

The extracellular matrix of connective tissue

Collagen and elastin

Proteoglycans, fibronectin, laminin

Biochemical markers of bone resorption and remodeling

Biochemistry II

Lecture 13

2008 (J.S.)

Connective tissues (soft connective tissue, ligaments, cartilage, bone) are of mesenchymal origin.

The **proportion of cells** is relatively small:

fibroblasts and **fibrocytes** (chondroblasts and chondrocytes, osteoblasts and osteocytes, too) produce the major components of extracellular matrix, **histiocytes** (fixed and free macrophages) scavenge foreign particles and tissue detritus, **mast cells** (mastocytes) releasing heparin and histamine, **adipose cells** (adipocytes), **leukocytes**, namely **lymphocytes**, **plasmocytes**, and **monocytes** (circulating macrophages, leaving vessels through diapedesis).

The **extracellular matrix** includes

a **fibrillar component** – fibrils of **collagen** and **reticulin**,
in some connective tissue types **elastin** fibres, and
an **interfibrillar component** (the amorphous ground substance) –
proteoglycans and a glycosaminoglycan **hyaluronic acid**,
fibronectin, **laminin** and other cell adhesion glycoproteins,
and, in bones, voluminous insoluble **mineral component**.

Collagen fibres

are included in all connective tissue types. They consist of bundles or networks of thin collagen microfibrils.

Collagen

is not a single protein but a major class of glycoproteins in the intracellular matrix of connective tissue. In mammals, collagen represents up to 25 % of all body proteins mass. **Nineteen collagen types (collagens I – XIX)** are known till now. They differ from each other in their occurrence, in part in the structure and function, too.

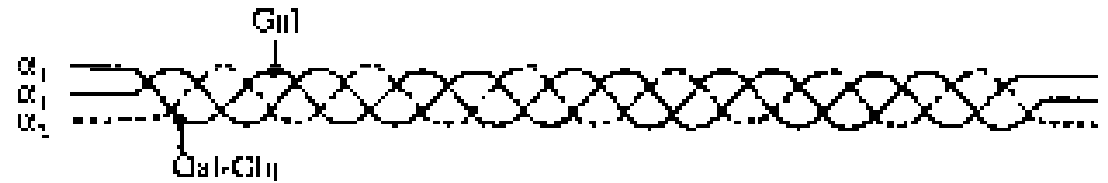
All collagen types include (at least in part) the characteristic **triple-stranded helix** (triple helix), which occurs in other proteins rather rarely.

Only **five collagen types** are **typical fibrillar proteins**, the molecules of which form long fibrous structures. In **the other collagen types**, parts of polypeptide chains are arranged into one or more **globular domains**.

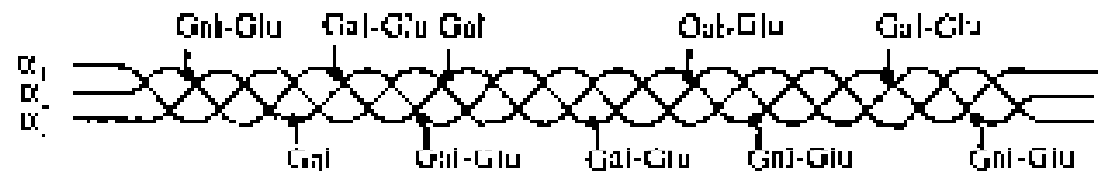
In the following text, most attention is paid to the **collagen type I**, the most prevailing collagen type in soft connective tissue and bones.

Some structural divergences in collagen types I, II, III, and IV

Collagen I is the most common type (**skin, bones, tendons, dentin**), resisting to tensile strength. Slightly glycosylated (< 1 % saccharides), no cysteinyl residues.



Collagen II is the major type present in the **hyaline cartilage** of joints. High degree of glycosylation, no cysteinyl residues.



Collagen III (skin, aorta, uterus) is an elastic type in the form of thin **reticuline fibrils**. Very low glycosylation, cysteinyl residues are present, small number of disulfide bridges.



Collagen IV is the typical type of **basement membranes** (among others renal glomeruli, capsule of the eye lens) forming the non-fibrillar network that stabilizes a thin membrane. Its flexible triple helices include some non-helical segments and at their C-ends there are globular domains. Saccharidic component about 15 %, cysteinyl residues and disulfide bridges are present.

The structure of collagen fibrils



electron optical picture of soft connective tissue

1 μm

0.3 - 3 μm



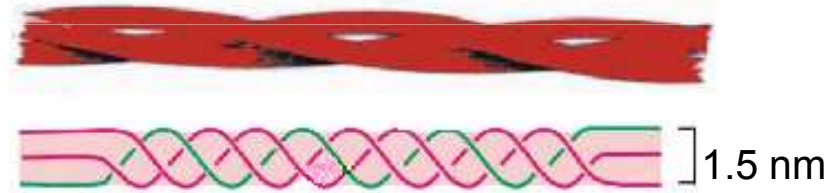
microfibrils – either a felty tangle or arranged bundles of protofibrils interacting with proteoglycans

protofibrils are formed through aggregation of tropocollagen units



10 - 30 nm

tropocollagen unit 300 nm x 1.5 nm
(triple helix, in type I collagen
two chains α_1 and one chain α_2)



1.5 nm

Triple-stranded helices of tropocollagen

Polypeptide chains of fibrillar collagen types (named α -chains) include the multirepeated
sequences Gly-X-Y.

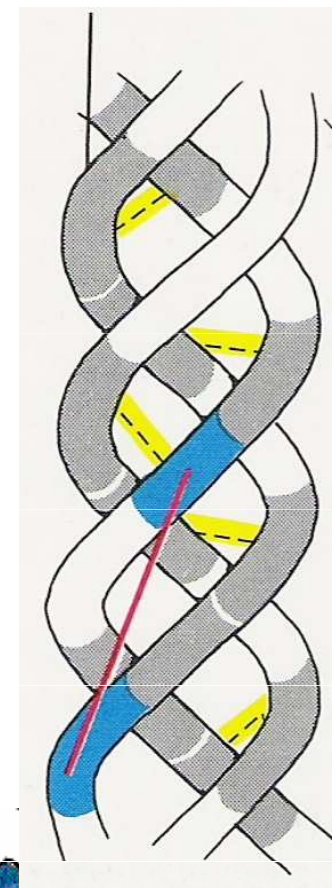
X and Y can be residues of any amino acids, but mostly prevail
in position X **proline**, 3-hydroxyproline, Glu, His, Leu, Phe,
in position Y **4-hydroxyproline**, Thr, Lys, Arg.

In α -chains, each third amino acid residue is residue of **glycine**, approximately one quarter of residues are residues of **proline or hydroxyproline**.

The secondary structure of α -chains is, due to the high incidence of proline or hydroxyproline residues, a structure called the **steep left-handed helix** with 3.3 amino acid residues per turn. Those chains cannot turn in the way that would enable stabilization by means of hydrogen bonds between the groups within the particular chain (as it is usual in α -helices).

The secondary structure of α -chains is stabilized by formation of trimers, three helical α -chains are wound together to form **right-handed triple helices** that are maintained by interchain hydrogen bonds between the NH-groups of glycine residues, and the carbonyl groups of residues X in an adjacent chain. These long trimers are called **tropocollagen units** (or molecules)..

At the both N- and C-ends of α -chains, short sequences occur, which are lacking in any regular structure; these segments are called **N-terminal** and **C-terminal telopeptides**.



Two or even all three chains of tropocollagen are of the same type. E.g., the type I collagen consists of two chains α_1 I, the third chain is distinct (α_2 I), type II collagen in the cartilage include three chains of the type α_1 I).

Within the helical regions, there are no interchain disulfide bridges. **Covalent interchain crosslinks** are formed in the course of collagen "ripening".

The synthesis of collagen in fibroblasts, chondroblasts, and osteoblasts:

INTRACELLULAR:

- The synthesis of separate **procollagen chains** (pro- α_1 , pro- α_2 , etc.)
- Cotranslational **hydroxylation** of prolyl and lysyl residues and **glycosylation** of hydroxylysyls (Hyl) within the endoplasmic reticulum
- Formation of procollagen from three chains (triple-helical middle parts).
- **Secretion** of procollagen by exocytosis.

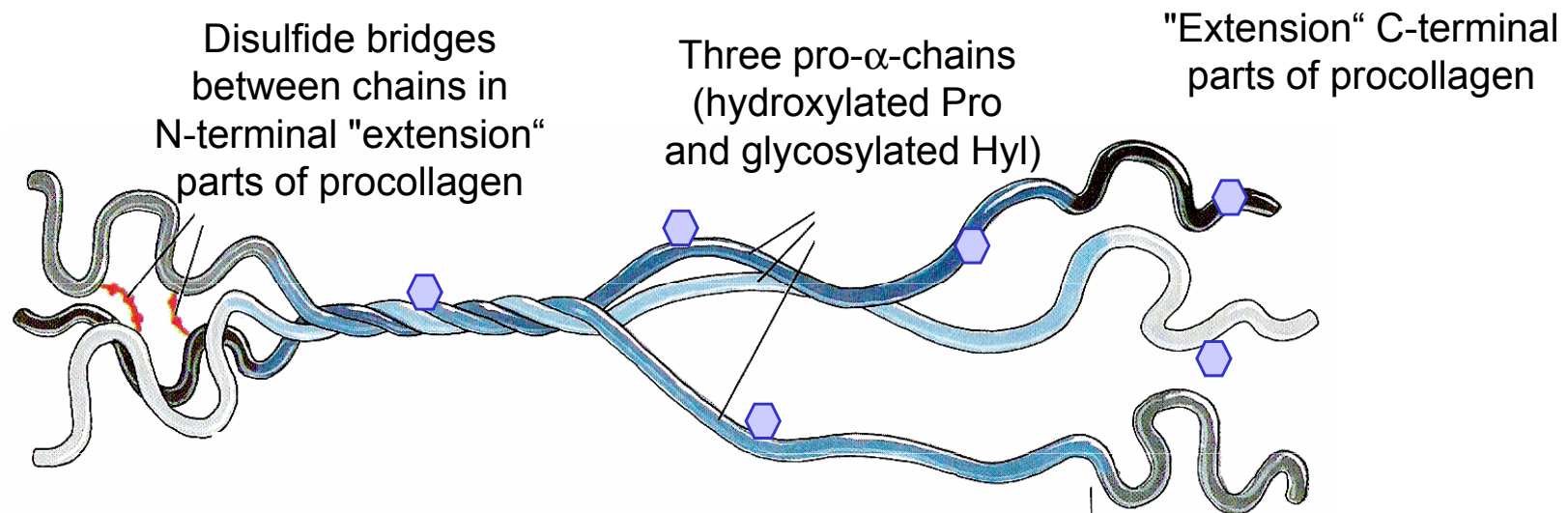
EXTRACELLULAR:

- Removal of N- and C-terminal propeptides – **tropocollagen** is formed
- Aggregation of tropocollagen units into **protofibrils**
- Interactions of protofibrils with proteoglycans – **microfibrils** are formed
- **Collagen ripening** – **oxidation of lysine** side chains to allysine, formation of aldimines and aldols, then also of hydroxypyridinium and other types of **intermolecular crosslinks**.
- **Degradation** of collagen is initiated specifically by collagenases (Zn²⁺-metalloenzymes), then by various non-specific proteinases.

Details of the collagen biosynthesis:

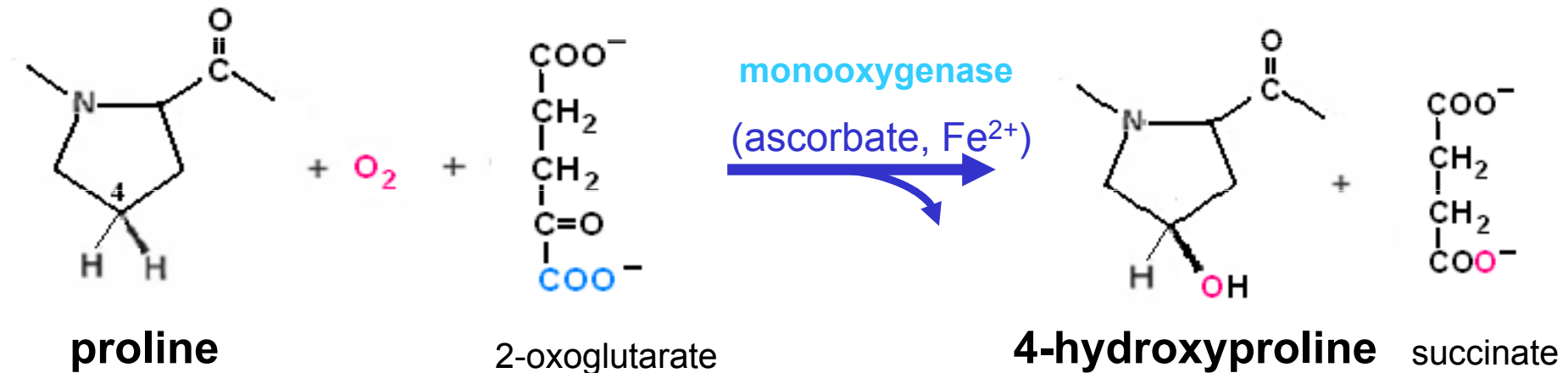
- Ribosomal synthesis of separate **procollagen** chains (pro- α_1 , pro- α_2 , etc.), the chains enter the endoplasmic reticulum.
- **Cotranslational hydroxylation** of prolyl and lysyl residues and **glycosylation** of hydroxylysyl (Hyl) residues:

Both hydroxylation of Pro and Lys and O-glycosylation of hydroxylysine in the middle parts (as well as N-glycosylation of C-terminal sequences) of procollagen chains is the prerequisite for the formation of disulfide interchain bonds within the N-terminal "extension" sequences of nascent procollagen chains, and even for the formation of disulfide bonds in their C-terminal parts. The resulting stabilization of globular structure of the terminal sequences supports winding of the chain middle parts and formation of the triple helix.



Hydroxylation of proline and lysine residues

is catalyzed by the **monooxygenase (hydroxylating) system**, in which **2-oxoglutarate** acts as an unusual reductant (it is oxidatively decarboxylated to succinate in the reaction). **Fe²⁺** ions and **L-ascorbate** are essential cofactors:

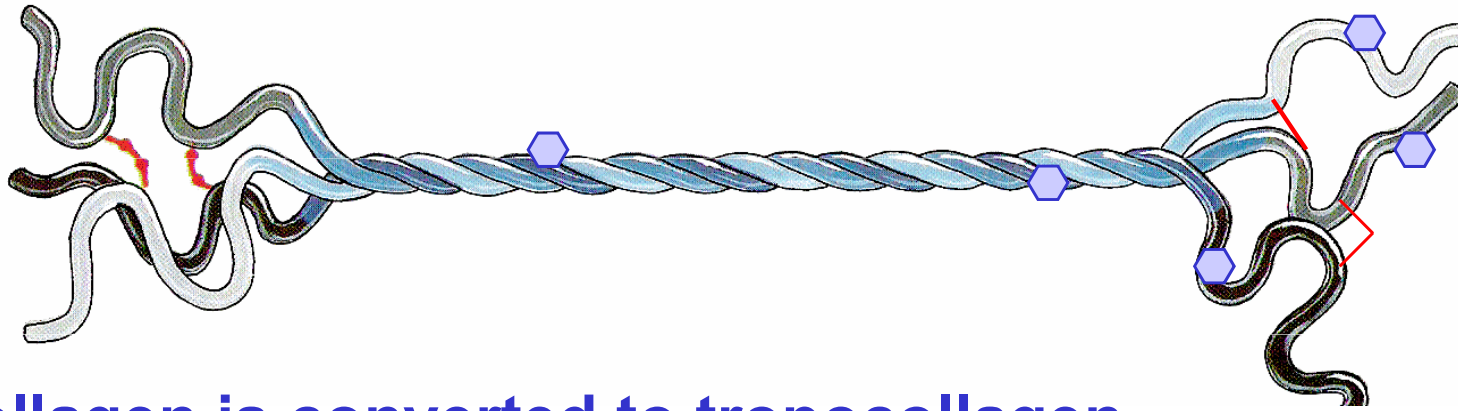


5-Hydroxylation of lysyl residues is analogous to the hydroxylation of proline.

Glycosylation of 5-hydroxylysine residues

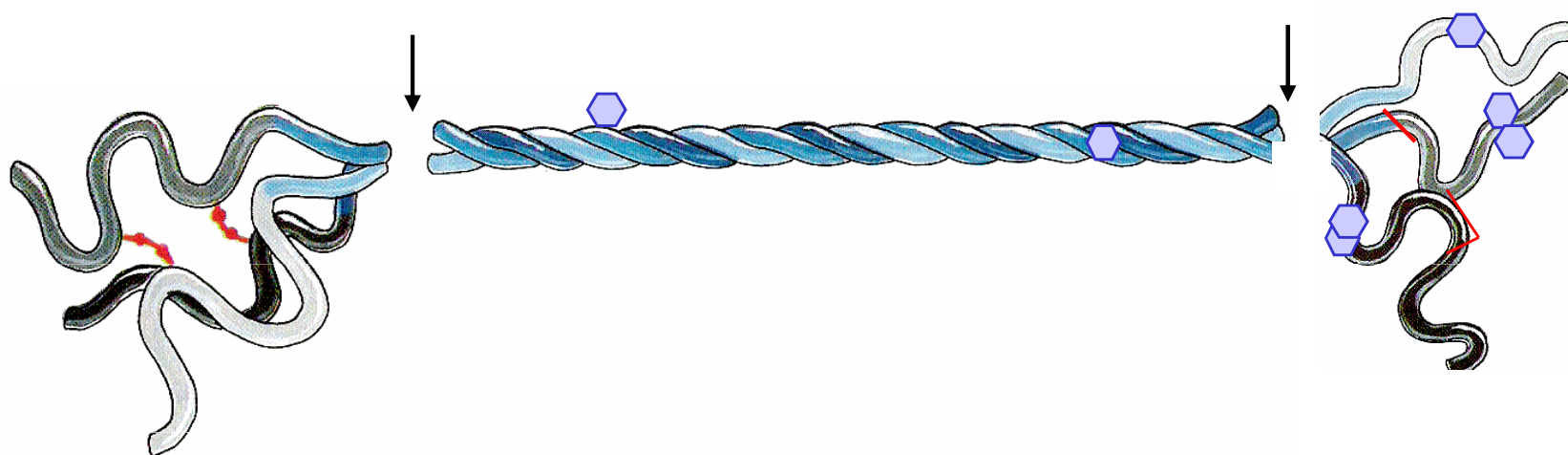
In type I tropocollagen, only a few glucosyl-galactosyl or galactosyl groups occur attached through O-glycoside bond to hydroxylysine side chain. The glycosylation is catalyzed by two specific **UDP-glycosyl:hydroxylysyl-glycosyltransferases**.

Winding of the procollagen middle parts results in the triple helix



Procollagen is converted to tropocollagen

Specific **procollagen peptidases** catalyze the removal of globular (and stabilized by disulfide bonds) **N-terminal and C-terminal propeptides** by hydrolysis within the non-helical segments. In clinical biochemistry, the abbreviations PINP and PICP are used for those propeptides.



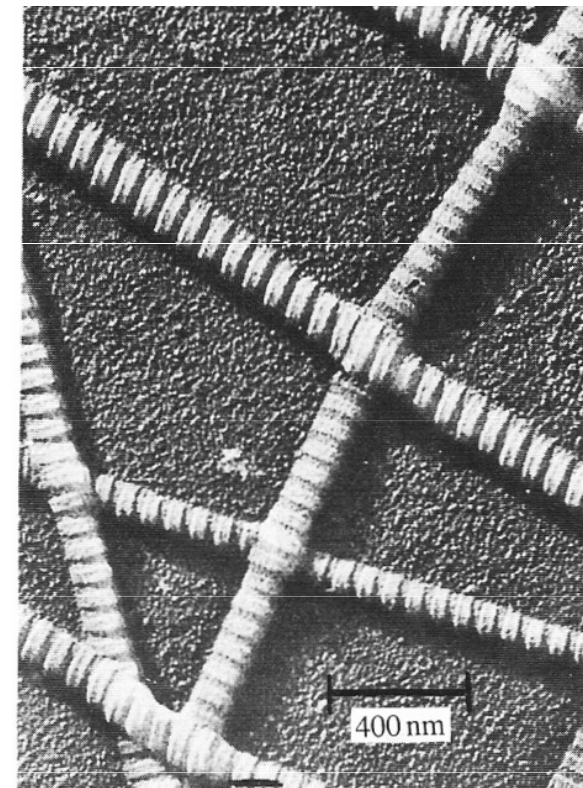
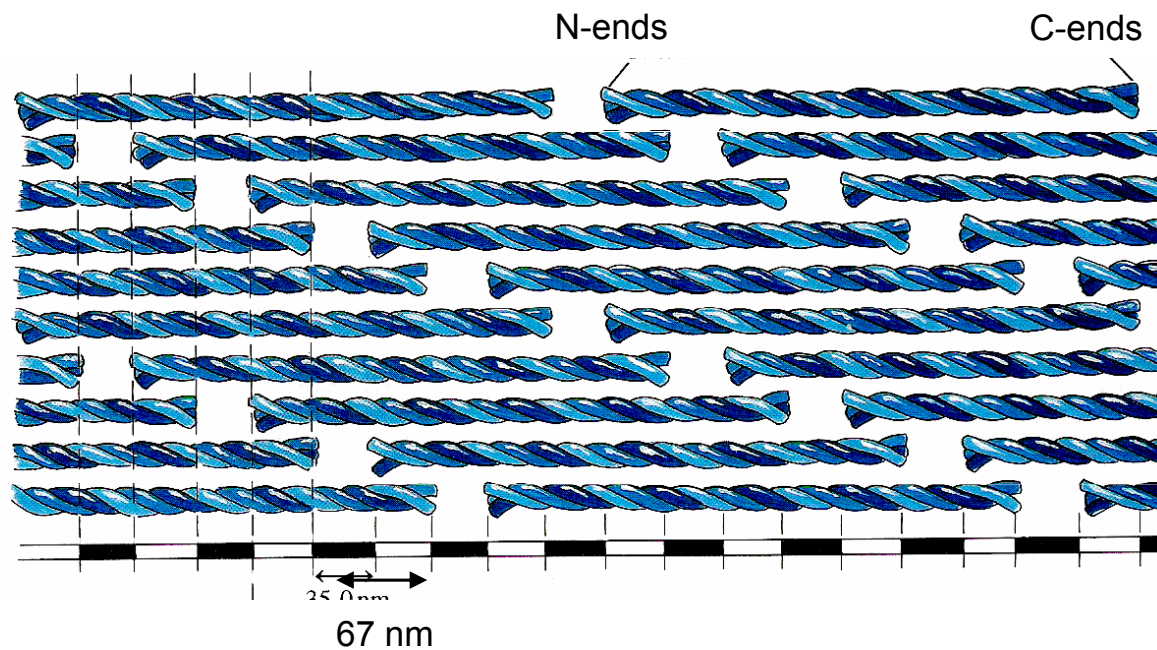
procollagen I N-terminal propeptides

tropocollagen

procollagen I C-terminal propeptides

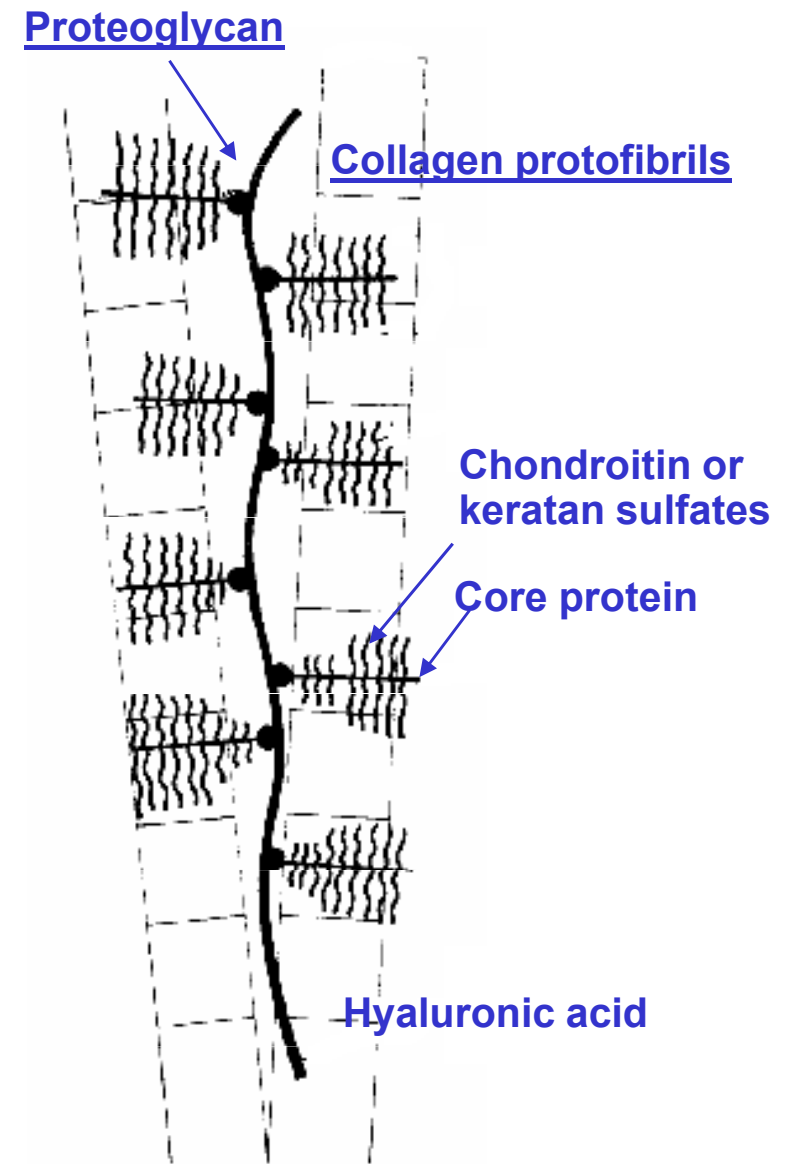
Aggregation of tropocollagen units – formation of protofibrils

After processing and close to the surface of fibrocytes, tropocollagen units associate spontaneously to form protofibrils, parallel bundles, the diameter of which corresponds to about 5 – 10 units. Tropocollagen units in protofibrils are shifted by $\frac{1}{4}$ of their length, so that the regularity of gaps and overlaps generates the **banding pattern** in the fibrils. .



Protofibrils of collagen interact with glycosaminoglycan chains of **proteoglycans**:

Bundles or felty tangles of protofibrils of fibrillar collagen types compose thick **microfibrils**.

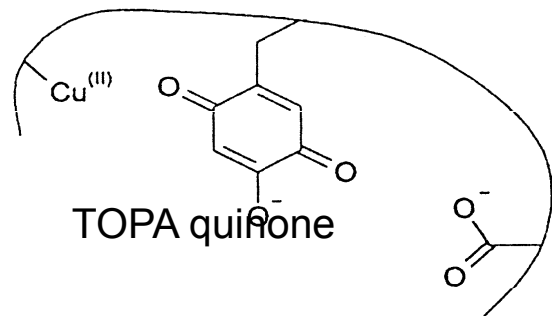


Further stabilization of collagen (maturing, ripening) – formation of covalent crosslinks

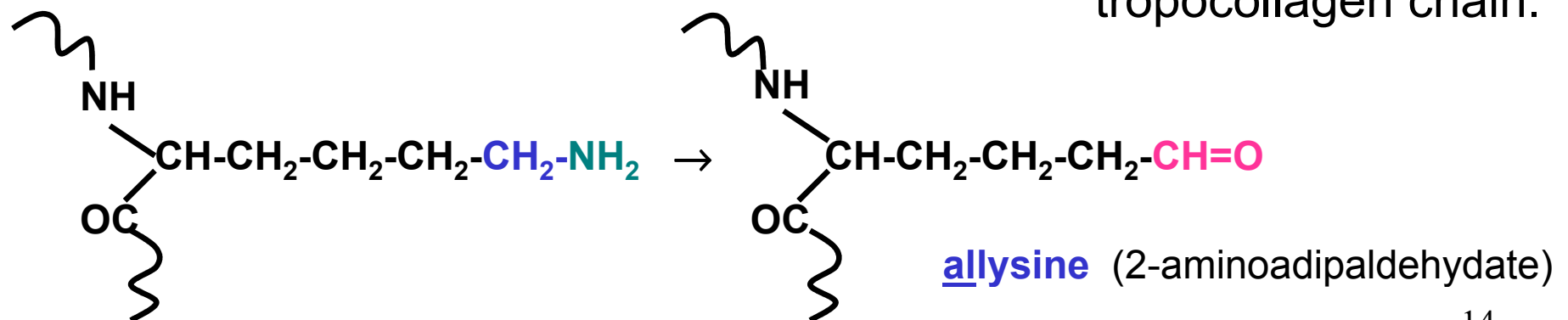
In crosslink formation, the decisive role belongs to the side chains of **lysine** or non-glycosylated **hydroxylysine** residues.

The initial reaction – the only one enzymatic reaction involved in crosslink formation – is the **oxidative deamination** of lysine or hydroxylysine side chains in the non-helical ends of fibril-forming tropocollagens, catalyzed by a specific extracellular aminooxidase **lysyl oxidase**.

Lysyl oxidase is copper-containing enzyme, coenzyme a quinone TOPA quinone:



Oxidative deamination of ϵ -amino group gives a reactive **aldehyde group** – the reactive residue of amino acid called **allysine** substitutes the residue of lysine in polypeptide tropocollagen chain.

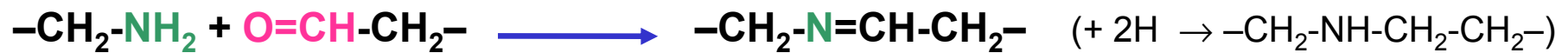


Interchain covalent crosslinks

originate in **non-catalyzed reactions** between the side chains of allysine and lysine residues.

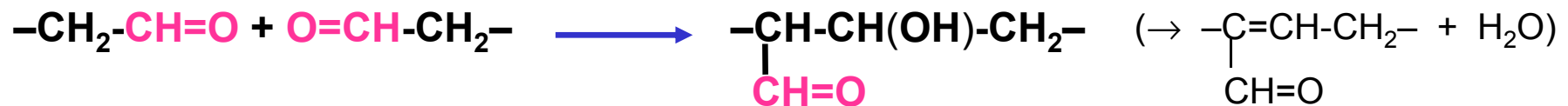
Simple covalent bridges join only two adjacent polypeptide chains, two types of those crosslinks are possible:

- **Aldimine type**, when the aldehyde group of allysine reacts with the ϵ -amino group of lysine. The product is an aldimine (Schiff base):



The reaction is rapid, but the product is unstable. It is stabilized very slowly by hydrogenation, the crosslink is then called **lysinoonorleucine** bridge.

- **Aldol type**, when two aldehyde groups of allysine react with each other (aldol condensation). One aldehyde group remains free in the bridge, so that it can take part in another reaction.

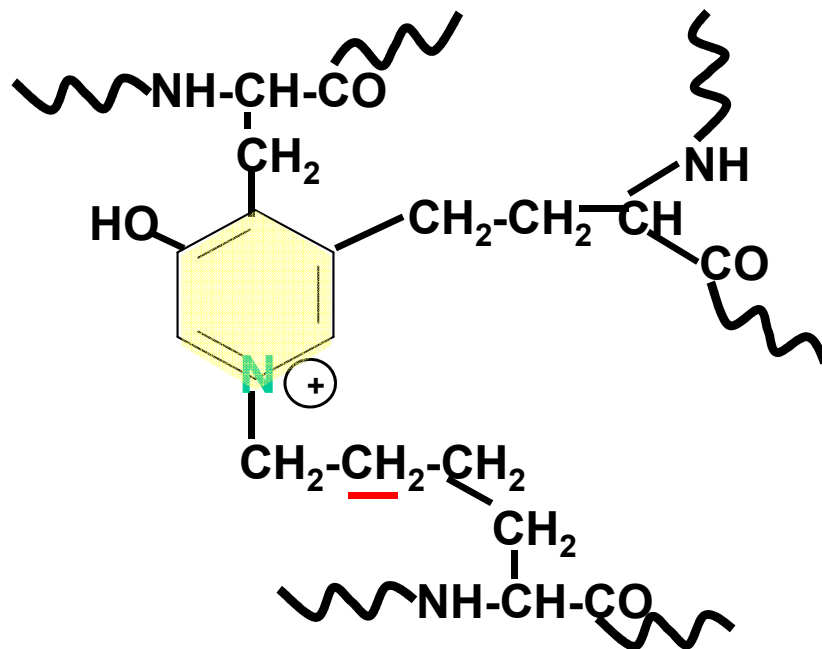


The resulting aldol is unstable, but it can be stabilized by elimination of water (a double bond is formed) to the **dehydroallysinealdehyde** bridge..

Aldimine bridges can react with the aldehyde group of another allysine side chain. Then the heterocyclic pyridinium ring is closed that binds **three polypeptide chains** together through stable covalent bonds – the **hydroxypyridinium type** of crosslink is formed..

In each tropocollagen unit, only four of all lysine residues (two near the N-ends and other two near the C-ends of the chains) can take part in formation of those hydroxypyridinium crosslinks.

Therefore, hydroxypyridinium crosslinks join N-ends of chains with C-ends of adjacent tropocollagen units.



The hydroxypyridine ring is a very stable structure. It can be determined even after hydrolysis of all peptide bonds in collagen fragments, e.g., excreted into the urine, as **deoxypyridinoline** or **pyridinoline**. The only difference between those two structures is the presence or absence of an alcoholic group on the carbon 5 (highlighted in the formula). This hydroxyl is present, when hydroxylysine was one of the reactants.

Elastic fibres

are characteristic components of **elastic connective tissue** in the arterial walls, pulmonary alveoli, skin, ligamentum nuchae, and **elastic cartilage**.

The structure and formation of elastic fibres

The elastic deformability of elastin derives from the presence of elastic fibres, which consist of spatial network (located in the fibre axis) formed by crosslinking of a soluble protein tropoelastin into insoluble "mature" **elastin**.

Elastin network in elastic fibres is surrounded by a microfibrillar sheath that consists of glycoprotein **fibrillin** and **fibromodulin**. During the formation of elastic fibres, those two proteins are arranged in the form of **oxytalan fibrils**, which serve as a mould that is then filled by tropoelastin, and in which tropoelastin is crosslinked to mature elastin.

Elastin

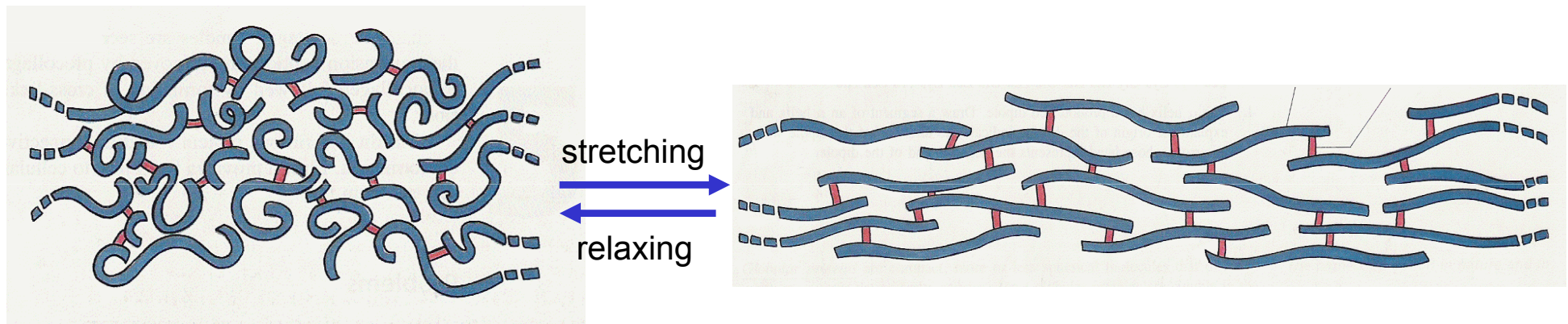
is an unusual protein. **Nonpolar amino acids prevail** distinctly. In the tropoelastin chains (about 750 AA residues - .33 % glycine, 10 % proline with a small portion of hydroxyproline, and over 40 % valine, alanine, and other hydrophobic amino acids). Hydroxylysine is absent, no saccharidic component. The characteristic feature of elastin is the alternation of large nonpolar segments with **segments including lysine**, important for crosslinking of the chains.

Elastin is not a typical fibrillar protein, even when it is the major component of elastic fibres and determines their elastic deformability.

The chains of soluble tropoelastin (taken sometimes as monomers or subunits of elastin) **don't have a regular secondary structure**, their conformation is usually declared as disarranged.

The tropoelastin chains are joined by means of numerous covalent crosslinks during the process of maturation. This **tropoelastin crosslinking** produces **insoluble elastin**, an elastic spatial network is formed. Hydrophobic interactions between numerous nonpolar amino acid residues (namely valine) support the elasticity.

Scheme of the elastic network of elastin

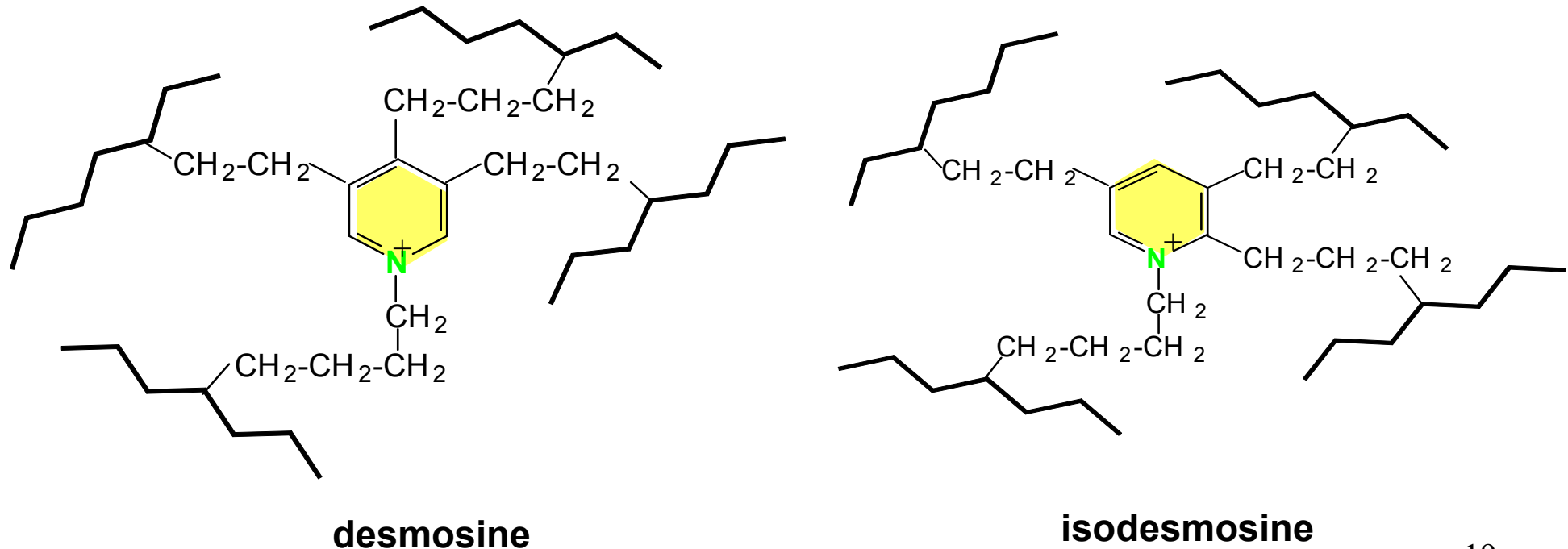


Covalent crosslinks in elastin

are formed in a similar way as in collagen – in non-catalyzed reactions between the side chains of lysine and allysine residues (allysine is produced by oxidative deamination catalyzed by **lysyl oxidase**).

Two chains can be bound through **simple aldol or aldimine bridge**, acyclic **merodesmosine** crosslink joins three side chains (the reaction of aldol with the lysine side chain).

Four side chains of allysine and lysine (usually of only two tropoelastin chains) form a tetrafunctional crosslink (the reaction of merodesmosine with allysine) with the stable pyridinium structure called either **desmosine** or **isodesmosine**:



Synthesis, ripening, and degradation of elastin

The synthesis of elastin occurs only in the last phase of foetal development, it is completed practically soon after the childbirth. The later synthesis of considerable amounts of elastin is not supposed.

INTRACELLULAR synthesis in **fibrocytes** and **leiomyocytes** of blood vessel walls

PROELASTIN

peptide fragments?

posttranslational processing
(incl. limited proline hydroxylation)

soluble **TROPOELASTIN**

SECRETION OF TROPOELASTIN into the **EXTRACELLULAR** matrix

partial degradation
of tropoelastin chains

incorporation into the fibrilin mould,
spatial arrangement (**coacervation**),
allysine is formed (**lysyloxidase**),
non-catalyzed formation of desmosines
(**crosslinking**), interactions with proteoglycans

insoluble **ELASTIN** (a very long biological half-life)

specific **elastases**
supporters of breakdown

ozone, (NO)_x, tobacco smoke,
in vessels – intercalation of **LDL**,
bile and fatty acids

antielastase factors

α₁-antitrypsin,
α₂-macroglobulin,
HDL in blood vessels

peptide fragments

(some of them are potent immunogens)

Elastin exhibits a very long biological half-life (the half-life of elastin in the wall of aorta is referred to be about 40 years). After the childbirth, the synthesis of elastin is distinctly reduced, if it is possible at all.

The loss of tissue elasticity

(namely of skin, large vessels, lung) in aging organisms is indisputable. Much attention is paid to the elastin degradation.

Wrinkles are the sign of low skin elasticity that decreases by 2 - 5 % per decade (extrapolated to neonatal period). Wrinkles represent rather a cosmetic problem, though it is extremely interesting from the commercial point of view to those who produce cosmetics and feign the knowledge how to remove wrinkles.

The loss of vessel wall elasticity in ageing doesn't quite depend on the low elastin content, but rather on elastin modification due to partial degradation caused by activity of elastase (released mostly by polymorphonuclear leukocytes), supported by the interactions of elastin fibres with LDL or bile acids.

The loss of lung tissue elasticity is associated directly with the degradation of elastin in the walls of alveoli. **Elastase** is released from circulating neutrophils but, under normal conditions, elastase is nearly completely inhibited by **α_1 -antitrypsin**. Inherited deficit of α_1 -antitrypsin or factors that diminish its effect (smoking, ozone in the ground smog) distinctly increase the risk of pulmonary emphysema.

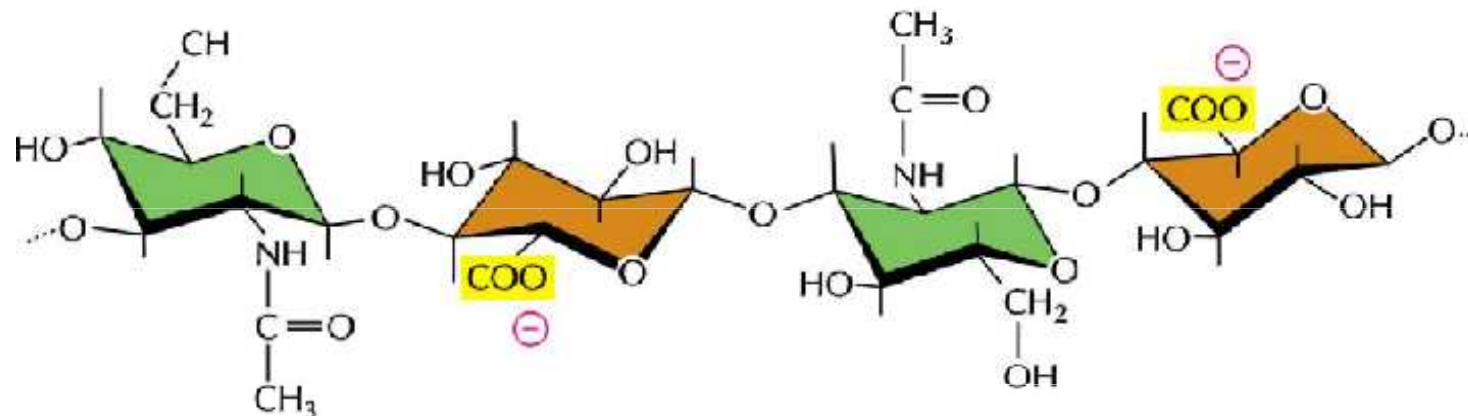
Proteoglycans

If not thinking of dense collagen connective tissue and bone, proteoglycans represent the most voluminous component of amorphous ground substance in connective tissue, which fill in the space among fibres and cells.

In proteoglycans, numerous chains of heteropolysaccharides – **glycosaminoglycans** (GAG) bind through glycosidic bonds the **core protein**.

Glycosaminoglycans

The common feature of various glycosaminoglycans (e.g. dermatan sulfate, keratan sulfate, hyaluronic acid) is the regular alternating of units *N*-acetylated amino sugars and glycuronic acids. Both types of units are oft sulfated (except hyaluronic acid):.



Segment of the hyaluronic acid molecule \rightarrow 3) GlcNAc (β 1-4) GlcA (β 1-3) GlcNAc (β 1-4) GlcA (β 1-3) \rightarrow

Hyaluronate is the only glycosaminoglycan that occurs in the ground substance as a heteropolysaccharide without any covalent bond to proteins. Other GAGs are attached to proteins through glycosidic bonds forming proteoglycans.

Proteoglycans

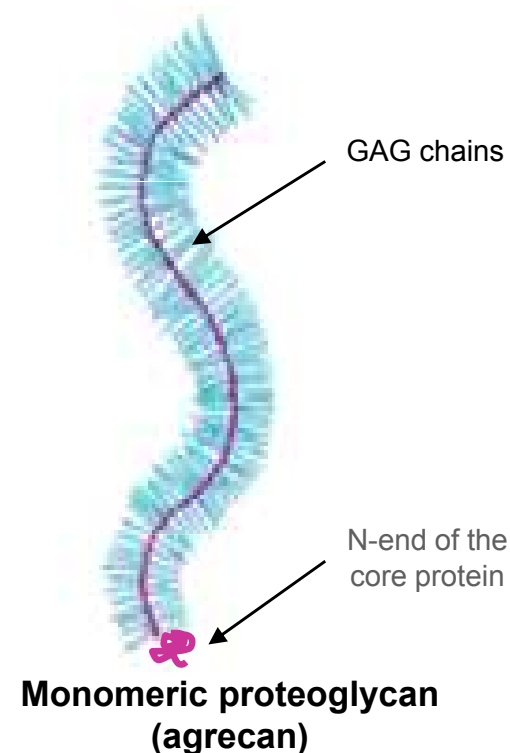
Core protein of proteoglycans is a relatively large protein, M_r about 200 000. In spite of that size, it represents only about 5 – 15 % mass of the proteoglycan.

At the N-end of the chain, there is a globular domain, through which proteoglycans can bind non-covalently (with the assistance of two other glycoproteins, link proteins) hyaluronic acid forming so complicated aggregates.

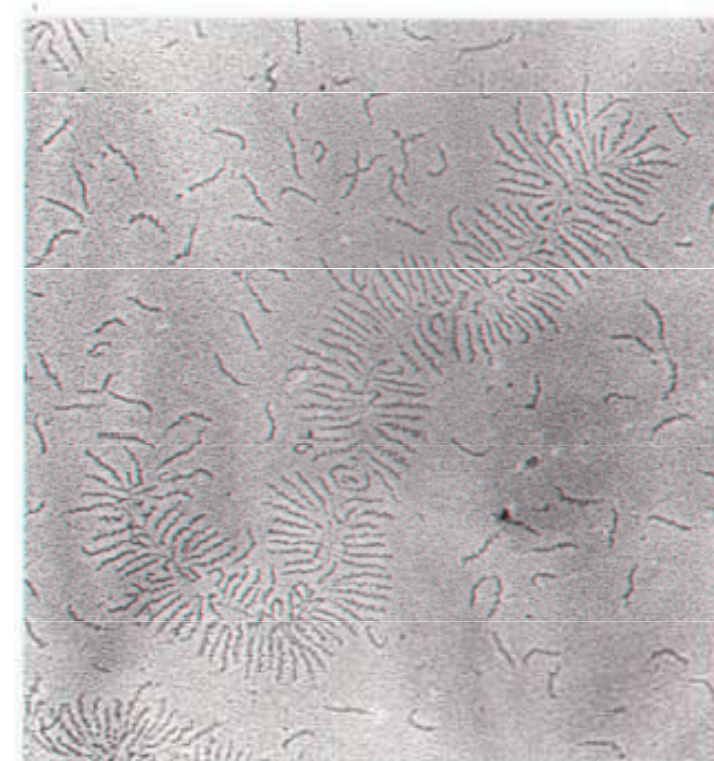
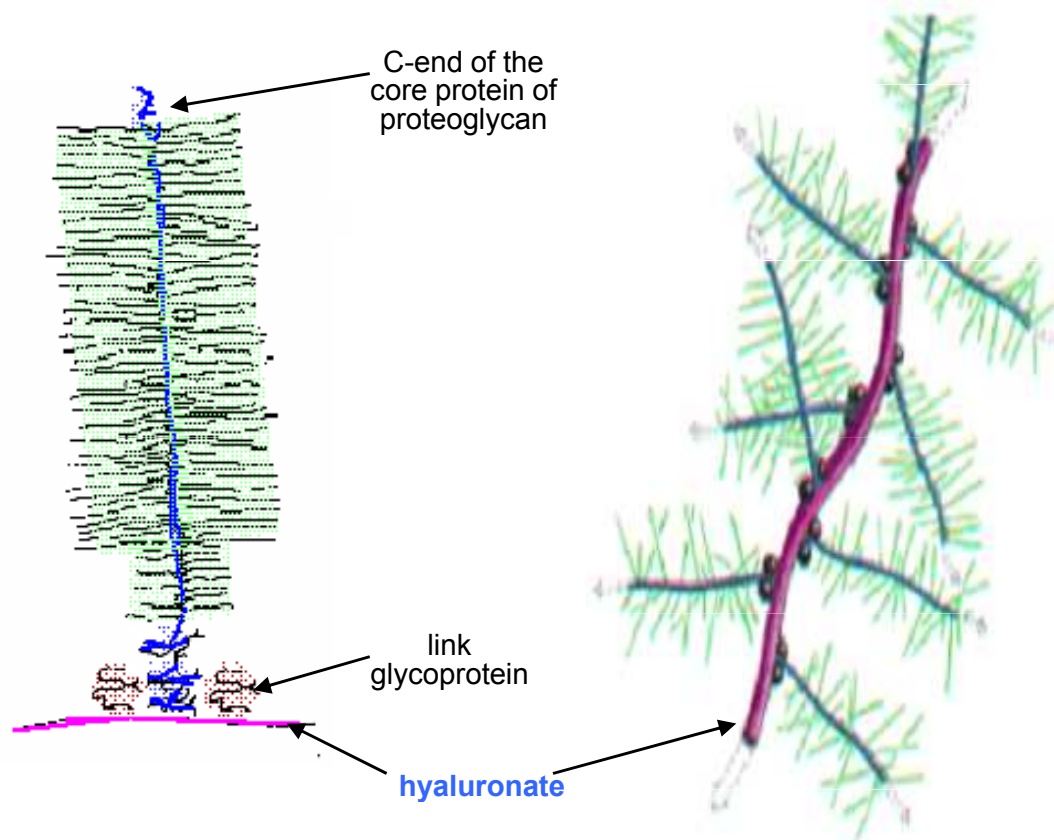
Glykosaminoglycans are linked to the core protein mostly by their sequence Gal-Gal-Xyl. Nearly all those bonds are **O-glycosidic bonds to serine** residues. The chains include 10 – 100 monosaccharide units.

The number of GAG chains in **monomeric proteoglycans** called **agrecans** is very approximately 100. The agrecan structure resembles a bottle-brush.

Proteoglycans are **highly hydrated**, and numerous carboxylate and sulfate groups bind due to negative electric charges large amounts of hydrated cations.



A large number of simple monomeric proteoglycans (agrecans) bind their globular domains of core proteins **non-covalently** to a long chain of **hyaluronic acid**, each of these interactions is supported by two molecules of link glycoproteins. **Huge aggregates** are formed in this way namely in hyaline cartilages. They contribute to the resistance of a cartilage to mechanical pressure and to its elasticity. The solidity of a cartilage depends on the number and arrangement of collagen fibrils.



Electron optical picture of proteoglycans in extracellular matrix of an cartilage

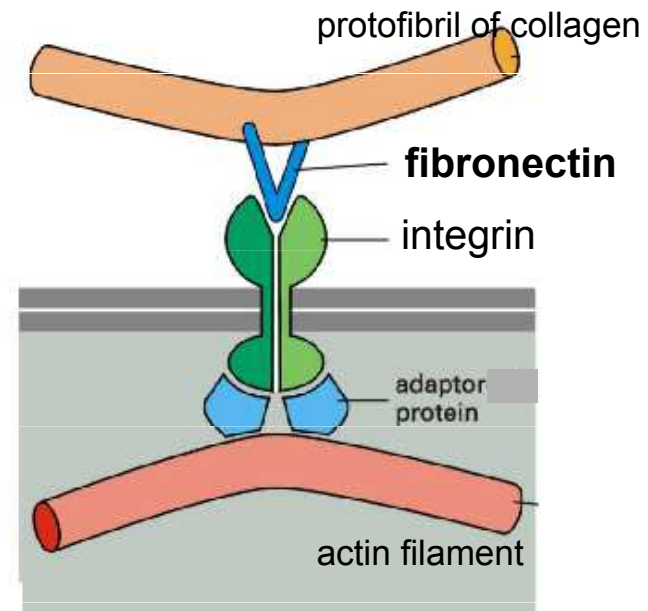
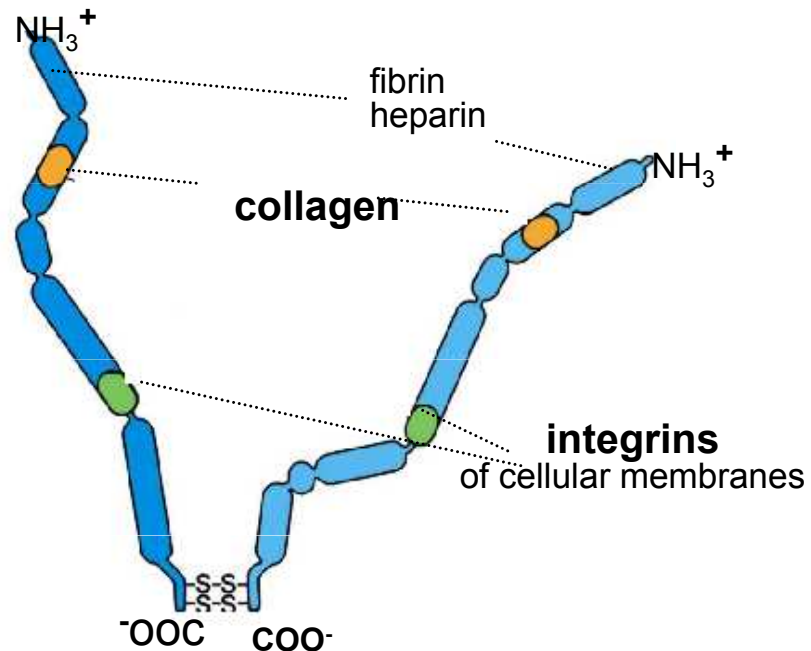
Fibronectin

is a glycoprotein of the extracellular matrix, released by fibrocytes, endothelial cells, and other cell types. The liver cells secrete fibronectin into the blood plasma (concentration 150 - 700 mg / l). It mediates **interactions between collagen, proteoglycans and cells**. Its name descends from Latin "fibra nectere", metaphorically speaking a cellular adhesive.

Two polypeptide chains (230 kDa) of fibronectin are joined by disulfide bonds and include binding domains composed of repeated sequences.

Deficit of fibronectin on the surface of tumour cells can support their release and formation of metastases.

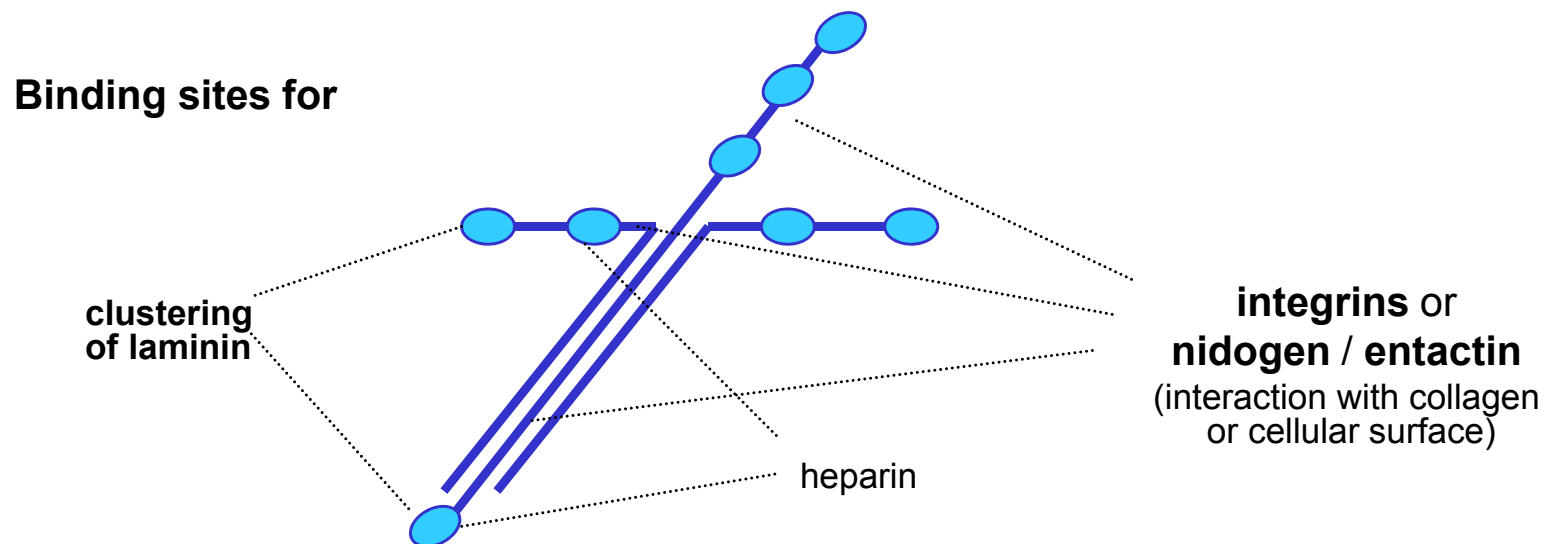
Binding domains for



Laminin

Laminins are members of the family of non-collagen glycoproteins in **basement membranes**. They are large heterotrimers (M_r 950 000). Three chains are joined through disulfide bridges and they are arranged into a cross-like form with four arms. Similarly to fibronectin, laminins include several binding sites, e.g. for integrins of cellular membranes, heparin, and heparan sulfate.

Laminin aggregates reversibly into a flat polymeric network at high concentrations of Ca^{2+} ions. Those polymers bind through another protein (either nidogen or entactin) **type IV collagen** and core proteins of proteoglycans. These interactions are the cause of anchoring **epithelial cells onto components of basement membranes**.



The bone

The bone is a tissue exhibiting very high metabolic activity, though it doesn't seem so at first glance.

The **unremitting bone remodeling**, both osteoresorption and bone formation, continues after the growth of bones is finished.

The bulkiness and consistency of bones depends on the balance between resorption and formation.

Composition of bones

Water 25 % in compact bone (12 % cementum, 10 % dentin, 1 % tooth enamel)

Organic components 30 %

Mineral components 40 %

In dry bone tissue organic components 40 – 45 %
mineral components 60 – 55 %

Extracellular matrix:

organic components - collagen type I
proteoglycans
sialoprotein
osteocalcin
citrate

mineral components - hydroxylapatite $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$
with octacalcium phosphate $\text{Ca}_8(\text{HPO}_4)_8 \cdot 5\text{H}_2\text{O}$
and amorphous calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$

on the whole 85 %

CaCO_3	10 %
CaF_2	0.3 %
CaCl_2	0.2 %
$\text{Mg}_3(\text{PO}_4)_2$	1 %
alkaline salts	2 %

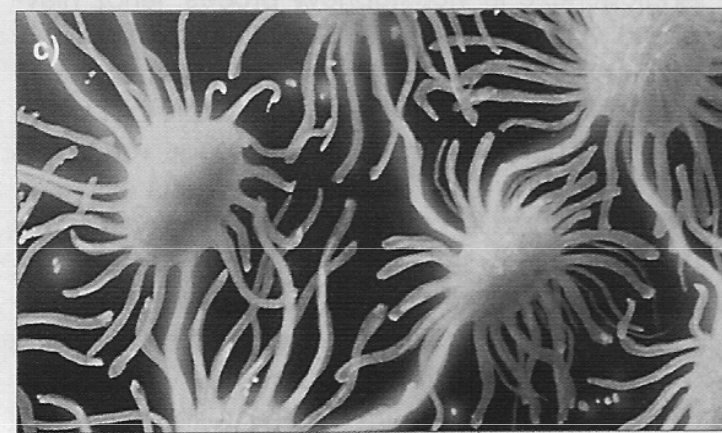
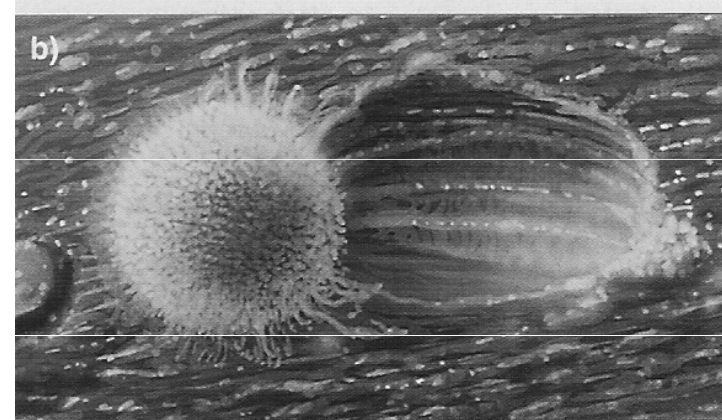
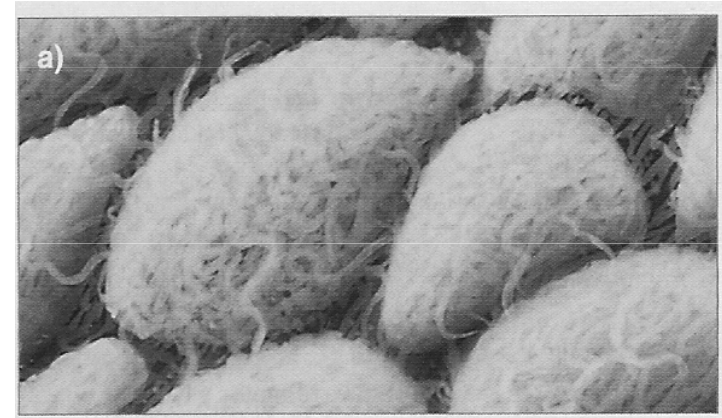
Bone cells: **osteoclasts** (modified macrophages, bone resorption),
osteoblasts (a type of fibroblasts, bone formation), and
osteocytes (transformed aged osteoblasts) affect the activity of
previous cell types by paracrine secretion of interleukins, tumour
necrosis factor (TNF), osteoprotegerin, prostaglandins and other
various growth factors.

Bone cells

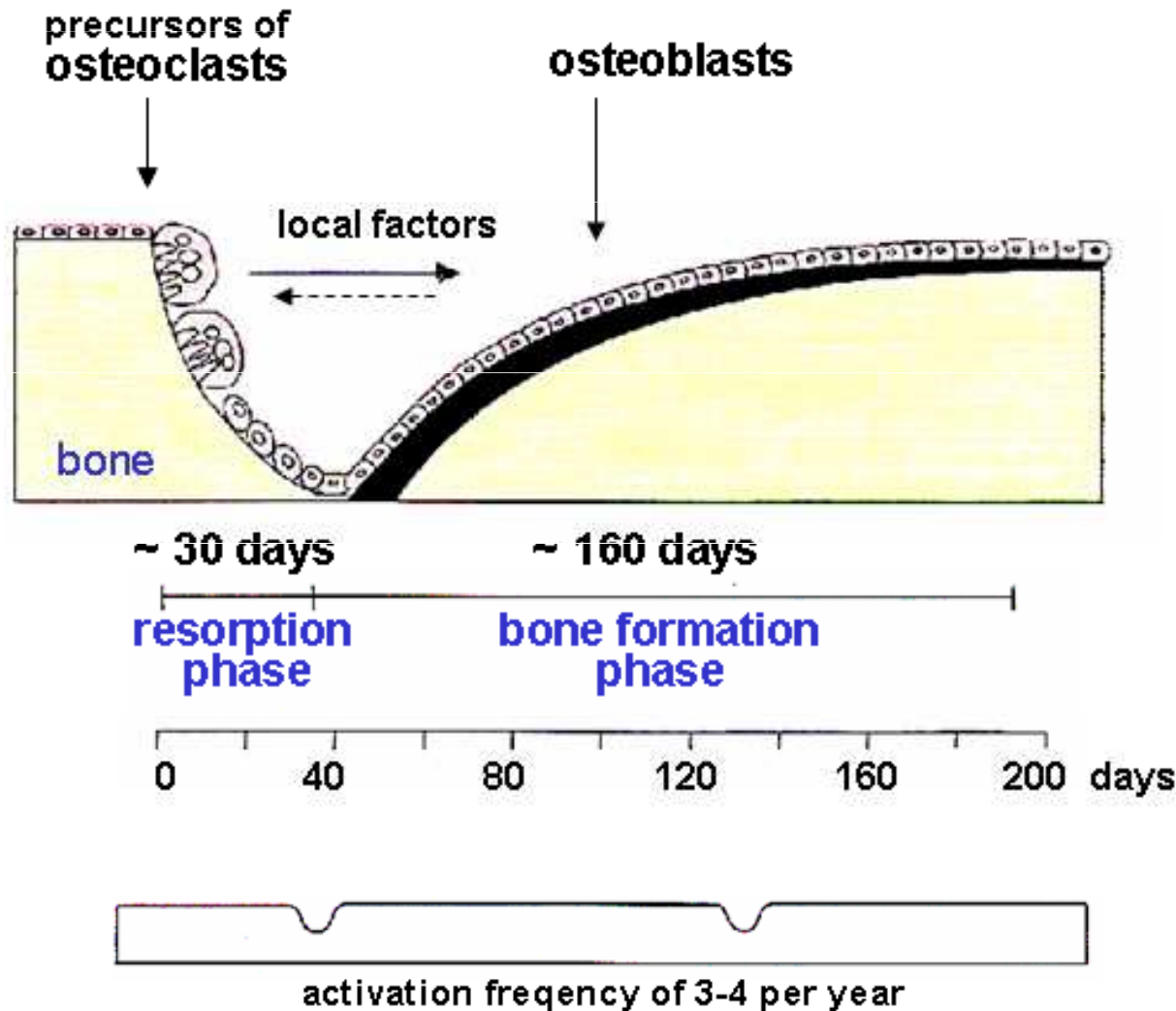
Osteoblasts occur on the surfaces of growing or remodelated bones, less frequently inside adult bones. They synthesize and insert osteoid (extracellular matrix), deposit the bone minerals, and consecutively settle and transform into osteocytes. The surface of osteoblasts binds molecules of alkaline phosphate that support mineralization of the matrix.

Osteoclasts occur at sites of active bone resorption, in resorption pits. They include many lysosomes exhibit high activity of acid phosphatase. Osteoclasts liquidate fragments of collagen and other organic components through phagocytosis.

Osteocytes are prevailing cellular elements of mature bone. They are dispersed in lacunas and form a cellular net by means of contacts between their projections. Life span of osteocytes is estimated to about 25 years, extinct cells initiated bone resorption.



Control of bone remodeling



Bone resorption

stimulated by parathyrin
calcitriol

inhibited by calcitonin
estrogens

Bone formation

stimulated by (parathyrin)
calcitriol

inhibited by androgens
estrogens
glucocorticoids

Biochemical markers of bone metabolism

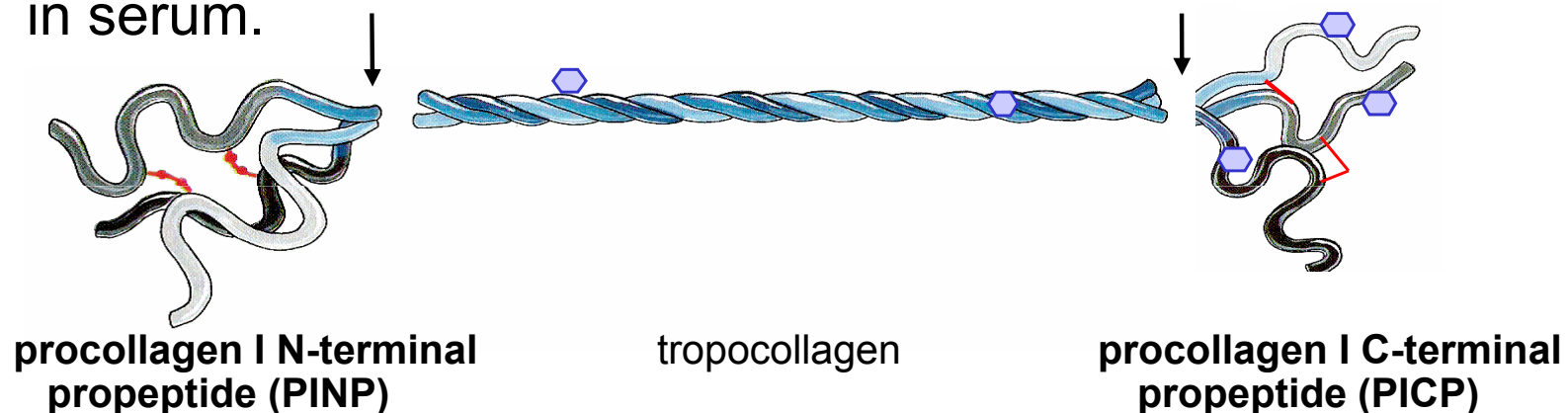
are useful in clinical biochemistry in tests of osteoporosis and other metabolic osteopathies.

Biomarkers of bone formation

Catalytic concentration of the **bone isoenzyme of alkaline phosphatase (ALP)** in serum – the isoenzyme is a marker of osteoblast activity, of which it is an ectoenzyme. Total activity of ALP can serve only as a very rough estimate due to prevalence of other isoenzymes.

Concentration of **osteocalcin** (bone Gla protein, BGP) in serum – osteocalcin is a modulator of bone remodeling secreted by osteoblasts, its production is induced by calcitriol. Osteocalcin is the main non-collagen protein in the extracellular matrix of bones.

Concentration of **N-terminal** or **C-terminal propeptides** of **procollagen I** in serum.



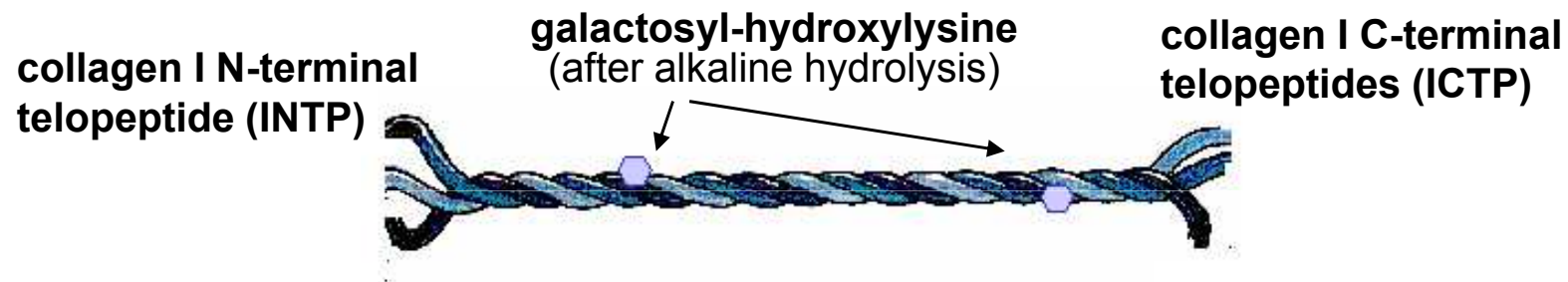
Biomarkers of bone resorption

Catalytic concentration of the **bone isoenzyme of acid phosphatase** (ACP) in serum. This isoenzyme is one of the six ACP isoenzymes (called also tartrate-resistant ACP) considered a marker of osteoclast activity.

Concentration of **C-terminal telopeptide of collagen I** (ICTP, or of C-terminal octapeptide, CTx) in serum or urine. C-terminal telopeptides are terminal non-helical sequences of tropocollagen I, without crosslinks.

Excretion of **N-terminal telopeptide of collagen I** (INTP, NTx) into the urine – N-terminal non-helical sequences of tropocollagen I, which may include some crosslinks, too..

Determination of **deoxypyridinoline** and **pyridinoline** in the urine, and determination of **galactosyl hydroxylysine** in the urine.



Excretion of **hydroxyproline** (free or total) in the urine was in common use for years, but currently it is taken as an obsolete test of bone resorption.