

ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

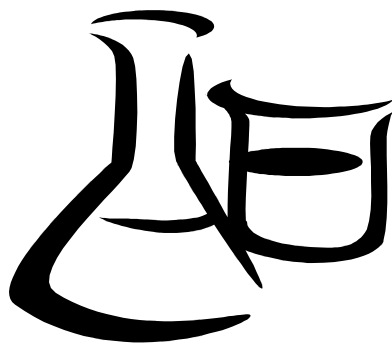
# Calcium and phosphorus Metabolism of bone tissue

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*General Medicine*

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2018/2019

# 1 Bone Tissue

Bone provides mechanical support and protection to soft organs, enables movement, hosts hematopoietic tissue, and serves as storage of calcium, phosphate, and magnesium ions.

From one third it consists of **protein matrix**, and from two thirds of the **bone mineral**.

- The bone protein matrix contains mostly type I collagen (90 %); together with other proteins such as osteocalcin, osteonectin, osteopontin, etc. (10 %).
- The bone mineral is composed from small crystals of hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ . Other compounds, such as calcium carbonate, calcium fluoride and magnesium phosphate, are present as well.

The metabolic activity of bone tissue is provided by bone cells. **Osteoblasts** form **osteoid** (the bone protein matrix), into which mineral salts are deposited. Other cell types include **osteoclasts**, whose main function is bone resorption, and **osteocytes**, which complement activity of osteoclasts by providing fine tuning of bone resorption.

During lifetime of an individual, the bone tissue is subject of perpetual **remodelation**, encompassing **osteoclastic bone resorption** followed by **osteoblastic bone formation**. Osteoclasts dissolve mineral components and degrade the bone matrix. On the other hand, osteoblasts intracellularly synthesize precursors of the bone matrix proteins. Functional coupling of bone resorption and formation ensures that the living bone tissue is constantly renewed.

Metabolism of bones is tightly coupled to metabolism of calcium and phosphorus.

## 2 Calcium

### 2.1 Homeostasis of calcium

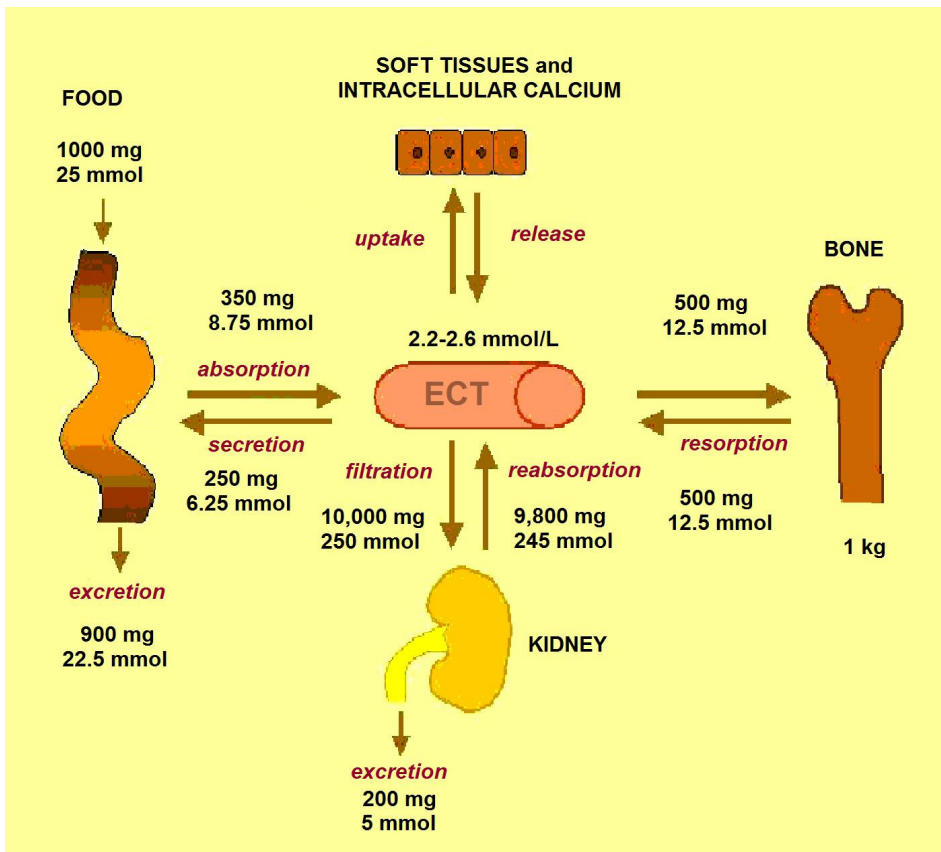
Human body contains about 1,000 g of calcium. Vast majority (98 – 99 %) of this amount is built in the hard tissues such as bones and teeth; the rest is located outside bones, largely extracellularly. Calcium is released from the hard tissues in response to body needs.

Very small amount of calcium is found in **intracellular fluid**. Inside the cell, 55 % of calcium is located in the endoplasmic reticulum, the rest in other cell organelles. Concentration of  $\text{Ca}^{2+}$  in the cytosol ( $10^{-7}$  mol/l) is four orders of magnitude lower than its concentration in blood plasma ( $10^{-3}$  mol/l); and this steep gradient between extra- and intracellular fluid is kept by many membrane transport mechanisms. Transient elevations of  $\text{Ca}^{2+}$  in the cytosol represent important signals for the cell, mediating the whole array of cellular functions and events (e.g. muscle contraction, transmission of nerve excitation, secretion of hormones, cell division).

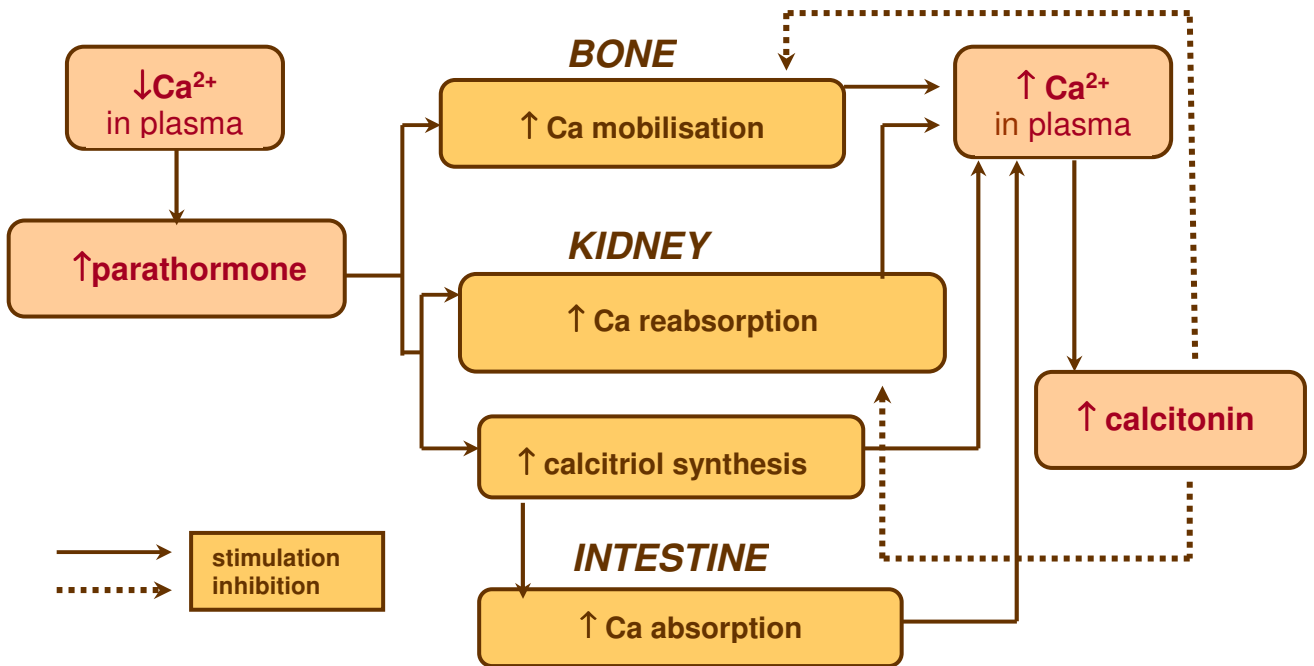
The dietary intake of calcium fluctuates around 1 g per day; in the periods of increased demand (growth, pregnancy, lactation) it can be as much as 1.5 g. Under physiological condition about 25-40 % of ingested calcium is absorbed in the small intestine. From the extracellular fluid the calcium passes mainly to bones where it becomes a substantial component of bone mineral. Exchange between bone tissue and extracellular fluid helps to regulate calcemia. Excretion of calcium (Fig.1) takes place in the intestine (80%) and kidney (20%). The renal excretion critically affects calcium balance in the body. In an adult, intake of calcium normally balances its excretion. Positive calcium balance is characteristic for childhood and adolescence, while in women after menopause and advanced age in general a negative calcium balance is found.

Level of calcium in the blood is regulated by **parathyroid hormone**, **1,25-dihydroxy-cholecalciferol (calcitriol)** and **calcitonin** (Fig. 2).

**Fig. 1 Daily calcium balance**



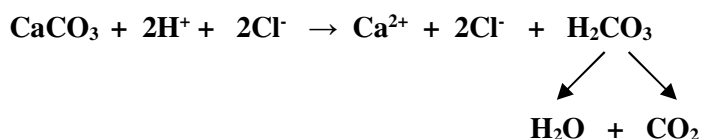
**Fig. 2 Regulation of calcium homeostasis**



## 2.2 Intestinal absorption of calcium

Various factors control the absorption of calcium in the intestine. In addition to the effects of hormones (especially calcitriol), the chemical form in which calcium occurs in the food as well as presence of other dietary components play major roles.

The **forms of calcium** differ in their biological availability, which is determined chiefly by **solubility in water**. In general, the water-soluble substances are easily absorbed. However, even poorly soluble calcium salts can be utilized thanks to the **acidic medium in the stomach**. Calcium carbonate, one of the poorly soluble compounds, can serve as an example. The hydrochloric acid in the stomach converts  $\text{CaCO}_3$  to a well soluble calcium chloride. The dissociated  $\text{Ca}^{2+}$  ions can then be absorbed in the intestine.



For this reason, calcium carbonate or calcium phosphate, which is also poorly soluble, should be taken together with meals. In patients with decreased production of gastric juice due to decline with age or stomach resection the calcium bioavailability can be reduced.

### List of selected calcium salts and their aqueous solubility

Salt	Solubility in water
Calcium carbonate	Less than 0.1 g/l $\text{H}_2\text{O}$
Calcium phosphate	Less than 0.1 g/l $\text{H}_2\text{O}$
Calcium citrate	0.2 g/l $\text{H}_2\text{O}$
Calcium gluconate	3.5 g/l $\text{H}_2\text{O}$
Calcium lactate	12.0 g/l $\text{H}_2\text{O}$
Calcium lactogluconate	45.0 g/l $\text{H}_2\text{O}$
Calcium chloride	740.0 g/l $\text{H}_2\text{O}$

The bioavailability of calcium is both positively and negatively affected by other food components that are ingested simultaneously with calcium. Presence of substances that form insoluble compounds with  $\text{Ca}^{2+}$  reduces its bioavailability. This is the case of foodstuffs with high contents of **oxalate** (e.g., spinach, rhubarb), **phytate** (e.g., cereals), and **fiber**. Likewise, some components of tea form poorly soluble calcium salts. That is why calcium tablets should not be rinsed with tea. Some fatty acids also react with calcium producing insoluble calcium soaps.

Other food components can increase absorption of calcium. These are complex-forming agents that keep calcium in a soluble form. Some amino acids and saccharides display this effect. For example, lactose forms a complex with calcium with stoichiometry 1:1.

## 2.3 Calcium in serum

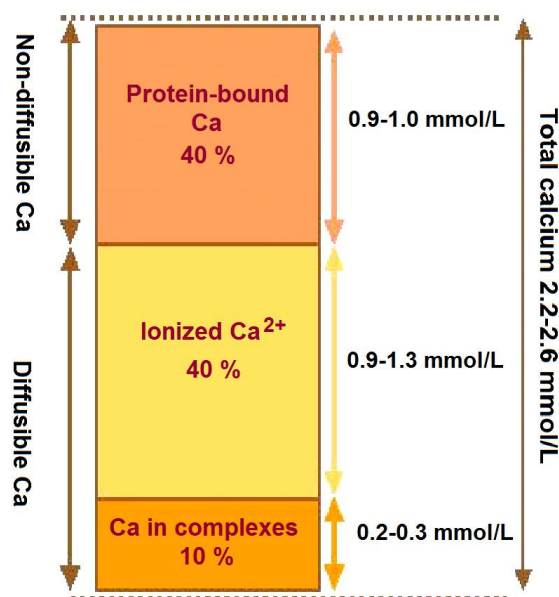
Estimation of serum calcium represents a basic screening examination for assessment of calcium homeostasis.

Calcium in serum exists in several forms (Fig. 3):

- 60 % of total calcium is **diffusible** – filtered by renal glomeruli. From this fraction:
  - 50 % of total calcium is in **free (ionized) form** (denoted as  $\text{Ca}^{2+}$ ). This is the biologically **active form** of calcium.
  - 10 % of total calcium occurs in **low-molecular-weight complexes** with citrate, phosphate or hydrogen carbonate
- 40 % of total calcium is **not diffusible** (does not pass the glomerular membrane) as it is **bound to plasma proteins** (90 % to albumin, 10 % to globulins). The protein-bound calcium is not biologically active, but rather it represents a **readily accessible reserve** from which calcium can be quickly released during hypocalcemia.

In hypoalbuminemia the calcium fraction bound to albumin decreases. Drop of plasmatic albumin of 10 g/l makes the total serum calcium level 0.2 mmol/l lower without any effect on the plasma concentration of ionized calcium. On the other hand, hyperproteinemia (e.g. in malign myeloma) may lead to a high increase in total calcemia, again without change in ionized calcium level. Therefore, both parameters, i.e. serum calcium and albumin, should be considered together. The amount of  $\text{Ca}^{2+}$  depends on pH: it decreases in alkalosis and increases in acidosis, due to mutual competition of  $\text{Ca}^{2+}$  and  $\text{H}^+$  ions for the binding sites on albumin.

Fig. 3 Forms of calcium in serum



### Reference values:

Normally, concentration of **total calcium in the serum (S-Ca, calcemia)** is kept within a rather narrow range of **2.25 – 2.75 mmol/l**. Calcemia around 4 – 5 mmol/l is considered as the upper limit of serum calcium concentration compatible with life. Death in hypercalcemia is due to heart arrest. On the other hand, the lower limit of calcemia still compatible with life lies around 1 mmol/l. Severe hypocalcemia leads to **convulsions (tetania)**.

**Ionized calcium (fS- $\text{Ca}^{2+}$ ): 0.9 – 1.3 mmol/l**

**Table 1: Some causes of hypercalcemia:**

Condition	Mechanism
Tumor of parathyroid gland: primary hyperparathyroidism	High secretion of parathyroid hormone
Some malign tumors, metastases into bones	Activation of osteoclasts: resorption of bone mineral prevails
Hyperproteinemia	Increased non-ionized calcium
Over-dosing by vitamin D	Increased calcium absorption from the intestine

**Table 2: Some causes of hypocalcemia:**

Condition	Mechanism
Removal or damage to parathyroid gland	Low secretion of parathyroid hormone
Gastrointestinal diseases	Impaired absorption of calcium in intestine
Hypovitaminosis of vitamin D: - impaired absorption of vitamin D - diet deficient in vitamin D - impaired conversion of vitamin D to active metabolites due to e.g. liver or kidney diseases	Decreased absorption of calcium in intestine
Disorder of tubular reabsorption of calcium in the kidney	Increased loss of calcium in kidney
High intake of phosphate	Compensatory decrease in calcium

## 2.4 Calcium in urine

The diffusible (ultrafiltratable) fraction of serum calcium in renal glomeruli passes into primary urine; subsequently in the tubules 98-99 % of this amount is reabsorbed, and the rest is excreted into urine.

Amount of calcium in the urine (calciuria) depends on:

- dietary contents of calcium and its absorption in the intestine
- degree of osteoresorption
- function of renal tubules

Calciuria can be examined in urine collected for 24 hours whose volume is recorded. Concentration of calcium is estimated in a sample of collected urine, multiplied by the volume of urine, and expressed as **daily loss of calcium in urine (dU-Ca)**.

$$\text{Urinary Ca loss (mmol/24 hours)} = \text{Ca in urine (mmol/l)} \times \text{Volume of urine/ 24 hours (l)}$$

The result depends on the accuracy of urine collection; therefore together with calcium also concentration of creatinine is estimated.

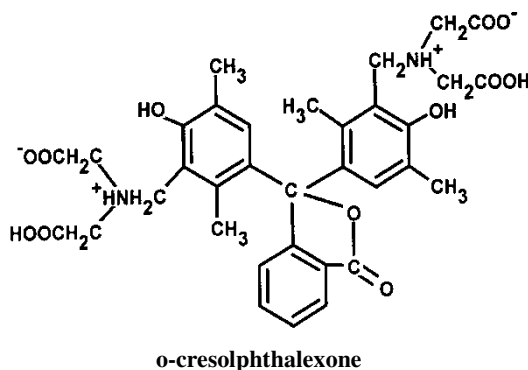
**Hypercalciuria** is found in 50 % of patients with **urolithiasis (urinary stones)**.

**Reference values of calciuria (dU-Ca): 2.4 – 7.2 mmol/ 24 hours.**

## 2.5 Principle of calcium measurement

The reference method for estimation of **total calcium** is **atomic absorption spectrometry**.

One of the methods recommended for routine measurements of total calcium in serum and urine is based on **spectrophotometry of colored complexes**, formed in a reaction of calcium with a suitable chelating substance. One such complex-forming substance in use is ***o*-cresolphthalexone**: with calcium ions in alkaline medium it produces a violet complex; the color of the solution is proportional to concentration of calcium ions. Another such complex-forming agent in use is a metallochromogene **arsenazo III**.



Estimation of total calcium in serum is one of the tests suffering from relatively big errors of measurement. The complex-forming agent does not bind all the calcium present in the blood sample because it has to compete with plasmatic proteins and other calcium chelating substances in blood. Significant deviations from normal composition of blood (e.g. pronounced dysproteinemia) may therefore produce false high or low values of calcemia.

The **free (ionized) fraction of calcium** can be measured by means of **potentiometry using ion-selective electrodes**. The major advantage of this technique is its rapidity. Another important fact is that this measurement provides information on the biologically active form of calcium regardless of the various factors influencing the levels of total calcium (e.g. severe hypoproteinemia, acid-base balance disorders etc.). On the other hand, the main difficulty is brought about by necessity to process only very fresh blood samples – unless analyzed immediately, the pH in blood sample quickly deteriorates which obviously affects also the availability of ionized calcium.

## 3 Phosphorus

### 3.1 Homeostasis of phosphorus

Phosphorus is an important **structural component of cells** (nucleic acids, phospholipids) as well as **bone tissue** (hydroxyapatite), participates in **energy conservation** in the form of macroergic phosphates (ATP, creatine phosphate), plays an important role in **regulation of enzyme activity** (phosphorylation and dephosphorylation of enzymes), and contributes to **buffering** of blood and urine in the form of hydrogen and dihydrogen phosphates.

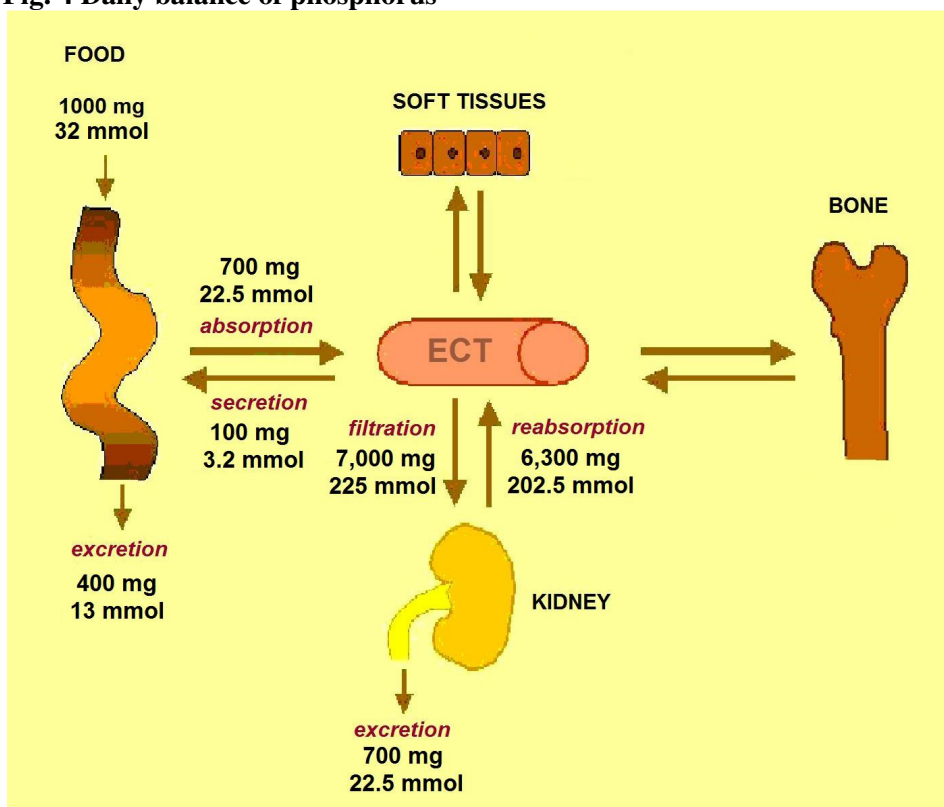
The human daily intake of phosphorus is about 1,000 mg. About 70-80 % of the dietary phosphorus is absorbed in the small intestine. The intestinal absorption of phosphorus is proportional to its contents in food, and in part is regulated by calcitriol. In addition to absorption there is also an intestinal secretion of phosphorus (about 100 mg daily). Majority of phosphorus is excreted by the kidney (see below) and this amount is controlled by parathyroid hormone (Fig. 4).

An adult human body contains about 700 g of phosphorus, which is found in:

- bones, 80 % of total, in hydroxyapatite
- muscles and visceral organs, 10 – 20 % of total
- extracellular fluid, 1 %

**Intracellular phosphorus** is present largely in organic esters of phosphoric acid (intermediates in conversions of saccharides and lipids), including ATP, 2,3-diphosphoglycerate and cAMP. The phosphates are the most abundant anions inside the cells. **Extracellular phosphorus** exists predominantly as inorganic phosphate. An exchange of phosphates takes place between the extracellular and intracellular fluids; this phosphate exchange can affect its serum levels (see below). The cellular uptake of phosphate is associated mainly with metabolism of glucose, where various glucose-phosphate esters are employed. Alkalosis also promotes entry of phosphate into the cells.

**Fig. 4 Daily balance of phosphorus**



### 3.2 Phosphorus in serum

The phosphorus exists in serum in two main forms:

- Organic phosphorus (about 70 %, mostly in phospholipids)
- Inorganic phosphorus (about 30 %, as phosphate<sup>1</sup>), most of which is free and only to a small extent it binds to serum protein (unlike calcium) or to calcium and magnesium. Only the inorganic phosphate is routinely estimated in clinical chemistry laboratories. At the physiologic pH 7.4 the ratio between hydrogen phosphate  $\text{HPO}_4^{2-}$  and dihydrogen phosphate  $\text{H}_2\text{PO}_4^-$  in serum is 4:1.

Concentration of serum phosphate depends on the function of parathyroid glands and the kidney (both glomerular filtration and tubular reabsorption). Parathyroid hormone decreases renal reabsorption of phosphate. Renal insufficiency in general results in elevation of serum phosphate.

<sup>1</sup> The terms 'phosphorus' and 'phosphate' are in fact fully interchangeable, as the inorganic phosphorus exists in the body as dihydrogen phosphate or monohydrogen phosphate.

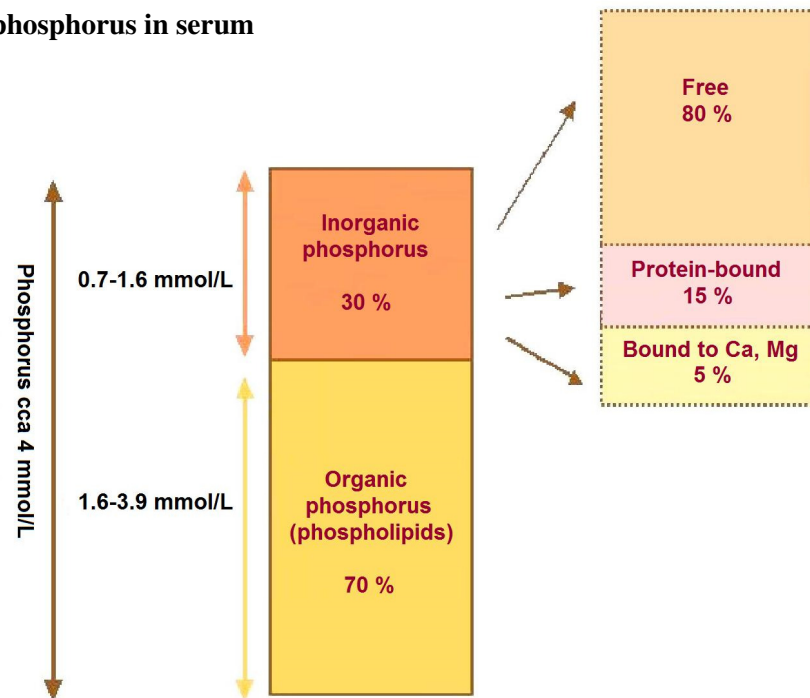


Next, serum phosphate concentration also reflects a balance between intake of dietary phosphate, and exchange of phosphate deposited in bone tissue.

Changes in serum phosphate levels often accompany changes in serum calcium. Usually an inversion relationship between the calcium and phosphate levels is observed, e.g. in primary hyperparathyroidism the blood calcium rises while phosphate decreases. However, in over-dosing by vitamin D both calcium and phosphate are increased together. It is worth keeping in mind that free phosphate can directly react with ionized calcium. A heavy hyperphosphatemia for instance can directly lead to significant drop in ionized calcium. An ionic product of plasmatic calcium and phosphate ion concentrations is also important. In general, this product is even at physiological condition above the value needed for precipitation of insoluble calcium phosphate, i.e. the blood is an over-saturated solution from this point of view. If the product increases further, which happens most often in kidney failure, the calcium phosphate can start to precipitate in soft tissues, typically in arterial media – metastatic calcifications are formed.

Serum concentrations of phosphate do not always properly reflect the amount of phosphorus in the intracellular fluid. Phosphate enters the cells for the needs of metabolic and energetic processes. On the other hand, if catabolism prevails the phosphorus moves from intracellular to extracellular space. It can result in hyperphosphatemia in severe catabolic states. On a shift to anabolic phase the cellular need for phosphorus markedly rises because of replenishment of ATP and other phosphorylated substrates and the serum level of phosphate decreases. In order to prevent hypophosphatemia, phosphate should be supplemented in these conditions.

**Fig. 5 Forms of phosphorus in serum**



**Reference values of serum inorganic phosphate (fS-P inorganic): 0.65 – 1.61 mmol/l**

The serum phosphate levels are physiologically higher in children, because of bone growth.

### 3.3 Phosphorus in urine

The kidney plays an important role in homeostasis of phosphorus. About 80 % of the phosphorus filtered by glomeruli is reabsorbed in the proximal tubulus, about 10 % in the distal tubulus, and the rest is excreted into urine.

In healthy persons the amount of urinary excretion of phosphate fluctuates considerably. Isolated estimation of phosphate in urine is insufficient for assessment of phosphate metabolism in the body, since with good kidney function phosphate excretion changes in accordance with dietary phosphate intake. An increased dietary intake results in increased excretion and vice versa.

Examinations of **clearance of phosphate** and **tubular reabsorption of phosphate (TRP)** are more informative. For their calculations also the values of creatinine in serum and urine are needed. For the TRP estimation a time-based collection of urine is unnecessary. The tubular reabsorption of phosphate decreases e.g. in hyperparathyroidism, and increases in hypoparathyroidism.

#### Daily renal output of phosphate:

$$\text{dU-inorganic phosphate (mmol/24 h)} = \text{U-inorganic phosphate (mmol/l)} \times \text{volume of urine (l/24 h)}$$

#### Clearance of phosphate:

$$C_P = \frac{U_P \times V}{S_P}$$

$U_P$ : concentration of phosphate in urine  
 $S_P$ : concentration of phosphate in serum  
 $V$ : diuresis in ml/sec.

#### Tubular reabsorption of phosphate:

$$\text{TRP} = 1 - \frac{U_P \times S_{Cr}}{S_P \times U_{Cr}}$$

$S_{Cr}$ : concentration of creatinine in serum  
 $U_{Cr}$ : concentration of creatinine in urine

*(All concentrations of serum and urine phosphate and creatinine must be in the same units).*

#### Reference values:

Daily renal output of phosphate:	<b>16 - 64 mmol/ day</b>
Clearance of phosphates:	<b>0.135 – 0.225 ml/sec</b>
Tubular reabsorption of phosphates:	<b>0.85 – 0.95</b>

### 3.4 Methods of phosphate estimation

The assay for phosphate in serum and urine is based on a reaction of phosphate with **ammonium molybdate in acidic medium** yielding a colorless phosphomolybdate complex, whose absorbance can be measured either directly in UV region or following its conversion to phosphomolybdate blue. Another possibility is a reaction of phosphate with ammonium molybdate and ammonium vanadate. In this case a yellow molybdate-vanadate-phosphoric acid is formed.

### 3.5 Solubility of calcium phosphate

One area where solubility of calcium phosphate needs to be taken into consideration is parenteral nutrition. A wrongly prepared mixture can give rise to precipitates of calcium phosphate that can severely threaten the patient. Stability of solutions containing calcium and phosphate salts depends among others on the *value of pH, concentrations of phosphate and calcium*, and also on the *choice of their salts* used for the parenteral nutrition:

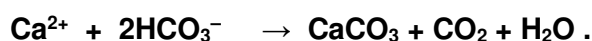
- **Low pH** prevents precipitation of calcium phosphate. Adjustment of pH towards fairly low values increases the proportion of  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ , which is well soluble in water (18 g/l), unlike the poorly soluble  $\text{CaHPO}_4$  (300 mg/l). Acidification can dissolve an already formed precipitate. Conversely, addition of hydrogen carbonate increases the susceptibility to precipitation.
- **Concentration of phosphates and  $\text{Ca}^{2+}$**  should be chosen with regard to calcium phosphate product, which should not exceed 75 ( $c^0 = \text{mmol/l}$ ):

$$\text{Ca}^{2+} (\text{mmol/l}) \times \text{phosphate} (\text{mmol/l}) < 75$$

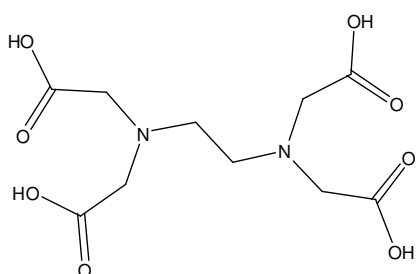
- Considering the salt choice, as a source of mono- and dihydrogen phosphates the sodium or potassium salts are used. Organic phosphates, such as glucose 1-phosphate or glycerol phosphate, display the lowest tendency to precipitation. Calcium chloride can serve as a source of calcium ions. Like in the case of phosphates, it is more suitable to choose an organic salt, such as calcium gluconate. Compared to the inorganic salts,  $\text{Ca}^{2+}$  in an organic salt dissociates less; hence the danger of calcium phosphate precipitation is reduced.

#### Calcium salts and hardness of water

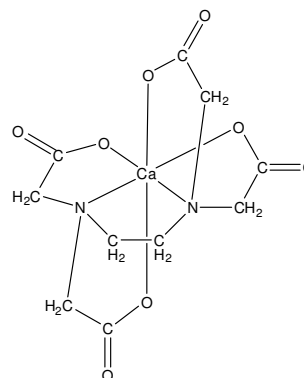
Total content of divalent ions of alkaline earth metals dissolved in water is denoted as hardness of water. It is mostly given by presence of calcium and magnesium ions. High hardness of drinking water is rather unwanted mainly from technical reasons – it leads to deposition of limestone inside vessels and equipment. Especially during heating of hard water a practically insoluble calcium carbonate is formed:



The limestone formation is often prevented by addition of ethylenediaminetetraacetic acid (EDTA) into household and cosmetic preparations. EDTA acts as a chelator; with calcium ions it forms very well soluble compounds. Calcium is firmly bound with six coordination covalent bonds rendering it unavailable for production of insoluble carbonate.



Ethylenediaminetetraacetic acid (EDTA)



Complex of EDTA with calcium

## 4 Biochemical Markers of Bone Metabolism

### 4.1 General features

The biochemical markers of bone metabolism are not specific for a particular disease; rather, they provide information on the metabolic status of the skeleton. They are assessed together with findings from tests on metabolism of calcium and phosphate.

These markers are suitable for:

- determination of degree of osteoresorption and bone formation
- differentiation of various bone diseases
- monitoring of therapeutic effects

The markers, further listed in two groups as markers of bone formation and bone resorption, in principle include:

- degradation products of bone matrix, released from the organic matrix due to osteoresorption
- bone matrix proteins and bone enzymes synthesized in osteoblasts or osteoclasts, passing into blood or urine

### 4.2 Markers of bone formation

The markers of bone formation include the following osteoblastic products in serum:

- *Osteocalcin*
- *Alkaline phosphatase and its bone isoform*
- *Propeptides of procollagen type I*

#### 4.2.1 Osteocalcin

**Osteocalcin** is the most abundant non-collagen bone polypeptide, also denoted as BGP (bone gla protein)<sup>2</sup>. This protein has a high affinity to hydroxyapatite. It is produced by **osteoblasts** and incorporated into extracellular bone matrix; in part it also enters the circulation. It is highly specific for the bone tissue, but it is also unstable and its estimation is not without pitfalls.

#### 4.2.2 Alkaline phosphatase (ALP) and its isoenzymes

The **alkaline phosphatase** (EC 3.1.3.1.) is an enzyme that catalyzes hydrolysis of monoesters of phosphoric acid in alkaline medium. ALP is present in all cells of the body attached to their membranes through its glycosyl-phosphatidylinositol anchor. The enzyme exists in several isoforms that are encoded by four genes. The **liver, bone and kidney isoforms** are all products of one, tissue unspecific gene. The other three genes encode **placental, intestinal and other isoenzymes**. Differences among isoforms encoded by the same gene originate from post-translational modifications of the carbohydrate part of enzyme molecules. In healthy adults, isoenzymes of liver origin prevail in the blood plasma, while in growing children the proportion of bone isoenzyme, produced by osteoblasts, is much higher. Estimation of ALP and its isoenzymes is useful mainly in diseases of the liver and biliary tract, and in bone diseases (Table 3).

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<sup>2</sup> gla means  $\gamma$ -carboxyglutamic acid, which is present in osteocalcin and binds calcium.

**Table 3: Isoenzymes of alkaline phosphatase:**

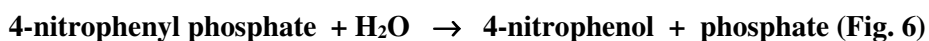
<b>ALP Isoenzyme:</b>	<b>Origin:</b>	<b>Examples of increase:</b>	<b>Comments:</b>
<b>Bone</b>	Osteoblasts	<ul style="list-style-type: none"> <li>- primary bone tumors (osteosarcoma)</li> <li>- secondary tumors (bone metastases of prostatic cancer, breast cancer)</li> <li>- healing of bone fractures</li> <li>- rachitis and osteomalacia</li> <li>- hyperparathyroidism</li> </ul>	<ul style="list-style-type: none"> <li>- increase indicates high osteoblastic activity</li> <li>- in adults less than half of total activity</li> <li>- much higher in growing children</li> </ul>
<b>Liver</b>	Membrane of hepatocyte, epithelium of biliary tract	<ul style="list-style-type: none"> <li>- biliary obstruction due to stone or tumor</li> <li>- damage to hepatocytes</li> <li>- hepatic tumors</li> </ul>	<ul style="list-style-type: none"> <li>- in healthy adults the dominant ALP isoform in plasma</li> </ul>
<b>Intestinal</b>	Enterocytes	<ul style="list-style-type: none"> <li>- some inflammatory intestinal diseases</li> </ul>	<ul style="list-style-type: none"> <li>- association with blood groups: occurs in B and O, while in persons with group A and AB normally undetectable</li> <li>- physiologically rises after meal</li> </ul>
<b>Placental</b>	Trophoblast		<ul style="list-style-type: none"> <li>- physiological in the 3<sup>rd</sup> trimester of gravidity</li> <li>- thermostable</li> </ul>
<b>Atypical</b>	Some tumors		<ul style="list-style-type: none"> <li>- e.g. Reagan's isoenzyme</li> </ul>

### Principle of ALP estimation

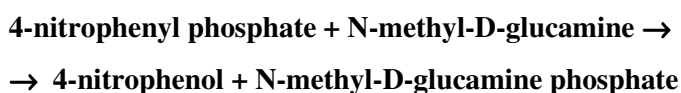
Phosphatases can catalyze **hydrolysis** of various organic phosphomonoesters to phosphate anion and the corresponding alcohol or phenol. In addition, the ALP mediates also a **transphosphorylation reaction**, in which the phosphate group is transferred to a suitable acceptor.

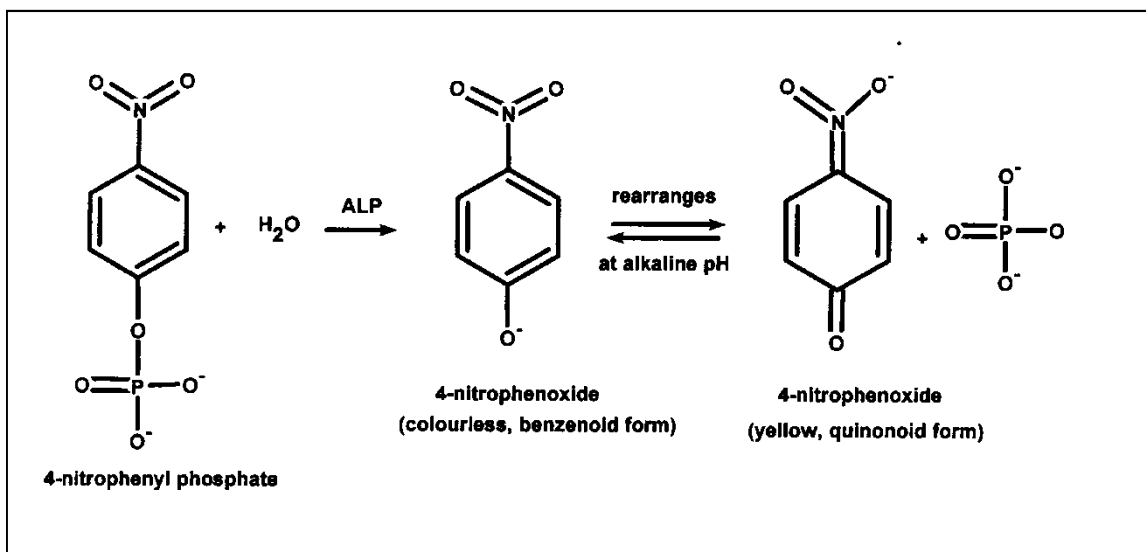
If 4-nitrophenyl phosphate is used as the substrate; the alkaline phosphatase catalyses the following reactions:

1) *hydrolysis:*



2) *transphosphorylation:*





**Fig.6: Principle of alkaline phosphatase estimation**

In the first reaction the phosphate is hydrolytically cleaved from the substrate (Fig. 6). In the second transphosphorylation reaction the N-methyl-D-glucamine acts simultaneously as a buffer and as an acceptor for phosphate; in this way the reaction is accelerated.

The substrate **4-nitrophenyl phosphate** is **colorless** in the alkaline medium, while product of its ALP-dependent hydrolysis has a quinone-like form (Fig. 6) at alkaline pH, which is intensely **yellow**. The released 4-nitrophenol, then, is a measure of ALP activity and is estimated photometrically using either kinetics or end-point approach (following termination of the enzyme reaction by an inhibitor). ALP is activated by sodium chloride.

### Estimation of ALP isoenzymes

Isoenzymes of alkaline phosphatase can be distinguished on a basis of their different physical, chemical, immunological and electrophoretic properties.

Methods for estimation of ALP isoenzymes utilize selective inactivation of some isoenzymes by heat, phenylalanine, leucine, or urea; or differences in electrophoretic mobility. Good separation of liver and bone isoenzymes is sometimes difficult. Recently, new methods for estimation of bone isoenzyme have appeared, based on immunochemistry, or binding of the bone ALP to a specific lectin followed by electrophoresis.

### Reference values:

Total catalytic concentration of ALP (fS-ALP,  $\mu\text{kat/l}$ ):

Adults	<b>0.66 – 2.2 <math>\mu\text{kat/l}</math></b>
Children (1 – 10 years)	<b>1.12 – 6.2 <math>\mu\text{kat/l}</math></b>
Children (10 – 15 years)	<b>1.35 – 7.5 <math>\mu\text{kat/l}</math></b>

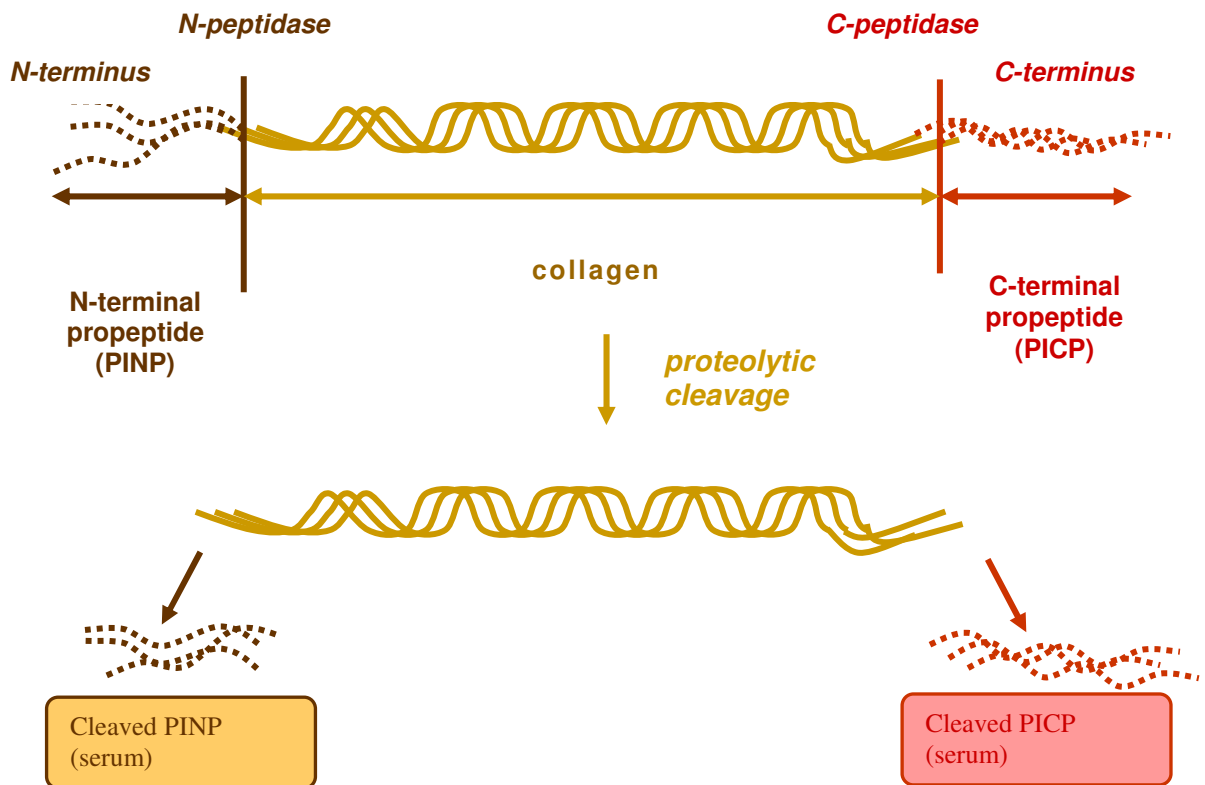
Bone isoenzyme of ALP:

Adults	<b>0.26 – 0.40 <math>\mu\text{kat/l}</math></b>
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### 4.2.3 Propeptides of procollagen type I

Collagen is in **osteoblasts** synthesized as procollagen; and before incorporation into bone matrix both procollagen termini are proteolytically cleaved. Thus, for each molecule of mature collagen, one **C-terminal propeptide** (PICP: procollagen I C-terminal propeptide) and one **N-terminal propeptide** (PINP: procollagen I N-terminal propeptide) are released.

**Fig. 7: Propeptides of procollagen**



### 4.3 Markers of bone resorption

During bone resorption its mineral component is dissolved and bone matrix is degraded. It is accompanied by release of calcium, phosphate, many enzymes and matrix degradation products into blood and urine.

The markers of bone resorption include:

- *Pyridinoline and deoxypyridinoline in urine*
- *N- and C-telopeptides of collagen type I*
- *Hydroxyproline in urine*
- *Bone isoenzymes of acidic phosphatase*

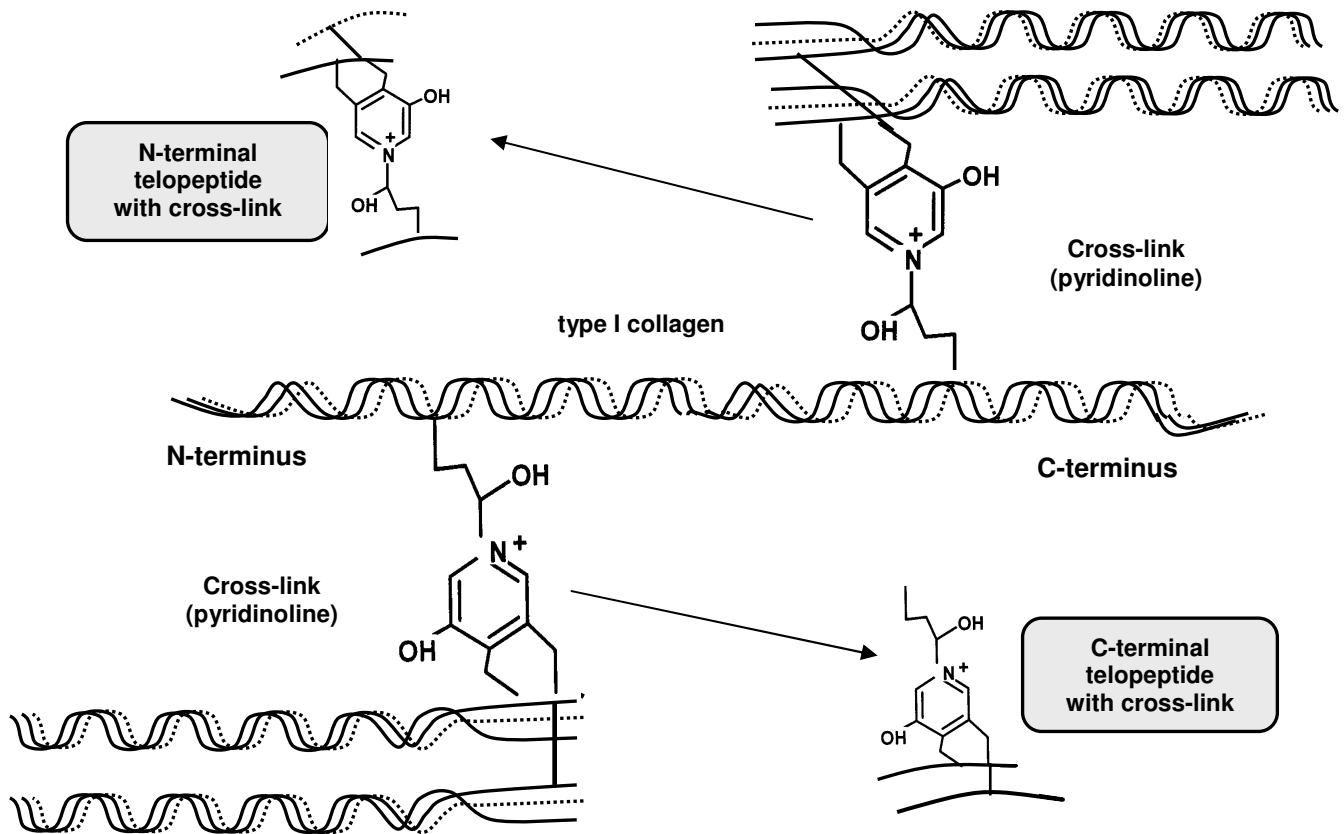
#### 4.3.1 Pyridinoline and deoxypyridinoline in urine, N- and C-telopeptides of collagen type I

Mature collagen and elastin are characterized by formation of cross-links, i.e. intermolecular connections of lysine or hydroxylysine side chains of collagen fibrils. **Pyridinoline** originates from connecting three hydroxylysine moieties, while two hydroxylysines with one lysine would produce **deoxypyridinoline**. If the collagen is degraded, these cross-links are released into circulation and

pass into urine, where they can be estimated. Amount of pyridinolines/deoxypyridinolines reflects intensity of bone resorption. In the blood they circulate either as free pyridinolines/deoxypyridinolines, or as part of **terminal portions of collagen**, denoted as **C-telopeptide** (ICTP: C-terminal telopeptide of collagen type I) and **N-telopeptide** (INTP: N-terminal telopeptide of collagen type I) (Fig. 8). Estimation of these substances is technically rather demanding; high-performance liquid chromatography (HPLC) or immunochemical techniques are used.

**Fig. 8: Telopeptides of collagen type I**

(According to Calvo et al., Endocrine Review, 17, 333 – 366, 1996)



#### 4.3.2 Hydroxyproline in urine

Collagen contains fairly high amount of **hydroxyproline**. During collagen degradation the hydroxyproline is released into circulation and partly excreted into urine; the rest is metabolized in the liver. Hydroxyproline in urine rises if bone resorption is increased (e.g. osteoporosis, hyperparathyroidism). However, it is one of less specific markers of osteoresorption as it can originate also from proteins of the complement and degradation of propeptides of procollagen. Hydroxyprolinuria can be affected by dietary intake of collagen (meat, bouillon, gelatin), and so for proper examination the patient should be at least one day on a diet excluding any collagen-containing food.



### 4.3.3 Acidic phosphatase (ACP) and its bone isoenzyme

**Acidic phosphatase** (E.C. 3.1.3.2.) hydrolyses various phosphate esters at pH below 7. It occurs in a variety of tissues (prostate, bones, liver, kidney, spleen, blood cells), and again, several isoenzymes may appear in the circulation:

- **Bone (resistant to tartrate)** isoenzyme (TRACP) is specific for the bone tissue. **Osteoclasts** contain a lot of this lysosomal enzyme, which is secreted into circulation during bone resorption. Unlike other markers of osteoresorption it is not excreted by the kidneys, and so estimation of the bone isoenzyme is especially valuable in patients with impaired renal function.
- **Prostatic (tartrate-sensitive)** is normally absent from blood, but appears in prostatic hyperplasia or cancer
- **Thrombocytic** isoenzyme is released due to blood clotting, that is why serum has higher levels of ACP than plasma
- **Erythrocytic** isoenzyme becomes significant if the serum sample is hemolytic.

Biochemical estimation of ACP employs cleavage of synthetic esters of phosphoric acid. One of the used substrates is 4-nitrophenylphosphate (the same as used in the ALP assay), which is cleaved by ACP to phosphate and 4-nitrophenol in acidic medium.

#### Reference values:

Total catalytic concentration of ACP in plasma:	Men	0 – 108 nkat/l
	Women	0 – 92 nkat/l
Bone isoenzyme of ACP		50 – 90 nkat/l
Prostatic (tartrate-sensitive) fraction:	Men	0 – 43 nkat/l

## 5 Examples of Metabolic Bone Diseases

Metabolic bone diseases typically affect the bones as a whole. They are caused by impairment in the organic component of bone tissue, the bone mineral, or both. The most significant metabolic bone disease is osteoporosis, but osteomalacia is also common.

### 5.1 Osteoporosis

Osteoporosis is characterized by a **decay of bone mass (both organic and inorganic bone components are decreased proportionally)** and disorder of bone micro-architecture, resulting in fragility and tendency to fractures. The decay of bone mass is more severe than the one corresponding to the age, gender, and race of a given individual.

Nowadays, osteoporosis is considered as a 'civilization disease', with a high incidence in industrial countries. It supposedly occurs in about 6 – 7 % of our citizens. Older women are predominantly affected. The osteoporosis results from an imbalance between escalated bone resorption and normal or decreased bone formation. Until the age of 50, contents and density of the bone mass change little with age in both men and women. Later, however, in post-menopausal women in connection to the loss of ovarial activity, the bone resorption prevails over bone formation, and this trend continues ever after. In men the bone mass also decays with age, but with much slower rate, and therefore men are less affected by osteoporosis and resulting fractures than women.

Diagnosis of osteoporosis is nowadays made on a basis of **bone mass measurement using imaging techniques**, but the **laboratory tests** can also be useful for disease recognition, assessment of its progression and prognosis. Markers of calcium and phosphate metabolism, together with bone resorption/formation are examined. In post-menopausal women, endangered by osteoporosis, typically **increased markers of osteoresorption**, unmatched by increased bone formation, are found.

## **5.2 Osteomalacia and rachitis**

Osteomalacia is characterized by a **decrease in the mineral component of the bone**, due to a **disorder in the mineralization process**. High amount of osteoid (non-mineralized organic matrix) that calcifies slowly or not at all is found.

**Rachitis (rickets)** is a specific term for an osteomalacia in children. Growth of bones is a prerequisite for development of rachitis.

Osteomalacia is most often caused by **vitamin D deficiency**, resulting from its lack in the diet, or disorders of its digestion and absorption. Lack of sunshine, and diseases of liver and kidney associated with impaired conversion of vitamin D to its active metabolites, would also lead to osteomalacia.

Laboratory testing in osteomalacia typically shows:

- *low calcemia*
- *low phosphatemia*
- *high catalytic concentration of alkaline phosphatase, especially its bone isoenzyme*

## **5.3 Paget's bone disease**

The Paget's disease typically affects only certain parts of the skeleton. It results from local uncontrolled bone resorption, associated with excessive and disorganized bone formation. The structure of produced bone is defective. Numbers of both osteoclasts and osteoblasts are elevated. The biochemical findings are dominated by an **increased value of alkaline phosphatase (bone isoenzyme)**. Estimation of bone resorption markers is also useful.