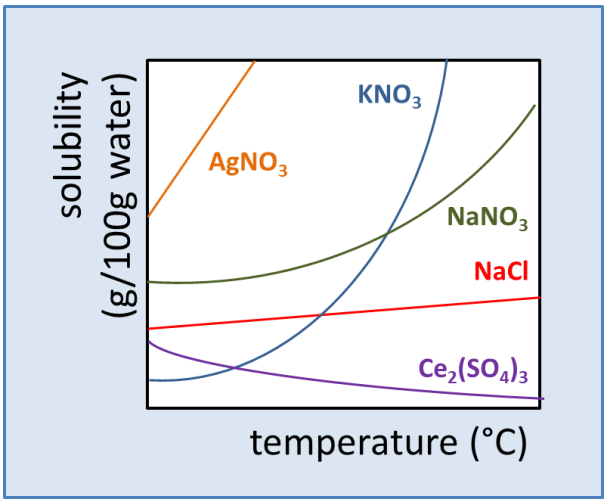
in case, dissolution is an endothermic process, compounds become more soluble with rising temperature.

Figure VI-3.: Dependence of solubility on the temperature



VI.4.3 Solubility of ionizable compounds

Solibility of ionizable substances depends on the pH of the solution. To calculate the solubility (S_{pH}) of the compound, using the *Henderson-Hasselbach equation*, knowledge of the intrinsic solubilty value (S_0) and the pK_a value of the molecule is required. The intrinsic solubility equilibrium constant characterizes the precipitation equilibria of the uncharged species.

In case of a *weak acid* the dissociation and the solubility can be defined as

$$HA \leftrightarrow H^+ + A^- \qquad K_a = \frac{[H^+][A^-]}{[HA]}$$

$$HA\,(s) \leftrightarrow HA \qquad S_0 = \frac{[HA]}{[HA(s)]} = [HA]$$

In a saturated solution, the effective solubility, at a particular pH is defined as the sum of the concentration of all of the compound species dissolved in the aqueous solution:

$$S_{pH} = [A^-] + [HA]$$

It is convenient to restate the equation in terms of only constants and with pH as the only variable.

$$S_{pH} = \frac{K_a[HA]}{[H^+]} + [HA]$$

$$S_{pH} = [HA]\left(\frac{K_a}{[H^+]} + 1\right)$$

$$S_{pH} = S_0(10^{-pKa+pH} + 1)$$

$$\log S_{pH} = \log S_0 + \log(10^{-pKa+pH} + 1)$$

In case of *weak bases* the correlations are similar to that of weak acids:

$BH^+ \leftrightarrow H^+ + B$ $\qquad K_a = \frac{[H^+][B]}{[BH^+]}$

$B\,(s) \leftrightarrow B$ $\qquad S_0 = \frac{[B]}{[B(s)]} = [B]$

$$S_{pH} = [B] + [BH^+]$$

$$S_{pH} = [B] + \frac{[H^+][B]}{K_a}$$

$$S_{pH} = [B]\left(\frac{[H^+]}{K_a} + 1\right)$$

$$S_{pH} = S_0(10^{+pKa-pH} + 1)$$

$$\log S_{pH} = \log S_0 + \log(10^{+pKa-pH} + 1)$$

In case of *diprotic ampholytes* there are two steps of ionization, solubility is influenced evidently by both pK_a values.

$H_2X^+ \leftrightarrow H^+ + HX$ $\qquad K_{a1} = \frac{[H^+][HX]}{[H_2X^+]}$

$HX \leftrightarrow H^+ + X^-$ $\qquad K_{a2} = \frac{[H^+][X^-]}{[HX]}$

$HX\,(s) \leftrightarrow HX$ $\qquad S_0 = \frac{[HX]}{[HX(s)]} = [HX]$

$$S_{pH} = [X^-] + [HX] + [H_2X^+]$$

$$S_{pH} = S_0(1 + 10^{-pKa2+pH} + 10^{+pKa1-pH})$$

$$\log S_{pH} = \log S_0 + \log(1 + 10^{-pKa2+pH} + 10^{+pKa1-pH})$$

On the following figures the solubility-pH profile of a weak acid, a weak base and a diprotic ampholyte can be seen.

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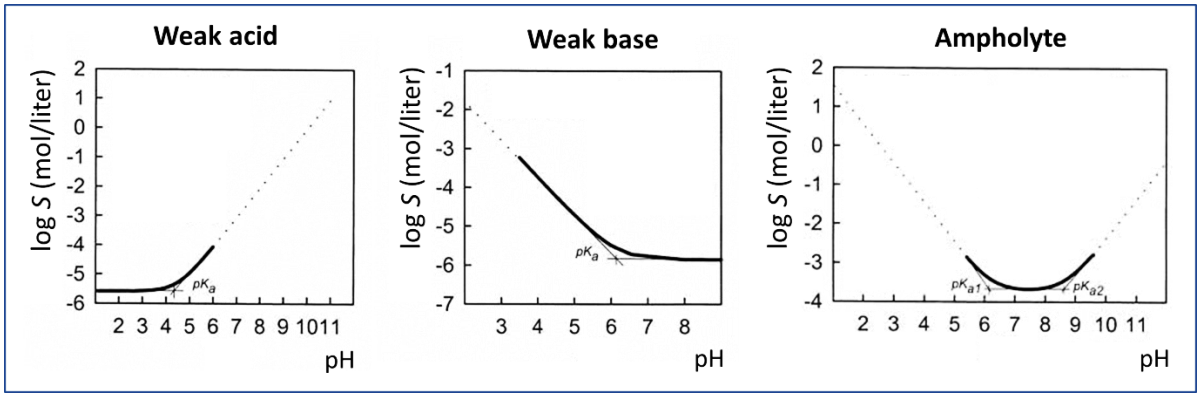
Figure VI-4.: Solubility-pH profile for a weak acid, a weak base and for an ampholyte



As is evident from the acid curve, for pH << pK_a the function reduced to the horizontal line log$S \approx$ logS_0. For pH >> pK_a, log$S$ is a straight line as a function of pH, exhibiting a slope of +1. At the pH lower than the pK_a solubility of a weak acid is minimal.

The bases shows a slope of -1. The pH at which the slope is half-integral equals the pK_a. A mirror relationship between the curve for an acid and the curve for a base can be seen on the figure. If the pH >> pK_a, than log$S \approx$ logS_0. Consequently, solubility of bases is at pH lower than pK_a value significant.

In case of diprotic ampholytes the solubility-pH profile is similar to a parabola. If pH << pK_{a1} the curve is similar to the curve of bases, but if the pH >> pK_{a2} the curve is comparable to the curve of acids. The minimum-point of the curve is the isoelectric point, at that pH is the solubility the lowest.

For the solubility speciation curves discontinuities can be seen. These are the transition points between a solution containing some precipitate and a solution where the sample is completely dissolved.

The pH of the gastrointestinal fluids is, therefore, one of the most important influences on the saturation solubilty of ionizable drugs. The pH of the gastrointestinal fluid varies widely with location in the gastrointestinal tract. Additionaly, gastric pH exhibits complex variation between the fed and fasted states.

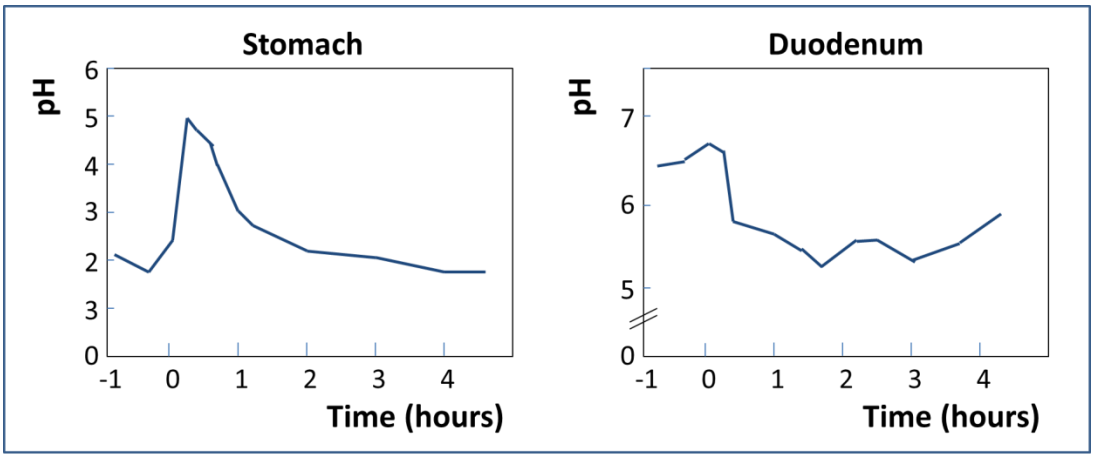
Table VI-2: Average pH values in healthy humans at various sites in the gastrointestinal tract

Location	Average pH	
	fasted state	fed state
Stomach	1.7	4.9 (0.1 óra)
Duodenum	6.5	5.4 (1 óra)
Jejunum	6.6	5.2 – 6.0
Ileum	7.4	7.5

Following ingestion of a meal the gastric pH initially increases because of the buffering effects of food components. However, 3-4 hours following the meal intake the

fasted condition pH returns to normal as a response to gastric secretion due to food ingestion. For instance, generally, weak bases such as *ketoconazole* (pK_{a1} = 6.5; pK_{a2} = 2.9) will be less soluble in the stomach if administered immediately after food intake because the gastric fluids are less acidic.

Figure VI-5.: Gastric and duodenal pH in the fasted state and after food intake



It is important to note, that other factors such as age, pathophysiological conditions (e.g. achlorhydria, AIDS) and concurrent drug therapy (e.g. H_2-receptor antagonists, proton pump inhibitors) will influence the pH of the luminal fluids. In the case of poorly soluble weak bases, such as the mentioned ketoconazole, elevated gastric pH in AIDS patients leads to a reduced rate of drug dissolution and consequently to malabsorption.

In response to a meal as well as to the arrival of chyme from the stomach, the small intestine pH at first decreases, however, the fasted state pH is re-established as soon as pancreatic bicarbonate is produced. Poorly soluble weak acids with pK_a values less than 6 (e.g. furosemide pK_a = 3.9; indomethacin pK_a = 4.5) are relatively insoluble in the preprandial gastric juice and dissolution occurs first in the upper small intestine. However, in the case of very weakly acidic compounds like paracetamol (pK_a = 9.5) or hydrochlorothiazide (pK_a = 8.8) the variations in pH in the gastrointestinal tract are irrelevant to their solubility because these compounds remain in the free acid form over the physiological pH range.

VI.4.4 **Presence of forign compounds, purity of substance and solvent**

The solubility of a compound may be influenced by the impurities of the solvent or of the impurities in the substance itself. Impurities which are more soluble than the compound investigated may cause big errors during measurments, especially when a non specific analytical method is used.

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VI.5 Experimental methods to determine the solubility

Several methods can be applied to determine the solubility. A distinction of methods should also be done whether kinetic or thermodynamic solubility will be determined.

The aim in the early discovery phase of drug development is to filter and rank the compounds, therefore, collection of informative data about solubility are adequate. It is required, that the methods used in the discovery should be very fast, to provide the possibility to screen hundredthousands of molecules, that is, the methods have to be high throughput (HT). Methods, which capacity is at least 50 molecules/day are usually called HT. Kinetic solubility is determined in the early discovery phase, where samples are introduced as 20-30 mM DMSO solutions. Little fractions of DMSO solution is added to the aqueous medium. When precipitation begins, concentration of the solution will be determined.

In the later phase of drug development, determination of the thermodynamic solubility is required as well. In principle, the solid compound will be added in excess directly to the aqueous medium, the solution will be incubated for a longer time and the concentartion of the saturated solution will be determined using an appropriate analytical method. The methods have to be precise and reproducible.

Table VI-3: Experimental methods to determine the solubility

Method	Type of solubility	Type of method	Detector	Limit of detection
Turbidimetric	kinetic	HT	UV	5 µg/ml
Nephelometric	kinetic	HT	laser nephelometer	5 µg/ml
Direct UV (µSOL)	kinetic/ thermodynamic	HT	UV	0.1 µg/ml
Saturation shake-flask	thermodynamic	not HT	UV	1 µg/ml
Miniaturized shake-flask	thermodynamic	HT	HPLC-UV or LC/MS	1 µg/ml
Generator column	thermodynamic	not HT	HPLC	0.1 µg/ml
DTT	thermodynamic	not HT	potentiometry	5 ng/ml
CheqSol	thermodynamic	HT	potentiometry	0.1 µg/ml

VI.5.1 Methods to determine the kinetic solubility

Turbidimetry detection-based methods have been introduced by *Lipinski* and co-workers. The sample is dissolved in DMSO. The stock solution is diluted with an aqueous buffer solution to form a dilution series. Usually, measurements are performed at one pH. In that solution where the solubility value is reached, precipitation will occur. The undissolved particles cause turbidity which results in increase of absorbance in the UV detector. Measurments are automated, a large number of samples can be investigated shortly. Disadvantages of the turbidimetric methods are: poor

reproducibility for very-sparingly water soluble compounds, use of excessive amounts (>1% v/v) of DMSO in the analate addition step and lack of standardization of practice.

In the so-called **nephelometric method**, intensity of the scattered light will be measured using a laser nephelometer, from which the concentration of the saturated solution can be calculated.

In the „**direct-UV method**" a known quantity of sample is added to a known volume of buffer solution with known pH. The amount of sample must be sufficient to couse precipitation resulting in forming a saturated solution. After separation of the solid phase, the UV spectrum of the clean solution will be registered on a spectrophotometer. A reference solution is also prepared by a dilution method. A known quantity of the sample is dissolved in a known volume of the solvent, making sure, that precipitation will not occur. Spectrum of the reference solution will be compared to the spectrum of the sample prepared by the dilution method. Evaluation is based on the areas under the curves.

In order to increase throughput, the classical saturation shake-flask method has been transfered to 96-well plate technology using a robotic liquid dispensing system. Analyses are performed with fast generic gradient reverse-phase HPLC.

The capacity of that methods is satisfying, they are able to determine the kinetic solubility. However, their accuracy is limited, since very small volumes are investigated, and usually on one pH. Further disandvantage of the methods is, that differences in solubility of different crystalforms and polymorphs disappear due to the dissolution in DMSO.

VI.5.2 Methods to determine the thermodynamic solubility

Saturation shake-flask method is a classical method to measure the equilibrium solubility. It is a relatively simple procedure. The drug, which is ususally crystallic, is added to a standard buffer solution (in a flask) until saturation occurs, indicated by precipitation. The thermostated saturated solution is shaken until equilibration between the two phases establishes. Generally it requires a long equilibration time (min. 24-48 hours). After microfiltration or centrifugation, the concentration of the substance in the supernatant solution is determined using HPLC, usually with UV detection. If a solubility-pH prifile is required, the measurments have to be performed in parallel in several different pH buffers. Disadvantages of the method are, that it is time consuming and requires a large amount of test material. Therefore the method is not suitable for addressing solubility in early drug discovery. When introducing and validating new methods the shake-flask method is often used as the reference method.

The high throughput method based on the shake-flask method using a 96-well microtitre plate format and plate UV spectrophotometry was also introduced. The aim is primarily to enhance the capacity and to decrease the amount of materials (sample, solvent).

Potentiometric methods for solubility measurement can be applied to investigate ionizable molecules as well. The so-called pSOL apparatus is based on dissolution template titration (DTT-method). The procedure takes as input parameters the measured (or calculated) pK_a and the measured (or calculated) $logP_{o/v}$ values. Tha latter parameter is used to estimate the intrinsic solubility (S_0). Using the pK_a and the estimated S_0, the DTT procedure simulates the entire titration curve before the assay. The simulated curve serves as a template for the instrument to collect individual pH

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measurments in the course of the titration. The pH domain containing precipitation is apparent from the simulation. Titration of the sample suspension is done in the direction of dissolution, eventually well past the point of complete dissolution. The rate of dissolution of the solid depends on a number of factors, which the instrument takes into account. For example, the instrument slows down the rate of pH data taking as the point of complete dissolution approaches, where the time needed to dissolve additional solid substantially increases. Only after the precipitate completely dissolves, does the instrument collect the remainder of the data rapidly. Typically, 3 - 10 hours are required for the entire equilibrium solubility data taking. An entire solubility-pH profile is deduced from the assay.

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VII Physicochemical properties of drugs - Lipophilicity and partition coefficient

Lipophilicity is one of the oldest and very important physicochemical properties of biological active molecules, which desribes the likehood of a molecule to participate into an apolar (lipophil) phase, with respect to a hydrophilic phase. The ability of drugs to diffuse passively through biological membranes is influenced mainly by their lipophilicity.

Therefore, lipophilicity has a wide impact on the oral absorption, distribution within the body, as well as metabolism and elimination of drug candidates. On the other side, lipophilicity is an extensively used parameter in the process of drug development, including drug design, drug formulation and drug-delivery studies.

VII.1 The partition coefficient

A pure substance may distribute itself between two immiscible or partially miscible solvents in intimate contact until equilibrium develops. The **partition coefficient** is the ratio of the activities of the compound in the two phases of the mixture at equilibrium. The partition coefficient is at permanent temperature and pressure a constant value. In the case of diluted solutions, when the concentartion is lower than 10^{-2} M, the partition coefficient can be expressed using the concentartion values instead of activities. To characterize the lipophilicity of a compound the most common term used is the *octanol/water partition coefficient*, with the symbol *P*. In the correlation "o" refers to the organic phase and "w" to the aqueous phase. Since *P* can range over many orders of magnitude, it is usually expressed as its decadic logarithm, log*P*.

According to the definition by *Nernst*:

$$P = \frac{\gamma_o c_o}{\gamma_w c_w}$$

In case of *dilute solutions*:

$$P = \frac{c_o}{c_w}$$

As agreed, concentration in the organic phase is the dividend, therefore the higher the partition coefficient the bigger the affinity of the substrate to the organic phase, that is the lipophilicity.

According to partition, drug molecules can be classified into two main groups: *neutral* (non-ionized) and *ionizable* (acids, bases) compounds.

The so-called ***true partition coefficient*** expresses the partition of nonionized, neutral particles (solute present in a single electrical state), and is – at a given temperature and pressure – undependent on the pH. However, the so-called ***apparent partition coefficient*** or ***distribution coefficient*** (*D* or P_{app}) is dependent on the

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proportion of all substances, all different electrical species present in the solution, which in turn depends upon the pH of the solution.

For neutral molecules, in so far as association does not occur, the true and the experimentally measured partition coefficients are always equal. On the other side, in case of ionizable molecules, ionization may occur depending on the pK_a value and the pH of the medium, therefore more than one electrical species are present in the solution, the true partition coefficient and the apparent partition coefficient differ from each other.

If the pK_a value of the compound is known, the true partition coefficient can be calculated using the following equations.

In the case of *acids:*

$$A^- + H^+ \rightleftharpoons HA \qquad K_a = \frac{[HA]}{[H^+][A^-]}$$

$$P = \frac{[HA]_o}{[HA]_v}$$

$$P_{app} = \frac{[HA]_o}{[HA]_v + [A^-]_v} = \frac{[HA]_o}{[HA]_v + \frac{[HA]_o}{[H^+]K_a}} = \frac{P}{1 + \frac{1}{[H^+]K_a}}$$

$$\log P = \log P_{app} + \log(1 + 10^{pH - pKa})$$

In the case of *bases:*

$$B + H^+ \rightleftharpoons BH^+ \qquad K_b = \frac{[BH^+]}{[H^+][B]}$$

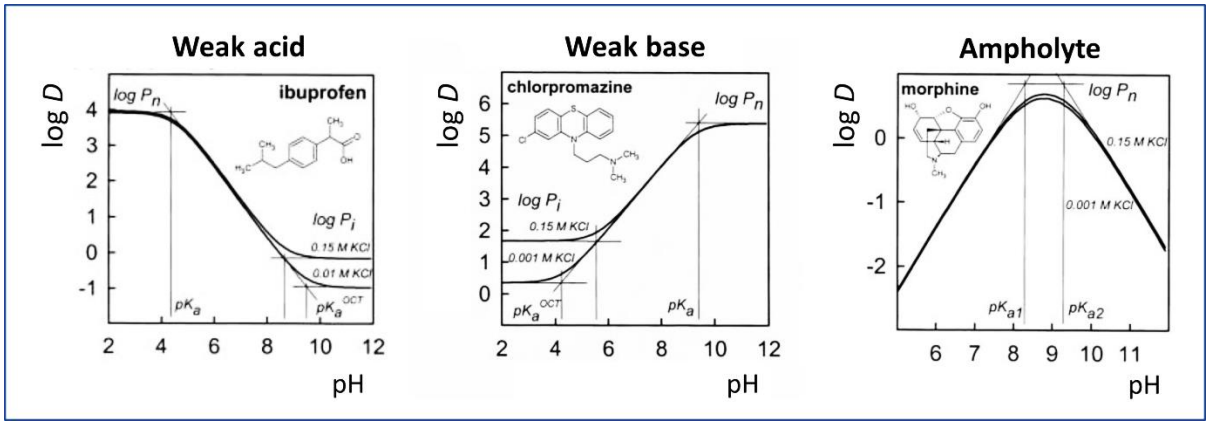
$$P = \frac{[B]_o}{[B]_v}$$

$$P_{app} = \frac{[B]_o}{[B]_v + [BH^+]_v} = \frac{[B]_o}{[B]_v + K_b[B]_v[H^+]} = \frac{P}{1 + K_b[H^+]} = \frac{P}{1 + \frac{[H^+]}{K_a}}$$

$$\log P = \log P_{app} + \log(1 + 10^{pKa - pH})$$

It is apparent, since weak acids are neutral at pH lower than the pK_a, their lipophilicity increases in a solution with lower pH. When the pH is higher than the pK_a, lipophilicity decrases. Obviously, in the case of bases correlation between pH and lipophilicity is reverse. For ampholytes lipophilicity shows maximum at pH between the two pK_a values.

Figure VII-1.: Lipophilicity profile of a weak acid, a weak base and an ampholyte



VII.2 Importance of the partition coefficient

The log*P* values of drug molecules are intuitive, manageable data, very useful to compare the lipophilicity of the compounds. Log*P* value of most drugs (about 90%) is between 0 and 5. Drugs with log*P* outside that range can be transfered only by specific transport mechanisms or show extreme pharamcokinetic character.

Some drug molecules with different log*P* values are collected in the following table, representing how lipophilicity influences the absorption.

Table VII-1: Log*P* value and characters of absorption of some drug molecules

Drug	log*P*	Absorption
Ascorbic acid	-1,85	not absorbed through passive diffusion, but through active transport
Methilhomatropine-bromide	-1,65	not absorbed, does not enter the CNS
Diazepam	2,82	good absorption through passive diffusion
Amiodarone	7,57	accumulates in the body (half time: 25 days)

From pharmaceutical point of view, knowledge of the true partition coefficient is relevant to predict the transport of drug molecules through biological membranes, because only nonionized, neutral molecules are able to be transfered by passive diffusion.

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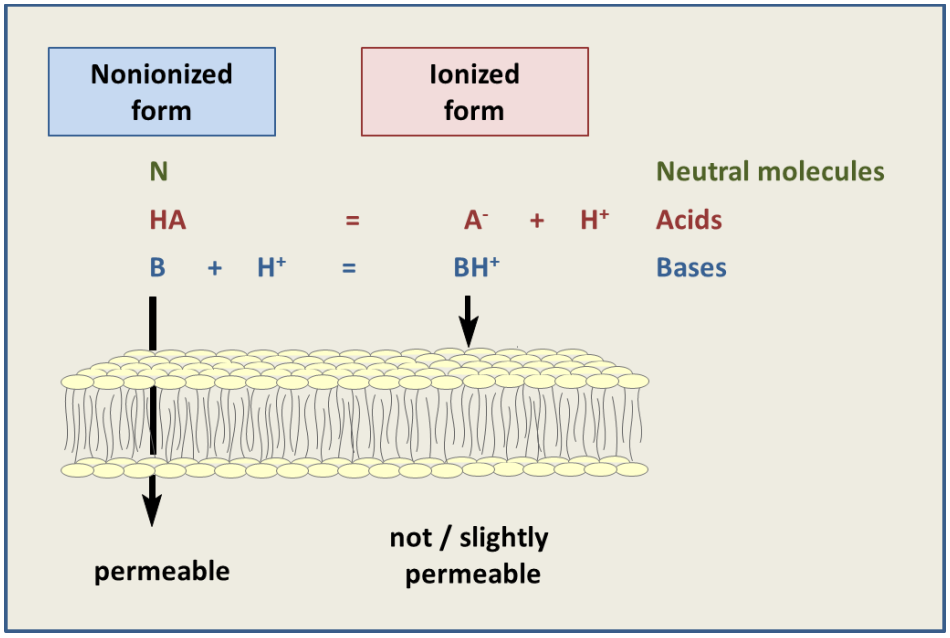
Figure VII-2.: „pH-partition hypothesis”



In the case of ionizable compounds knowledge of the pH dependence of the apparent partition coefficient is essential to understand their fate in the body. The partition coefficient of ionizable molecules alters in the body as a function of the pH of the compartment, allowing the prediction of place and rate of absorption.

However, it should be noted, only a general, not very precise prediction of transport is possible, because oral bioavailibility of a drug will be influenced by a lot of parameters

Log*P* values are also required to solve several analytical and technological problems.

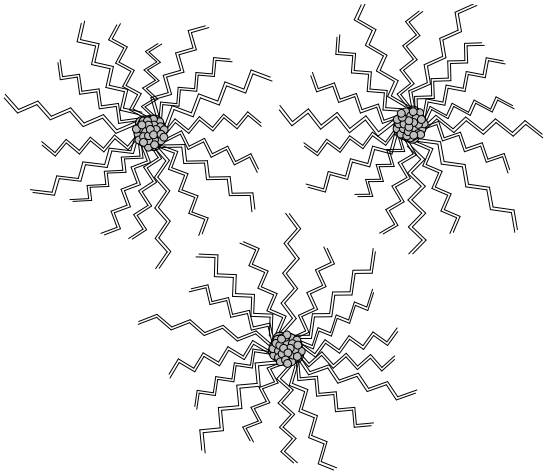
VII.3 Lipophilicity and phospholipophilicity

VII.3.1 The octanol/water system

In pharmaceutical chemistry lipophilicity is conventionally expressed as the logarithm of the octanol/water partition coefficient. Octanol ($C_8H_{17}OH$) is the organic solvent of choice as due its amphiphilic property (apolar hydrocarbon chain and polar hydroxyl functional group) it is able to mimic the interactions between drugs and the lipid layer or protein components of the membrane. This solvent system has been proved to be a good predictor of biological distribution.

Octanol is immiscible with water, however it is able to bind a significant amount (2.36 M) of water. Octanol molecules form micelles in water.

Figure VII-3.: Octanol micelles



Since biological membranes are diverse, only one solvent system is not really able for modeling biological distribution, therefore nowadays more solvent systems are used, for instance the „critical quartett" of solvent system (octanol/water, alkane/water, chloroform/water and propyleneglycol-dipelargonate (PGDP)/water).

Table VII-2.: „Critical quartett" of solvent system

Solvent	Type of membrane
octanol	amphipathic
alkane (cyclohexane)	inert
chloroform	mainly proton donor
propylenglycol-dipelargonate	mainly proton acceptor

VII.3.2 **Phospholipophilicity**

Relevance of liposome/water and liposome/buffer systems is also increasing. Liposomes consists of phospholipides (phosphatidylcoline, phosphatidylserine, phosphatidylinositol) and other molecules (fatty acids, cholesterol, bile acids etc.). In such systems partition of ionic forms is also significant.

Phospholipophilicity characterizes mainly the absorption of drug molecules from the gastrointestinal tract. It provides additional informations about absorption, because phospholipophilicity takes, besides the hydrophobic interactions, the ionic-ionic interactions and hydrogen bonding also into consideration.

VII.4 Determination methods of partition coefficients

Methods to determine log*P* values can be classified in two groups: the direct methods and the indirect, chromatographic methods. In addition, prediction methods are also available, which are used to predict lipophilicity due calculation.

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Table VII-3: Experimental methods to measure log*P*

Method	Substance needed (mg)	logP range	Type of method	Capacity (compound/day)
Direct				
shake-flask method	10-50	-2 - +3	not HT	2
96-well titre plate	1-5	-2 - +4	HT	100
potentiometry	5-10	-2 - +6	not HT	20
Indirect				
RP-TLC	1-3	0 - +5	not HT	50
RP-HPLC – normal	1	-1 - +6	not HT	120
RP-HPLC – HT method	0,01	-1 - +6	HT	100
microemulsion elektrokinetic chrom.	1	-1 - +6	HT	120

VII.4.1 **Direct methods**

Traditional shake-flask method

The generally accepted standard method for log*P* measurment is still the shake-flask method. The essential of this method is the intense partition of the compound between two immiscible solvent phases at a constant, controlled temperature.

Before measurment, the two phases, the aqueous and the organic phase (usually octanol), are saturated with each other to avoid later error. The substance investigated is dissolved in the aqueous phase. After determination of the exact concentration of the aqueous solution, the organic phase is added and intense mixing by shaking or stirring is performed. When the equilibrium state is reached, the two phases are separated by centrifugation, and the concentration of the distributed substance in the phases is determined with a suitable analytical method. Usually spectrophotometry is the method of choice if the spectral properties of the compound make it possible.

In order to receive reproducible results, the standardization of the method is required, that is the following experimental conditions have to be controlled: the time of presaturation of the partitioning phases, temperature, checking whether equilibrium has set in, permanent ionic strength.

Under validated conditions the method has an average error of ± 0.05 to 0.10 log units in the -2 to +3 log*P* interval. Log*P* of very hydrophilic or lipophilic compounds (log*P* lower than -2 or higher than +4) can not be determined very accurate.

The traditional shake-flask method is still a reference method despite the disadvantages of it (work- and time-consuming, big volume of solvents is needed, compounds in narrow log*P* range can be investigated etc.).

Potentiometric methods

Essential of potentiometric methods is that two consecutive titrations are performed under the same conditions: the first one in aqueous medium without organic

phase, and the second titration in the presence of two phases. If the substance investigated distributes between the two phases, the two potentiometric titration curves differ from each other.

Two dissociation constants can be determined, the pK_a value and the p_oK_a value, the later is calculated from the titration which is performed in the presence of the organic phase. Calculation of partition coefficient is based on the difference of dissociation constants.

In case of *acids:*

$$P = (10^{\mathrm{p_oK_a - pK_a}} - 1)\mathrm{R}$$

In case of *bases*:

$$P = (10^{\mathrm{pK_a - p_oK_a}} - 1)\mathrm{R}$$

where R is the ratio of the volumes of the aqueous and organic phases.

The method is accurate and very useful for investiagtion of ionizable compounds. Substances which log*P* is in the range from -1 to +6 can be measured.

VII.4.2 **Indirect methods**

Indirect techniques are actually the most used methods to determine lipophilicity. Chromatographic methods are fast and relative simple, a lot of compounds can be investigated in a short time, which is important in the early phase of drug development. The methods can also be used to investigate very lipophilic and hydrophilic compounds, when they are unsuitable for the traditional techniques.

The most important indirect techniques are **reversed phase chromatographic methods** (thin-layer chromatography RP-TLC and high pressure liquid chromatography RP-HPLC).

The theoretical base for the determination of log*P* by chromatographic methods is the relation between the partition coefficient and a chromatographic retention parameter (R_M or log*k*') based on liquid/liquid partition.

TLC:

$$R_M = \log\left(\frac{1}{R_f} - 1\right) = \log\left(\mathrm{K_{TLC}}\frac{V_S}{V_M}\right)$$

HPLC:

$$\log k' = \log\left(\mathrm{K_{HPLC}}\frac{V_S}{V_M}\right)$$

where R_f is between 0 and 1, R_M (calculated from R_f) can vary between +∞ to -∞, logk' is the logarithm of the capacity factor at HPLC, V_S and V_M is the volume of the stationary and the mobile phase, respectively (V_S/V_M is the phase ratio, constant for the given chromatographic system), K_{TLC} and K_{HPLC} are the chromatographic partition coefficients.

R_M values (calculated using R_f) and log*k*' values are in linear correlation with logP values in a suitable chromatographic system:

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$$\log P = \mathrm{a}R_M + \mathrm{b}$$

$$\log P = \mathrm{a}\log k' + \mathrm{b}$$

The essence of chromatographic log*P* determination is thus the determination of the regression parameters of the above equations by using a set of standards with known octanol/water log*P* values. Based on these equations the log*P* of other compounds can be calculated.

Lipophilicity of geometric isomers can also be investigated using the HPLC method.

The so-called IAM Chromatography is a very promising chromatographic method, where silica resin is modified by covalent attachment of phospholipid-like groups to the surface. The retention parameters mimic the partitioning of drugs into phospholipid bilayers.

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VIII Physicochemical properties of drugs - Permeability

Extent and rate of absorption of a drug molecule is mainly inluenced by the rate of dissolution and by the rate of transport through the biological membranes. **Permeability** charecterizes the transport of molecules by passive diffusion through phopholipid bilayers (not just partitioning into them).

Importance of permeability is increasing, since that parameter is nowadays the best predictor of absorption from the gastrointestinal tract and thereby of the oral bioavailibility.

VIII.1 Definition of permeability

Permeability is the extent of penetration of molecules through the cell membrane. It is a kinetic parameter, signed as P_0 (inrinsic permeabaility of the neutral particles) or as P_e (effective permeability of the ionized particle). Unit of peremeability: cm/sec.

VIII.2 Biological membranes and transport mechanisms

VIII.2.1 Structure of biological membranes

Biological membranes, in the form of cell membranes, consist of a phospholipid bilayer. The major class of membrane lipids are phospholipids, namely phosphoglycerides and sphingomyelin. Phosphoglycerides, including phosphatidylcoline, phosphatidyl-etanolamine, phosphatidylserine and phosphatidylinositol consist of glycerol, two molecules fatty acids, phosphoric acid and an alcohol. Usually, the fatty acid connected to the first carbon atom of glycerol is saturated, but the fatty acid bondend to the second carbon of glycerol contains a double bound. The conformation of the double bound is *Z*-conformation, therefore the hydrocarbon chain bends.

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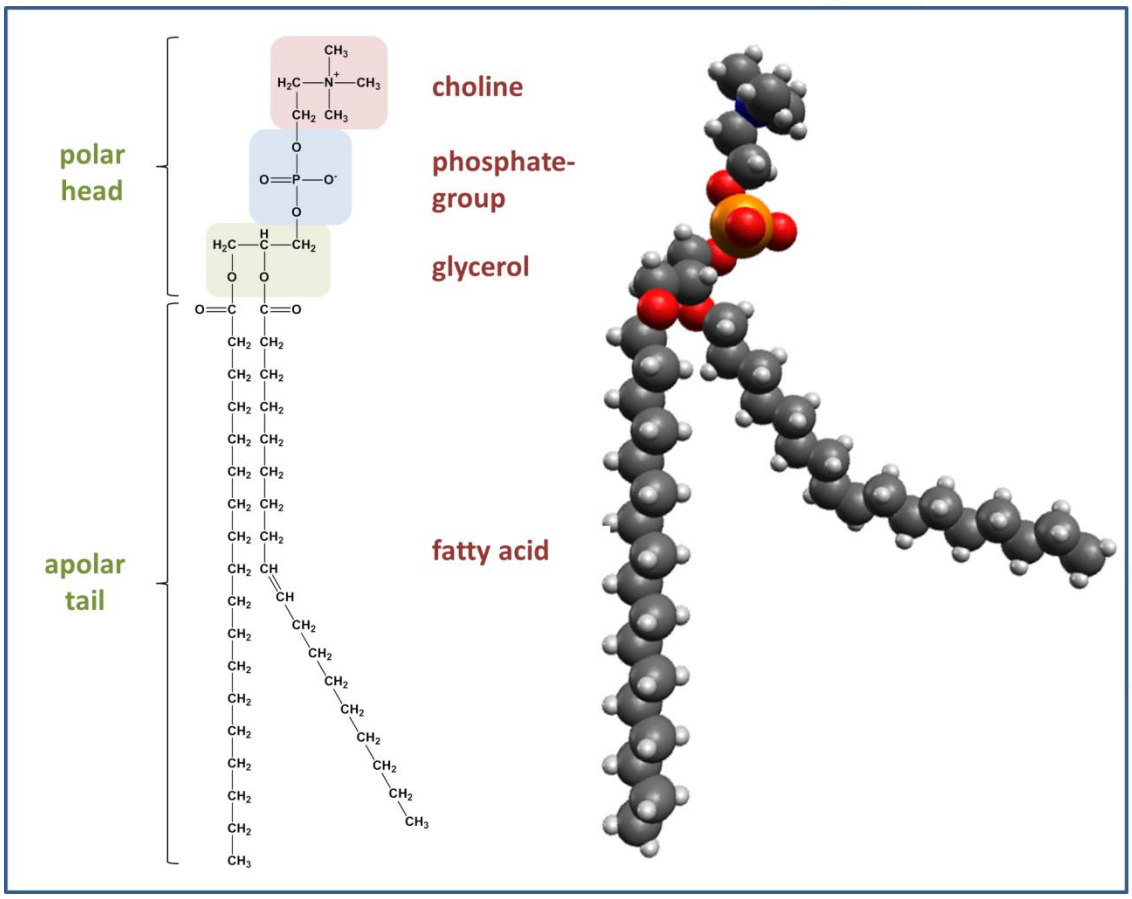
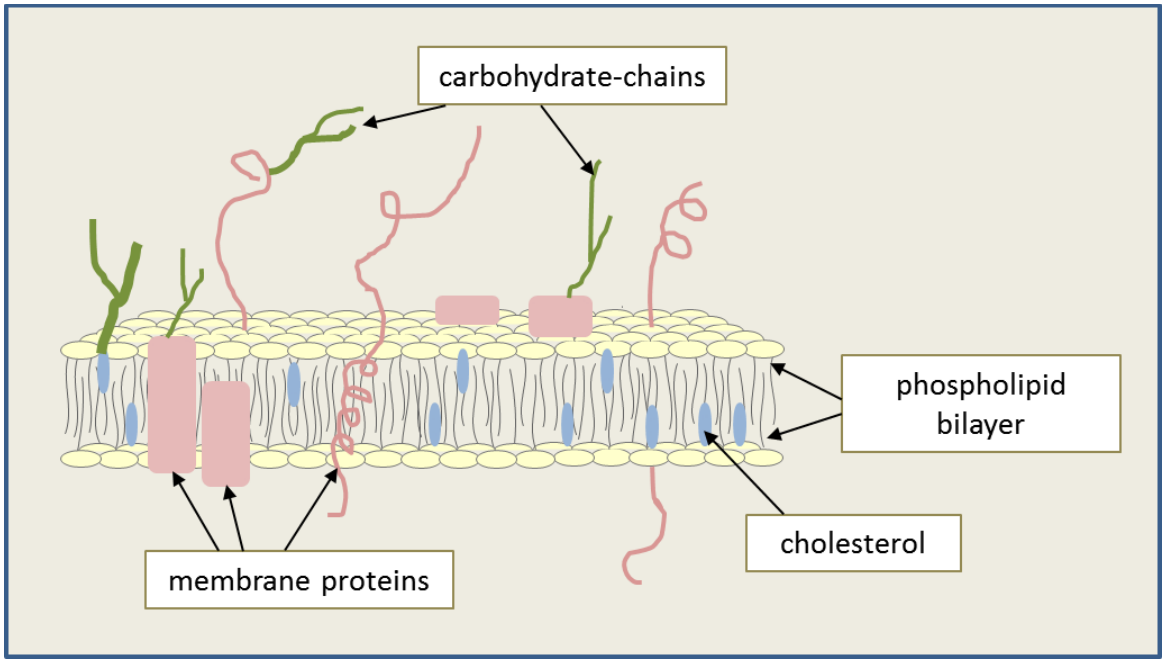
Figure VIII-1. Structure of phosphatidylcholine



Lipids are amphiphilic: they have one end that is soluble in water (polar) and an ending that is soluble in fat (apolar). By forming a double layer with the polar ends pointing outwards and the nonpolar ends pointing inwards membrane lipids can form a *lipid bilayer* which keeps the watery interior of the cell separate from the watery exterior. The interactions of lipids, especially the hydrophobic tails, determine the lipid bilayer physical properties such as *fluidity*.

Cholesterol is also an important component in the cell membrane. Cholesterol forms secondary bonds with the lipids and therefore takes part in stabilizing the membrane. *Membrane proteins* are proteins that interact with biological membranes. Membrane proteins perform a variety of functions vital to the survival of organisms. Membrane receptor proteins relay signals between the cell's internal and external environments. Transport proteins move molecules and ions across the membrane. Membrane enzymes may have many activities, such as oxidoreductase, transferase or hydrolase. Cell adhesion molecules allow cells to identify each other and interact.

Figure VIII-2. Structure of biological membranes



VIII.2.2 **Mechanisms of penetration**

Penetration through the cell membrane may occur according several mechanisms. The so-called ***paracellular*** diffusion occurs through the tight junction complexes, mainly small and hydrophyl molecules (maximal molecular size about 250 Da) are transported this way. ***Transcellular transport*** occurs through the membrane or the pores in the membrane, it possesses more types.

Passive transport processes

Passive transport does not require an input of chemical energy, being driven by the growth of entropy of the system. The rate of passive transport depends on the permeability of the cell membrane, and the concentration gradient.

Simple passive diffusion is the passive movement of solute from a high concentration to a lower concentration until the concentration of the solute is uniform throughout and reaches equilibrium. Rate of diffusion is mainly influenced by the lipid/water partition coefficient, it depends on the concentration gradient, on the thickness and area of the membrane. *Fick's first law* describes the rate of diffusion:

$$\frac{dc}{dt} = \Delta c \frac{AD}{l}$$

Δc is the difference in concentration (mol/m^3), D is the diffusion constant (m^2/s), A is the area of the membrane (m^2) and l is the thickness of the membrane (m). Only nonionized molecules are able to pass through the membran by passive diffusion.

Facilitated diffusion, also called carrier-mediated diffusion, is the movement of molecules across the cell membrane via special transport proteins that are embedded

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within the cellular membrane. Many molecules, which are insoluble in lipids and too large to fit through the membrane pores will bind with the specific carrier proteins, and the complex will then be bonded to a receptor site and moved through the cellular membrane. Facilitated diffusion is a passive process, the solutes move down the concentration gradient and do not use extra cellular energy to move.

Filtration is movement of molecules across the cell membrane pores and intracellular gaps due to hydrostatic pressure. Depending on the size of the membrane pores, only solutes of a certain size may pass through them. Rate depends ont he concentration gradient.

Active transport

Active transport is the movement of molecules across a cell membrane in the direction against their concentration gradient, i.e. moving from an area of lower concentration to an area of higher concentration. If the process uses chemical energy, such as from adenosine triphosphate (ATP), it is termed *primary active transport*. *Secondary active transport* involves the use of an electrochemical gradient. Unlike passive transport, active transport uses cellular energy. Active transport is usually associated with accumulating high concentrations of molecules that the cell needs, such as ions, glucose and amino acids.

Endocytosis is an energy-using process by which cells absorb molecules (such as proteins) by engulfing them. It is used by all cells of the body because a lot of substances are large polar molecules that can not pass through the cell membrane. The opposite process is **exocytosis**.

13.3. Importance of permeability

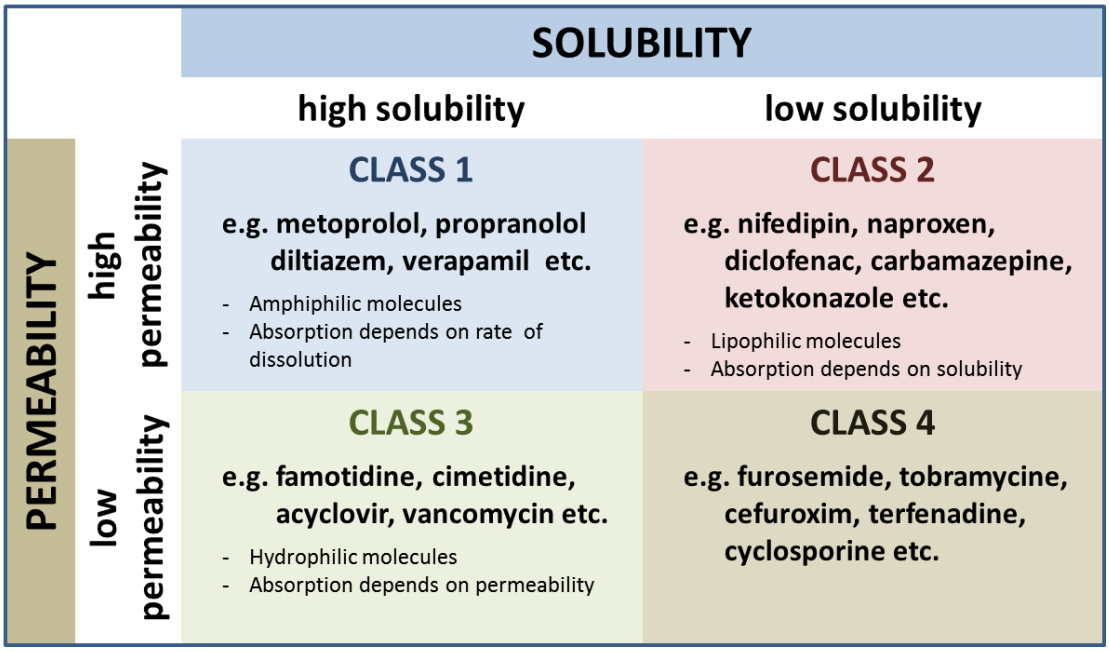
Transport through the cell membrane is influenced by a lot of parameters. One of that parameters is the lipid/protein and lipid/cholesterol ratio. In order to modellize different tissues the mentioned ratio can be modified under experimental conditions.

Passive transport is mainly influenced by the lipophilicity of the molecule, because the apolar/apolar interactions between the substances and fatty acids play an important role in the transport processes. However, the acid-base properties of a drug also have to be taken in account, since they are relevant in forming ionic interactions. In addition, molecule size, structure, stereochemistry influence permeability as well.

Determination of permeability is crucial, since it is the best predictor of absorption from the gastrointestinal tract, and therefore of oral bioavailibility.

The so-called *Biopharmaceutics Classification System* (BCS) allows estimation of the likely contributions of three major factors: dissolution, solubility and intestinal permeability, which affect oral drug absorption from immediate-release solid oral products. The next figure shows the four BCS classes, based on high and low designations of solubility and permeability.

Figure VIII-3. Biopharmaceutics Classification System (BCS)



VIII.3 Experimental methods to determine permeability

In vivo investigation of permeability would be the most effective method, but that kind of investigations are impossible in the early phase of drug development. Several *in vitro* methods are used in the practice, which are basically cellular models or non-cellular models.

VIII.3.1 **The PAMPA-model**

The PAMPA (*Paralell Artificial Membrane Permeability Assay*) applies synthetic membranes (containing phospholipides) to investigate penetration.

A multi-well microtitre plate is used for the donor and the acceptor compartment, which are separated by a special membrane (125 μm thick); the whole assembly is commonly referred to as a "sandwich".

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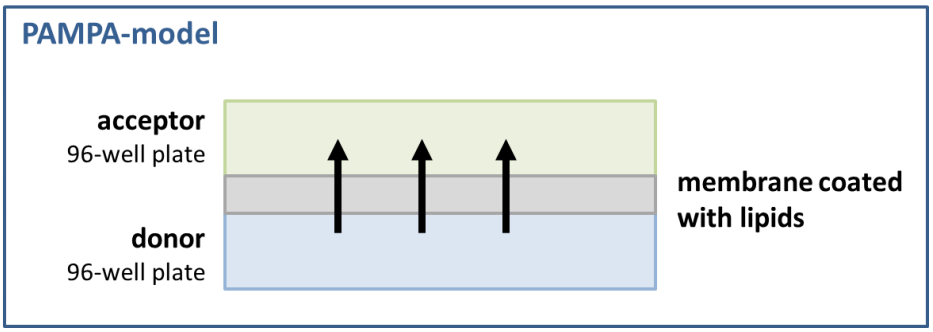
Figure VIII-4. Schematic structure of PAMPA-model



Membranes used can be various, for example dioleylphosphatidylcholine or phosphatidyletanolamine dissolved in an indifferent organic solvent (e.g. *n*-dodecane), which is fixed on the microfilter plate. To modellize the apical and basolateral side of the membrane, buffers with appropriate pH are filled into the donor and acceptor side. At the beginning of the test, the drug is added to the donor compartment, and the acceptor compartment is drug-free. After an incubation period which may include stirring, the sandwich is separated and the amount of drug is measured in each compartment, using the adequate analytical method UV plate reader or LC/MS). Effective permeability can be calculated using the next equation:

$$P_e = \frac{-2{,}303}{A(t - t_s)} \times \left(\frac{V_A V_D}{V_A + V_D}\right) \times log\left(1 - \left(\frac{V_A + V_D}{(1 - R)V_D}\right) \times \left(\frac{c_D^t}{c_D^0}\right)\right)$$

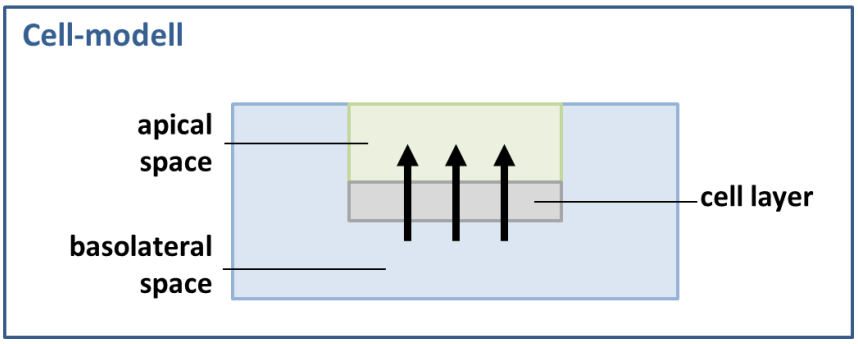
where A is the area of the filter (cm^2), t is the incubation time (s), t_s is the time to rich the stationary state of the membrane (s), V_A is the volume of the solution in the acceptor compartment (cm^3), V_D is the volume of the solution in the donor compartment (cm^3), R is the ratio of the compound absorbed in the lipid membrane (retention factor), C_D^t is the concetration in the donor compartment after t time (mol/cm^3) and C_D^0 is the initial concentration in the donor compartment (mol/cm^3).

Measurments on the PAMPA systems are reproducible, reliable, relatively low costs and big capacity are characteristic for them. They can be automated and are therefore high throughput methods. An important advantage is, that many parameters are variable (lipid components, incubation time, pH, solvents etc.). However, disandvantage of the technique is, that it provides informations only about passive transport.

VIII.3.2 **Cellular models**

Application of a cellular model allows the investigation – besides the passive transport – of active transport, efflux and some metabolic processes as well.

Figure VIII-5. Schematic structure of cell-model



The *Caco-2 model* contains a monolayer of heterogeneous human epithelial colorectal adenocarcinoma cells. Caco-2 cells express a number of enzymes and transporters (e.g. P-glycoprotein, dipeptide transporter), consequently, the permeability data determined in Caco-2 model takes also the active transport mechnisms into consideration.

The *MDCK-cells* (*Madin-Darny Canine Kidney*) are often used in cellular models, too. The cells express less transporters, but the specialty of MDR1-MDKC (cells transfected with the P-gp encoding gene) is, that the P-glycoprotein, the efflux protein is widely present ont he surface of the cells. Therefore, the MDR1-MDCK permeability assay is a valuable tool for the identification and characterisation of P-gp substrates and inhibitors.

Application of cellular models allow the determination of the so-called apparent peremeability constant:

$$P_{app} = \frac{dC_r}{dt} \times \frac{V_r}{AC_0}$$

where dC_r/dt means the alteration of substance concentration on the basolateral side as a function of time (mol/dm^{3}s), V_r is the volume of the basolateral space (cm^3), A is the area of the cell membrane (cm^3) and C_0 is the initial concentrationof the compound in the apical space (mol/cm^3).

Application of cellular models require a lot of time and preparation. Costs are relative high, and the experimental parameters can only be changed in a small range. However, it is a big advantage, that it provides informations about both passive and active transport, trans- and paracellular transport mechanisms. Nowadays high throughput 96-well versions of Caco-2 are also been used.

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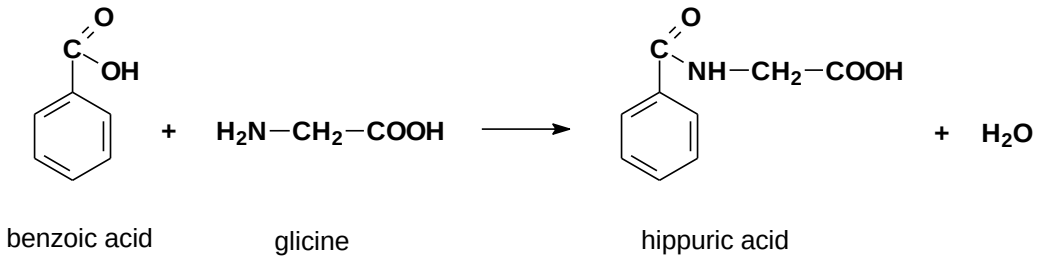
IX Drug metabolism

Most of the drugs and foreign substances (xenobiotics) which get into the body is chemically transformed. The physico-chemical properties and the biological effects of the transformed compounds are typically different from the parent compounds. Accordingly, knowledge of metabolic transformations of drugs (xenobiotics) is essential to understand their biological actions at the molecular level.

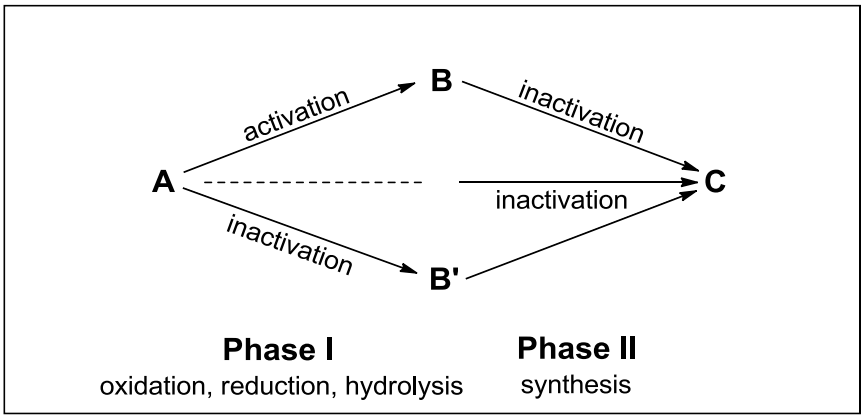
Recognition and study of metabolism of drugs is dated back to the first part of the 19th century. Historically, one of the metabolites first isolated was *hippuric acid*, the glycine conjugate of benzoic acid. It was structurally characterized by *V. Dessaignes* in 1845 (Figure XII-1).

Figure IX-1. Reaction of conversion of benzoic acid to hyppuric acid**.**



The first summary of results on the field was made by *R.T. Williams* who reviewed the pathways of detoxication of natural and synthetic compounds in the book titled "*Detoxification Mechanisms*" published in 1947. In the second edition of the book the author described a general scheme of metabolic transformations of xenobiotics which were divided into Phase I (oxidation, reduction, hydrolysis) and Phase II (synthesis) groups (Figure XII-2).

Figure IX-2. The two possible pathways of transformation of xenobiotics.



Investigation on biotransformation of drugs became a central field of research of pharmacology, pharmaceutical chemistry and toxicology. Knowledge of Phase I and Phase II transformations of xenobiotics largely contributed to introduction new drugs to with less side effects than those developed earlier. Namely, studies at molecular level of pathways of biotransformations showed that detoxifying pathways (reactions that result

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in faster elimination of xenobiotics) can produce reactive intermediates which can be responsible for development of toxic effects.

From biochemical point of views biotransformation pathways of xenobiotics can be classified as follows.

a.) Non enzyme-catalyzed reactions
b.) Enzyme-catalyzed reactions
    2.1. Reactions catalyzed by microsomal enzymes
    2.2. Reactions catalyzed by non-microsomal enzymes

Classification of enzymes catalyzing metabolic transformations into microsomal and non-microsomal groups is based on nomenclature of fractions of cell homogenates obtained by ultracentrifugation. Enzymes found in the microsomal fractions are membrane-bound while those found in the supernatant are cytosolic in the intact cells.

IX.1 Phase I – or functionalization reactions

It is the oxidation reactions that possess the highest importance among the three main groups – oxidation, reduction and hydrolysis - of Phase I metabolic transformations. Due to their chemistry, however, both reductions and hydrolytic processes can play important role in formation of metabolites facilitating activation and elimination of the parent drugs. Enzymes catalyzing Phase I reactions can be classified as microsomal and non-microsomal based on their cellular localization (Table XII-1).

Table IX-1. The most important Phase I reactions and cellular localization of the enzymes catalyzing of them.

Reaction	Enzyme	Localization
Oxidation	Cytochrome P450	microsomal, mitochondria
	Flavin monooxygenase	microsomal
	Prostaglandin-H syntetase	microsomal
	Monoamine-oxidase	mitochondria
	Aldehid-dehidrogenase	mitochondria, cytosol
	Alcohol-dehydrogenase	cytosol
	Xanten-oxidase	cytosol
Reduction	Azoreduktase	intestinal flora, microsome, cytosol
	Nitroreduktase	intestinal flora, microsome, cytosol
	Carbonyl reductase	cytosol
	Quinone reductase	cytosol
Hydrolysis	Esterase	microsomal, cytosol, lysosome
	Peptidase	lysosome
	Epoxide-hydrolase	microsomal, cytosol

The next two sections introduce the two most important families of enzymes of oxidative transformations.

IX.1.1 **Oxidation reactions**

IX.1.1.1 The cytochrome P450 (CYP450) enzymes

It is the family of cytochrome P450 (CYP450) enzymes, which is the most important among the "mixed function oxidases" catalyzing oxidative transformations of xenobiotics. Some of them catalyze biotransformation of specific endogenous substrates (e.g., steroids, fatty acids, bile acids). Others show lower substrate specificity and catalyze oxidative transformation of exogenous substances (xenobiotics).

CYP enzymes are hem-containing proteins, in which the iron-protoporphyrin is linked to the CYP protein. Each CYP enzyme is composed of a single chain protein, which is linked to the iron-proporphyrin ring though hydrophobic, electrostatic and coordinative bonds. The molar weight of the isoenzymes falls into the 45000-60000 D range. The coordination number of the central iron ion in the iron-protoporphyrin complex is six.

The enzymes are named after their characteristic absorption band about 450 nm, which can be observed when the reduced iron (II) form of the central iron ion binds carbon monoxide (CO) instead of molecular oxygen (O2).

Family of the CYP enzymes is comprising isoenzymes coded by different genes. Each isoenzyme can be characterized and identified by similarity of primary structures of the respective proteins. According to internationally accepted system, enzymes showing higher than 40% homology are belonging to the same family. This is indicated by an Arabic number following the CYP abbreviation (e.g., CYP1, CYP2, etc.). Enzymes showing higher than 55% homology are forming subfamilies in a particular family. This is indicated by a latin capital letter following the arabic number in the code generally acknowledged for identification of the CYP enzymes (e.g., CYP1A, CYP2C, CYP3A). Individual proteins (P450 genes) are distinguished by additional arabic number following the latin letter (e.g., CYP1A2, CYP2A6, CYP2E1).

Expression of CYP enzymes is the highest in the liver, however, they are ubiquitous in the lung, the small intestine, the kidney, the skin, the placenta and the brain. The CYP3A4 and 3A5 isoenzymes amount to about one-third of the total CYP proteins in the liver and about two-third of them in the small intestine. Biotransformation of about one-third of the clinically important drugs is catalyzed by the two CYP450 isoforms.

The electrons needed for the CYP450 enzymes embedded in the endoplasmic reticulum are provided by NADH or NADPH. The CYP450 enzymes form a *multienzyme complex* with the enzymes (NADPH-cytochrome P450 reductase, NADH-cytochromb_5-reduktase) catalyzing the electron transfer and catalyze the following reactions:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The most important types of reactions catalyzed by the CYP450 enzymes as follows.

1. *Hydroxylation* of *aromatic* rings.

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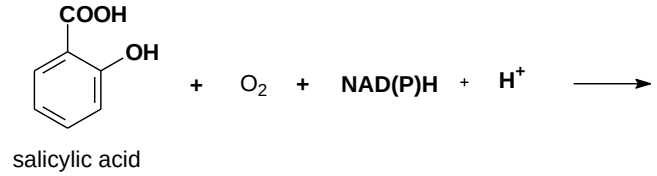
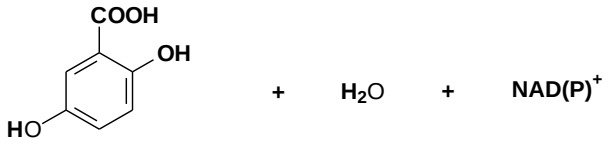
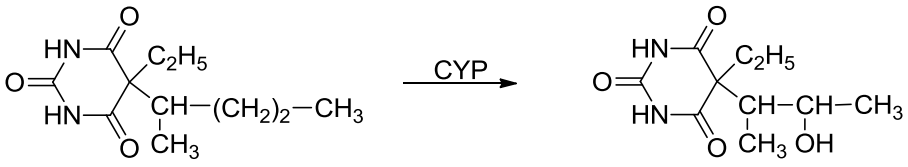


salicylic acid



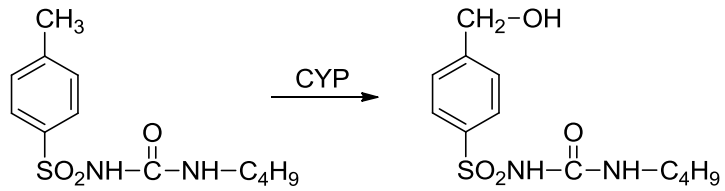
gentizic acid

2. Hydroxylation of aliphatic carbon atom.



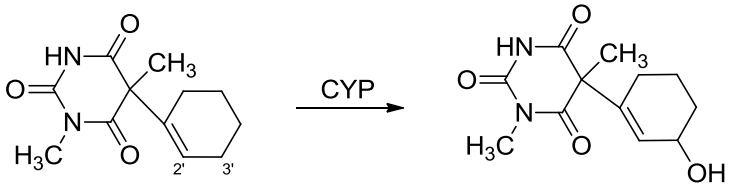
phenobarbital — side-chain-oxidized phenobarbital

3. Hydroxylation of benzylic carbon atom.



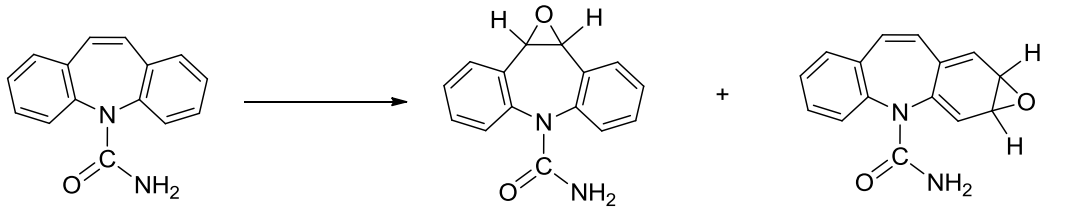
tolbutamid — side-chain-oxidized tolbutamid

4. Hydroxylation of allylic carbon atom.



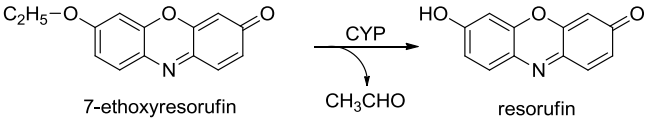
hexobarbital — 3'-hydroxihexobarbital

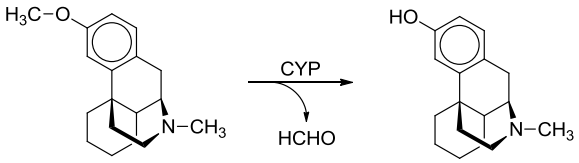
5. Epoxidation of carbon-carbon double bond.



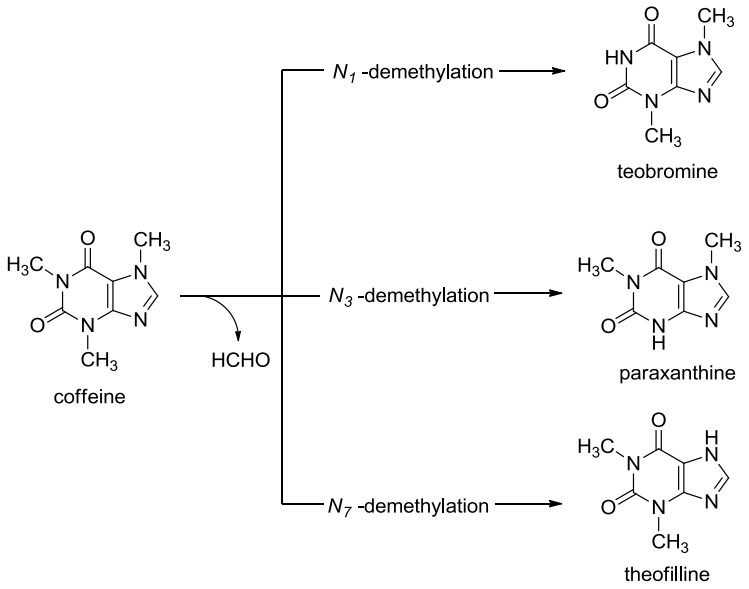
carbamazepin — carbamazepin-10,11-epoxide (stable epoxide) — carbamazepin-2,3-epoxide (instable arene oxide)

6. *O-Dealkylation*

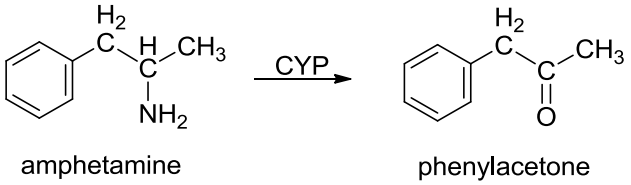




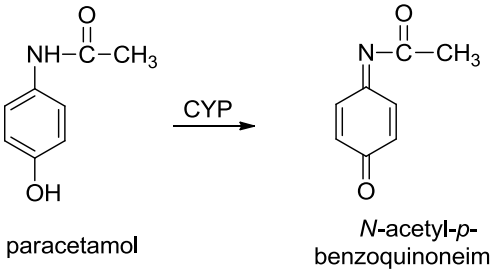
7. *N-Dealkylation*



8. *Oxidative deamination*



9. *Dehydrogenation*



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IX.1.1.2 Flavin-dependent monooxygenases

Further microsomal enzymes playing important role in oxidative Phase I reactions are the flavin-dependent monooxygenase (FMO) enzymes. The flavin-dependent monooxygenases can catalyze oxidation of xenobiotics in the presence of flavin mononucleotide (FMO) or flavin- adenine dinucleotide (FAD), molecular oxygen (O2) and NAD(P)H cosubstrates.

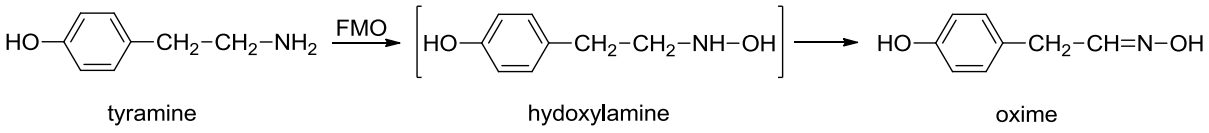
Structure of the FMO enzymes and mechanism of the catalyzed reactions are substantially different from those catalyzed by the CYP450 enzymes. Therefore, substrate specificity of the FMO enzymes is much wider than that of the CYP450s. Contrary to the CYP450 enzymes – which are preferentially catalyzing oxidative processes at *carbon* atoms – FMO enzymes prefer oxidations at *nitrogen*, *sulphur*, *phosphorous* and *selenium* atoms.

As far as the mechanism of the KMO enzymes is concerned, it should be mentioned that activation of the dioxygen molecule – unlike the CYP enzymes –is accomplished through binding the O_2 molecule to the flavin moiety in hydroperoxide form.

Contrary to the large number of CYP450 isoenzymes, there have described only five different forms of the mammalian FMO enzymes. It is the FM3 isoform that is expressed in human liver in the largest amount. Besides FMO3, FMO4 and FMO5 are the other isoforms that are expressed in small amount. Among the further, extrahepatic forms it is FMO1 is to be mentioned, whose expression is the highest in the kidney and in the mucosal cells of the small intestine.

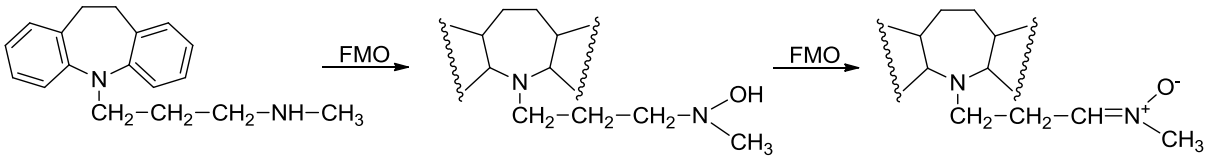
The most important reactions catalyzed by the FMO enzymes as follows.

1. Oxidation of *aliphatic primer amines* to hydroxylamine derivatives.



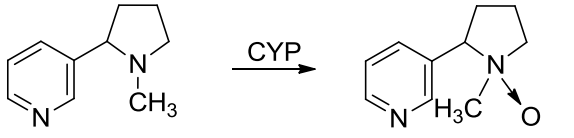
tyramine hydoxylamine oxime

2. Oxidation of *aliphatic secondary amines* to hydroxylamines and nitrones.



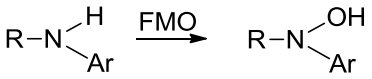
dezmethyl-imipramine nitrone

3. Oxidation of *aliphatic tertiary amines* to N-oxides.

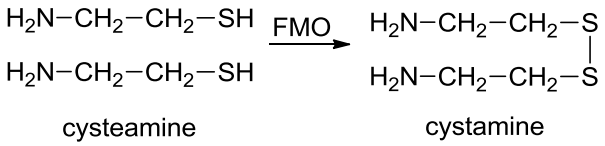


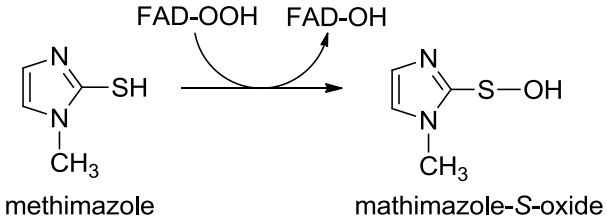
nicotine nicotine-1'-*N*-oxide

4. Oxidation of *N-alkyl-arylamines* to hydroxylamine derivatives.



5. Oxidation of *sulphur containing compounds.*





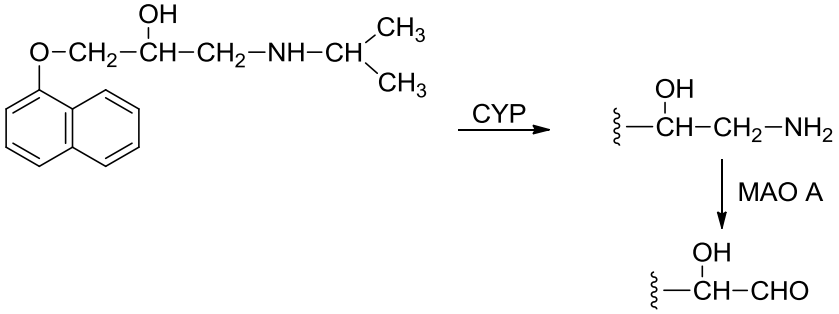
IX.1.1.3 Non microsomal oxidations (selection))

XII.1.1.3.1. Monoamine oxidase (MAO) enzymes

Monoamine oxidase (MAO) enzymes – together with the diamino oxidases (DAO) and polyamine oxidases (PAO) – are catalyzing oxidative deamination of primary, secondary and tertiary amines. Xenobiotics are oxidized mostly by the MAO enzymes.

MAOs are expressed in the liver, the kidney, the small intestine, the brain and the platelets, embedded into the outer membrane of mitochondria. There are two known forms of the enzymes (MAO A and MAO B) humans having some different substrate preferences. *Serotonin, norepinephrine* and the dealkylated derivative of *propranolol* are examples of preferred substrates of the MAO A isoform. The CYP-catalyzed N-dealkylation followed MAO A-catalyzed oxidation of propranolol is shown on Figure XII-3.

Figure IX-3. Metabolism of propranolol.



Among the preferred substrates of MAO B isoform non-phenolic beta-phenylalkylamines and benzylamines are to be mentioned.

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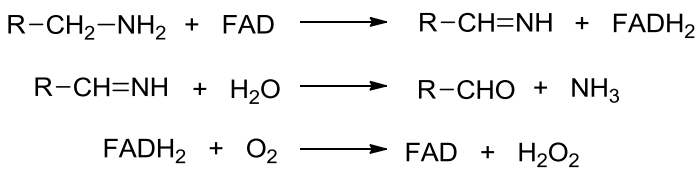
The MAO enzymes flavin-adenin dinucleotide (FAD) containing enzymes. The catalytic cycle of the MAO enzymes is shown on Figure XII-4.

Figure IX-4. The catalytic cycle of the MAO enzymes.



In the first step of the catalytic cycle, the substrate is oxidized to the respective imine derivatives while FAD is reduced to $FADH_2$. In the next step – as a result of hydrolysis of the imine – incorporation of the oxygen atom of a water molecule occurs while the substrate is converted into aldehyde and an ammonia molecule is released. The closing step of the catalytic cycle is regeneration of FAD by means of molecular oxygen (O_2) while it is reduced to hydrogen peroxide.

IX.2 Phase II – or conjugation reactions

The characteristic feature of the Phase II reaction is that the parent molecules – or a previously structurally modified derivate of them – react with small molecular weight endogenous molecules to form a less lipophilic (more water-soluble) and less active conjugation, addition or substitution products. The most important types of reactions, the enzymes and cellular localization of enzymes catalyzing the reactions are summarized in Table XII-2.

Table IX-2. The most important metabolic Phase II transformations and the subcellular localization of the enzymes catalyzing them.

Pathway	Enzyme	Localization
Conjugation with glucuronic acid	UDP-glucuronosyltransferase	microsomal
Sulphate conjugation	Sulphotransferase	cytosol
Glutathione conjugation	GSH-S-transferase	cytosol, microsomal
Amino acid conjugation	Acyl-CoA: amino acid N-acyl transferase	cytosol, mitochondria
Acetylation	N-acetyl-transferase	cytosol
Methylation	Methyltransferase	cytosol, microsomal

Conjugation reactions represent the most important type of Phase II metabolic transformations. Most of the drugs are lipophilic in nature. Therefore, they absorb well but their excretion is rather slow. In most of the conjugation reactions ionizable hydrophilic molecules are linked to the parent compound (or to its metabolite formed in a Phase I reaction) resulting in formation of a derivative with increased water solubility. The largest body of the drug molecules is excreted in bile and/or in urine as conjugates.

Products of the two main conjugation reactions (conjugation with glucuronic acid and sulfate) has been considered to be pharmacologically inactive/hardly active

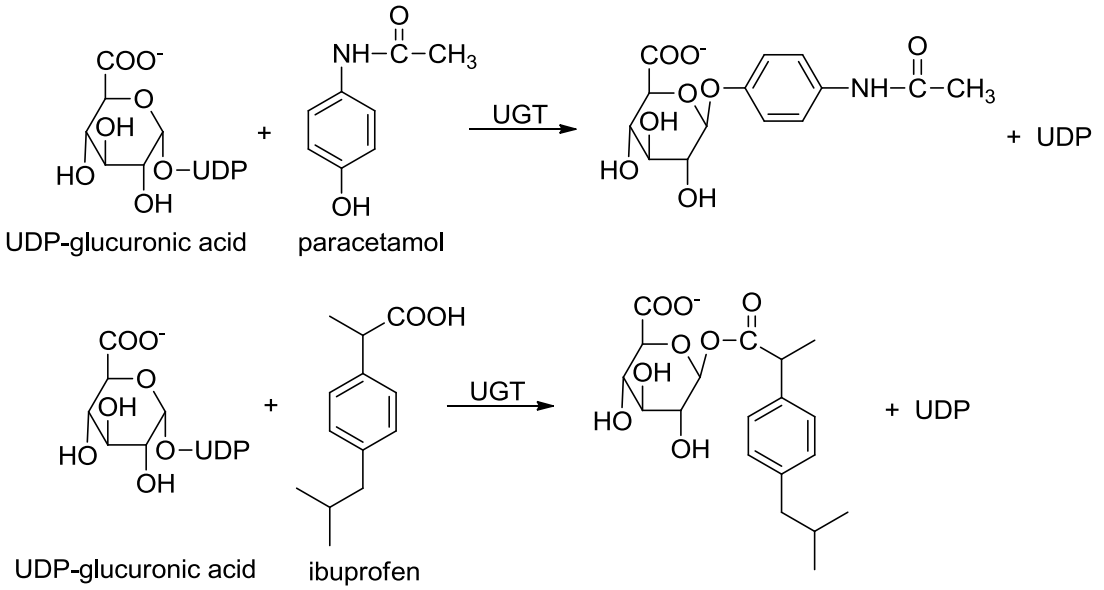
metabolites for a long period of time. This simplified picture, however, has been changed. Thus, it was recognized, for example, that morphine-6-glucuronide is more potent major analgesics than morphine and it is the sulfate conjugate of minoxidil, which is the pharmacologically active form of the parent drug. Most of the conjugates are less toxic than the respective parent drugs. There are metabolites (e.g., the glucuronide conjugate of diclofenac), however, which are responsible for development of toxic side effects.

IX.2.1 **Conjugation with glucuronic acid**

Conjugation with glucuronic acid can be considered as the most important Phase II reaction. It importance is mostly due to the high glucuronic acid supply of the liver and to the relatively high number of drug molecules carrying functional groups (e.g., carboxyl, hydroxyl, mercapto and amino) that can be taken into account as substrates.

The pioneering work of *G. J. Dutton* and *I. D. E. Storey* played an important role in discovery of molecular mechanism of the conjugation reaction. They recognized that it is *uridine diphosphate glucuronic acid* (UDP glucuronic acid) as cofactor and the enzyme *UDP-glucuronosyltransferase* (UGT) play important role in the conjugation reaction. The UDP-glucuronosyltransferase (UGT)-catalyzed conjugation reaction of paracetamol and ibuprofen is shown on Figure XII-5.

Figure IX-5. The UDP-glucuronosyltransferase (UGT)-catalyzed conjugation reaction of paracetamol and ibuprofen.



The glucuronide conjugates are polar, water soluble metabolites, which are excreted in the bile or the urine. It is basically the molecular mass of the parent (non-conjugated) molecules that determines whether the conjugates are excreted in the bile or the urine.

On the other hand, epithelial cells of the intestine possess *beta-glucuronidase* activity, the enzyme that can hydrolyze the glucuronide conjugates. As a result of such hydrolysis, the parent lipophilic molecules are liberated from the glucuronide conjugates that are excreted into the small intestine in the bile. Thus, the liberated lipophilic drugs can be reabsorbed from the gastrointestinal tack. Such a transfer of the

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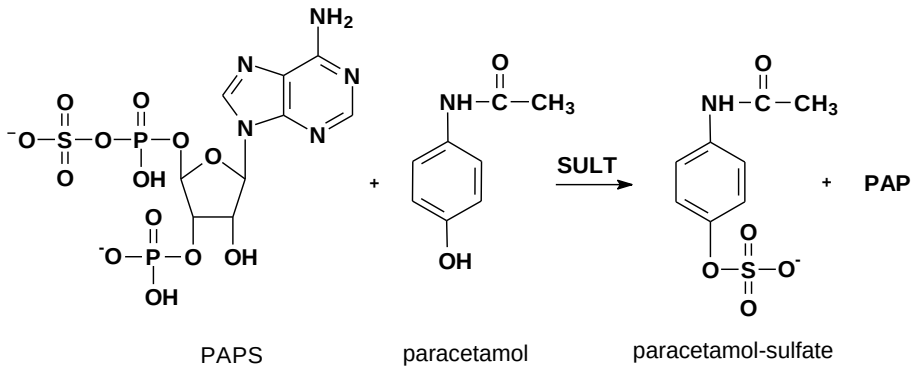
drug molecules between the liver (the site where the metabolites are synthesized) and the gastrointestinal track (where they are hydrolyzed) is called *enterohepatic circulation.*

IX.2.2 **Sulfate conjugation**

Conjugation with sulfate is the second most frequent transformation among the Phase II reactions. Sulfate conjugation is an important metabolic pathway of phenolic drugs and other xenobiotics as well as several endogenous compounds such as steroid hormones, bile acids, thyroid hormones, and the catecholamine neurotransmitters. The most important feature of the sulfate conjugates is their increased aqueous solubility and readiness to be excreted. Although the metabolic transformation is known since 1876, it was only in the 1950s when the mechanism of the reaction started to be investigated. It was recognized that it is 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as cofactor and the enzyme sulforansferase (SULT) play important role in the conjugation reaction. The sulfoltransferase (SULT)-catalyzed conjugation reaction of paracetamol is shown on Figure XII-6.

Figure IX-6. The sulfoltransferase (SULT)-catalyzed conjugation reaction of paracetamol



Sulfate-conjugation is primarily the metabolic transformation of phenolic compounds. The rate of the reaction is determined by the actual PAPS and inorganic sulfate supply. The PAPS supply is rate limiting since the sulfate supply is generally low. Thus, contribution of sulfate conjugation in comparison to glucuronide conjugation is decreasing with increased dose of the xenobiotic.

Sulfatases, the enzymes catalyzing hydrolysis of the sulfate conjugates, of the epithelial cells can hydrolyze the sulfate conjugates excreted in the bile resulting enterohepatic circulation of the sulfate conjugates formed in the epithelial cells and the liver.

IX.2.3 **Conjugation with amino acids**

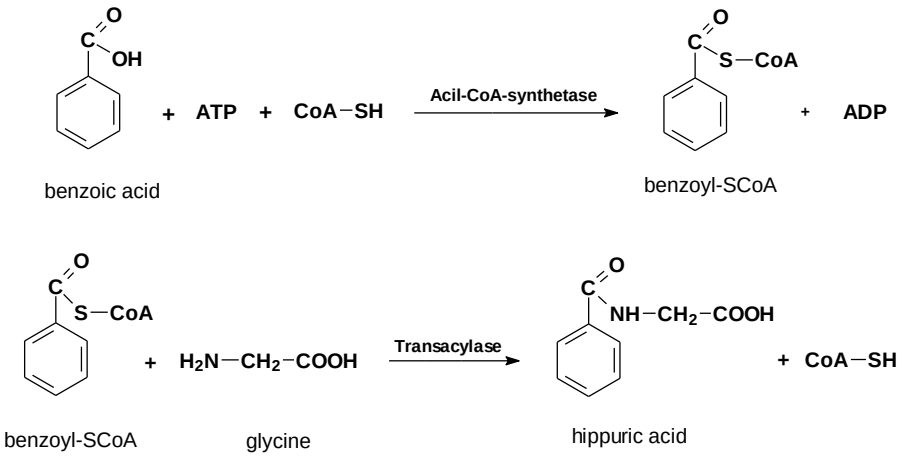
The first experimental evidence of metabolic transformation of foreign substances in the body was provided by conjugation of benzoic acid with glycine. Since this very first observation (1841) several amino acid conjugate of other aromatic, branched chain aliphatic, aromatic-aliphatic and heterocyclic carboxylic acids have been identified providing evidence of importance of conjugation of xenobiotics with amino acids. Conjugation with glycine, glutamine and taurine is the most frequent types of

transformation, the reaction can proceed, however, with arginine, histidine and serine among others.

The first step of the conjugation reactions is activation of the carboxyl group of xenobiotics through conjugation with coenzyme A (CoA-SH) to form the respective thioester (acyl-SCoA) derivative. Synthesis of the acyl-SCoA derivatives is an ATP-dependent reaction. Reaction between the amino acid and the activated carboxyl group is catalyzed by the transacylase (acyl-CoA: amino acid N-acyltransferase, Nat) enzymes which are localized in the cytosol or in the mitochondria.

The transacylase-catalyzed reaction of benzoic acid with glycine to form hyppuric acid is shown on Figure XII-7.

Figure IX-7. The transacylase-catalyzed reaction of benzoic acid with glycine to form hyppuric acid.



IX.2.4 **Conjugation with glutathione**

Glutathione (GSH) is a tripeptide (gamma-glutamyl-cysteinyl-glycin) which can be found in relatively high concentration (1-10 mM) in the cytosol. Due to its thiol functional group it (a) is a strong *nucleophilic* reactant and (b) can be oxidized to its disulfide (GSSG) derivative. As a consequence of its oxidizabilty it can act as reducing (*antioxidant*) reactant. The oxidation is reversible; GSSG can be reduced back to GSH by means of NADP(H)-dependent enzymes. Due to its nucleophilic reactivity GSH plays an important role in protecting chemical modification of nucleophile centers (O, N and S atoms) of cellular macromolecules (e.g., proteins, DNA) by reactive electrophilic metabolites (cytoprotective effect). On the other hand, the GSH/GSSG reversible redox system plays and important role in maintaining the redox homeostasis of the cells (antioxidant effect).

According to the above, reduced glutathione (GSH) is readily react with those xenobiotics or with their metabolites which carry electron-deficient (electrophilic) centers. The reactions are catalyzed by the *glutathione-S-transferase* (GST) enzymes. The GST-catalyzed conjugation reaction of ethacrynic acid with reduced glutathione (GSH) is shown on Figure XII-8.

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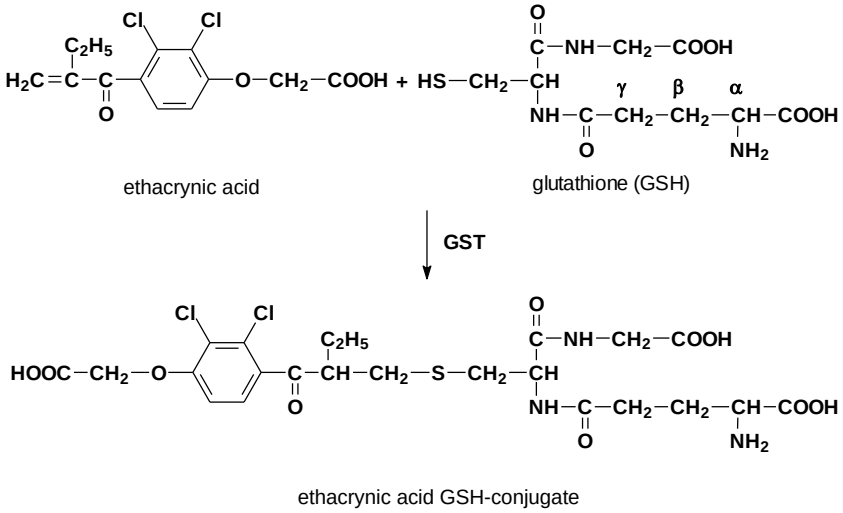
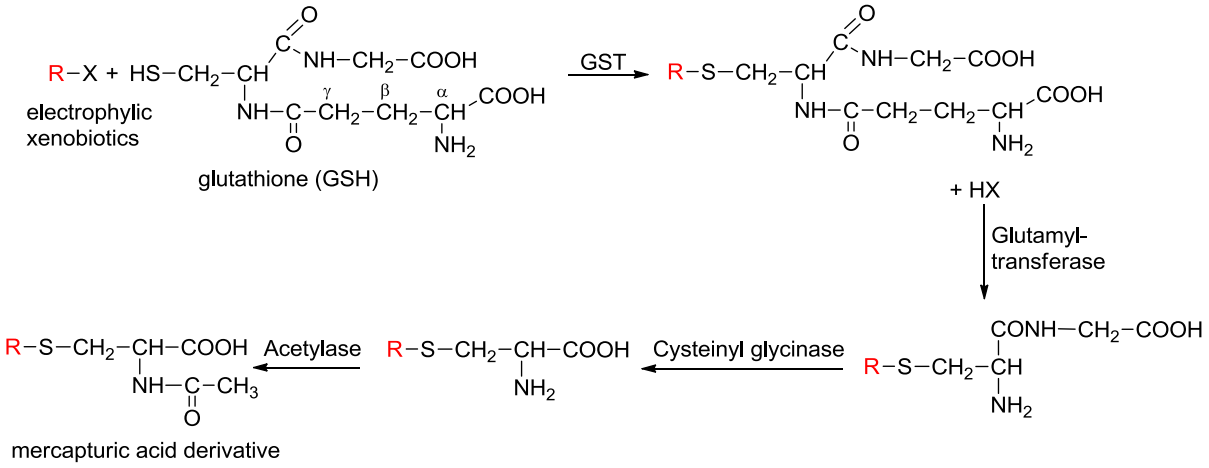
Figure IX-8. The glutathione-S-transferase (GST)-catalyzed conjugation reaction of ethacrynic acid with reduced glutathione (GSH)



Glutathione-S-transferases are expressed in the highest levels in the liver, the intestine, the kidney and the lungs, where they are localized in the cytoplasm (>95%) and the endoplasmic reticulum (<5%).

The GHS-conjugates are excreted in the bile or – after transformation to the respective mercapturic acid derivatives in the kidney- in the urine. During the course of mercapturic acid formation the glutathione conjugates – through consecutive hydrolysis of glutamic acid and glycine – are transformed to the corresponding cysteine conjugates, which are converted to the respective N-acetylcysteine (NAC) (mercapturic acid) derivatives. This latter reaction is catalyzed by the acetyl-SCoA: amino acid N-acyltranszferase (NAT) enzymes (Figure XII-9).

Figure IX-9. Reaction path of transformation of glutathione conjugates to the respective mercapturic acid conjugates



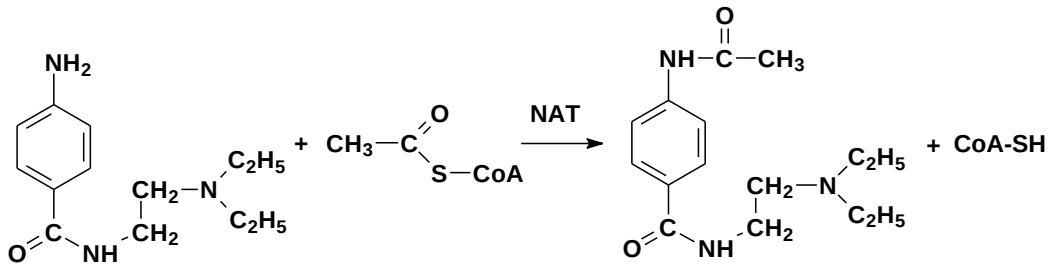
IX.2.5 **Acetylation**

Acetylation is primarily the acylation reaction taking place between the acetyl group of acetyl-SCoA and the amino group of amino acids, aromatic primary amines (Ar-NH_2) and aromatic hydrazines (Ar-NH-NH_2). The first N-acetyl metabolites was

discovered by *R. Cohn* (1893) who described metabolism of *m*-nitrobenzaldehyde to N-acetyl-*m*-aminobenzoic acid and excretion of the metabolite in the urine in the rabbit.

The pioneering work of *F.A. Lipmann* played an important role in discovery of molecular mechanism of acetylation reactions as well as acylation reactions of xenobiotic carboxylic acids (conjugations with amino acids). Reaction between the amino groups and the activated acetyl group (acetyl-SCoA) is catalyzed by the acetyl-CoA: amino acid N-acyltranszferase (NAT) enzymes. The NAT-catalyzed reaction of procainamide with acetyl-SCoA is shown on Figure XII-10.

Figure IX-10. The NAT-catalyzed reaction of procainamide with acetyl-SCoA



procainamide acetyl-SCoA N-acetyl-procainamide

Genetic polymorphism of the NAT enzymes has a great impact on rate of acetylation reactions of drugs and other xenobiotics. In respect to the kinetics of the acetylation reactions slow- and fast (wild type) acetylator phenotypes can be distinguished. These differences affect pharmacokinetics of drugs that are metabolized by the NAT enzymes and the amount of DNA-reactive metabolites of N-acetyl derivatives of genotoxic arylamines.

IX.2.6 **Methylation**

Methylation is a well-known biochemical transformation being more important metabolic pathway in the fate of endogenous than that of exogenous substances. Discovery of molecular mechanism of methylation reactions is heavily associated with works of *G. L. Cantoni* who discovered the role of *S-adenosyl methionine* (SAM) acting as cofactor in the reactions.

S-Adenosyl methionine (SAM) (Figure XII-11) is a carbonium-type compound, which can react with nucleophilic centers (O, S and N atoms) of the acceptor molecules. Consequently, there are phenols, catecholamines, aliphatic and aromatic amines, nitrogen containing heterocycles and thiols the most important class of substrate molecules.

The O-methylation reaction of norepinephrine catalyzed by catechol-O-methyltransferase (COMT) is shown on Figure XII-11.

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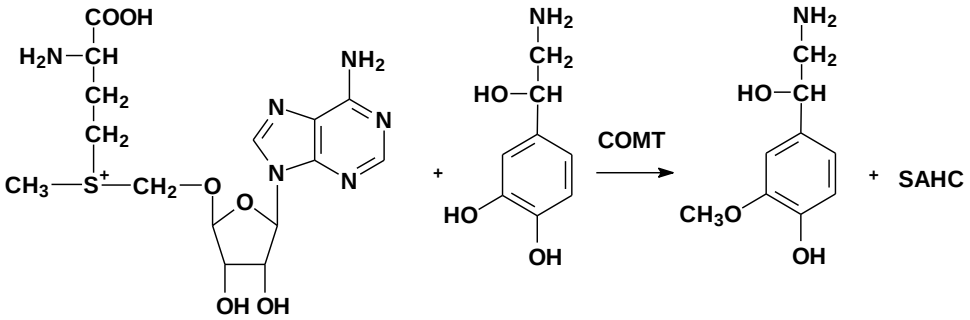
Figure IX-11. The O-methylation reaction of norepinephrine catalyzed by catechol-O-methyltransferase (COMT).



SAM norephinephrine ephinephrine

SAM= S-adenosyl-methionine
SAHC= S-adenosyl-homocysteine

Methylation – similar to acetylation – generally reduces aqueous solubility of the parent molecules and blocks reactivity of their nucleophilic centers that can be involved in other Phase II reactions that result in formation of metabolites in increased aqueous solubility. One of the exceptions is N-methylation of pyridine derivatives that form pyridinium cations with full positive charge.

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