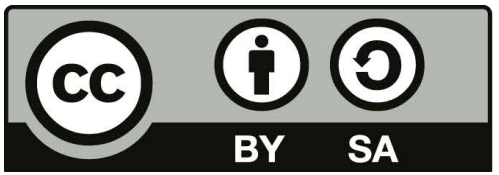
EVROPSKÁ UNIE
Evropské strukturální a investiční fondy
Operační program Výzkum, vývoj a vzdělávání

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Introduction



What is advanced biochemistry?

Biochemistry has been a part of chemistry curriculum at UCT Prague (previously ICT Prague) as Biochemistry I and Biochemistry II till 2011. That time there was only a 5-year “inženýr” program. Biochemistry I included structure and function of proteins, enzymes, nucleic acids and other components of life. This was followed by Biochemistry II, which was about metabolism.

Since 2011, studies were split into bachelor and master studies. This resulted into change of biochemistry to a basic and advanced biochemistry. The basic biochemistry is the part of curriculum of all students. Advanced biochemistry is the part of the curriculum of students of biochemistry, biotechnology, food sciences and related specializations.

Both courses cover similar topics, but the advanced biochemistry goes to details. **Basic knowledge of biochemistry is needed for advanced biochemistry**.

What is advanced biochemistry?

1. Introduction
2. Non-covalent Interactions in Biological Sciences
3. Oxygen
4. Essential Factors: Vitamins, Cofactors, Coenzymes and Prosthetic Groups
5. Regulation of Enzymatic Activity
6. Signal Transduction – Receptors
7. Biochemistry of Human Organs and Tissues I
8. Biochemistry of Human Organs and Tissues II
9. Metabolism of other Organisms
10. Gene Technologies I
11. Gene Technologies II
12. Clinical Biochemistry
13. History of Biochemistry
14. Testing your Knowledge

What is biochemistry about?

Biochemistry is chemistry of living systems. In last decades this term was evolving.

Molecular biology studies genetic information and its expression (replication, transcription and translation) at the molecular level.

Cell biology studies signaling in the cell and its structural elements (receptors, organels, cytoskeleton, cell cycle etc.).

Biochemistry is nowadays mostly understood as a chemistry of metabolism.

Biotechnology applies results of these disciplines in technology.

Omics approaches study biological systems in high throughput.

All these fields are overlapping.

What is biochemistry about?

The main role of molecular and cell biology, biochemistry, omics and other fields is to find cures to diseases. How can biochemistry (metabolism research) contribute? It seemed for many years that biochemistry is dead. Basic metabolic pathways have been determined decades ago. Since that time it seemed that there is nothing to discover. Metabolic enzymes were believed as poor targets for drugs. If you inhibit a metabolic enzyme you inhibit or kill the cell, sick as well as healthy. However, in last decade or so biochemistry is back in Vogue due to these findings.

IDH1 story
Isocitrate dehydrogenase (IDH) catalyses oxidation of isocitrate by NAD^+ followed by decarboxylation, producing 2-oxoglutarate. It is an enzyme of citrate cycle, which implies that it is an “innocent” enzyme in cancer, poor target of drugs etc.

However, it was found that IDH1 and IDH2 is frequently mutated in cancers. This implies that mutation provides some advantage for cancer cells.

In 2009 it was found that mutant IDH1 and IDH2 catalyses alternative reaction producing D-2-hydroxyglutarate.

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IDH1 story
D-2-Hydroxyglutarate accumulate in cancer cells. This causes:

- changes in methylation of DNA

- changes in modification of histons

- hypoxia and D-2-hydroxyglutarate support each other

Cancer cells are more aggressive due to different gene expression and due to tolerance to lack of oxygen.

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IDH1 story
2009 – discovery of production of D-2-hydroxyglutarate by mutant IDH1

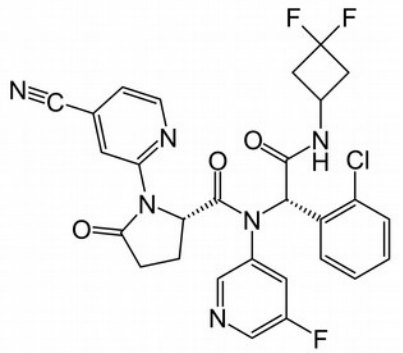
2011 – D-2-hydroxyglutarate alters DNA methylation

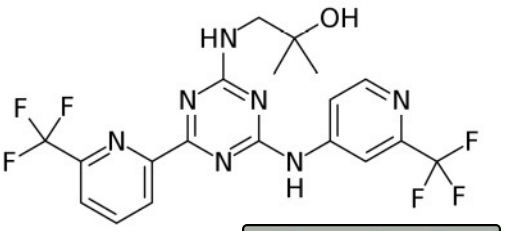
2011 – D-2-hydroxyglutarate alters histon modification

2015 – D-2-hydroxyglutarate levels is elevated by hypoxia

2018 – Ivosidenib – phase III clinical trial for acute myeloid leukemia and cholangiocarcinoma

2018 – Enasidenib (IDHIFA) – approved for acute myeloid leukemia





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“Global enzymes” story

Replacement of fossil fuels by biofuels caused rise of food prices in last decade. Therefore researchers study tools for production of biofules from cheap and waste materials, such as straw, saw dust, wooden chips and other materials.

Cheep materials for biofuel production contain saccharides in the form of cellulose. There is a never-ending struggle in nature between plants and cellulolytic microgranisms. Plants have evolved highly durable cell walls containing cellulose, other polysaccharides, lignin and other protective compounds that make enzymatic hydrolysis of cellulose difficult.

On the other hand, cellulolytic microorganisms have develop sophisticated cellulases, carbohydrate-binding modules, other cellulose-degrading enzymes (e.g. oxygenases), cellulosome and other mechanisms.

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“Global enzymes” story

Cycles of elements in nature have become a hot topic due to climate change and other environmental topics. Nitrogen cycle involves nitrogen fixation forming ammonium, nitrification of soil ammonium to nitrites, their nitrifying to nitrates and decomposition (denitrifying) of nitrates to nitrogen. Ammonium, nitrites and nitrates can be assimilated by plants or microogranisms. Ammonium can be produced by microbial biomass degraders. Similar cycles driven by microorganisms exist for sulfur, phosphorus and other elements. These cycles take place in huge quantities in soil, in oceans and in other environments and can be sensitive to changes of conditions.

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Synthetic biology

The concept of synthetic biology has been known for a long time, but recently it became real owing to solution of problems related to co-expression of multiple enzymes. It is possible to clone whole metabolic pathways from one organism to another. This requires knowledge of metabolism and knowledge of enzyme kinetics.

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Synthetic biology

As a curiosity, we can mention a report (*Nature*, **521**, 281-283, 2015) that two groups of researchers independently cloned the first and the second half of morphine biosynthesis pathway from poppies into yeast *S. cerevisiae*. This has risen concerns that leaking of these yeast strains to public would enable home “brewing” of opiate drugs.

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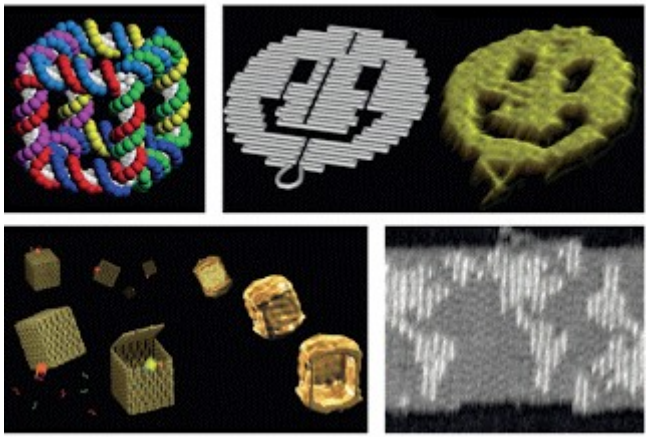
New tools and techniques

Nanotechnology



http://philipball.blogspot.com/2014/06/programmable-matter-kicks-off.html

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New tools and techniques

Gene silencing and editing

RNA interference (RNAi) – specific degradation of mRNA

Zinc finger nucleases, TALENs, CRISPR/Cas9 –
specific disruption of a gene or its specific modification

Cheep sequencing

High throughput sequencing methods

Electron and optical microscopy at high resolution

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Holy grails of biochemistry

Full understanding of human genome – detailed understanding of all genes, their functions, relationships between genes, population differences etc.

Detailed models of cells and organisms – doing “experiments” on mathematical models

Medicine based on genomics, individual medicine, systems medicine

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Holy grails of biochemistry

More drug targets – discovery of new drugs is slow, partially due to lack of new drug targets, old targets have been explored

Protein folding – atomistic simulations of protein folding on a routine basis

Keeping track with bacterial, viral and cancer resistance

Enzymatic production of cheep energy sources

Required knowledge

Biochemistry

Building blocks of biomacromolecules:
Amino acid structure, properties (polar/nonpolar/acidic/basic ...), peptide bond,
Nucleic acid bases, nucleosides, nucleotides
Basic monosaccharides (glucose)

Structure of biomacromolecules:
Primary, secondary, tertiary and quaternary structure of proteins, phi and psi angle, Ramachandran plot
DNA structure (chemical structure, formation of double helix, orientation of strands)
Basic polysaccharides (glycogen, starch)

Structure of other biomolecules
Phospholipids, membranes

Triacylglycerols

Required knowledge

Biochemistry

Metabolism, catabolism, anabolism

ATP structure

How is ATP produced, membrane and substrate phosphorylation

How is ATP used, biosynthesis, motion, pumping across a membrane

Required knowledge

Biochemistry

Hydrolysis of proteins, nucleic acids, polysaccharides and lipids

Glycolysis, glukoneogenesis, pentose phosphate pathway

Krebs cycle, electron transport chain

Lipid metabolism (degradation and synthesis pathway)

Amino acid metabolism (transamination, glytamate dehydrogenase, ammonium metabolism)

Location of these processes

Required knowledge

Molecular biology

Gene, genome

Replication basics, DNA polymerase

Transcription basics, RNA polymerase

Translation basics, ribosome, tRNA

Organization of a cell, organels, compartmentization

Biology

Human organs (digestive tract, blood and immune system, liver,
adipose, kidney, muscles, connective tissues, brain, senses)

Final exam

Test

Written test with 10 questions (10 % each), typically short or few sentence answers, thinking is more important than memorizing

50 % required in order to pass to the oral exam

Oral exam

Discussion on the test results, clarification of your answers, extending questions, questions testing your in-deep knowledge

Non-covalent Interactions in Biological Sciences

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What are major non-covalent interactions in biological systems?

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electrostatic (Coulombic) interactions – interactions between charged groups, partial charges or dipoles

van der Waals (London, dispersion) interactions – much weaker interactions between charged or uncharged atoms

hydrogen bonds – they are not special, they can be viewed as a combination of electrostatic and vdW, but play important role in structures of biomolecules

alternative hydrogen bonds and other interactions (pi-pi, CH/pi, cation/pi, halogen bonds...)

hydrophobic interactions – are not real (physical) interactions measurable in vacuum at 0 K, but rather an interplay between solvation and desolvation

Where are non-covalent interactions important in biological systems?

Where are non-covalent interactions important in biological systems?

protein structure formation, protein-protein, protein-nucleic acid, protein-ligand interactions, cytoskeleton, membranes,
formation of DNA double-helix, RNA structure formation, antibody-antigen, receptor hormone,
drug-target interactions, enzyme-substrate interactions

How could be non-covalent interactions exploited?

How could be non-covalent interactions exploited?

application of antibodies (identification of proteins, bacteria etc., immunochemical assays, flow cytometry, immunohistochemistry, fluorescence microscopy, affinity purification, therapeutic antibodies etc.)

drug design, drug targeting, enzyme inhibition, receptor activation/inhibition

diagnostics

affinity-based isolation of biomolecules, chromatography

...

How could be non-covalent interactions studied?

How could be non-covalent interactions studied?

Structural biology, calorimetry, affinity measurements, optical measurements, affinity chromatography, enzyme assays

Determination of 3D structures of molecular complexes,
determination of binding thermodynamics (delta *H*, *G*, *S*),
saturation thermodynamics measurements (by measuring any optical, NMR, electrochemical or other variables differing for bound and unbound state)
determination of retention times in affinity chromatography
measurement of *Km* or *Ki* for enzyme substrates/inhibitors
direct measurements of concentrations of equilibrium mixture components

How non-covalent interactions stabilize native structures of proteins and nucleic acids?

How they can be denaturated?

How non-covalent interactions stabilize protein structure?

secondary structure – hydrogen bonds, alpha-helices and beta-sheets are formed by hydrogen bonds between the main chain atoms

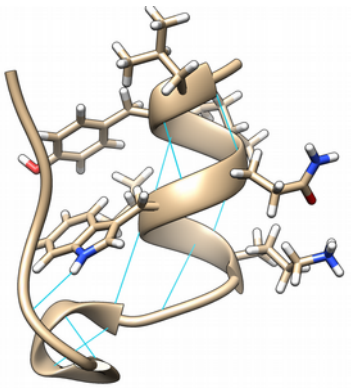
tertiary structure – all kinds of interactions, hydrophobic effect as the major driving force

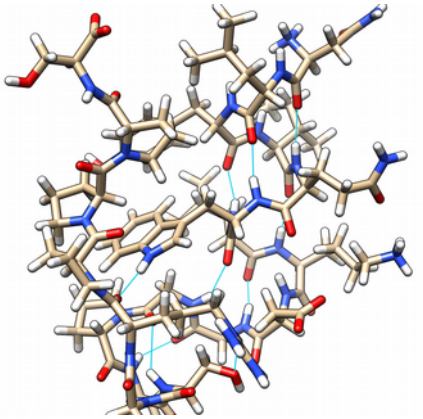
quaternary structure – all kinds of interactions

Denaturation:
- heat
- urea, guanidinium
- surfactants, non-polar solvents

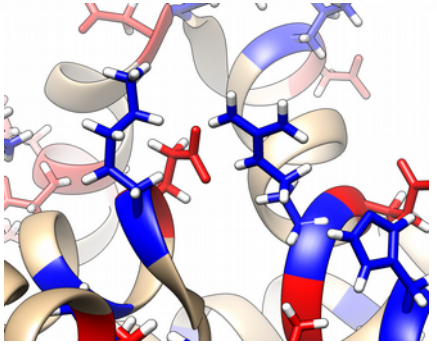
How non-covalent interactions stabilize protein structure?

Hydrogen bonds

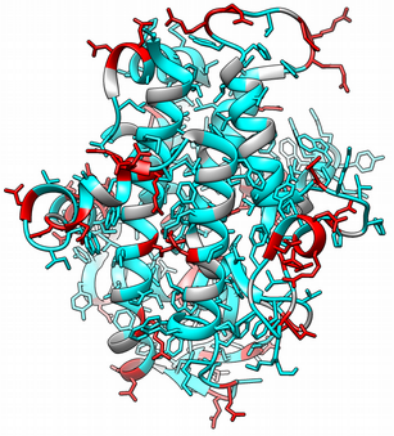




Electrostatic interactions



Hydrophobic



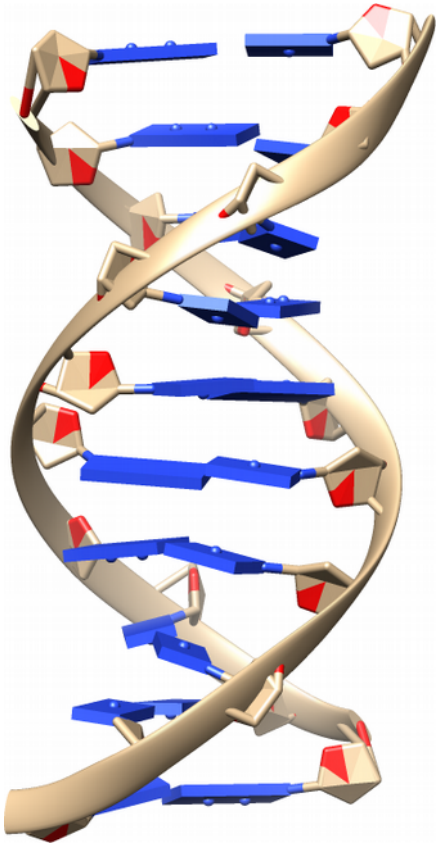
How non-covalent interactions stabilize nucleic acids?

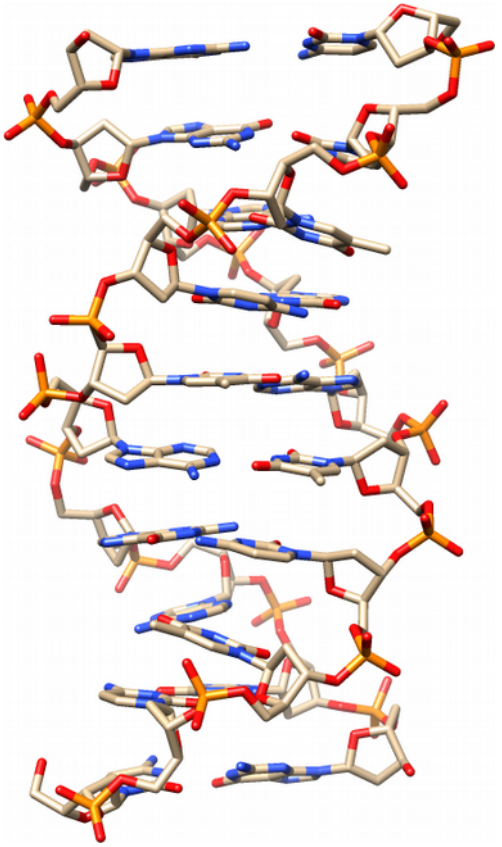
base pairing – hydrogen bonds between A-T (two) and G-C (three), that is the reason why GC-rich nucleic acids are more stable

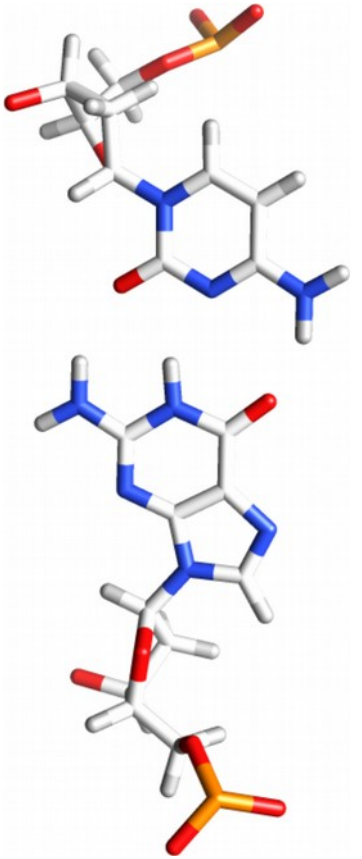
pi-pi interactions – between DNA stacks

Denaturation:
- heat

How non-covalent interactions stabilize nucleic acids?







How non-covalent interactions stabilize membranes?

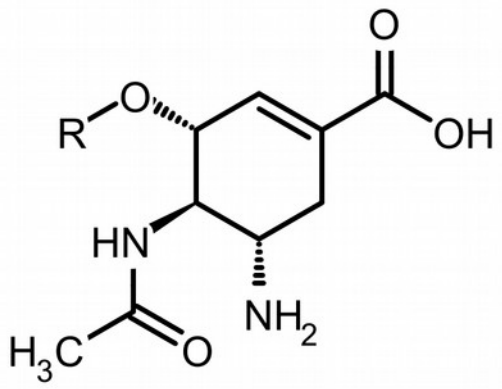
hydrophobic effect

https://www.youtube.com/watch?v=Im-dAvbI330

Membranes are stabilized by hydrophobic effect of hydrophobic fatty acid chains.

How non-covalent interactions stabilize drug-target interactions?

Tamiflu



R:	K*i* (nM)	R:	K*i* (nM)
–H	6 300	$-CH_2CH_2CH_2CH_3$	300
–CH	3 700	$-CH_2CH(CH_3)_2$	200
$-CH_2CH_3$	2 000	$-CH(CH_3)CH_2CH_3$	10
$-CH_2CH_2CH_3$	180	$-CH(CH_2CH_2CH_3)_2$	16
$-CH_2CH_2CF_3$	225	–cyklopentyl	22
$-CH_2OCH_3$	2 000	–cyklohexyl	60
$-CH_2CH{=}CH_2$	2 200	–fenyl	530
		$-CH(CH_2CH_3)_2$	1

The process of drug discovery can be seen as optimization of non-covalent bonds to achieve highest possible affinity.

Affinity chromatography

Antibody-antigen – antibody can be immobilized on a resin in a column, magnetic beads or other material. This material can be used to isolate corresponding proteins.

Protein-ligand – same can be done for ligands to isolate proteins or other way around

enzyme-substrate (or substrate analogue)

Famous interaction pairs

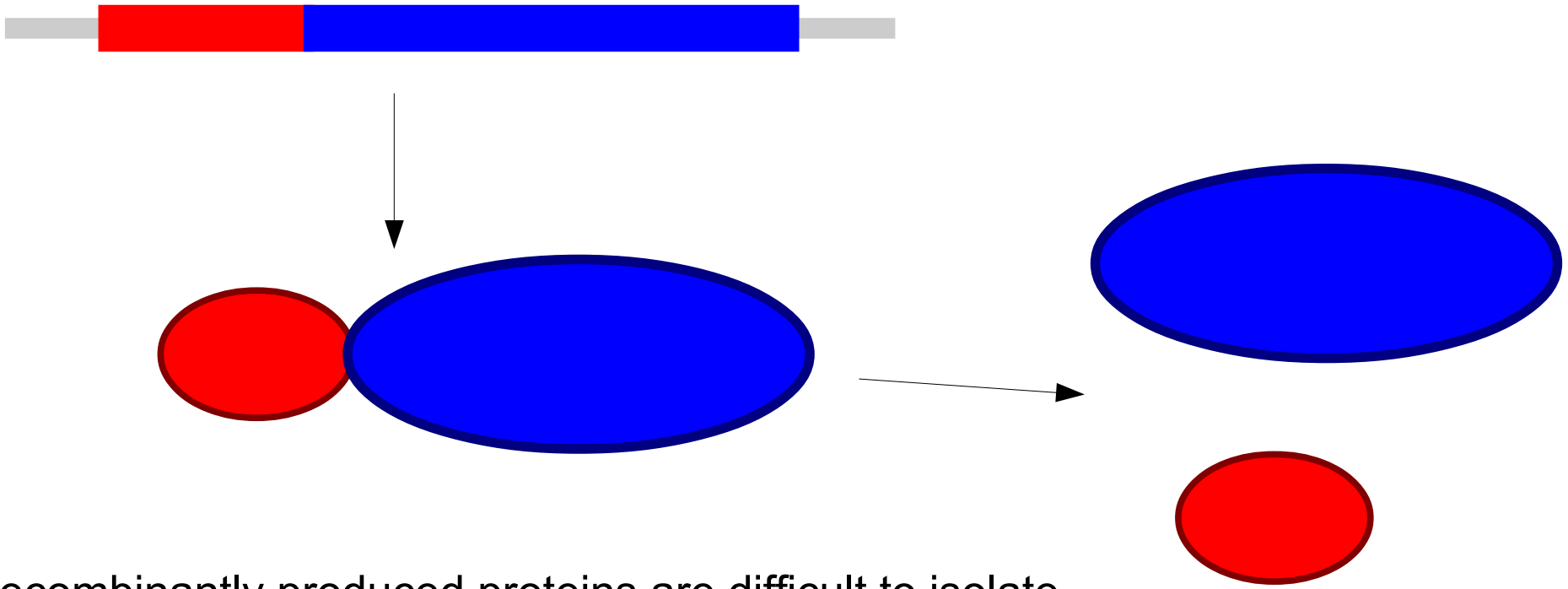
streptavidin-biotin, avidin-biotin – extremely high affinity, often used as affinity tags in molecular biology

protein A – IgG antibody – protein A is a protein with affinity to IgG produced by *S. aureus*

affinity tags in recombinant protein production – next page

Affinity tags in recombinant protein production

poly-His – Ni^{2+}
maltose-binding protein – amylose
glutathion-S-transferase – glutathione



Recombinantly produced proteins are difficult to isolate because producing organisms produce many other protein. Therefore, it is useful to modify their genes to produce a chimeric protein composed of the protein and a tag on N- or C- terminus. The resulting chimeric protein can be isolated and the tag can be removed by a protease, if necessary.

How can be affinity quantified?

equilibrium (dissociation/association) constants

$$P + L \rightleftharpoons PL$$

$$K_{dissoc.} = \frac{[P][L]}{[PL]} \qquad K_{assoc.} = \frac{[PL]}{[P][L]}$$

Gibbs/Helmholz free energy, Affinity

$$\Delta G_0 = -RT \ln K$$

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

How does formation of a complex depend on concentration(s)?

Saturation:

binding of a ligand to a protein

enzyme kinetics

$$saturation = \frac{[L]}{[L] + K_S}$$

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

Binding of ligand to protein or substrate to enzyme follows saturation thermodynamics. At low concentrations of ligand/substrate there is a quasi-linear relationships between concentration and saturation. Absolute saturation would be achieved at infinite concentration.

Oxygen

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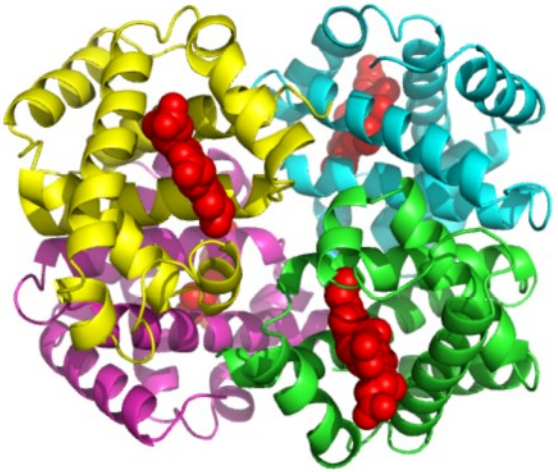
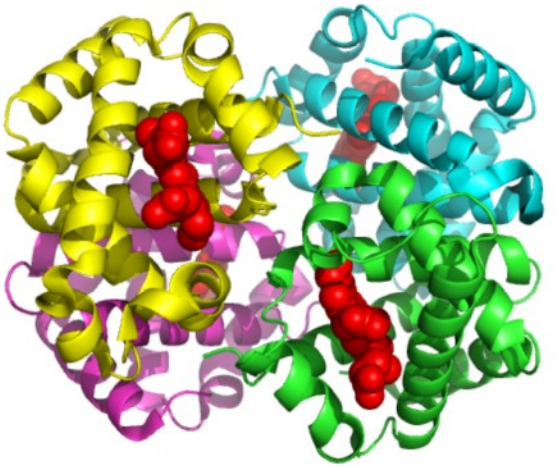
Hemoglobin (haemohlobin in UK)



R – relaxed (1RVW)

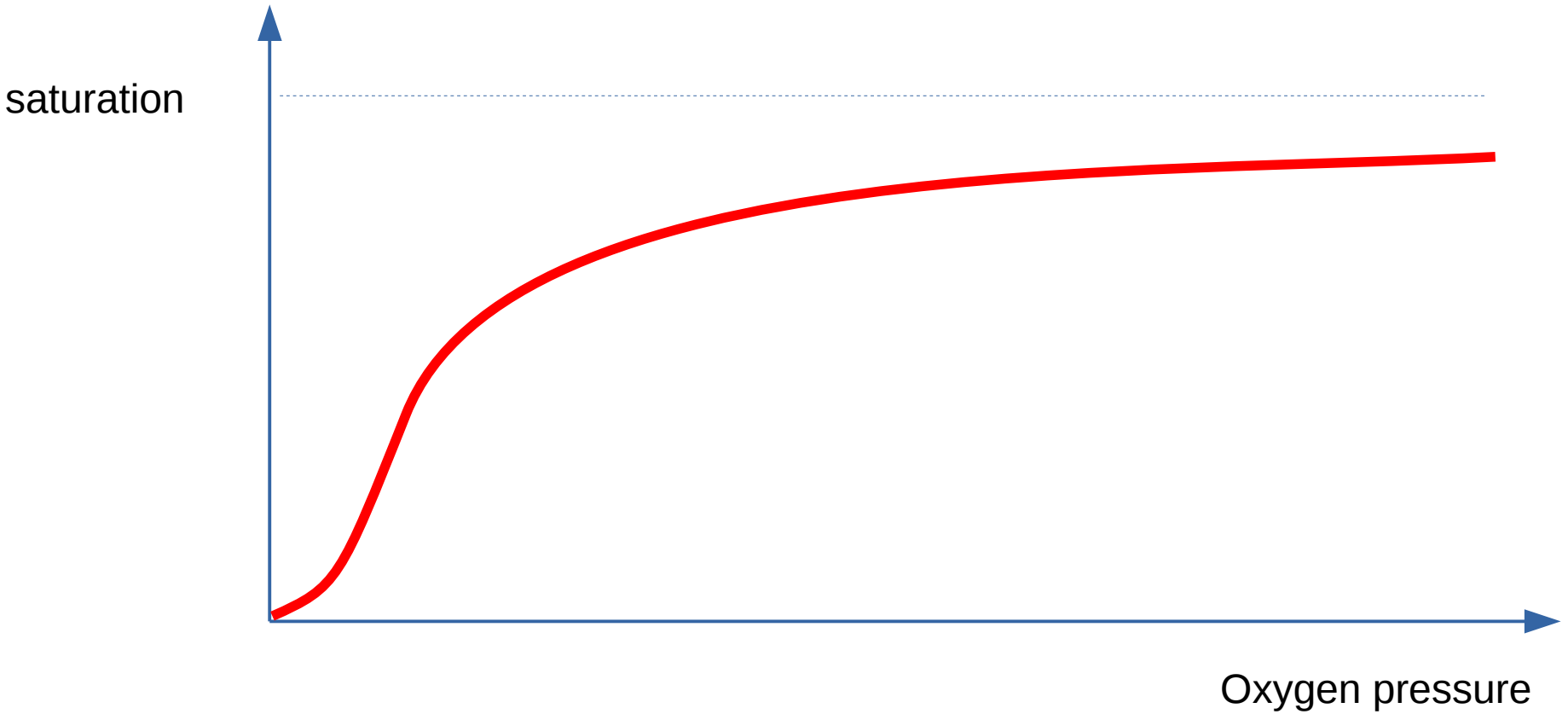


T – tense (2D5Z)

Hemoglobin is homotetramer. Each subunit contains heme that binds oxygen via iron atom. It can exist in two states R and T with a slight conformational difference. In absence of oxygen it prefers R. Binding of oxygen stabilizes T. The T form shows higher affinity to oxygen than R. As the result, binding of single oxygen increases affinity of the remaining three sites. Binding of another oxygen increases affinity of other two sites etc.

The fact that binding of oxygen in one site influences binding in another distant site gives the name of this effect – allosteric (“different” “site”).

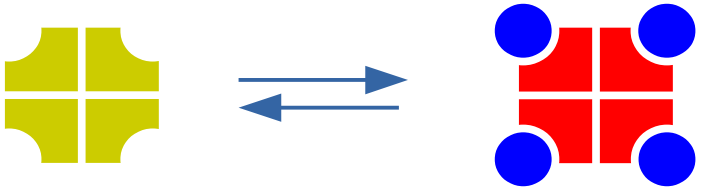
Hemoglobin



Unlike other binding of ligand by other proteins, hemoglobin does not show hyperbolic saturation curve. The curve of hemoglobin is sigmoid due to the allosteric effect. It binds oxygen weakly at low concentration and strongly at high concentrations. This is important for physiology. Hemoglobin can be almost fully loaded in lungs (high oxygen concentration) and it can release almost all oxygen in other tissues (low oxygen concentration). This cannot be achieved without allostery.

Hemoglobin

Hill's model

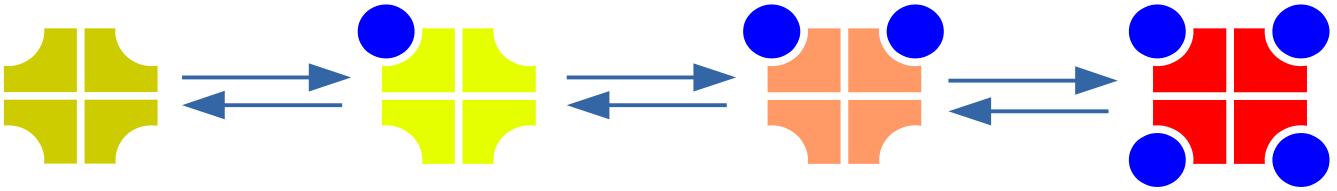


$$S = \frac{[A]^{\kappa}}{[A]^{\kappa} + K_S}$$

The oldest model of allostery is Hill's model. According to this model hemoglobin can bind either no oxygen or four oxygen molecules in all four sites. This model is not realistic, it is not probable that five molecules meet at the same moment. Saturation follows the Hill's equation (above). Standard saturation curve corresponds to the Hill's equation with κ=1. The coefficient κ is theoretically equal to four for hemoglobin. In reality it is the range 1.7-3.2. Hill's model cannot explain values of κ different from the number of binding site as well as non-integer values.

Hemoglobin

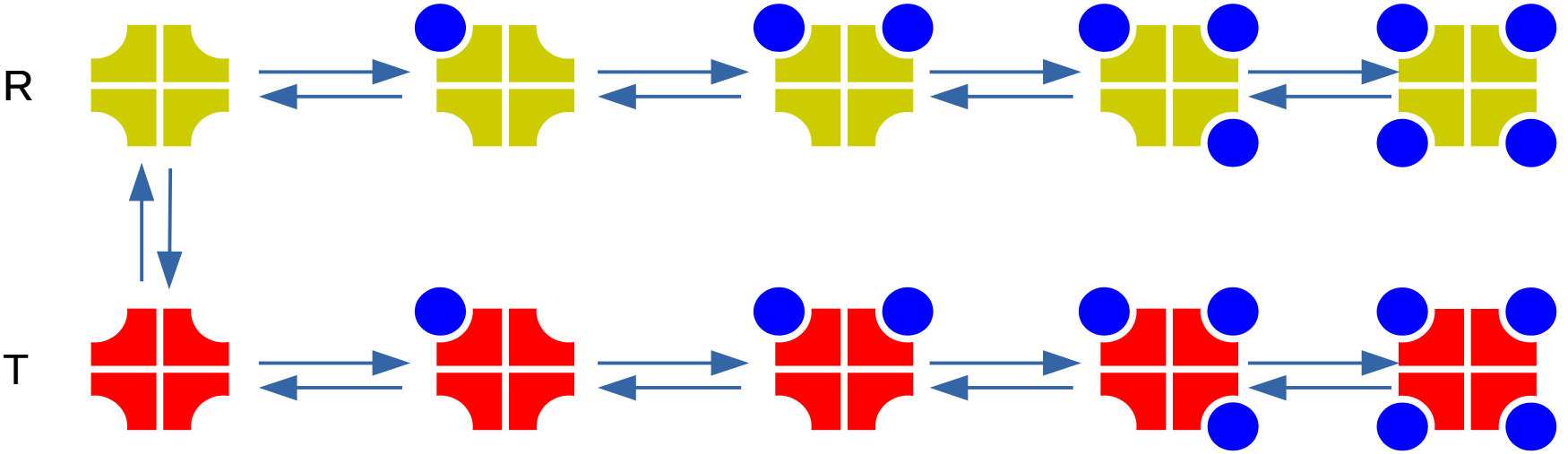
Aldair's model



Aldair's model of allostery is more advanced and it can explain different values of κ. Binding of each oxygen has different equilibrium constant. However, this model is bit chicken-hearted because it is easy to explain anything by quite complicated model. Aldair's model also adds little to explanation of the structural basis of allostery.

Hemoglobin

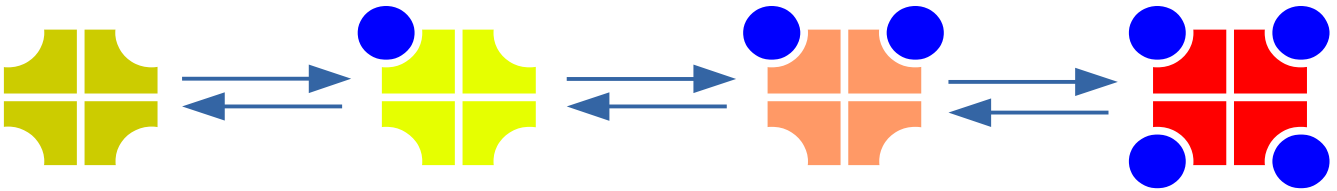
Monod-Wyman-Changeux (MWC)



One of most respected model is the one by Monod, Wyman and Changeux (MWC). It correctly predicted existence of the two forms – relaxed and tense. According to this model hemoglobin exists in two states (conformations) R and T. R is more favored in absence of oxygen. It has also weaker affinity towards oxygen. The tense form is unpopulated in absence of oxygen. Since T binds oxygen more strongly binding of oxygen shifts R-T equilibrium in favor of T. MWC model can explain experimental values of Hill's coefficients.

Hemoglobin

Koshland-Némethy-Filmer



Koshland-Némethy-Filmer model is similar to Aldair's model. It is extended in the way that it adds a structural explanation to Aldair's model. It introduced the term induced fit, i.e. binding of oxygen introduces the changes in the hemoglobin structure.

Hemoglobin

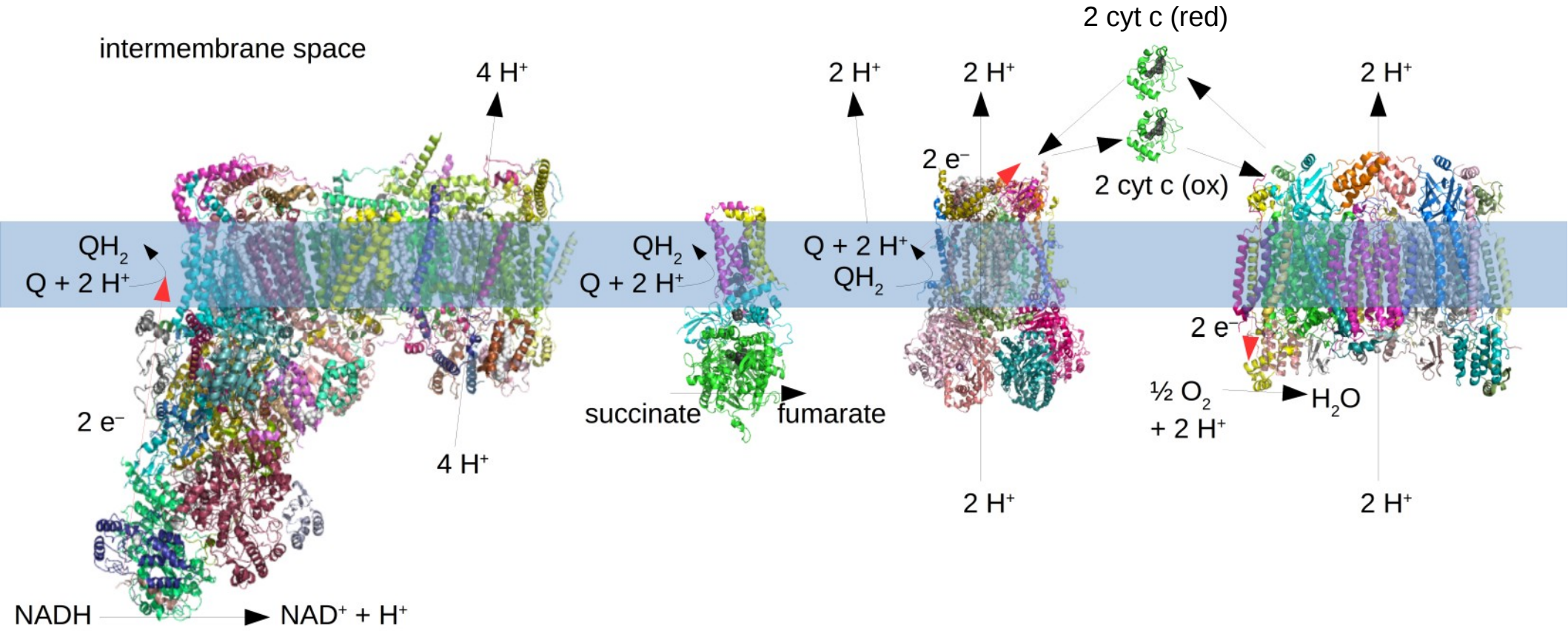
Affinity of hemoglobin for oxygen is also influenced by:

Charge state of iron – only Fe2+ binds oxygen, Fe3+ (methemoglobin) can be enzymatically reduced to Fe2+ by NADH. This reaction consumes most NADH produced in erythrocytes (since erythrocytes do not have mitochondria they produce little NADH).

Carbon dioxide and **acidity** (Bohr effect) shifts the dissociation curve right. It supports release of oxygen in tissues with high carbon dioxide content (and thus high acidity).

2,3-Bisphosphoglycerate also shifts the dissociation curve right. It is an intermediate of glycolysis in erythrocytes (not in usual glycolysis). It connects glucose metabolism with oxygen binding. By this erythrocytes can respond to hypoxia or other conditions.

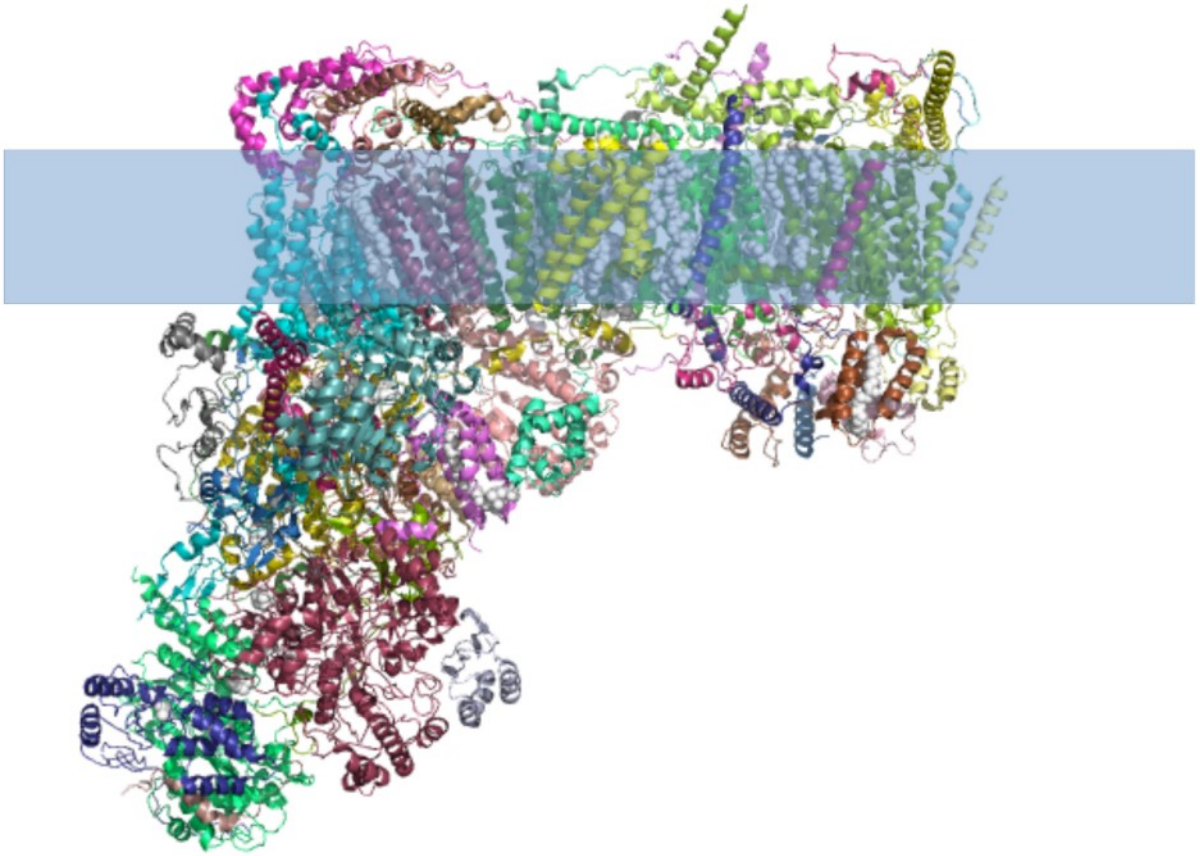
Respiratory chain



NADH from citric acid cycle and other mitochondrial processes (e.g. fatty acid degradation) is recycled in respiratory chain (also known as electron transport chain). In this case electrons flow via complex I, III and IV. At the end they convert oxygen to water. The result is pumping of protons across the membrane.

Respiratory chain

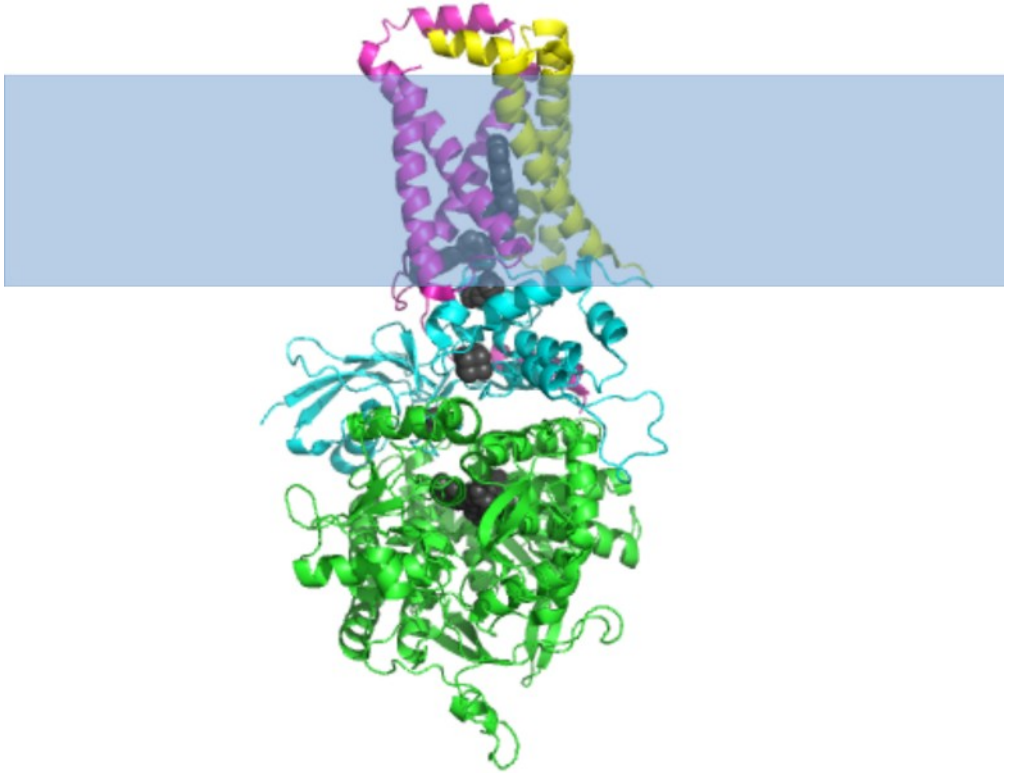
Complex I



NADH dehydrogenase (NADH:ubiquinone oxidoreductase, EC 1.6.5.3)
NADH + ubiquinone + H^+ → NAD^+ + ubiquinone-H_2 (+ 4 H^+ transported)
contains FMN and Fe-S clusters

Respiratory chain

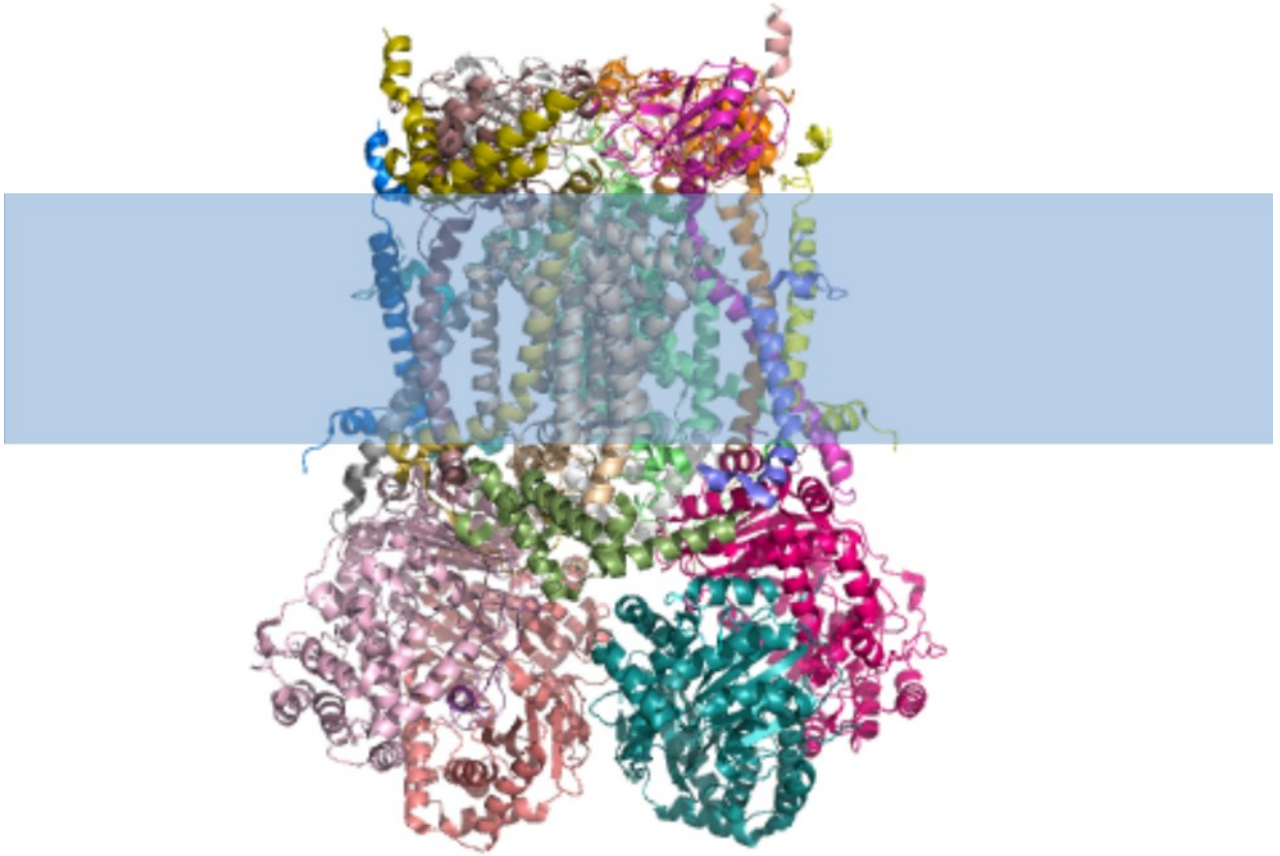
Complex II



succinate dehydrogenase (succinate:quinone oxidoreductase, EC 1.3.5.1)
succinate + ubiquinone → fumarate + ubiquinone-H_2
contains FAD, Fe-S clusters and heme

Respiratory chain

Complex III



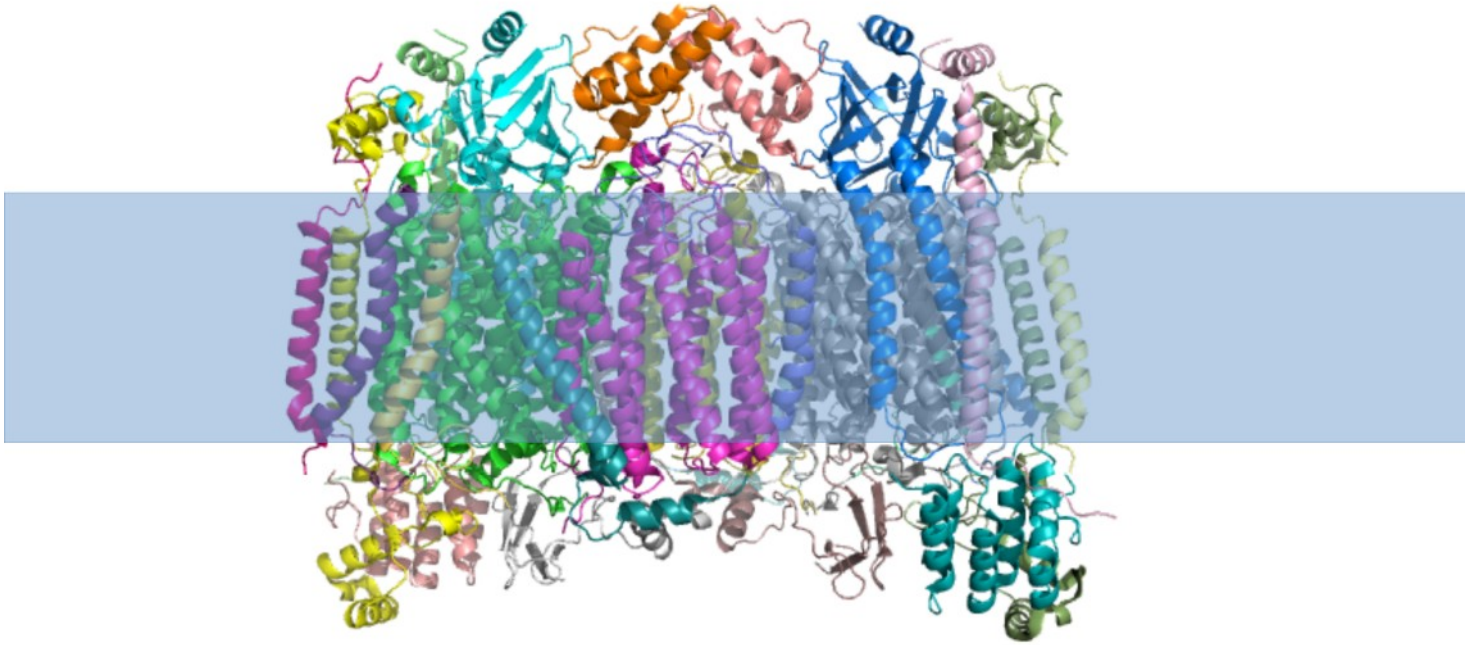
cytochrome bc_1 complex (quinol:ferricytochrome-c oxidoreductase, EC 1.10.2.2)

ubiquinone-H_2 + 2 ferricytochrome c → ubiquinone + 2 ferrocytochrome c +
2 H^+ (out) (+ 2 H^+ transported)

contains Fe-S cluster and hemes

Respiratory chain

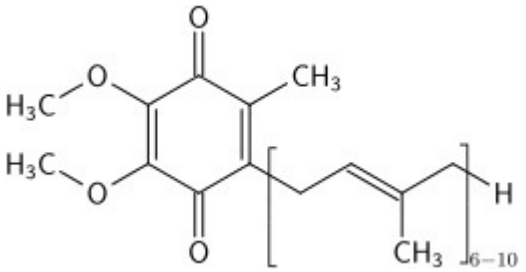
Complex IV



cytochrome *c* oxidase (ferrocytochrome-c:oxygen oxidoreductase, EC 1.9.3.1)
2 ferrocytochrome *c* + ½ O_2 + 2 H^+ → 2 ferricytochrome *c* + H_2O (+ 2H^+ transported)
Contains heme and copper centres

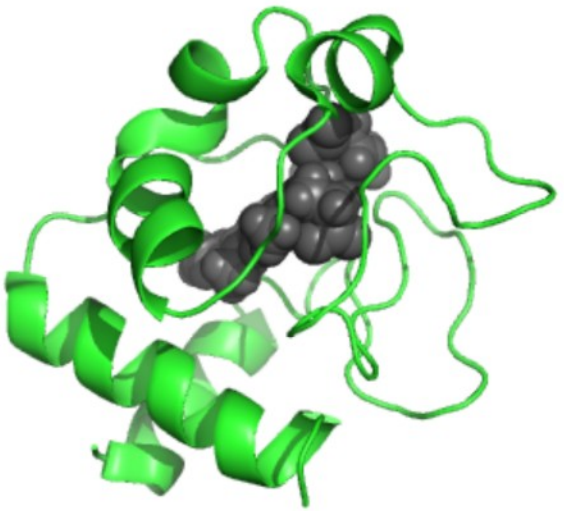
Respiratory chain

Ubiquinone (coenzyme Q_{10})



Can be reduced to hydrochinonic form

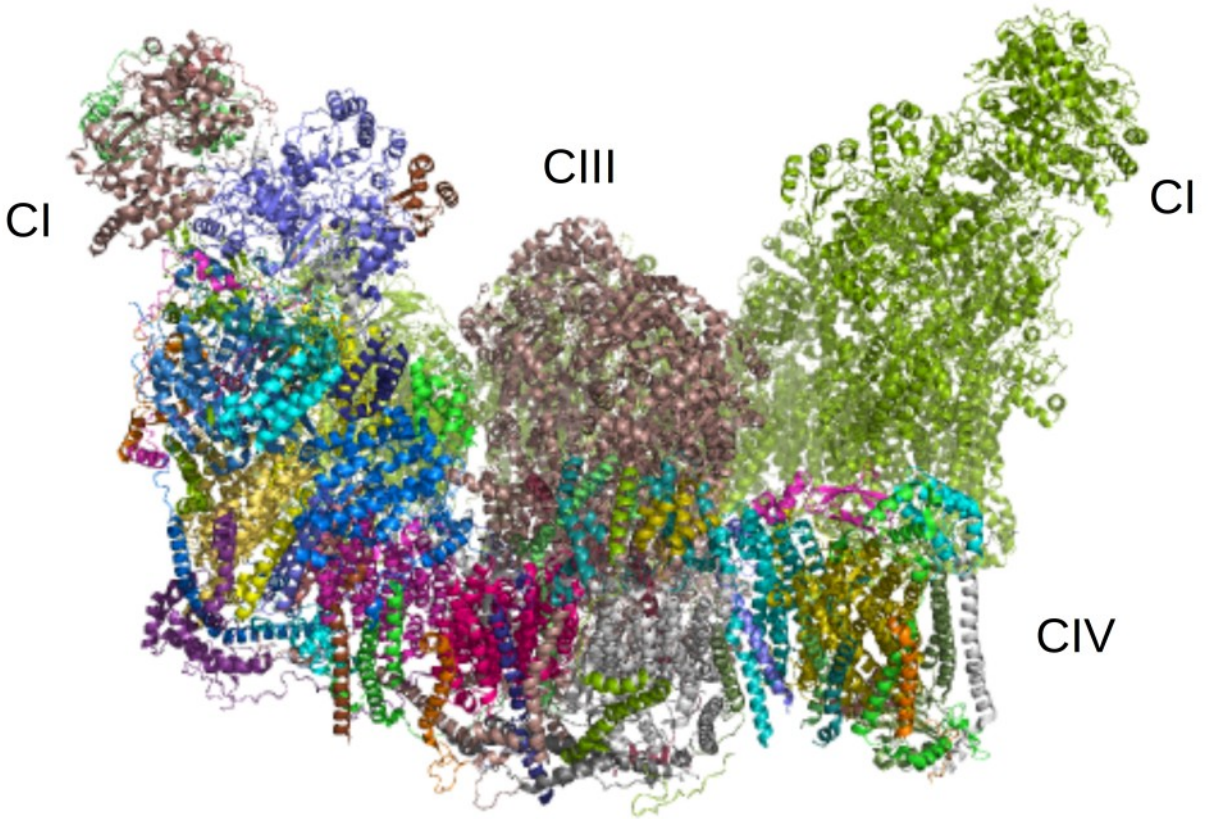
cytochrome *c*



oxidized: ferri-
reduced: ferro-

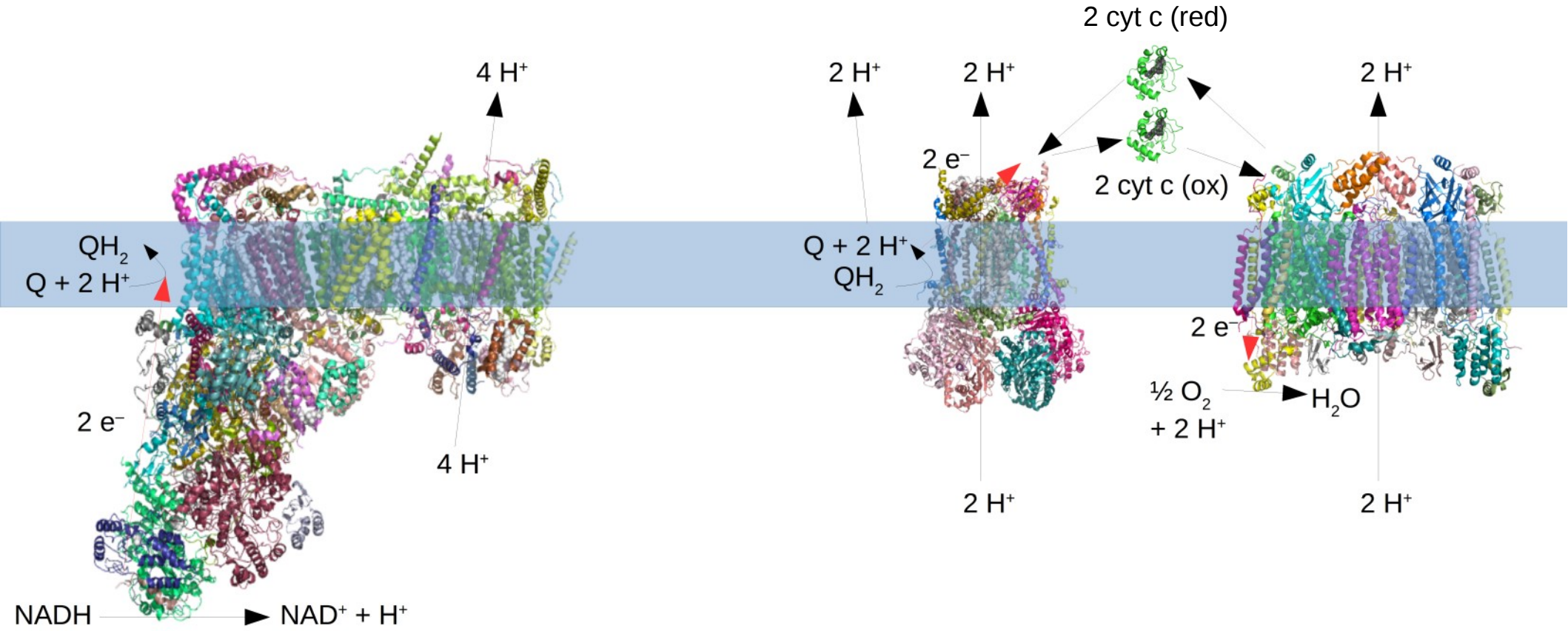
Respiratory chain

Megacomplex



Respiratory chain c
a large megacomp
carriers.

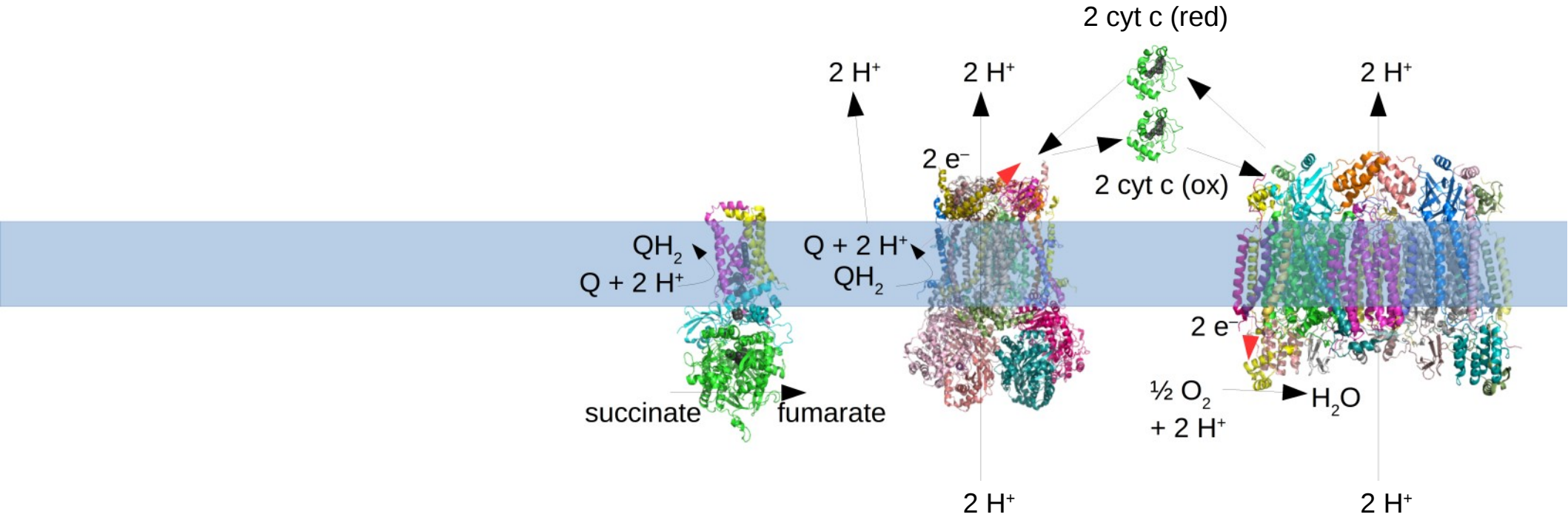
Respiratory chain



NADH as the substrate

$NADH + ½\ O_2 + 3\ H^+ (in) \rightarrow NAD^+ + H_2O + 2\ H^+ (out)\ (+ 8\ H^+ transported)$

Respiratory chain

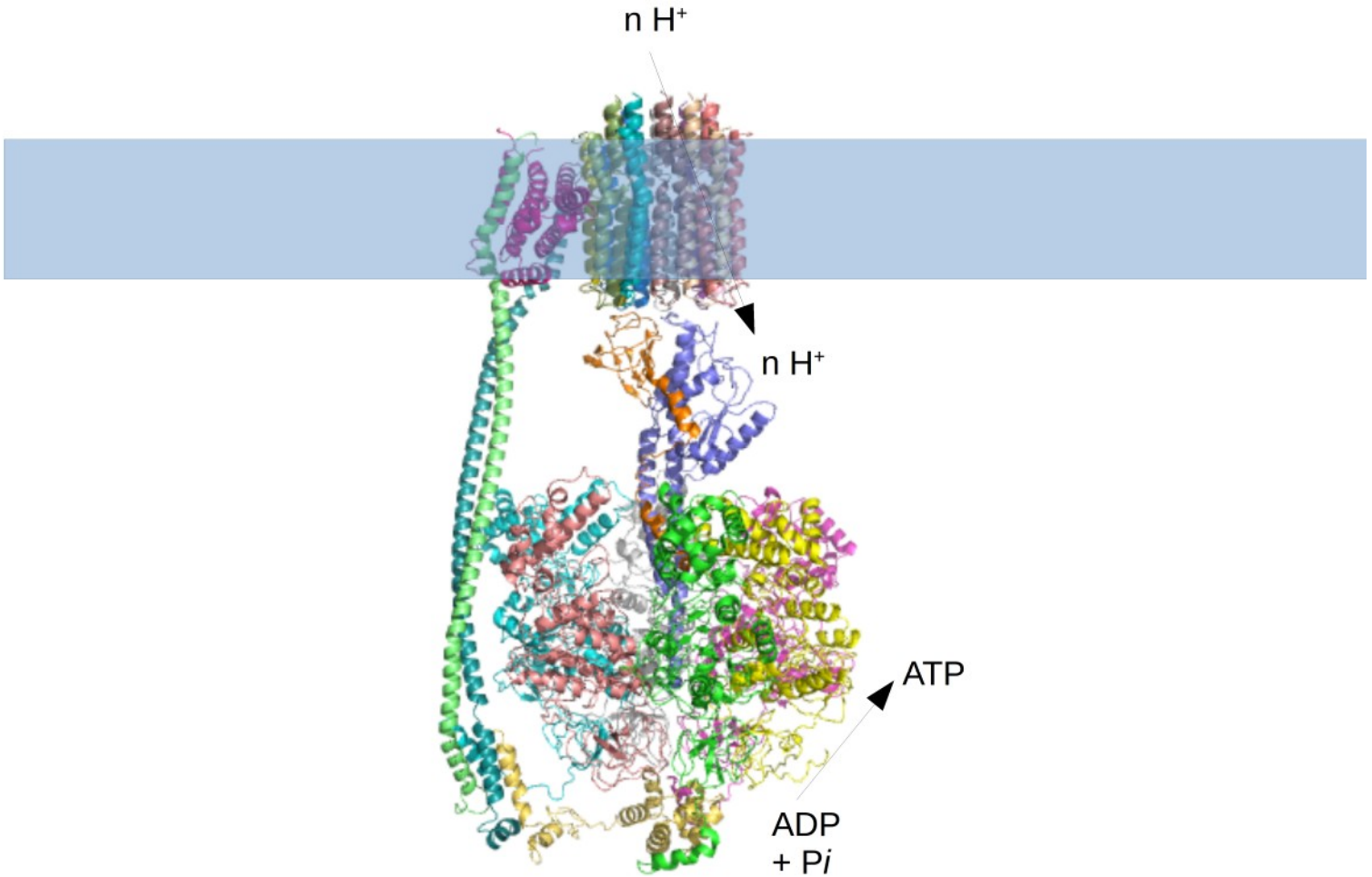


succinate as the substrate

succinate + ½ O_2 + 4 H^+ (in) → fumarate + H_2O + 2 H^+ (out) (+ 4 H^+ transported)

Respiratory chain

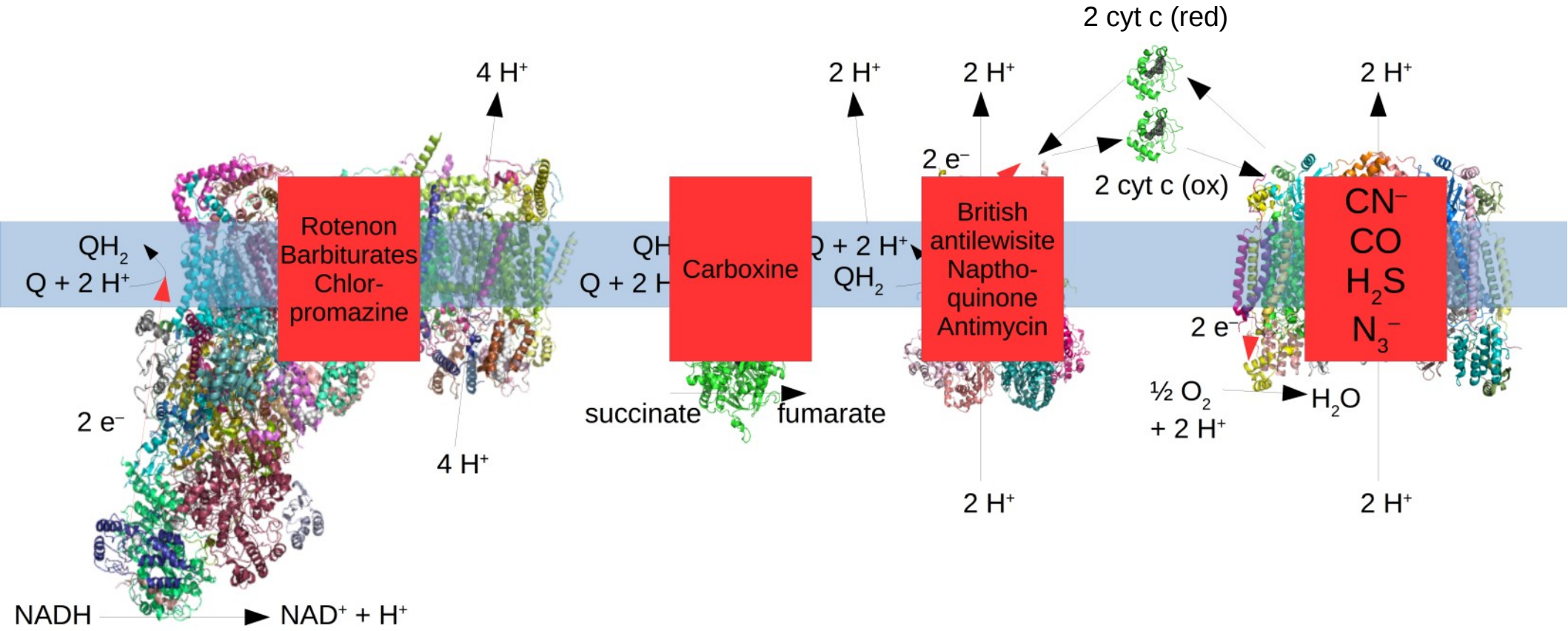
F-ATPase



https://www.youtube.com/watch?v=oFgM1dVRi6I

Respiratory chain creates the gradient of protons on the membrane. This is associated with the concentration gradient as well as membrane potential. Energy stored in this (proton motive force) can be used to create ATP by F-ATPase. This enzyme is also known as FoF1-ATPase (o for oligomycin inhibitor, sometimes written as F0F1). This enzyme converts proton motive force to rotation (watch the movie!!!) and rotation to ATP synthesis.

Respiratory chain



Research

Respiratory chain was deciphered using various approaches. Application of inhibitors turned out to be very useful. For example it is possible to prepare cytochrome C in oxidized or reduced form. It is also possible to monitor the form of cytochrome C by spectrophotometry. If you isolate complex IV and supply it with reduced cytochrome c, it will oxidize it. If you add cyanide, it will stop. If you add cyanide to whole mitochondria, it will still reduce cytochrome c. This shows that 1. cyanide inhibits complex IV and 2. complex IV is the terminal step. Similarly it is possible to test various inhibitors to determine the order of complexes.

Respiratory chain

Chemiosmotic theory (theory involving formation of proton motive force and its conversion to ATP synthesis) had been controversial for a long time. One of evidence supporting it is the function of decouplers. These are compounds reducing the proton motive force. Examples are:

2,4-dinitrophenole: Can pass the membrane in both forms and thus transport protons along the gradient. This ceases proton motive force.

Valinomycin: This peptide-based compound makes pores in the mitochondrial membrane. These pores can transport potassium cations. This does not influence concentration gradient of protons (protons cannot pass) but it ceases electrostatic potential (charge gradients). Potassium cations move into the mitochondria to compensate charge gradient caused by protons exported by respiratory chain. Therefore, valinomycin makes only partial effect compared to 2,4-dinitrophenole.

Human newborn infants and some hibernating animal can decouple proton motive force in non-shivering thermogenesis in brown adipose tissue. They can switch on the protein **thermogenin**, which leaks protons out of mitochondria, while converting proton motive force to heat. This process is controlled by GPCR (β-adrenergic receptor), which triggers formation of cAMP, cAMP activates protein kinase, this activates a lipase which produces a fatty acid. The fatty acid activates thermogenin.

Essential Factors: Vitamins, Cofactors, Coenzymes and Prosthetic Groups

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Vitamins

essential exogenic factors for certain group of organisms, low daily consumption (compared to e.g. essential amino acids)

Pro-vitamins

vitamin precursors

Cofactors

low-molecular weight compounds required for enzymatic catalysis

Coenzymes

low-molecular weight compounds carriers of chemical groups

Prosthetic groups

a cofactor strongly (often covalently) bound to an enzyme

Vitamins

A – retinol
B1 – thiamine
B2 – riboflavin
B5 – panthotenate
B6 – pyridoxal
B12 – cobalamin
C – ascorbic acid
D – calciferol
E – tocoferol
H – biotin
K – phylloquinon
PP – niacin

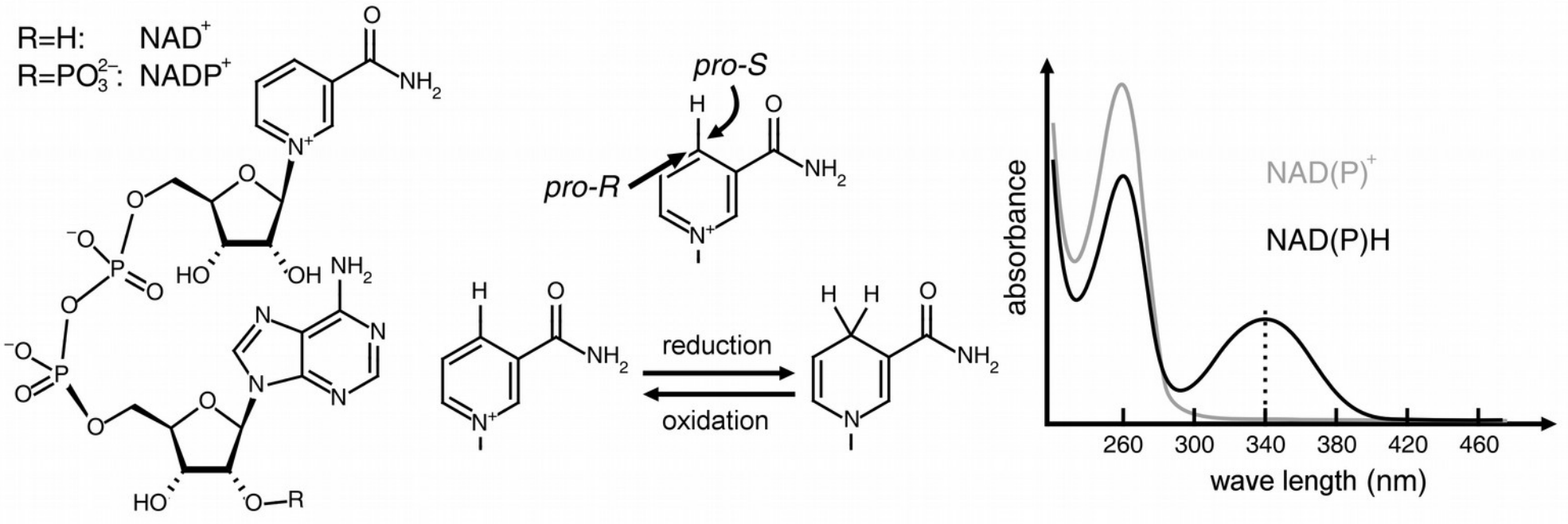
Vitamins, coenzymes, cofactors, prosthetic groups

The terms “vitamins”, “coenzymes”, “cofactors” and “prosthetic groups” are somehow blurred. This is because vitamins were discovered in times when their biochemical function was not clear. Chemists discovered vitamins by analyzing food and by studying biological of their deficiencies. In the other words, vitamins were discovered as nutrients with low daily intake, yet important for health. Much later it was found that most (but not all) vitamins are cofactors, prosthetic groups or their precursors.

Also, the terms “coenzymes”, “cofactors” and “prosthetic groups” are somehow blurred. Coenzymes are substrates of enzymatically catalyzed reactions in cell. They can exist in two or multiple forms (e.g. oxidized and reduced) and in some reactions they are converted from one form to another (e.g. oxidized) and in some reactions they are converted back (e.g. reduced). The fact that they are recycled in metabolism makes it possible that they are present in relatively low concentrations in a cell. Prosthetic groups are compounds bound to enzymes (covalently or non-covalently) and their change from one form to another and back takes place in a single catalytic cycle. The term cofactors unites coenzymes and prosthetic groups. CAUTION: Some researchers use the term prosthetic group for covalent bound-only prosthetic groups and cofactors for other prosthetic groups.

NAD+

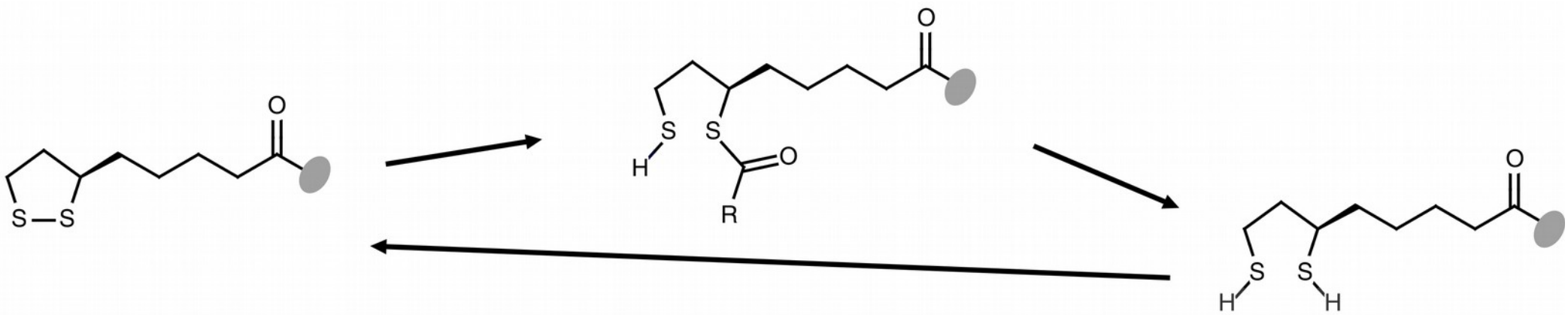
$\varepsilon_{NAD(P)H,340}$ = 6,22.10^3 l/mol/cm



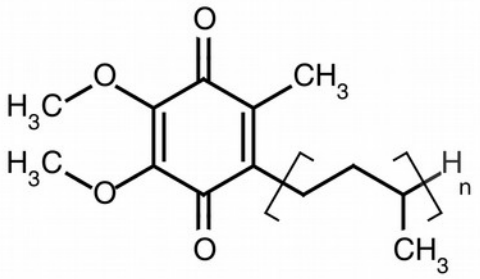
Nicotinamide (niacin, vitamin PP)

NAD+ or NADP+ are typical coenzymes of oxidoreductases (interestingly, it can be a prosthetic group of a hydrolase – S-adenosylhomocystein hydrolase). Niacin is its precursor. Most enzymes are NAD+ or NADP+ specific and these coenzymes play different roles in metabolism. Interesting feature of NADH or NADPH is its absorbance maximum at 340 nm. Thanks to this it is possible to measure rates of enzymatic reactions using these coenzymes. This is important in clinical diagnostics.

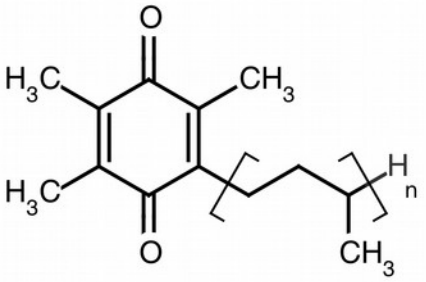
lipoic acid



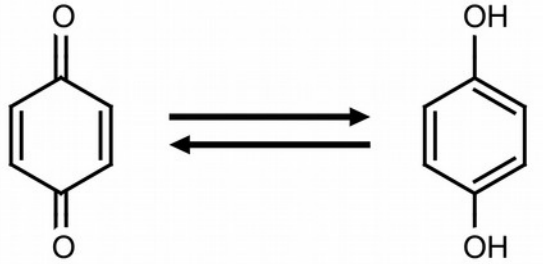
quinons



ubiquinon

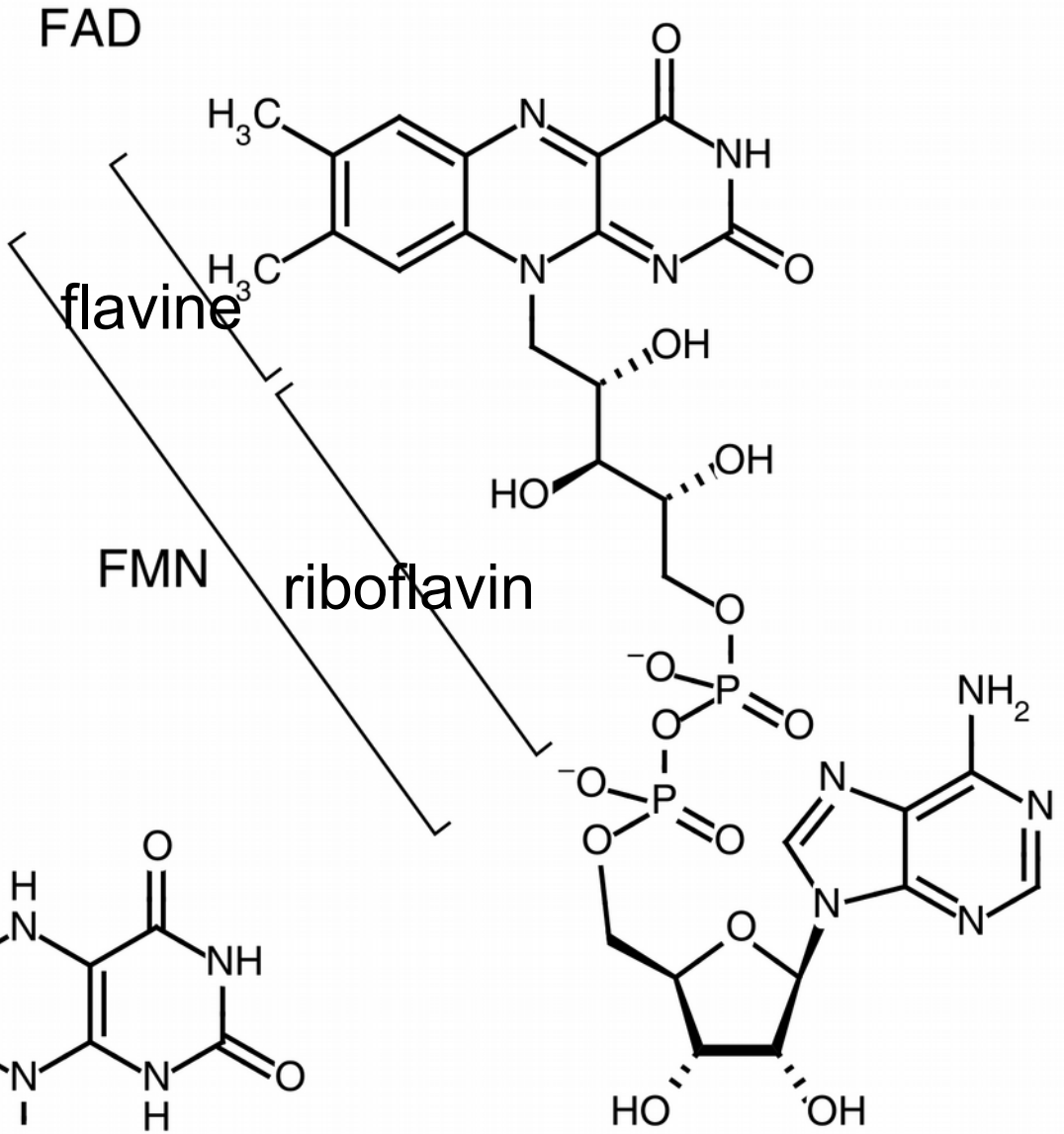


plastoquinon



Pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenases complex use a specific prosthetic group called lipoic acid. It is bound by amidic bond to Lys side chains. It can exist in oxidized, reduced and acylated reduced form (with acetyl or succinyl as the acyl). Ubiqinon and plastoquinon are quinonic coenzymes. They are highly non-polar. Unlike usual coenzymes, which are dissolved and diffuse in the 3D space of the cell, quninons are dissolved and diffuse in the 2D space of the membrane of mitochondria or thylakoid, respectively.

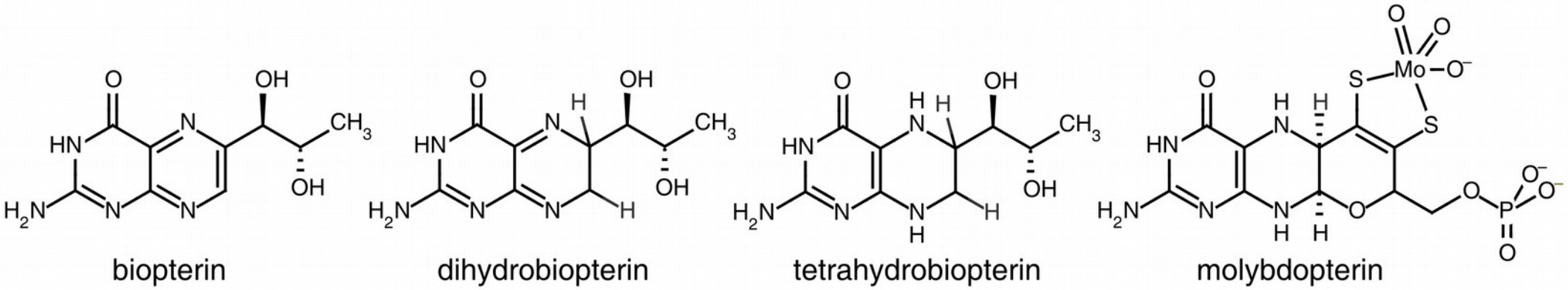
FAD/FMN



Riboflavin – vitamin B2

FAD is a typical prosthetic group of oxidoreductases. Riboflavin (vitamin B2) is its precursor. It can exist in oxidized and reduced forms or in other forms (some enzymes use FAD in more complicated reactions). Similarly to NAD+ and NADP+ it is possible to distinguish between oxidized and reduced forms, but it is not (so much) useful as for NAD(P)+ because FAD is a prosthetic group.

pterins



$$Phe + O_2 + THB \rightarrow Tyr + H_2O + DHB$$

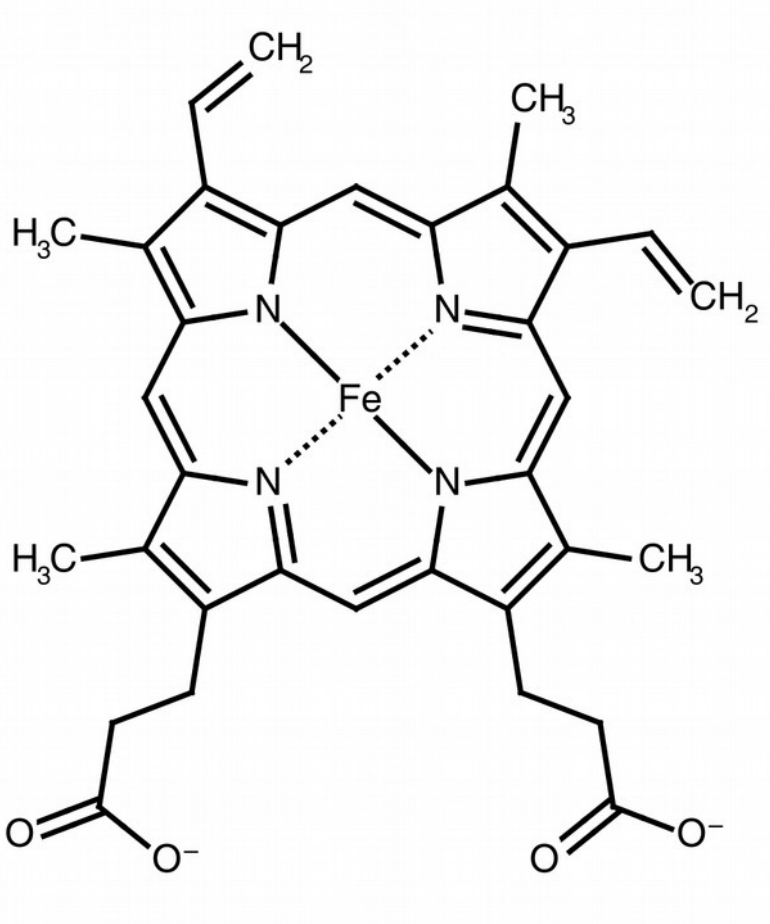
Pterins are interesting cofactors of oxidoreductases. (Di/tetrahydro)biopterin is a coenzyme of phenylalanine hydroxylase. This enzyme is famous for its inborn deficiency causing phenylketonuria. THB has been approved to treat this disease. Molybdopterin is a prosthetic group of xanthine oxidase. It is an interesting example of molybden-containing compound and enzyme in human body.

heme

binding to a protein - non-covalent/coordination-covalent
- covalent (addition of SH to vinyls)

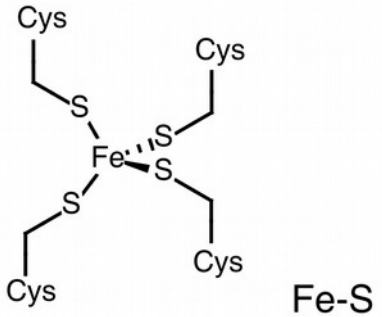
role in proteins - transport of oxygen
- electron traffic
- catalysis

cytochromes

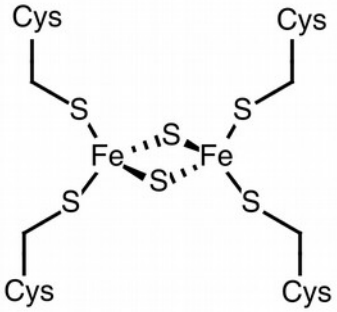


Heme is famous for its role in hemoglobin. Hemoglobin is an oxygen-binding protein, not enzyme. Heme plays important role in many enzymes, mostly oxidoreductases. Iron can exist in 2+ or 3+ states. It can also exist in high-spin states. Furthermore, it can bind many ligands by coordination covalent bond.

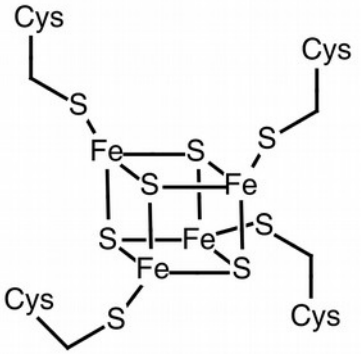
FeS proteins



Fe-S



2Fe-2S



4Fe-4S

Glutathione

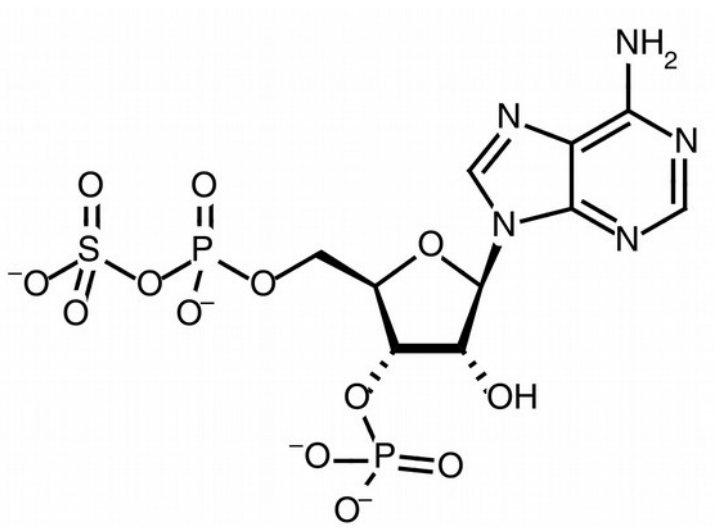
γ-Gly-Cys-Gly

Iron in iron-sulfur clusters of FeS protein can exist in 2+ or 3+ states. These clusters can act as a kind of “electric wiring” in proteins and enzymes. 4Fe-4S can be also found in aconitase enzyme (one cysteine is missing).
Glutathione is a peptidic coenzyme. The reduced form is a tripeptide. The oxidized form is its disulfide-linked dimer. It participates in many spontaneous reactions with oxidative species. It can be reduced by glutathione reductase using NADPH.

ATP
- transfer of phosphate
- can work as a cofactor

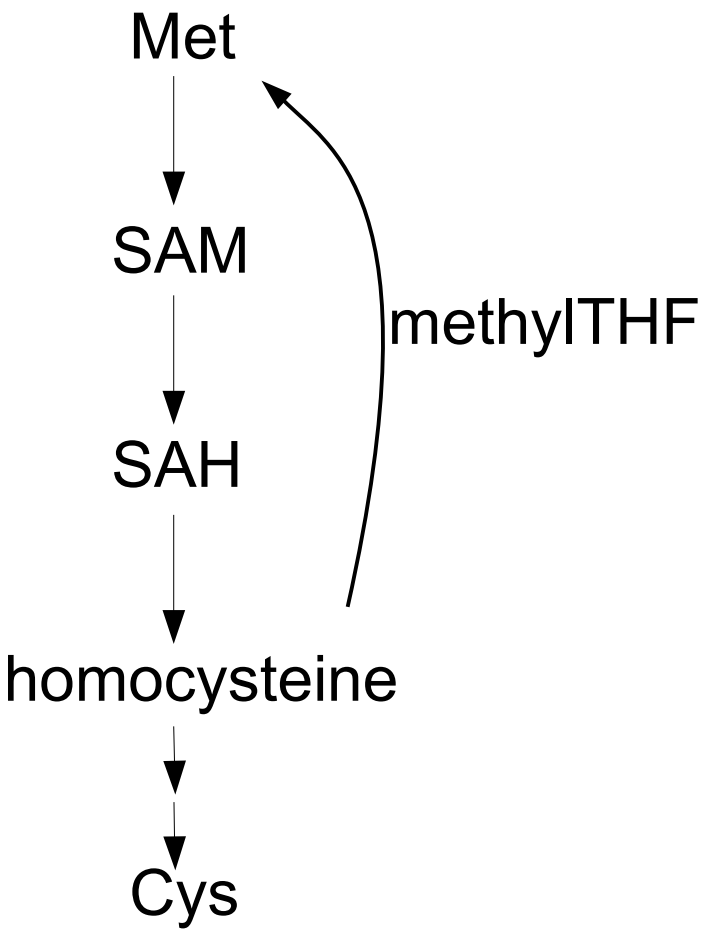
Activated monosaccharides, phospholipid heads etc.

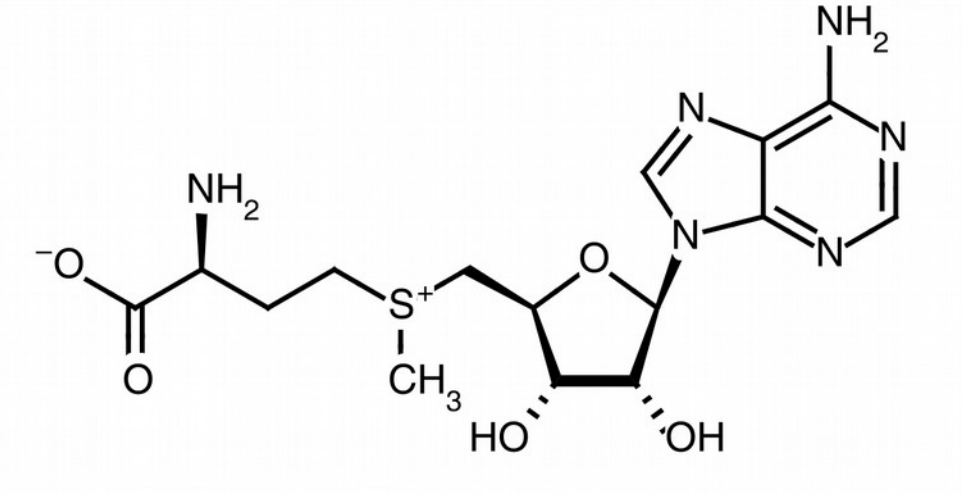
PAPS – 3'-phosphoadenosine-5'-phosphosulphate



ATP is source of energy and coenzyme of transferases. Phosphotransferases are called kinases. Some complex molecules, such as saccharides or phospholipids are synthesized from building blocks such as CDP or UDP (e.g. UDP-glucose). PAPS is a coenzyme of sulfotransferases. It can be used in synthesis of sulfated carbohydrates such as heparins.

SAM (S-adenosylmethionine, AdoMet)



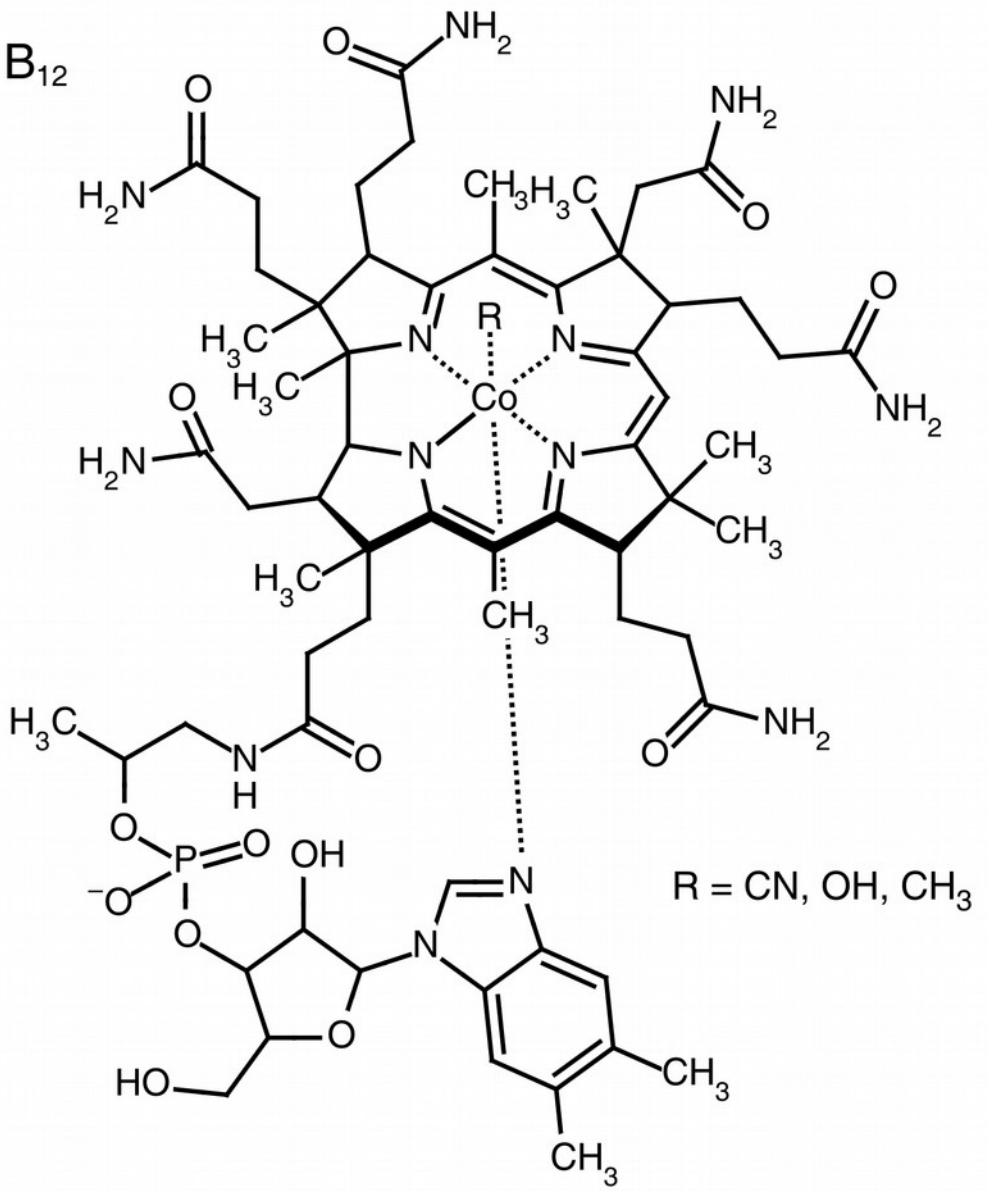


Methylating agent in cells is S-adenosinemethionine (SAM or AdoMet). It is an intermediate of methionine metabolism. Some proteins, DNA or folates (see later) can be methylated.

Cobalamin (B12)

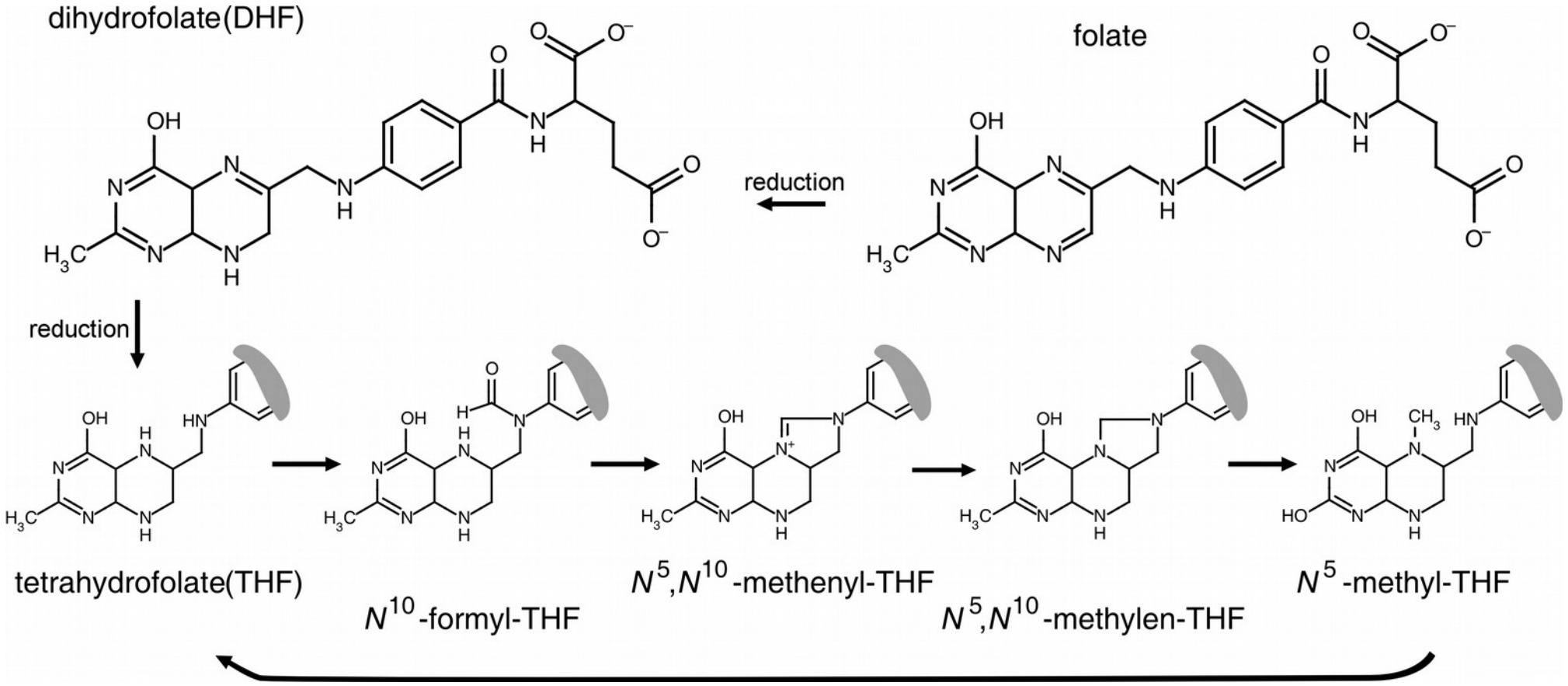
transfer of methyl group
(maleinate → succinate)
NDP → dNDP

carriers:
- haptocorrin
- intrinsic factor
- transcobalamin



The most complicated vitamin with lowest daily income is B12. It plays important role in conversion of maleinate to succinate in degradation of fatty acids with odd number of atoms. It also participates in methionine biosynthesis. It is highly non-polar. Therefore it cannot circulate in body in a bare form and must be bound to one three of protein carriers.

folic acid



purine biosynthesis
uracyl → thymine
Gly → Ser,Thr

vitamin of B-group – folacin

methothrexate

Folates participate in many reactions as carriers of single-carbon moieties such as methyl, methylene or formyl. Its derivative methothrexate inhibits some of folate metabolism reactions. It is used as anticancer and immunosuppressive drug.

Biotin (vitamin H)

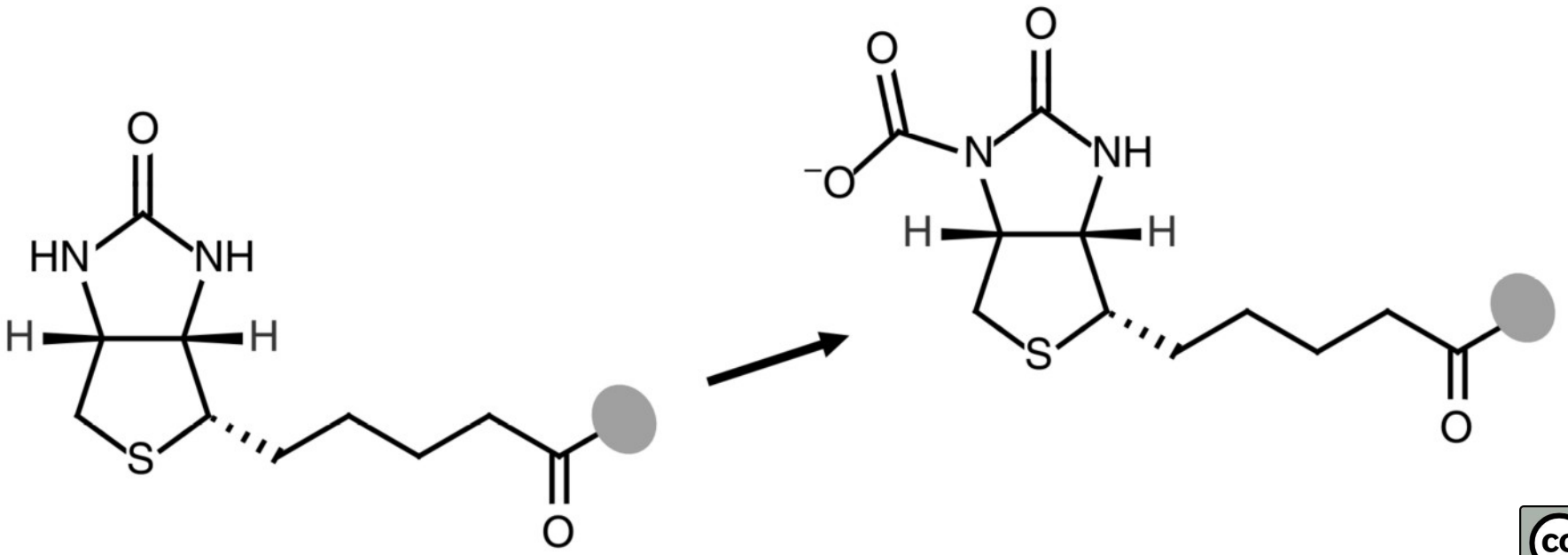
transfer of CO_2

HCO_3^- + ATP → HCO_3-P

HCO_3-P + biotin-BC →
carboxylated biotin-BCCP

avidin, streptavidin

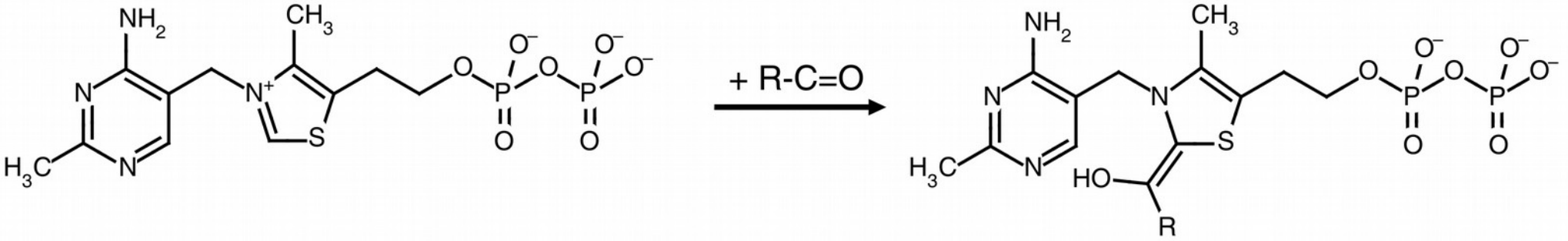
Biotin caries a carboxylic group. It is a prosthetic group bound to the enzyme (or biotin carboxyl carrier protein, BCCP) covalently by amidic bond to Lys side chain. There are two proteins with extreme affinity to biotin – avidin from eggs and bacterial streptavidin. These proteins are often used in molecular biology research for affinity techniques.



thiaminediphosphate

thiamine = vitamin B1

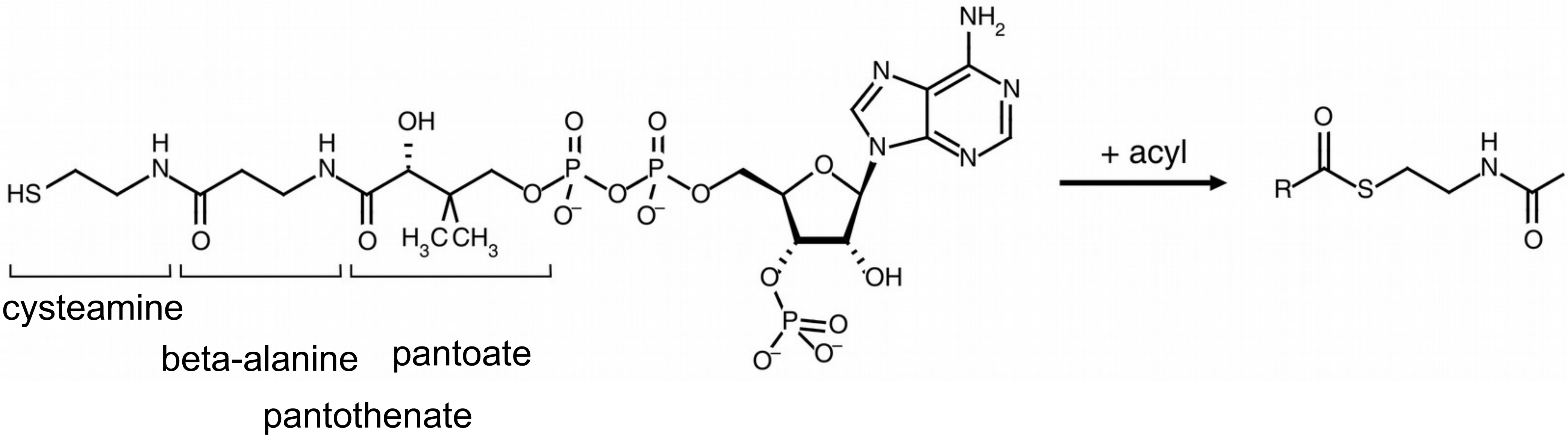
pyruvate dehydrogenase
2-oxoglutarate dehydrogenase
pyruvate decarboxylase



TPP participates in decarboxylation of pyruvate or 2-oxoglutarate in pyruvate or 2-oxoglutarate dehydrogenase complex, pyruvate decarboxylase and other enzymes.

Coenzyme A

pantothenate = vitamin B5



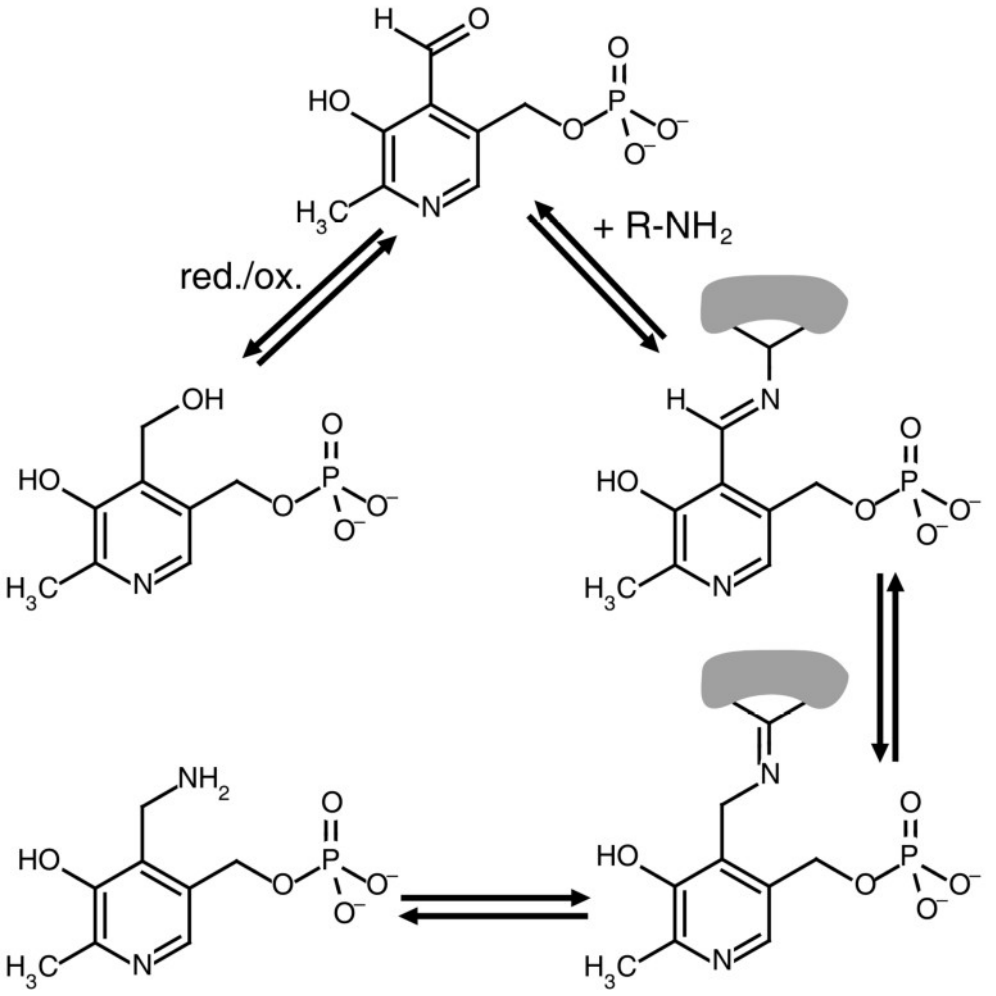
acyl carrier protein

Coenzyme A is a carrier of acyl groups. It is composed of ADP, pantioc acid, beta-alanine (pantoic acid and beta-alanine form pathothenic acid) and cysteamine. Cysteamin and beta-alanine are decarboxylation products of Cys and Asp.

pyridoxalphosphate

pyridoxal/ol/amin = vitamin B6

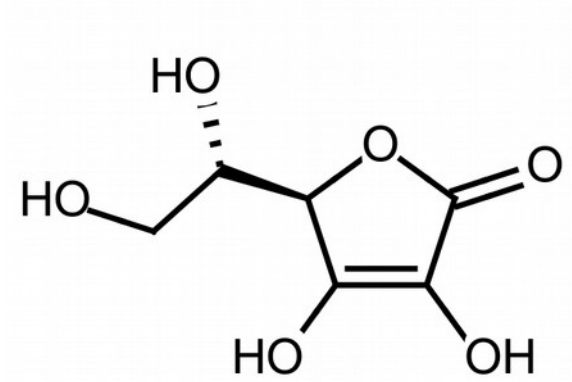
Pyridoxalphosphate is very versatile prosthetic group. It is usually bound noncovalently, but in the resting states of some enzymes it binds covalently via a Shiff base to a Lys sidechain. It can participate in transamina-tion reaction depicted here. First, the amino acid (e.g. Ala) binds, double bonds are rearanged and oxoacid (pyruvate) is released. Next, the whole procedure repeats in oposite direction and with a different amino/oxo-acid. Usually, 2-oxoglutarate binds and Glu is produced. PLP participates in many other reactions of amino acids (deamination, racemisations, decarboxylations) and other reactions.



pyridoxalphosphate

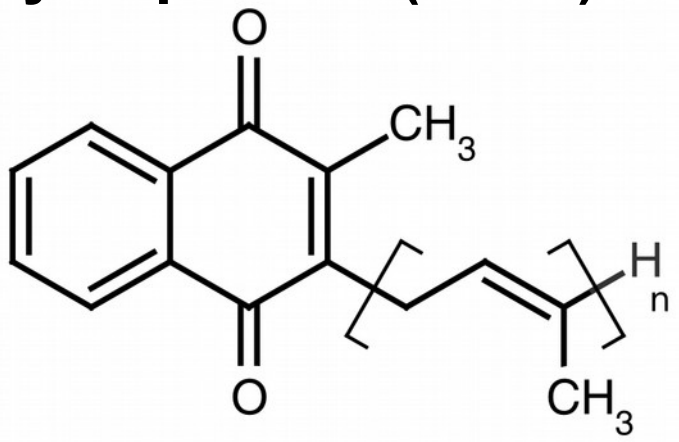
- transamination
- decarboxylation
- racemisation
- deamination (Ser → pyruvate)
- amino acid transport through a membrane
- nicotinic acid biosynthesis
- phosphorylase

Ascorbic acid



reducing agent:
dopamin + ascorbate + O_2
→ norepinephrin
+ dehydroascorbate
prosthetic group:
hydroxylation of collagen

Fylloquinons (vit. K)



Glu → γ-carboxy-Glu
blood coagulation
warfarin
heparin

Tocoferrol (vitamin E)
antioxidants

Retinol/retinal (vitamin A)
- biochemistry of vision
- signaling molecule

Calciferol (vitamin D)
-hormonal function in calcium metabolism

As mentioned at the beginning, at the time of vitamin discovery there was little known about their biochemical function. As found later, most vitamins are precursors cofactors and cofactors, but not all. Vitamins A, C, D, E and K do not fall into the group of cofactor vitamins. Vitamin A is a part of rhodopsin – light-sensing G protein-coupled receptor (GPCR). It is also important hormone in tissue development. Vitamin C is reducing agent in norepinefrine biosynthesis. In hydroxylation of collagen it keeps iron ion in 2+ state. Vitamin D plays hormonal role in calcium metabolism. Vitamins E are antioxidants. Vitamins K is involved in carboxylation of glutamyl residues in key blood coagulation factors. It is therefore important for blood coagulation. Vitamin K analogues – warfarins – are used to treat thrombosis.

Regulation of Enzymatic Activity

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Regulation of Enzymatic Activity

no “real” regulation:

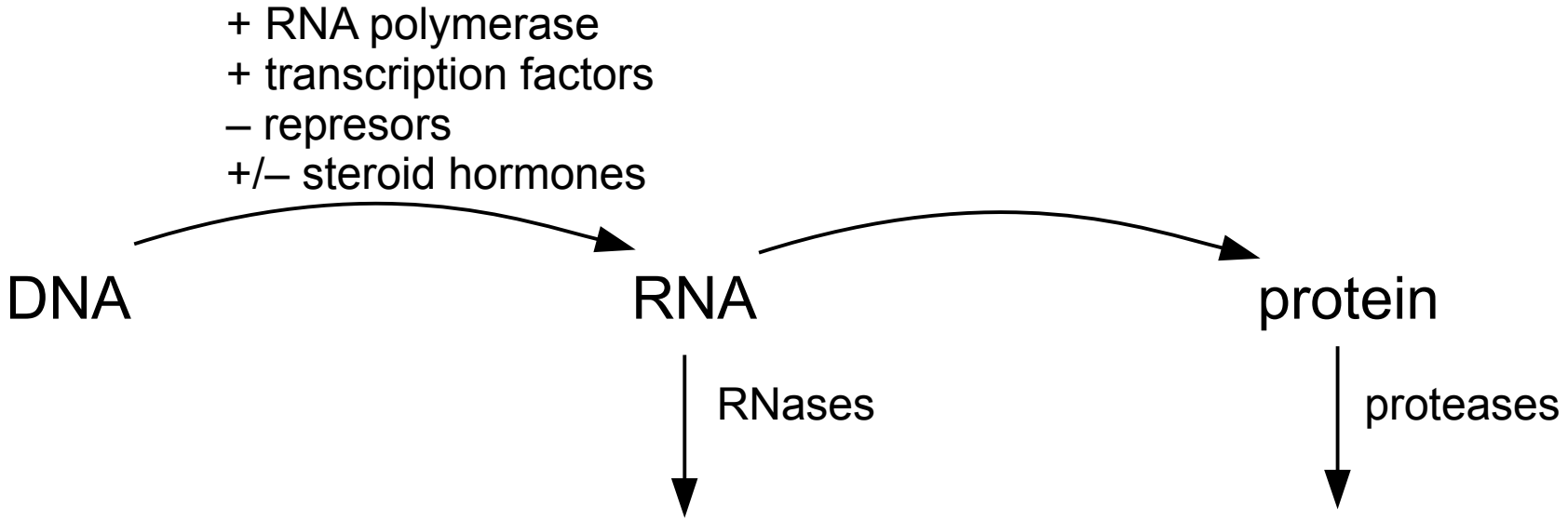
- regulation of enzyme expression and turnover
- control of enzyme trafficking
- supply of cofactors

“real” regulation of enzymatic activity:

- activation/inhibition by small-molecule ligands
- activation/inhibition by protein binding
- activation/inhibition by regulatory domains
- activation/inhibition by covalent modifications
- activation by proteolytic cleavage
- regulation by physical conditions

Enzymatic activity can be regulated by controlling concentration of the enzyme (no “real” regulation) or by controlling its function (“real” regulation)

Regulation of enzyme expression and turnover and control of enzyme trafficking



No “real” regulations include regulations of transcription of the gene of the enzyme and regulation of its degradation or degradation of its RNA. For most biochemist it is not natural to say that this controls the activity of the enzyme because it controls its **concentration**. However, many molecular biologists do not hesitate to say that something “activates” or “inhibits” an enzyme, despite it activates or inhibits transcription of its gene.

Activation/inhibition by small-molecule ligands

regulation: activation/inhibition

regulation: non-allosteric/allosteric

inhibition: competitive, noncompetitive, acompetitive, covalent

parameters: saturation constant, inhibition constant, IC50

Many small molecule compounds such as second messengers (e.g. cAMP) bind non-covalently to an enzyme and modulated its activity. Some compounds activate. For this it is possible to apply saturation kinetics
activity = max.activity [A]/([A]+Ks)
For inhibitors it is possible to apply standard models of enzyme inhibition (competitive, noncompetitive, acompetitive). Allosteric effect may play a role in activation as well as inhibition and may cause behavior different from standard model.

Activation/inhibition by small-molecule ligands

second messengers:

- cAMP, cGMP
- diacylglycerol
- IP_3 and other inositolphosphates
- Ca^{2+}, calmodulin
- prostaglandins
- NO, H_2S, CO
- ethylene, ppGpp, others

Second messengers are small molecule compounds involved in signal transduction from receptor to cellular proteins. Their concentrations indicate different physiological states of the cell. Beside signaling enzymes, such as protein kinases, they may regulate activity of metabolic enzymes. Most famous secondary messengers are cAMP, diacylglycerol or inositol phosphates. They also include gaseous signaling molecules or plant and bacteria-specific molecules.

Activation/inhibition by small-molecule ligands

feedback regulation of metabolic pathways:

Pasteur effect: anaerobic glycolysis is faster than aerobic

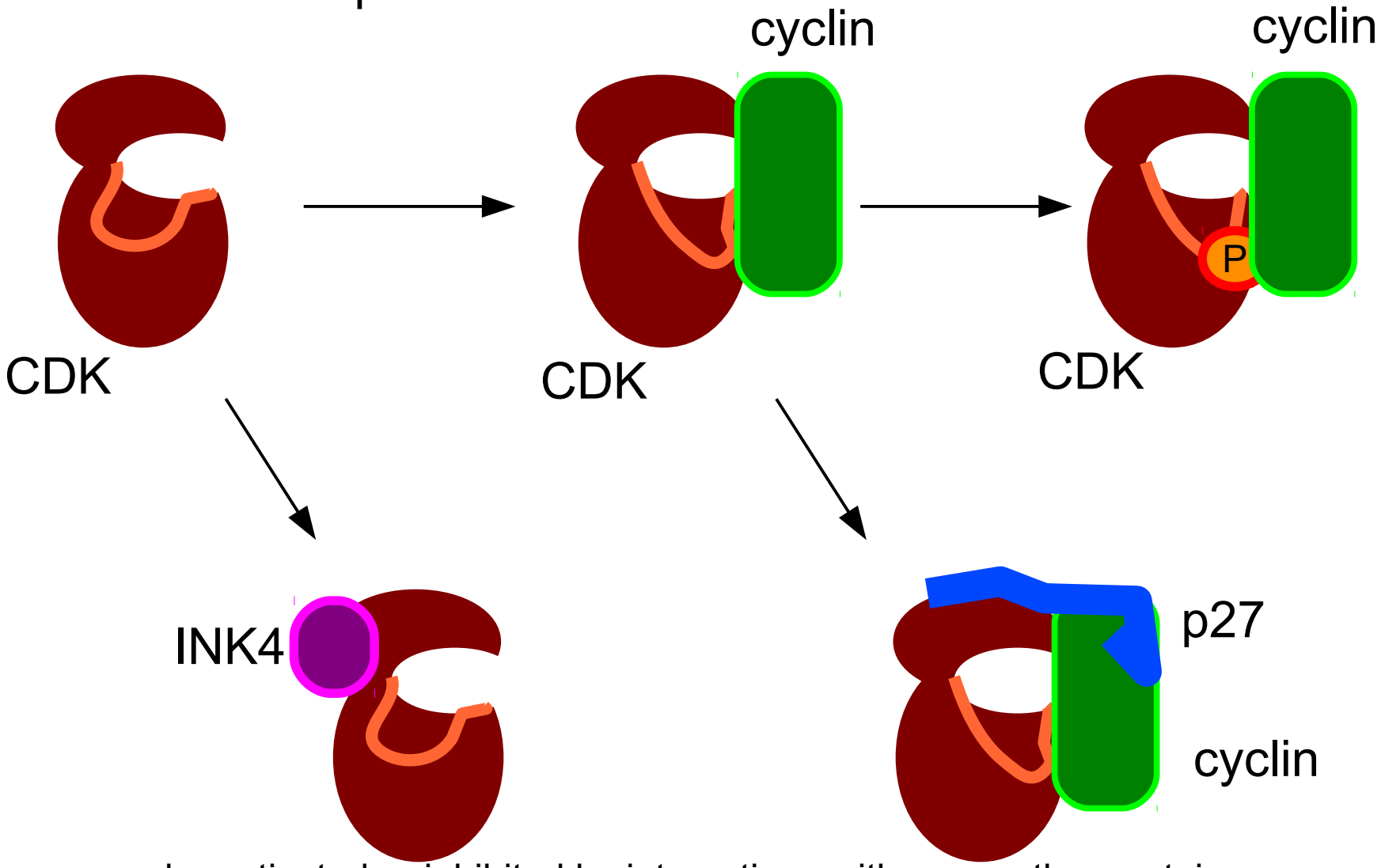
- phosphofructokinase is activated by ADP, AMP, fructose-2,5-bisphosphate, inhibited by ATP and citrate

- pyruvate kinase is inhibited by ATP

In 1857 Pasteur discovered that oxygen increases the rate of growth of yeast. This can be explained by two types of glucose metabolism in yeast. In aerobic metabolism glucose is oxidated to carbon dioxide and water. This yields approx. 32 mols of ATP per one mol of glucose. Under anaerobic conditions yeast produce ethanol yielding approx. 2 mols of ATP per one mol of glucose. In order to sustain comparable production of ATP yeast slow down aerobic and accelerate anaerobic glycolysis. This is done by inhibition of phosphofructokinase by ATP or citrate, which are present in high concentrations under aerobic conditions. In contrast, AMP and ADP accelerate glycolysis by activation of phosphofructokinase.

Activation/inhibition by protein binding

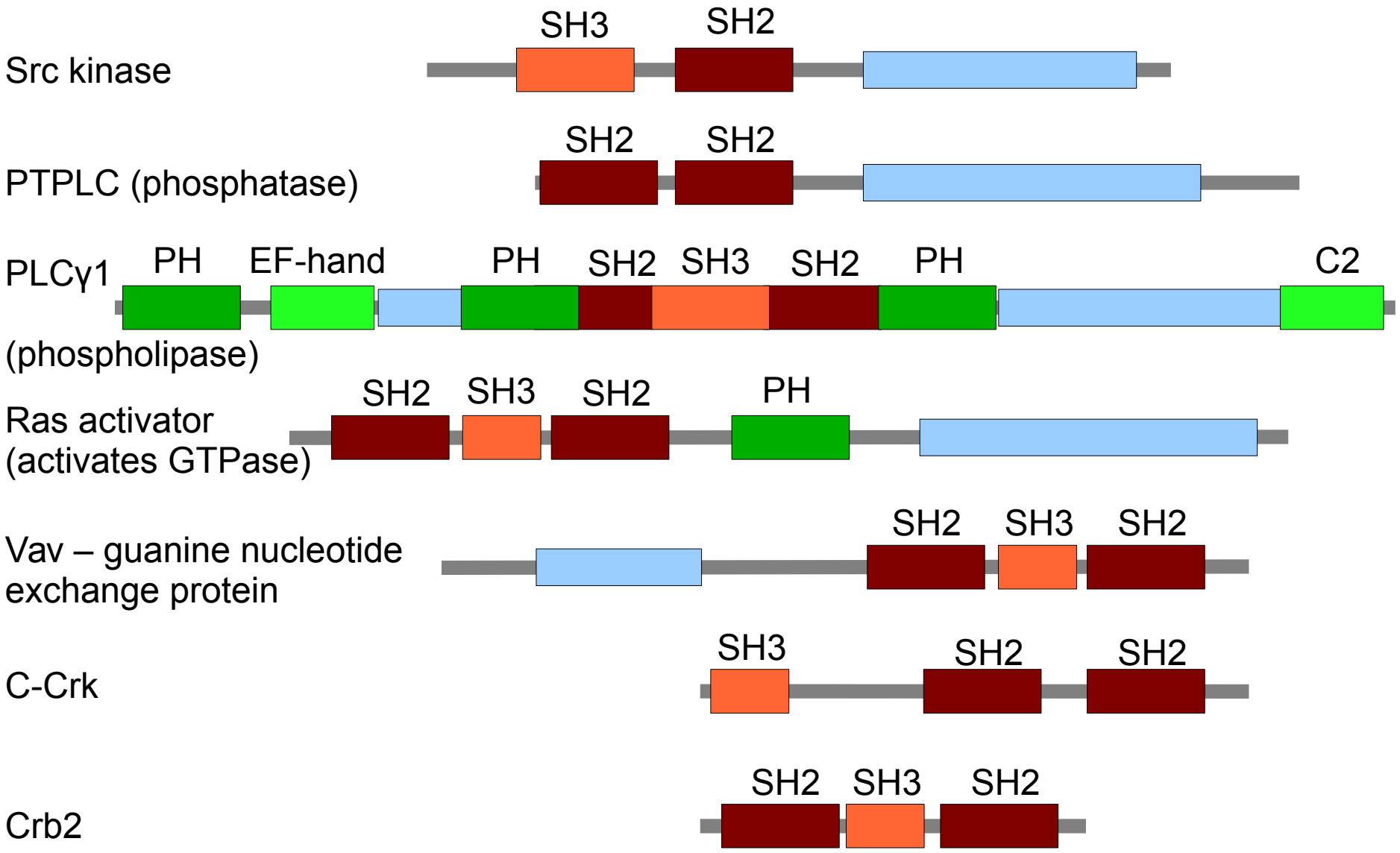
- activator/inhibitor proteins



Enzymes can be activated or inhibited by interactions with some other proteins. For example, cyclins regulate activity of cyclin-dependent kinases. This control cell cycle.

Activation/inhibition by regulatory domains

regulatory (adaptor, interaction) domains

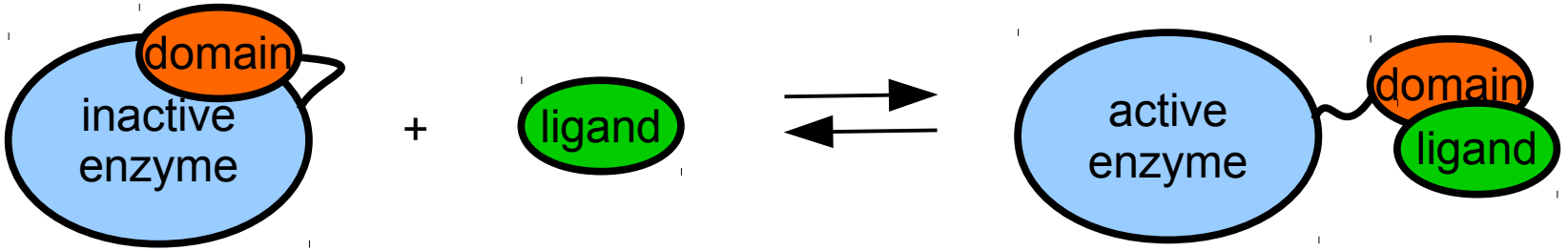


Activation/inhibition by regulatory domains

regulatory (adaptor, interaction) domains

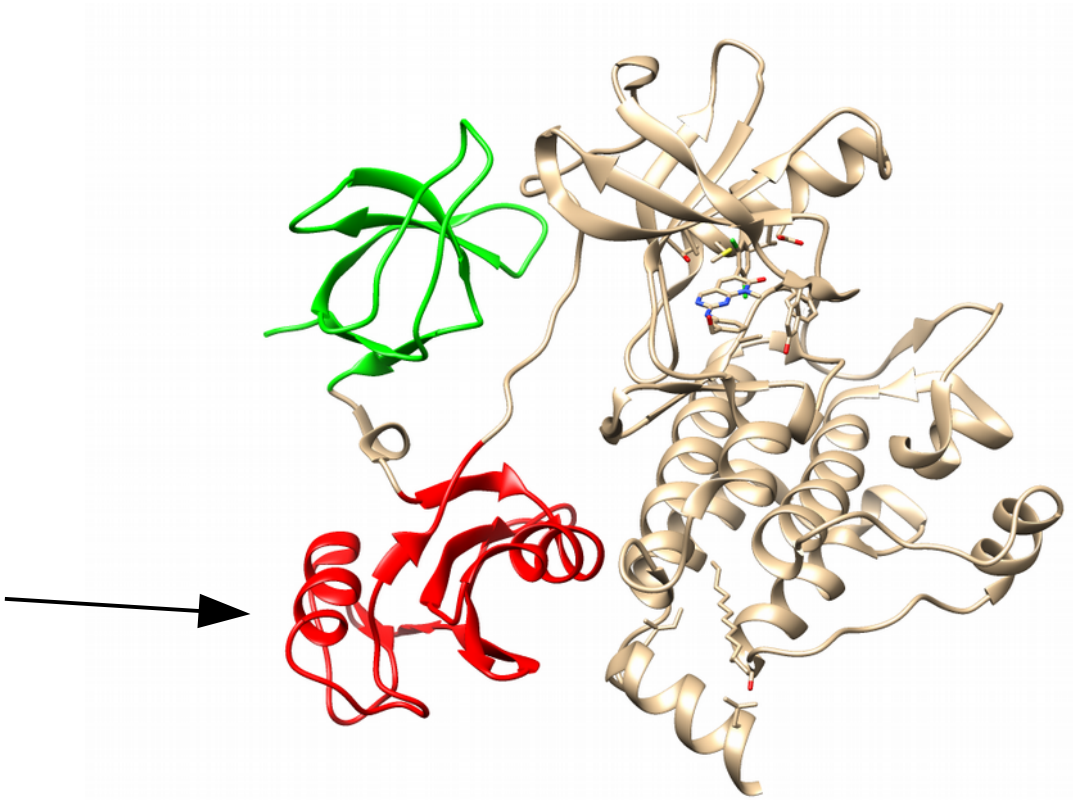
Cyclins are examples of proteins regulating enzymatic activities by inter-molecular interactions with enzymes. Production of cyclins and cyclin-dependent kinases (CDKs) is controlled at the level of transcription. Transcription controls cycline concentrations, they control activities of CDKs and they in turn control transcription. This forms a complicated cell cycle regulation system.

However, activities of some enzymes can be controlled by intra-molecular interaction with its regulatory domains. We will use the term domain as a smaller part of larger protein (as a one polypeptide chain) with some function. It was found that many eukaryotic proteins are composed from such regulatory domains as a kind of “molecular Lego”. For example SH2 domain was found in 111 human proteins (with minor differences in amino acid sequences). It competes for binding either to the enzyme to which it is attached or to a concurrent protein containing phosphotyrosine motif.



Activation/inhibition by regulatory domains
regulatory (adaptor, interaction) domains

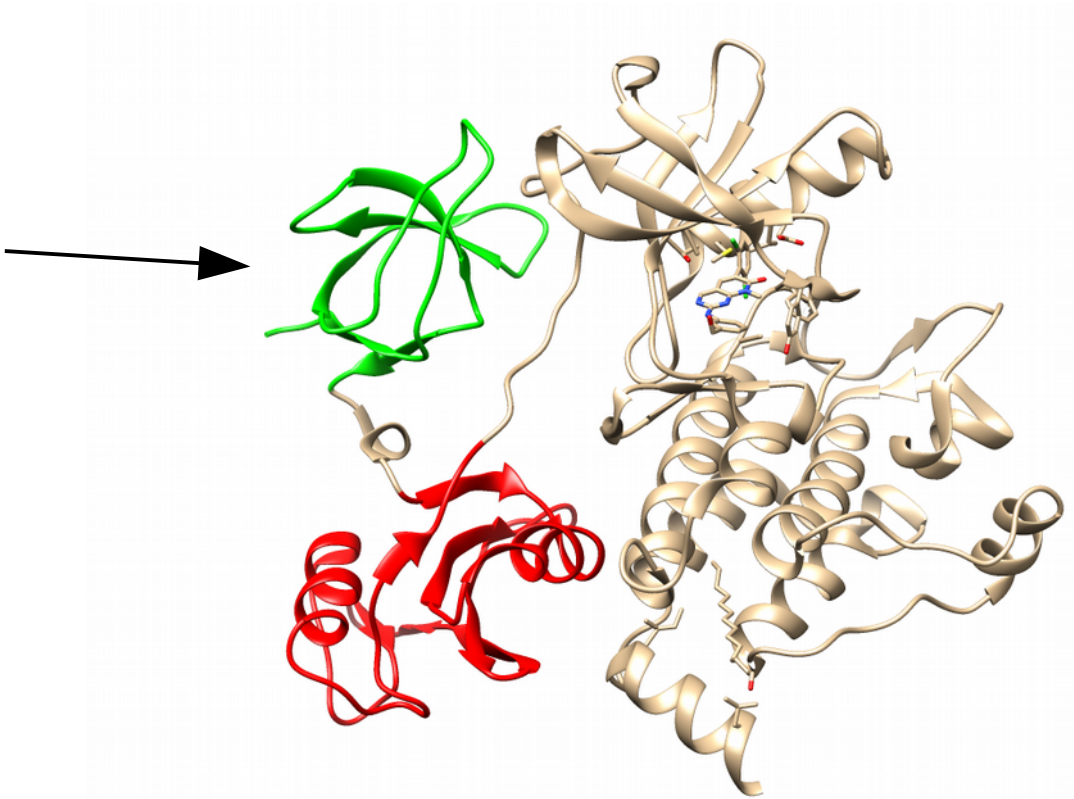
SH2 domain (Src-homology type 2)
protein kinases, protein phosphatases, phospholipases, regulatory proteins and others



-pY–x–x–Φ-

Activation/inhibition by regulatory domains
regulatory (adaptor, interaction) domains

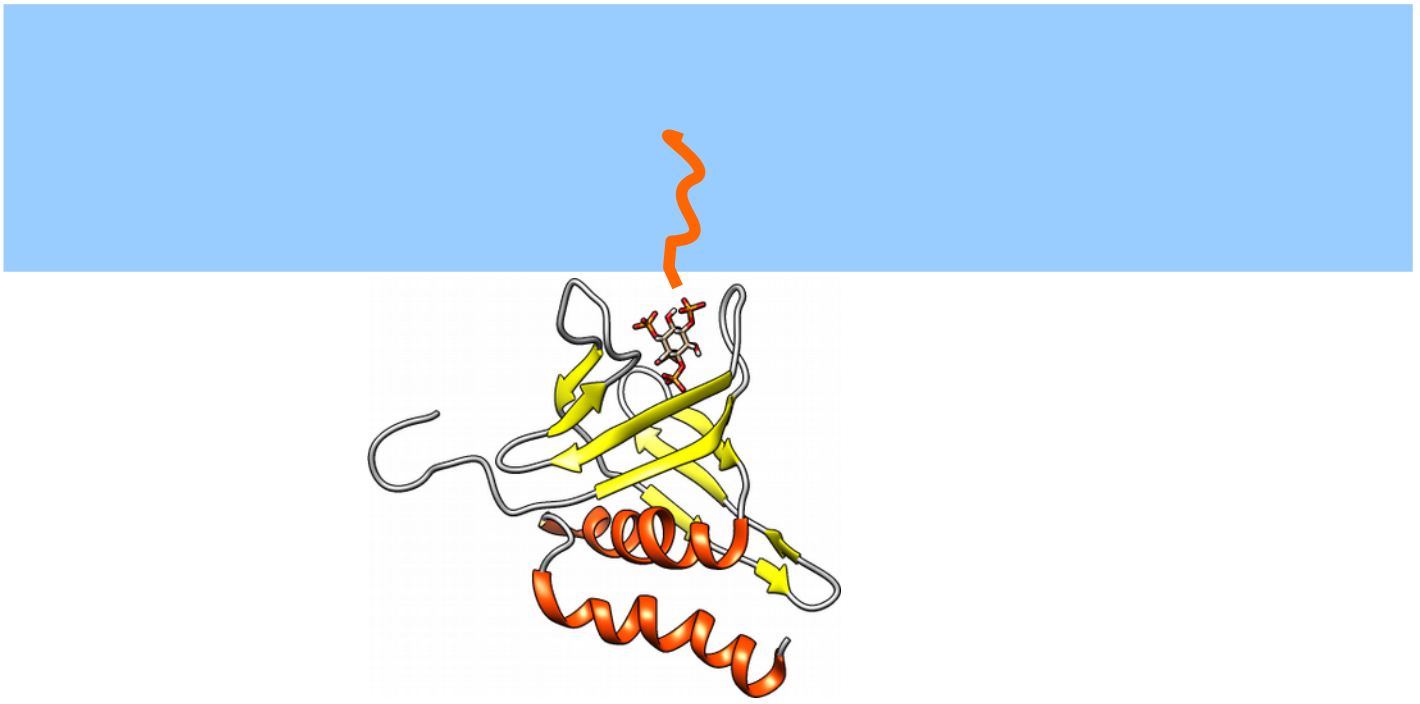
SH3 domain (Src-homology type 3)
protein kinases, protein phosphatases, phospholipases, regulatory proteins, myosin, spectrin and others



-R/K–x–x–P–x–x–P-
-x–P–x–x–P–x–R/K-

Activation/inhibition by regulatory domains
regulatory (adaptor, interaction) domains

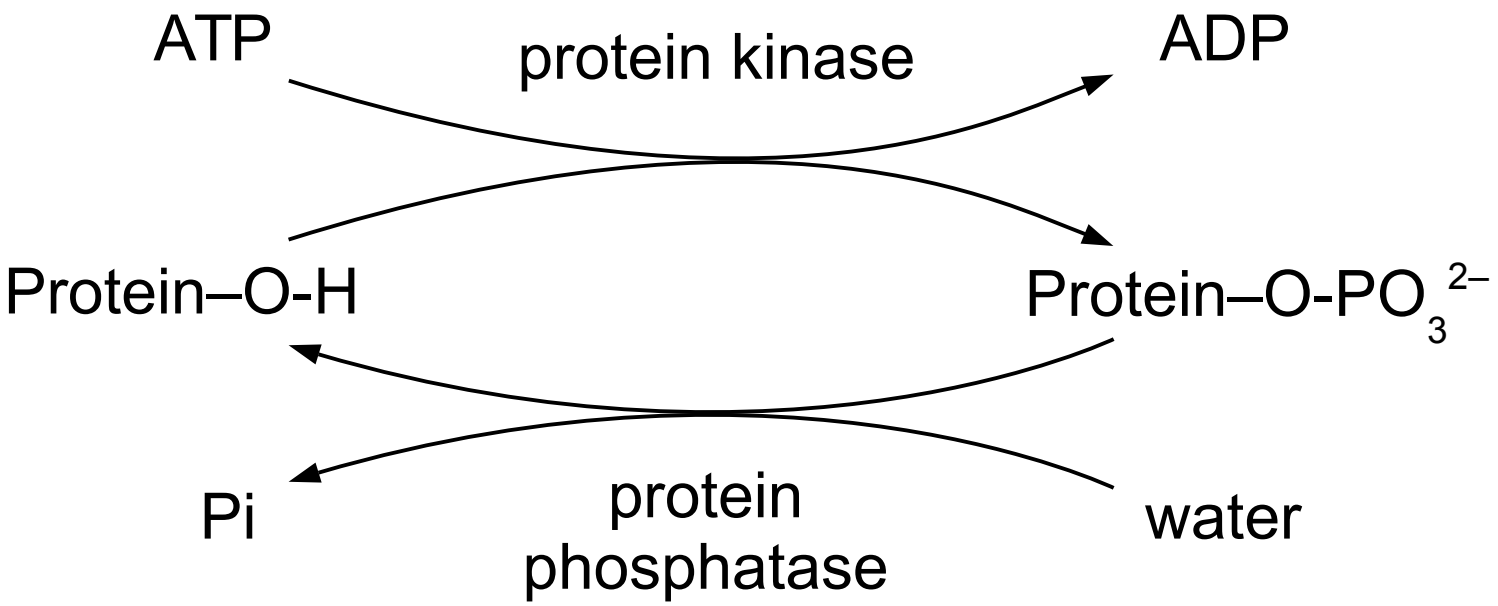
PH domain (Pleckstrin-homology)
phospholipases, protein kinases and others



inositole phosphates (PIP_2, PIP_3), free or in phospholipids

Activation/inhibition by covalent modification

phosphorylation/dephosphorylation



Protein phosphorylation is one of most important system for regulation of enzymatic activities. Protein kinases phosphorylate either Tyr, Ser or Thr residues (other PKs exists but are rare). This changes the activity of the enzyme. Phosphorylation can be reverted by protein phosphatases. Protein kinases have been challenging targets for drug discovery, but it was believed that there are so many ATP-binding proteins that makes development of specific inhibitor impossible. However, discovery of Imatinib (Gleevec) have shown that it is possible. This sparked further interest in protein kinases.

Activation/inhibition by covalent modification

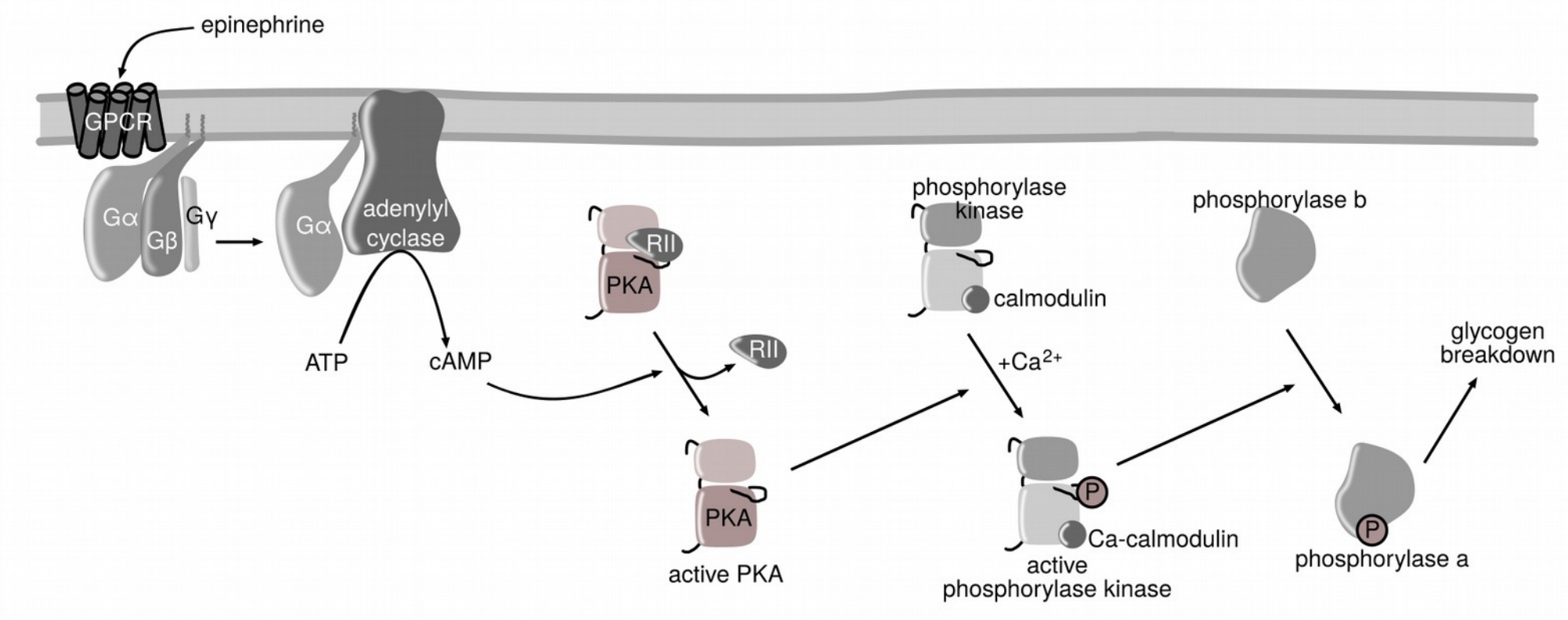
phosphorylation/dephosphorylation

Enzyme	**Activator**
Protein kinase A (PKA)	cAMP
Protein kinase B (PKB)	PtdInsP3
Protein kinase C (PKC)	DAG
Ca^{2+}/calmodulin-PK	Ca^{2+} - calmodulin
AMPK	AMP
MAPK	phosphorylation
Insulin receptor	insulin
Src, Abl, Jak ...	phosphorylation and others

There are more than 500 PKs in human genome. PKs can be divided into two evolutionary related families – Ser/Thr and Tyr PKs. There are also receptor PKs such as insulin receptor.

Activation/inhibition by covalent modification

phosphorylation/dephosphorylation



Function of PKs is shown on the example of glycogen breakdown regulation. A small molecule epinephrine binds to its receptor from GPCR family. This activates G proteins. Galpha protein activates adenylyl cyclase. This enzyme produces cAMP from ATP. cAMP activates PKA, PKA activates phosphorylase kinase and phosphorylase kinase activates phosphorylase. This enzyme degrades glycogen by phosphorolysis. Important feature of this pathway is that each activated molecule of enzyme may activate large number of other molecules, so the signal is amplified along the pathway. A similar pathway exists for insulin, which counteracts this pathway.

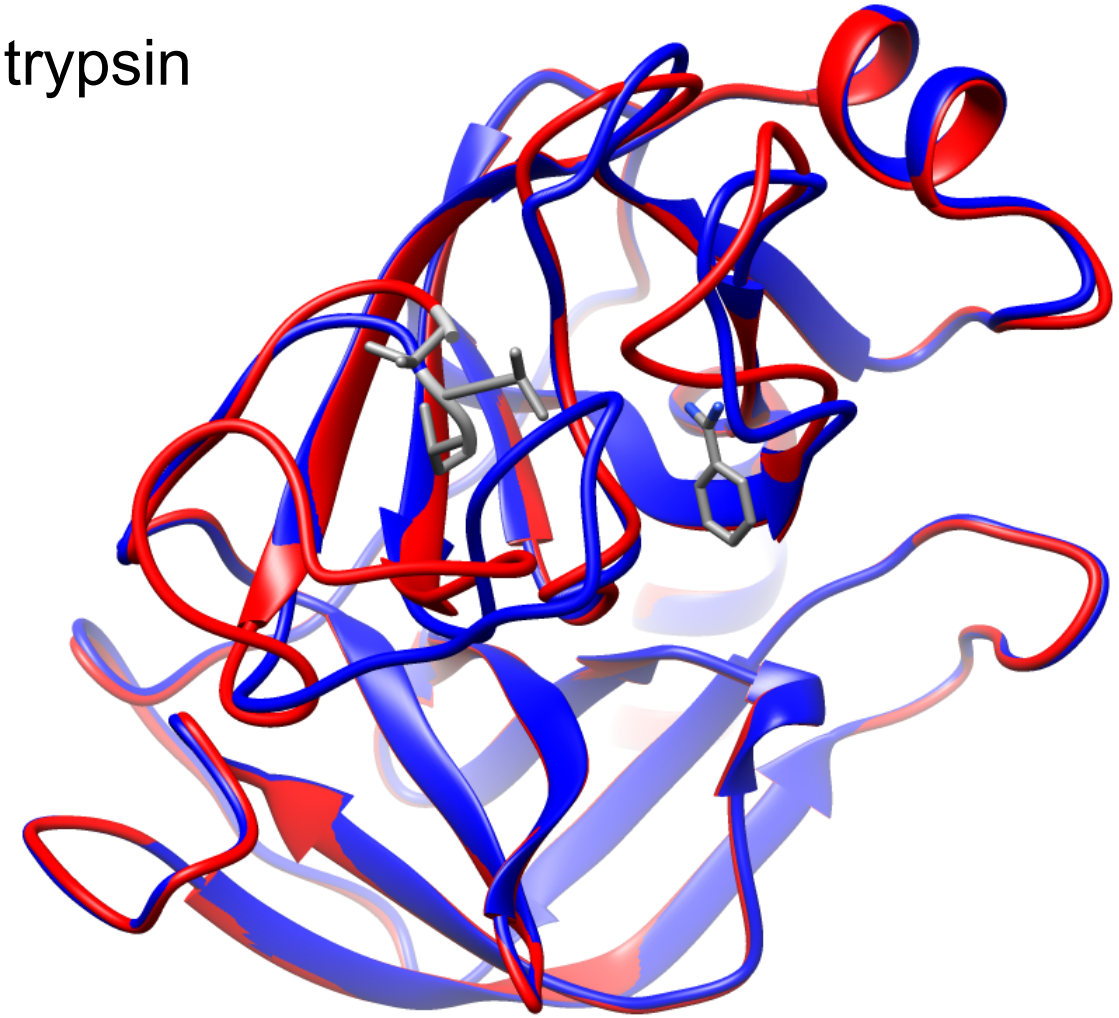
Activation/inhibition by covalent modification

other modifications

- ADP-ribosylation on Arg
- adenylation on Tyr
- others

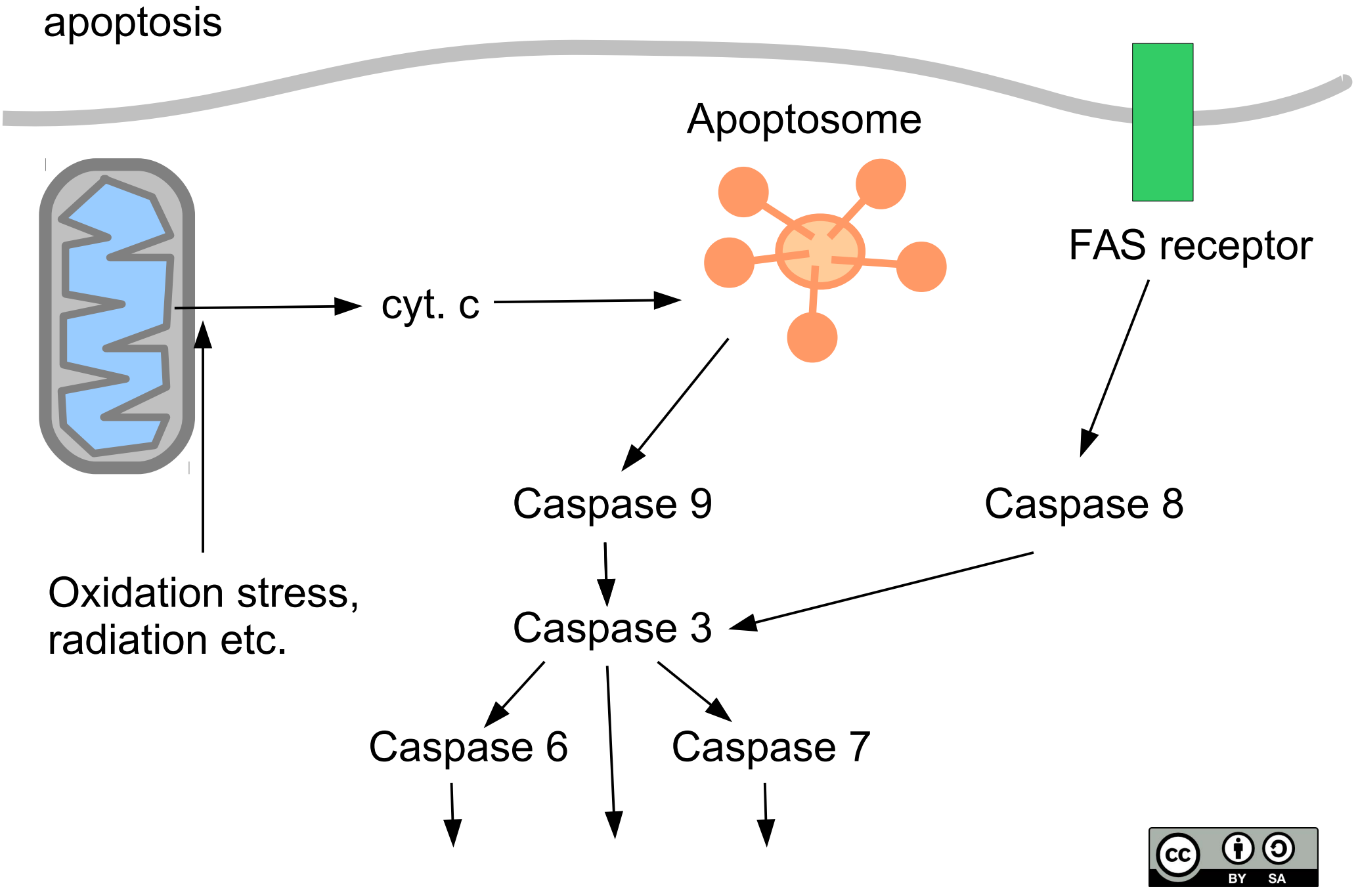
Activation by limited proteolysis

trypsinogen → trypsin



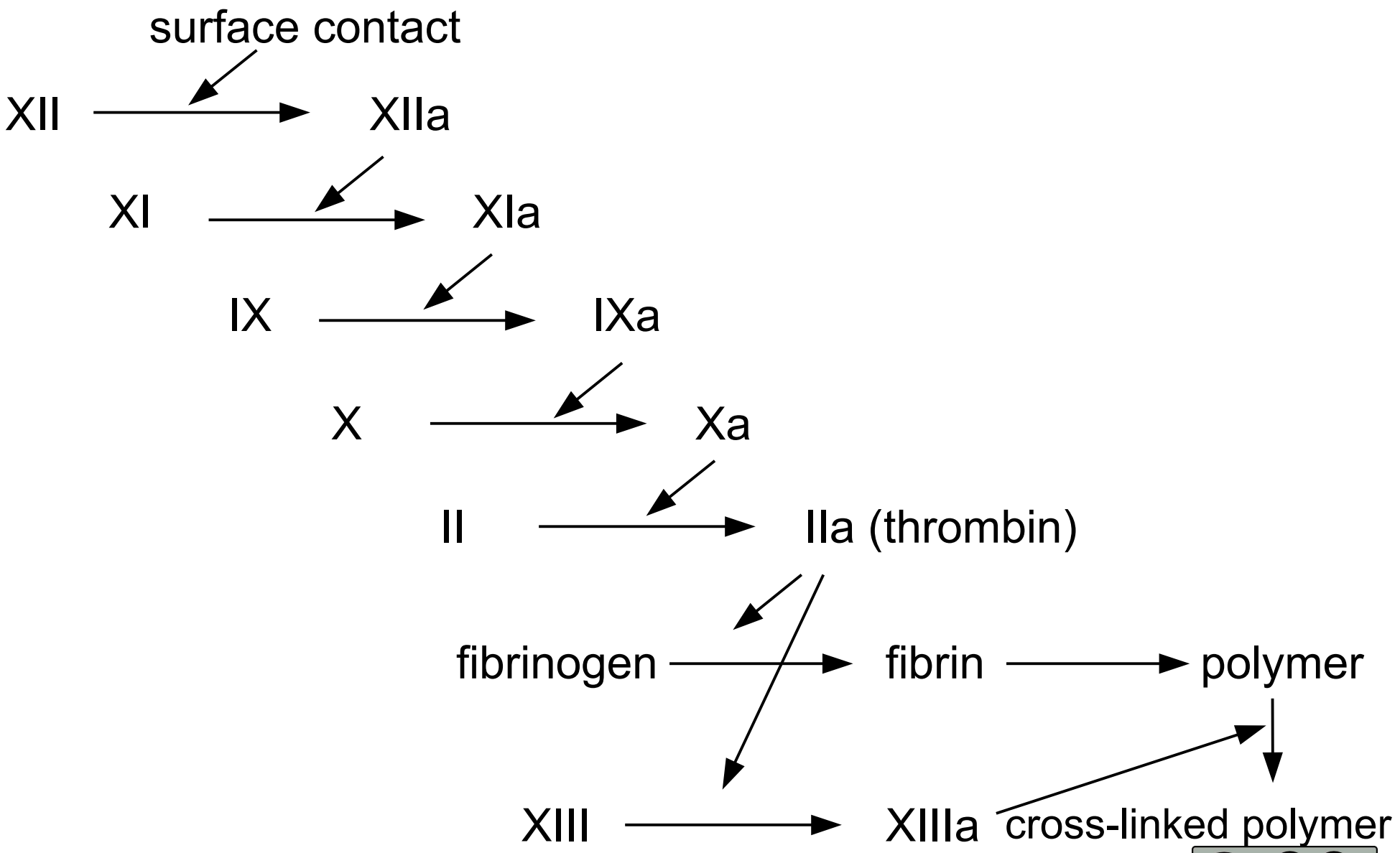
Some enzymes are activated by partial proteolysis. Trypsin is synthesized in pancreas as an inactive precursor called trypsinogen (blue). It is then activated by removal of N-terminal residues (gray) to obtain active trypsin (red). Other pathways regulated by partial proteolysis include apoptosis (with Caspases) and blood coagulation pathway.

Activation by limited proteolysis



Activation by limited proteolysis

blood coagulation



Allosteric effect

homo/heterotropic

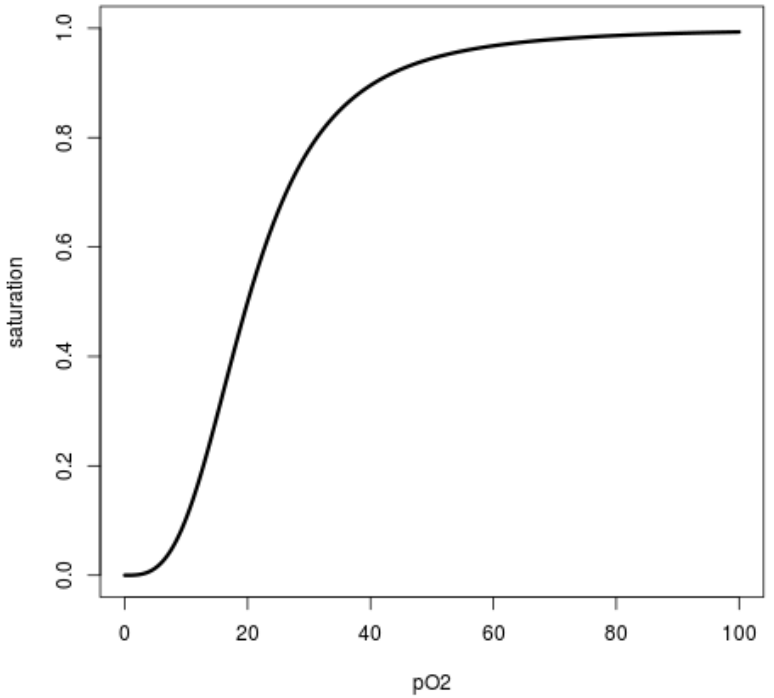
positive/negative

Important role in regulation of enzymatic activities is played by allosteric effect. The word allosteric means “different place”. It means that something happens at one side of protein and it makes an effect on the other side of protein. For example, binding of a ligand into the binding site A changes the shape of a distant active site B of the enzyme, thus it changes its activity. It can be homotropic (the same molecule is ligand of A and ligand or substrate of B) or heterotropic (different molecules bind to A and B). It can be also positive (binding to A increases affinity or activity in B) or negative.
Allosteric effect is usually accompanied by change in the 3D structure of the protein. It was not possible to demonstrate the allosteric effect directly in the times when determination of protein structures was not possible or its possibilities were limited. It was demonstrated indirectly. In this sense, homotropic allosteric effect and hemoglobin play the key role in demonstration of allostery phenomenon.

Allosteric effect

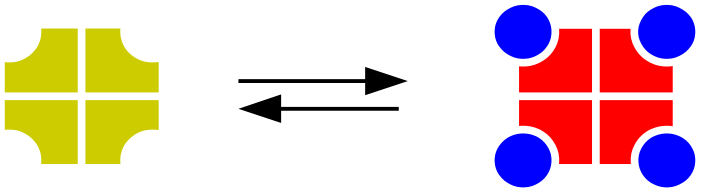
Hemoglobin

Hemoglobin binds oxygen with low affinity at low concentrations (or low partial pressure) and with high affinity at high concentrations. Therefore it can be almost fully loaded in lungs and it releases almost all oxygen in tissues. This makes oxygen transport highly efficient. Different affinity is achieved by following mechanism. Hemoglobin is homotetramer. Binding of oxygen to the first subunit supports binding of the second oxygen to the second subunit and so forth. The saturation curve is therefore not hyperbolic but rather sigmoid:



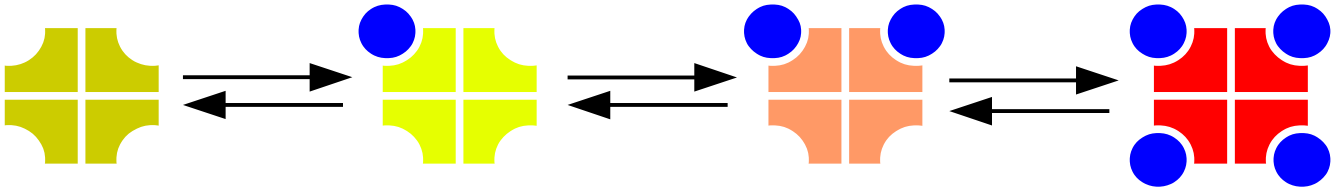
Allosteric effect

Hill's model

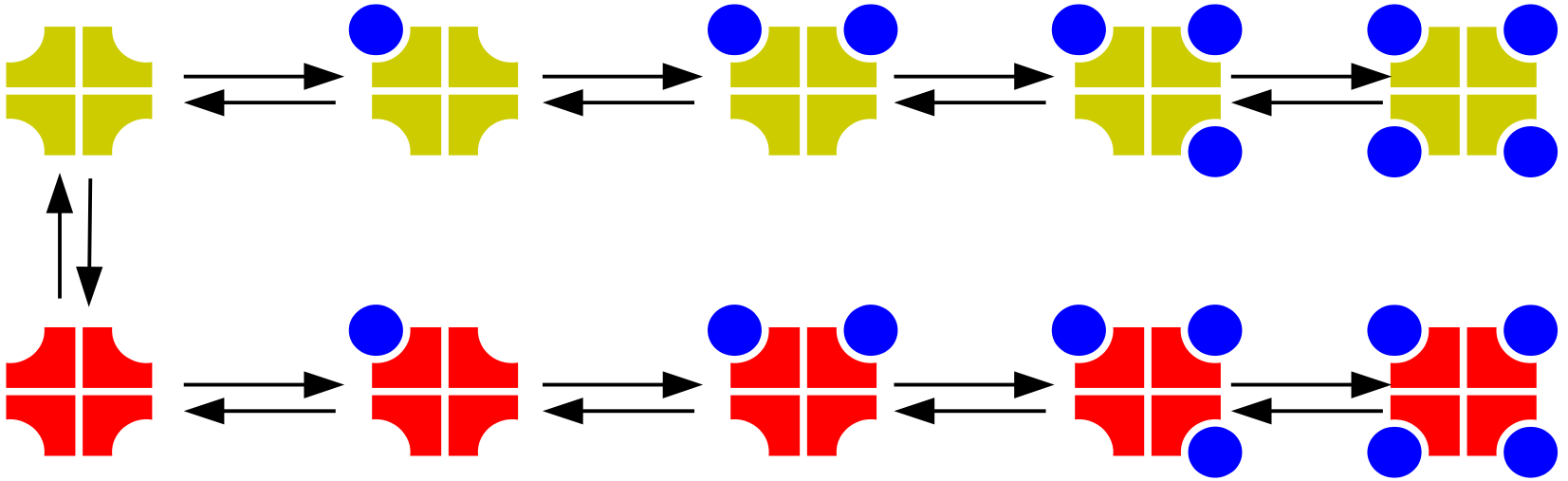


$$S = \frac{[A]^{\kappa}}{[A]^{\kappa} + K_S}$$

Aldair's model



Monod-Wyman-Changeux model



Koshland's model

Allosteric effect

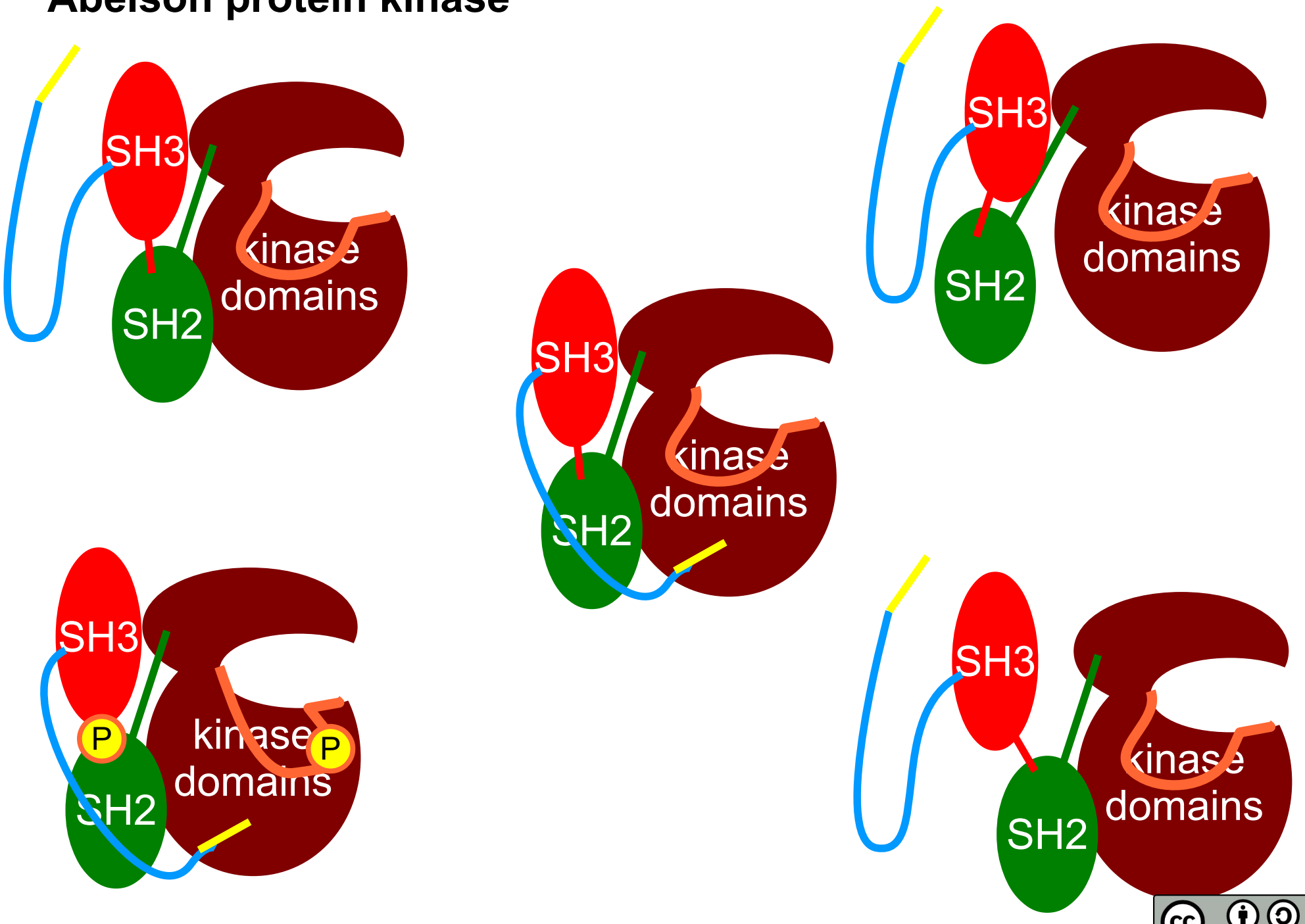
Hemoglobin

Several models were proposed to explain allosteric effect in hemoglobin. Hill's model assumes that hemoglobin can bind either no or certain number (κ) of molecules. The problem of Hill's model is that elementary reactions with more than two reactants are kinetically unfavorable, it cannot explain non-integer values of κ and it cannot explain negative allosteric effect.

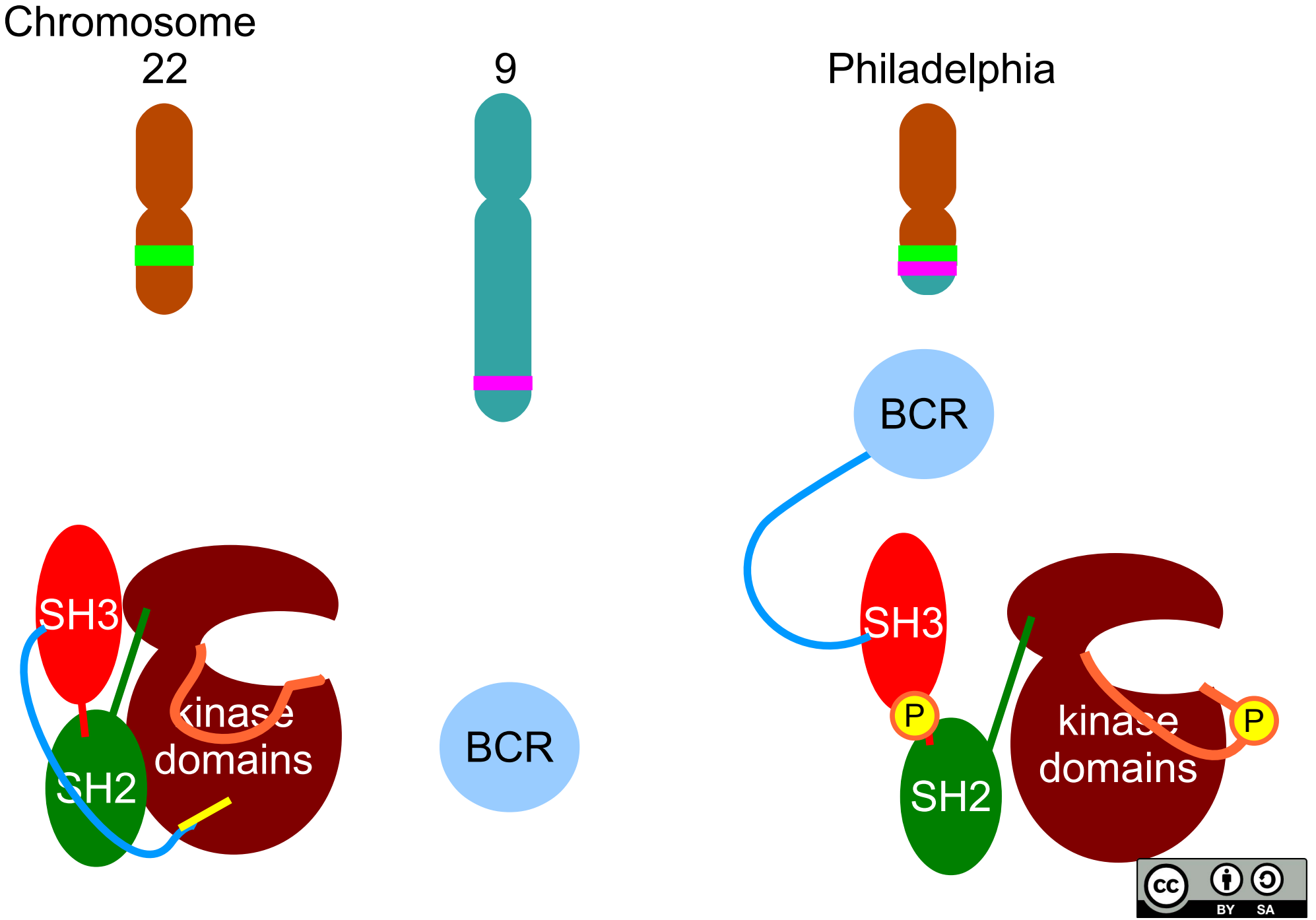
Aldair's model simply uses different dissociation constants for binding of the first, second, third and fourth oxygen. This model was further developed by Koshland in order to provide a structural explanation. Disadvantage of Aldair's model is a high number of parameters.

The model developed by Monod, Wyman and Changeux assumes that hemoglobin exists in two forms – relaxed (R) and tense (T). The relaxed form has lower and tense form higher affinity towards oxygen. There is an equilibrium between the relaxed and tense form and it is shifted towards the relaxed form in absence of oxygen. Oxygen binds strongly to the tense form and thus shifts the equilibrium towards the tense form. The terms relaxed and tense is also used in other allosteric proteins.

Abelson protein kinase



Abelson protein kinase



Abelson protein kinase

Abelson protein kinase is a tyrosine protein kinase involved in regulation of processes of immune response. It is tightly regulated and its regulation demonstrates different modes of enzyme activity control mechanisms. In the “default” form it is packed. It is modified by N-terminal myristyl chain. Association to the membrane may cause release of myristyl and its binding to the membrane. It also contain two regulatory domains SH2 and SH3. It can be therefore activated by binding to SH2 or SH3 ligands. Finally, it can be phosphorylated by another kinase or by another molecule of the same protein.

Abelson kinase is associated chronic myeloid leukemia. In patients suffering this disease there is a Philadelphia chromosome formed by fusion of chromosomes 9 and 22. This causes that the N-terminus of the Abelson kinase gene is replaced by another protein. The first step of its activation is therefore already finished at the time of its proteosynthesis. The enzymatic activity is therefore activated, which causes high phosphorylation of cellular proteins, its uncontrolled growth and cancer. Inhibitor of Abelson kinase – Imatinib (Gleevec) – can be used to treat chronic myeloid leukemia.

Signal Transduction - Receptors

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Types of receptors

- G protein-coupled (GPCR, 7H)

- ligand-gated ion channels

- voltage-gated ion channels

- receptor tyrosine kinases

- other kinase-associated receptors

- integrins, selectins, cadherins...

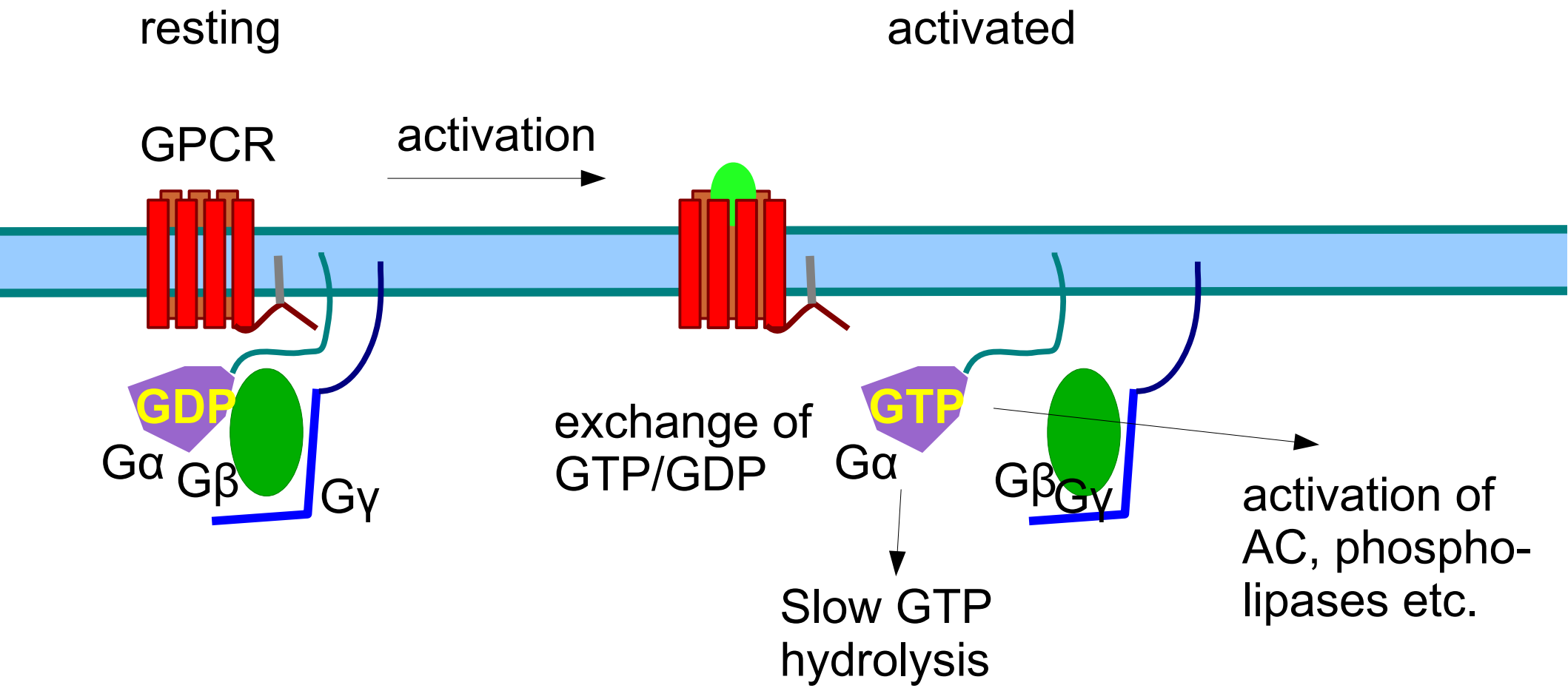
The term receptor was introduced for cellular elements that sense some signals, typically hormons. In this talk we will limit to receptors present on the surface of a cell that transduce the signal into the cell. Intracellular receptors, such as steroid hormone receptors, will not be discussed (steroids can pass the membrane and go to nucleus, where these receptors are present). There are several above mentioned types of receptors, many of them important drug targets.

G protein-coupled (GPCR, 7H)

- biogenic amines (epinephrine, norepinephrine, dopamine, serotonin, GABA)
- amino acids (glutamate)
- nucleoside (adenosine)
- peptides (glucagon, angiotensins, oxytocin, vasopresin, ...)
- proteins (complement, interleukines, ...)
- odorants, flavours
- light (rhodopsin)
- other

GPCRs are important drug targets. Approximately 1/3 of drugs target GPCRs. They can act either as agonists, i.e. they bind to a receptor and activate it, or they can act as antagonists, i.e. they bind to receptors and block it from binding of an endogeneous agonist, or they can act by alternative ways. Examples of drugs targetting GPCRs include beta-blockers (to treat hypertension), antihistaminics (to treat allergy), analgetic opioids and many others. It si believed that recent development in structural biology of GPCRs may open the door for new GPCR-based therapeutics.

G protein-coupled (GPCR, 7H)



GPCRs are embedded in the membrane. Binding of a ligand (or a drug) triggers a conformational change in the receptor. This causes release of activated G proteins. G proteins exchange GDP for GTP. GTP is slowly hydrolysed and after hydrolysis the G protein is deactivated. During this period G proteins activate either adenylyl cyclase or phospholipase C. Activation of these enzymes triggers production of second messengers. Alternative mechanisms also exist.

G protein-coupled (GPCR, 7H)

Structural biology of GPCRs:

Bacteriorhodopsin (1996)

Rhodopsin (2000)

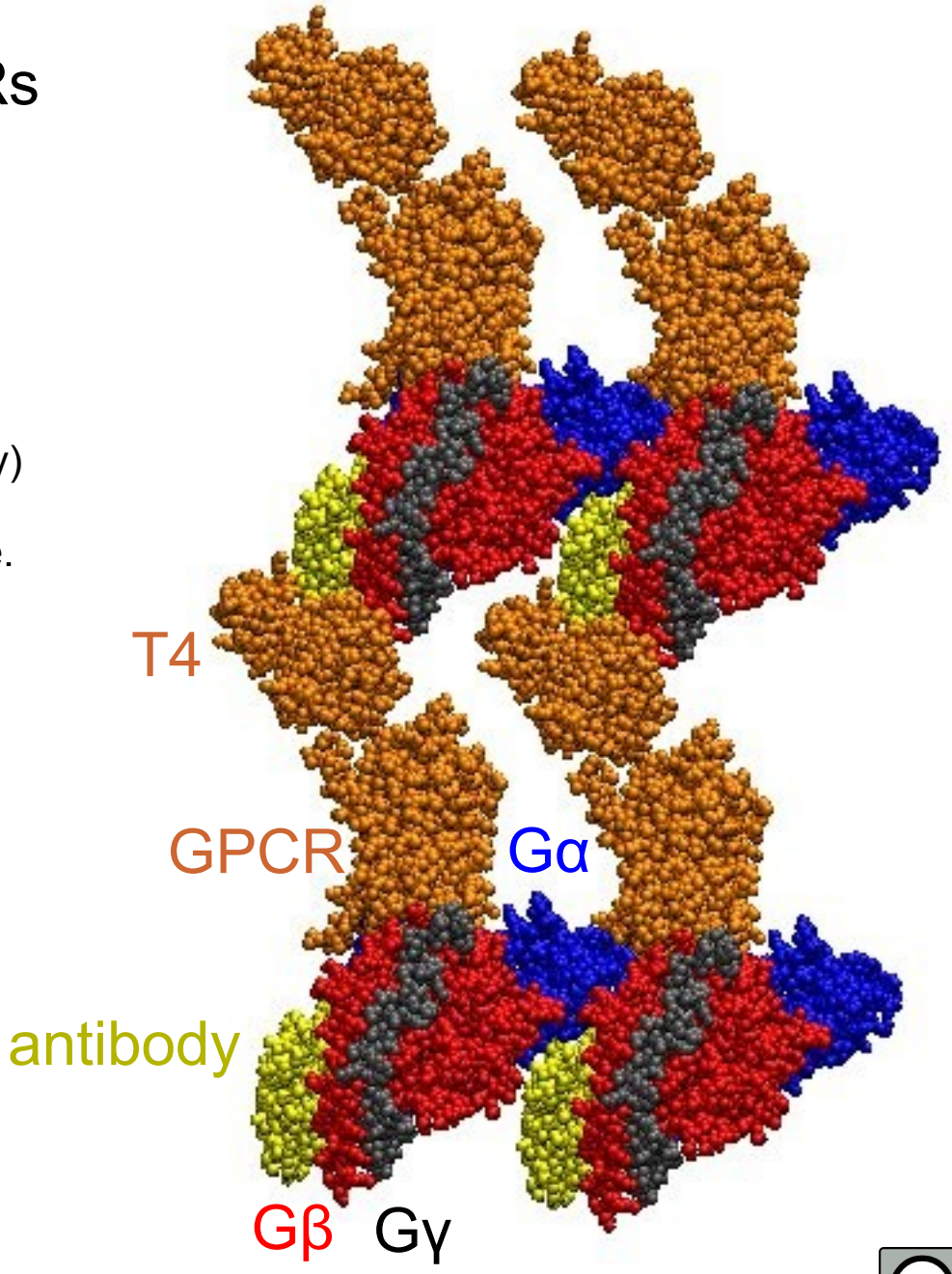
adrenergic receptor (2007)
GPCR-G-proteins complex (2011)
Nobel prize 2012 – B.K. Kobilka

Determination of protein structure is extremely difficult for membrane proteins. Therefore, determination structures of GPCRs was impossible for many years. Bacteriorhodopsin – a distant homologue of GPCRs – was determined in 1996, rhodopsin in 2000 but major breakthrough was achieved by Brian Kobilka and others. Several “tricks” were developed to determine structures of GPCRs, namely using of chimeric proteins with easy to crystallize proteins, application of recombinant antibodies or preparation of thermostabilized GPCRs.

G protein-coupled (GPCR, 7H)

Structural biology of GPCRs
β-adrenergic receptor

Complex of β-adrenergic receptor with G-proteins was determined by Kobilka group in 2011. It used a chimera “trick” (combined with a vital T4 lysozyme) and a nanobody (small recombinant antibody) trick. Membrane environment was mimicked by lipid cubic phase technique.



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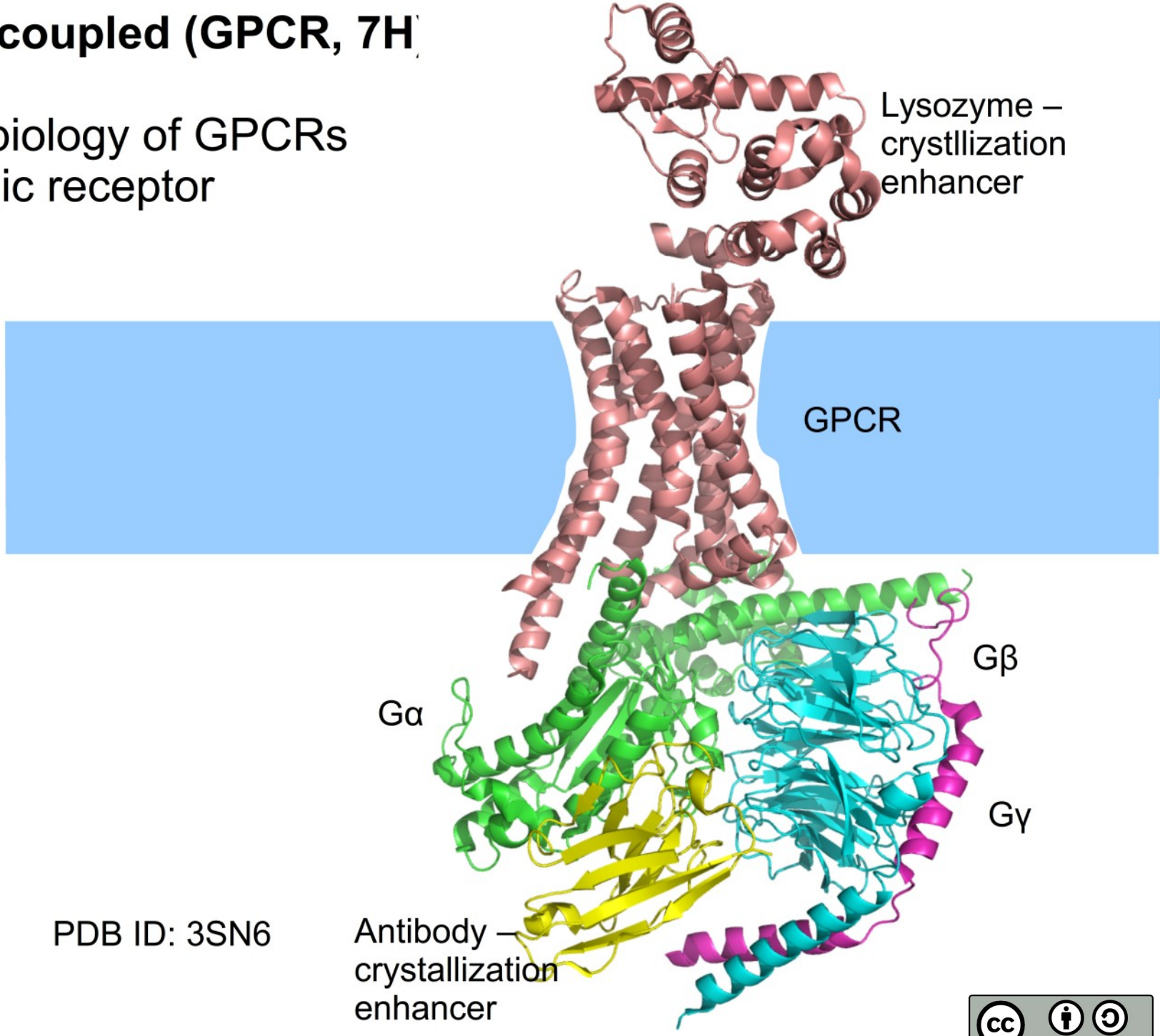
G protein-coupled (GPCR, 7H

Structural biology of GPCRs
β-adrenergic receptor



G protein-coupled (GPCR, 7H)

G proteins:

Gα – member of the family of small GTPases together with Ras, Rho, elongation factors etc., major activator of downstream processes, myristylated

Gβ – beta-propeller, in complex with Gγ also activates downstream processes

Gγ – anchors the Gβγ complex to a membrane

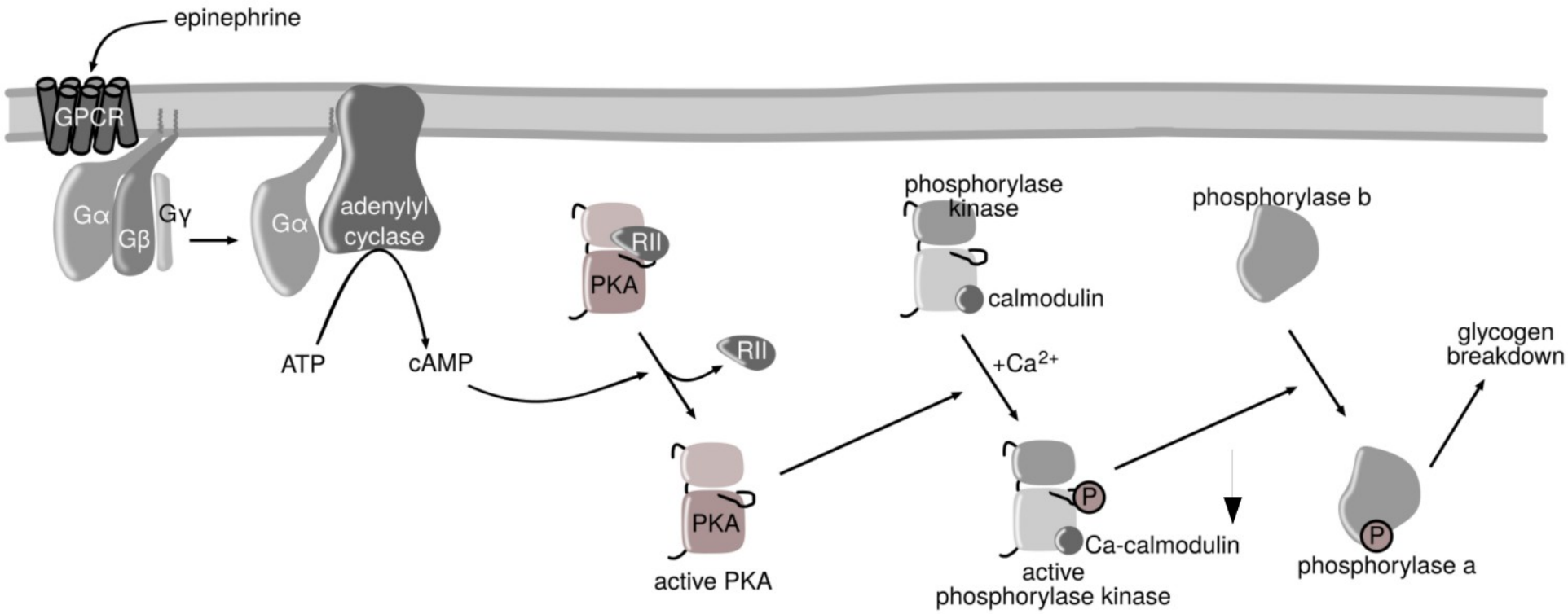
There are three G protein types and many other subtypes.

G protein-coupled (GPCR, 7H)

Downstream signaling:

Adenylyl cyclase (cAMP), guanylyl cyclase (cGMP)

Phospholipases

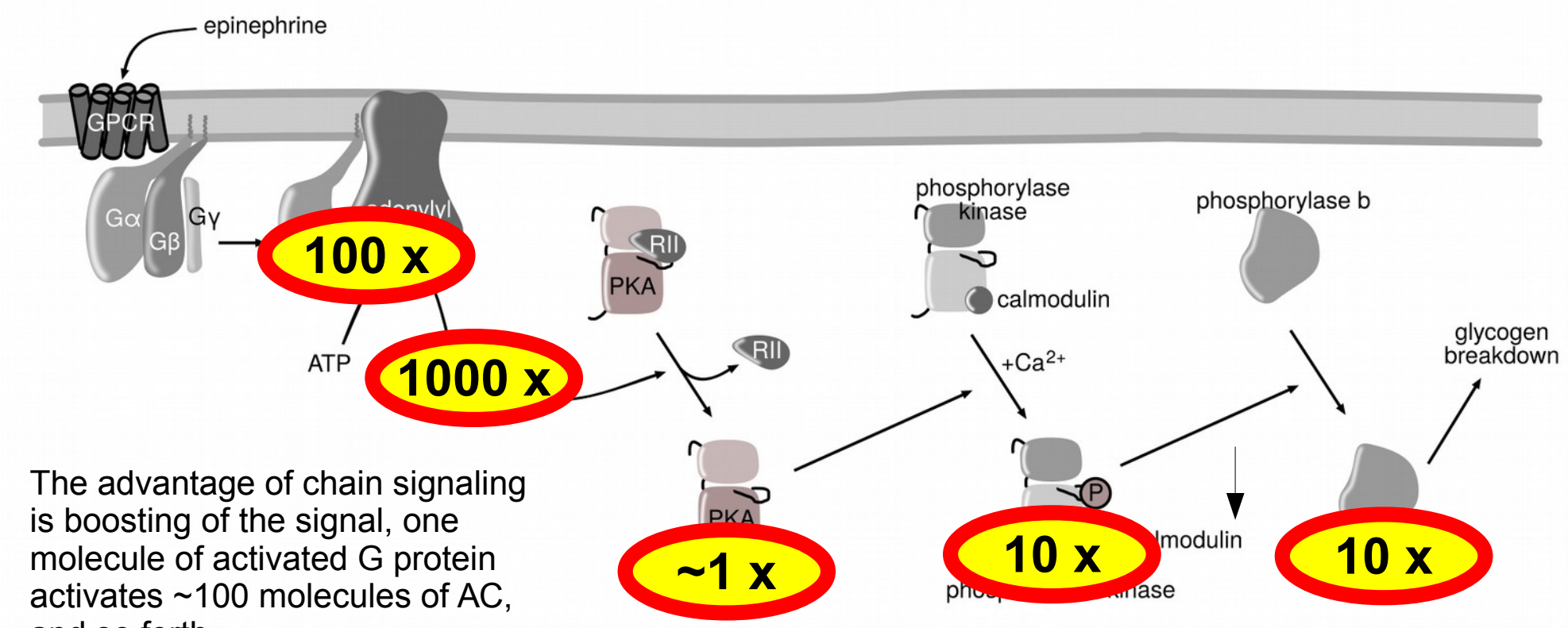


G-protein coupled (GPCR, 7H)

Downstream signalling:

Adenylyl cyclase (cAMP), guanylyl cyclase (cGMP)

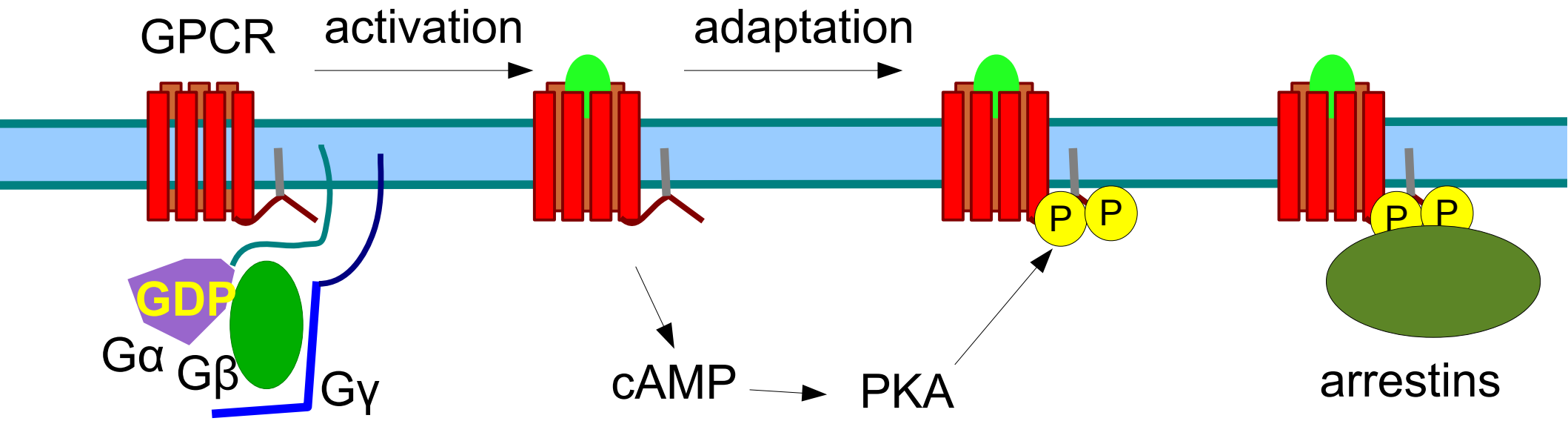
Phospholipases



The advantage of chain signaling is boosting of the signal, one molecule of activated G protein activates ~100 molecules of AC, and so forth.

G-protein coupled (GPCR, 7H)

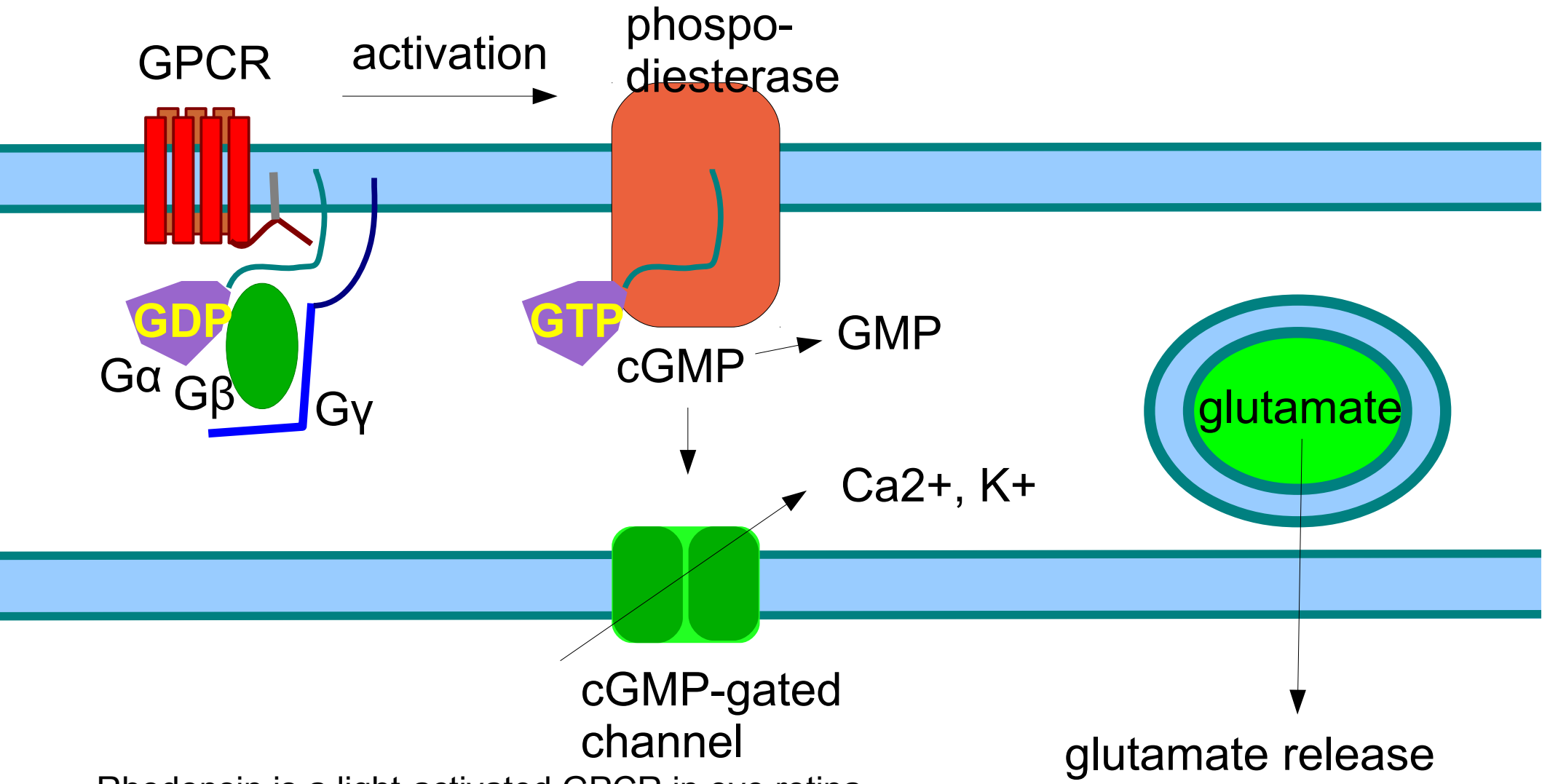
Adaptation



It is important to reduce sensitivity of GPCR activation in an exces of ligand. When the GPCR is fully activated for a long time it activates PKA. PKA phosphorylates the receptor. A protein called arrestin binds to phosphorylated receptor and blocks it from function. It also trigers internalization of the GPCR (excision of membrane vesicle containing the receptor and its transport to lysosome). Arrestin plays also important role in addiction to drugs of abuse.

G-protein coupled (GPCR, 7H)

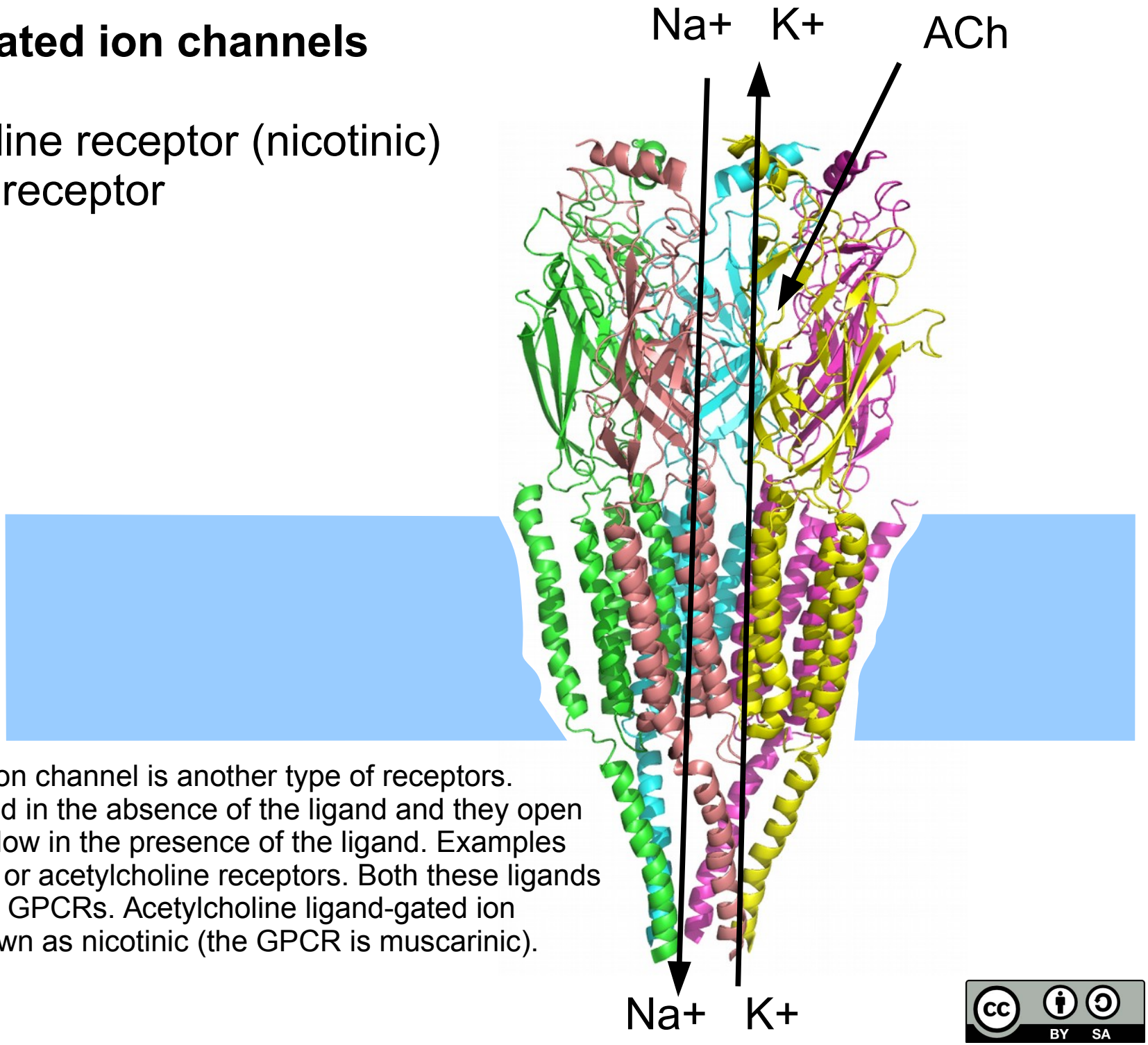
rhodopsin (activated by light)



Rhodopsin is a light-activated GPCR in eye retina.

Ligand-gated ion channels

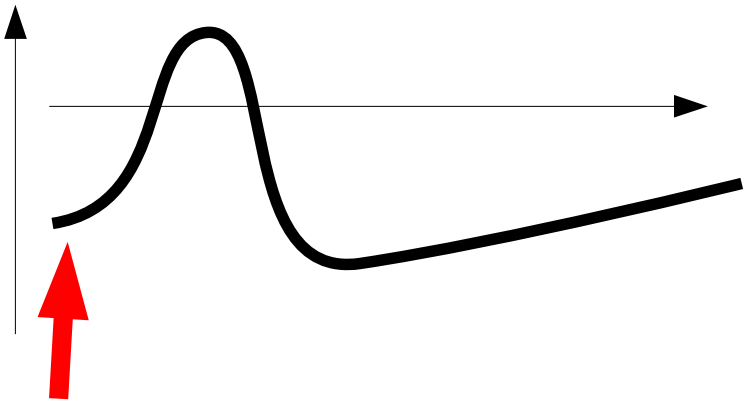
acetylcholine receptor (nicotinic)
serotonin receptor

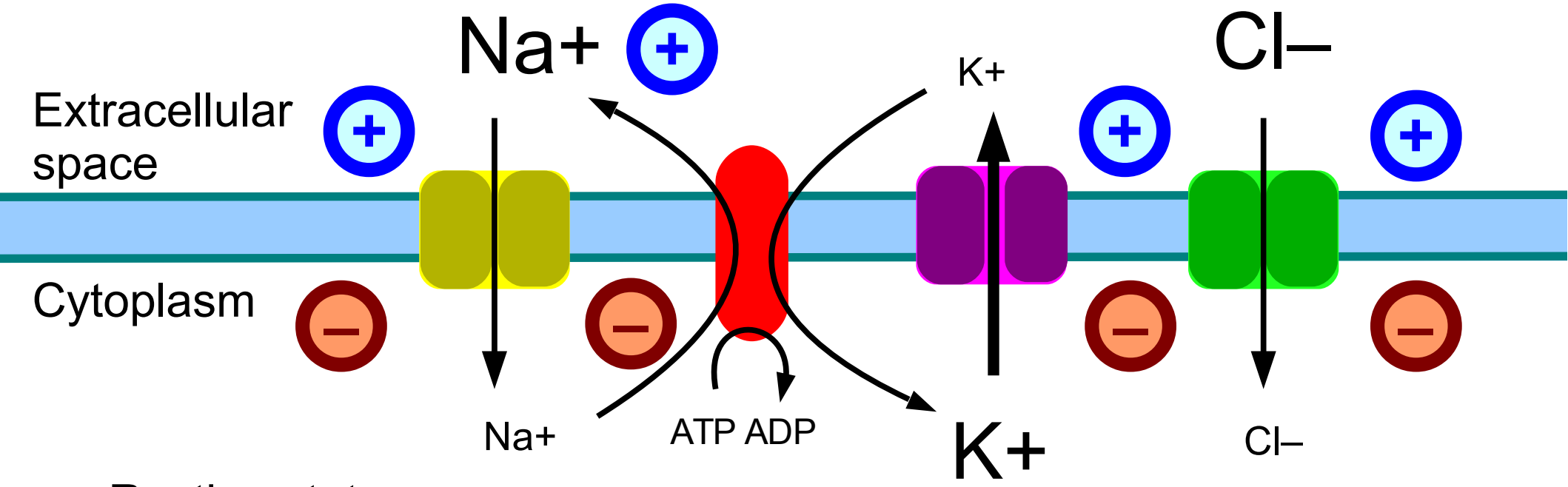


Ligand-gated ion channel is another type of receptors. They are closed in the absence of the ligand and they open and triger ion flow in the presence of the ligand. Examples are serotonine or acetylcholine receptors. Both these ligands have also their GPCRs. Acetylcholine ligand-gated ion channel is known as nicotinic (the GPCR is muscarinic).

Voltage-gated ion channels

voltage-gated potassium channel
voltage-gated sodium channel



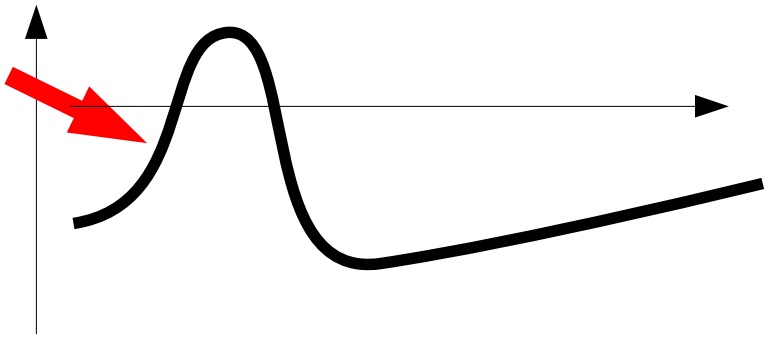


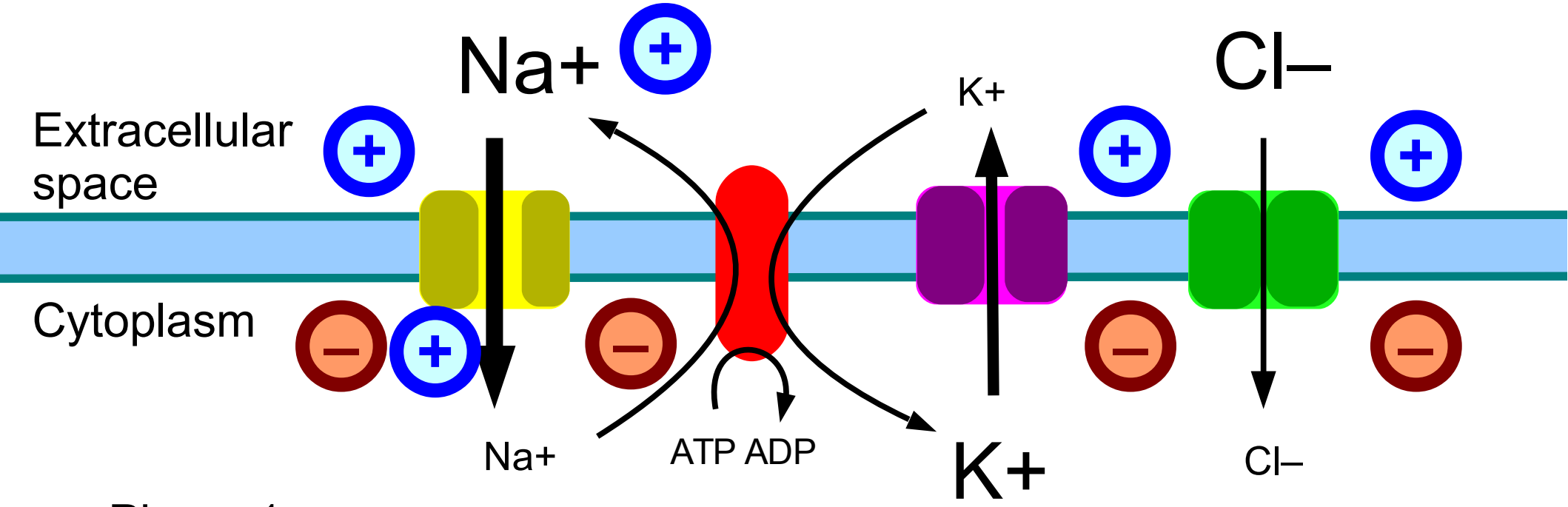
Resting state

In the resting state Na,K-ATPase creates a gradient of Na+ and K+ (there is high Na+ concentration out and K+ in). The K+-channel leaks, which causes excess of charge out (negative membrane potential).

Voltage-gated ion channels

voltage-gated potassium channel
voltage-gated sodium channel



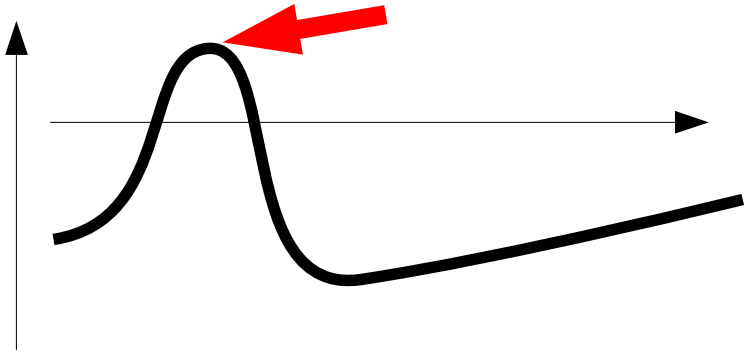


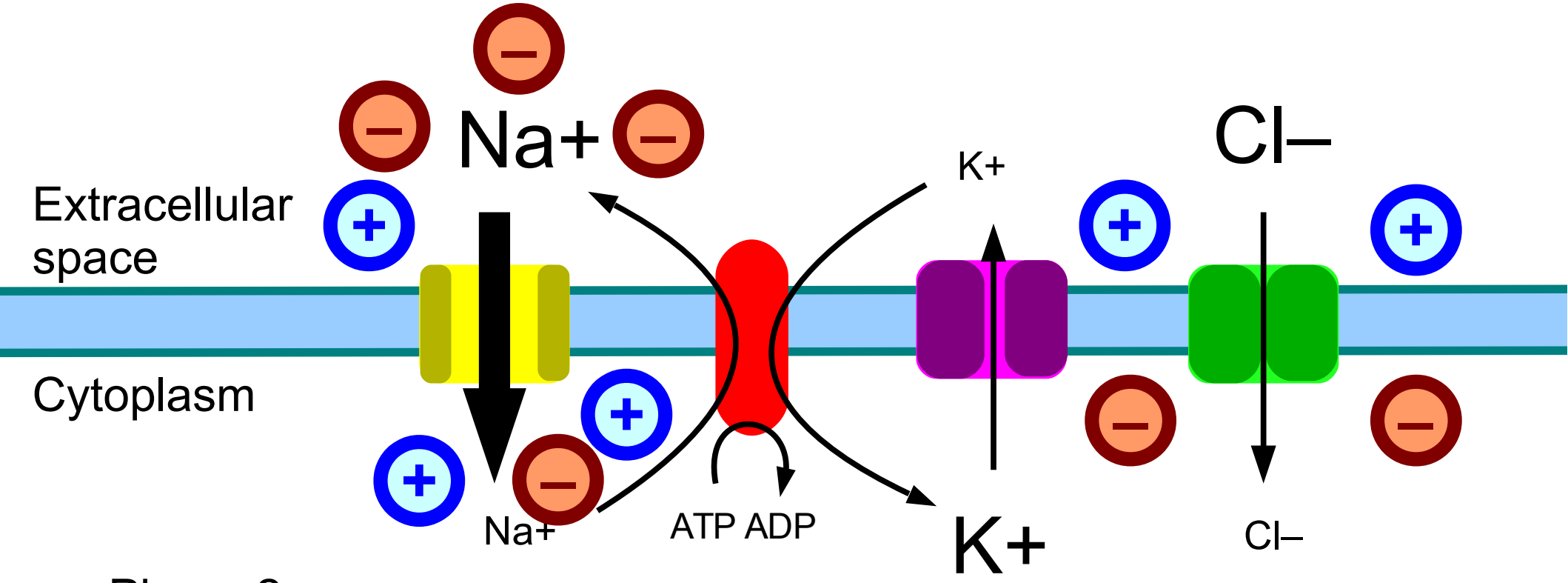
Phase 1

In the first phase of the signal transduction the Na+ channel opens as a result of change in the potential. Na+ goes in.

Voltage-gated ion channels

voltage-gated potassium channel
voltage-gated sodium channel



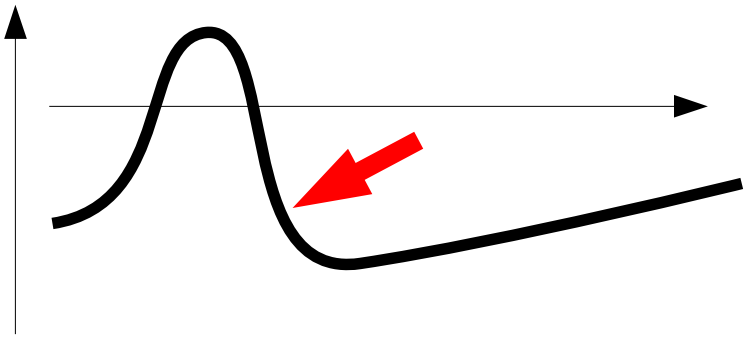


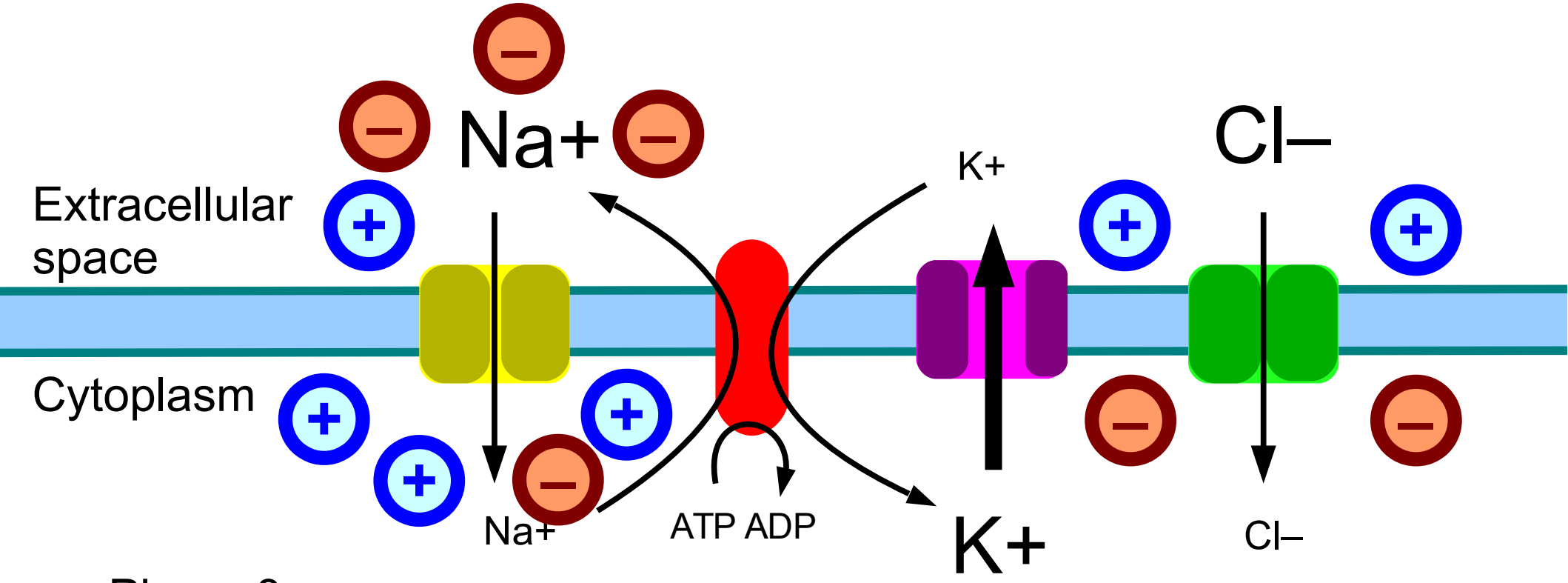
Phase 2

Transport of Na+ switches the potential from negative to positive. Once the potential is positive, it opens the K+-channel.

Voltage-gated ion channels

voltage-gated potassium channel
voltage-gated sodium channel



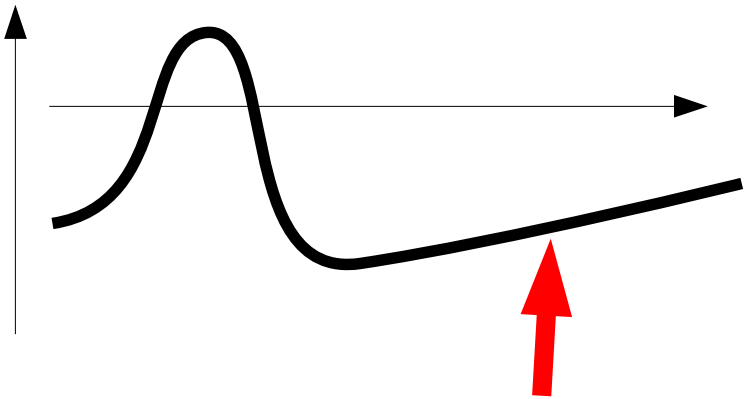


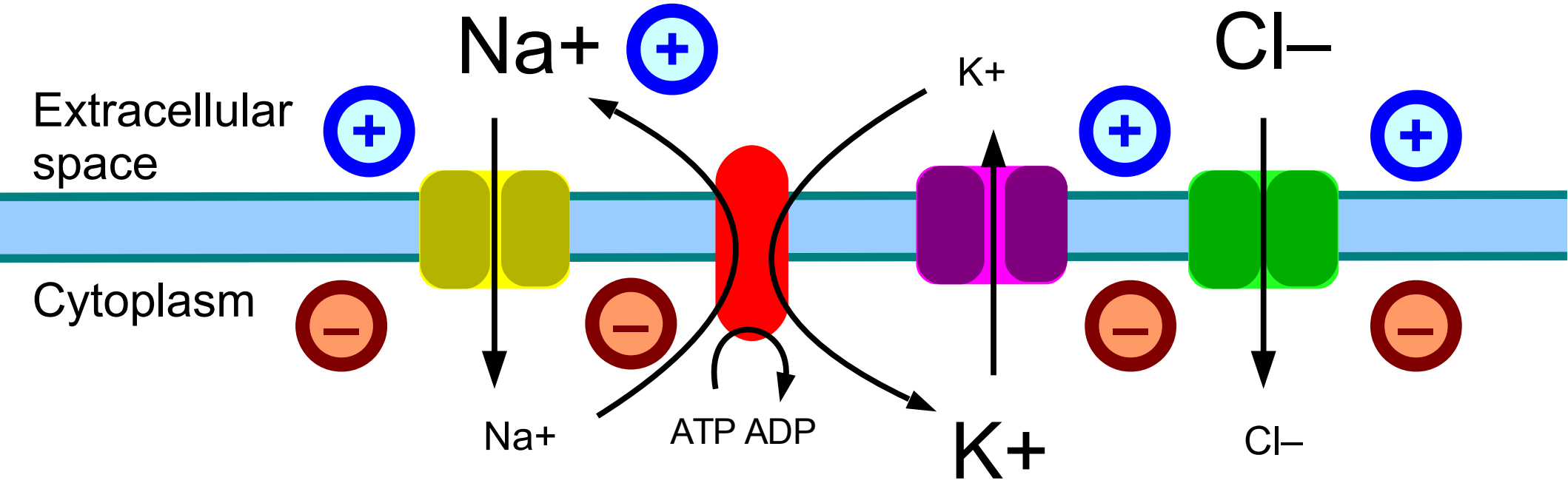
Phase 3

As K+ goes out, the membrane potential returns towards the initial state.

Voltage-gated ion channels

voltage-gated potassium channel
voltage-gated sodium channel

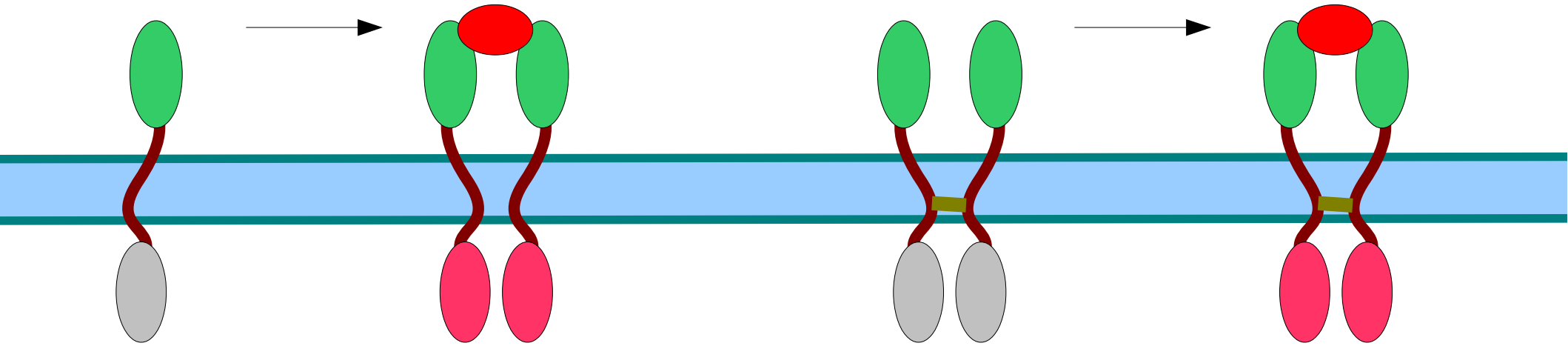




Phase 4

In the last phase the cell returns into the initial state. Since the neurons are long tubular cells, the process described on last 5 slides propagates linearly, as electric current, along the neuron.

Receptor tyrosin kinases



EGFR

Insulin rec.

Other types of receptors are represented by receptor protein kinases. When a ligand (epidermal growth factor for EGFR or insulin for insulin receptor) binds to extracellular domain (this may or may not be associated with dimerization). This triggers protein kinase activity in the intracellular domian. Then the kinase domain can phosphorylate various intracellular proteins.

Other types of receptors

- integrins, selectins, cadherins...

Integrins mediate interactions of the extracellular matrix and the cell. They also sense signals from the extracellular matrix. They influence cell growth, differentiation and programmed cell death (apoptosis).

Selectins bind sugars on cell surfaces.

Cadherins are calcium-dependent cell adhesion molecules.

Biochemistry of human organs and tissues I

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Organs and tissues

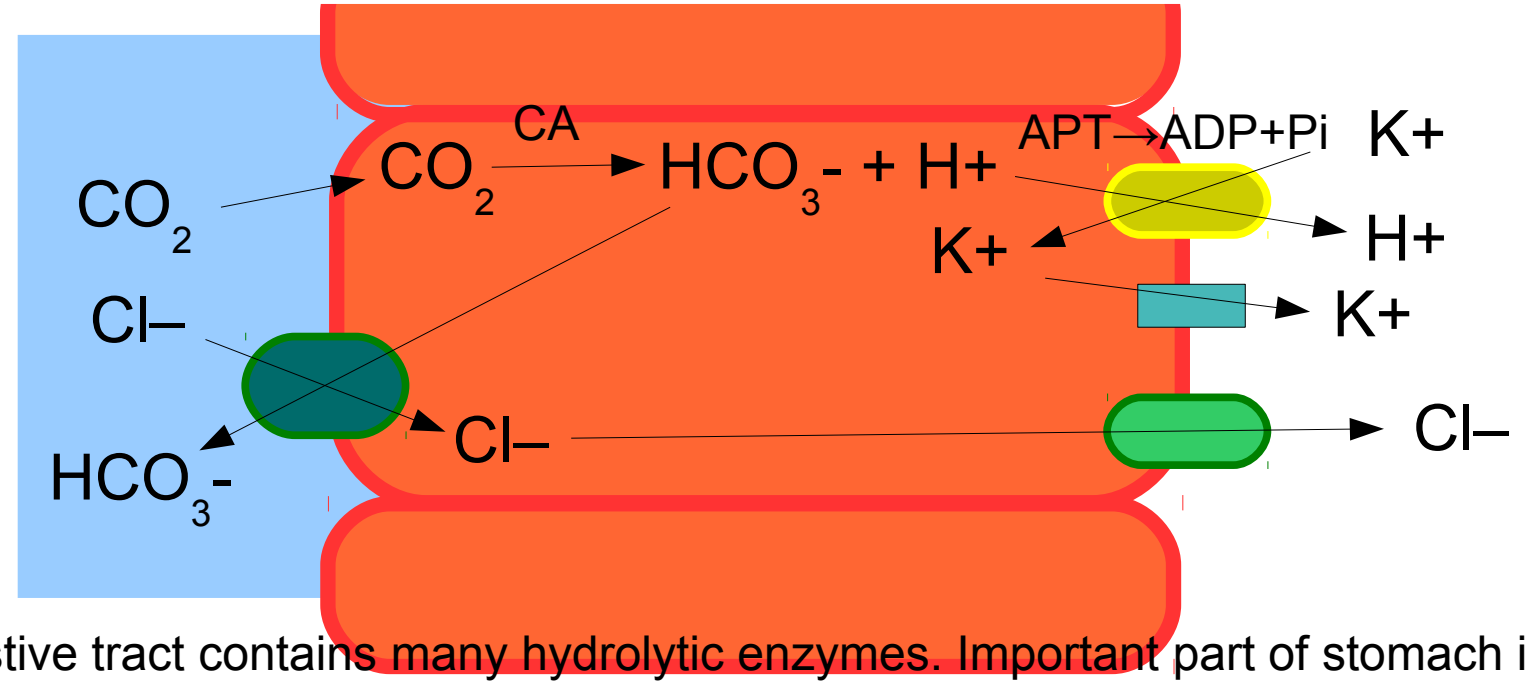
- digestive tract
- blood and immune system
- liver
- adipose
- kidney
- muscles
- connective tissues
- brain, senses

In this pair of lectures we will show biochemistry typical for human organs and tissues. We will start in the digestive tract and we will follow nutrients to blood circulation. Next we show other features of blood such as immunity or coagulation. Then we will mention biochemistry of liver, adipose tissue, kidney and muscles. At the end we will look at neural system and hormonal signalling.

Digestive tract

Saliva - digestive function (α-amylase)
- protective function (antibodies, lysozyme)
- other functions (mucin – rheological properties)

Gastric acid - digestive function (HCl, pepsin, chymosin, lipase)
- absorption of nutrients (haptocorrin, intrinsic factor)
- other functions (mucin)

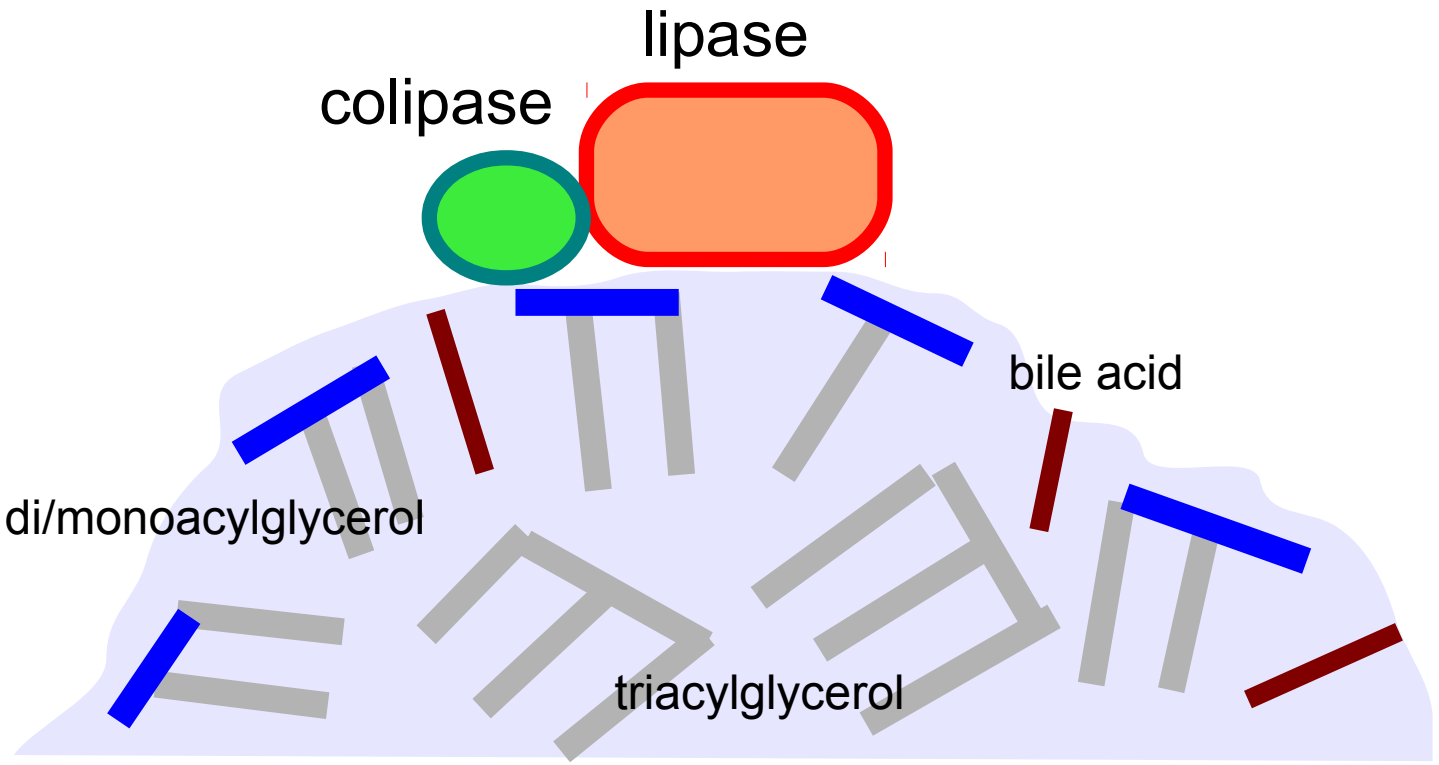


Digestive tract contains many hydrolytic enzymes. Important part of stomach is gastric acid containing HCl. HCl is produced by active transport of protons into the stomach. Other proteins such as potassium and chloride channel or carbonic anhydrase (CA) participate in this process.

Digestive tract

Pancreatic juice

- neutralization of gastric acid (HCO_3^-)
- digestive function (trypsin, chymosin, elastase, carboxypeptidase, α-amylase, lipase, colipase, phospholipase, nucleases, …)

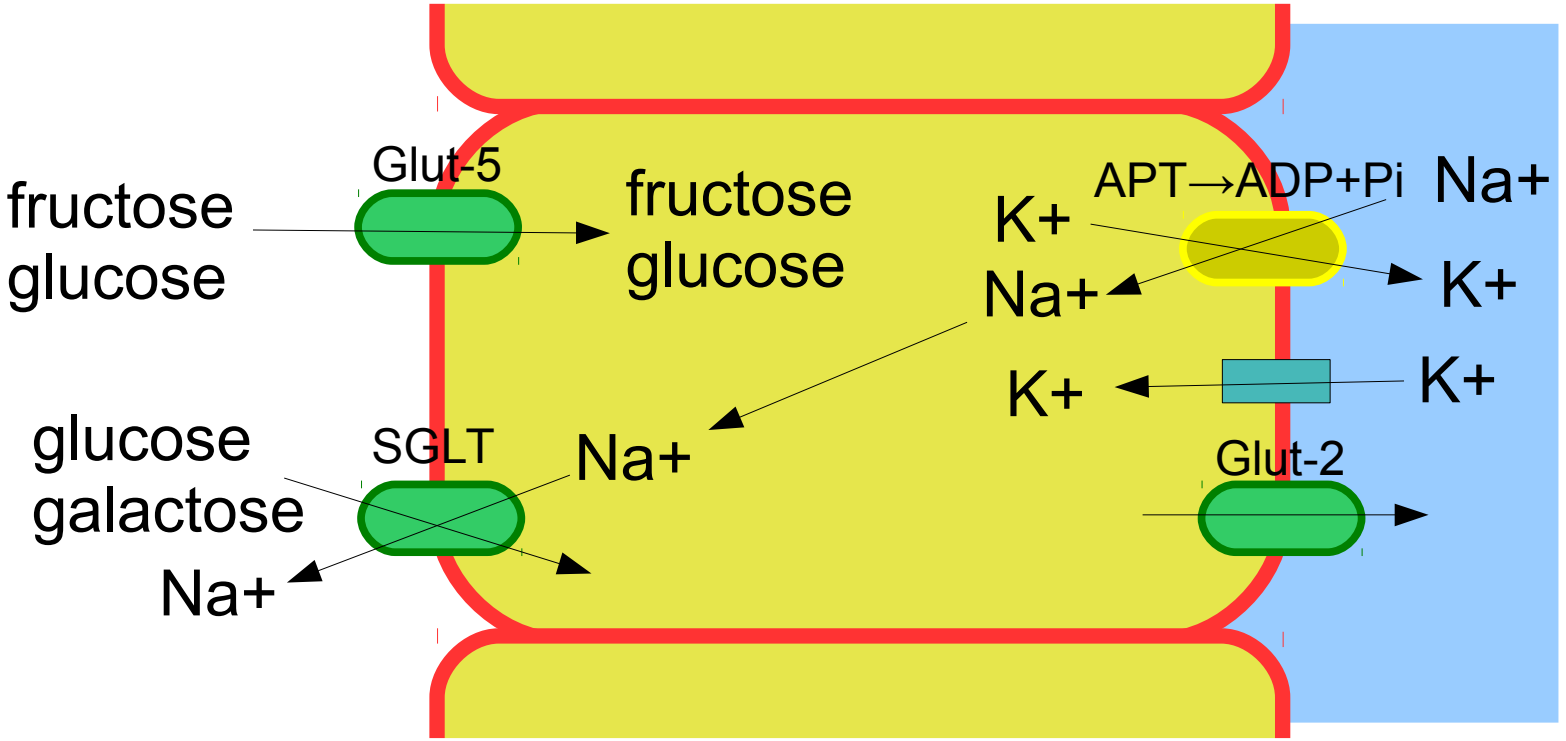


Pancreatic juice hydrolyses many nutrients. Lipase (with its helper colipase) is an interesting enzyme because it works at the phase interface.

Digestive tract

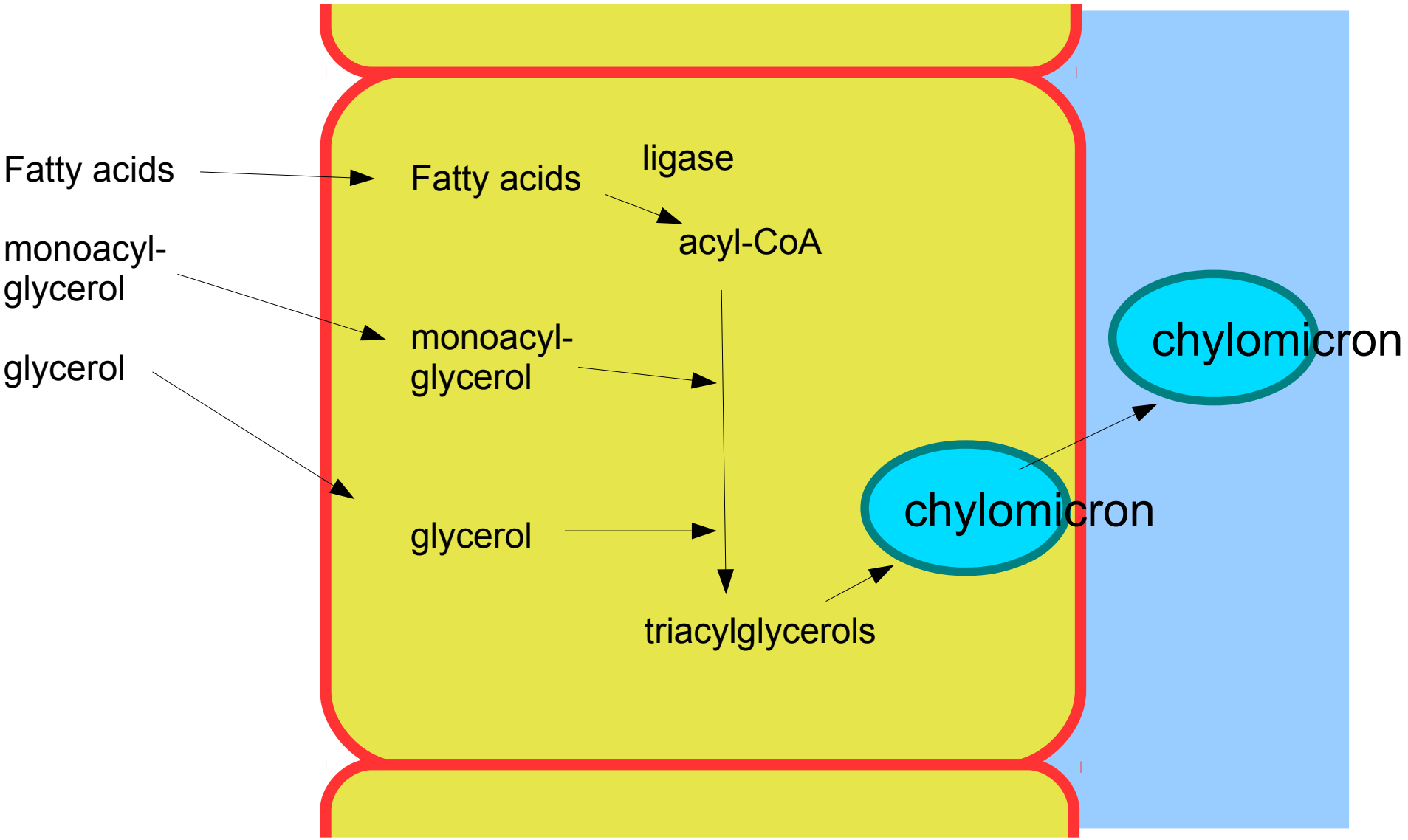
Bile - emulgation (bile acids)
- neutralization of gastric acid (HCO_3^-)

Intestinal juice
- digestive function (peptidases, glycosidases, lipases, phospholipases, nucleases etc.)



Bile neutralizes gastric acid, emulgates lipids (by bile acids) and provide protection against many microorganisms. Transport of saccharides may be active (SGLT) or passive (Glut).

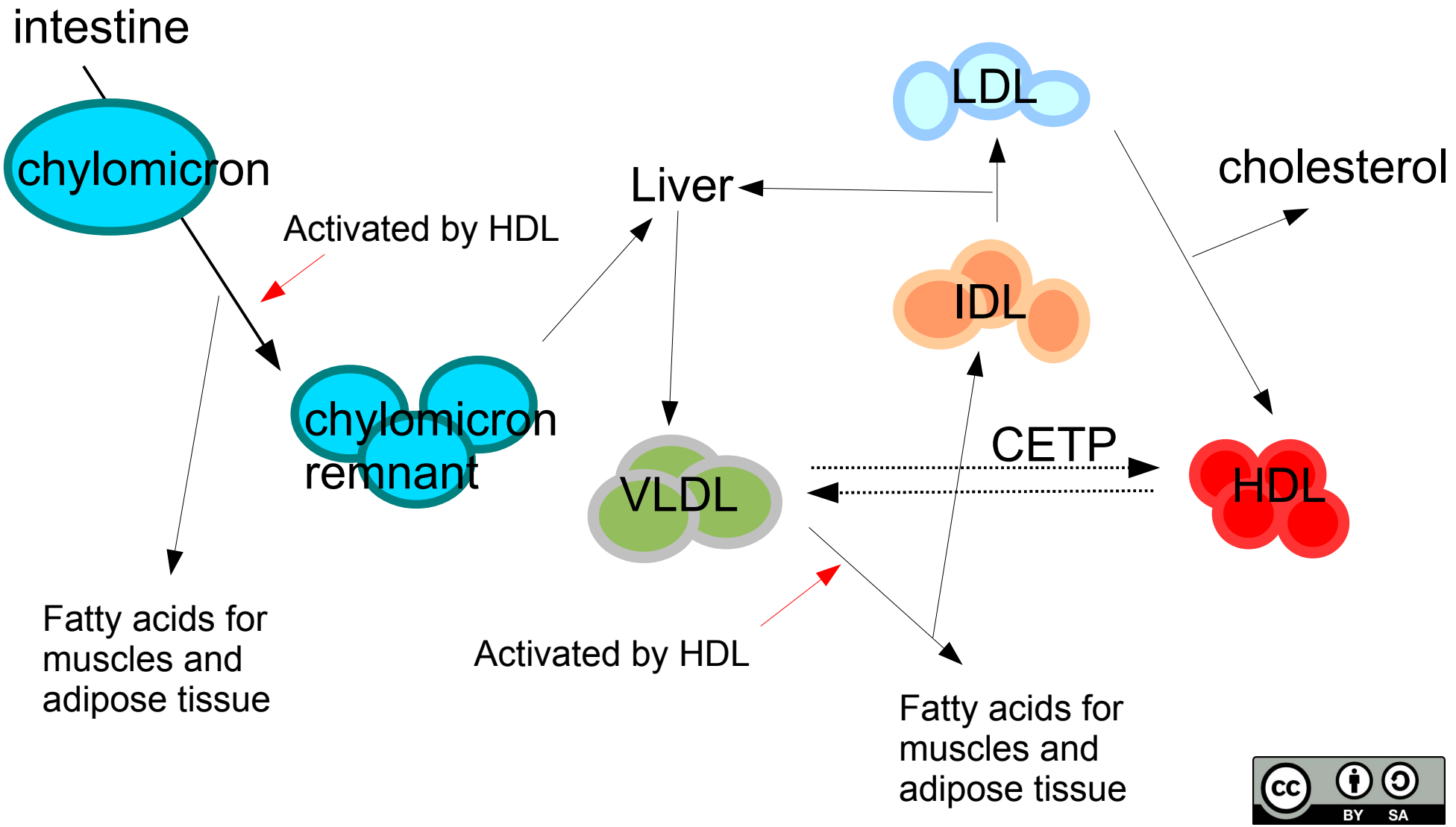
Lipid resorption, resynthesis, lipoproteins



Lipids are insoluble in water cannot be simply transported by the blood stream. Instead they are transported in the form of lipid-protein complexes – lipoproteins. In order to control lipoprotein formation, composition, release etc. intestinal cells hydrolyse tryacylglycerols and then resynthesize them. Largest lipoprotein particles produced by digestive tract are chylomicrons.

Lipid resorption, resynthesis, lipoproteins

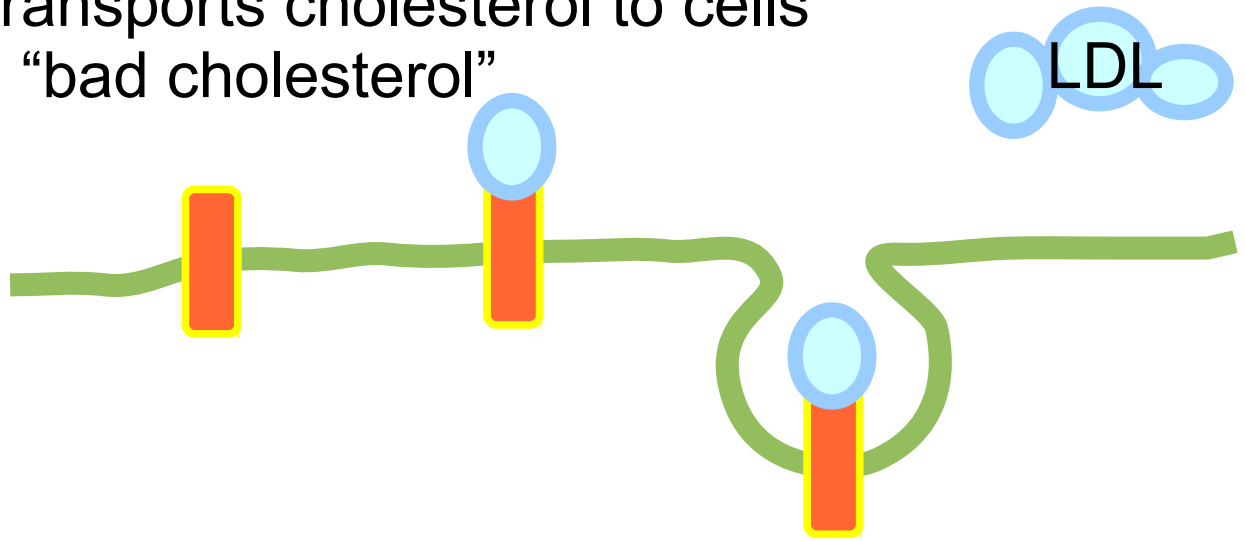
Composition: triacylglycerols, cholesterol, phospholipids, proteins (1-50 %, apoproteins)



Lipid resorption, resynthesis, lipoproteins

HDL: transports cholesterol to liver for degradation
"good cholesterol"

LDL: transports cholesterol to cells
"bad cholesterol"



CETP – cholesterol ester transfer protein

Torcetrapib:

November 30, 2010 – "*This will be one of the most important compounds of our generation*" Jeff Kindler, Pfizer's chief executive

December 2, 2010 – Pfizer announces halt of phase III clinical trials of Torcetrapib

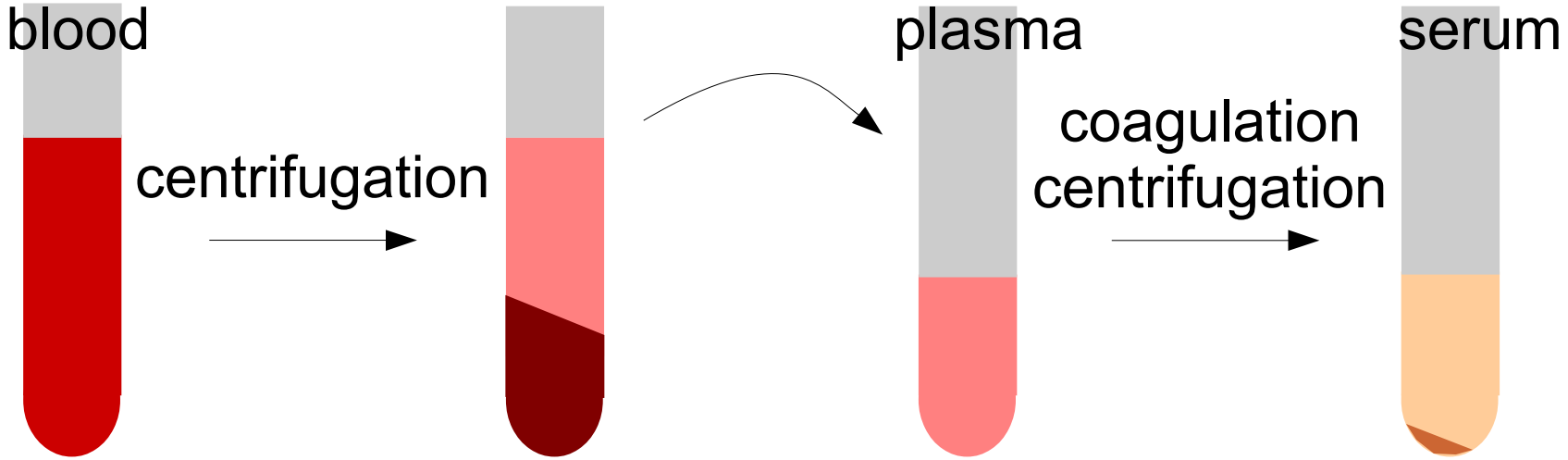
Lipid resorption, resynthesis, lipoproteins

Lipoproteins are microscopic (5-1000 nm) particles composed of triacylglycerols, phospholipids, cholesterol, acylated cholesterol (cholesterol esters) and proteins (known as apoproteins). Largest chylomicrons deliver triacylglycerols from digestive tract to muscles and adipose tissue and to liver. In this process they lose lipid content and become chylomicron remnants. These are uptaken by liver. Liver synthesizes very low density lipoproteins (VLDL). These deliver triacylglycerols to muscles and adipose tissue. In this process they lose triacylglycerols and change to intermediate (IDL) and low density lipoproteins (LDL). These particles can be reuptaken by liver. Another lipoprotein particles are high density lipoproteins (HDL). They act as scavengers of cholesterol from the body. Then they are reuptaken by liver and cholesterol is degraded. While LDL are associated with atherosclerosis (“bad” cholesterol), HDL is protective (“good” cholesterol). Hypercholesterolemia is treated by cholesterol lowering drugs called statins. They inhibit hydroxymethylglytaryl-CoA dehydrogenase in the cholesterol biosynthesis pathway. However, they lower good as well as bed cholesterol. Therefore cholesteryl ester transfer protein (CETP) was proposed as a new drug target. This protein facilitates exchange of triglycerides from VLDL or LDL for cholesterol esters from HDL, and vice versa. Several compounds inhibiting the function of this protein have been developed, but their medicinal application was disappointing.

Blood

Roles: - transport of oxygen, carbon dioxide, other nutrients
- water distribution
- acidobasic homeostasis
- thermoregulation
- immunity
- self-protection (blood coagulation)

Composition: ~45% of cellular elements



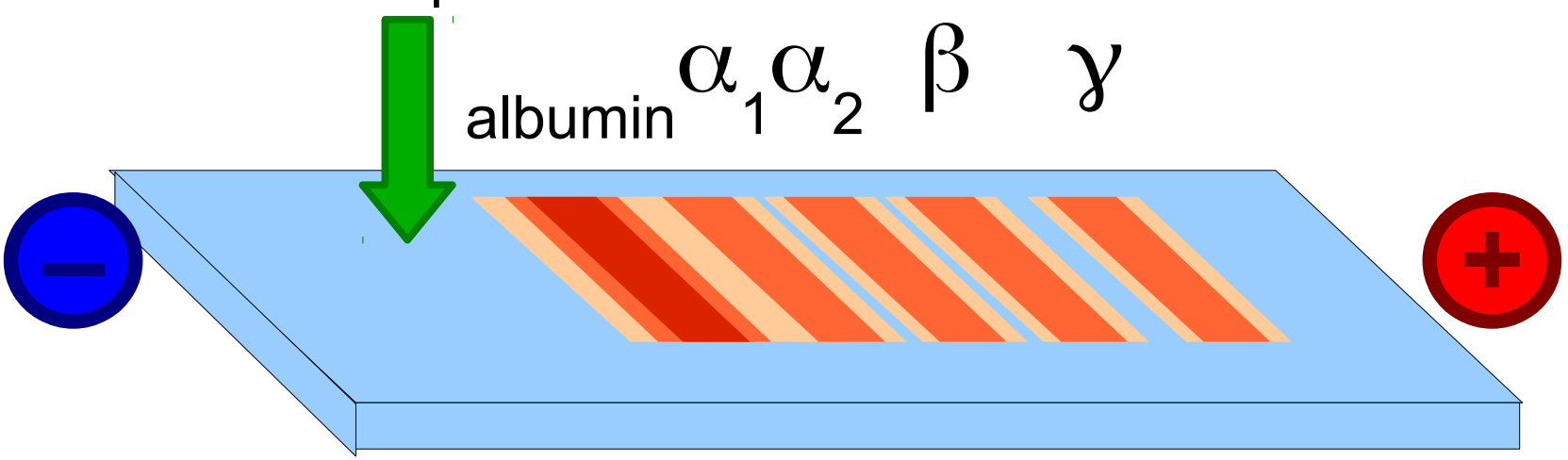
Hematopoiesis: bone marrow, fetal spleen and liver

Blood after removal of cells is called plasma. Plasma naturally coagulates and after removal of coagulated proteins it is called serum. It is also possible to avoid coagulation by addition of metal chelating agents such as EDTA.

Blood

Composition of serum:

- salts (mostly Na+, Cl-, 0.15M NaCl, 0.9% NaCl)
- glucose, lactate, urea, amino acids, triacylglycerols, cholesterol
- proteins 6-8%

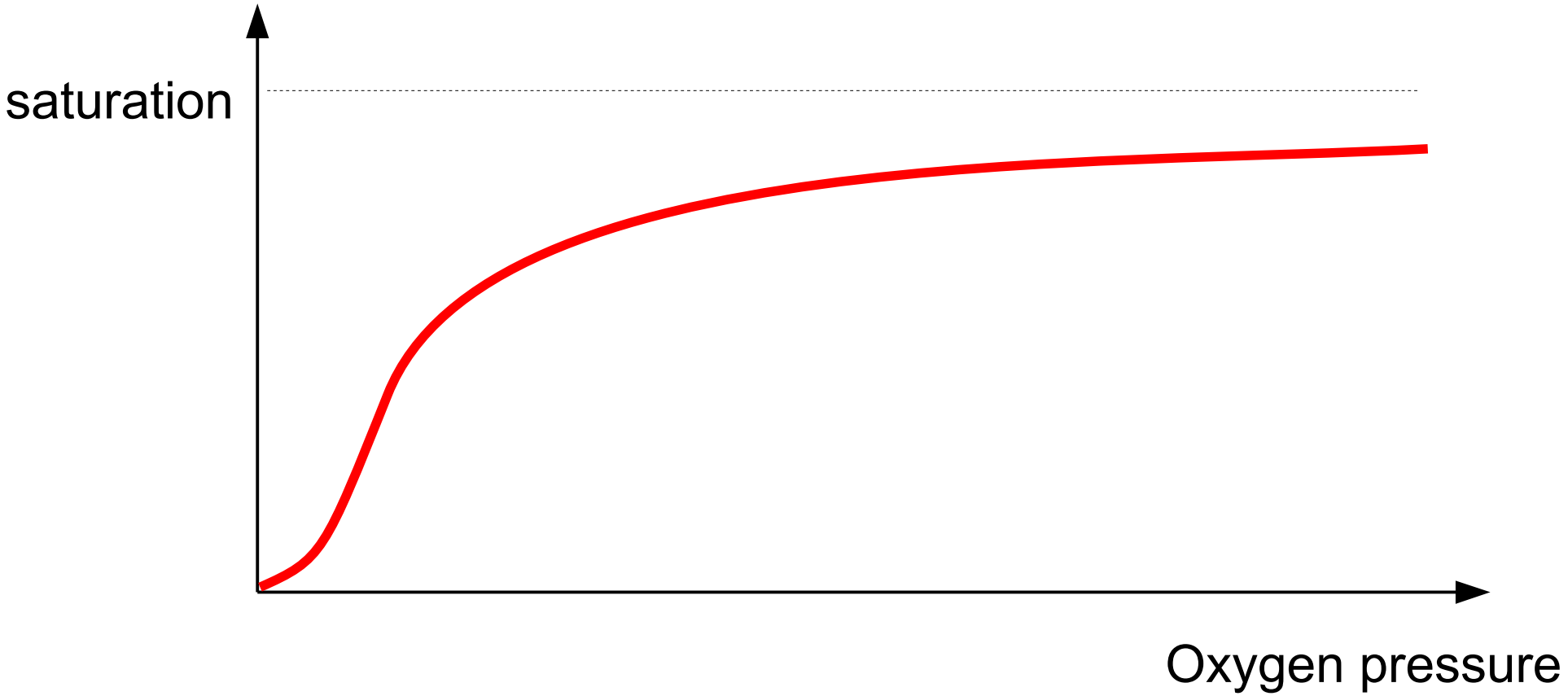


albumin – colloidal, transport and osmotic function

Serum proteins can be separated by a traditional electrophoresis procedure in agarose gel. Agarose (purified algal polysaccharide) is mixed with buffer and heated. While cooling down it forms a gel. It is possible to make a well in the gel, load it with sample and apply electric field. After separation it is possible to visualize separated proteins by stain them by a dye or by precipitation with antibodies.

Blood

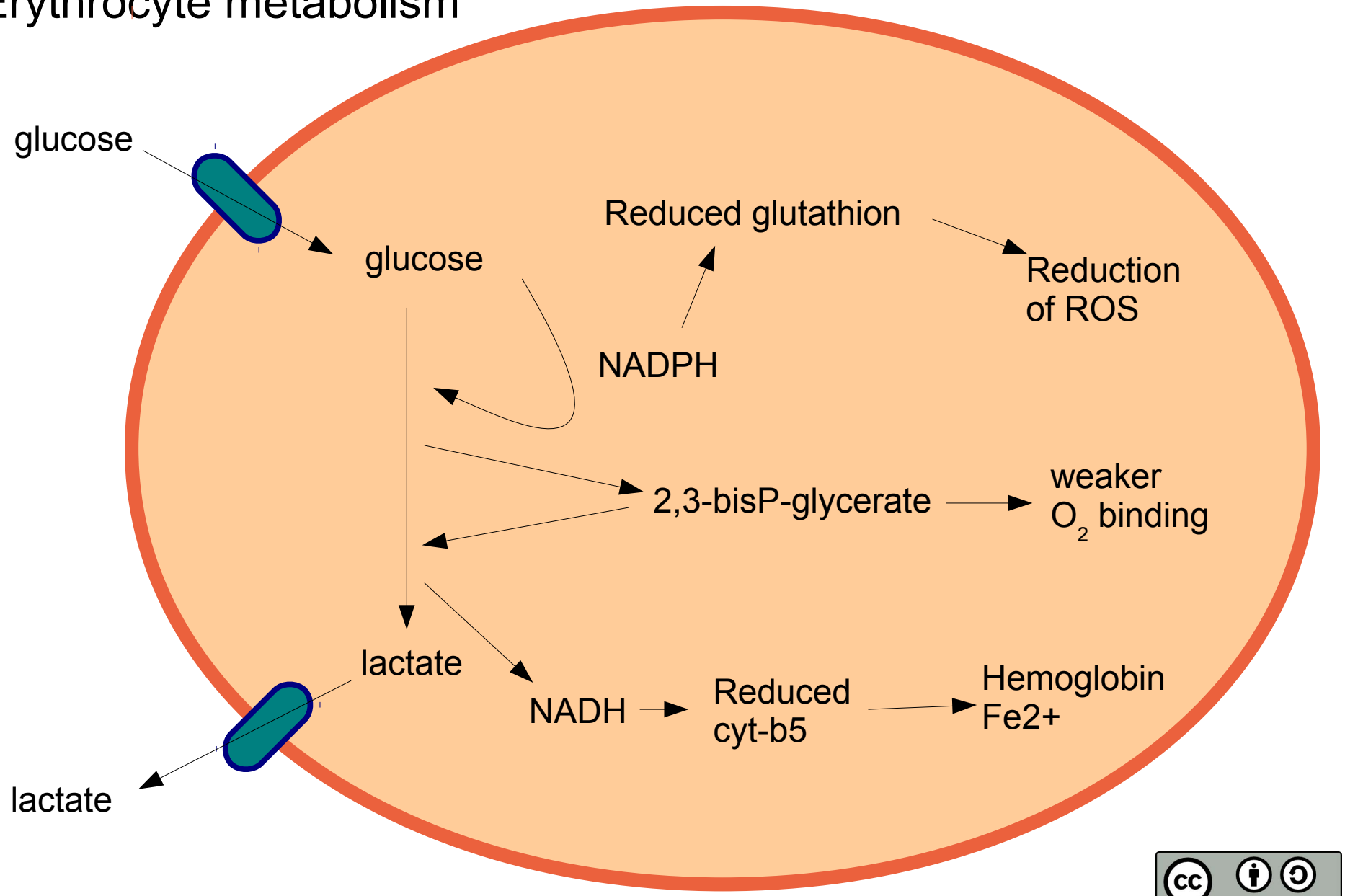
Oxygen transport
hemoglobin – allosteric effect



Blood transport oxygen. Saturation curve of hemoglobin-oxygen complex is not hyperbolic (as for usual binding proteins). Instead it is sigmoid. This makes it possible to almost fully load hemoglobin in lungs and release almost all oxygen in tissues. The reason behind this was explained in the lecture dedicated to regulation of enzymatic activities.

Blood

Erythrocyte metabolism



Blood

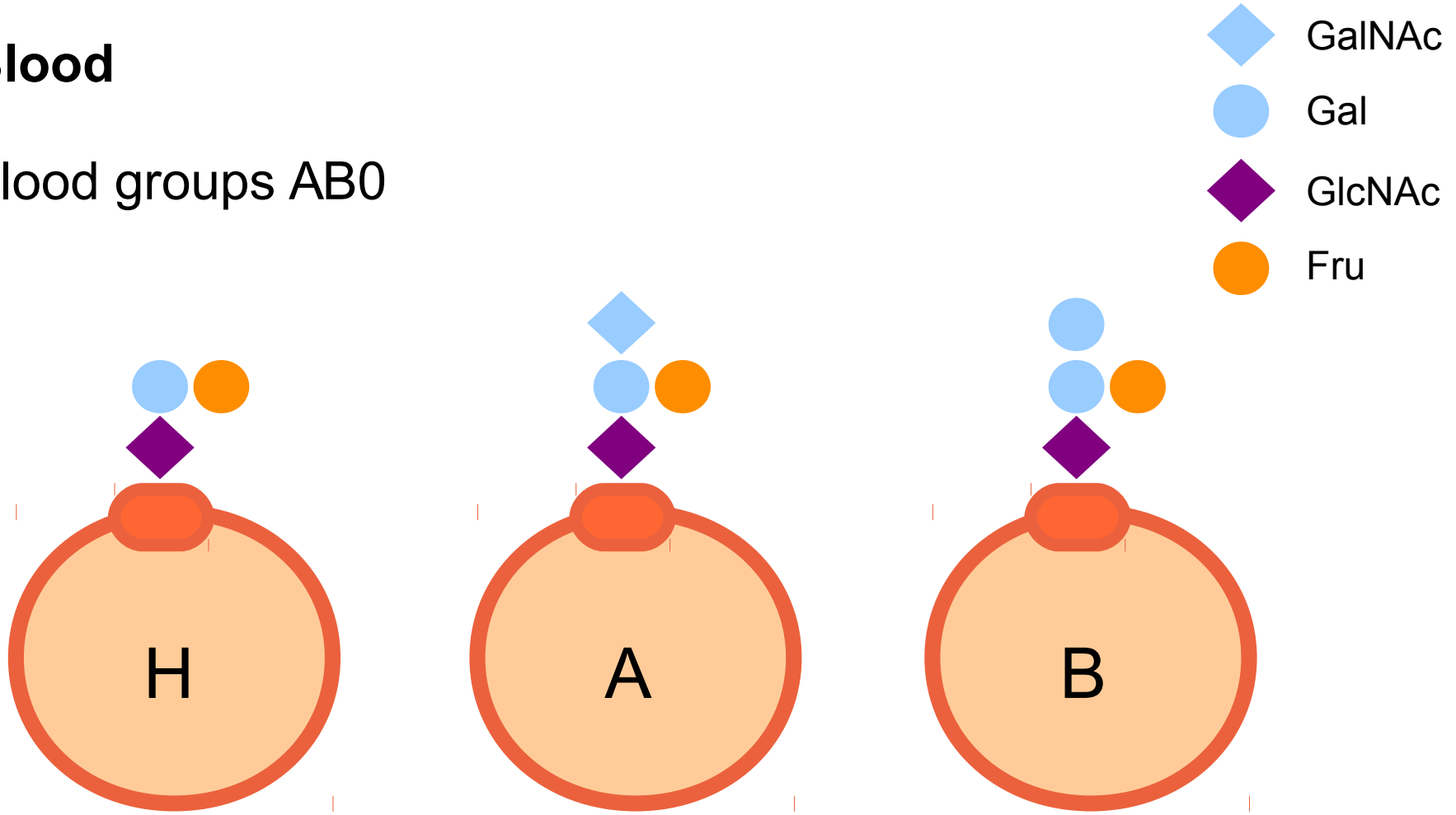
Erythrocyte metabolism

Erythrocytes are strange “cells”. “Cells” (but not cells) because they lose nucleus and mitochondria in the process of hematopoiesis. Therefore they cannot divide, respirate, degrade fatty acids etc. Erythrocytes metabolize glucose to lactate. Produced ATP is used to fuel ion pump, which maintain concentration gradients on the cytoplasmatic membrane. Etyrthrocye glycolysis differ from standard glycolysis in 2,3-bisphosphoglycerate as an intermediate. This compound influences the function of hemoglobin. Some NADH produced can be used to reduce iron in hemoglobin to 2+ form. Pentose phosphate pathway is also very intensive in erythrocytes. It produces NADPH, which reduces glutathion. Glutathion reduces oxidative species, which are widespread in erythrocytes.

Erythrocytes carry blood group antigens. Depending on person’s genome, antigen H, A, B or both A and B can be present. People with just antigen H (blood group 0) produce antibodies against A and B. Therefore they can receive blood transfusion only from blood group 0 donors. In contrast, people with antigens A and B (blood group AB) produce no antibodies and they can receive blood from any donor. Mixing of incompatible bloods causes precipitation (aglutination) of red blood cells. This is visible in a test tube and can be used in diagnostics.

Blood

Blood groups AB0



acceptor donor	A (anti-B)	B (anti-A)	AB (-)	0 (anti-A, anti-B)
A	-	A	-	A
B	A	-	-	A
AB	A	A	-	A
0	-	-	-	-

Immunity

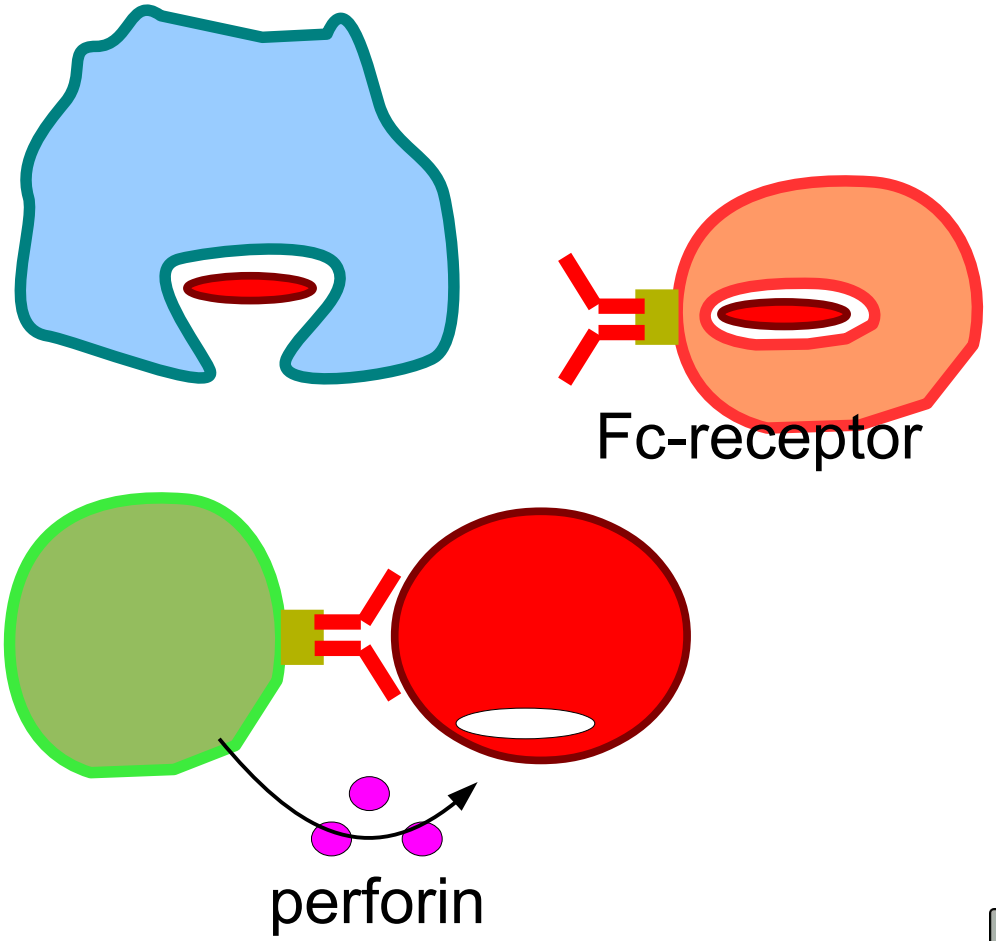
Innate immunity *vs.* adaptive immunity

cellular immunity *vs.* humoral immunity

Innate immunity:
macrophages

granulocytes

NK-cells

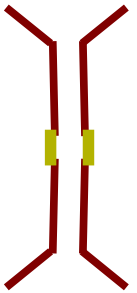


Blood

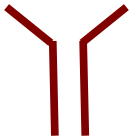
Immune system

Antibodies

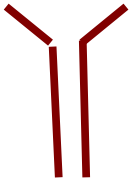
IgA – mostly in intestinal fluid and other secrets



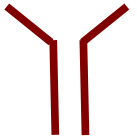
IgD – B-lymphocyte receptor



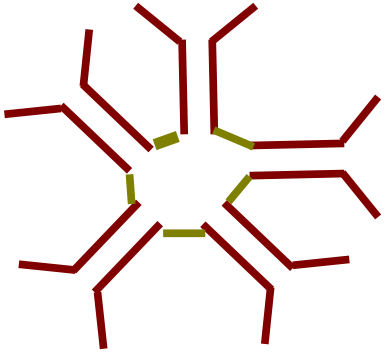
IgE – allergy-related



IgG – ~75 % of all Igs in blood

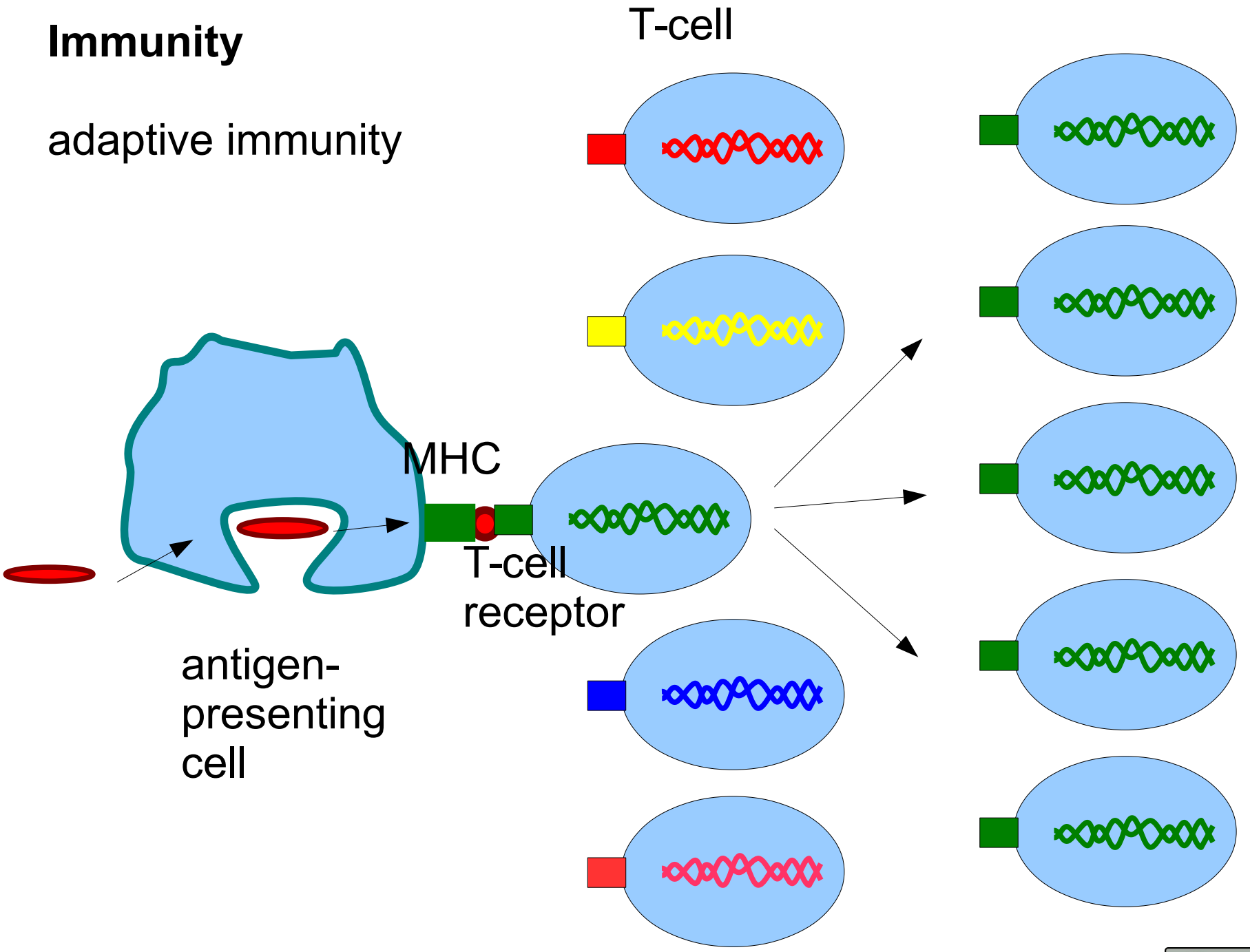


IgM



Immunity

adaptive immunity



Immunity

adaptive immunity

antigen-presenting cell	macrophage B-lymphocytes	almost all cells
MHC	MHC class II.	MHC class I.
T-lymphocyte	helper	cytotoxic
role	activation of antigen-presenting B-cells, production of soluble antibodies	attacking of viral-infected or cancer cell by perforin and granzyme, start of apoptosis

Immunity

Immune system targets all elements that are foreign to the body and simultaneously it does not target element that are own to the body. This mechanism is highly specific (difference between own and foreign might be very small). Occasionally it may happen that the immune system targets own elements, which causes autoimmune diseases. Immunity can be classified as innate (non-adaptive) and adaptive. Adaptive immunity can “learn” to recognize new antigens. Human genome is in general same for all somatic cells, but there are exceptions from this rule. The gene coding antibodies is an example of such exception. This gene undergoes hypermutation, which causes that certain part of the gene (the one coding variable region of antibodies) is variable. The resulting proteins are displayed on surface of these cells. When these cells get in the contact with antigen they are activated to grow and produce antibodies. Specificity for foreign and not own antigens is achieved by elimination of cells coding for antibodies against own antigens in the prenatal development. Only cells not coding for antibodies against own antigens can mature.

Immunity can be also classified as cellular or humoral. Humoral immunity uses antibodies as soluble proteins. These proteins – immunoglobulins – can be applied in many fields including diagnostics, analytical chemistry, affinity purifications and therapies.

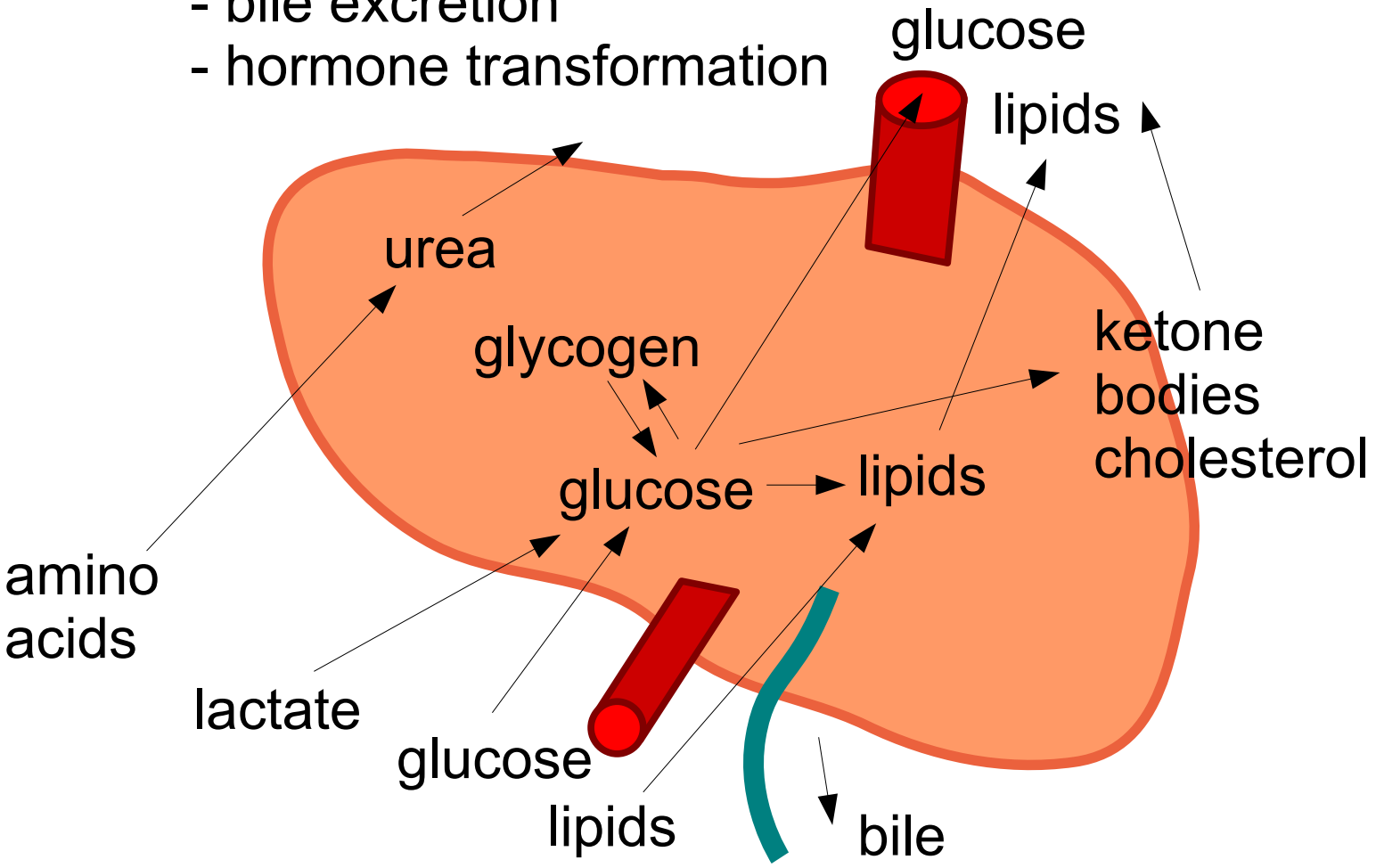
Biochemistry of human organs and tissues II

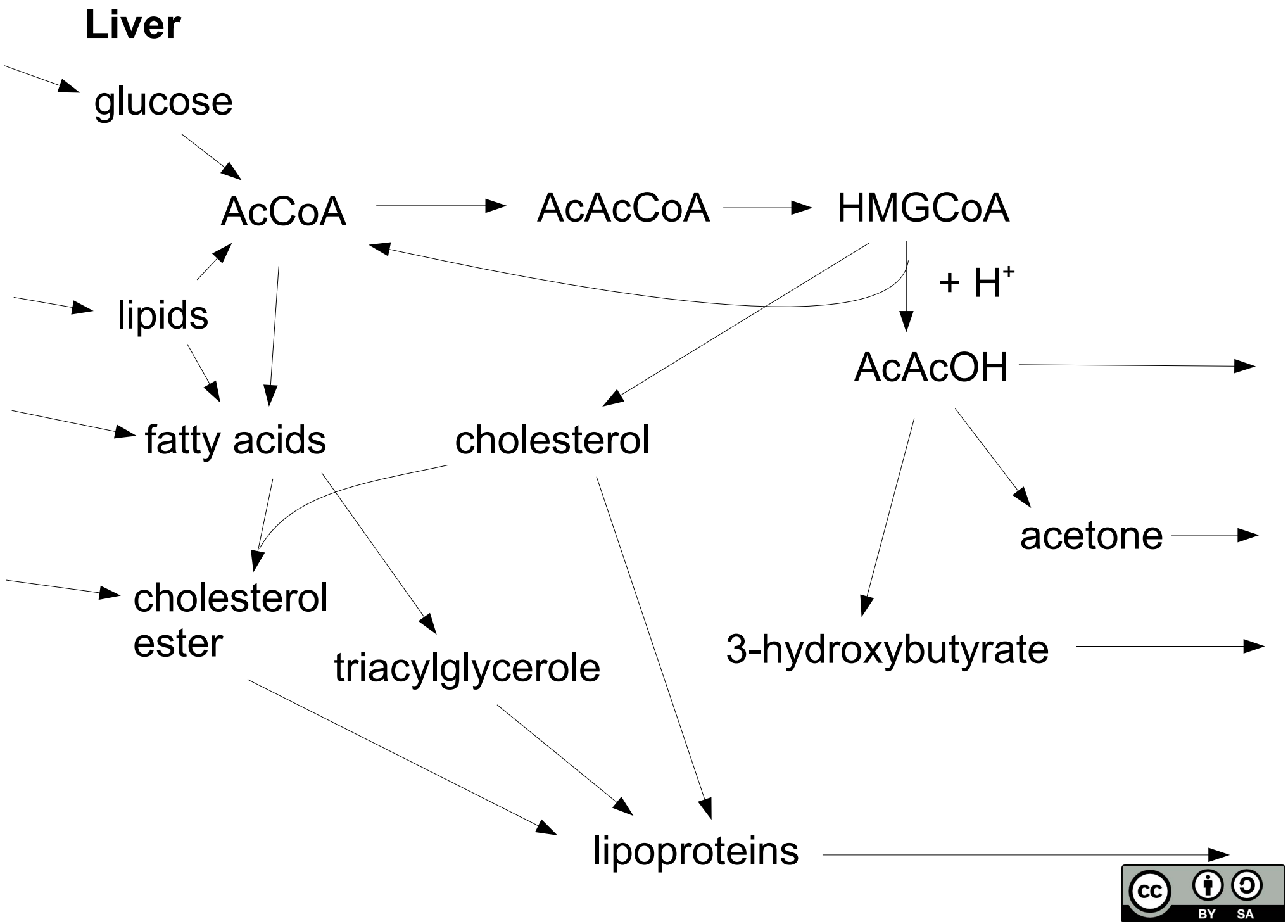
EVROPSKÁ UNIE
Evropské strukturální a investiční fondy
Operační program Výzkum, vývoj a vzdělávání

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Liver

Function:
- input of nutrients from small intestine
- metabolism of saccharides, lipids, amino acids etc.
- storage (glycogen, B12, iron)
- detoxification
- bile excretion
- hormone transformation

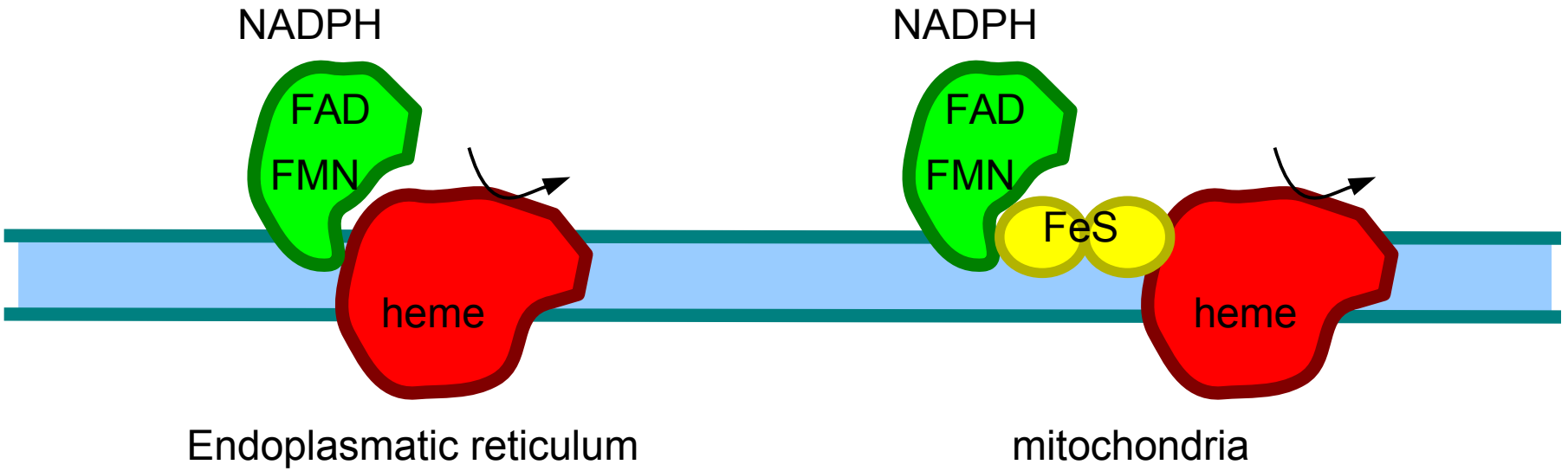



Liver
glucose
AcCoA
AcAcCoA
HMGCoA
+ H+
lipids
AcAcOH
fatty acids
cholesterol
acetone
cholesterol ester
triacylglycerole
3-hydroxybutyrate
lipoproteins

Liver

Cytochrom P450

$$R\text{-}H + O_2 + NADPH + H^+ \rightarrow R\text{-}O\text{-}H + H_2O + NADP^+$$



hydroxylation, epoxidation, dealkylation, oxidation of N, S, P etc.
~60 isoforms
CYP 3A4, 2C19, 1A2, 2A6, 2B6 are major drug metabolizes
sterole metabolism

Liver

ethanol metabolism

high energy input
high NADH/NAD+ ratio
suppression of citric acid cycle
lactate synthesis, acidosis
lactate in blood reduces clearance of uric acid
high production of keton bodies
high lipid production
degradation of ethanol by microsomal alcohol oxidase
(one of CYP isoenzyme)

Liver

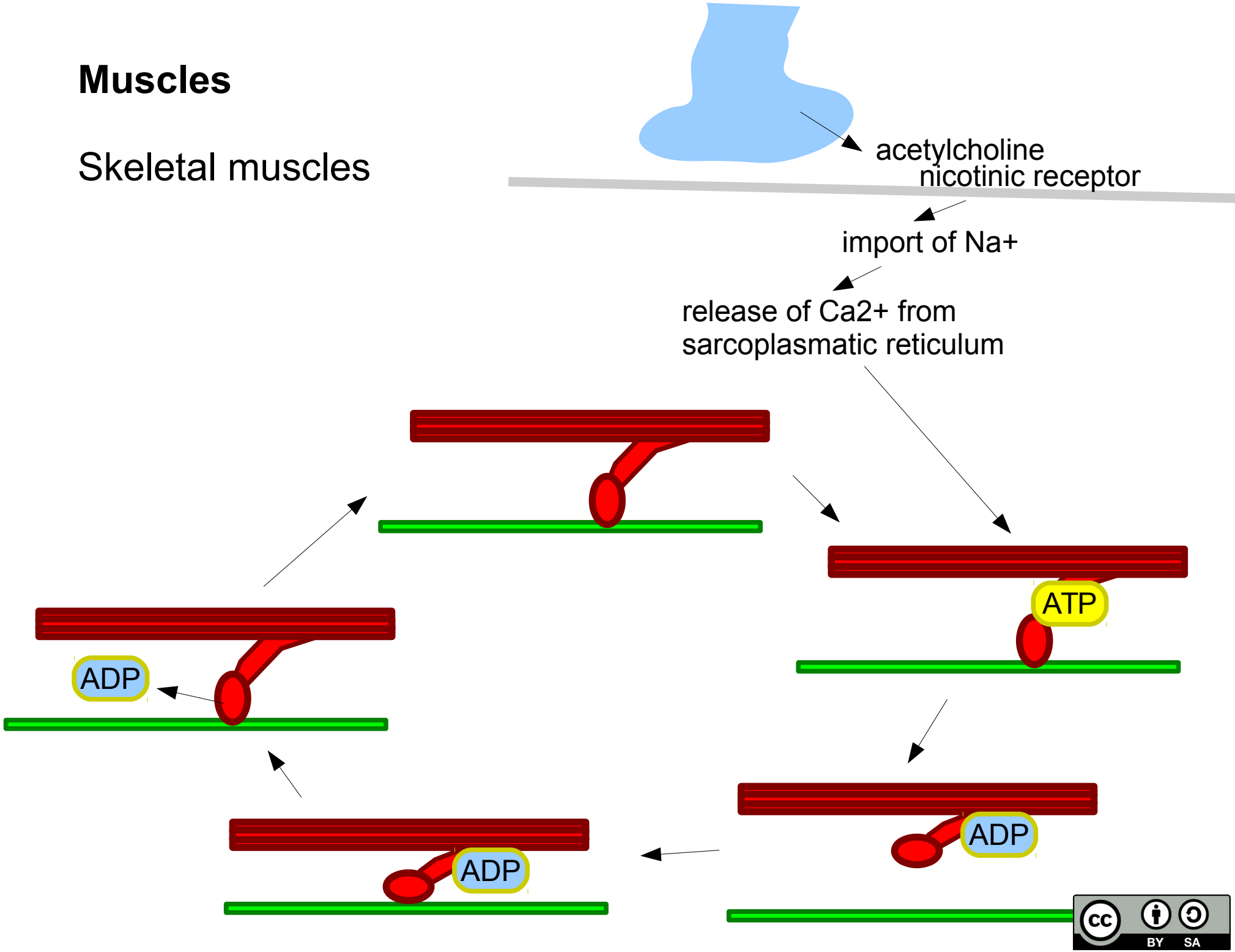
Liver plays many roles in human metabolism. It participates in metabolism of fatty acids and cholesterol and in control of lipoproteins (see previous lecture). It also participates in gluconeogenesis of glucose from lactate. Muscles under intensive work with lack of oxygen produce lactate from glucose. Lactate is transported by blood to liver where it is converted back to glucose. Glucose can go back to muscles. This is known as Cori cycle. Liver also stores glycogen. It also produces ketone bodies. Condensation of AcCoA produces acetoacetat, 3-hydroxybutyrate and acetone. These compounds are known as ketone bodies. They can be used by other tissues as a source of energy. High production of ketone bodies is typical for starving, low-carbohydrate high-fat diet, alcohol intoxication or improperly treated type 1 diabetes. Liver also metabolizes amino acids.

Cytochrome P450 (CYP, named for its absorbtion at 450 nm in complex with CO) is a complex of multiple liver enzyme with a broad substrate and reaction specificity. In nature they metabolize natural secondary metabolites from plants and other food. In medicine it plays an important role in metabolism of drugs. In general it oxidases non-polar exogenous molecules to more polar, and thus more soluble, products. More soluble products can be excreted more easily. There are many isoenzymes of CYP. For a new drug it is important to determine which CYP isoenzyme metabolize it because this influences its half live in body, genetic differences in drug function or interactions between drugs. Some drugs (as well as some natural products) are known to inhibit some CYPs, which may prolong the effect of other drugs.

Liver metabolizes ethanol by alcohol dehydrogenase and other enzymes. High ethanol consumption causes production of ketone bodies, acidosis and high lipid production. It can also cause that ethanol becomes oxidized by an alternative microsomal ethanol oxidizing system.

Muscles

Skeletal muscles

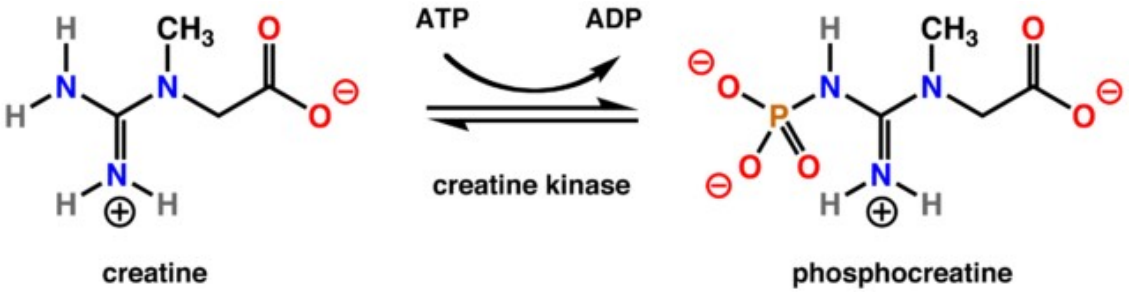


Muscles

creatine

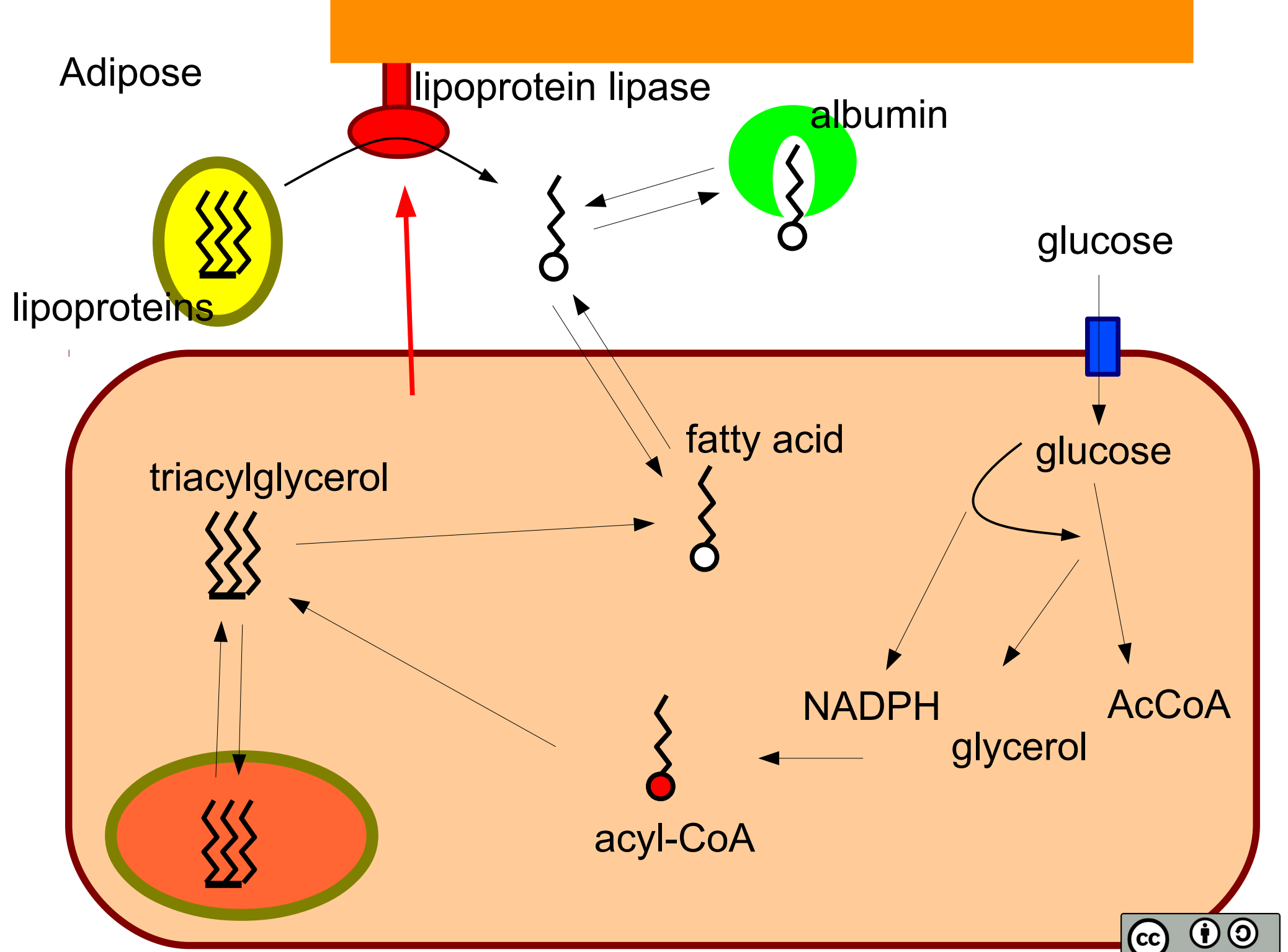
Arg + Gly → ornitine + guanidinoacetate

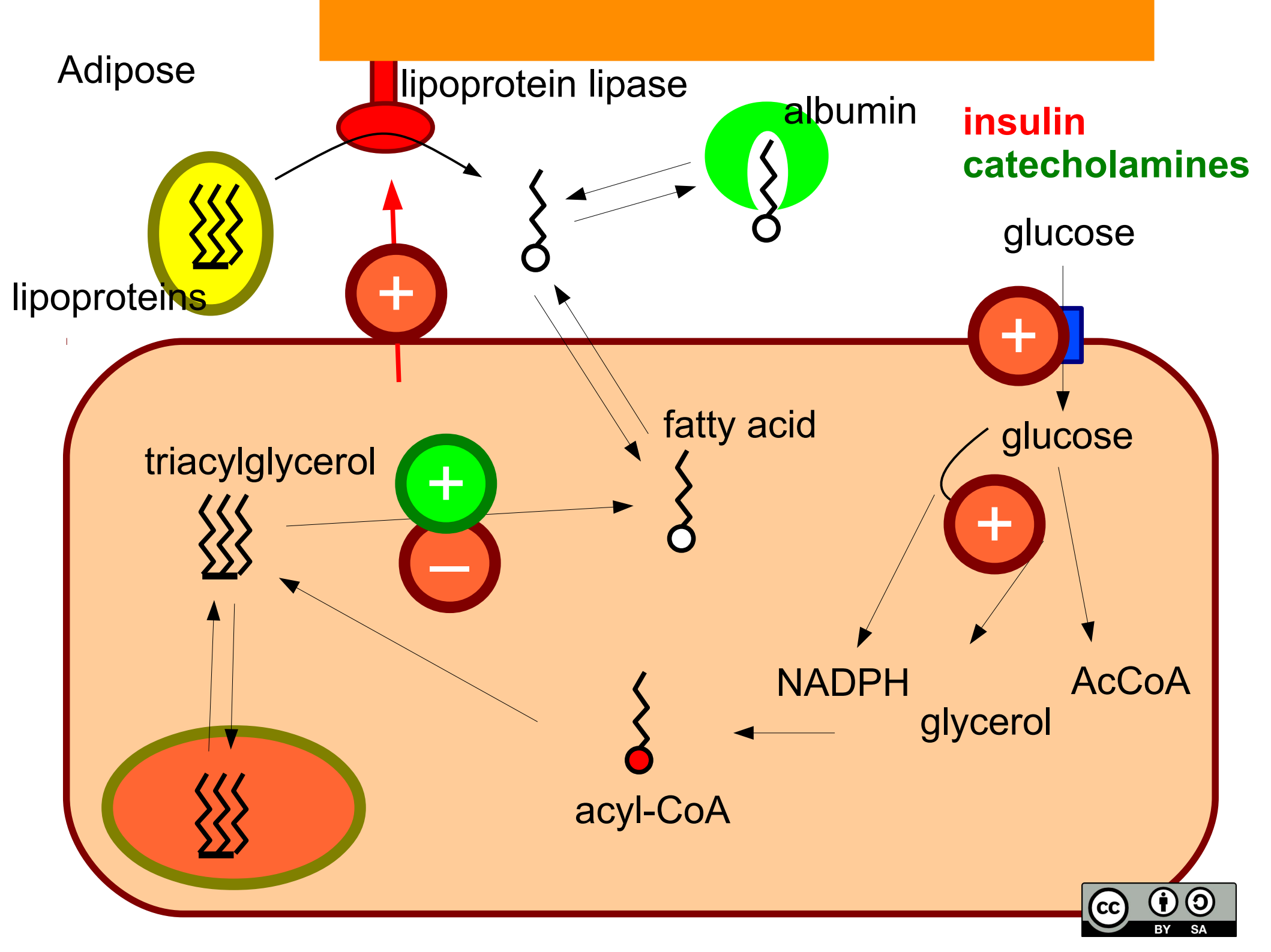
guanidinoacetate + SAM → creatine + SAH



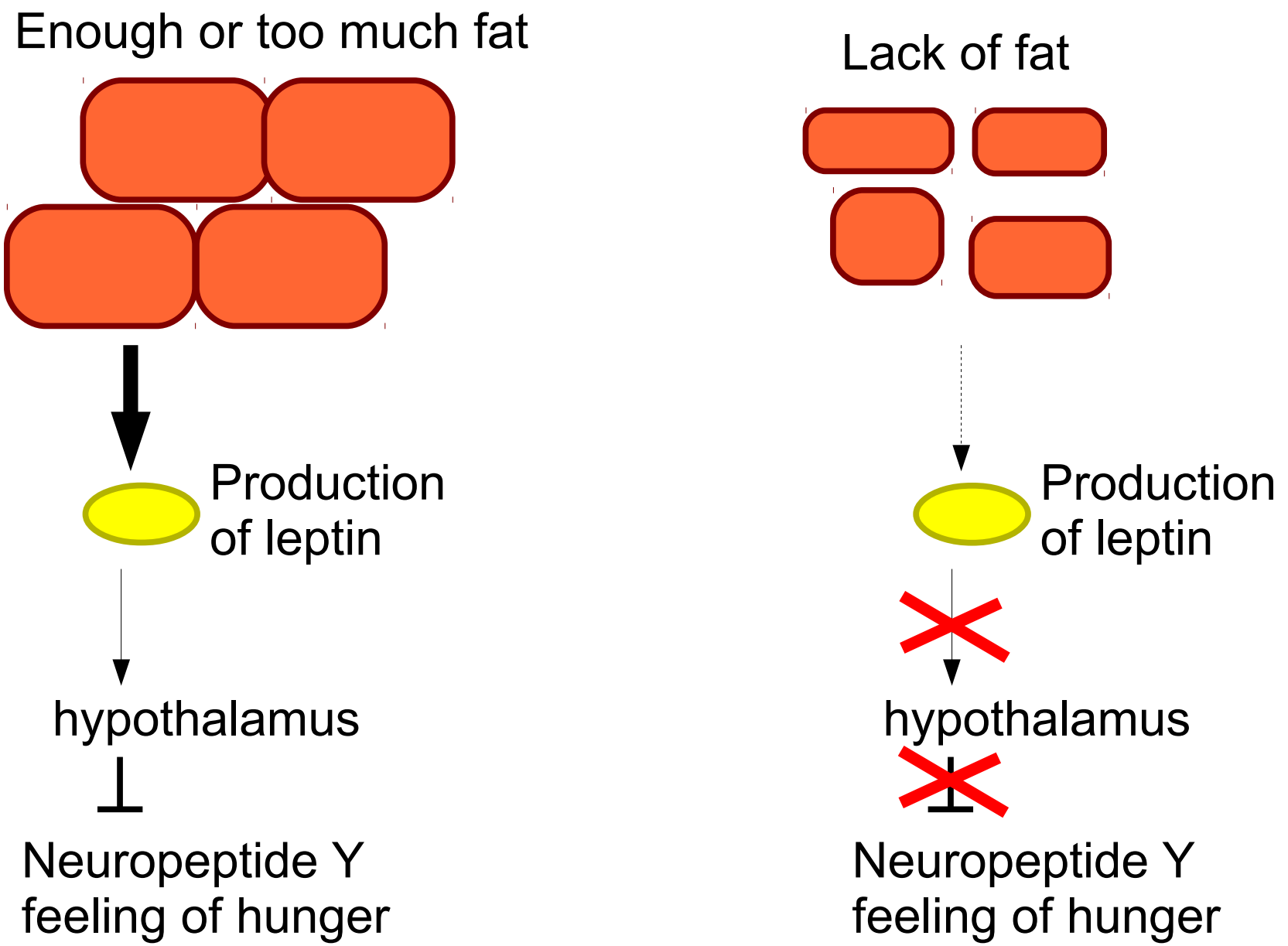
Skeletal muscles convert ATP to mechanical work. It receives the signal from neuron in the form of acetylcholine neurotransmitter. This activates ligand-gated ion channel (nicotinic receptor) which releases flow of Na+ into the cell. This releases Ca2+ from sarcoplasmatic reticulum (special endoplasmatic reticulum for Ca2+ storage) into cytoplasm. This activate actin-myosin system. Actin is rather static filamental protein. On the other hand, myosin is a molecular motor that can “walk” on actin. It is activated by Ca2+. While hydrolysing ATP it can make one step on actin filament.

Muscles need lot of energy and often do not have enough oxygen for this. To solve this they can produce lactate. Beside this they may store energy by reversible formation of phosphocreatin.


Adipose
lipoprotein lipase
albumin
lipoproteins
glucose
fatty acid
triacylglycerol
glucose
NADPH
AcCoA
glycerol
acyl-CoA


Adipose
lipoprotein lipase
albumin
insulin
catecholamines
lipoproteins
glucose
fatty acid
glucose
triacylglycerol
NADPH
AcCoA
glycerol
acyl-CoA

Adipose



Adipose

Insulin resistance:

obesity, high concentration of free fatty acids in blood

↓

switch from glucose metabolism to fatty acid metabolism in many tissues

↓

lack of absorption of glucose

↓

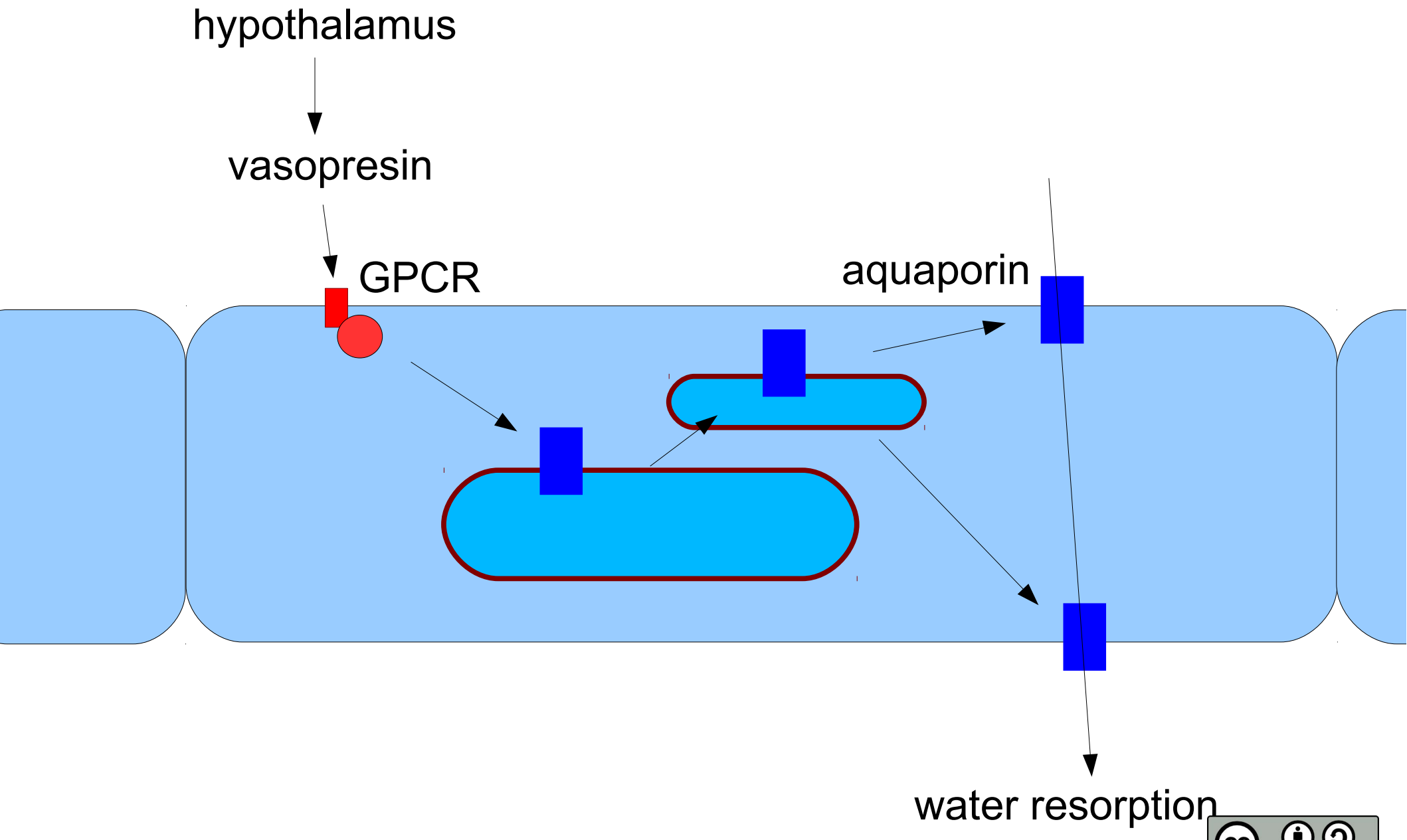
high glucose concentration in blood, type II diabetes

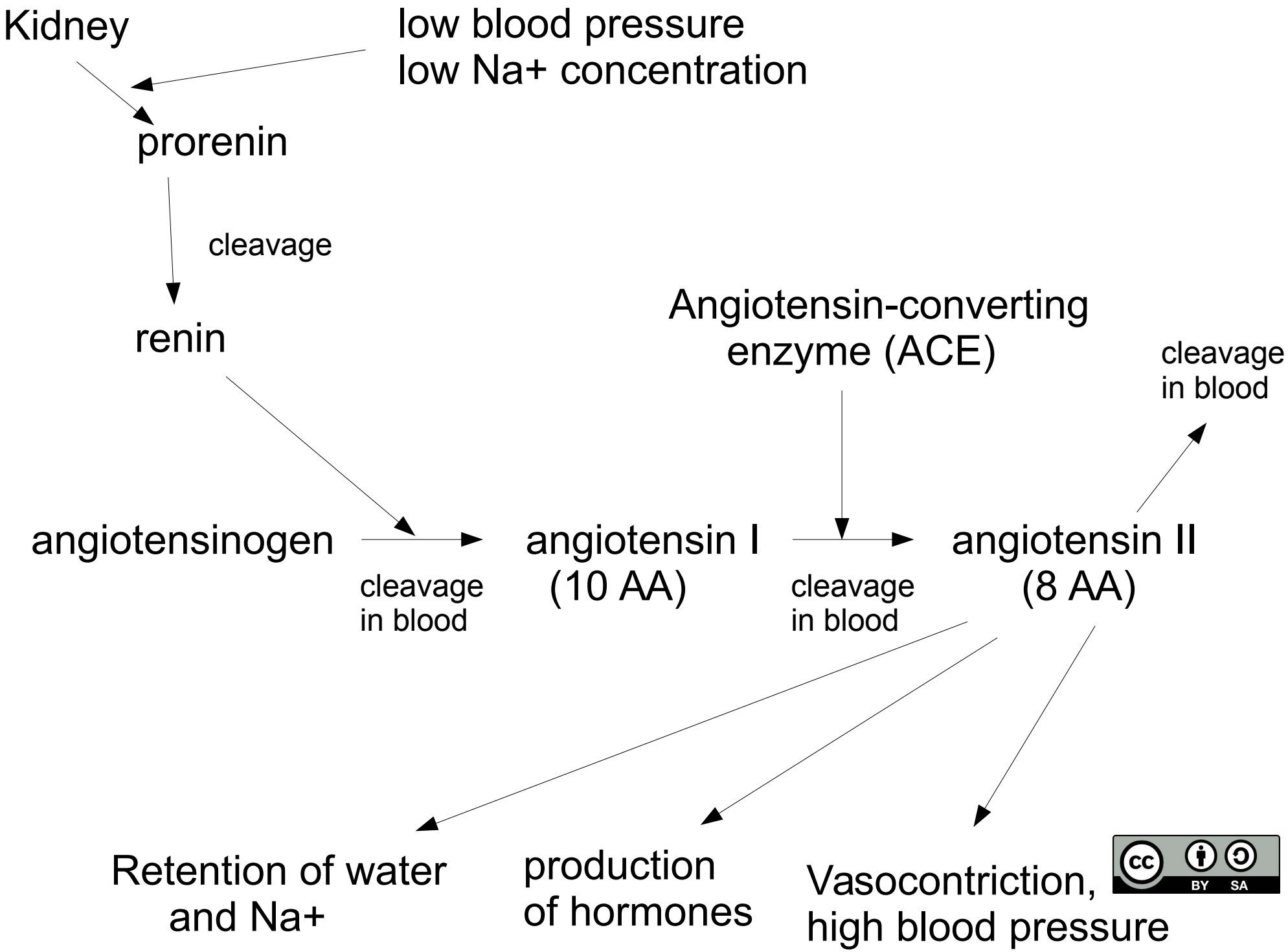
Adipose

Adipose tissues store lipids. It takes glucose from blood and convert it to AcCoA. It also produces NADPH by pentose phosphate pathway. Next it uses these two components to produce fatty acids and triacylglycerols. These are stored in lipid droplets stabilized by phospholipid monolayer and interface proteins. These triacylglycerols can be utilized by a lipase and fatty acids can be released to blood. Free fatty acids are produced in blood by lipoprotein lipase and they are immediately ingested by muscle or other cells and utilized as a source of energy. Free fatty acids can also circulate in blood bound to albumin. Insulin promotes intake and metabolism of glucose by adipose cells. It also enhances production of lipoprotein lipase. Catecholamines support cellular hydrolysis of triacylglycerols.

Adipose tissue plays also a hormonal role. For example it produces the hormone called leptin with hunger-inhibiting function. In healthy individuals there is a reasonable amount of fat in the adipose tissue. Its reduction by starving reduces amount of produced leptin, which in turn supports hunger. On the other hand, high amount of adipose produces high amount of leptin and inhibits hunger. This balances the amount of adipose tissue. This is also the reason why fast loss of fat may cause reduction of leptin, which triggers hunger. In contrast, ghrelin hormone produced by stomach causes hunger. Some stomach surgery used to treat obesity may reduce production of ghrelin and thus inhibit hunger.

Kidney




Kidney
low blood pressure
low Na+ concentration
prorenin
cleavage
renin
Angiotensin-converting
enzyme (ACE)
cleavage
in blood
angiotensinogen
cleavage
in blood
angiotensin I
(10 AA)
cleavage
in blood
angiotensin II
(8 AA)
Retention of water
and Na+
production
of hormones
Vasocontriction,
high blood pressure

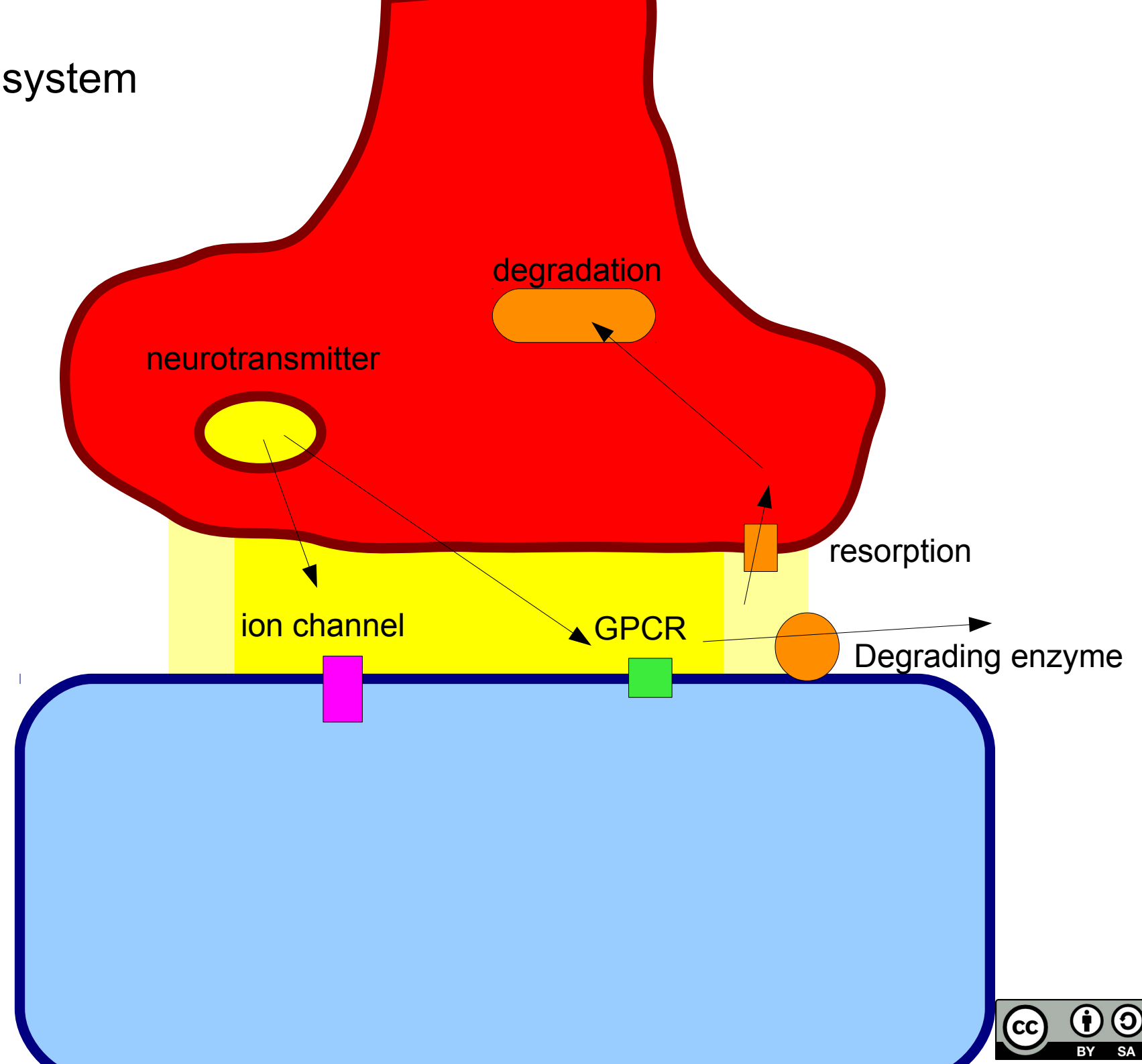
Kidney

Kidney filters blood. It separates cells and large proteins and retains them in blood. Water, small molecules and ions can pass. Next, they are resorbed by special mechanisms. Kidney play important role in acidobasic and osmotic homeostasis and blood pressure control.

Peptidic hormone vasopressin binds to its receptor (GPCR). The signal causes higher production and insertion to membrane of protein aquaporin, which transports water molecules (but not ions) through the membrane. Because of different concentrations of ions on different sides of membranes, water is resorbed.

Kidney is also related to renin-angiotensin system. Protein angiotensinogen is produced by liver. In blood it is cleaved by protease renin produced by kidney. This leads to a peptide angiotensin I. This can be further hydrolysed by angiotensin converting enzyme (ACE) to angiotensin II. Angiotensin II supports vasoconstriction and increases blood pressure. It also influences kidney and renin production. ACE is very important target of drugs used to treat hypertension.

Nervous system



Nervous system

Neurotransmitters

acetylcholine	+/–	M,I	CNS and PNS	AChEsterase
glutamate	+	I	CNS	resorption
glycine	–	I	CNS	resorption
GABA	–	I	CNS	resorption, metab.
dopamine	+/–	M	CNS and PNS	resorption, metab.
noradrenalin	+/–	M	CNS and PNS	resorption, metab.
adrenalin	+/–	M	CNS and PNS	resorption, metab.
serotonin	+/–	M,I	CNS	resorption, metab.
histamine	+/–	M	CNS	resorption, metab.
endorphins, enkephalins				
dynorphins	–	M	CNS	cleavage
adenosine, ATP	helper	M	CNS	resorption

+ activatin
– inhibiton
I ionotropic (Na+, K+, Cl–)
M metabolotropic (cAMP, Ca2+)

Nervous system

Neurotransmitters

Paralytic gasses
antialzheimer
drugs

acetylcholine	+/–	M,I	CNS and PNS	ACh
glutamate	+	I	CNS	resorption
glycine	–	I	CNS	resorption
GABA	–	I	CNS	resorption, metab.
[illegible]	+/–	M	CNS and PNS	[illegible]
[illegible]	+/–	M	CNS and PN	[illegible]
[illegible]	+/–	M	CNS and PN	[illegible]
serotonin	+/–	M,I	CNS	[illegible]
histamine	+/–	M	CNS	resorption, metab.

Amphetamins, antidepressants

Amphetamins, kokain

Amphetamins

antidepressants

[illegible]phalins

opiates

[illegible]	–	M	CNS	cleavage
[illegible]	helper	M	CNS	resorption

coffein

+ activatin
– inhibiton
I ionotropic (Na+, K+, Cl–)
M metabolotropic (cAMP, Ca2+)

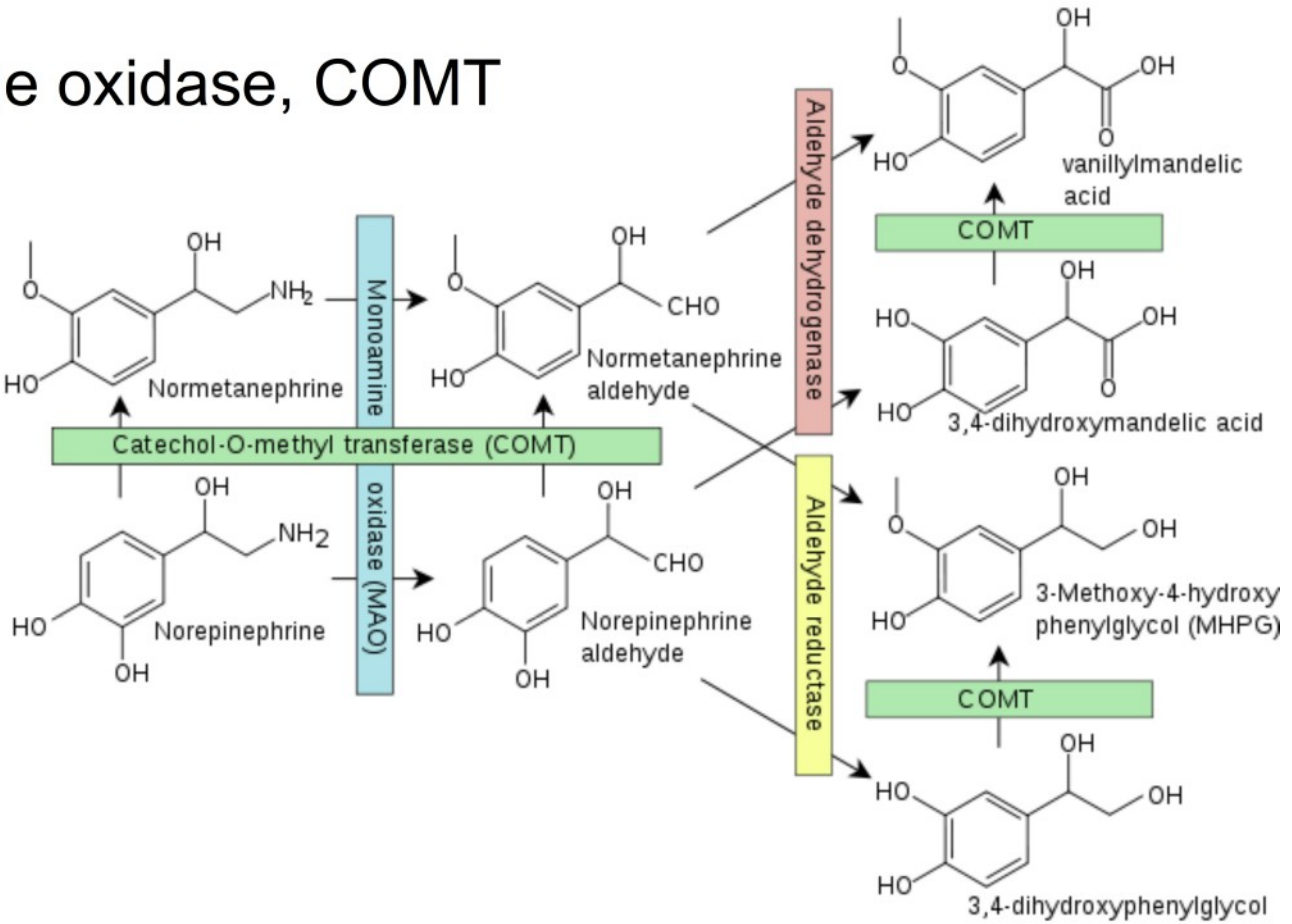
Nervous system

Neurotransmitter degraders

Acetylcholine esterase

acetylcholine + $H_2O \rightarrow$ acetate + choline

monoamine oxidase, COMT



Nervous system

A signal is transduced within a neuron in form of electric current as explained in the lecture dedicated to receptors. Transduction of a signal from one neuron to another neuron or to other target cell is facilitated by neurotransmitters. In general, the neurotransmitter is stored in the neuron in vesicles. Activation by electric signal causes that these vesicle fuse with the membrane and release its content into the space between cells (synaptic cleft). On the target cell it bind to receptors (GPCRs or ion channels) and activates other mechanisms. Often it is necessary to terminate this signalling. Otherwise once activated cell would be active for a long time. Termination of the signal can be done by degradation of the neurotransmitter, by its resorption or by combination of both processes. Acetylcholine esterase act directly in the synaptic cleft by hydrolysing acetylcholine. Monoamine oxidase and catechol-O-methyltransferase metabolize neurotransmitters (catecholamines) inside the neuronal cell.

Many chemical compounds interfere these processes. For example neurotoxic gasses inhibit acetylcholine esterase and thus prolong neuronal signalling by acetylecholine. Cocaine prolong the effect of dopamine by inhibiting its reuptake. Amphetamines activate some GPCRs, inhibit reuptake of catecholamines and may also inhibit metabolism of catecholamines. Some other drug of abuse, such as opiates or LSD, act directly on GPCRs.

Hormones

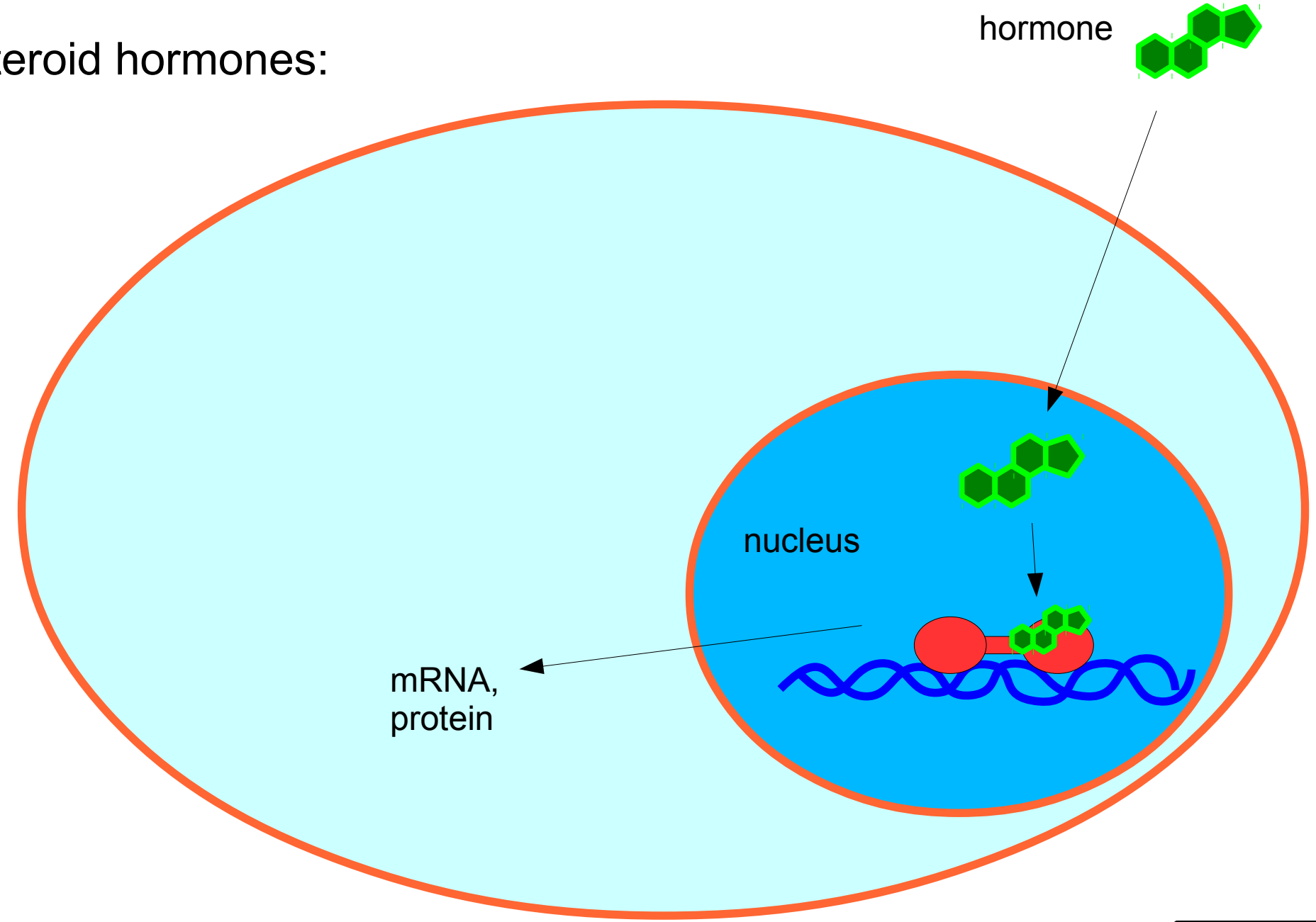
Chemistry:
- proteins, peptides, steroids, amino acid derivatives

Role:
- metabolism, growth, differentiation, homoeostasis, digestion etc.

Distance of action:
- endocrine, paracrine, autocrine

Hormones

Steroid hormones:



Hormones

Steroid hormones:
Corticosteroids – produced by adrenal cortex
- cortisol – lipolysis, proteolysis, gluconeogenesis, immunosuppressive
- aldosterone – function of kidney, reabsorption of Na+

Sexual steroids:
♂ Androgens – testosterone
♀ Estrogens – estradiol
♀ Gestagens – progesterone

Hormones

Peptide hormones:
Insulin – 30+21 amino acids – glycogen metabolism
Glucagon – 29 amino acids – glycogen metabolism
Angiotensin II – 8 amino acids – blood pressure
Vasopressin – 9 amino acids – blood pressure, water resorption
Oxytocin – 9 amino acids – uterine contraction, milk ejection
Growth hormone, ACTH, Ghrelin, Neuropeptide Y, ...

Other hormones:
Thyroxine – thermogenesis, basal metabolism, embryonic development

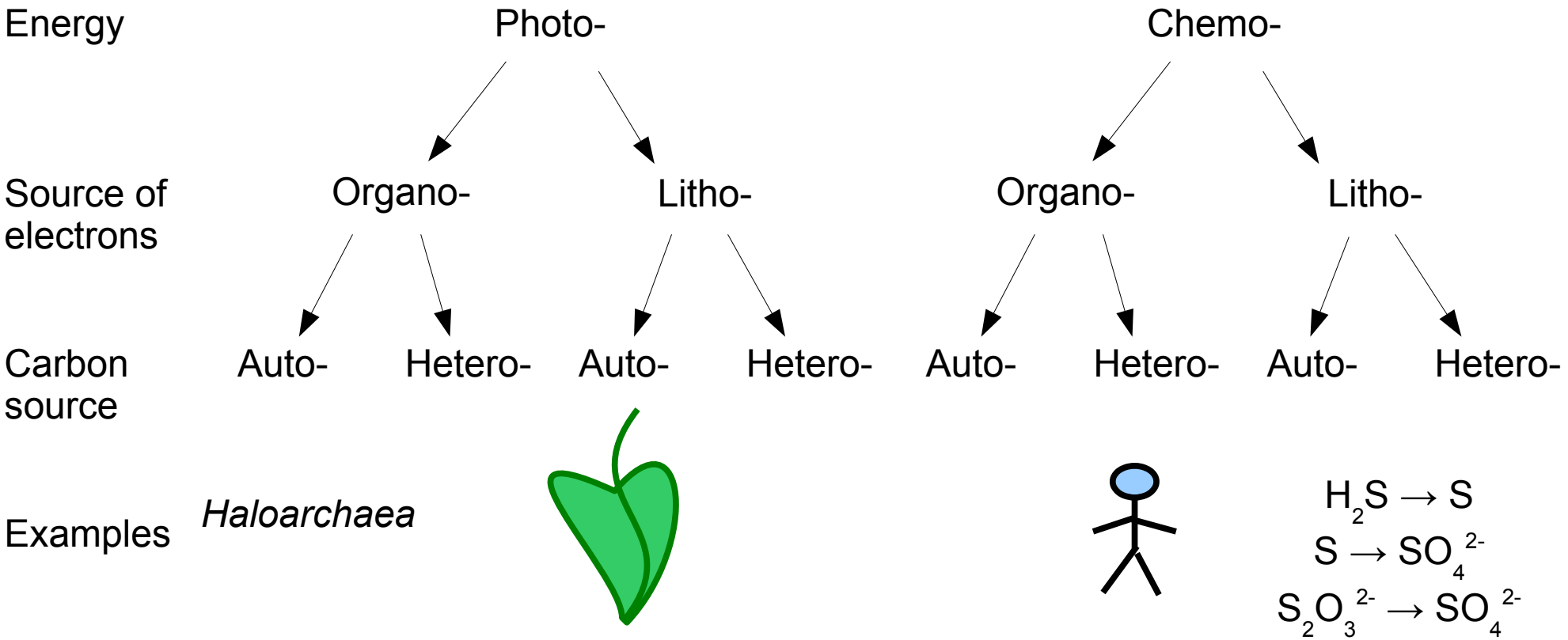
Hormones

Hormones have been discovered as compounds present in blood with some effect on physiology of some organs. From the chemical point of view they are diverse. They include proteins, peptides, steroid hormones and amino acid derivatives. Protein and peptide hormones usually influence target tissues by binding to membrane receptors, mostly GPCRs (glucagon, angiotensin, oxytocin, vasopressin and many others) or less commonly receptor protein kinases (insulin). Steroid hormones use completely different mechanism. These compounds are so hydrophobic that they can spontaneously pass through the membrane and reach nucleus. Here they bind to special receptors and directly regulate transcription.

Biochemistry of microorganisms and plants

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY



$H_2S \rightarrow S$

$S \rightarrow SO_4^{2-}$

$S_2O_3^{2-} \rightarrow SO_4^{2-}$

$NH_3 \rightarrow NO_2^-$

$NO_2^- \rightarrow NO_3^-$

$Fe^{2+} \rightarrow Fe^{3+}$

$CH_4 \rightarrow CO_2$

$CH_3OH \rightarrow CO_2$

Organisms can be classified based on energy source, source of electrons or source of carbon. Humans are chemo-, organo- and heterotrophs. They cannot use photosynthesis, they oxidize organic compounds and they eat other organisms. Plants are photo-, litho- and autotrophs. Chemolithotrophs oxidize inorganic compounds. Some organisms are capable of anaerobic respiration, i.e. they can use inorganic compounds other than oxygen as acceptors of electrons (oxidating agents). *Haloarchaea* use a primitive photosynthesis to produce ATP, but not NADPH.

Fermentation

Fermentation is anaerobic metabolism of sugars. Most common chemoorganothrophs,including humans, metabolize sugars (e.g. glucose) under aerobic conditions to water and carbon dioxide. This produces lot of energy in the form of ATP. However, this is not possible under anaerobic conditions. Fermentation process is chemically speaking a disproportionation reaction, i.e. redox reaction converting the substrate into one oxidized and one reduced product. For example yeast convert glucose to reduced ethanol and oxidized carbon dioxide.

Glycolysis produce little energy in the form of ATP (two molecules per one molecule of glucose). Moreover, pyruvate as the terminal product of glycolysis is oxidized relative to glucose. Glycolysis therefore requires conversion of NAD^+ to NADH. Absence of oxygen or other mechanism oxidizing NADH would lead to accumulation of NADH, lack of NAD^+ and termination of glucose metabolism. It is necessary to oxidize NADH by other mechanism.

Intensively working human or animal muscle, erythrocytes or some cancer cells use NADH to reduce pyruvate to lactate.

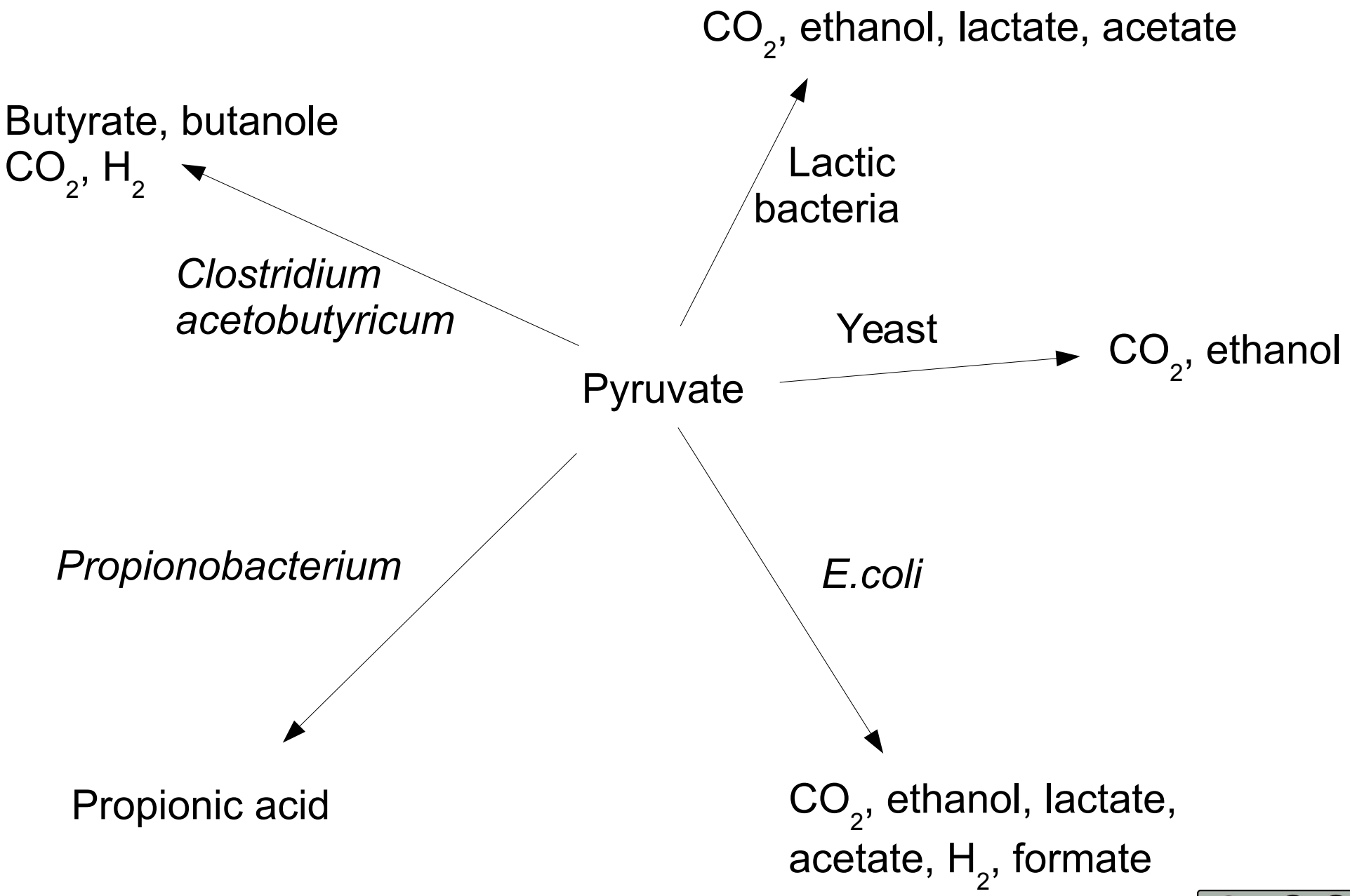
Yeast reduce acetaldehyde to ethanol.

E. coli and some other bacteria produce acetate, formate and/or hydrogen.

Propionobacterium produces propionate.

Clostridium acetobutylicum produces butanol.

Fermentation



Fermentation
Yeast

Side products:

biacetyl

CH_3-CO-CO-CH_3

acetoin

CH_3-CO-CHOH-CH_3

butandiol

CH_3-CHOH-CHOH-CH_3

Pyruvate decarboxylase requires thiamine diphosphate. As an alternative product it can produce biacetyl, acetoin or buthandiole. These compounds are sometimes favoured, sometimes disfavoured in fermented products.

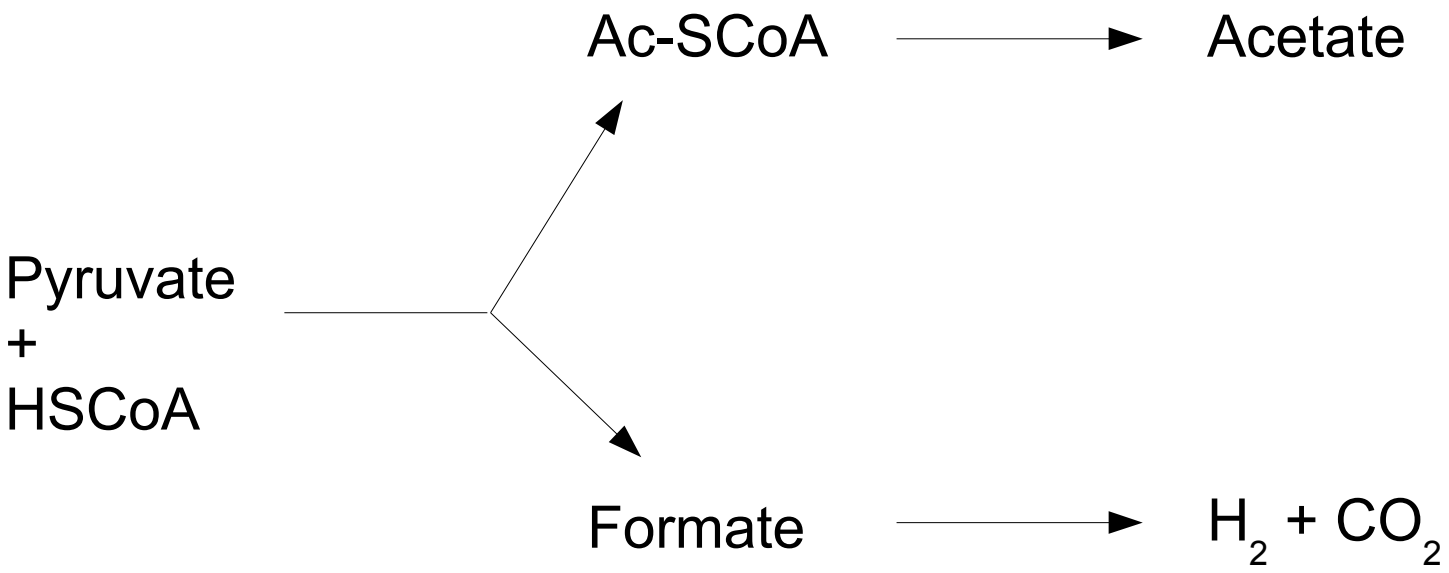
Fermentation
Lactic acid bacteria

Lactic acid bacteria ferment glucose to lactate in similar way as human muscles under anaerobic conditions. This is done by *Lactococcus, Enterococcus, Streptococcus* and *Pediococcus*.

Leuconostoc or *Weissella* use partial pentose phosphate pathway. They convert glucose to Glc-6-P. This is oxidized to 6-phosphogluconate and then decarboxylated to pentose-5-phosphate. This molecule is decomposed into glyceraldehyde-3-P and acetyl phosphate. Glyceraldehyde-3-P goes to truncated glycolysis to form lactate. Acetyl phosphate is reduced to ethanol.

Some lactic acid bacteria produce peptidic antibiotics bacteriocins.

Fermentation
E.coli



E. coli and similar gut microorganism growing on a low-sugar and high-protein (or high-peptide, high-amino-acid) medium stinks. When growing on sugar-rich medium it produces acetate and formate, so it smells like something pickled. This is also used to identify microorganisms in microbiological diagnostics. *E. coli* and similar organisms are often tasted based on their ability to hydrolyse oligosaccharides. For example, *E. coli* can hydrolyse lactose. When growing on a medium containing lactose it produces acids and this causes change of colony colour due to presence of acidobasic indicator. In contrast, *Salmonella* cannot ferment lactose, so it metabolizes protein hydrolysates producing basic amines.

Fermentation
Propionobacterium

Pyruvate → oxalacetate → malate → fumarate

↓

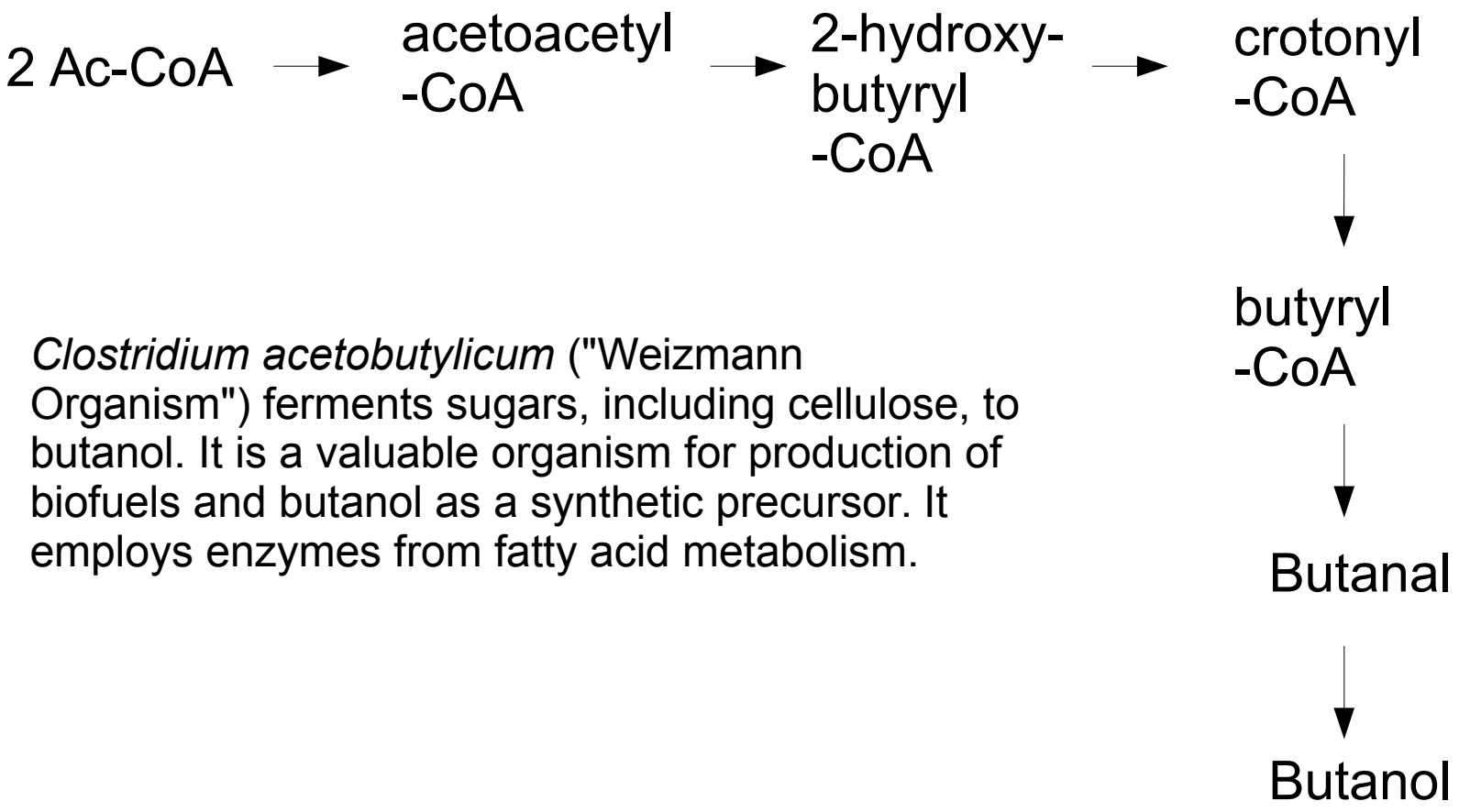
succinate

↓

Propionate + CO_2

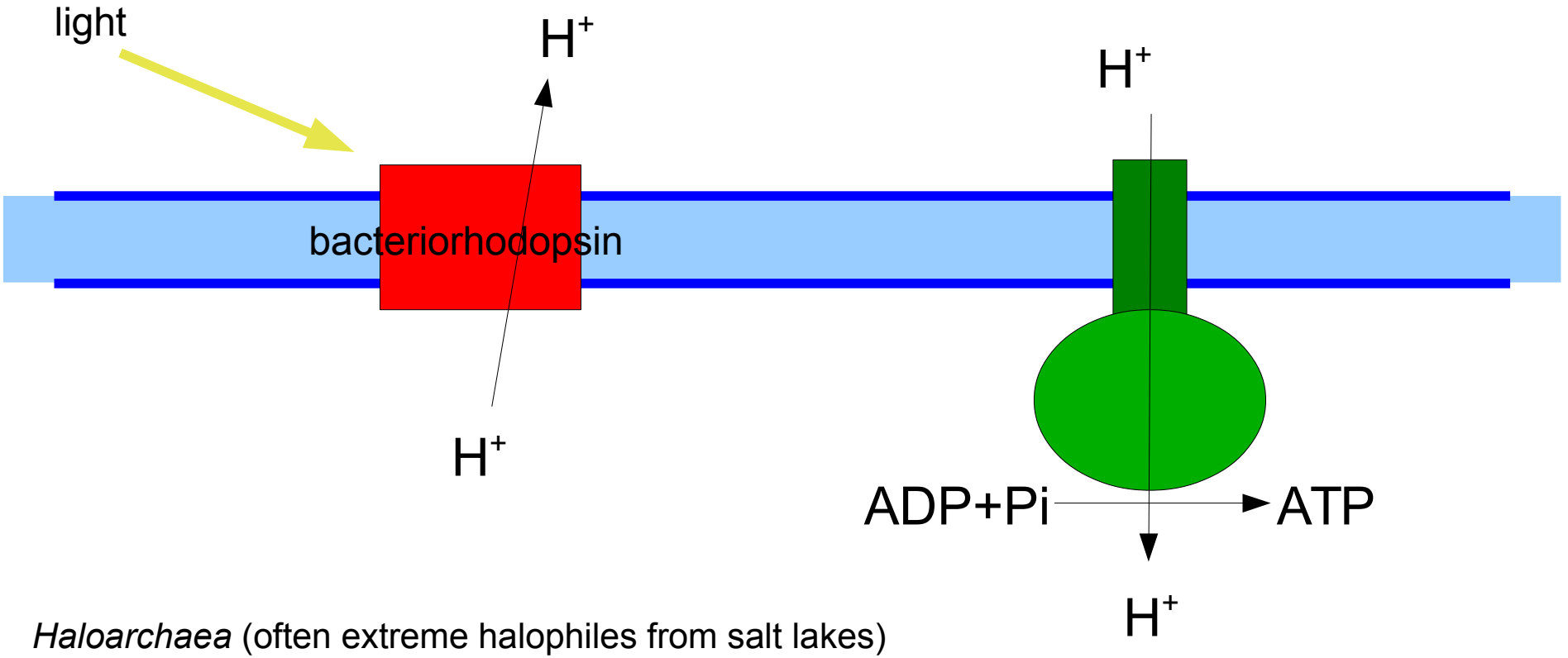
Propionobacterium uses some reactions from the Krebs cycle to produce succinate, which is in turn decarboxylated to propionate. This compound is found in some types of cheese, especially in Emmental, due to fermentation by this microorganism.

Fermentation
Clostridium acetobutylicum



Clostridium acetobutylicum ("Weizmann Organism") ferments sugars, including cellulose, to butanol. It is a valuable organism for production of biofuels and butanol as a synthetic precursor. It employs enzymes from fatty acid metabolism.

Photosynthesis
Haloarchaea



Haloarchaea (often extreme halophiles from salt lakes) can run a primitive photosynthesis. Light is converted by bacteriorhodopsin to produce proton motive force, which is used to produce ATP. Unlike plants they do not photolyze water and produce NADPH. Membranes of these microorganims are packed with bacteriorhodopsin, which is in a quasi-crystalline form. This enabled determination of its structure in 1990.

Photosynthesis

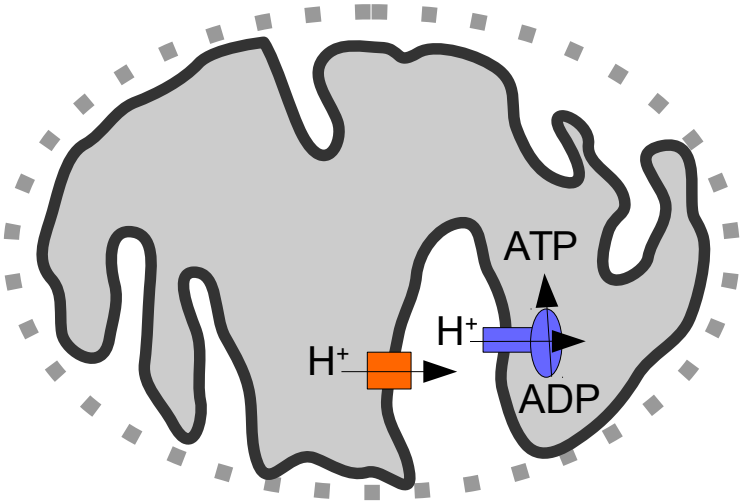
There is a widely accepted theory that mitochondria used to be a prokaryotic microorganism that invaded some pro-eukaryotic cell, started to live there in a symbiosis and later lost most of its genetic information.

Beside physical similarity of mitochondria and prokaryotic cell it is also mitochondrial genome with few intronless genes that supports the theory.

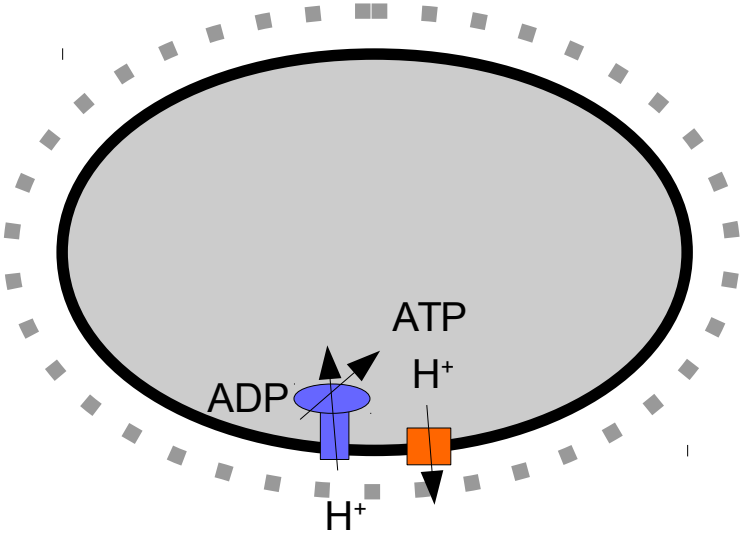
Mitochondria pump protons across its inner membrane into the intermembrane space. Protons return via F_OF_1-ATPase producing ATP.

Also prokaryots pump protons out. Protons produce energy on their way back.

mitochondrion



prokaryotic cell



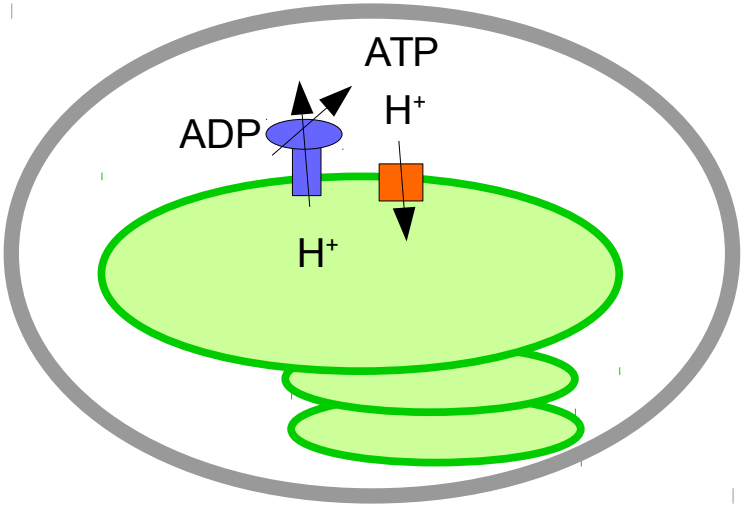
Photosynthesis

Analogously, chloroplasts are believed to be former photosynthetic prokaryots. Here, comparison with *Halloarcheum* is provided.

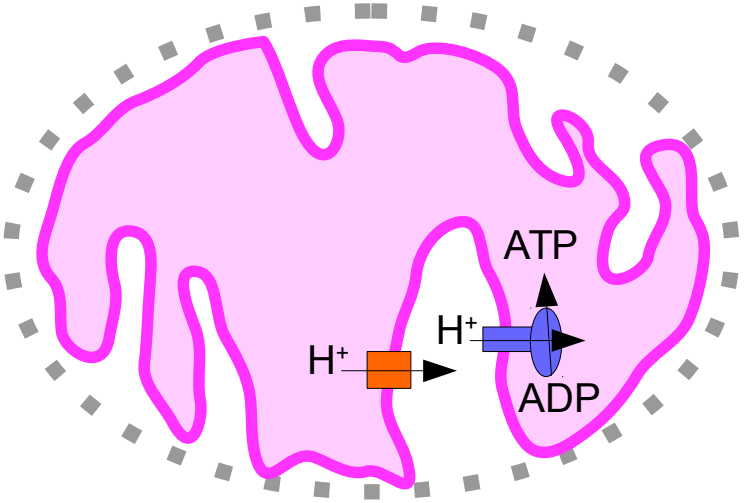
Chloroplast also has its own genome.

In contrast to mitochondria, chloroplasts contain disk-shaped membrane compartments called thylakoids. Protons are not pumped outside chloroplasts, instead they are pumped into thylakoids. Thylakoids are arranged to maximize the surface for efficient harvesting of light. The interior of thylakoid is called lumen.

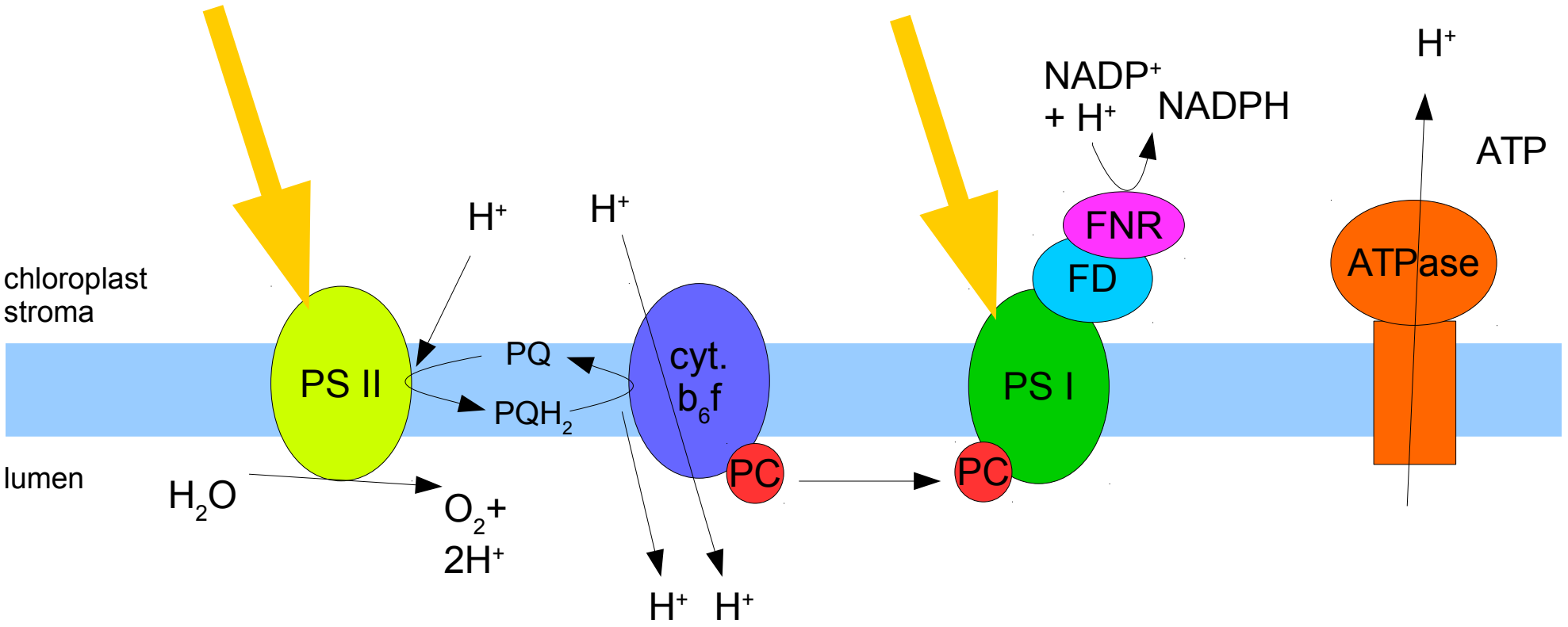
chloroplasts



Halloarcheum

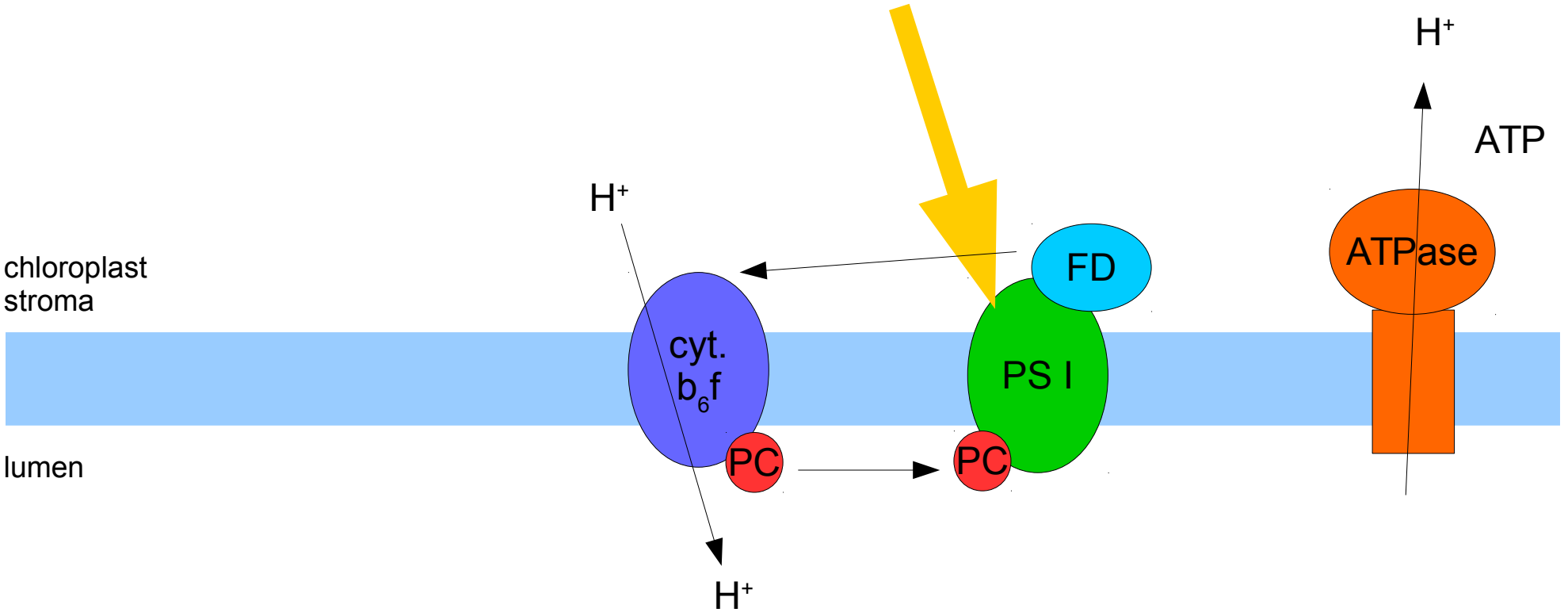


Photosynthesis
Plants, alga



In plant non-cyclic photosynthesis photons are captured by photosystem II. The energy is used to photolyze water to oxygen and H^+. Electrons are used to reduce plastoquinone (plant analogy to Q10). This travel to cytochrome b6f where electrons are transferred to plastocyanine (plant analogy to cytochrome C). These electrons travel to photosystem I where other photons are captured and finally they are used to reduce $NADP^+$ to NADPH. Protons transported across the membrane are used to produce ATP by ATPase.

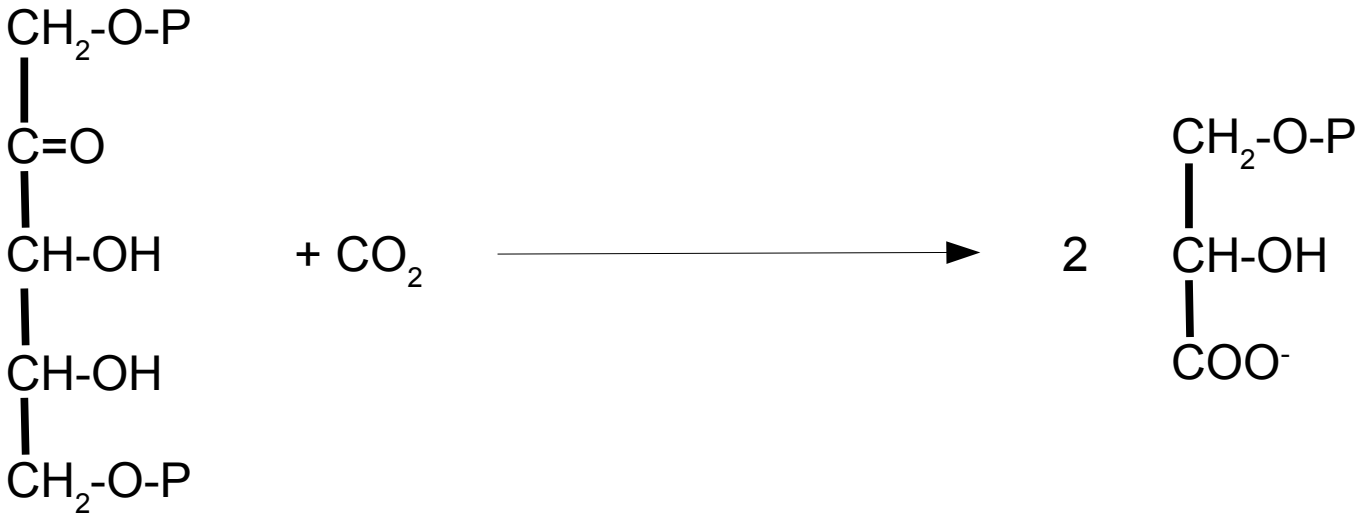
Photosynthesis
Plants, alga



In plant cyclic photosynthesis photons are captured only by photosystem I and electrons travel from cyt. b6f and PS I. Protons transported across the membrane are used to produce ATP by ATPase, but no water is photolysed and no NADPH produced. Plants can regulate it to fulfil their ATP and NADPH needs.

Photosynthesis
Plants, alga

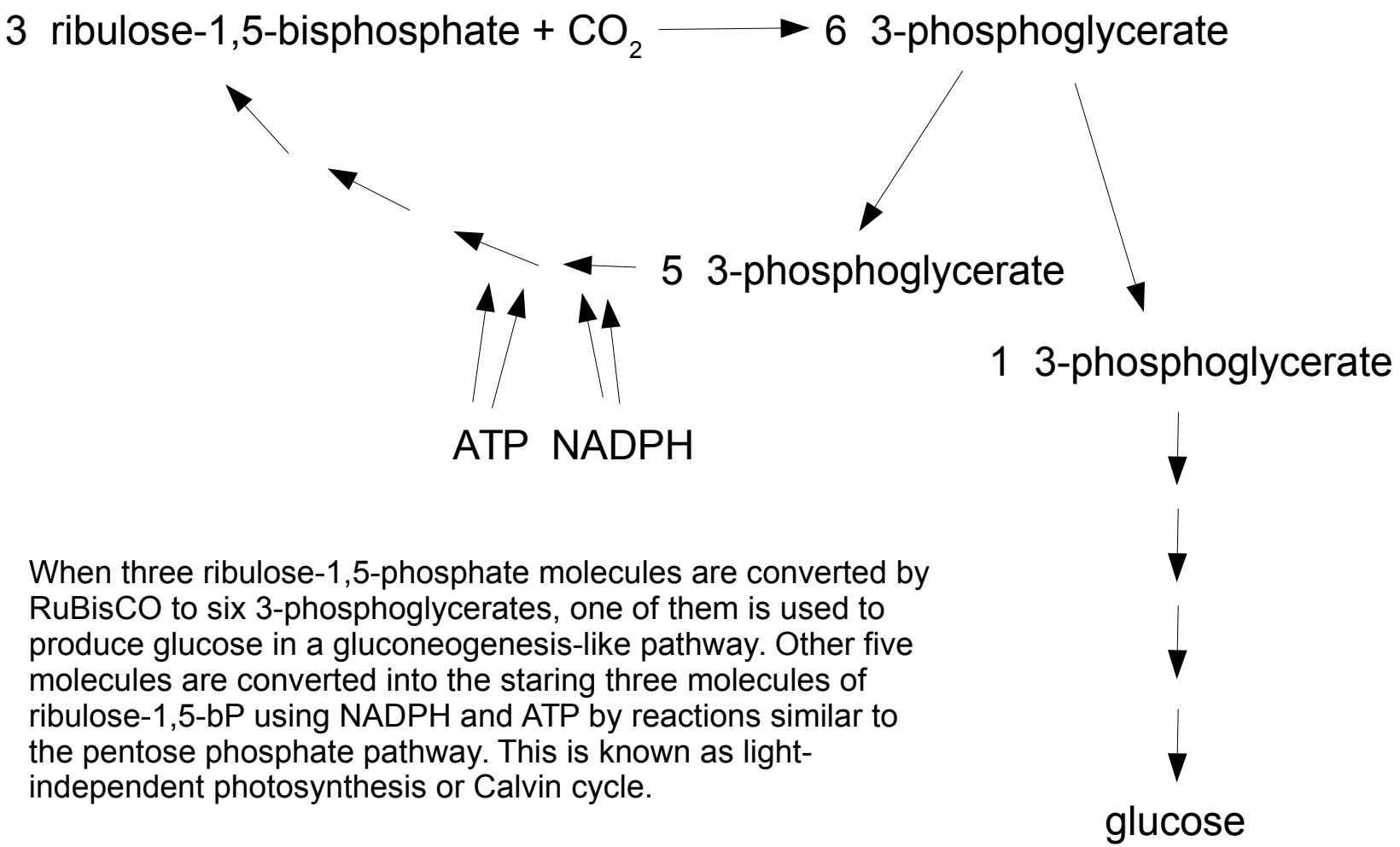
Ribulose-1,5-bisphosphate carboxylase/oxygenase – RuBisCO



RuBisCO is the most common protein on Earth. It catalyses carboxylation reaction (C in RuBisCO, depicted) or oxygenase reaction (O in RuBisCO, producing 3-phosphoglycerate and phosphoglycolate)

Photosynthesis
Plants, alga

Calvin cycle



When three ribulose-1,5-phosphate molecules are converted by RuBisCO to six 3-phosphoglycerates, one of them is used to produce glucose in a gluconeogenesis-like pathway. Other five molecules are converted into the staring three molecules of ribulose-1,5-bP using NADPH and ATP by reactions similar to the pentose phosphate pathway. This is known as light-independent photosynthesis or Calvin cycle.

Photosynthesis
Plants, alga

Photorespiration

RuBisCO may produce one 3-phosphoglycerate and one phosphoglycolate by its alternative oxygenase reaction. The role of this reaction is bit enigmatic, if there is any role. The process recovering phosphoglycolate is known as photorespiration.

Phosphoglycolate is hydrolysed to glycolate and then transported to cytoplasm. There it is oxidized to glyoxylate. By condensation with ammonium it produces glycine. Two glycine molecules produce serine in mitochondria. Serine is converted to glycerate and finally 3-phosphoglycerate.

Photorespiration causes loss of energy (up to 25 %), produced hydrogen peroxide and makes other negative impacts. The reasons for photorespiration is unclear. Regulatory, redox homeostasis and other explanations have been proposed.

Photosynthesis
Plants, alga

Photorespiration

Oxygenase reaction of RuBisCO can be reduced by high concentration of carbon dioxide. Some plants, called C4 and CAM plants, use different tricks to do this.

Most plants are called C3 plants. The name comes from the product of carbon dioxide fixation, which is 3-phosphoglycerate with 3 carbons.

C3 plants do not have any special mechanism to increase carbon dioxide concentration in cells. The level of photorespiration is high. These plants cannot adapt to hot and dry areas.

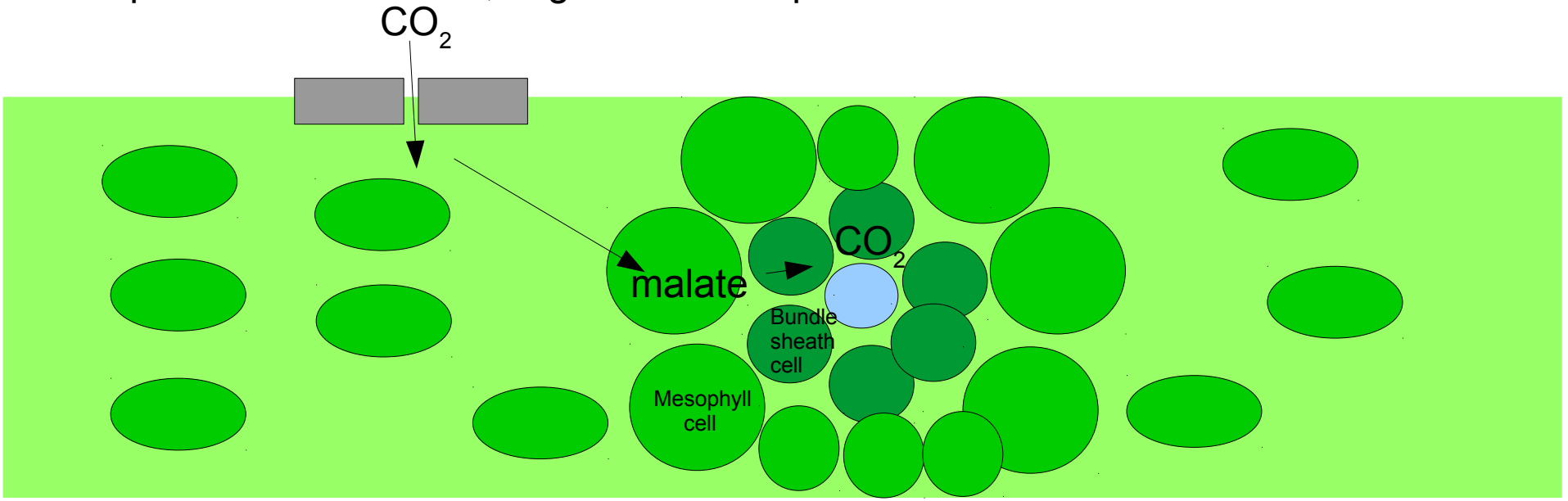
It is possible to recognize sugars produced by C3 and C4 plants from isotopic signatures due to kinetic isotope effects in reactions catalysed by corresponding enzymes. It is for example possible to reveal adulteration of maple syrup (C3) by saccharose from corn or sugar cane (C4).

Photosynthesis
Plants, alga

Photorespiration

C4 plants separate carbon fixation and RuBisCO reaction in the space. At some cells they catalyse reaction of carbon dioxide with phosphoenolpyruvate catalysed by phosphoenolpyruvate carboxylase. This forms oxalacetate. Oxalacetate (C4) is reduced to malate and transported to other cells where it serves as a source of carbon dioxide. These pathways may differ (some involve aspartate).

C4 plants include maize, sugar cane or rape.



Photosynthesis
Plants, alga

Photorespiration

CAM (Crassulacean acid metabolism) plants separate carbon fixation and RuBisCO reaction not only in the space, but also in time. In night these plants open stomata (“air vents”) and accumulate CO2 to form malate. Malate is stored in vacuols.

During the day plants close their stomata. CO2 is released from malate and used in photosynthesis.

CAM plants are tropical plants such as pineapple.

Gene technologies I

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Tools of gene technologies

1. Restriction enzymes
   Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.
2. Other enzyme tools
   Other enzymes in gene technologies include DNA ligase, reverse transcriptase and others.
3. Polymerase chain reaction
   Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.
4. Sequencing
   Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.
5. Microarrays
   Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.
6. Gene cloning
   Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.
7. RNAi
   RNA interference can be used to specifically knock out a gene.
8. Gene editing
   CRISPR/Cas system can be used in gene editing to knock out or modify a gene.
9. Gene therapy
   Modification of human genome for disease treatment.

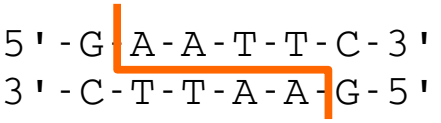
Tools of gene technologies

1. Restriction enzymes

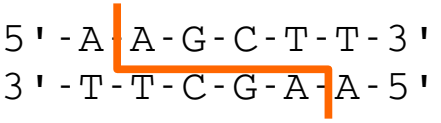
Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.

Examples:

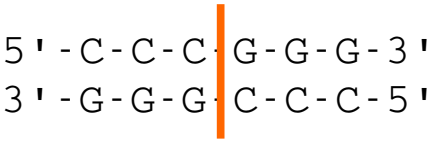
*Eco*RI (from *E. coli*)



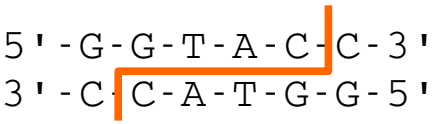
*Hind*3 (from *Haemophilus influenza*)



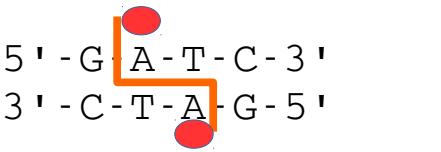
*Sma*I (from *Serratia marcescans*)

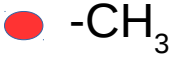


*Kpn*I (from *Klebsiella pneumoniae*)



*Nde*I (from *Diplococcus pneumoniae*)





Tools of gene technologies

1. Restriction enzymes

Restriction enzymes (restriction endonucleases) are commonly used to digest DNA in specific sites for analytical as well as preparatory purposes.

Various bacteria have evolved mechanism to specifically cleave viral DNA. They specifically cleave DNA containing certain sequence. At the same time they mask such sequences in their own DNA by methylation, binding of proteins etc. Restriction endonucleases most commonly used in research are type II restriction endonucleases. They recognize a palindomatic sequence and they cleave both strands within this sequence. They are abbreviated by the name of organism, e.g. *Eco*RI is from *E. coli*, *Bam*HI from *Bacillus amyloliquefaciens* etc. They are often produced recombinantly. They are supplied as solutions with high concentration of glycerol, so they are liquid at –20 ºC and they can be repeatedly used without thawing and freezing.

Practically, they are used to verify a sequence. If we know the sequence of some fragment, but we are not sure, it is possible to predict size of fragments produced by cleavage by endonuclease and compare them with sizes determined by electrophoresis (usually agarose gel electrophoresis). Restriction enzymes can be used to specifically cleave DNA into fragments, that can be combined by DNA ligase or other enzymes into a longer constructs. This can be used in gene cloning.

*Eco*RI (from *E. coli*) and *Hind*3 (from *Haemophilus influenza*) are examples of enzymes producing sticky 5'-overhanging ends. *Sma*I (from *Serratia marcescans*) is an example of enzymes producing blunt ends. *Kpn*I (from *Klebsiella pneumoniae*) is an example of enzyme producing sticky 3'-overhanging ends. *Nde*I (from *Diplococcus pneumoniae*) cleaves only methylated DNA.

Tools of gene technologies

2. Other enzyme tools

Other enzymes in gene technologies include DNA ligase, reverse transcriptase and others.

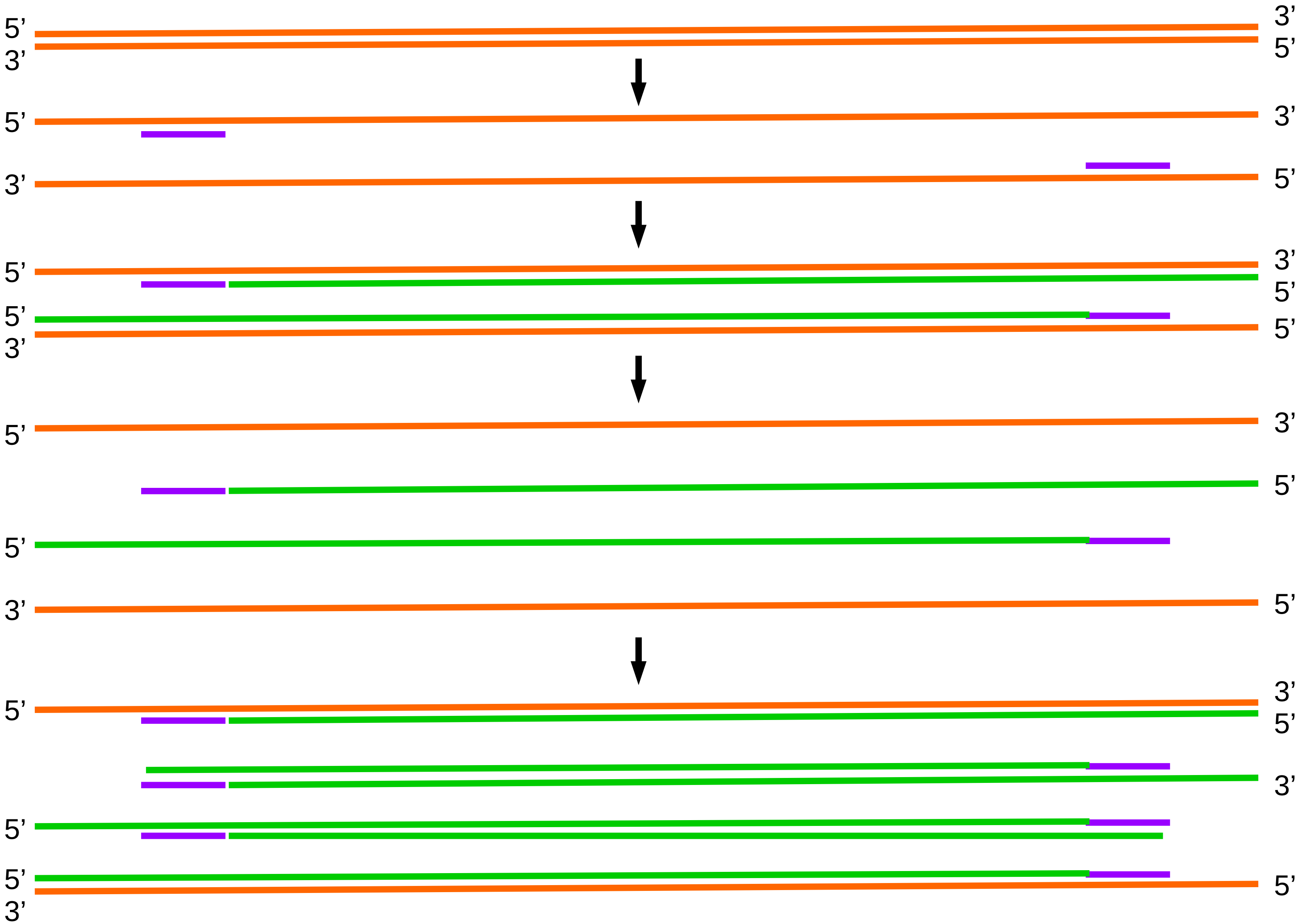
DNA ligase joints two fragments of DNA. Energy required for this is obtained by cleavage of ATP. Natural role of this enzyme is in DNA replication or repair. Typically T4-phage DNA ligase is used in laboratory practice. DNA ligase can easily join two fragments with complementary sticky ends, typically fragments produced by the same enzyme. It can also join two fragments with blunts ends, but this is more complicated and different protocol is needed.

Reverse transcriptase (RNA-directed DNA polymerase) is a viral enzyme produced by retroviruses. It can be used to rewrite RNA into a complementary DNA. It is usually applied in sequencing, cloning and quantification of mRNA, but also in other applications. For example, most amino acid sequences of protein were not determined by direct sequencing of proteins. Instead, mRNA was converted to a complementary DNA (cDNA) by reverse transcriptase, isolated and sequenced. The protein sequence was then obtained by simple translation. The cDNA can be inserted into a suitable vector for recombinant production of the corresponding protein.

Tools of gene technologies

3. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.



Tools of gene technologies

3. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.

DNA polymerases are used by cell in DNA replication, repair and other processes. Very often they have not only 5'→3' polymerase but also the 3'→5' and 5'→3' exonuclease activity for "proofreading" and "nick translation". First DNA polymerase used to amplify DNA in vitro was Klenow fragment (DNA pol I from *E. coli* cleaved by protease to avoid 5'→3' exonuclease activity. This enzyme can synthesize the second strand to a single stranded DNA. Unfortunately it cannot survive denaturation of DNA by high temperature, because high temperature also denaturates the enzyme.

This problem was solved by introduction of thermostable enzymes from thermophilic microorganisms. PCR requires a DNA sample (template), DNA polymerase, magnesium cations, primers (short synthetic oligonucleotides defining the start and the end of the amplified DNA) and building blocks (dATP, dTTP, dGTP and dCTP). It is done in cycles (typically tens) comprising 1. denaturation of DNA by high temperature, 2. annealing of primers onto the template, 3. extension of primers by DNA polymerase. All these steps are done in programmable thermostats (thermocyclers). Amplified DNA is selected by design of primers. Various companies sell custom primers for quite low costs.

PCR can be used to detect (e.g. in forensics or diagnostics), amplify (e.g. isolate gene from an organism), quantify, label, mutate or shuffle DNA.

Tools of gene technologies

3. Polymerase chain reaction

Polymerase chain reaction (PCR) is one of most common DNA technology. It can be used to detect, isolate, quantify or label nucleic acids in research, diagnostics, forensics, biotechnologies and other fields.

PCR can be made quantitative by different approaches. For example, the amount of the product can be monitored by a fluorescent dye that binds onto a double-stranded and is fluorescent only when bound. The level of fluorescence indicates the concentration of the product and can be used to estimate the concentration of the template.

Alternatively, it is possible to exploit the 5'→3' exonuclease activity. Beside the two primers, another oligonucleotide complementary to the middle of the amplified fragment is added to the reaction mixture. This oligonucleotide is labeled by a fluorescent dye and a quencher of fluorescence. This oligonucleotide is not fluorescent because of proximity of both moieties. Cleavage by the exonuclease activity releases the fluorescent dye which becomes really fluorescent. This phenomena is known as fluorescent resonance energy transfer (FRET).

Quantitative PCR is used to quantify certain biological material in another biological material (e.g. contamination and adulteration of one food by another food) or in diagnostics. It is also used together with reverse transcription to quantify RNA, mostly mRNA.

Tools of gene technologies

4. Sequencing

Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.

Template

```
5′-GCGCATGCGCCAATTATAGCTACGATCGATCGGCATCCGATCCGCT-3′
3′-CGCGTACGCGGTTAATATCGATGCTAGCTAGCCGTAGGCTAGGCGA-5′
```

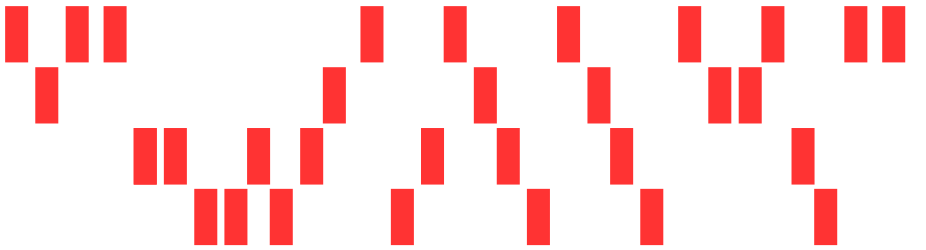
Primer

```
 5′-CGCATG-3′
3′-CGCGTACGCGGTTAATATCGATGCTAGCTAGCCGTAGGCTAGGCGA-5′
```

Products with ddCTP

```
CGCATGC●
CGCATGCGC●
CGCATGCGCC●
CGCATGCGCCAATTATAGC●
CGCATGCGCCAATTATAGCTAC●
CGCATGCGCCAATTATAGCTACGATC●
CGCATGCGCCAATTATAGCTACGATCGATC●
CGCATGCGCCAATTATAGCTACGATCGATCGGC●
CGCATGCGCCAATTATAGCTACGATCGATCGGCATC●
CGCATGCGCCAATTATAGCTACGATCGATCGGCATCC●
```

ddCTP
ddGTP
ddATP
ddTTP



Tools of gene technologies

4. Sequencing

Determination of nucleic acid sequence underwent a huge progress since its introduction, especially in terms of throughput and price per base.

The first DNA sequencing was done by Sanger using DNA polymerase, radioactively labeled primers and dideoxynuclotide triphosphates (ddNTP). The reaction was carried out in parallel in four test tubes. Each contained a template, labeled primer, dNTP and one of ddNTP (ddATP, ddTTP, ddGTP and ddCTP). ddNTP cannot be extended and causes termination of DNA synthesis. Finally, each reaction is analysed by electrophoresis. Termination by specific nucleotides makes it possible to read the DNA sequence.

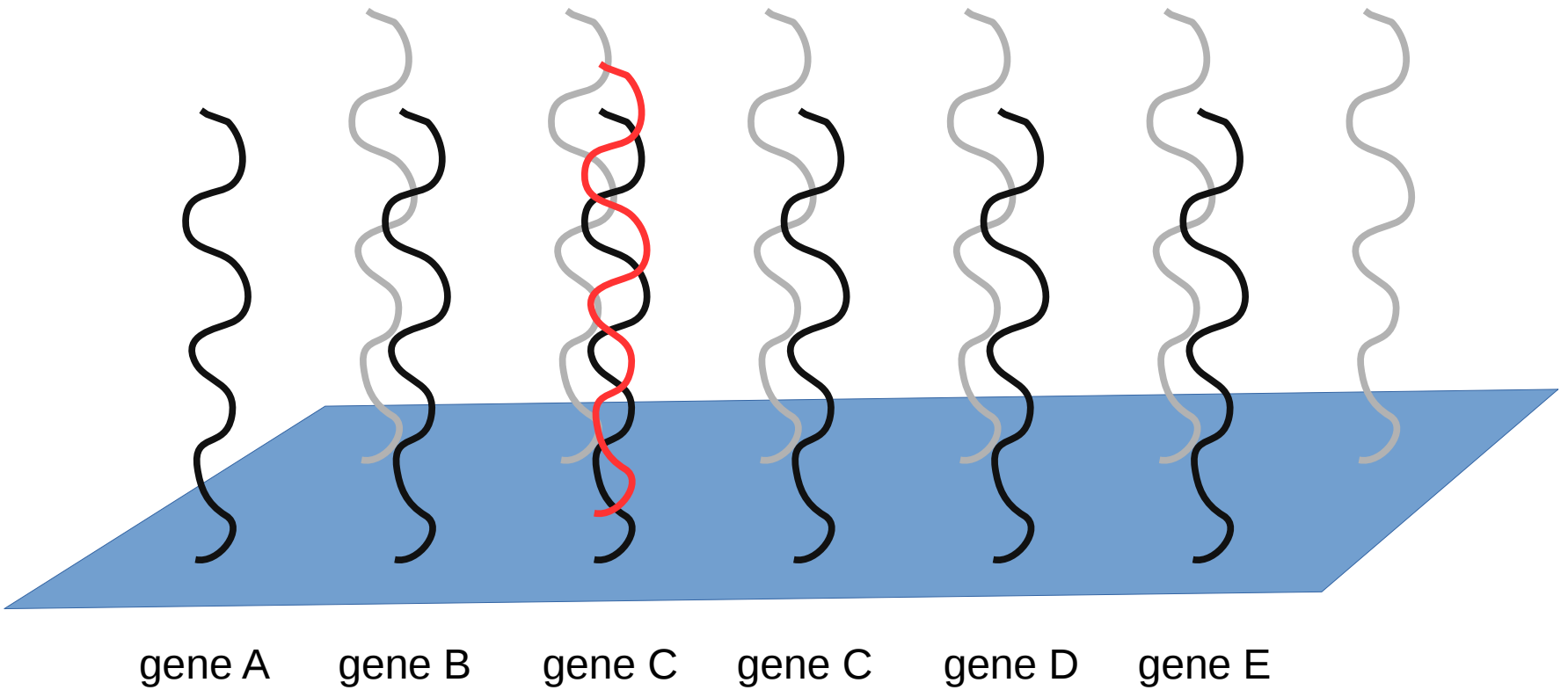
DNA sequencing was later improved by fluorescent labeling (replacement of each dNTP by a color-coded terminating building block) and capillary electrophoresis. This technology was used to sequence human genome.

In last decade, new parallel techniques were introduced. These techniques provide high throughput by parallelization of sequencing reactions. They use reversible fluorescently labeled terminators (Illumina), detection of polymerase reaction using luciferase (454 system) of electrochemistry (Ion Torrent). DNA-polymerase-independent technologies include DNA-ligase-based sequencing (SOLiD) and nanopore-based sequencing (MinION). These modern technologies made it possible to sequence with much higher throughput and at much lower costs.

Tools of gene technologies

5. Microarrays

Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.



Tools of gene technologies

5. Microarrays

Microarrays have been used to parallel measurement of concentration of specific nucleic acids, especially mRNA.

DNA microarrays (or DNA chips) have been used to determine concentration of various nucleic acids in parallel. A DNA microarray contains numerous spots. Each spot contains a different DNA with known sequence. The sample with labeled nucleic acids is loaded onto the microarray. DNA on the microarray hybridizes with the DNA in the sample. The chip is then scanned to measure intensity of label on each spot. This intensity is proportional to the concentration of the nucleic acid in the sample.

DNA microarrays can be produced by separate synthesis of DNA probes and their application on the microarray surface using micropipetting. Alternatively, DNA probes can be synthesized in situ by photochemically activated DNA synthesis.

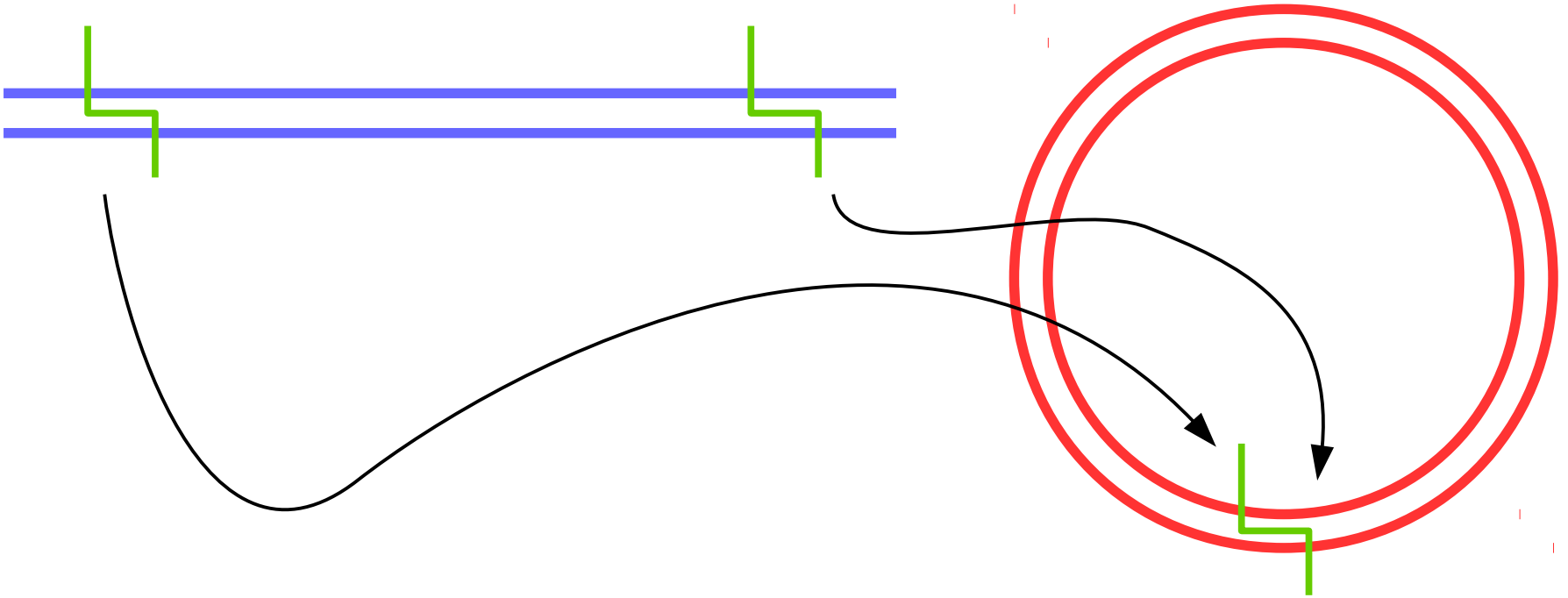
DNA microarrays are mostly used to measure mRNA concentration (gene expression). mRNA is first converted into cDNA and labeled by reverse transcriptase and then hybridized onto a DNA microarray. Less frequently it can be used to measure DNA content (e.g. to study genome instability in cancer) or to identify DNA molecules recognized by proteins by their coprecipitation by antibodies.

Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

DNA can be isolated directly from an organism (by use of proteases and RNases, detergents, heat, chromatography etc.) or by PCR. Next, it can be digested by a restriction endonuclease. Also a vector DNA can be digested by the same restriction endonuclease. These fragments have compatible sticky ends so they can be ligated by DNA ligase. Alternatives for a DNA ligase reaction exist. The resulting construct can be introduced into an organism.



Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

DNA can be used to produce proteins. Isolation of a protein from a natural source is often very difficult or impossible. First, natural material is often difficult to obtain. Second, a cell contains hundreds or thousands of proteins. Complicated series of chromatographic and other separations are necessary. The result can be hampered by losses due to protein instability. As an alternative, the gene coding a protein can be inserted into a suitable vector and produced recombinantly. Usually a molecule of mRNA is converted to cDNA and then inserted to vector to avoid problems with splicing. Prokaryotic expression systems provide high yields, but they are not good for “spoiled” proteins (difficult to fold, with post-translational modifications etc.). As an alternative, it is possible to use yeast (*Pichia*), insect (*Spodoptera*) or mammalian (e.g. chinese hampster ovary – CHO) cells. Protein isolation can be simplified by adding a sequence tag at N- or C- terminus of the protein. Oligo-His, maltose-binding protein (MBP) or glutathion-S-transferase (GST) are most popular sequence tags. They can be isolated by affinity for nickel ions (oligo-His), amylose (MBP) or glutathion (GST). The tag can be cleaved.

Gene technologies are used to produced enzymes or antibodies used in biotechnology or as drugs (biotech drugs).

Tools of gene technologies

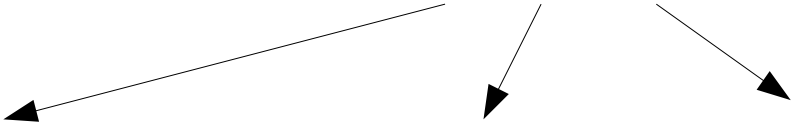
6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

Recombinant antibodies are biotech drugs with high potetnial

Nomenclature:

Alemtuzumab



-tu-	-u-	-mab
different malignancies	human	monoclonal antib.
-col- = colorectal	-o- = mouse	
-mel- = melanoma	-a- = rat	
-mar- = breast cancer	-e- = hamster	
-pr(o)- = prostate cancer	-i- = primate	
-lim- = immune system	-xi- = chimera	
-bac- = bacterial inf.		
-cir- = cardiovascular		

Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

Biotech drugs

Insulin – porcine (1923), chemically or enzymatically humanized (seventies) and recombinant (1982) hormone for diabetes treatment

Bevacizumab (Avastin) – recombinant humanized mouse monoclonal antibody against VEGF (vascular endothelial growth factor) for treatment of colon cancer (inhibits angiogenesis)

Infliximab (Remicade), Adalimumab (Humira) – humanized mouse and human, respectively, antibody against TNF-α (tumour necrosis factor) for treatment of rheumatoid arthritis and other autoimmune diseases

Etanarcept (Enbrel) – fusion protein of TNF-α receptor and Fc fragment of antibody

Epoetin α (Epogen, Procrit, Eprex, Espo) – recombinant human erythropoietin for treatment of anaemia

Tools of gene technologies

6. Gene cloning

Isolation of DNA (e.g. gene) from one organism and its insertion to another organism is nowadays routinely used in research, biotechnology and other areas.

Other special applications of gene cloning include:
Generation of fusion proteins – two or more proteins can be fused into a single polypeptide chain for research purposes

Fusing with green fluorescent protein (GFP) – example of fusion is fusion with GFP or other fluorescent proteins. GFP is naturally produced by a jellyfish. It can be used to label proteins and to visualize them or track their trafficking or degradation in the cell.

Yeast two-hybrid system – This is a special system to detect protein-protein interactions

Mutagenesis – mutation, random or specific, can be introduced into a gene in a suitable vector

Tools of gene technologies

7. RNAi

RNA interference can be used to specifically knock out a gene.

Neutralization of mRNA by a complementary DNA or RNA used to be a popular as a tool to reduce expression of genes. When doing similar experiments, Craig C. Mello and Andrew Fire found that it is not necessary the neutralizing strand, but it is a double-stranded RNA which causes reduction of gene expression by triggering its degradation. The double stranded RNA is recognized and cleaved by a RISC complex.

This phenomenon can be used to selectively and reversibly knock-down a gene of interest. This can be used to develop model organisms in order to test hypothesis on drug targets. For example, if researchers want to treat some disease by inhibition of an enzyme, they can use RNAi to mimic enzyme inhibition by knocking-down the gene coding the enzyme. It is been also being tested in clinical trials to treat viral diseases or cancer.

Tools of gene technologies

8. Gene editing

CRISPR/Cas9 system can be used in gene editing to knock out or modify a gene.

It was found that bacterial DNA contains repetitive sequences (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR). They are formed by repeating alternation of variable and conserved blocks. Later it was found that variable blocks are similar to DNA of viruses infecting bacteria. It was shown that this is a bacterial adaptive immune system! Bacteria infected by a virus can digest and incorporate a piece of its DNA into its genome into CRISPR. These fragments are inherited to the offspring of the bacteria. The CRISPR codes short RNA molecules (guide RNA – gRNA) containing a variable and constant part of each repeat. In case of viral infection this RNA can bind to a viral DNA and target it to Cas9 nuclease.

It can be used to specifically knock-out the gene by introducing the gRNA into the cell or into the genome. Several technologies have been developed to modify (introduce deletions, insertions or modifications) into the targeted gene.

This technology is currently used to modify DNA of various organisms or to correct DNA in therapy.

Tools of gene technologies

9. Gene therapy

Modification of human genome for disease treatment.

Gene therapy has been considered for a long time as a viable strategy to treat various diseases. It is possible to replace nonfunctional gene (due to its absence or mutation) by a functional one. It is also possible to introduce genes neutralizing unwanted genes. It is also possible to introduce a “suicide” gene into the diseased tissue.

Unfortunately the progress of gene therapy was very slow. This is due to fact that introduction of some gene into sensitive areas of genome can cause other complications, even a cancer. This was the case of the clinical trial for treatment of X-linked severe combined immunodeficiency in 2003. Several patients treated by this therapy developed T-cell leukemia. Since then, specificity of gene delivery was improved. Several gene therapies have been approved since 2016 (Strimvelis, Tisagenlecleucel, Yescarta, Luxturna).

Gene technologies II

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Omics projects

1. Genomics
   Determination of genome sequence

2. Exomics and transcriptomics
   Determination of mRNAs and their concentrations

3. Proteomics
   Determination of protein variants and their concentrations

4. Lipidomics, glycomics, ...
   Determination of lipid or carbohydrate composition or composition of other classes of molecules.

5. Metabolomics
   Determination of metabolite composition

Omics projects

1. Genomics

    Determination of genome sequence

First genome was sequenced in 1981 (mitochondrial genome)

Haemophilus influenzae genome was sequence in 1995

Yeast *S. cerevisiae* genome was sequenced in 1996

Human genome was sequenced 1990 – 2003, but it was continuously precised till 2007.

Before introduction of next generation sequencing sequenced genomes included: human genome, genomes of important model organisms (*E. coli*, yeast, *Arabidopsis thaliana*, *Caenorhabditis elegans*), important pathogens and extremophiles.

Knowledge of human genomes make is possible to understand human diseases.

Knowledge of genomes of model organisms makes it possible to design experiment employing these species.

Knowledge of genomes of pathogens makes it possible to identify their weak points for antibacterial or antiviral therapy.

Knowledge of genomes of extremophiles makes it possible to identify, clone and produce novel enzymes and other proteins, for example thermostable ones.

Omics projects

1. Genomics

    Determination of genome sequence

Next-generation sequencing made genomics relatively cheap. The number of microbial genomes has grown to tens of thousands. It also introduced genomics of individuals or tissue-targeted and even single-cell genomics.

For human genome and genomes of common animals go to: http://www.ensembl.org

For other species go to:

- http://metazoa.ensembl.org
- http://plants.ensembl.org
- http://fungi.ensembl.org
- http://protists.ensembl.org
- http://bacteria.ensembl.org

Omics projects

1. Genomics

    Determination of genome sequence

Conventional genomics isolates DNA from certain organism and sequences it.

Metagenomcs was developed as an alternative. It isolates DNA from some environment and sequences it, no matter from which organism it comes from.

The advantage of metagenomcs is that many organisms are hardly or not cultivable, nevertheless their DNA can be isolated.

Metagenomcs has been applied on various environments such as soil, ocean water, extreme environments, contaminated environments etc. This can be used to determine which organisms live in such environments and how microbial composition evolves and responds to external stimuli.

Metagenomics can be also used to find some new enzymes. Does not matter that we do not know the latin name of the source.

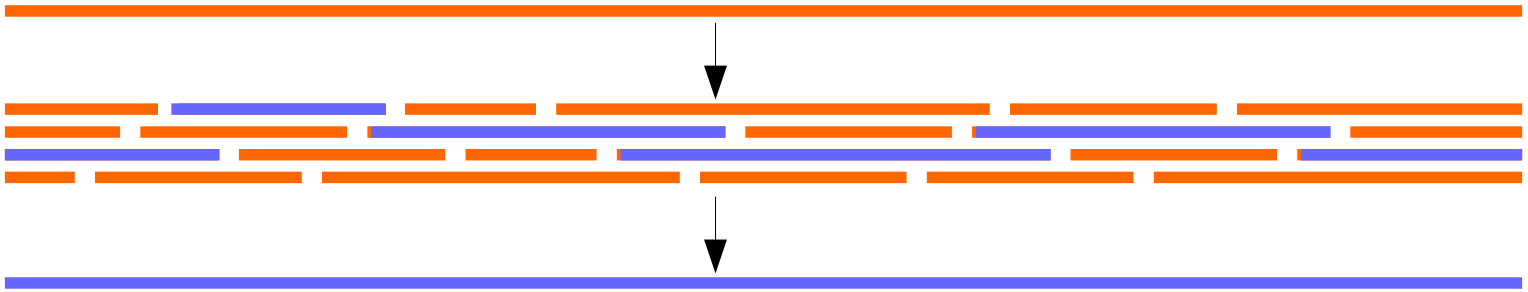
Metagenomics also shapes the research in human microflora, especially gut microflora.

Omics projects

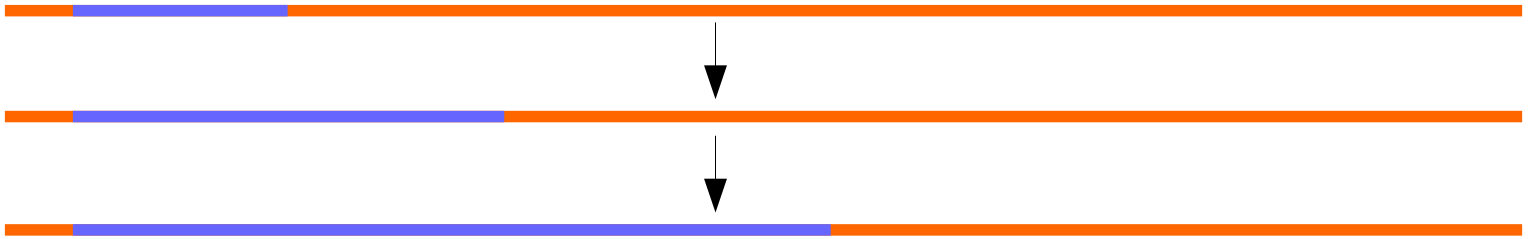
1. Genomics

Determination of genome sequence

Shotgun



Walking



Omics projects

1. Genomics

    Determination of genome sequence

DNA sequencing technologies can sequence only relatively short reads (hundreds of base pairs). In order to sequence longer DNAs it is necessary to use one of strategies described bellow.

Shotgun sequencing requires digestion of the DNA into overlapping fragments and their sequencing. The final sequence can be assembled from the fragments. Walking strategy sequences a short read. The resulting sequence is used to design a primer for the next read and so forth until the whole sequence is determine. Sequencing by the former approach can lead to incompleteness. The second approach is low-throughput. Therefore, highly sophisticated strategies combining both approaches have been developed for large genomes.

Since many genomes have been sequenced it is possible to map reads onto a reference genome instead assembling it *de novo*. For example, reads of a genome of a patient with a genetic disease can be mapped onto standard human genome.

Omics projects

2. Exomics and transcriptomics

Determination of mRNAs and their concentrations

Exomics determines sequence of all mRNA of certain cell types. This is useful for identification protein sequences and splicing.

Transcriptomics determines concentrations of individual mRNAs. It is used to:
find the difference between tissues, unstressed and stressed cells, sick and healthy cells etc. This can be used to understand the molecular basis of the tissue differentiation, stress response and disease. It can be also used in diagnostics to identify disease markers.

Traditional method for determination of mRNA concentration is Northern blot. More modern is a quantitative PCR with reverse transcription.

However, these techniques can be applied on few genes. As a high throughput it is possible to use DNA microarrays. Recently, next-generation sequencing is used to sequence all mRNA. Concentration of mRNA is determined from the number of sequence reads on given gene.

Omics projects

3. Proteomics

Determination of protein variants and their concentrations

Exomics approach neglects the fact that a single mRNA can be translated into different variants of a proteins, differing mostly in posttranslational modifications.

Transcriptomics approach neglects the fact that concentration of a protein is not fully correlated with the concentration of mRNA. There is a decent correlation, but there are important exceptions. For example, concentration of hypoxia inducible factor (HIF), involved in low oxygen response, is mostly controlled by its degradation and not by the level of mRNA. Therefore, systematic protein identification and measurement has been developed, known as proteomics.

Concentration of an individual protein is usually determined by Western blot. Determination of all protein is cell can be done by 2-dimensional electrophoresis, with isoelectric focusing in one and SDS-PAGE in the second dimension. More modern are approaches based on mass spectrometry such as MALDI or electrospray, usually together with proteolytic sample digestion and/or liquid chromatography.

Omics projects

4. Lipidomics, glycomics, ...

    Determination of lipid or carbohydrate composition or composition of other classes of molecules.

Healthy and sick cells differ in composition of other classes of molecules such as lipids or carbohydrates.

Lipids can be studied by chromatographic methods with mass spectrometry.

Similar methods can be used to determine carbohydrates. Many proteins in eukaryotic cells are glycosylated. Glycosylation patterns may differ between healthy and sick cells.

Omics projects

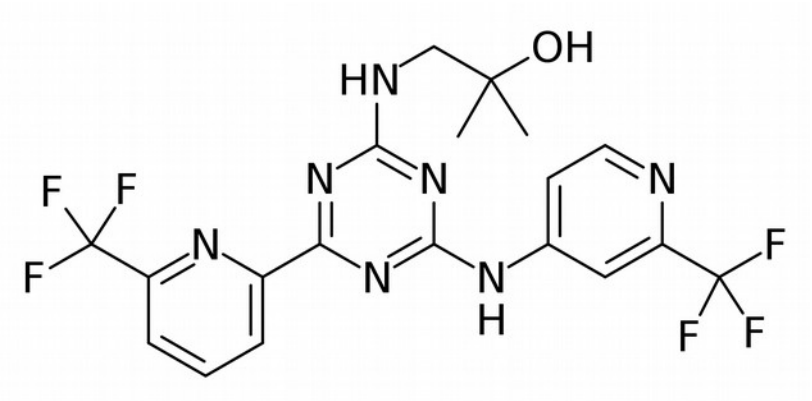
5. Metabolomics

Determination of metabolite composition

Cells differ also in metabolite composition. This can be studied by chromatography, mass spectrometry, NMR and other techniques.

For example, it was found that some cancer cells contain high concentrations of D-2-hydroxyglutarate. Later it was found that the compound is produced as a side reaction by a mutant version of isocitrate dehydrogenase. This compound influences DNA and histon methylation and low-oxygen response, making cancer cells stronger.

This is a nice example how the omics project can identify a new target for therapy. Inhibitors of isocitrate dehydrogenase have been developed and subjected to clinical trials. Now the drug is marketed as Enasidenib.



Other fields of research

Bioinformatics
Systems biology
Epigenetics
Comparative genomics
Population genomics
Functional genomics
Interactomics
Synthetic biology

Other fields of research

Bioinformatics

Bioinformatics provides an informatics service for genomics, molecular biology and related fields. This discipline comprises:

- analysis of raw genomic data to determine complete genome (assembly, mapping)

- finding and identification of genes in the genome

- linking DNA, mRNA and protein sequences

- analysis of transcriptomics, proteomics and other omics data

- storing of sequences of genes and complete genomes in databases

- prediction of function of genes

- prediction of structural elements (e.g. domains) in protein structure

- prediction and analysis of 3D structures

- other analyses of sequences and 3D structures

Other fields of research

Systems biology

Systems biology is aimed at conversion of genomic information into functional models of cells and organisms.

For example, it is possible to identify all metabolic enzymes in the genome of an organism. This makes it possible to reconstruct metabolic pathways in the cell. Experiments providing time-resolved concentration changes in metabolites, enzyme kinetics etc. can be used to model time-course of metabolic processes. This makes it possible to simulate response to enzyme inhibition, enzyme mutation, excess of metabolite etc.

Moreover, it is possible to identify different signaling molecules (receptors, adaptor proteins, protein kinases and other signaling enzymes). Next, it is possible to reconstruct signaling pathways. Similarly to metabolism, signaling pathways can be simulated to predict the outcome of receptor activation or inhibition, mutation of a signaling proteins, inhibition of signaling enzymes etc.

Other fields of research

Epigenetics

It was found that there is a heritable information that is not encoded in DNA sequence. These changes involve:

- covalent modification of DNA, especially DNA methylation

- covalent modification of histons, especially methylation or acetylation of Lys side chains

- presence of different RNAs

DNA methylation can be studied by bisulfite sequencing. Bisulfite (HSO_3^{2-}) converts cytosine, but not methylated cytosine, to uracyl. Parallel sequencing of treated and untreated sample provides information about DNA methylation.

Drugs altering methylation (epigenetic drugs) have been approved for cancer treatment.

Other fields of research

Comparative genomics

Similar organisms have similar genomes in terms of presence and sequences of genes. However, they can differ in genome architecture. For example, human and chimpanzee proteins typically differ in two amino acids, but some genes are moved on a chromosome and one human chromosomes is split into two in chimpanzee.

Comparison of genomes is therefore not straightforward and a whole new genomics discipline called comparative genomics has been developed.

Other fields of research

Population genomics

High throughput sequencing made it possible to sequence genomes at reasonable costs. First it was applied to genomes in populations of various species. The 1000 Genomes Project was launched in 2008 and in 2012 it provided 1,092 genomes of human individuals from various countries of the world.

Population genomics makes it possible to identify variability of a genome.

In medicine, the term mutation is used to describe a difference from the “standard” genome which causes a disease.

In contrast, the term polymorphism is used to describe a variability or a difference from the “standard” genome which causes differences between organisms, but not (directly) a disease.

Other fields of research

Functional genomics

Functional genomics is used to determine the function of genes. The most common tool is gene knockout. Knocking out of a gene leads to disruption of a metabolic or regulatory pathway or other response, which can be used to determine the function of the gene. Traditionally, homologous recombination is used to knockout genes. More recently, zinc-finger nucleases, Transcription activator-like effector nuclease (TALENs) and CRISPR have been developed.

The term functional genomics is also used to describe approaches aimed at integration of genomics, transcriptomics, interactomics etc.

Other fields of research

Interactomics

Many biological signaling processes involve interaction between proteins.

Affinity between two proteins can be determined by their co-isolation (pull-down assay). One protein is immobilized onto a suitable matrix. This is mixed with a cell extract. Proteins with the affinity can be isolated with the matrix and identified by chromatography with mass spectrometry.

As an alternative it is possible to use a yeast two-hybrid system. One gene is inserted into one vector, the second into second vector. If these proteins interact it starts a signal (e.g. enzymatic activity). Transcriptomic yeast two-hybrid studies of all pairs of genes have been carried out for several organisms.

It was found that a typical protein interacts with 10 – 100 other proteins.

Other fields of research

Synthetic biology

Building of new organisms is behind the idea of synthetic biology. One of most promising applications is cloning of whole metabolic pathways to “teach” some microorganism to produce some metabolite. Cloning multiple genes is similar to cloning a single gene, but much more complicated due to numerous reasons. Recently many of these obstacles have been solved.

Media has reported in 2015 (*Nature*, **521**, 281-283, 2015) that two groups of researchers independently cloned the first and the second half of morphine biosynthesis pathway from poppies into yeast *S. cerevisiae*. This risen concerns that leaking of these yeast strains to public would enable home “brewing” of opiates.

Today, most antibiotics and many other drugs are discovered as secondary metabolites of microorganism. Researchers isolate microorganisms from environment. Next, they are cultivated and compounds are isolated from the medium. These compounds are tested for biological activity. This excludes microorganisms that cannot be cultivated. As an alternative, some researchers test sequencing of genomes and identification of microorganisms producing interesting compounds, cloning of their metabolic pathways and synthesis of compounds into a suitable organism.

Clinical Biochemistry

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Clinical biochemistry

Assays:
- elements, ions, gases
- small molecules
- enzymes
- proteins
- others

Materials:
- serum
- plasma
- urine
- others

Any analytes related to patient's health could be analyzed and used in diagnostics. Many elements, ions, dissolved gases, small molecules, enzymes and proteins are routinely assayed in clinical laboratories. They can be assayed in blood (serum or plasma), urine or other samples (stool, liquor and others). Sample type may be encoded by the color of a test tube. These colors differ from country to country. Most blood analytes can be assayed in serum. Serum is sampled into red test tubes (in CZ). Violet test tubes contain disodium EDTA, which binds Ca^{2+} and blocks coagulation. These samples are used for hematological testings (counting of blood cells). Sodium citrate also chelates Ca^{2+}, but reversibly. It is contained in black or blue test tubes for sedimentation rate test or for blood coagulation tests, respectively (concentrations are different). Green test tubes contain NaF. It also binds Ca^{2+}, but also intracellularly. Therefore it blocks glycolysis. It is used to measure glucose and related analytes.

Clinical biochemistry

Units:
- M, mM, µM, g/l
- katal (kat)
- mmHg
- osmole (1M of osmolytically active ions)
- others

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

Essential ions, dissolved gases, small-molecule compounds

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

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

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

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

H_2O_2 + peroxidase substrate → color product *peroxidase*

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

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

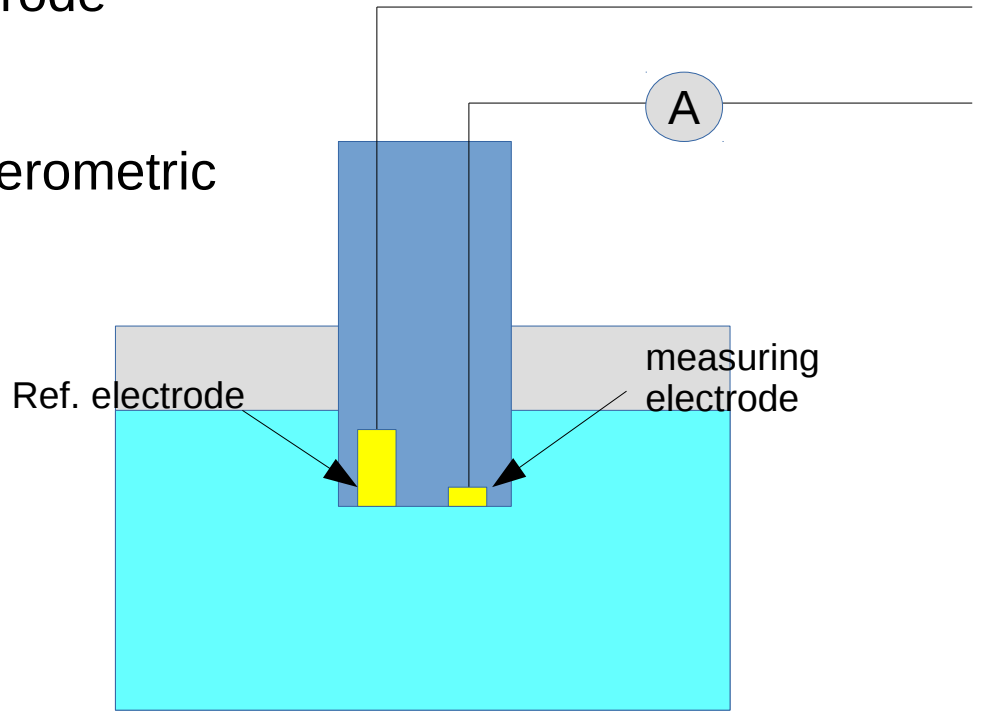
Alternatively, glucose can be assayed by phosphorylation by ATP by hexokinase followed by oxidation by NADP+ by glucose-6-phosphate dehydrogenase.

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

General amperometric sensor



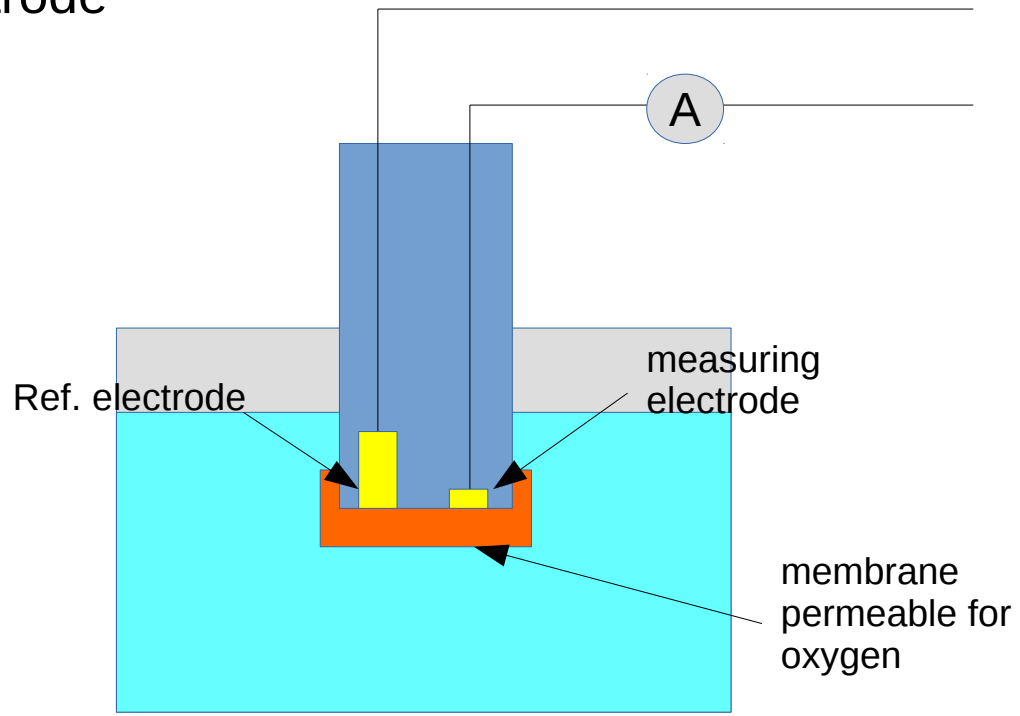
Glucose oxidase reaction can be also used in an amperometric sensor (enzyme electrode). A general amperometric sensor can be composed of measuring and reference electrode. It is immersed into the solution and potential is applied on electrodes. The current is limited by the rate of electrochemical reaction,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

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

Oxygen sensor



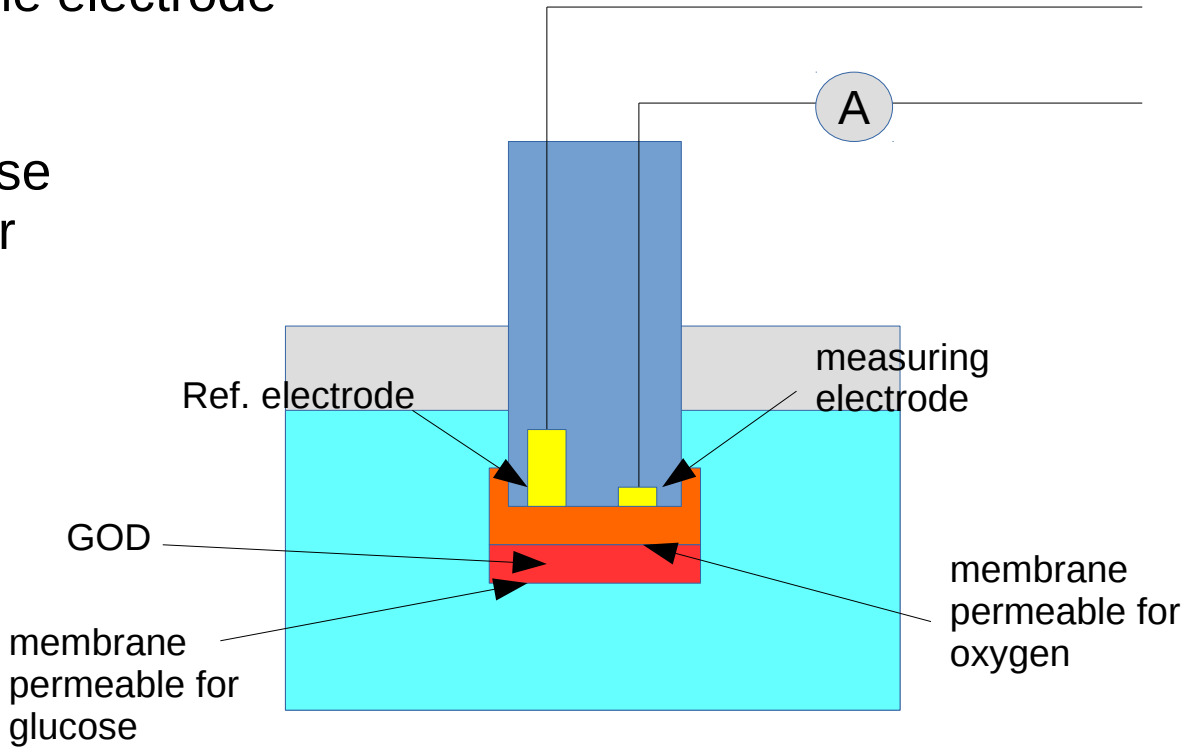
By adding a compartment separated by a membrane permeable only for oxygen it is possible to restrict reactions only to oxygen. Such sensor is used to measure oxygen concentrations in clinical biochemistry, biotechnology, fish production etc.

Essential ions, dissolved gases, small-molecule compounds

- Glucose – diabetes

enzyme electrode

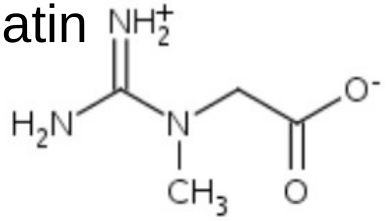
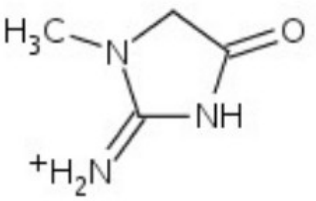
Glucose sensor



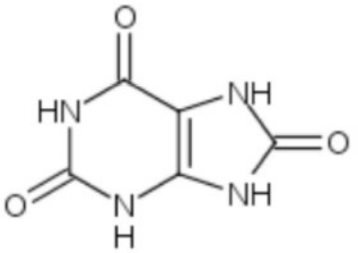
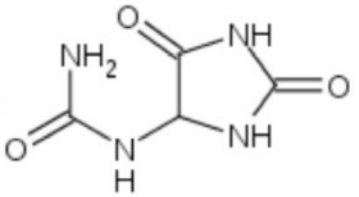
By adding another compartment separated by a membrane permeable only for glucose with glucose oxidase it is possible to measure glucose. These sensors are used in clinical labs or by diabetic patients.

Essential ions, dissolved gases, small-molecule compounds

Creatinin – reaction with picric acid

creatin  creatinin 

Uric acid – higher in gout

uric acid + O_2  allantoin + H_2O_2 

Bilirubin (direct, conjugated) – liver diseases, hemolysis

- diazocoupling with reagent

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

Essential ions, dissolved gases, small-molecule compounds

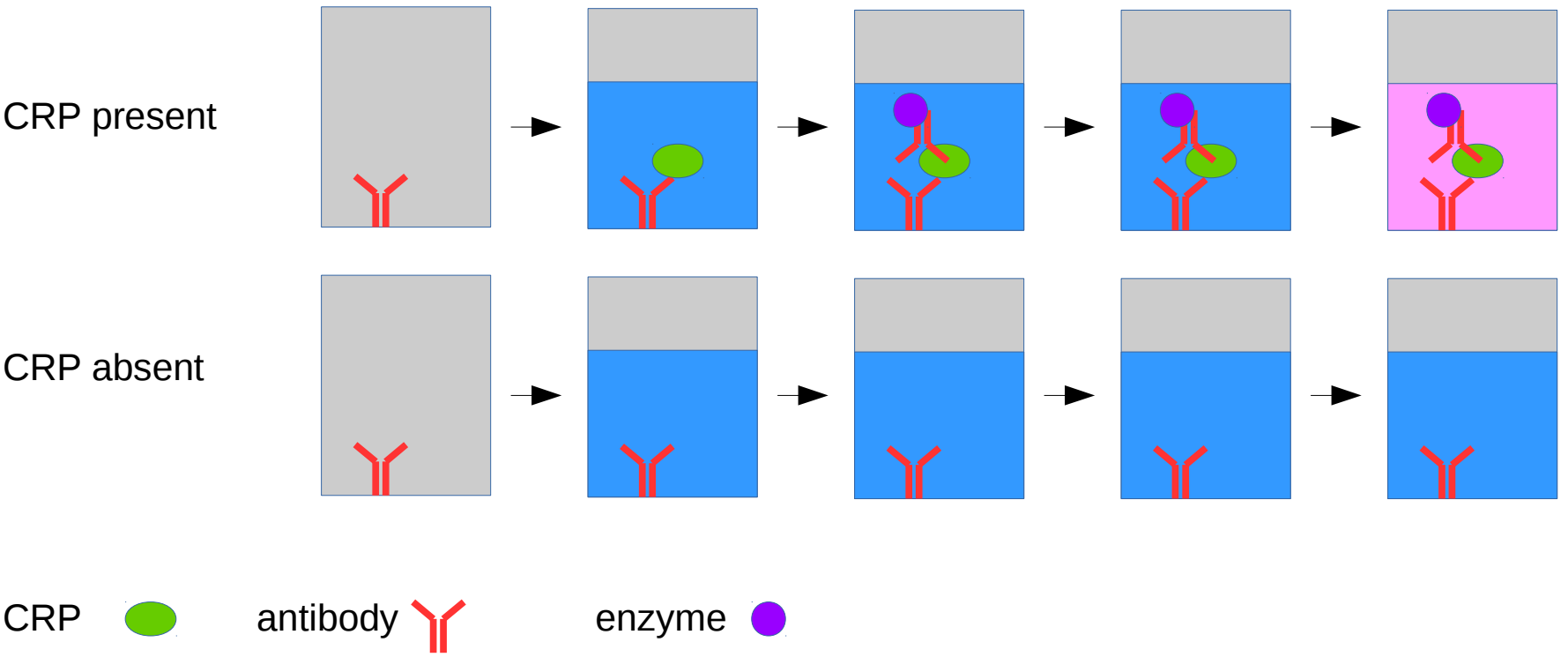
- Lactate – lactate dehydrogenase – low oxygen supply

Lactate can be assayed by lactate dehydrogenase and NAD+.

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

Proteins

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



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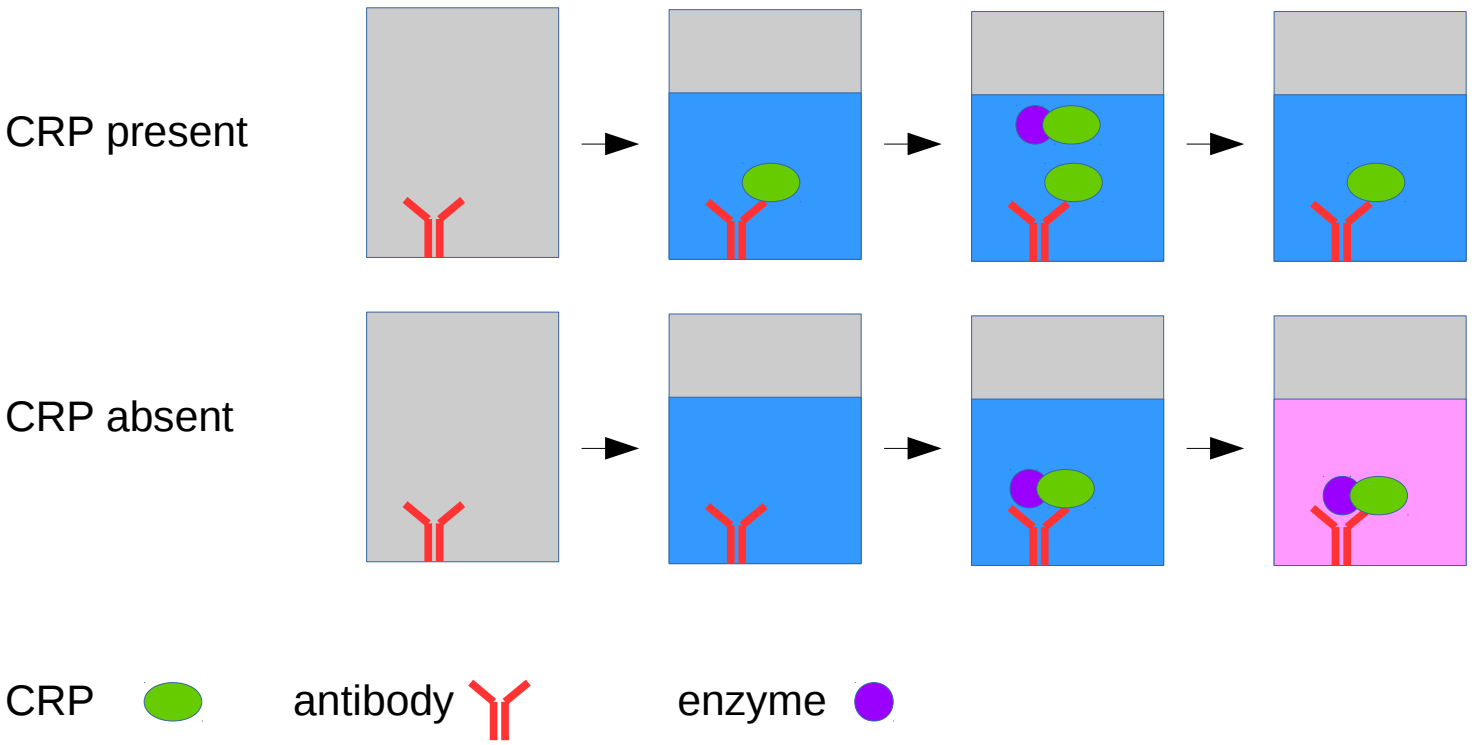

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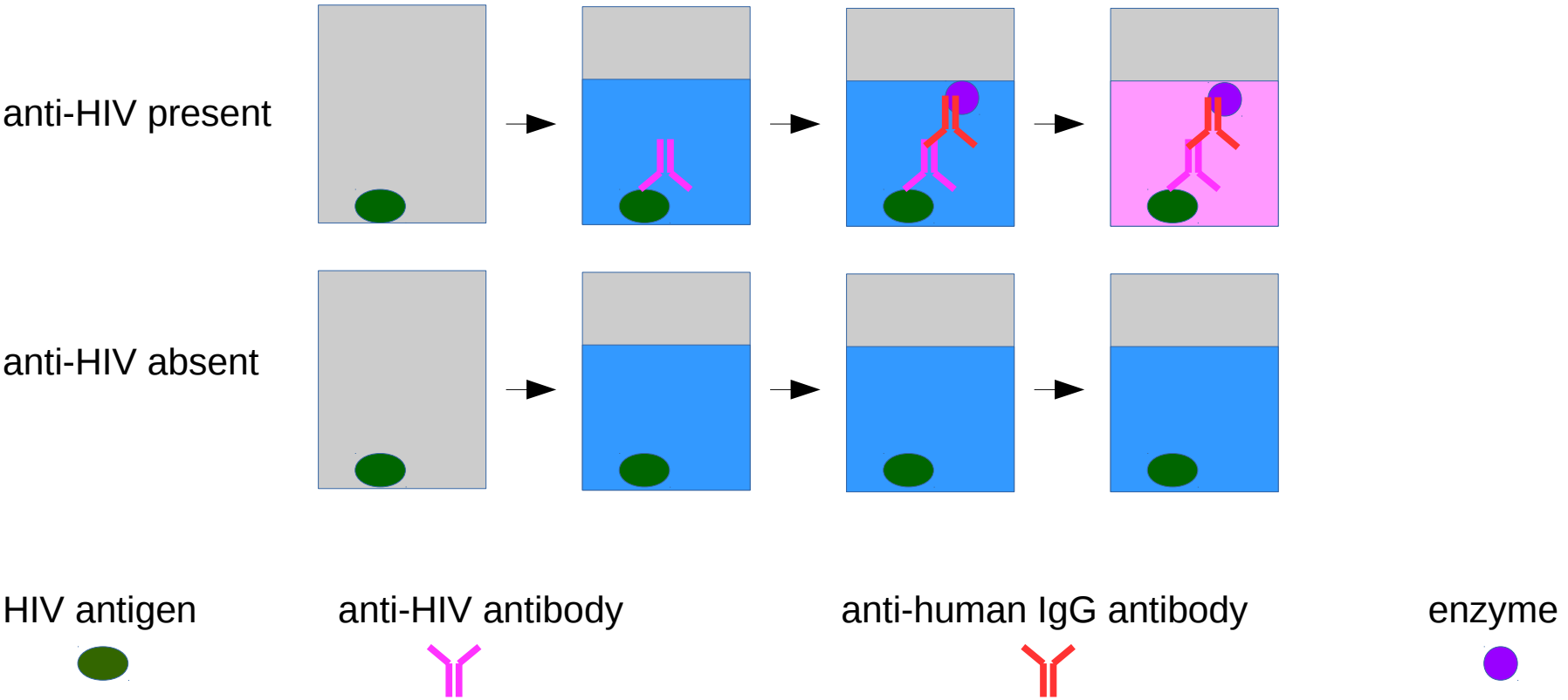

Proteins

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

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

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

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

Enzymes

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

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

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

Enzymes

- Alanine transaminase (ALT) – liver diseases

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

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

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

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

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

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

Enzymes

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

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

- Acid phosphatase – prostate cancer
  chromogenic substrate, also immunochemically

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

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

Enzymes

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

creatine phosphate + ADP → creatine + ATP *CK*

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

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

or

creatine + ATP → creatine phosphate + ADP *CK*

ADP + PEP → ATP + pyruvate *pyruvate kinase*

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

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

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

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

Lipids

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

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

Other methods

Flow cytometry
Histology, histochemistry
Cultivation of pathogens
Immunoassays
DNA-based methods
MRI
PET, SPECT

History of Biochemistry

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MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

Sources

Nobel prices in chemistry

Nobel prices in physiology

Human Genome Project, Celera Genomics

others

(indicated decades and periods are approximate)

History of Biochemistry

18 century

discovery of starch digestion by saliva or meat digestion by stomach acid

History of Biochemistry

19 century

Louis Pasteur – fermentation is accelerated by ferments (enzymes)

Anselme Payen – discovery of diastase (amylase)

Wilhelm Kühne – word “enzyme”

Eduard Buchner – fermentation takes place in a cell-free free extract

Hans Karl August Simon von Euler-Chelpin – glycolysis is the action of enzymes

History of Biochemistry

19 century

Friedrich Miescher – existence of DNA

Albrecht Kossel – isolation of DNA, identification of some components

History of Biochemistry

1st half of 20 century

Gustav Embden, Otto Meyerhof and Jakub Karol Parnas –
discovery of glycolysis, isolation of glycolytic enzymes, identification of individual steps

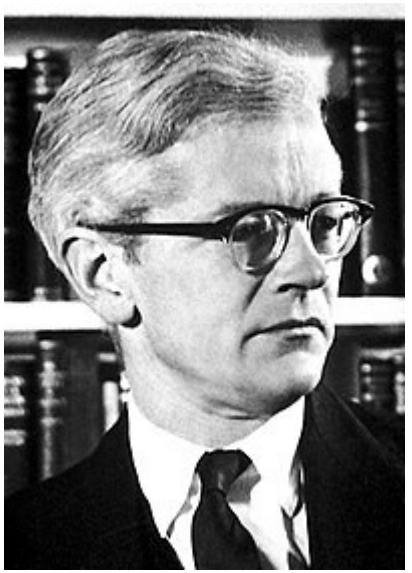
Hans Adolf Krebs – Krebs cycle, urea cycle, glyoxylate cycle

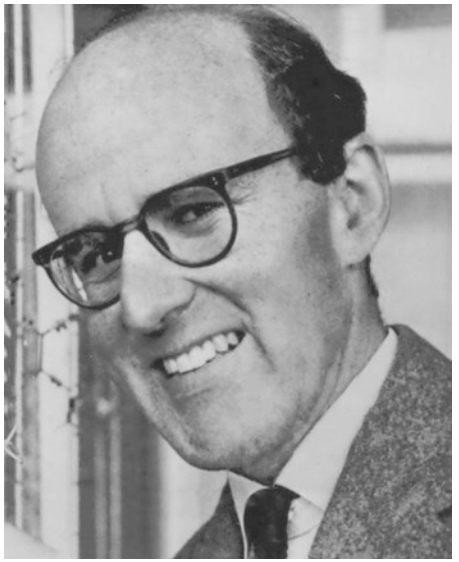
History of Biochemistry

1st half of 20 century

James B. Sumner – urease and catalyse are proteins, they can be crystallized

John Cowdery Kendrew and Max Perutz – X-ray structure of myoglobin





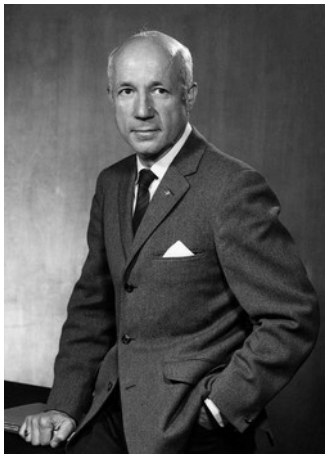
Phoebus Levene – identification of DNA building blocks

Jean Brachet – RNA discovery

History of Biochemistry

1st half of 20 century

Melvin Calvin - photosynthesis



Luis F. Leloir – sugar metabolism

History of Biochemistry

1st half of 20 century

John Howard Northrop and Wendell Meredith Stanley – crystalization of proteins, enzymes and viruses

Alexander R. Todd – structure and synthesis of nucleosides, nucleotides and coenzymes

Dorothy Crowfoot Hodgkin – 3D structure of penicilin, insulin, proteins

History of Biochemistry

1950s

Robert Brainard Corey, Linus Carl Pauling – secondary structure of proteins

Max Perutz – structure and function of hemoglobin

Rosalind Franklin and Raymond Gosling – X-ray diffraction of DNA

Francis Crick, James Watson, Maurice Wilkins – DNA structure

Francis Crick – central dogma of molecular biology

Frederick Sanger – sequencing of insulin

History of Biochemistry

1960s

Christian B. Anfinsen – protein folding

Frederick Sanger – sequencing of RNA

Peter D. Mitchell – chemiosmotic mechanism of ATP synthesis

Paul Berg – recombinant DNA

Robert Bruce Merrifield – solid-phase synthesis of peptides/protein

Johann Deisenhofer, Robert Huber and Hartmut Michel –
3D structure of membrane proteins

Sidney Altman and Thomas R. Cech – catalytic RNA

History of Biochemistry

1970s

Kary B. Mullis and Michael Smith – PCR, side-directed mutagenesis

Jens C. Skou, Paul D. Boyer and John E. Walker – Na^+/K^+-ATPase

John B. Fenn – electrospray MS

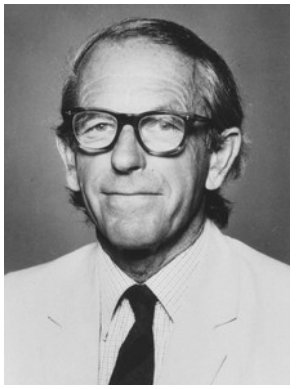
Koichi Tanaka – MALDI MS

Kurt Wüthrich – NMR in structural biology

History of Biochemistry

1970s

Frederick Sanger – sequencing of DNA



J. Andrew McCammon and Martin Karplus – first simulation of protein dynamics

Roger D. Kornberg – RNA polymerase structure and function

Bruce A. Beutler and Jules A. Hoffmann – innate immunity

Ralph M. Steinman – dendritic cells

Sydney Brenner, H. Robert Horvitz and John E. Sulston – apoptosis

History of Biochemistry

1980s

Peter Agre – aquaporins

Roderick MacKinnon – structure of ion channels

Aaron Ciechanover, Avram Hershko and Irwin Rose
– ubiquitin, proteasom

Robert Lefkowitz – function of G protein-coupled receptors

Yoshinori Ohsumi – autophagy

Françoise Barré-Sinoussi ans Luc Montagnier - HIV discovery

Barry J. Marshall and J. Robin Warren – *Helicobacter pylori*

Leland H. Hartwell, Tim Hunt and Paul M. Nurse – cell cycle

Stanley B. Prusiner – prions

History of Biochemistry

1990s

Osamu Shimomura, Martin Lee Chalfie and Roger Y. Tsien
– Green Fluorescent Protein

Eric Betzig, Stefan Hell and William E. Moerner
– ultra-high resolution microscopy

Tomas Lindahl, Paul L. Modrich and Aziz Sancar – DNA repair

James E. Rothman, Randy W. Schekman and Thomas C. Südhof
– vesicle traffic

John B. Gurdon and Shinya Yamanaka – pluripotency

Andrew Z. Fire and Craig C. Mello – RNA interference

History of Biochemistry

Human Genome Project

Launched in 1990

Human Genome Project funded by National Institute of Health (USA) and Welcome Trust (UK)

Celera Genomics started a concurrent project with a lower budget, but they used public data from HGP

Published: *Nature* 2001, **409**(6822): 860–921 (HGP).
Science 2001, **291**(5507): 1304–1351 (Celera).

History of Biochemistry

Human Genome Project

www.ensembl.org

Version GRCh38.p12 (2013)

3,609,003,417 base pairs

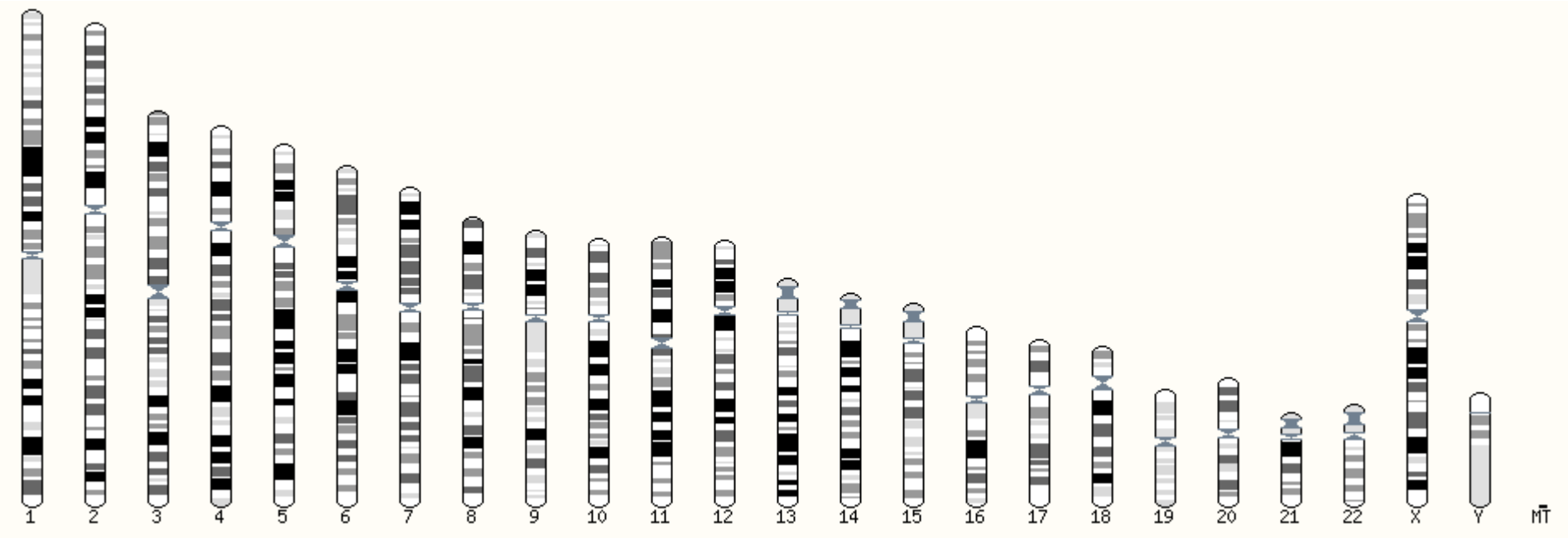
20,376 coding and 22,305 non-coding genes

203,903 gene transcripts (e.g. splicing variants)

History of Biochemistry

Human Genome Project

www.ensembl.org



History of Biochemistry

2000s

Venkatraman Ramakrishnan, Thomas A. Steitz and Ada Yonath
– 3D structure and function of ribosome

Brian Kobilka – structure of G protein-coupled receptors

Jacques Dubochet, Joachim Frank and Richard Henderson
– high-resolution cryoEM

Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak
– telomere and telomerase

History of Biochemistry

2010s

CRISPR/Cas9

Next generation sequencing of DNA

Cell reprogramming - induced pluripotent cells

Synthetic biology

CryoEM as emerging structural biology tool

Integrative omics, ENCODE

Single cell omics

Single molecule studies

Questions and Answers

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

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

How to design PCR primers?

PCR primers are short oligonucleotides (approx. 20 bp). The DNA sequence is conventionally written from the 5’ to 3’ strand. The complementary sequence can be obtained in two steps. First, replace A→T, T→A, C→G and G→C. Second, put the resulting sequence into the reverse order.

The forward primer can be obtained by simply taking a short sequence from the left side of the template.

The reverse primer can be obtained by taking a short sequence from the right side of the template and making a complementary sequence.

Primers must fulfill several other properties such as length, melting temperature, low self-complementary etc. There are special tools available for primer design. Many companies provide custom primer synthesis. Often you can analyze primers on their web site before ordering them.

```
5’-GCATGTGCAGACGGCGATATGCGACGTACGATCGATCGACTATCACGCAACGGCACGACCGCACT-3’
 5’-CATGTGCAGACG-3’                                                3’-CCGTGCTGGCGTG-5’
```

DNA polymerase does not “know” where to terminate the length of the PCR product, nevertheless PCR produces products of exact length. How?

In the first cycle of the PCR the DNA polymerase indeed does not know where to terminate the DNA synthesis. This leads to a product with variable length (its 5’ terminus is defined by the primer, but its 3’ terminus is variable). However, this product is synthesized directly from the template and its concentration therefore grows linearly. PCR usually uses very small amount of the template. In a PCR with 20 cycles you can reach 20x concentration of the template, which is still low concentration.

The product with the fixed length (its both thermini are defined by primers) is produced either from the product with variable length or from another molecule of the product with the fixed length. After the step 1 there is no product with the fixed length. After the step 2 there is the same concentration as the template, after the step 3 it is 2x, after the step 4 it is 4x and after the step 20 it could be concentration 524,288x higher than template.

We can assay activities of many enzymes using the fact that NAD(P)H absorbs at 340 nm but NAD(P)$^+$ does not. S-adenosylhomocystein hydrolase is an exceptional enzyme because it uses NAD$^+$ as the prosthetic group, not coenzyme. What can we learn about this enzyme from absorbance at 340 nm?

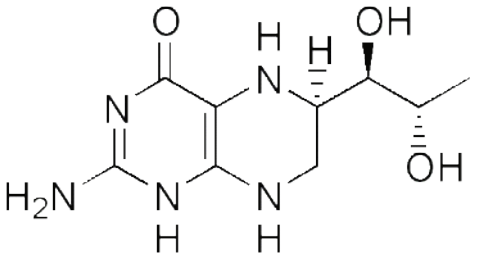
Most NAD(P)$^+$ dependent enzymes use this molecule as a coenzyme, i.e. a substrate that is present in the cell in low concentrations and is permanently recycled by various reactions.

S-Adenosylhomocystein hydrolase is different. It uses NAD$^+$ to oxidize the substrate and goes to NADH. Hydrolysis of the oxidized substrate is easier. After hydrolysis the oxidized product is reduced. NADH is oxidized back to NAD$^+$ in this step. NAD$^+$ therefore exits the catalytic cycle unchanged. It is an integral part of the catalyst and can be considered prosthetic group (caution: some researchers use the term prosthetic group to refer to covalently bound cofactors).

Unlike other NAD$^+$-dependent enzymes (dehydrogenases) we cannot assay S-adenosylhomocystein hydrolase activity by measuring absorbance at 340 nm. If we isolate pure S-adenosylhomocystein hydrolase saturated by NADH/NAD$^+$ and we remove free NAD$^+$ or NADH we can use absorbance to measure NADH/NAD$^+$ ratio in the active site under various conditions such as substrate concentration. By this we can learn important facts about the mechanism of the reaction.

Tetrahydrobiopterin has been approved for treatment of the disease called phenylketonuria. Try to explain its function.

The name phenylketonuria indicates abnormally high concentration of phenylketones in urine. It is inborn error of metabolism caused by lack of phenylalanine hydroxylase activity. Patients cannot metabolize phenylalanine to tyrosine. Instead they produce phenylpyruvate, phenylacetate and other metabolites that cause physical problems. There are many mutations that may cause low or no activity of this enzyme. Many of them are destabilizing enzyme. Tetrahydrobiopterin is coenzyme of this enzyme. It can bind to mutation-destabilized enzyme, stabilize it in the native state and thus rescue its activity.



Many signaling pathways work in the way that the molecule A activates molecule B, B activates C and so forth until it activates Z. What is the advantage compared to direct activation of Z by A?

Typically, one molecule of A may activate multiple molecules of B, each B may activate multiple C and so forth. As the result, one molecule of A may activate a huge number of molecule Z. The signaling is therefore very sensitive and rapid.

This is example of GPCR signaling, apoptosis, blood coagulation and many other processes.

The compound 2,4-dinitrophenole was used to treat obesity in 1933-1938, but was discontinued due to its toxicity. The compound can freely diffuse through a membrane in the protonated $(O_2N)_2$-C_6H_4-OH as well as in deprotonated form $(O_2N)_2$-C_6H_4-O^-. It was found that it reduces production of ATP. Try to explain how 2,4-dinitrophenole works.

2,4-Dinitrophenole can freely diffuse through membranes in both forms. It can therefore get protonated in the intermembrane space of mitochondria and then pass into the mitochondria, lose its proton and again diffuse out of mitochondria. This eliminates the proton motive force and stops production of ATP. The cell catabolizes nutrients but anabolism is reduced.

What is the advantage of the positive homotropic allosteric effects in nature?

The term homotropic allosteric effect means that the same impulse (such as compound binding) is the cause as well as the outcome of the effect. For example, binding of oxygen to hemoglobin influences its affinity to other molecules of oxygen.

Hemoglobin is the example of the positive homotropic allosteric effect, because binding of oxygen enhances affinity to other oxygen. The physiological consequence is that it binds oxygen strongly at high concentrations (in lungs) and weakly at low concentrations (in the rest of body). Oxygen can be efficiently loaded in lungs and most of it is released in the body.

Let us imagine a fictive hemoglobin without allostery. Strong binding in lungs can be achieved by strong affinity to oxygen. However, such hemoglobin would release only a small fraction of oxygen cargo in the body and oxygen would circulate in blood without being used. Release of oxygen in body can be achieved by weak affinity to oxygen. However, it means low saturation in lungs.

What is the advantage of the negative homotropic allosteric effects in nature?

Negative homotropic allosteric effect is bit more complicated than the positive one. We can find it, for example, in some bacterial represors such as catabolic activator protein (CAP). CAP binds cAMP with negative homotropic allosteric effect. The result is that binding is extremely strong at low concentration. The saturation is steeper at low concentrations and gentle at high concentrations of cAMP. This is important because the cell must sense very low concentrations of cAMP.

Is there any example of GPCR activated by some action other than ligand binding?

GPCRs are mostly studied as drug targets. Binding of natural as well as artificial ligands is therefore the most explored mechanism of GPCR activation. However, GPCRs can be activated by other mechanisms. The one covered by slides is activation of rhodopsin by light. There are reports of GPCRs activated by proteolytic cleavage, change of temperature or mechanically. GPCR signaling by ligands can be also influenced by membrane potential.

If you feed a laboratory animal with cocaine why it does not produce antibodies against cocaine?

Cocaine is a small molecule (303.353 g/mol) and the immune system does not produce antibodies against small molecules. Production of antibodies requires presentation of antigen using some MHC and small cocaine cannot be presented. Organism may used other mechanisms to get rid of cocaine, for example cytochrome P450.

If you want to produce antibody against cocaine you can bind it covalently onto some mouse protein and inject it back to mouse. Mouse does not produce antibodies against the protein because it is a mouse protein, but it produces antibodies against its modification, i.e. against cocaine. Such polyclonal antibody can be used in cocaine assay.

Is DNA in all cells in the body the same or are there any exceptions?

Indeed, there are exceptions. T cells contain the gene for the T cell receptor. This gene differs among the population of T cells in the body. This is achieved by shuffling short segments of the DNA in the gene. This makes a large repertoir of T cells receptors that can bind almost any antigen. Adaptive immunity is based on the fact that the cell with the right gene can be selected.

There are some other exceptions, for example one use in brain development to avoid self junctions of neurons.

It is not good idea to inject pure water into blood stream. 0.15M NaCl is used instead. Why?

Blood contains approximately concentration of NaCl equal to 0.15 mol/l. Blood cells and other cells in the body contain similar salt concentrations (composition of slats may depend on the cell type). Lower concentration of salts would cause high osmotic pressure on cell membranes. Cells would tend to absorb water and increase its size. This causes cell disruption, in particular hemolysis.

Why are volunteers of clinical trials asked not to eat grapefruits?

Citruses, and especially grapefruits, contain high concentrations of furanocoumarins that inhibit the main drug metabolizing enzyme in the body, which is cytochrome P450 (in particular its isoenzyme 3A4). This causes that metabolism of a drug is slower and its effect may be stronger and/or longer. This may influence the results of a clinical trial. Patients using some drugs may be also instructed not to consume grapefruits due to interaction with the drug (interaction in the pharmacological meaning of the word).

Compounds used in doping of athletes include testosterone and erythropoietin (EPO). Testosterone can be applied on arms and legs as a gel. EPO must be injected. Explain what is the difference between the two compounds, so that they require different administration.

Testosterone is a small molecule, steroid hormone, highly nonpolar and barely soluble in water. When applied as a gel on arms and legs it can distribute into muscles, penetrate cell membranes, go to nuclei, bind to nuclear receptors and regulate transcription.

In contrast, EPO is a protein (21 kDa). It must be therefore delivered by injection. Moreover, EPO acts on blood cell formation.

Maple syrup may be adulterated by addition of cane sugar. This can be detected by mass spectrometry due to different composition of carbon isotopes in maple and cane sugars. Why isotopic composition of sugar from these plants differ?

Maple tree is a C3 plant. Sugar cane is a C4 plant. Isotopic composition of carbon dioxide in the air is more or less constant. However, rates of enzymatic reactions (and chemical reactions in general) might be slightly dependent on isotope composition. Enzymatic reactions can slightly enrich some isotopes in their products. Carbon dioxide fixation is done by different reactions in maple and cane, so the isotopic composition is different and can be resolved by mass spectrometry.

Ephedrine was produced in Czechoslovakia by fermentation of brewers yeast with benzaldehyde. This produced the compound CH_3-C(=O)-CH(OH)-C_6H_5.

This can be converted to ephedrine chemically by reaction with methylamine and reduction. Try to explain why yeast produce the above mentioned compound.

This compound resembles acetoin (CH_3-C(=O)-CH(OH)-CH_3), which is together with biacetyl and butandiol a side product of pyruvate decarboxylase reaction. These compounds can be found in alcoholic drinks and other food products, sometimes favored and sometimes disfavored. Pyruvate decarboxylase decomposes pyruvate to carbon dioxide and acetaldehyde. As a side product, the already formed molecule of acetaldehyde may condense with a nascent acetaldehyde molecule to form acetoin. When the first molecule of acetaldehyde is replaced by beznaldehyde it produces the above written molecule. This biotechnology was intensively used in Czechoslovakia to produce ephedrine and is responsible for today dominance of the Czech Republic in illegal metamphetamine production.

Activity of pancreatic lipase can be assayed using a fluorogenic substrate. This substrate is composed of glycerol esterified by two fatty acids and and one glutaric acid. On the second carboxylic group of the glutaric acid there is a covalently bound fluorescent compound (not fluorescent when bound). The substrate is not fluorescent. Lipase can hydrolyze the bond between the glycerol and glutaric acid. The resulting conjugate of glutaric acid with the fluorescent compound decomposes and releases the fluorescent compound. However, lipase can hydrolyse the two fatty acids as well, maybe even at higher rate. How is it possible that this substrate can be used in clinical biochemistry despite the fact that it measures only a fraction of real enzymatic activity?

When some washing powder developer wants to add lipase into a washing powder he/she might be interested in the “real” activity of the enzyme, i.e. how much of lipids it can decompose in the washing cycle under conditions of washing machine. On the other hand, clinical biochemist is usually not interested in the “real” activity of the enzyme. The fact that some enzyme is present in blood indicates that some organ was damaged and the enzyme leaked. The activity of the enzyme is used in clinical biochemistry as a measure of enzyme concentration (under stable conditions such as pH, temperature etc. enzyme activity is linearly dependent on enzyme concentration). It does not matter that the substrate measure only for example 10 % of the “real” activity if it is enough for accurate measurement and if it is always 10 %.

Immunochemists often use for example goat anti-mouse antibody labeled by horseradish peroxidase. Why is such antibody useful?

If you want to use some immunochemistry technique, for example ELISA, to detect concentration of some protein you can use mouse antibody against this protein and label it by enzyme, for example by horseradish peroxidase. However, conjugation of enzyme and antibody is quite difficult job. It make sense to do it for a commercial kit but not for single laboratory experiment.

Instead it is possible to use another antibody (referred to as secondary antibody) to detect the first antibody. The secondary antibody, for example goat anti-mouse antibody labeled by horseradish peroxidase, is commercially available and already labeled from the factory.

Why is NADH assay (absorbance at 340 nm) so popular in clinical assays?

The wavelength 340 nm falls to UV range. It is possible to use special plastic disposable cuvettes, which is important when working with infectious material. Moreover, most blood components do not absorb at 340 nm. DNA and RNA absorb at 260 nm, proteins at 280 nm. Molar extinction coefficient is reasonably high, which makes these assays sensitive. Finally, it is one wavelength for many assays, so it is possible to use one equipment for multiple purposes.