ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK A VFN

Amino acids – reactions and conversions. Nitrogen balance

Practical lesson on medical biochemistry

General Medicine

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1 Colored reactions of amino acids and proteins

Amino acids are characterized by a simultaneous presence of carboxyl group –COOH and amino group $–NH_2$. In addition, some amino acids contain in their side chains other functional groups such as –SH, –OH, guanidine group or aromatic ring. Presence of these structures enables various color reactions that can be employed for amino acid detection. Thus, for instance, free α-amino group of any amino acid reacts with **ninhydrin**. Aromatic rings in structures of tyrosine, phenylalanine or tryptophan give **xanthoproteic reaction**. Phenolic (tyrosine) and imidazole (histidine) groups can react with **diazonium salts**. -SH group of cysteine can be demonstrated by reaction with **Pb^{2+} ions**.

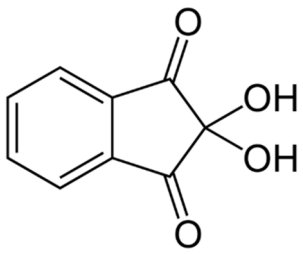
Amino acids are connected by peptidic bonds in peptides and proteins. Proof of peptidic bond by **biuret reaction** with Cu^{2+} ions is utilized for estimation of total protein concentration in a sample.

The functional groups in the side chains of amino acids joined to polypeptidic chain react in a similar way as the free amino acids do. It means that reactions specific only for certain amino acids can be employed as a test whether given peptide or protein contains the specific amino acid or not. In this way we will compare two proteins in our practical lesson: **egg albumin** and **gelatin**. The albumin is an example of 'full-blown' protein, containing all amino acids. On the other hand, gelatin is a product of denaturation and partial hydrolysis of collagen; its prevailing amino acids are glycine, proline, hydroxyproline and glutamic acid, whereas the content of aromatic amino acids phenylalanine and tyrosine is very low, and tryptophan as well as cysteine is absent.

1.1 Ninhydrin reaction

Ninhydrin is a reagent for ammonia and **primary aliphatic amines**; hence also for any amino acid that contains a free amino group. In dependence on the kind of **amino acid** its reaction with ninhydrin results in a **blue-violet to brown** product. The imino acids **proline** and hydroxyproline react with ninhydrin as well, but to a different product that has a **yellow** color.

In peptides and proteins the amino acids amino groups are bound in the peptidic bonds, but the ε-amino groups of lysine or terminal $-NH_2$ groups can still give the ninhydrin reaction.

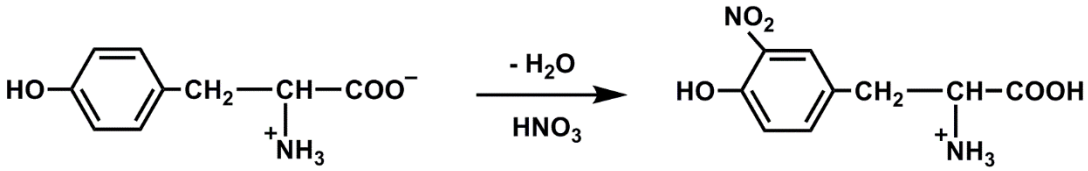


Structure of ninhydrin

The reaction with ninhydrin is widely used for amino acid detection. In medicine it is employed in examination of disorders of amino acid metabolism, such as phenylketonuria. Ninhydrin reaction can be used for instance as a detection reaction in qualitative tests for amino acids in blood or urine by means of paper or thin-layer chromatography. Another example is a quantitative estimation of the amino acid composition of polypeptides. In this technique the analyzed polypeptide is first hydrolyzed, i.e., all its peptidic bonds are cleaved. The resulting mixture of amino acids is separated by ionex chromatography and the subsequent detection of particular amino acids is facilitated by reaction with ninhydrin.

1.2 Xanthoproteic reaction

Nitration by concentrated nitric acid is a reaction typical for aromatic ring. The resulting nitro derivatives have an intense yellow color (Greek *xanthos* = yellow). Amino acids that possess an aromatic ring – tyrosine, tryptophan and (weakly) phenylalanine react in a similar way. Likewise, majority of proteins in dependence on their aromatic amino acid contents give a positive reaction with concentrated nitric acid. Following addition of concentrated nitric acid to a protein solution a white precipitate of denatured protein appears that turns yellow after boiling.



Tyrosine **Yellow color**

Yellow spots on the skin that develop after sullying with nitric acid are also due to xanthoproteic reaction, this time with aromatic amino acids present in epidermal proteins.

1.3 Reaction of cysteine – proof of sulfur in protein molecules

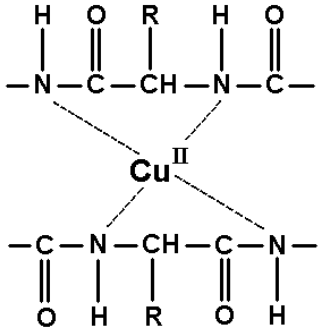
Cysteine, as well as proteins containing significant amount of this amino acid, in strongly alkaline medium release hydrogen sulfide, which can be demonstrated by reaction with lead(II) acetate. A brownish-black precipitate of lead(II) sulfide develops:

$$\text{R-SH} \xrightarrow{OH^-} \text{R-OH} + \text{H}_2\text{S}$$

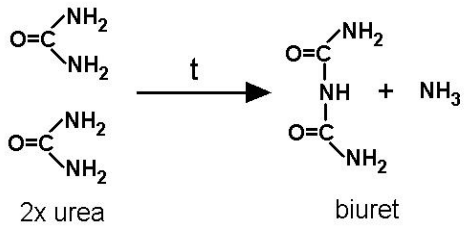
$$\text{H}_2\text{S} + \text{Pb(CH}_3\text{COO)}_2 \longrightarrow \text{PbS}\downarrow + 2\ \text{CH}_3\text{COOH}$$

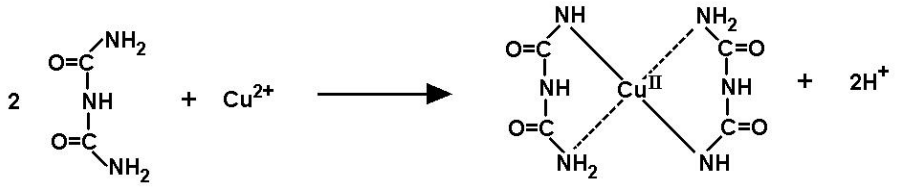
1.4 Biuret reaction

Proteins give a characteristic violet color when mixed with copper(II) ion in alkaline solution. The color change results from complexation of copper(II) by nitrogen atoms of adjacent peptide bonds:



The reaction depends on the presence of peptidic bonds, not on the properties of amino acid side chains; hence, all proteins indiscriminately react. In general, any substance that has at least two adjacent groups -CO-NH_2 (amide) or at least two peptide bonds -CO-NH-, will give the reaction. Thus, the simplest reacting compounds are oxamide H_2N-CO-CO-NH_2, or biuret (bis-urea, dimer of urea) that gave the reaction its name:





The biuret reaction is still commonly employed as a quantitative assay for protein in a biological sample, for instance a patient's serum.

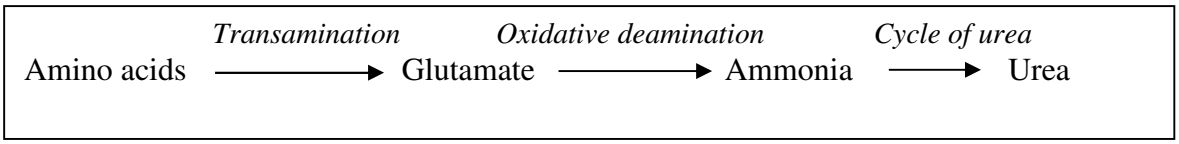
N.B.: The reaction is called after biuret because this compound also reacts. However, when the biuret reaction is used to measure proteins, which is the commonest case, the compound biuret as such is actually not used at all, since it is neither in the sample nor in the reagent.

2 Aminotransferases

Catabolism of amino acids in the body usually starts with **transamination** – a reversible transfer of the amino group from an amino acid to a keto acid. It is performed by a group of enzymes called **transaminases**. Pyridoxal-5'-phosphate (a vitamin B6 derivative) serves as an essential cofactor.

The transaminases are specific for one pair of amino acid/keto acid, but unspecific for the other. As the catalyzed reaction is freely reversible, it can serve both for amino acid disposal and the biosynthesis of non-essential amino acids from the citric acid cycle or glycolysis intermediates.

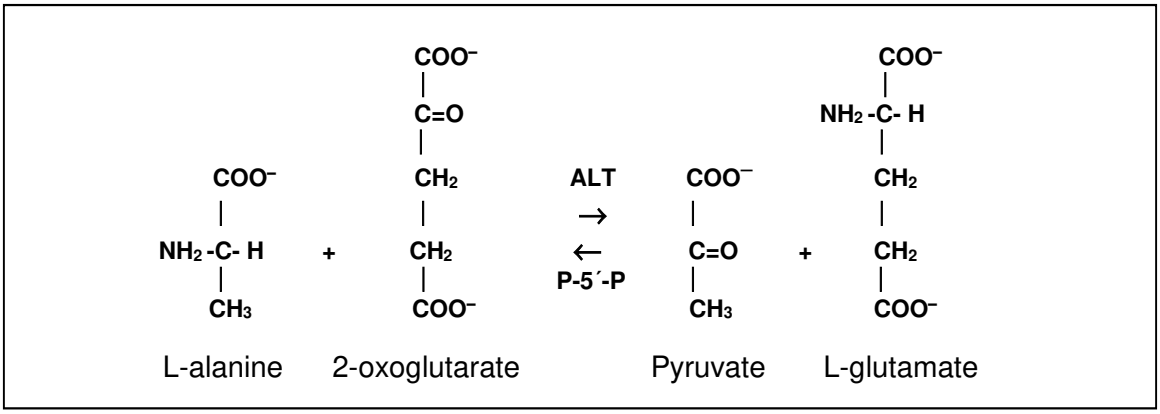
In catabolism of amino acids the combined action of transaminases generally leads to transfer of the amino acid nitrogen to 2-ketoglutarate, producing glutamate. The subsequent oxidative deamination of glutamate liberates the nitrogen as ammonia, which is then disposed by the liver urea cycle.



In clinical chemistry, estimations of **alanine aminotransferase (ALT)** and **aspartate aminotransferase (AST)** in the blood plasma have become widely used as markers of **liver cell damage**. The enzymes function within the liver (or other) cells, but once the cells die and spill out their contents, their enzymes transitionally circulate in the blood. If more cells leak their contents or die because of a disease, we will measure higher activities of these enzymes.

2.1 Alanine aminotransferase (ALT)

ALT catalyzes a reversible transfer of amino group from alanine to 2-oxoglutarate:



ALT is present mostly in the **liver**; its activity in other organs (skeletal muscle, myocardium, etc.) is much lower. Unlike AST it localizes **only to the cytosol**. In addition to the roles in metabolism of amino acids mentioned above, its action is necessary for the **glucose-alanine cycle**. It is an exchange between the liver and skeletal muscle: during starvation the muscle produces alanine by transamination of pyruvate; the liver takes alanine form blood, transaminates back to pyruvate, which is then used for gluconeogenesis.

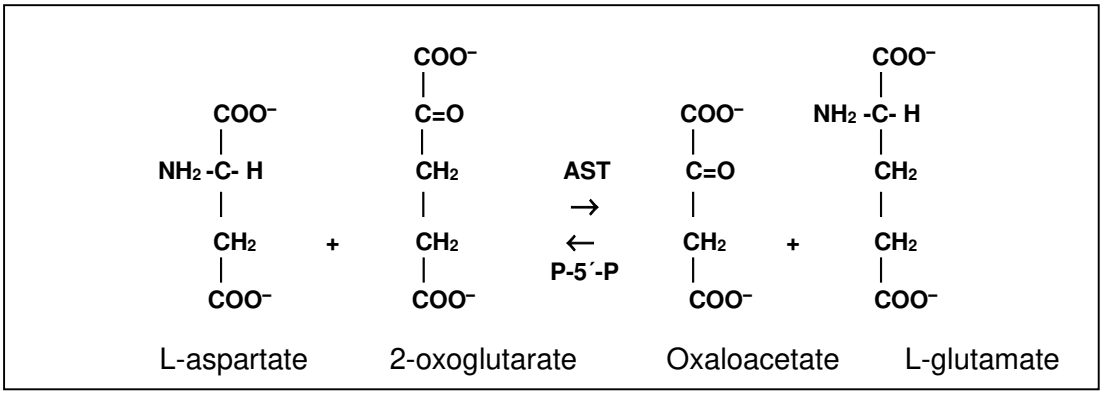
Estimation of ALT is a sensitive and relatively specific test for hepatocyte damage. Its activity in serum rises even in a **small damage of the liver cell**, caused by **increased permeability of the cell membrane**. In inflammation of the liver (viral hepatitis), for instance, elevation of ALT is the earliest indicator that the hepatocyte cell membrane integrity is compromised. Repeated ALT estimation is suitable for monitoring course of the disease.

Reference values:

Catalytic concentration of serum ALT (S-ALT):
Men up to 0.80 µkat/l
Women up to 0.60 µkat/l

2.2 Aspartate aminotransferase (AST)

AST catalyzes a reversible transfer of amino group from aspartate to 2-oxoglutarate:



AST occurs in numerous organs: **liver, heart, skeletal muscle, kidney, pancreas,** and **red blood cells**. It is so widely distributed because of its participation in the **malate shuttle**, a mechanism for an effective transfer of reducing equivalents from cytosolic $NADH+H^+$ to the mitochondrial respiratory chain.

It exists in two isoenzymes: **mitochondrial** (about 70 %), and **cytosolic** (about 30 %). Cytosolic fraction is readily released into circulation due to mild alterations of hepatocyte cell membrane permeability. In contrast, the mitochondrial fraction is released only after **destruction (necrosis) of the hepatocyte**. Therefore, a high increase of serum AST is a marker of hepatocyte destruction, because both isoenzymes are likely to participate in the increase.

Since AST is not specific for the liver tissue, it can be elevated also in **damage of skeletal muscle and myocardium.** AST in blood rises in acute myocardial infarction (heart stroke) and following heart surgery, but also due to a long lasting strenuous physical exercise.

Hemolysis of the sample can cause false positive results of AST estimation, since quite high levels of the enzyme are present in the erythrocytes.

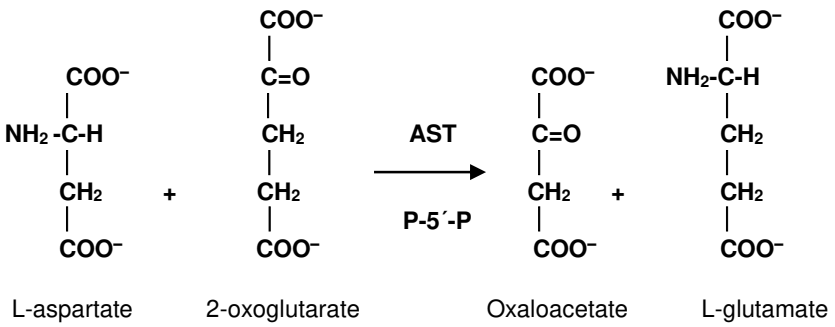
Reference values:

Catalytic concentration of serum AST (S-AST):
Men up to 0.85 µkat/l
Women up to 0.60 µkat/l

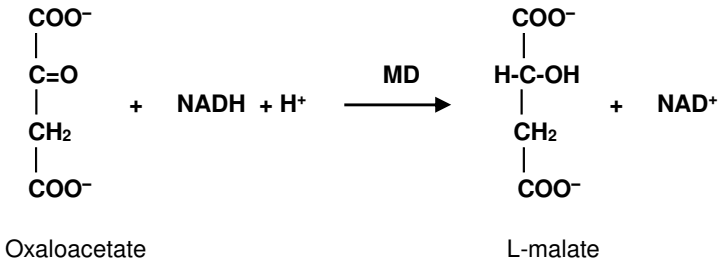
2.3 Methods for estimation of aminotransferases

Aspartate aminotransferase:
The estimation of AST is based on the Warburg optical test. In the first enzyme reaction, catalyzed by AST from the sample, oxaloacetate is formed. In the next, indication reaction, malate dehydrogenase (MD) reduces oxaloacetate to malate and simultaneously oxidizes NADH to NAD^+. Activity of AST is determined kinetically as a decrease in absorbance of reduced NADH at 334, 340 or 365 nm.

First enzymatic reaction – *formation of oxaloacetate:*



Second indication reaction – *quantification of oxaloacetate:*

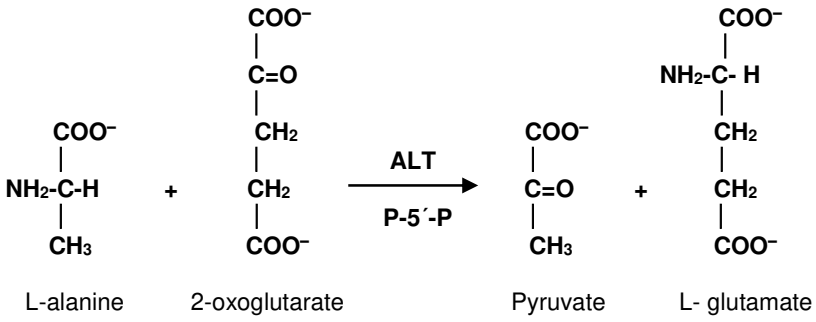


The reaction mixture for AST assay contains in addition to the substrates (L-aspartate and 2-oxoglutarate), NADH and malate dehydrogenase also pyridoxal-5'-phosphate and lactate dehydrogenase. Addition of pyridoxal-5'-phosphate saturates the enzyme with its essential cofactor and ensures full enzyme activity. Lactate dehydrogenase is necessary for reduction of any other NADH-dependent keto acids in the sample; otherwise false high values could be obtained. These

reactions take place during 5–15 min. pre-incubation of the mixture without 2-oxoglutarate. Next, the AST reaction is started with 2-oxoglutarate and the change in absorbance (ΔA) is read in one-minute intervals for several minutes.

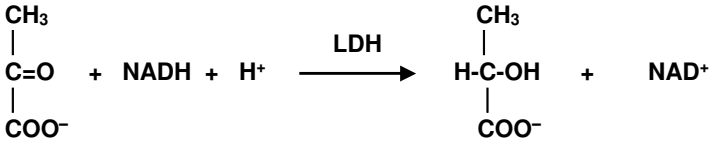
Alanine aminotransferase:
The assay for ALT is again based on the Warburg optical test and is in principle very similar to the estimation of AST, except that in the first enzyme reaction, alanine serves as donor of amino group instead of aspartate, and pyruvate is formed. The indication reaction is catalyzed by lactate dehydrogenase that simultaneously reduces any endogenous keto acids. Just as for AST, the procedure requires 5–15 minutes of pre-incubation, then the reaction is started with 2-oxoglutarate.

First enzymatic reaction – *formation of pyruvate:*



L-alanine + 2-oxoglutarate → Pyruvate + L- glutamate

Second indication reaction – *quantification of pyruvate:*



Pyruvate + NADH + H⁺ → Lactate + NAD⁺

2.4 Evaluation of examination of aminotransferases

Aminotransferases are widely used in the diagnostics of liver diseases. In the **acute viral hepatitis**, typically both AST and ALT activities are markedly increased. Two- to three-fold elevation is noted as early as in the prodromal stage, it peaks (up to 100 μkat/l) the 7^{th} – 12^{th} day after appearance of icterus, and normalizes usually the 5^{th} – 8^{th} week. Strong (even 10-fold) but transient increase of aminotransferases is observed in severe gallstone colics. Other hepatic lesions are usually accompanied by rather moderate elevations (max. 5-fold) of ALT and AST. In chronic liver diseases, such as liver cirrhosis, the activities of aminotransferases are often only slightly above the upper reference limits.

In general the extent of aminotransferase increase correlates well with severity of liver damage. However, if a large part of hepatic tissue has been lost due to e.g. liver cirrhosis, the number of cells that can still release the aminotransferases may be so low that serum levels of aminotransferases do not exceed the reference limits even in a severe liver damage.

Moderate non-specific increase of both aminotransferases can also be observed after strenuous physical exercise (release from skeletal muscle), and also in obese persons.

The severity of hepatocyte damage can also be assessed from the ratio AST/ALT, also known as **De Ritis Quotient**. A value higher than 1 is considered as a sign of unfavorable prognosis.

3 Urea

The transamination and oxidative deamination processes in the amino acid catabolism release the amino acid nitrogen as **ammonia, NH_3/NH_4^+**. This is a highly water soluble, but toxic substance, and terrestrial organisms need a safe way of its disposal. Mammals depend on the **liver urea cycle** that convert the toxic ammonia to also highly water-soluble, but non-toxic **urea, $H_2N\text{-}CO\text{-}NH_2$**.

In quantitative terms, urea is the most significant degradation product of protein and amino acids in the human body. It is actually one of several **non-protein nitrogen compounds**, in addition to urea they encompass creatinine, uric acid, ammonia and amino acids. All these substances remain in the solution after precipitation of serum proteins by deproteination reagents. Estimation of non-protein nitrogen compounds in blood and urine is important for monitoring of function of the **liver**, where most of their metabolic pathways are localised, and **kidney**, which is the dominant place of their excretion.

Urea diffuses freely through cell membranes and so its concentrations in plasma and intracellular fluid are equal. It is excreted from the body mainly in the kidney by a combination of glomerular filtration and tubular resorption. The latter is variable: lower at higher diuresis while increasing when diuresis is low.

The **concentration of urea in the blood** in general depends on: **amount of protein in the diet**, **excretion by the kidney**, and **metabolic function of the liver**.

For instance, serum urea can increase due to **high protein intake in the food**. One gram of protein (dietary or endogenous) can give rise to 5.74 mmol (0.34 g) of urea. Increased concentration of serum urea without changes in other non-protein nitrogen compounds (esp. creatinine) is a hallmark of an **intense protein catabolism**, which occurs e.g. in starvation, febrile state or malignancy. Children have lower catabolism of protein, and show demonstrably lower serum urea levels.

Serum urea increases in **diseases of the kidney that lead to marked restriction of glomerular filtration** (below 30 %); simultaneously, high levels of creatinine are found. Unlike creatinine, estimation of urea is not suitable for an early detection of decrease in glomerular filtration. However, it is useful for monitoring of patients in chronic hemodialysis treatment.

In **liver function failure**, synthesis of urea falls down, and so its concentration in the serum.

Some causes of changes in serum urea concentration:

Serum urea increased:	***Serum urea decreased:***
Impaired kidney function	Low-protein diet
High-protein diet	Impaired liver function
High catabolism of protein	Late pregnancy (growing foetus demands protein)
Dehydration	

Reference values (fS-Urea): **Women:** **2.0 – 6.7 mmol/l**
Men: **2.8 – 8.0 mmol/l**

Output (loss) of urea in urine (dU-urea):

An adult excretes daily 167-583 mmol/ 24 hours, depending on protein uptake in food and protein catabolism. Urinary urea output is calculated according to the formula:

dU-urea(mmol/24 hours) = U-urea (mmol/l) × Volume of urine per 24 hours (l)

Principle of urea estimation in serum and urine:

In general, urea in biological fluids can be estimated either directly, or indirectly as ammonia. In the indirect assay, first an enzyme ***urease*** cleaves urea to carbon dioxide and ammonia that in aqueous medium exists as ammonium ion. Next, the amount of ammonium is estimated by the ***Berthelot's reaction***: ammonium ion with sodium hypochlorite and phenol or salicylate form a colored product. The reaction is catalyzed by sodium nitroprusside.

Nowadays, the recommended routine method for measurement of the ammonium ions generated by the urease reaction utilizes conversion of α-ketoglutarate to glutamate. The reaction is catalyzed by ***glutamate dehydrogenase***, and coupled to oxidation of NADH to NAD^+ (Warburg's optical test).

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\textit{Urease}} 2NH_4^+ + CO_2$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow[\textit{dehydrogenase}]{\textit{Glutamate}} \text{L-glutamate} + NAD^+ + H_2O$$

4 Nitrogen balance

Human body continuously synthesizes and degrades proteins. **Nitrogen balance** refers to the difference between intake of nitrogen in food and amount of nitrogen excreted during a given period of time. It provides a basic information on the overall **turnover of body proteins**.

While intake of nitrogen can be estimated quite accurately, excretion of nitrogen compound is rather difficult to measure since nitrogen is excreted not only in urine as urea, but also in stool, by skin, or by other body secretions.

In order to obtain the value of nitrogen balance for a given patient, we need to know:

1. **Intake of nitrogen in grams per 24 hours**
2. **Excretion of nitrogen in grams per 24 hours**

Ad 1: Calculation of nitrogen intake is based on the amount of protein in food, found in the dietary tables, or on the amount of amino acids administered in parenteral nutrition.

Proteins or amino acids contain on average 16 % of nitrogen, hence

Intake of nitrogen (g/24 hours) = intake of proteins/amino acids × 0.16

Ad 2: Excretion of nitrogen (catabolic nitrogen) is given by metabolism of proteins taken in food as well as degradation (turnover) of body proteins. It consists of a variety of nitrogen-rich catabolites: mostly urea (about 80 %), but also creatinine, uric acid and others.

The major pathway of protein catabolism is production of urea in the liver and its excretion in the kidney. This portion of excreted nitrogen is found from estimation of urea output in urine per given time period.

In addition to the kidney, nitrogen is excreted also by other extrarenal ways. Contents of nitrogen in stool and loss by skin are not measured directly, rather, a rough estimate is taken into account.

Normal value of catabolic nitrogen is about **10 g/day**. Estimation of catabolic nitrogen is used e.g. for determination of dosage of amino acids in parenteral nutrition.

Calculation of catabolic nitrogen:

Catabolic nitrogen = Urea in urine (mmol/l) × V (L) × 0.028 × 1.2
+
Δ urea in serum (mmol/l) × 0.028 × Body weight (kg) × Factor of body water
+
Loss of urea by stool and skin

The calculation contains three items:

- Amount of urea excreted per 24 hours obtained by collecting urine during a given time period and estimating concentration of urea in the urine. This value is extrapolated to total urine nitrogen on the assumption that urea forms 80-84 % of urine nitrogen (the rest is excreted as ammonia, uric acid, creatinine etc.) and that 1 mole of urea contains 28 grams of nitrogen.

- Difference in urea nitrogen in the total body water. Change in urea concentration in serum at the beginning and at the end of the examined period is considered here, e.g., if urea concentration increases during the examined day, it means that some urea has not been excreted. Urea diffuses through all body membranes, so its distribution volume is total body water.

- A rough estimation of the nitrogen lost by stool and skin is added.

0.028 … Factor converting mmol of urea to g of N (1 mmol of urea contains 0.028 g N).
V (l)… Volume of urine per day in liters (diuresis).
1.2 … Factor converting total urine N on the assumption that urea contributes 80-84 %.
Δ urea in serum (mmol/l)….difference in serum urea at the beginning vs. end of the examination period
Factor of body water … 0.6 for men, 0.55 for women.
Loss by stool and skin… depends on the body temperature:

37 ˚C	1 g
37-38 ˚C	1.3 g
38-39 ˚C	1.5 g
39-40 ˚C	1.8 g

Finally, if both intake and excretion of nitrogen are known, the balance can be calculated:

Nitrogen Balance = Intake of Nitrogen (g/24 hrs) – Catabolic Nitrogen (g/24 hrs)

Evaluation of nitrogen balance:

Nitrogen balance	*Characterisation*	*Occurrence*
About zero (balanced)	Intake of nitrogen matches its excretion	• Healthy adult
Positive	Intake of nitrogen exceeds excretion – **anabolism of proteins prevails**	• Growing organism • Recovery following trauma, illness, surgery, etc. • Pregnancy
Negative	Intake of nitrogen is lower than its excretion – body protein is used as source of energy, **catabolism of protein prevails** (loss of nitrogen means loss of protein). 1 g of net nitrogen loss means loss of about 6.25 g of body protein (25-30 g of muscle mass)	• Serious diseases (infections, operations, tumors, burns, gastrointestinal diseases, etc.) • Starvation • Lack of essential amino acids in diet (lack of one essential amino acid is enough!) • Advanced age