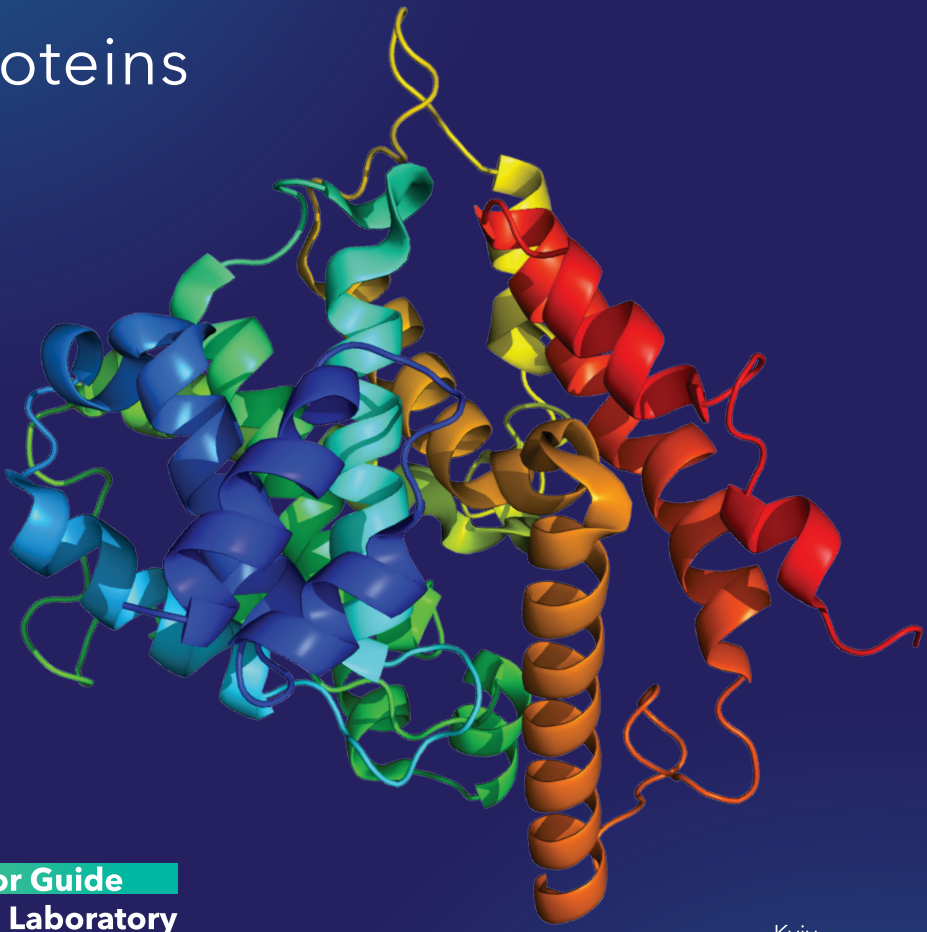
Ministry of Education and Science of Ukraine
National Academy of Sciences of Ukraine
Junior Academy of Sciences of Ukraine

FUNDAMENTALS OF BIOCHEMISTRY

Proteins



Tutor Guide and Laboratory Manual

Kyiv
2023

Ministry of Education and Science of Ukraine
National Academy of Sciences of Ukraine
National Centre "Junior Academy of Sciences of Ukraine"

FUNDAMENTALS OF BIOCHEMISTRY. PROTEINS

Tutor Guide
and Laboratory Manual

Kyiv
National Centre
"Junior Academy of Sciences of Ukraine"
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F97

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F97

The educational and methodical publication is intended for deepening knowledge and carrying out laboratory work on the theme "Fundamentals of Biochemistry. Proteins". The publication generalizes modern theoretical knowledge on the structure and functions of amino acids and their polymers – proteins in living organisms. The practical recommendations for the isolation, identification and research of proteins are presented in the form of laboratory works.

The publication is intended for the students of the Junior Academy of Sciences of Ukraine and the educational establishments in which chemistry is the subject of in-depth study. The materials of this textbook and lab manual may be used by tutors in practical work on the school curriculum, in planning and carrying out the research projects by high school K-12 students within school curriculum and during the work of the chemical (chemical and biological) sections of after-school education institutions, academic research centres, etc. as well as by chemistry and biology teachers as an auxiliary publication for teaching activities.

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INTRODUCTION

The surrounding world consists of a great variety of tiny particles – atoms and molecules. They are moving and changing, interacting and breaking down all the time. However, the life cycle of these "bricks" is subjected to the laws of nature and the rules that are the basis of the Universe from the beginning. The interactions between atoms and molecules and their continuous transformations have caused their complexity and diversity and led to the appearance of life on our unique planet.

Imagine the difference between a simple mixture of molecules, like air, soda or sugar solutions, even seawater with dissolved inorganic salts, organic compounds, air gases, suspended mineral particles, and a small living cell with its extreme variety of chemical compounds, which performs a define function (nutritional, respiratory, protective, signalling, reproductive, etc.). Their purpose is to provide this small unit living functions. Complexity of the processes ensuring the organism life makes it possible establish the science combining biology and chemistry.

So, we've come close to understanding what this amazing science called ***biochemistry*** or ***biological chemistry*** (whichever you prefer). And you realize that ***biochemistry*** is a science that studies the structure of chemical substances which are essential for organisms and the chemical reactions, as well as any transformations that take place with the participation of these substances. All these issues are integrated into living processes. The list of these substances is very long, ranging from the simplest (oxygen, water, metal ions) to the extremely complex, such as proteins. And they are extremely significant because every substance has a set of functions and performs them constantly.

It is impossible to study each of them in this book, otherwise it will turn into a multi-volume edition. We suggest to learn more about the proteins that are the most complex and interesting compounds in biochemistry, their isolation from other substances. their structure, properties and functions performed by them in living organisms.

"Fundamentals of Biochemistry. Proteins" book compiled for educational purposes and consists of theoretical and practical parts. Theoretical part presents

modern general information about the chemical and supramolecular structure, properties and function of proteins.

In practical part we collect the series of labs that will help you develop your practical skills needed for chemical experiments, confirm high level of your knowledge about proteins, get more detailed information on the main means of isolation, identification and studying the structure of proteins as well as evaluation of their chemical and enzymatic properties.

It should be understood that any laboratory-based work does not involve a mechanical set of actions, which are essential for observation any unusual effects (unexpected changes of colours, gas bubbles appearance, precipitating and other "Wow!"). Conducting the experiments and its final results should always be accompanied by proper conclusions that demonstrate understanding the interrelation between observations, obtained results and theoretical knowledge!

The focus of the experimenter must be on each experiment cognitive value, that is, the ability to observe the characteristics of the reactions, note the significant and minor changes, their sequence, and understand why these changes occurred and what their reasons were.

If you want to improve your knowledge about proteins, you need to know the basics of Periodic Law, the structure of inorganic and organic compounds, the types of chemical bonds, the general classification of chemical compounds, the types and conditions of chemical reactions. It is also necessary for sure and thorough performing of every laboratory-based work from this collection.

Before starting any laboratory-based work you should revise the Basic Safety Rules and the Basic First Aid Procedures. It is necessary for doing experiments safely and exclude the main dangers of any chemical procedure.

Modern chemical terminology and nomenclature, the notation system, which is recommended by the International Union of Fundamental and Applied Chemistry (IUPAC) and the National Commission on Chemical Terminology and Nomenclature, are used in this book.

The publication is intended for students of the Junior Academy of Sciences of Ukraine and general secondary education establishments in which chemistry is the subject of in-depth study.

The materials of this textbook and lab manual may be used by tutors in practical work on the school curriculum, in planning and carrying out the research projects by high school K-12 students within school curriculum and during the work of the chemical (chemical and biological) sections of after-school education institutions, academic research centres, etc. as well as by chemistry and biology teachers as an auxiliary publication for teaching activities.

CHEMICAL AGENTS AND RULES FOR HANDLING THEM

Chemical reagents are substances used for chemical reactions, analysis and synthesis.

Depending on the degree of purity, chemical reagents are classified as technical grade, pure, analytical grade, extra pure etc. Additional purity grades should be stated out by any country. Chemical reagents purity and quantity of impurities are regulated by the National State standards and technical conditions for their production and uses. The pure and analytical grade reagents are applicable for using in most chemical experiments.

The following rules should be observed while handling chemical reagents:

- reagents should be kept in closed vessels or containers to avoid laboratory contamination;
- solutions and solids should be taken in quantities required by the defined experimental technique;
- solid reagents should be taken with a spatula;
- liquid solutions or reagents should be taken with pipettes or drip dispensers;
- excess of a reagent should not to be put back into the original container which it was taken out to prevent reagent contaminating;
- keep the solutions of concentrated acids and alkalis, other dangerous and toxic substances in ventilated reagent box;
- all the experiments with dangerous reagents should be carried out in laboratory fume hood.
- Everybody should follow these rules to prevent accident during chemical experiments.

GENERAL LABORATORY SAFETY RULES

1. ***General provisions***

It is absolutely forbidden to have lunch or a drink in the chemical laboratory.

You should check the number and operability of laboratory equipment, accessories, glass and chemicals, personal protective equipment before starting any experiment.

Working with defective equipment is forbidden.

The aim, theoretical data and the stages of the chemical experiment should be well-known at its beginning. You should start the experiment just after the supervisor's permission.

Cleanliness and order on the workplace must be observed while working in the laboratory. Safety rules must be followed.

Messiness and untidiness during chemical experiment often result in accidents, namely harm to the health, damaging of equipment and clothing, incorrect results of experiments.

If the experiment is too long, the work should be organized in such a way when some other required experimental procedures can be performed simultaneously.

2. ***Basic rules of work in a chemical laboratory***

2.1. Chemicals, distilled water, gases, electricity should be used reasonably in the laboratory.

2.2. The instructions of equipment using should be read and kept to.

2.3. It is necessary to be careful while working with chemical equipment.

2.4. The test tubes with liquid must be heated carefully to avoid spraying that may cause scalds and loss of the solution.

2.5. During heating the test tube hole must be directed away from yourself and others. You should not look inside a test tube with any liquid that is being heated.

2.6. When it is necessary to feel an odour the substances in test tubes or cups, the air flow from the container should be directed by easy palm movement to yourself. You can smelt chemicals very carefully.

2.7. The experiments with unpleasant smell substances or reagents that produce

gases harmful to the health should be conducted in the fume hood. It is forbidden to work with these chemicals on the lab desk.

2.8. The waste solutions of acids, alkalis, etc., must be poured into container with wastes. If the solutions contain the compounds of silver, mercury, cerium, lead, other heavy, scattered and precious metals, they should be poured into special containers for further regeneration.

2.9. The most reactions conduct when the appropriate conditions are applied. The reagent should be added only to the solution prepared for defined experiment.

The uncontrolled volume of acid or alkali solution cannot be added to the solution.

The solutions of reactive highly reactive chemicals must be mixed dropwisely. Most of the chemicals are mixed with a glass rod.

The pH of solutions is often controlled using glass rod and peace of indicator paper.

2.10. To verify the possibility of precipitation, put 3 drops of analytic solution and 3 drops of the reagent into the test tubes.

2.11. The deposition completeness in a separate supernatant portion should be checked after full reagent addition and precipitation. If precipitation in the control sample is in progress, an additional quantity of reagent must be added to the reaction medium. When supernatant control sample does not give a positive reaction with the reagent the deposition is finished.

2.12. Deposition is generally done at elevated temperatures resulting in larger sediment particles which are easily separated from the supernatant.

3. ***Handling acids and alkalis***

3.1. When working with concentrated acids and alkalis, you should be careful to prevent their getting on your eyes, skin and clothes.

3.2. When you mix concentrated sulphuric acid, ***you should pour small portions of the acid into the water, not vice versa***. The hydration reaction of sulfuric acid is highly exothermic! If water is added to concentrated sulfuric acid, it can boil and spray dangerously outside.

3.3. While transferrin high volumes of concentrated acids and alkalis:

- put on the rubber gloves, aprons and safety glasses;
- large containers of liquids should be placed on the stand and then inclined and poured over the funnel into pure and dried vessel very slowly;
- defined volume of solutions should be measured with a pipet or a graduated cylinder;
- solid alkalis and acids are taken with a spatula; during their grinding your eyes are protected with safety glasses.

4. ***Handling harmful and poisonous substances***

4.1. Harmful and noxious substances (cyanides, barium, mercury, lead, arsenic, copper and their salts) should not get into your body via skin, lungs, gastrointestinal tract.

4.2. Washing hands is needed after handling such substances.

4.3. The mercury-filled instruments should be placed on special pallets in order to avoid spreading spilled mercury on the desktop or floor when the instruments are damaged.

If a spill occurs, you should gather small mercury balls very carefully using copper wire, foil or plate. Then you have to place the mercury-covered copper metal into a beaker and fill it with fine sulphur powder or $FeCl_3$ solution.

5. ***Handling combustible and flammable substances***

5.1. To use flames is forbidden in the laboratories where combustible and flammable substances are handled. Combustible, flammable and high volatile substances mustn't be stored closed to the sources of flame or heated electrical instruments (thermostats, electric ovens, heaters etc.). These substances should be stored in tightly closed small vessels in special laboratory box.

5.2. Water baths are preferred sources for heating hydrocarbons, alcohols, esters, esters and other flammable substances. The heating must be done in a condenser-equipped flasks in the fume hood.

5.3. You have to avoid contact of alkali or alkali-earth metals with water due to their high chemical reactivity. They should be stored under a moisture-free layer of highly boiled hydrocarbons or special silicone oil. After finishing your work, these metals are put into a special container and utilize by covering with alcohols.

6. ***Handling explosive mixtures***

6.1. Some gases (hydrogen, ethane, carbon monoxide, etc.) and highly volatile substances (hydrocarbons, alcohols, CS_2 and others) form explosive mixtures with air (oxygen) while evaporating. Before starting work with them, the ventilation should be switched on for preventing explosive fumes stockpiling in the laboratory.

6.2. Forming explosive mixtures substances (chlorates, perchlorates, persulphates and other oxidants) are prohibited to heat, hit or store with other substances (organics, reductants).

7. ***Putting out laboratory fires***

If there's a fire in the lab, all electrical items should be switched off, and the gas supply should be turned off immediately. The fire source should be covered with a sand layer or a fire blanket and treated with a fire extinguisher. In the laboratory the water for extinguishing fires is used carefully because in some cases a water can intensify a fire.

FIRST AID IN CASE OF ACCIDENTS

Labour protection instructions and safety regulations in the chemical laboratory should be followed. Accidents (scalds, burns, injuries, poisoning, etc.) could be prevented by careful handling chemical reagents, instruments and equipment.

If the accident did occur, the victim must be provided with first aid:

1. After getting any acid on the skin, you should flush it off with clean cold water and then the injured area must be treated with 2% baking soda ($NaHCO_3$) solution to neutralize the acid residues.

2. After getting some alkali solutions on the skin, you should flush it off with clean cold water and then treated with 2% boric or citric acid solution.

3. After getting some phenol, bromine and similar aggressive substances on the skin, you should flush off the chemicals with organic solvents (alcohols, esters, etc.).

4. After chlorine, bromine, nitrogen oxides poisoning, the injured should breathe diluted ammonia solution vapour and drink much milk.

5. After thermal skin burning, the sterile dressing must be put onto the burnt area and the person should be taken to hospital.

6. After cutting, the wound must be washed with 3% aqueous hydrogen peroxide solution, the skin must be treated around the wound with an alcohol solution of iodine and a sterile dressing must be put on the injured area.

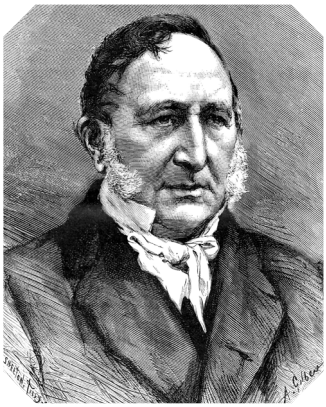
7. When the first aid procedures are finished the person should be taken to hospital.

PROTEINS. STRUCTURE AND PROPERTIES

The term ***protein*** is derived from the Greek word ***πρώτα***, meaning "primary" or "first place". The famous Swedish scientist Jens Berzelius discovered this type of chemical compounds in 1838. He proposed to use this term for the group of the most important for life organic compounds. Later that year, the German chemist G. Mulder used the term "proteins" for high molecular nitrogen-enriched compounds which can be found in any living organism, animal or vegetable species.

Jens Jacob Berzelius
(1779–1848)
a Swedish chemist, one of the founders of chemistry as a science

He discovered a series of chemical elements, like Ce, Se, Th; obtained Si, Ti, Ta and Zr substances; determined the atomic mass of 46 chemical elements; deduced the formulas of acetic, tartaric and succinic acids.



Gerrit Jan Mulder
(1802–1880)
a German chemist

He studied proteins, invented the methods of isolating proteins from animal or vegetable species, and deduced the element composition for proteins.

In fact, the proteins are the most complex molecules in living organisms due to their structure and functional purpose. No wonder, when you realize that the chemical structure of each protein molecule has been "created", "polished", "tested" and "implemented" by nature for million years of its evolution.

But before considering the mysterious and unique features of these large polymeric (yes, exactly polymeric!) molecules, you should get to know more about the specific "bricks" from which proteins are built. They are called amino acids.

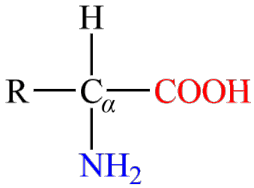
Why does it matter? The sequence, in which each amino acid is placed along the chain of chemically bounded amino acids that make up the proteins, determines the 3D structure of the protein molecule and its function in the cells of the living organism.

Amino Acids

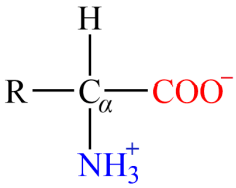
Amino acids are organic compounds containing at least one organic acid group (carboxyl, –COOH) and one basic group (amino, $–NH_2$). Today about 300 amino acids are known among a lot of natural organic compounds. But not all of them are needed to build up the protein molecules.

Slightly over 20 amino acids have overcome evolutionary selection and – COOH and $–NH_2$ groups are attached to the same carbon atom in the structure of their molecules (as a rule, it is denoted by a Greek letter *"α"*). Amino acid molecules that have this structure are called ***α-amino acids***. The R group in the structure of the amino acid denotes an organic radical providing specific features of each amino acid. Such features are chemical properties, protein structure-forming function and its biological role.

Different ways of α-amino acids depicting



non-ionized
(formal)



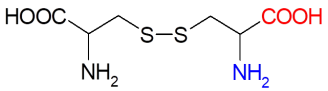
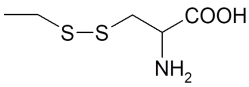
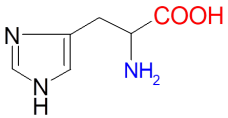
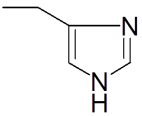
ionized
(actual)

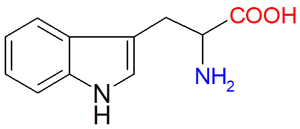
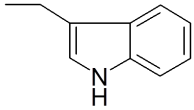
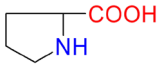
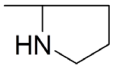
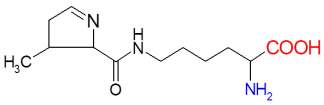
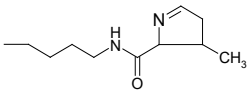
α-Amino acids always exist in an ionized form. In this case, either the amino group has a charge (+) and the molecule is in a cationic form or the carboxyl group

has a charge (-) and the molecule turns into an anion. If both groups are charged, the molecule forms zwitterion (double ion).

Table 1 shows all α-amino acids from which protein (peptide) molecules are built by nature.

Table 1
Amino Acids Found in Protein Molecules

Name	*Short symbols*		*Chemical structure*	*R group*
Glycine	Gly	G	H-CH(NH_2)COOH	–H
Alanine	Ala	A	H_3CCH(NH_2)COOH	–CH_3
Valine	Val	V	$(CH_3)_2$CHCH(NH_2)COOH	–CH$(CH_3)_2$
Leucine	Leu	L	$(CH_3)_2$CHCH_2CH(NH_2)COOH	–CH_2CH(CH3)$_2$
Isoleucine	Ile	I	C_2H_5CH(CH_3)CH(NH_2)COOH	–CH$(CH_3)C_2H_5$
Serine	Ser	S	HOCH_2CH(NH_2)COOH	–CH_2OH
Threonine	Thr	T	CH_3CH(OH)CH(NH_2)COOH	–CH(OH)CH_3
Cysteine	Cys	C	HSCH_2CH(NH_2)COOH	–CH_2SH
Cystine	–			
Methionine	Met	M	$CH_3S(CH_2)_2$CH(NH_2)COOH	–$(CH_2)_2SCH_3$
Aspartic acid	Asp	D	HOOCCH_2CH(NH_2)COOH	–CH_2COOH
Asparagine	Asn	N	H_2NCOCH_2CH(NH_2)COOH	–CH_2CONH_2
Glutamic acid	Glu	E	HOOC$(CH_2)_2$CH(NH_2)COOH	–$(CH_2)_2$COOH
Glutamine	Gln	Q	H_2NCO$(CH_2)_2$CH(NH_2)COOH	–$(CH_2)_2CONH_2$
Lysine	Lys	K	$H_2N(CH_2)_4$CH(NH_2)COOH	–$(CH_2)_4NH_2$
Arginine	Arg	R	NH=C(NH_2)NH$(CH_2)_3$CH(NH_2)COOH	–$(CH_2)_3$NHC(NH_2)=NH
Histidine	His	H		
Phenylalanine	Phe	F	$C_6H_5CH_2$CH(NH_2)COOH	–$CH_2C_6H_5$
Tyrosine	Tyr	Y	HO$C_6H_4CH_2$CH(NH_2)COOH	–$CH_2C_6H_4$OH

Name	*Short symbols*		*Chemical structure*	*R group*
Tryptophan	Trp	W		
Proline	Pro	P		
Selenocysteine	Sec	U	$HSeCH_2CH(NH_2)COOH$	$-CH_2SeH$
			Discovered in 2000s in some enzymes	
Pyrrolysine	Pyl, O			
			Contained in enzymes of methanophilic organisms	

In general, all α-amino acid sets can be divided into several classes according to their chemical structure and properties. Due to complex structure and differences in chemical behaviour same amino acid may belong to different groups. Let's take a thorough look at different ***types of the basic α-amino acids classification*** to find out the similarity, difference and peculiarities of every protein "brick".

1. Chemical structure

A) ***Amino acids with the aliphatic R group***. The simplest amino acids are glycine, alanine, valine, leucine and isoleucine. The last three have a branched hydrocarbon substitute.

B) ***Amino acids with aromatic R substituent***. This group includes phenylalanine and tyrosine (both of them have a benzene ring structure), tryptophan (aromatic indole bicyclic group) and histidine (imidazole aromatic ring).

C) ***Hydroxyl-containing amino acids***. The hydroxyl group (alcoholic or phenolic) is in the structure of serine, threonine and tyrosine.

D) ***Sulfur-containing amino acids***. They are cysteine (thiol group –SH), methionine (thioether group –S–) and cystine (disulfide –S–S– group).

E) ***Heterocyclic amino acids***. Histidine, tryptophan, proline and pyrrolysine have heterocyclic fragments in their chemical structure

F) ***Imino acids***. Proline is the only amino acid that contains the secondary

amino group >NH, instead of the primary amino group $-NH_2$. Therefore, it is more properly to call it ***α***-imino acid.

2. Acid-base properties

A) ***Amino acids with the mainly acidic properties***. Amino acids with two carboxyl –COOH groups belong to this sub-class. They are aspartic and glutamic acids and their amide derivatives, asparagine and glutamine.

B) ***Amino acids with mainly basic properties***. Three amino acids as lysine (with an additional amino group), arginine (with guanidine group) and histidine (with imidazole group) have relatively strong basic properties.

3. The polarity of molecules (polarity is the ability to form a dipole with a clear separation of charges (+) and (-) within a molecule, analogues to a battery). This property of amino acids, which are the part of protein molecules, affects their shape and functionality considerably.

A) ***Non-polar*** amino acids with uncharged R substituent. These amino acids are also called hydrophobic ("repelling" water). This sub-class of amino acids includes alanine, leucine, isoleucine, valine, methionine, phenylalanine, tryptophan and proline.

B) ***Polar*** amino acids with R substituent that has no charge, but there are atoms with high electronegativity in the structure (O, N or S, or if R = H). This group includes glycine, serine, threonine, cysteine, glutamine, asparagine and tyrosine.

C) ***Polar*** amino acids with a positively charged R substituent. There are only three amino acids having this feature, namely lysine, arginine and histidine.

D) ***Polar*** amino acids with a negatively charged R substituent. Such amino acids contain two carboxyl groups – aspartic and glutamic acids.

4. Nutrition and biosynthesis value. *α*-Amino acids are involved in protein biosynthesis and other biological processes. The human body's need for some amino acids can be met by synthesizing them on its own. However, some other essential amino acids cannot be synthesized by the body so they must be obtained from the diet. By that yardstick, amino acids are divided into essential and nonessential ones.

A) ***Essential amino acids*** cannot be synthesized by a human, so protein-containing food having these amino acids must be obtained. They are important for preventing multiple childhood and adult malnutrition-related illnesses, pathologies and developmental defects. This group includes 10 amino acids: arginine, valine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and tryptophan. The two amino acids in this list, like arginine and histidine, are semi-essential because can be synthesized only by adults. So, a variety of high-quality protein sources should be included in a child's diet.

B) ***Nonessential amino acids*** are glycine, alanine, serine, cysteine, aspartic acid and asparagine, glutamic acid and glutamine, tyrosine and proline. The human

body is able to synthesize these amino acids for its own use. So, there is no need to get these amino acids from outside (proteins that contain them). But preferable, because of their synthesis is highly dependent from variety of other biologically active and nutrition components.

5. The metabolism (biochemical transformation in the body). Living organisms may use the carbon chain of amino acids as a feedstock for synthesizing of some other substances.

A) ***Glycogenic amino acids***. The human body uses these compounds for glucose and glycogen synthesizing. There are alanine, aspartic acid, glycine and methionine.

B) ***Ketogenic amino acids***. They are exclusively involved in endogenous (inside cells) fat synthesis processes. This group includes leucine and lysine.

C) Four amino acids are ***glyco-ketogenic*** or universal ones. Isoleucine, phenylalanine, tryptophan and tyrosine belong to this group.

Properties of Amino Acids

Both physical and chemical properties of amino acids are directly related to their chemical structure.

Physical Properties

Solubility. Due to the existence in the ionized state, amino acids are well soluble in water, but they are often barely soluble in most organic solvents.

Melting point. Due to the ionic nature of molecules, amino acids become liquid at elevated temperatures only. For most amino acids, the melting point is about 200 °C or above.

Taste. Human taste receptors make amino acids taste different. Glycine, alanine, valine have sweet taste. Leucine has no taste. Isoleucine and the sodium salt of glutamic acid have salty taste.

By the way, you know this compound quite well as modifier of taste, glutamate or sodium glutamate. It is usually added to meat, mushroom and fish dishes to enhance their flavour and taste. It is grossly abused in Chinese restaurants. Some people have low digestibility of this compound, so it is accompanied by a rapid manifestation of bowel disorder. These "surprises" for a Chinese restaurant visitor are called "Chinese restaurant syndrome".

Chemical Properties

Chemical properties of amino acids depend on the presence of carboxyl, amine and other functional groups in their structure.

General properties

Acid-base properties. Due to the simultaneous availability of acidic and basic functional groups, amino acids have both acidic and basic properties. That is why amino acid molecules are almost non-existent in uncharged state.

At low pH (in an acidic medium) amino groups are ionized, and the amino acid molecules have a positive (+) charge. In alkali medium (pH>7), the carboxyl groups are ionized and amino acid molecules become anions and acquire a negative (-) charge.

However, at a certain pH (different for each amino acid) the molecules have both positively charged $-NH_3^+$ groups and negatively charged $-COO^-$ groups. That is, amino acid molecules exist in a bipolar state or in the form of zwitterions (*from German* ***Zwitter***, means "***bipolar*** or ***hybrid***"). In this state, despite the presence of ionized (charged) groups, the total charge of the molecule is "0" and the molecule is electroneutral. The pH, at which this state is achieved, is called ***the isoelectric point (pI)*** of the amino acid. For example, leucine molecules become electro-neutral if pH = 6.0 (i.e. pI of leucine is 6.0).

The isoelectric point can be defined as the mean value of the dissociation constants (given as a negative decimal logarithm) of carboxyl and amino groups of defined amino acid.

Dissociation constant of –COOH group of leucine $K_{d1} = 4.0 \times 10^{-3}$

In accordance, $pK_{d1}\ (-COOH) = -\log (4.0 \times 10^{-3}) = 2.4$

Dissociation constant $-NH_3^+$ of leucine $K_{d2} = 2.5 \times 10^{-10}$

In accordance, $pK_{d2}\ (-NH_3^+) = -\log (2.5 \times 10^{-10}) = 9.6$

$$pI = x = \frac{pK_{d1(-COOH)} + pK_{d2(-NH_3^+)}}{2} = \frac{2.4 + 9.6}{2} = 6.0$$

If the amino acid consists of some other functional groups that are capable of ionizing, all their *pK* should be taken into account.

To sum it up, it can be assumed that amino acids behave as polybasic compounds during titrating.

The properties related only to –COOH groups:

a) ***neutralization reaction***. Salts (like –COONa) are formed as the result of interaction of amino acids with strong bases;

b) ***esterification***. Esters ($-COOR^1$) are formed as the result of interaction with alcohols (R^1OH);

c) ***interaction with ammonia and amines***. Depending on initials and conditions, the result of the reaction is a formation of salts ($-COONH_4$) or amides ($-CONH_2$, –CONHR);

d) ***decarboxylation***. This reaction produces a variety of biologically active amines in the cells, such as histamine from histidine, tyramine from tyrosine, and γ-aminobutanoic acid from glutamic acid. Each of these compounds plays an important role in a cell life.

Properties associated exclusively with –NH_2 groups:

a) ***neutralization reaction***. Salts (for example, –$NH_3^+Cl^-$) are formed after the interaction with strong acids, like HCl;

b) ***amidization***. Amides are formed after the interaction of with carboxylic acids, (–NHCOR[1]);

c) ***transamination***. The catalytic reaction of the transferring –NH_2 group from amino acids to other compounds plays an important role in cell metabolism;

d) ***oxidative deaminating***. This reaction produces ammonium –NH_4^+ and O-containing acid derivatives.

Amino Acid Sequence and Shape of Protein Molecules
Primary Protein Structure

Proteins are known to consist of 20 different amino acids. Each of them has its own specific properties. Each amino acid gets attached to an adjacent one by a strong covalent peptide (***amide***) –CO–NH– bond (*fig. 1*).

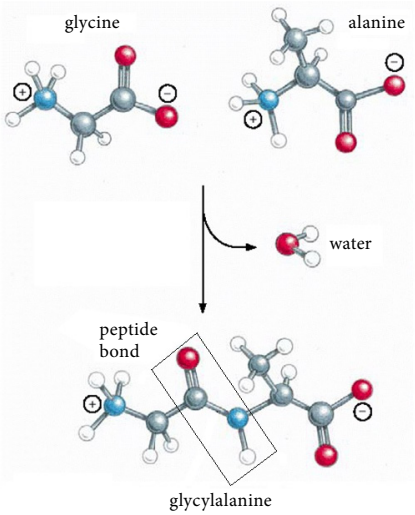


Fig. 1.
Scheme of peptide bond formation

This covalent bond is formed by interacting of the carboxyl group of one amino acid with the amino group of another one. As the result of this chemical reaction (called ***condensation***) a water molecule is eliminated and the peptide bond is formed. In organic chemistry, this reaction is similar to ***amidization*** process, that is, the interaction between carboxylic acids and amines that causes the formation of a new class of organic compounds called ***amides***. It is widely used for industrial producing a separate class of polymers, polyamides, which are used to make casings, nylon threads, fabrics, sport accessories and even the artificial sausages casings.

The name of this bond in protein chemistry is "peptide". That's why protein molecules are sometimes called ***polypeptides*** ("poly" means "many"). Each protein has an unique amino acid sequence that is accurately reproduced in each of its molecules. Today, there are thousands of proteins, and each of them has its unique amino acid sequence as any person has own unique fingerprint.

A repeating sequence of atoms along a polypeptide molecule is called a ***polypeptide backbone*** or ***polypeptide chain***.

Side groups R of amino acids do not take part in the formation of the peptide bond, but give each amino acid its unique properties. The diversity of these R groups is equal to the number of amino acids attached to this regular sequence of amino acids of the polypeptide chain.

Some of these side groups are known to be non-polar or polar, hydrophilic or hydrophobic, uncharged or charged (positively or negatively), non-reactive or reactive, etc. (*see Table 1* and Amino Acid Classification Chart). Each protein molecule differs in its sequence and number of amino acids, as well as in its sequence of chemically different R groups. In a short form, the chain forming process of a protein molecule is presented in *fig. 2*.

As is known from the theory of atom and molecule structure, all atoms behave as solid spheres with a certain radius (Van der Waals radius). The possible coupling angles in the polypeptide chain are limited by the repulsive forces and the absence of a mutually overlapping space between two separate atoms.

The spatial structure of the polypeptide chain depends on the three main bonds (*fig. 3*, shown in red) of each amino acid. The peptide bond has a flat structure (*fig. 3*, shown in grey), the C=O and N–H groups are unable to rotate around this bond axis. A variety of possible locations of atoms in a protein molecule is rather limited by these and some other spatial (***steric***) constraints. But despite these obstacles, a long flexible polypeptide chain of protein molecules can choose any of many other possible ways of packing.

The additional spatial constraints of the protein chain cause many non-covalent bonds formed between individual fragments of different parts of the chain. These bonds appear between the atoms in the main polypeptide chain and the atoms of the lateral amino acid fragments.

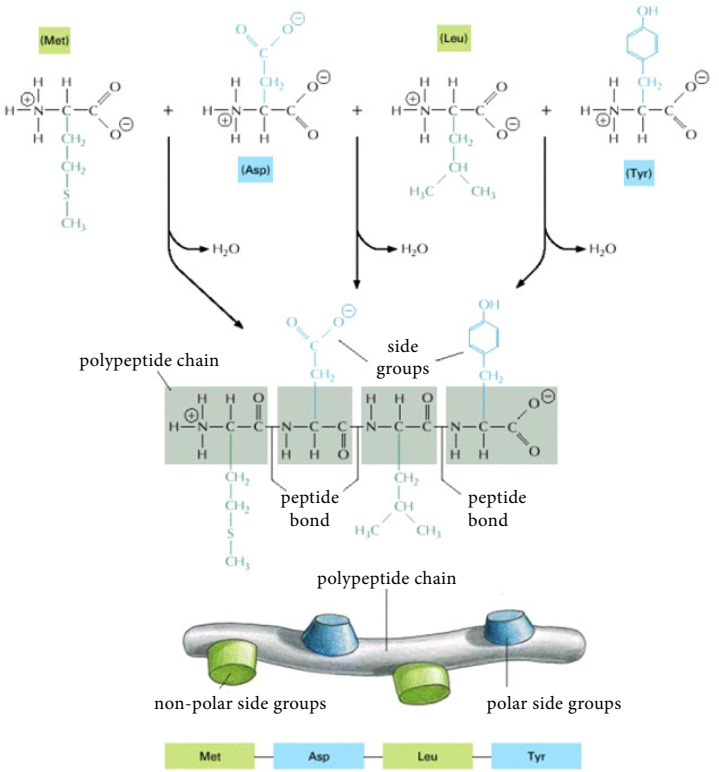


Fig. 2.
Building of polypeptide chain

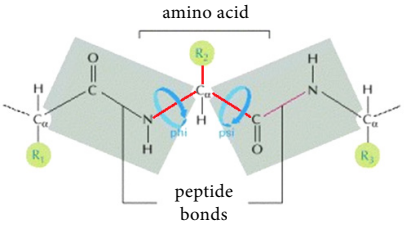


Fig. 3.
Bulk conformation of polypeptide chain fragment

A variety of non-covalent bonds playing important role in formation of protein molecules includes:

- hydrogen bonds (between an atom H, which is covalently bounded to an electronegative atom, and another electronegative atom);
- ionic or dipole-dipole bonds (between charged carrier groups);
- physical Van der Waals bonds (van der Waal attraction forces).

Non-covalent bonds are known to be 30–300 times weaker than covalent ones. However, their large number and variety compensate this "weakness" and help to keep firmly the adjacent parts of the polypeptide chain. Thus, the structural stability of each component of the protein molecule is determined by the total strength of a great number of weak bonds (*fig. 4*).

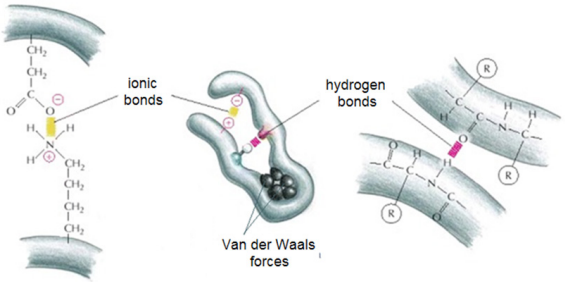


Fig. 4.
Impact of non-covalent bonds in a protein molecule folding

Hydrophobic interactions are another influential factor playing a key role in the shape of the protein molecule. In the aquatic environment, the hydrophobic parts of the molecules (non-polar side groups of some amino acids) are close up to one another to minimize destructive solvating effect of water molecules via formation of hydrogen, ionic and dipole bonds with different parts of the polypeptide chain. Therefore, the distribution of polar and non-polar amino acids along the polypeptide backbone (*fig. 5*) is a very important factor for regulating shape of any protein molecule.

The non-polar (hydrophobic) R groups of such amino acids as phenylalanine, leucine, valine and tryptophan are localized inside of the protein molecule and they form a tightly packed hydrophobic "nucleus" (as small droplets of hydrophobic oil drops are gathered together in water and form large droplet). This property allows them to avoid contact with the water molecules surrounding proteins inside the cell's biological environment.

Unlike this process, the polar R groups contained in arginine, glutamine, histidine are located on the outer part of a large protein molecule. They form hydrogen and ionic bonds with water molecules and other polar molecules in aquatic biological media.

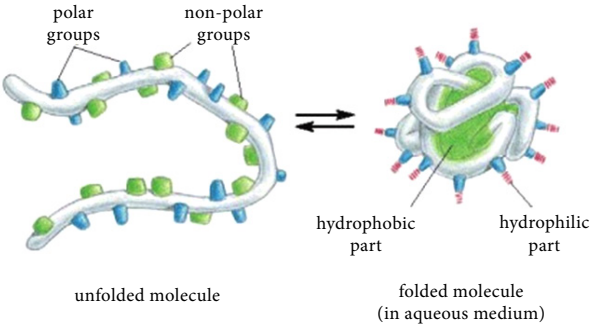


Fig. 5. Sequence of amino acid residues and a shape of protein molecule

If the polar groups of amino acids are located in the interior of the protein molecule, they usually form hydrogen bonds with the polar groups of other amino acids or the peptide groups of protein backbone and the large protein molecule gets a certain specific shape.

Secondary Protein Structure (α-Helix and β-Structure)

As a result of all possible interactions, each protein comes to be a special three-dimensional (3D) structure, determined by the amino acid order in its molecule. The finite spatial structure (or ***conformation***), which any polypeptide chain gets, naturally has minimal surface energy[1].

The pattern of molecular formation has been studied in vitro using specially purified proteins. The most characteristic form of protein molecules can be changed (unfolded) after heating, interaction with solvent or other chemicals that being capable of breaking down non-covalent bonds that hold the chain in a certain way (*fig. 6*). This treatment leads to the natural shape loss and converts the protein molecule into a more flexible polypeptide chain (called ***denaturation***).

It should be noted that the spatial form of the protein molecule is related to its functionality. Therefore, any changes in native shape of the protein molecules lead to loss of its useful functions.

In some cases, after the denaturing factor removal, the polypeptide chain is able to "refold" in a certain way, and the protein molecule can once again take its natural form (this phenomenon is called ***renaturation***) and functions. This behaviour indicates that the "information", which the protein needs to determine its own spatial structure, is in the sequence of amino acid residues in its molecule.

[1] It means that a molecule has got the most advantageous spatial structure (it is composed accurately and appropriately to its amino acid composition and sequence), and to change this shape the molecule must get the maximum amount of energy from the outside (i.e., by heating).

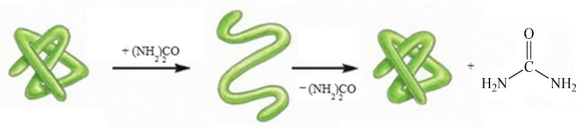


Fig. 6.
Denaturation and renaturation of protein molecule

Although the polypeptide chain can take its inherent shape (***conformation***) again without any assistance in a living cell, during the folding process the protein molecule is assisted by other "special" proteins, called ***molecular chaperones*** (chaperone as "to accompany" or "to assist"). These proteins bind to partially folded polypeptide chains non-covalently and help these chains to obtain the most energetically favourable form.

They are very important "helpers" of proteins in the cytoplasm of cells filled with a lot of different compounds. The main function of chaperones is preventing uncontrolled binding of temporarily unbound hydrophobic groups of amino acids just synthesized polypeptide chains to unnecessary aggregates of protein molecules forming.

Considering the necessary spatial structure of protein molecules is determined only by the sequence of amino acids, the chaperone proteins make the process of protein folding more reliable.

Protein molecules are known to be of different shapes and range within 50–2,000 amino acid residues. Large protein molecules consist of several different smaller polypeptide chains, which sometimes can be synthesized independently of each other. The detailed structure of any protein is quite complicated, so several different ways of its depicting have been invented to simplify it. Each of them emphasizes different features of the protein structure.

The difference in the depiction of polypeptide chains in three-dimensional structures (this type of structure of protein molecules is called ***secondary structure***) can be seen in the example of protein triosephosphate isomerase. This enzyme consists of eight helical structures (***α-helices***) located on the outer surface of a molecule and eight parallel planar structures (***β-structures***) located inside of a molecule.

In general, the similar structure of the protein molecule is called ***αβ-barrel***. In *fig. 7* several ways of depicting protein molecules are shown:

- on the left a "stick" model with the image of all atoms and bonds between them (atoms of each element are painted in a certain colour);

- on the middle a "spiral" model with the image of the elements of the secondary structure, like α-spirals and β-structures (individual elements of the secondary structure are of different colours);
- on the right a "ball" model based on the construction of images based on Van der Waals radii of atoms.

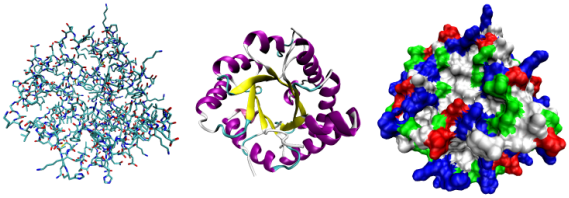


Fig. 7.
Different models of 3D structure of protein molecules

When the three-dimensional structure of different protein molecules are compared, it can be concluded that the overall conformation of each protein is unique but both secondary structure types can be found in some parts of proteins. For the first time, these features were found as a result of analysis of hair and silk proteins.

The first folding structure to be discovered was called α-helix. This structure was found in the structure of α-keratin protein in the outer skin layer (epidermis) and its derivatives such as hair, nails, and horns.

Not long after the discovery of the α-helix, a second folded structure (called the β-structure) was discovered in the fibroin protein that is the main constituent of silk. These two methods of polypeptide chain folding are the most common, because they result from hydrogen bonding between the N–H and C=O groups of a polypeptide backbone without involving the side chains of the amino acids into the process. As a result, α-helices and β-structures can be formed independently of the amino acid sequence and the nature in the protein molecule (*fig. 8*).

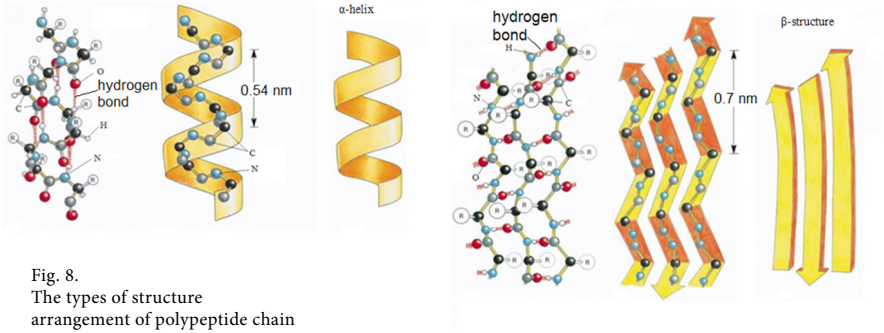


Fig. 8.
The types of structure arrangement of polypeptide chain

The core of many proteins contains large regions of β-structure conformation. As shown in *fig. 9*, a β-structure conformation can be formed either from adjacent polypeptide chains with the same orientation (parallel chains) or a polypeptide chain folding back and forth upon itself as harmonics. At the same time, each section of the chain running in the direction opposite to that of its nearest "neighbours" (antiparallel chain). Both β-structures types make a very rigid structure, consisting of adjacent polypeptide chains held together by hydrogen bonds.

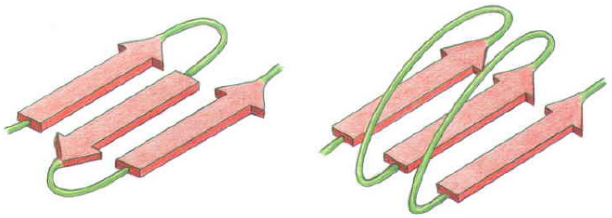


Fig. 9.
Bulk orientations of β-structures in protein molecules

A α-helix conformation is formed when a single polypeptide chain is twisted around on itself to form a rigid cylinder-like structure (*fig. 8*). In this type of folding, a hydrogen bond is formed between every fourth –NH–CO bond, linking C=O group of one peptide bond to the N–H group of another peptide bond. It gives rise to a regular helix having a complete turn that consists of 3.6 amino acids residues (or, if we turn this number into "whole" molecules; 5 complete helix turns are formed by 18 amino acids residues). Abundant α-helix proteins are located in cell membranes and such proteins have transport or receptor functions.

For example, the transmembrane protein crosses the lipid bilayer of the cell membrane with the part that has a α-helix conformation and consists predominantly of non-polar R groups of corresponding amino acids. In this case, the hydrophilic polypeptide chain "twists" itself in α-helix with the aid of hydrogen bonds and this α-helix is shielded from hydrophobic lipid layer around the protein by its own non-polar lateral groups. In some other proteins, α-helices wrap around each other to form a particularly stable structure known as a ***double helix***.

This structure can be formed when the two (in some cases three) α-helices have got most of their non-polar side R groups on one side, so they can twist around one axis with these hydrophobic R groups facing inward a new complex (*fig. 10*).

The rigid base for many proteins with reduced rigidity of polypeptide chains is formed by double helices. The example of it is α-keratin that forms the intracellular fibrous aggregates strengthening the outer skin layer and its formations. Another one is myosin protein, which is responsible for muscle contraction and make them to be strong.

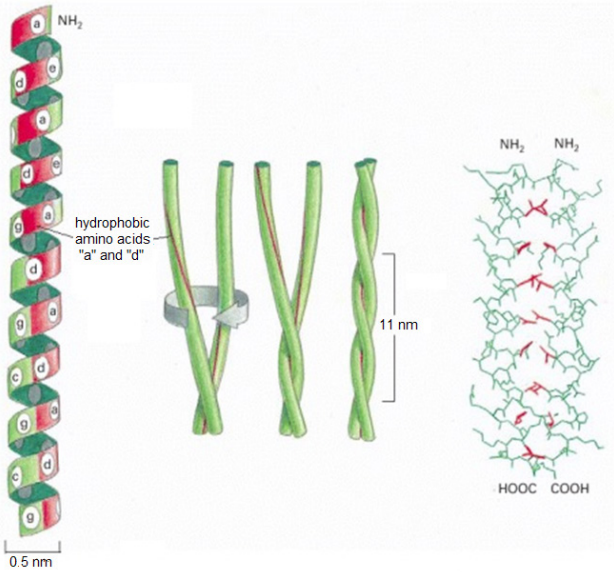


Fig. 10.
α-Helices based double spiral fold in some proteins

Protein Tertiary Structure

Even a small protein molecule is built from thousands of atoms linked together by precisely oriented covalent and non-covalent bonds in space. The complex structure is almost impossible to depict without a three-dimensional image. Modern chemistry distinguishes four levels of structural organization of protein molecules.

We have already learnt some of them.

The sequence of amino acids connected by peptide bonds is known as the ***primary protein*** structure. The formation of α-helices and β-structures by the fragments of polypeptide backbone is identified as a ***secondary protein*** structure. Pushing to the next level of understanding of the spatial structure of proteins requires some addition knowledge.

The spatial organization of the entire polypeptide chain of the protein molecule is called the ***tertiary structure*** of the protein. The conformation, functionality, and protein evolution studies have found another structural unit determining the feature of the structure of defined part of the protein molecule and being separated from previously mentioned types of protein structural organization. This structural unit is called a ***domain***.

It can be formed by any large part of the polypeptide chain. This part is capable of self-folding autonomously into a compact stable structure independently from the other parts of this polypeptide molecule. The domain generally contains 40–350 amino acid residues and it is "a module" that can be build up in a lot of other proteins. In other words, "a module" is a part of the constructor as a basic element of the design of various toys (*fig. 11*). Well, is that clear?

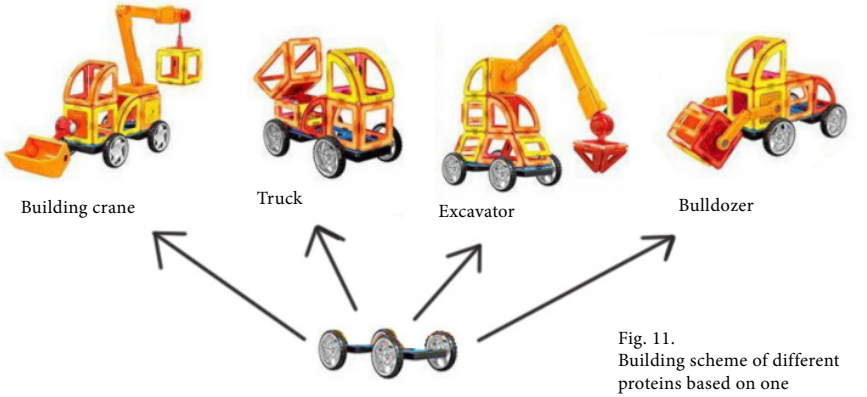


Fig. 11.
Building scheme of different proteins based on one domain (module unit)

Different protein domains often have different functions. For example, the structure of kinase (an enzyme that connects orthophosphoric acid residue to some proteins) contains four domains. The large and small protein kinase domains are responsible for the enzymatic activity, while SH2 and SH3 domains play regulatory roles. So, they influence on the catalytic activity (increase or decrease it per need) of the first two enzyme domains (*fig. 12*).

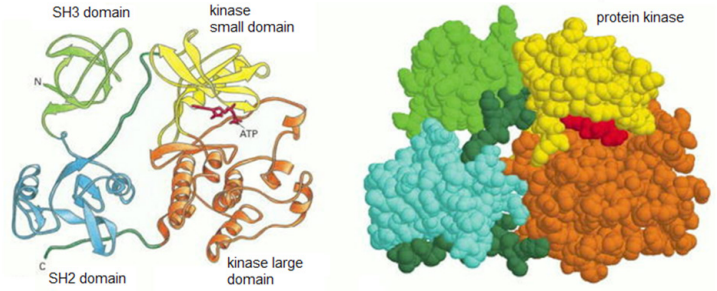


Fig. 12.
Domain structure of kinase

Small proteins contain only one domain, but larger proteins may contain dozens of domains, which are usually linked to each other by short and almost unstructured polypeptide fragments. *Fig. 13* shows examples of three different domains. The central nucleus of the protein domain can be composed of α-helices, β-structures or variety of their combinations. Almost 1,000 combinations of domain structures, which form about 10,000 different functional proteins, have been studied in detail.

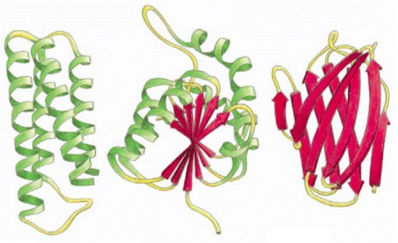


Fig. 13.
3D structure
of different domains

Evolution of Proteins

20 amino acids are known to be required for making up proteins. Each of them has its own structure and properties. A variety of proteins are made by a large number of combinations of these amino acids in a single polypeptide chain.

For example, to made up of peptide chain from only four amino acids, there are 20^4 or 160,000 of possible combinations of amino acid sequences. When the polypeptide chain consists of $\boldsymbol{n}$ number of amino acids, 20^n different combinations of amino acids can be formed. Typically, a molecule of an "ordinary" protein contains about 300 amino acid residues. Therefore, the theoretical number of combinations of amino acid sequences, which can build such protein, is equal to 20^{300}.

If you want, you can do the math and write the result in your exercise book. But don't waste your time and efforts. There aren't enough atoms in the Universe to create all possible molecules of this protein!

But implementing all structures of this protein in practice is not necessary, because only a tiny part of this huge number of polypeptide chains can acquire stable three-dimensional conformation (it is said to be less than one in a billion). And all available conformations have absolutely different chemical properties. So all the proteins in the cells are unique, and each of them has one stable conformation.

How is that possible? The answer lies in the elaborate mechanism of evolution: it is natural selection.

We do not need particular knowledge to understand that proteins with the unpredictable structure and chemical activity will not help in functioning and surviving of a complex natural object as a living cell. Finally, these useless proteins are eliminated by natural selection. As a result of the on-going evolutionary process, the protein molecule has got an specific amino acid sequence and due to it the chain acquires only one stable conformation. Thanks to it, the protein fulfils some certain clearly defined catalytic, structural or other functions.

Proteins are built so precisely that changing of a few atoms in one amino acid can lead to structural breakdown and loss of protein properties. So, we know that each protein has a stable three-dimensional structure and its characteristic properties. But this does not mean that the process of a protein molecule "development" has reached its completion.

Its structure and properties may continue to change and acquire new functions over the course of further evolution if it is necessary for the better living cell functioning.

In the past, it happened quite often, so there are a lot of known proteins today. They are classified into many families where each protein has a similar amino acid sequence and three-dimensional structure. To better understand it, we're going to study a pretty large family of proteins, serine proteases. This group of proteolytic (scission) proteins consists of digestive enzymes involved in the blood clotting process. There are chymotrypsin, trypsin and elastase, and several other proteases.

If we compare these enzymes, it can be found out that some parts of their amino acid sequences match each other quite well. Their three-dimensional conformation similarity is extraordinary because most twists and turns in polypeptide chain containing several hundred amino acid residues are almost identical (*fig. 14*).

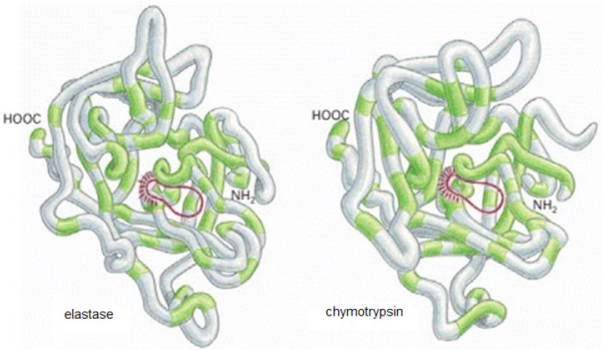


Fig. 14.
Three-dimensional structure of proteins of different functionality

However, all of these proteases have different chemical properties (enzymatic activity). They break down different proteins or peptide bonds between different amino acids. So, each enzyme has a special, but different function in the living organisms.

This example is not an exception. The same is true for hundreds of other protein families. In many cases significantly larger differences can be found in many amino acid sequences. In order to conclude what family the protein belongs to, the three-dimensional structure of the protein must be clearly compared.

In large protein families the proteins often have different functions. Some changes in the structure or sequence of amino acids that sometimes make the members of the same family be different, no doubt, are the result of evolution that has usefully altered their biochemical activity. Otherwise, many other changes have neither useful nor useless for the protein structure and properties.

It's worth bearing in mind that protein structure alterations (in other words, mutations) is a random process. So, in the evolution, a lot of negative transformations occurred which provide undesirable change the three-dimensional structure of protein molecules. Such "damaged" proteins largely being unsuitable for performing the necessary cell functions, the cell "got rid of" them as a waste.

Quaternary Structure of Proteins. Structure of Protein Complexes

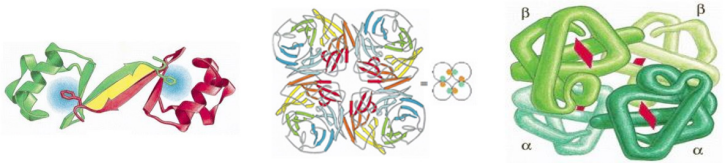
The weak non-covalent bonds let the polypeptide chain form the required three-dimensional conformation. By this way, some protein molecules can be bonded together creating massive cellular protein complexes. A small part of any protein molecule that can interact and bind to another molecule by non-covalent bonds is called a ***binding site***.

Different small and large protein molecules may have one or few binding sites. If the binding site recognizes the surface of another protein, the strong binding of these two polypeptide chains forms a larger molecule of a new complex protein with an accurate shape. In this complex protein, each polypeptide chain is called a ***protein subunit***.

Formation of complex protein by more than one polypeptide chain depicts a ***quaternary protein structure***. At its simplest, two identical joined polypeptide chains are arranged to each other according to a scheme "***head-to-head***", or "***DCBA-ABCD***". Thus, a ***dimer*** is formed that is a symmetric complex of two protein subunits, which joined due to the interaction of two identical binding sites. The ***cro-repressor*** (***repressor*** – suppressive) is a typical dimer protein that bound to DNA molecule. It is one of many other symmetric protein complexes formed by many copies of a

particular polypeptide chain that contained in each living cell. The neuraminidase enzyme is composed of four identical protein subunits. Each of them is linked to the next one "***head-to-tail***" ("***ABCD-ABCD***") creating a closed circle structure.

Many proteins in cells contain two or more types of polypeptide chain. A well-studied example of such protein complexes is haemoglobin that is a protein acting as a carrier of O_2 in erythrocyte blood cells. It contains two identical α-globin subunits and two identical β-globin subunits arranged symmetrically. The proteins containing several different subunits are very common situation in cells and the size of such protein structures may be quite large. *Fig. 15* illustrates the examples of proteins containing several subunits.



Cro-repressor Neuraminidase Haemoglobin

Fig. 15. Proteins are composed of few subunits

You can see some examples of complex proteins whose exact spatial structures are already known, so one can compare their size, shape and relative complexity (*fig. 16*). Let's just try to determine the importance of each of these proteins that you may not know.

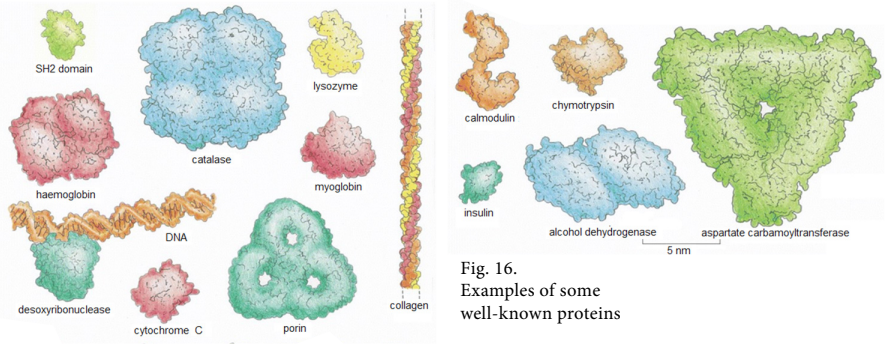


Fig. 16. Examples of some well-known proteins

SH2 domain is a part of protein kinase enzyme (we've got some information about it above) (*fig. 12*).

Lysozyme is an enzyme with strong antimicrobial properties, because it can destroy the peptidoglycan that is the main component of cell walls of some gram-positive pathogenic bacteria.

Catalase is an enzyme catalysing the reaction of hydrogen peroxide decomposition (with releasing H_2O and O_2) formed during biological oxidation processes. It also oxidizes low molecular alcohols and NO_2^- ions in the presence of H_2O_2.

Haemoglobin is the protein of red blood cells. It reversibly binds O_2 molecules in the lungs and transports them to the body tissues, and also transports CO_2 molecules in the opposite direction that is from the tissues to the lungs for CO_2 removing.

Myoglobin is a protein of the skeletal and heart muscles that binds and restrains O_2, creating some storage and releasing it in case of a temporary shortage.

Deoxyribonuclease is an enzyme that hydrolyses the phosphodiester bonds in the DNA molecule.

Collagen is a major component of connective tissue. Collagen gives it strength and elasticity.

Cytochrome C is an electron carrier in oxidative-restorative processes of cellular respiration. If necessary, it is able to activate cell ***apoptosis*** (programmed death).

Porin is the membrane protein that forms channel-like structures in the cell membrane through of which the various useful for its functioning molecules can transfer into the cell.

Calmodulin is one of the Ca^{2+} ions protein carriers. It does not have its own catalytic activity. As a subunit, it is a part of many enzymes, protein kinase in particular.

Chymotrypsin is an enzyme catalysing the reaction of peptide bond hydrolysis. It is most effective to cleave peptide bonds of aromatic amino acids such as tyrosine, tryptophan and phenylalanine.

Insulin is a hormone protein activating the enzymes that are responsible for the reducing concentration of glucose in the blood.

Alcohol dehydrogenase is an enzyme that catalyses the oxidation of alcohols and acetals to aldehydes and ketones respectively.

Aspartate carbamoyltransferase catalyses one of the reactions of pyrimidine nucleotides synthesis, which are basic components for DNA synthesis.

Proteins with Long Helical Fiber Structure

Some protein molecules are capable of linking together to form filament structures that can be located along an entire cell. Most simply, a long chain of identical protein molecules can be formed if each protein has at least two binding sites on different sides of the molecule that are complementary to each other as puzzle parts (*fig. 17*).

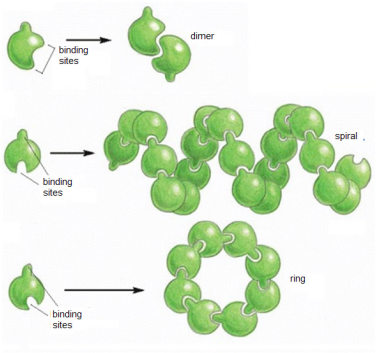


Fig. 17.
Formation of protein complexes through binding sites

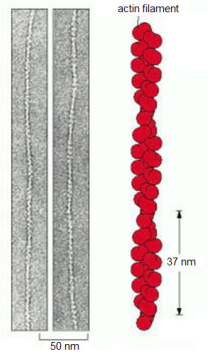


Fig. 18.
Building of actin filaments

Fig. 19.
Left- and right-handed protein helices

A good example is the actin filament (*fig. 18*). It has a long helical structure formed by many molecules of the protein actin that is one of the proteins of eukaryote cells performing a reinforcing function in the cytoskeleton.

Why is a helix such a common structure in nature?

As we can see, biological structures are often made by linking very similar elements (such as amino acids or proteins) forming long chains of repetitive elements. If all elements of the "constructor" are identical, the neighbouring structural elements (subunits) in the chain are linked in the way to minimize the free energy of the contact between them. As a result, each subunit is positioned in exactly the same way as the previous one along the entire chain in advance.

It is very rare for subunits to be joined up in a straight line, so mainly their arrangement results a helix. Depending on the twist direction, a helix can be either right-handed or left-handed (*fig. 19*).

All known protein α-helices are right-handed. A number of elements of a helix, which form a complete turn, can differ because this number depends on the "brick" structural features (dimensions, density of linking). Helices occur commonly in biological structures.

They can be formed ether by small molecules joined together by covalent bonds (such as the amino acids in the protein α-helix), or by larger protein molecules joined together by non-covalent bonds (such as actin molecules in actin filaments). Not surprisingly, that a helix is a typical structure formed by placing many similar units in the frequently repeated strict order.

Fibrous Proteins

The majority of proteins, which have already been studied, are ***globular*** (ball shaped) proteins in which the polypeptide chain folds up into a compact spherical structure with an irregular (very uneven) surface.

Enzymes tend to be globular proteins and have a ball shape, although they are sometimes very large and consist of few subunits (*fig. 16*). Some proteins, which have other functions and need to be in a constant motion within the living cell media, tend to have a simple elongated three-dimensional structure. They're called ***fibrous proteins***.

Alpha-keratin, or α-keratin, and similar to it, but less known collagen, belong to one of the large families of intracellular ***fibrous proteins***. The keratin fibrils are extremely stable. They are the main component in the "long-term" protein structures, which are form hair, horns and nails. An α-keratin molecule is a dimer of two identical subunits in which long α-helices are twisted together. This double helix ends are capped by globular domains containing a free binding site. Due to it, keratin assembles in large rope-like aggregates which are an important component of the cytoskeleton in cells giving them a particular shape and allowing to keep it together.

Being the main component of a jelly-like extracellular matrix that binds cells together to form tissues, fibrous proteins are most common outside of living cells.

Extracellular proteins are produced by cells and secreted into an extracellular biological medium, where they often assemble in sheet aggregates or long fibres. Collagen is the most common of these proteins in animal tissues. A collagen molecule consists of three long polypeptide chains, each of them contains the nonpolar amino acid glycine at every third position. This regular structure allows these chains to wind around each other, forming a long triple regular helix (*fig. 20*).

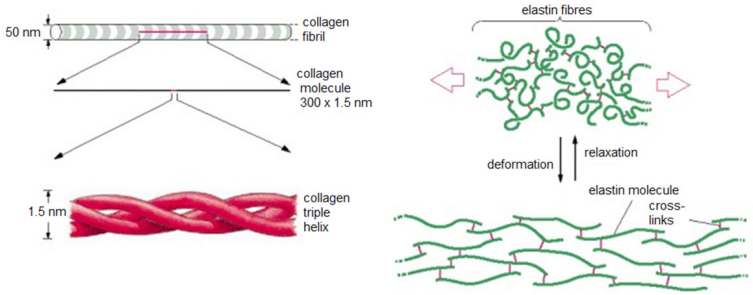


Fig. 20.
Examples of some fibrous proteins of extracellular matrix

A large number of collagen molecules then are bound side-by-side and end-to-end to form long and very strong structures. They are called ***collagen fibrils*** and responsible for the tissue tensile strength.

We'll learn more about another fibrillar protein in the extracellular matrix, elastin. Elastin molecules are formed from relatively unstructured polypeptide chains that are covalently linked in a rubber-like elastic net (*fig. 20*). Unlike most other proteins, the elastin chains have almost no definite structure, but they are able to interconvert one conformation to another one. These elastic fibres allow the skin and other tissues (such as arteries and lungs) to stretch and recoil without damage.

Chemical Structure Peculiarities of Extracellular Proteins

Most extracellular proteins are either attached to the outside of the plasma membrane of the cell or secreted (removed) in the extracellular media (matrix).

The extracellular matrix affects such proteins. To maintain the three-dimensional structure stability, the polypeptide chains of such proteins are often stabilized by covalent cross-linking. This linking can either join two amino acids in one polypeptide chain, or join the polypeptide chains of the separate protein subunits.

The covalent disulfide S–S bonds are the most common cross-linkages in extracellular proteins (*fig. 21*). They are formed when the protein can drift into the extracellular media. Disulfide bonds are formed when two side -SH groups of cysteine joining due to the reaction catalysed by a special enzyme in the cell's endoplasmic reticulum.

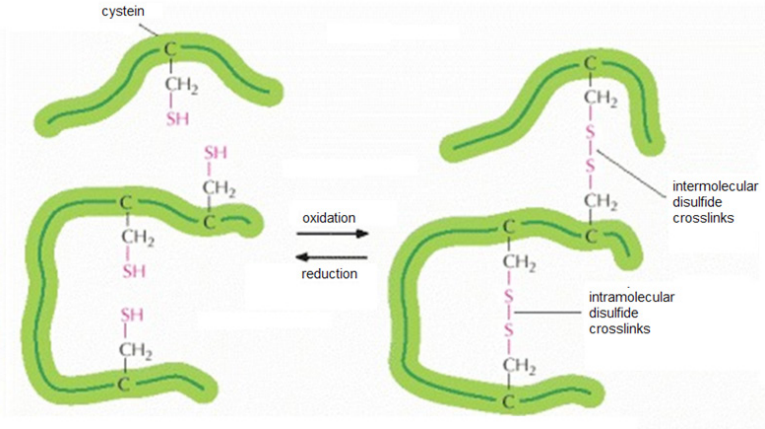


Fig. 21.
Principles of structure stabilization of extracellular proteins

Disulfide bonds do not change the protein molecule conformation, but strengthen its molecule three-dimensional structure. For example, lysozyme is the contained in tears enzyme that dissolves the bacteria cell wall. It can maintain long-term protective activity due to stabilization of the molecular structure by disulfide bonds. Disulfide bonds are usually not formed in cell cytosol. It is prevented by the high concentration of restorative substances that converts such S–S-bonds back to the thiol (–SH) groups.

The protein molecules are supposed not to require some additional spatial structure reinforcement inside their cells, where they are protected from the extracellular environment.

Functions of Proteins

Proteins perform many different functions, each of which is important not only for the proper cell functioning but the body as a whole.

Let's try to find out the main ***functions*** performing by a large number of different proteins that provide complex human life support.

1. ***Structural*** function. Some proteins are involved in forming of many cell components. Along with lipids, they are a part of cell membranes.

2. ***Catalytic*** function. Due to the structure, all biocatalytic enzymes are proteins.

3. ***Transportation*** function. Oxygen in various tissues is carried by haemoglobin and myoglobin containing in blood cells. Some other transport proteins form the complexes with lipids and carry them by blood and lymph.

4. ***Mechano-chemical*** function. The muscular work and other types of movement in the body are performed by special contractive proteins (actin, myosin) using the energy of chemical bonds accumulated in a body (ATP).

5. ***Regulatory*** function. Some hormones and other biologically active substances (insulin, ACTH) are proteins by their chemical nature.

6. ***Protective*** function. The antibodies (immunoglobulins) are proteins. Protein collagen is found in skin, keratin is found in hair. Skin and hair protect the body's internal environment from an external negative impact. Mucoproteins are found in mucous and synovial fluid (thick elastic mass in the joint cavity).

7. ***Supportive*** function. Tendons, joint surfaces, bone joints are mostly formed by proteins (collagen, elastin).

8. ***Energy*** function. Amino acids of proteins are involved in the glycolysis reaction providing the cells with energy.

9. ***Signal*** function. Many proteins are involved in some selective recognition processes, that is, as the result of an environmental variation (chemical, temperature, electrical, etc.). These proteins change their tertiary structure back, that can be detected as a signal to action by a cell.

Proteins as Building Blocks of Massive Supramolecular Structures

The same principles that enable some individual parts of a protein molecule to associate and form globular or fibrous structures are also applied for generating of much larger cell-like objects, supramolecular structures. These objects are enzyme complexes, ribosomes, protein fibrils, membranes and viruses. There are quite large objects compared to atoms and molecules. These objects are not made as a single structure of covalently linked molecules as well. They are formed by assembling of many different protein molecules, subunits, into a particular structure by means of intermolecular non-covalent bonds (the principle of ***self-organization***).

The use of smaller protein subunits for building such large structures has some advantages.

1. A large structure, which built from one or few smaller subunits, requires to store a small amount of genetic information.

2. A series of reversible processes can easily control both assembling and disassembling of this structure because the subunits are joined by low-energy linkages.

3. The errors in building large structure can be avoided using their own protective mechanisms, which remove defective subunits during assembling a complex molecule structure.

But how does the change in structure or the defects in subunits affect the final result? Look at *fig. 22*. Some protein subunits are assembled into the flat structures in which globules are arranged in the hexagonal unit cell. Specialized membrane proteins are sometimes arranged in this way in lipid layers. With a minor change in the geometry of some subunits, the planar structures, which are assembled from hexagonal elements, can convert into the tubular structures. With more changes, they convert into a hollow sphere.

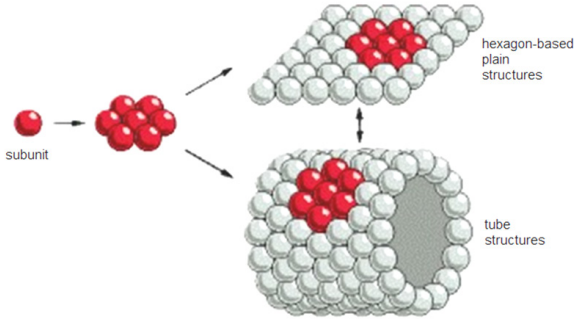


Fig. 22.
Proteins self-assembly
to supramolecular structures

What does such a shape look like? The tubular and spherical structures are able to wrap and bind specific RNA and DNA molecules and form the shells of well-known virus-like particles and viruses.

The formation of closed supramolecular structures, such as rings, tubes, and spheres, provides some additional stability of the spatial structure by increasing the number of bonds between protein subunits. Such structure is created by highly interdependent and cooperative interactions between subunits, it can be driven to assemble or disassemble by just a little change that can affect each individual subunit.

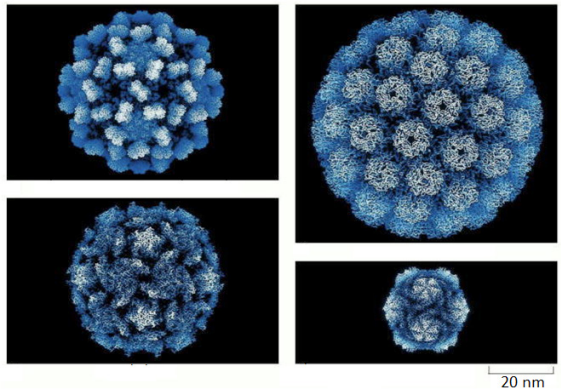


Fig. 23.
Visualization of different virus particles

These principles of molecular self-organization are vividly illustrated in the protein shell (capsid) of many common viruses taking the empty sphere form (*fig. 23*). The capsid is often composed of many identical protein subunits covering and protecting the viral nucleic acid (*fig. 24*). The proteins in such a capsid have a specifically adapted structure which provides:

- forming the different types of bonds for creating a spherical shell;
- enabling to release nucleic acid and other components of the virus particles for initiating replication of the virus (producing its own copies) as soon as the virus enters into the target cell.

In many viruses, identical protein subunits are assembled together to create a spherical shell that covers the native viral genome that consists of ether RNA or DNA. For geometric reasons, not more than 60 identical subunits must be assembled to make an accurate symmetric shell. If some little structural defects occur, a larger number of subunits can be used for a larger capsid formation.

For example, the tomato bushy stunt virus (TBSV) (*fig. 23*) has a spherical shape about 33 nm in diameter. The capsid is composed of 180 identical protein subunits, each containing 386 amino acids. Genome of this virus particle consist of RNA molecule that built from 4,500 nucleotides.

In order to construct such a large capsid, each protein must be structured in the way to provide its integrating into appropriate part of the capsid surface layer (in *fig. 24*, three forms of the subunit are specified by the colour).

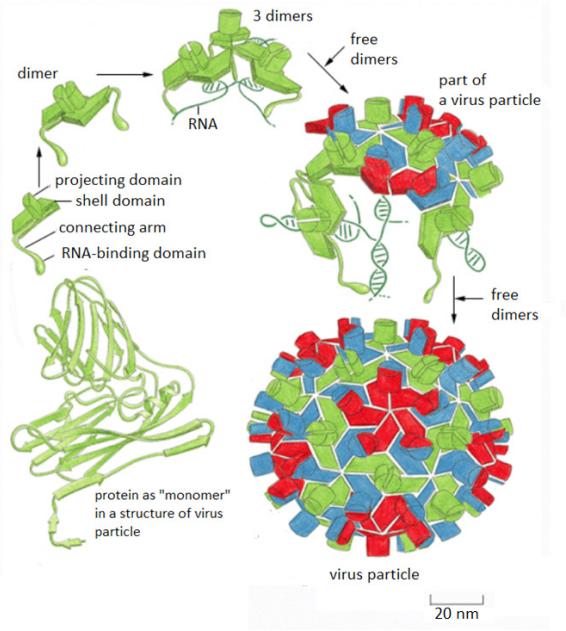


Fig. 24.
Composing typical virus particle from protein subunits

Lastly, we will discuss the structure of the infamous SARS-CoV2 virus that caused the outbreak global pandemic of coronavirus disease 2019 (COVID-19) in late 2019. The structure of this virus is composed of lots of different proteins. Each of them has a specific function and provides a high efficiency of entering the cell and its reproduction. In *fig. 25* you can see the images of a SARS-CoV2 virus taken with an electron microscope (on the left), a structure scheme of virus particle (on the right) and an image of the viruses entering through the cell membrane.

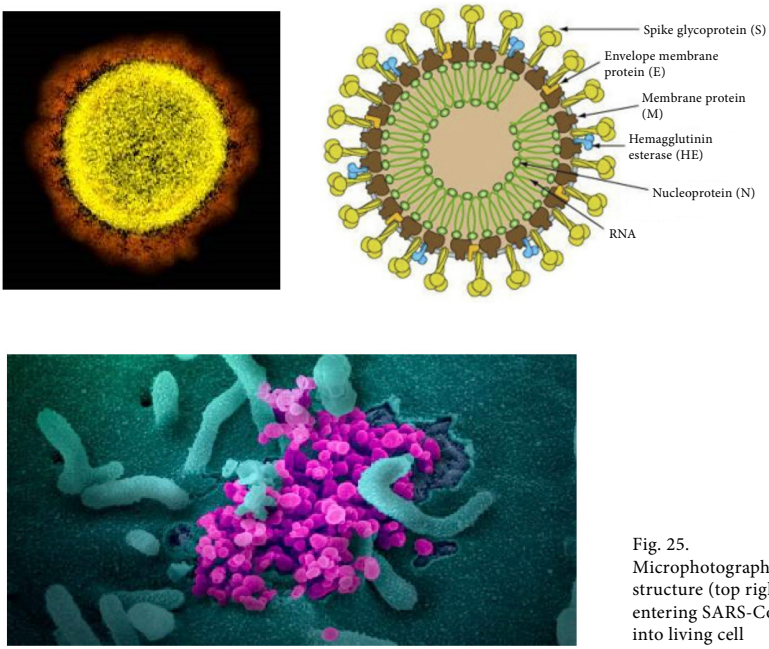


Fig. 25.
Microphotograph (top left), structure (top right) and entering SARS-CoV2 virus into living cell

What proteins does SARS-CoV2 consist of?

Glycoprotein S is a trimer by its structure. It consists of three polypeptide chains and has fungi-like shape. These complex proteins are arranged on the surface of a virus particle. Glycoprotein S is responsible for binding the virus to a target cell.

The ***membrane protein M*** consists of three transmembrane domains and is responsible for shape and curvature preserving of the viral particle surface. This protein supports the internal virus structure by non-covalent bonds.

Membrane protein E, which is little enough, also has a structure forming function in the capsid and participates in moving into the target cell through its membrane.

Nucleoprotein N consists of two domains both of which are linked to the RNA virus. Both ***nucleoprotein N*** and ***membrane protein M*** act together to form the spatial structure of the virus internal part.

Thus, those quite small virus particles are made of many protein-based molecules. Their chemical structure provides a unique way of their assembling and functioning. Due to it, this created by nature and invisible by non-equipped eye assembles becomes unique. The proteins have got a well-deserved name, because

they have a primary function of maintaining the existence of life. Nowadays we know almost everything about their chemical composition, features of molecular structure and supramolecular arrangement, chemical properties and functions. But it is still impossible to produce any of well-known proteins using convenient chemistry methods, which have already been known for many years.

And it once again confirms the unattainable level of nature development and the uniqueness of the objects it has created!

To compile the Chapter the theoretical materials and graphical data from follow sources were used

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ISOLATION, IDENTIFICATION AND CHEMICAL PROPERTIES OF PROTEINS

Experiment 1

Proteins from Animal or Vegetable Sources Methods of Isolation and Preparation for Testing

Basic notes!

Protein solutions are unstable and can be stored for 2–3 days. Meat protein solutions are the most unstable.

A 1 drop of toluene could be added to protein solution to extent their stability for additional 1–2 days.

Myosin (globulin of meat) and casein solutions will be obtained in concentrated state, while proteins of vegetable species and water-soluble milk proteins will be prepared in highly diluted state.

For some studies, alkaline protein solutions should be neutralized to obtain the opaque or weakly acidic solution.

Part A. Isolation of chicken egg albumin

Materials and apparatus:		***Reagents:***
Conical flask 500 mL with stopper	1	Chicken egg
Conical flask 200 ml	1	Distilled water
Beaker 50/100 ml	2	
Filtration funnel	1	
Peace of textile 10 × 10 cm		

Procedure:

1. Carefully separate white egg from yolk.
2. Obtained white egg (~ 25 ml) put into a 500 ml conical flask, add 100 ml of distilled, close with stopper and intensively shake.
3. Filter the mixture through textile-covered funnel.

4. Obtained slightly opaque transparent solution contains mainly albumin with small quantity of globulin. The main part of globulin as well as clots and films will remain on textile filter.
5. Filtered solution of egg albumin could be used for testing the properties of protein (see below).

Part B. Proteins of meat

Experiment B1. Isolation of meat albumin

Materials and apparatus:

Conical flask 100 ml	1
Beaker 100 ml	1
Filtration funnel	1
Glass rod	1
Filtration paper	

Reagents:

Minced fresh lean meat
Distilled water

Procedure:

1. Weight in a beaker appox. 25 g of minced meat.
2. Add 50 ml of distilled water.
3. Leave the mixture for 20–30 min, mix sometimes with glass rod.
4. Filter the mixture through paper filter.
5. Obtained solution contains meat albumin with small quantity of globulin. Blood pigments dyes the filtrate in red color. The presence of blood pigments gives the filtrate a red color, which will not interfere with further experiments.

Experiment B2. Isolation of meat globulin

Materials and apparatus:

Conical flask 100 ml	1
Beaker 100 ml	1
Filtration funnel	1
Glass rod	
Filtration paper	

Reagents:

Albumin-free meat from Exp. B1
Ammonium chloride, NH_4Cl, 15% (by weight) aqueous solution
Distilled water

Procedure:

1. Filtered minced meat from Exp. B1 wash 2–3 times with distilled water by portions of 10–15 ml and press to separate from excess of water.
2. Obtained whitened meat fibers transfer to a beaker and add 60–70 ml of 15% NH_4Cl solution.
3. Leave a mixture for 30–40 min, mix sometimes with glass rod.

4. Filter a mixture through dry folded filter (process of filtration occurs quite slowly).
5. The filtrate contains meat globulin – myosin. A myosin solution uses for follow experiments.

> ***Note! Do not dilute myosin solution with water because it initiates precipitation of the protein!***

Experiment B3. Gelatin

Materials and apparatus:		*Reagents:*
Conical flask 100 ml	1	Pork skin
Beaker 100 ml	1	Sulfuric acid, H_2SO_4, 0.05 M
Filtration funnel	1	Sodium hydroxide, NaOH, 0.1 M
Scalpel		Distilled water
Glass rod		
Filtration paper		

Procedure:

1. Weight in a beaker 15–20 g of cutted pork skin.
2. Add to a beaker 5 ml 0.05 M H_2SO_4 and 40 ml of distilled water. Boil the mixture for 4 hrs.
3. Monitor the boiling process and every 20 min add appropriate quantity of a water to maintain a level of the mixture at initial point.
4. To filter the hot mixture using folded paper filter.
5. Adjust pH of obtained gelatin solution to 5–7 by slow addition (dropwisely) of 0.1 M NaOH. Control the pH with universal indicator strips or pH-meter.

Part C. Milk proteins

Experiment C1. Milk albumin

Materials and apparatus:		*Reagents:*
Conical flask 100 ml	1	Fresh milk
Beaker 200 ml	1	Ammonium sulfate, $(NH_4)_2SO_4$,
Filtration funnel	1	saturated solution
Glass rod		Distilled water
Filtration paper		

Procedure:

1. Mix 50 ml of fresh milk with 50 ml of saturated $(NH_4)_2SO_4$ solution.
2. After 10 min filter the mixture through folded paper filter.

3. Non-soluble proteins, like casein and globulin, with fat drops remain on a paper filter.
4. Transparent milk albumin solution getting through the filer could be used for follow experiments.

Experiment C2. Casein

Materials and apparatus:

Conical flask 100 ml	2
Mortar with pestle	1
Filtration funnel	1
Pasteur pipette	3
Balances	
Glass rods	
Filtration paper	

Reagents:

Fresh milk or sour milk cheese
Sodium hydroxide, NaOH, 5 M
Distilled water

Procedure:

1. Non-filtered precipitate (see Experiment C1) consists of mainly casein and a fat. To obtain a casein solution the precipitate from the filter paper shoul be transferred to a mortar (or weight 5–6 g of cheese and grind it with 10–15 ml of water).
2. Add to the mortar 1.5 ml of 5 M NaOH solution and additionally grind the mixture adding 15 ml of water by small portions.
3. To filter the mixture through wet folded paper filter.
4. The first portions of turbid filtrate turn back to a filter. This approach allows to remain fine fat drops on a paper filter.
5. Obtained transparent supernatant is an alkaline solution of casein.

Experiment C3. A mixture of milk albumin and globulin

Materials and apparatus:

Conical flask 100 ml	2
Beaker 200 ml	1
Filtration funnel	1
Pasteur pipette	3
Glass rods	
Peace of textile 10 × 10 cm	

Reagents:

Fresh milk
Acetic acid, CH_3COOH, conc.
Distilled water

Procedure:

1. Measure 50 ml of fresh milk and mix with 50 ml of distilled water.

2. To the mixture add dropwisely 0.2–0.5 ml of concentrated CH_3COOH till the clots form.
3. After 10 min filter the mixture through a peace of textile (*see Part A. Isolation of chicken egg albumin*).
4. Filtration process occurs quite fast. The first portions of turbid filtrate require extra filtration through the same textile filter.
5. Obtained transparent albumin solution contains a small quantity of globulin and milk carbohydrate lactose could be used for follow experiments.

Part D. Isolation of plant albumin leucosin

Materials and apparatus:		***Reagents:***
Conical flask 100 ml	1	Wheat flour
Beaker 200 ml	1	Distilled water
Filtration funnel	1	
Glass rods		
Filtration paper		

Procedure:

1. In a beaker mix 25 g of wheat flour and 100 ml of water.
2. Stir the mixture occasionally with glass rod for next 30–40 min.
3. Filter the mixture through paper filter. A first portions of tubbid filtrate should be filtered again through the same paper filter.
4. Filtration process occurs quite fast. Obtained transparent solution contains protein of wheat seeds – leucosin.

Part E. Extraction of a mixture of plant proteins

Materials and apparatus:		***Reagents:***
Conical flask 100 ml	1	Dried plants
Filtration funnel	1	Sodium hydroxide, NaOH, 0.1 M
Centrifuge tubes 15 ml		Acetic acid, CH_3COOH, 0.5 M
Mortar with pestle	1	Distilled water
Pasteur pipette	3	
Glass rods		
Filtration paper		
Centrifuge		

Procedure:

1. Weight 3 g of dry plants and grind it in a mortar with 30 ml of 0.1 M NaOH solution.

2. Remain the mixture in a mortar and occasionally grind it for next 2 hrs.
3. Filter the mixture through folded paper filter and precipitate the proteins by addition of CH_3COOH solution till pH adjust to 4.0–4.5.
4. To wash the precipitate twice with diluted CH_3COOH (pH = 4.0) and centrifuge at 3000 min^{-1} for 15 min.
5. For the next experiments a mixture of proteins could be dissolved in a water that contains few drops of NaOH solution.

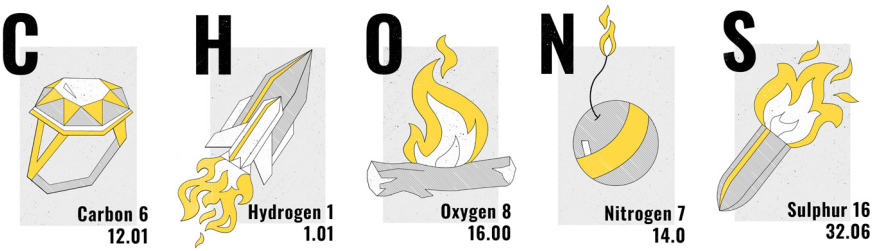
Discussion:

1. Could you explain what chemical characteristic of protein molecules requires using distilled water to extract them?
2. What is the purpose of holding up some time before further processing a mixture of natural raw materials and distilled water, when proteins are extracted? Does it apply to the protein molecule structure?

Experiment 2

Elemental Analysis of Proteins

Protein molecules consist mainly of five basic chemical elements:



- Carbon, C, its mass fraction is about 50%;
- Hydrogen, H, its content is about 7%;
- Oxygen, O, its content does not exceed 23%;
- Nitrogen, N, its content is ~16%;
- Sulphur, S, its mass fraction can range from 0 to 3%.

The simplest way to verify the qualitative elemental composition of a protein sample is pyrolysis and following identification of destruction products. When a protein sample is heated and the air access is limited,

some specific chemical transformations occur. In these circumstances, the main products of the destruction of protein molecules formed by amino acid residues are:

- ammonia NH_3, which indicates the nitrogen and hydrogen in the chemical structure of proteins;
- water, H_2O as the proof of a presence of oxygen and hydrogen in protein structure;
- char coal, C as the result of high carbon content in protein backbones;
- hydrogen sulphide, H_2S demonstrates sulphur and hydrogen in protein composition.

As a rule, high-precision analytical techniques, which require complex laboratory apparatus and experience for conducting the analysis, are typically used for the quantitative element analysis. However, the qualitative determination of protein composition can also be realized via some chemical transformations that anyone can perform.

Materials and apparatus:	***Reagents:***
Test tubes	Protein sample
Test tube holder	Cobalt (II) chloride, $CoCl_2$, 10% aqueous solution
Plastic pipettes	Lead (II) acetate, $Pb(CH_3COO)_2$, 5% aqueous solution
Gas burner or spirit lamp	Universal indicator paper
	Distilled water

Procedure:

Note! The experiments require appropriate quantity of protein (approx. 1 g).

1. A sample of protein (0.5–1 g) puts into dry test tube and heats carefully by a gas burner or spirit lamp.
2. After a while, it can be seen a clear changes in a protein sample due to degradation process and elimination of some resulting products.
3. Products of protein degradation and element composition of protein sample could be identified using *Table 2*.

Table 2

Chemical elements	***The product identified***	***Identification approach***	***Observations***
N, H	NH_3	Wet universal indicator paper	Color change: Orange → green to blue
			Write your observation:

Chemical elements	*The product identified*	*Identification approach*	*Observations*
O, H	H_2O	Visual	Drops of colorless liquid on cold part of tube
			Write your observation:
		Water test paper prepared by soaking paper in a solution of $CoCl_2$ followed by drying	Color change: Blue → pink
			Write your observation:
S, H	H_2S	Lead test paper prepared by soaking paper in a solution of $Pb(CH_3COO)_2$	Color change: white → grey to black
			Write your observation
C	Carbon	Visual	Black colored bulk char coal at a bottom of test tube
			Write your observation:

Discussion:

1. Can you explain why heating is the simplest and direct way for determination of element composition chemical in measuring the total protein quickly enough?
2. Can we determine the individual compounds in a mixture of thermally decomposing proteins, which are the identifiers for the elemental composition of protein molecules, without using chemicals, but only with the help of our senses? Could you explain how does it happen in details?

Experiment 3

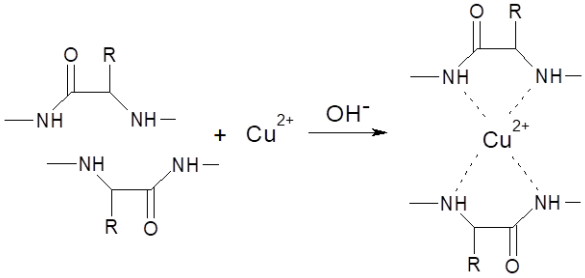
Chemical Methods for Identification of Proteins

All proteins contain large in size molecules which have a complex chemical structure. The presence in their structure such characteristic functional groups as carboxyl (–COOH), amine (–NH_2 or >NH), amide or peptide (–NH–CO–) and other, as well as larger functional fragments (aromatic rings, –C_6H_5, indole bicyclic structure fragments, etc.) gives containing them proteins some special chemical properties. By means of chemical reactions, so-called chemical identification reactions, in which these functional groups and structure fragments react, it is

possible not only to identify proteins in the test samples, but also to determine the peculiarities of chemical structure.

Biuret Test

The biuret test is a common way to identify any proteins. It's got its name from the product of the condensation of urea, a biuret $H_2NCONHCONH_2$, whose amino-groups form a complex compound of bright purple or violet colour with Cu^{2+} cations. Such a purple or violet complex is formed by Cu^{2+} cations with compounds[2] having peptide –NH–CO– functionality in an alkaline environment in accordance with scheme:



Only polypeptides with at least two neighboured peptide bonds in a molecule are capable to form the complex. Free amino acids and their dimers (dipeptides, that contain one peptide bond only) do not react in this way with the biuret test solution. The colour intensity is proportional to the number of peptide bonds involved in the complex formation. Therefore, this reaction is also used to quantify the protein content in the solution by colorimetric analysis.

Materials and apparatus:

Test tubes
Plastic pipettes

Reagents:

Protein solutions
Amino acid solution
Sodium hydroxide, NaOH, 3 M
Copper (II) sulfate, $CuSO_4$, 0.1 M
Distilled water

Procedure:
Slight coloration of protein solution do not prohibit conducting the experiment.

[2] There are few other compounds that also give a similar reaction with Cu^{2+} ions, such as oxamide or oxalatic acid diamide, $H_2NCOCONH_2$.

1. Three test tubes fill with 1 ml of amino acid solution (test tube #1), known protein solution (test tube #2) and unknown solution (test tube #3).
2. Add 1–2 ml of 3 M NaOH to the each of test tubes.
3. Swirl the test tubes content and add 1 drop of 0.1 M $CuSO_4$ to the each test tube. Observe color changes in test tubes.
4. In a case of quite low protein content (plant protein or milk albumin solutions) the experiment procedure should be changed. In a test tube you must mix 2 ml of unknown protein solution and an equal volume of 3 M NaOH. You must tilt the test tube and add very carefully with a pipette 0.5–1 ml of 0.1 M $CuSO_4$. In a test tube, $CuSO_4$ must form an upper layer that do not mix with another liquid. The purple ring appears at the interface (between two layers).
5. Fill *Table 3* with the results of experiment.

Table 3

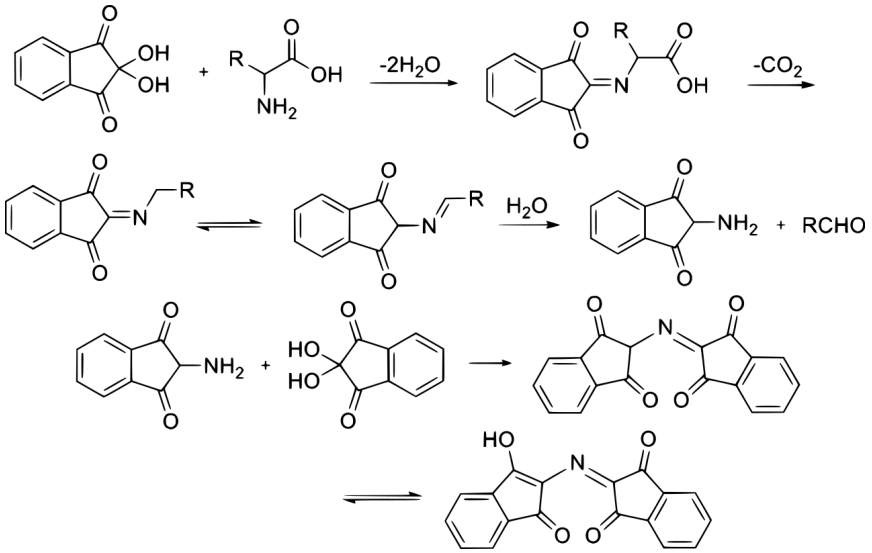
Test tube	***Amino acid or protein sample***	***Volume of 3 M NaOH added***	***Volume of 0.1 M CuSO4 added***	***Observations***
1				
2				
3				
4				

Discussion:

1. What chemical feature of protein molecules causes purple coloration after adding copper salt to the protein solution in alkaline environment? Could the colour-change reaction be observed with other substances that are not proteins?
2. Why the Biuret test can be used for both qualitative and quantitative protein analysis?

Ninhydrin Test

Ninhydrin or 2,2-dihydroxyindane-1,3-dione is an organic compound that is classified as ketone, alcohol and condensed carbocyclic compounds and has strong oxidative properties. It reacts actively with primary and secondary amines, amino acids, proteins, peptides and ammonia, forming brightly coloured compounds. The example of the reaction of coloured compounds forming is given below.



Ninhydrin decomposes the amino acid to aldehyde, ammonia, and CO_2 during a series of reactions. As a result, ninhydrin is partially transforms to reduced form and reacts with another ninhydrin molecule results in formation of intensively coloured dye. Ammonia, primary amines, amino acids and proteins form the different purple-coloured products. These compounds are known as "Ruhemann purple" dye. They were discovered in the early 20th century and named after their discoverer. The reaction was first paid attention to after the hand skin being stained with ninhydrin.

Secondary amines and amino acids, which have secondary amino-groups (proline, hydroxyproline), interact with ninhydrin to form yellow-coloured products.

The reaction of proteins with ninhydrin is often used in forensic investigation technique to identify fingerprints and sweat on different surfaces (especially porous ones).

Materials and apparatus:

Test tubes
Plastic pipettes
Water bath
Electric heater

Reagents:

Protein solutions
Ninhydrin, 0.2% ethanol solution
Distilled water

Procedure:

1. A test tube fill with 1 ml of protein solution and add 5 drops of ninhydrin solution.
2. Intensively swirl the test tube content and put into boiled water bath for 2 min.
3. Dyeing the mixture to violet color demonstrate a presence of ***α***-amino acids or proteins in a solution.
4. Fill *Table 4* with the results of observations.

Table 4

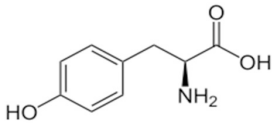
Test tube	***Protein sample***	***Volume of ninhydrin solution added***	***Observations***
1			
2			
3			
4			

Discussion:

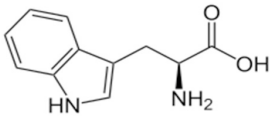
1. What chemical properties of ninhydrin allow it to be used to identify proteins, amino acids and other amine-containing compounds?
2. Why does ninhydrin dye the skin after getting on it? How is this characteristic used for practical purposes?

Xanthoproteic Test

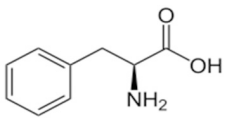
Xanthoproteic reaction (from a Greek word *xanthos* – yellow) is used to identify free aromatic amino acids and aromatic amino acids containing proteins. Chemically, amino acids containing aromatic structures (phenyl group, $-C_6H_5$) are considered to be benzene derivatives:



tyrosine

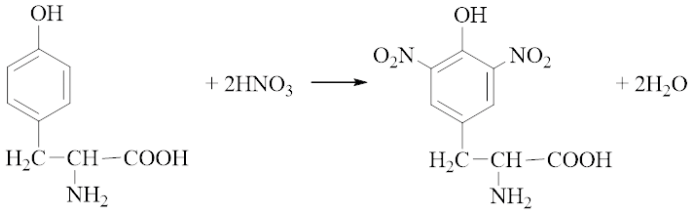


tryptophan

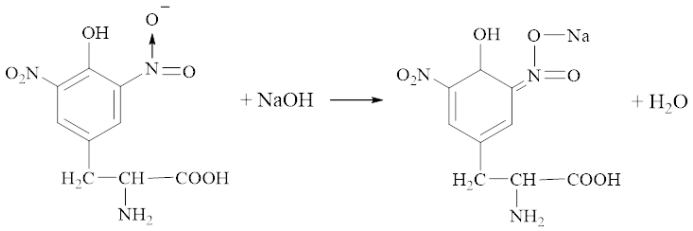


phenylalanine

So, such amino acids have some characteristics of aromatic hydrocarbons. For example, the interaction of benzene derivatives with nitric acid result in producing intensively yellow-coloured nitro compounds:



Characteristic change of colour to deep orange in the alkali medium is due to the formation of anionic form of nitro compounds:



These schemes reflect the pathway of the xanthoproteic reaction. Tyrosine and tryptophan are amino acids with aromatic fragments in the molecule that can easily be nitrated under "mild" conditions.

Phenylalanine is another aromatic amino acid. Its molecule also contains a benzene ring, but phenylalanine is not reacting with nitric acid under ordinary conditions.

What is your idea about the reason for this unusual behaviour?

The fact of the matter is that the aromatic ring of tyrosine and tryptophan are activated by -OH and -NH groups. These groups are directly attached to the benzene ring (*see above*). The aromatic ring gets an increased reactivity in many reactions (including nitration). Quite the opposite, the phenylalanine aromatic ring does not contain active substituents, so it is nitrated under quite hard conditions that are not allowed with protein sample. Therefore, nitration of phenylalanine or its fragments in proteins is impossible at xanthoproteic reaction conditions.

To conclude, proteins that do not contain tyrosine or tryptophan, like gelatin, do not give a positive xanthoproteic test reaction.

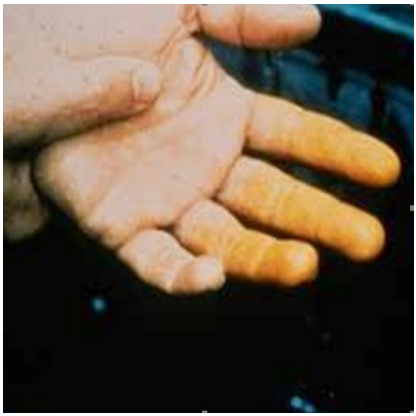


Fig. 26.
Xanthoproteic reaction
at chemical injury
of skin by nitric acid

When concentrated HNO_3 gets on the skin the products of the xanthoproteic reaction appears as yellow stains on the skin (*fig. 26*). Skin proteins contain tyrosine and tryptophan amino acids, which can easily be nitrated, result in corresponding yellow-coloured proteins of skin and products of their degradation.

It should be kept in mind that the changing of skin colour is not the only result of getting some concentrated nitric acid on the skin. This acid can cause severe injuries. It destroys proteins and other biomolecules, living tissues and skin layers, which is our body's highly efficient protective system. Therefore, acids should be handled carefully using all necessary protective equipment and accessories.

Materials and apparatus:

Test tubes
Plastic pipettes
Water bath
Electric heater

Reagents:

Protein solutions
Nitric acid, HNO_3, conc.
Sodium hydroxide, NaOH, 3 M
Distilled water

Procedure:

The experiment should be conducted in fume hood only!

1. Fill a test tube with 1 ml of protein solution and add 0.3 ml (8–9 drops) concentrated HNO_3. The mixture becomes turbid or precipitate appears due to denaturation of protein molecules.
2. The mixture boils carefully for 2 min till the precipitated protein dye into deep yellow colour. Hydrolysis of proteins could be also observed that resulted in partial dissolution of the precipitate without losses in colour intensity of the mixture. An appearance of yellow colour is due to presence of nitro substituted peptides and amino acids.
3. When the mixture cools down add excess of NaOH solution (1.5–2 ml) into the test tube. Interaction of alkali with nitro compounds results in producing deep orange-coloured anionic nitro compounds.
4. All the results of experiment should be generalized in *Table 5*.

Table 5

Test tube	***Protein sample***	***Volume of HNO_3 added***	***Volume of 3 M NaOH added***	***Observations***
1				
2				
3				
4				

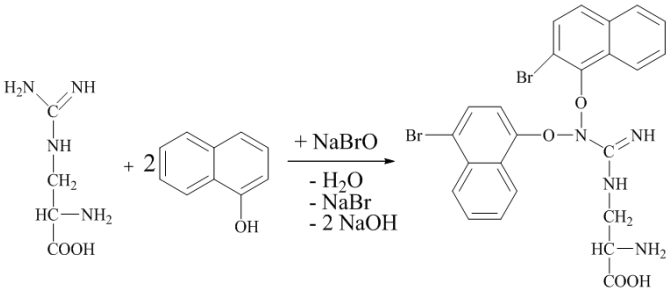
Discussion:

1. Could you explain the chemical nature of the xanthoproteic reaction? Where is it used in industry? What types of products can be produced using this acid?
2. What reaction can occur during the xanthoproteic test without causing discoloration or changing in colour intensity?

Sakaguchi Test

This reaction is specific for the guanidine -NH-C(=NH)-NH_2 group and uses for their determination in organic compounds[3]. Arginine is unique α-amino acid containing in own structure guanidine group. Therefore, the positive Sakaguchi test indicates the presence of arginine or its residue in the unknown sample.

According to reaction pathway free arginine or arginine residue in the protein react with α-naphthol in a presence of oxidant, e.g. sodium hypochlorite, NaClO or sodium hypobromite NaBrO in alkaline medium to form a red-colored compound.



Materials and apparatus:

Test tubes
Plastic pipettes

Reagents:

Protein solutions
Sodium hydroxide, NaOH, 10% aqueous solution
α-Naphthol, 1% ethanol solution
Sodium hypochlorite, NaClO, or
sodium hypobromite, NaBrO, 3% aqueous solution
Distilled water

Procedure:

1. Fill a test tube with 1 ml of unknown protein sample.
2. Add 0.5 ml of NaOH solution and 0.5 ml α-naphthol solution.
3. Swirl the mixture for 1 min.
4. Add 0.3 ml NaClO (or NaBrO) solution to the mixture.
5. Dyeing the mixture in red color is an evidence of presence of free arginine or its residues in a structure of protein sample.
6. Fill *Table 6* with results of the experiment.

[3] Guanidine substituents are used for producing the polymers, like ion-exchange resins, cosmetic goods, solid propellants, explosives, antifungal additives, fire retardants for treatment of textiles and other materials.

Table 6

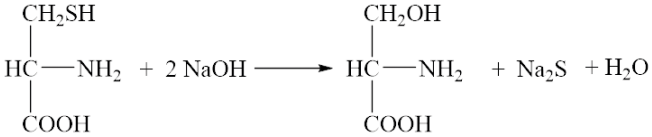
Test tube	*Protein sample*	*Volume of NaOH added*	*Volume of α-naphthol added*	*Volume of NaClO or NaBrO added*	*Observations*
1					
2					
3					
4					

Discussion:

1. Why is the Sakaguchi test is a specific reaction for arginine containing proteins?
2. Can this reaction be used to analyse other organic compounds except arginine and its structure fragments? What are they?

Sulfur Test

Sulfur test is a qualitative method of protein analysis for detecting sulfur-containing amino acids in the protein. Boiling the sample in alkaline medium provides the hydrolysis of protein molecules to form sulfide ions and other side products. Under alkaline condition, sulfur-containing amino acid, like cysteine, transforms into serine and Na_2S eliminates in accordance the scheme below:



Then, sodium sulfide reacts with lead (II) acetate, $Pb(CH_3COO)_2$ to form the black precipitate of lead sulphide, PbS:

$$Pb(CH_3COO)_2 + 4\,NaOH \longrightarrow Na_2[Pb(OH)_4] + 2\,CH_3COONa$$

$$Na_2S + Na_2[Pb(OH)_4] \longrightarrow PbS\downarrow + 4\,NaOH$$

Materials and apparatus:	***Reagents:***
Test tubes	Protein solutions
Plastic pipettes	Sodium hydroxide, NaOH, 10% aqueous solution
Water bath	Lead (II) acetate, $Pb(CH_3COO)_2$, 5% aqueous solution
Electric heater	Distilled water

Procedure:

1. Fill a test tube with 1 ml of unknown protein sample and add 2 ml of 10% NaOH solution. Boil carefully the mixture in the test tube for 3 min.
2. Cool down the mixture to ambient conditions and add 1 drop of $Pb(CH_3COO)_2$ solution.
3. An appearance dark grey or black color is the evidence of free sulfur containing amino acids or its residues in the structure of proteins.
4. Fill *Table 7* with results of the experiment.

Table 7

Test tube	***Protein sample***	***Volume of NaOH added***	***Volume of $Pb(CH_3COO)_2$ added***	***Observations***
1				
2				
3				
4				

Discussion:

1. To identify a resence of sulfur by the Sulfur Test, the solution of lead salt is typically used after partial degradation of proteins in alkali. What other reagents instead of Pb^{2+} can be used for qualitative identification of sulfur in protein samples?
2. Why is Pb^{2+} salts considered to be one of the best reagents for Sulfur Test reaction? Why are Cu^{2+} or Zn^{2+} not used?

Acree-Rosenheim Test

This reaction is specific for indole groups, which contain only one amino acid, tryptophan. For testing, the proteins which contain tryptophan residues should be degraded via hydrolysis process using a strong acid (e.g. sulfuric acid, H_2SO_4) to mixture of free amino acids. Interaction free tryptophan with formaldehyde, HCHO produces purple coloured condensation products. Thus, this reaction makes it possible to determine the presence of tryptophan residues in protein molecules.

Materials and apparatus:

Test tubes
Plastic pipettes
Water bath
Electric heater

Reagents:

Protein solutions
Formaldehyde, HCHO, 35% aqueous solution
Iron (III) chloride, $FeCl_3$, 2% aqueous solution
Sulfuric acid, H_2SO_4 conc.
Distilled water

Procedure:

1. Fill a test tube with 0.5 ml of unknown protein solution.
2. Add 2 drops of formaldehyde solution and 2 drops of $FeCl_3$ solution into test tube.
3. Swirl the test tube content.
4. Add 5 drops of concentrated H_2SO_4 carefully on the wall of the test tube to form top and bottom layers in the liquid mixture.
5. In a presence of tryptophan in the protein sample a violet or purple ring appears.
6. Fill *Table 8* with results of the experiment.

Table 8

Test tube	*Protein sample*	*Volume of HCHO added*	*Volume of $FeCl_3$ added*	*Volume of H_2SO_4 added*	*Observations*
1					
2					

Test tube	*Protein sample*	*Volume of HCHO added*	*Volume of $FeCl_3$ added*	*Volume of H_2SO_4 added*	*Observations*
3					
4					

Discussion:

1. Can the Acree-Rosenheim reaction identify other tryptophan-like compounds, such as indole in jasmine oil or indole-3-acetic acid in plant hormone?
2. Could you explain the role of sulfuric acid in the Acree-Rosenheim test and most other reactions in which aldehydes take part?

Experiment 4

Chemical Properties of Proteins

Acid-Base Properties

By nature, proteins are amphoteric polymer electrolytes and exist in the solution as colloidal particles. Their base properties are caused by a presence of amine ($-NH_2$ та >NH) functionalities and their acid properties are caused by a presence of carboxylic (–COOH) groups.

$$RNH_2 + H_2O \rightleftharpoons RNH_3^+ + OH^- \qquad RCOOH \rightleftharpoons RCOO^- + H^+$$

For most proteins, their dissociation constant (K_d) as an acid is slightly higher than the K_d of the same protein as the base. As a result, protein molecules in an aqueous solution tend to have a negative charge.

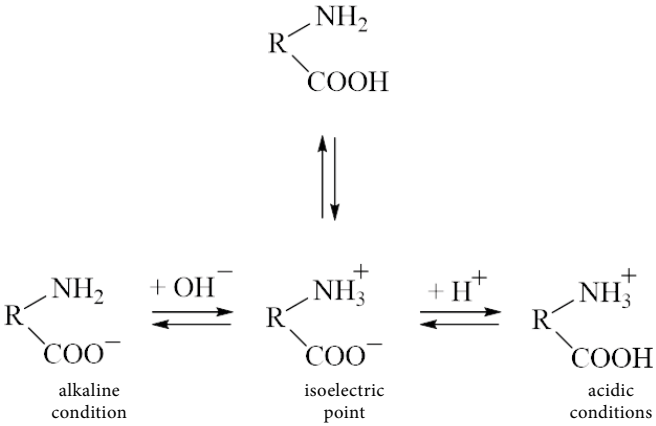
If an acid is added gradually to this protein solution, a dissociation of protein -COOH groups is supressed by excess of H^+. However, dissociation of proteins as bases due to its changing to salt form increases. So, the negative charge of the protein molecules decreases and finally disappears completely.

The colloid particles of protein molecules in the solution lose a charge and becomes less stable. As a result, protein molecules coagulate easily, so they coalesce and form large aggregates and even precipitate.

The pH value causing the coagulation of molecules is characteristic of each protein and it called an ***isoelectric point***.

Addition the excess acid (further reduction of pH value below the isoelectric point) increases proteins dissociation as the bases. The protein molecules get a positive charge and the colloidal solutions of proteins stabilize again.

The stability of negatively charged protein molecules increases in the alkaline condition.



Interaction of Proteins with Alkalis and Acids

Materials and apparatus:	***Reagents:***
Test tubes	Protein solutions
Plastic pipettes	Acetic acid, CH_3COOH, conc.
Water bath	Sodium hydroxide, NaOH, 0.1 M
Electric heater	Ammonium sulfate, $(NH_4)_2SO_4$, 15% aqueous solution
	Distilled water

Procedure:

1. Two test tubes (#1 and #2) fill with 3 ml of protein solution.
2. Test tube #1 heat to a boil. Most of proteins at such hard conditions denature and a precipitate forms.

3. To the test tube #2 add dropwisely concentrated CH_3COOH at continuous swirling. During acid addition a solution became turbid or precipitate appears (except a gelatin). Extra volumes of acid initiates dissolution of precipitated protein aggregates.
4. Obtained acidic transparent solution separate to 2 other test tubes #3 and # 4).
5. The test tube #3 heats to boil. Do you observe precipitation of protein molecules? Explain the main reason.
6. To the test tube #4 add dropwisely NaOH solution till alkaline medium. What changes do you observe? Write your observations below.
7. When transparent protein solution at high pH forms the test tube #4 boils for 1 min. What changes do you observe?

Protein sample _____

Addition of conc. CH_3COOH _____

_____

Boiling acidic solution _____

_____

Addition NaOH till alkaline medium _____

_____

Boiling acidic solution _____

Discussion:

1. What is the structure peculiarity of protein molecules make them dissolve in a water?
2. What conditions cause proteins either to dissolve in a water or become insoluble?

Buffer Properties of Proteins

Due to amphoteric properties, proteins in solution exhibit their buffering properties, i.e. they can bind both excess of H^+ ions, reducing the acidity of medium and excess of OH^- ions, reducing the alkalinity of the solution.

Materials and apparatus:	*Reagents:*
Test tubes	Natural protein solutions (prepared without addition of acids or bases)
Plastic pipettes	Hydrochloric acid, HCl, 0.1 M
Water bath	Sodium hydroxide, NaOH, 0.1 M
Electric heater	Universal indicator
	Phenolphthalein
	Distilled water

Procedure:

1. Fill a test tube with 3 ml of distilled water and add 1 drop of HCl solution.
2. In another test tube dissolve 1 drop of freshly prepared acid solution in 2 ml of distilled water
3. Add 1 drop of universal indicator to the las solution and observe pink color due to weak acidity of highly dissolved HCl solution.
4. Add slowly 2 ml of protein solution by few portions to pink colored acidic solution and observe the changes in color of resulting solution.
5. Similarly prepare very diluted alkaline solution and test buffer properties of proteins at excess of OH^- ions. Phenolphthalein could be used for tentative evaluation of pH changes.

Protein sample _____

Color of diluted HCl (universal indicator) _____

Color of diluted HCl after protein addition (universal indicator) _____

_____

Color of diluted NaOH (phenolphthalein) _____

Color of diluted NaOH after protein addition (phenolphthalein) _____

_____

Discussion:

1. What are the buffer properties? Why do protein molecules show them?
2. Why are the protein buffers good for living organisms?

Precipitation of Proteins

Protein molecules in a solution are stabilized not only by a positive or negative charge, but also by hydrate shell, i. e., a layer of water molecules oriented towards the protein molecule charge (if the charge is negative, H atoms of H_2O directed to protein molecule; if the charge is positive, O atoms of H_2O directed to protein molecule). The presence of hydrate shell keeps proteins molecules from aggregating (fusion) and precipitation (*fig. 27*).

In order to separate proteins from the solution (solvent), it is necessary to destroy both stabilization factors, such as surface charge and the hydration shell. Depending on the used reagents or the influence of external deposition factors, denaturation (precipitation) of proteins can be reversible and irreversible.

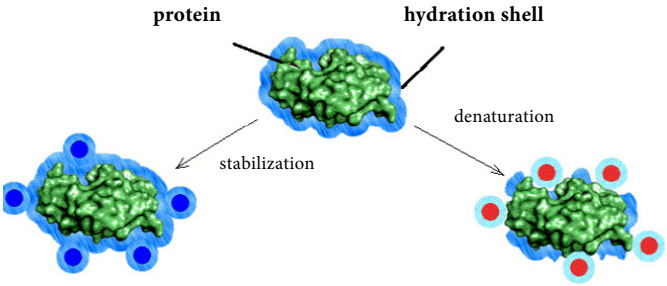


Fig. 27. Visualization of protein denaturing process

Salting out of Proteins

At high concentration the salts of alkali metals, ammonium and magnesium can precipitate (salt out) most of the proteins from their solutions. The main reasons for such behaviour of protein molecule are:

- discharging colloidal particles of protein molecules by adsorption of oppositely charged salt ions on their surface;
- disrupting of hydrate shells of hydrophilic protein molecules by the addition of sufficient quantities of salt ions because small ions have high hydration ability in comparison with large protein molecules.

Both factors reduce the stability of protein colloidal solution by facilitating the aggregation (coagulation) of protein molecules resulting in appearance of protein precipitate. Ammonium sulphate is a most preferred inorganic salt for precipitation of proteins. This salt is able to salt out the proteins from both acidic and neutral media. If other salts are used to salt out the proteins, the medium needs to be more acidified.

Salts of alkali metals and magnesium does not change the structure of protein molecules at salting out procedure, so this salting out is a reversible process. The resulting protein precipitates do not lose their ability to be dissolved when some excess of water is added.

Materials and apparatus:	***Reagents:***
Test tubes	Protein solutions
Plastic pipettes	Ammonium sulfate, $(NH_4)_2SO_4$, 40% aqueous solution
	Distilled water

Procedure:

1. Fill a test tube with 2 ml of protein solution, add 2 ml of $(NH_4)_2SO_4$ and swirl carefully the mixture.
2. Obtained mixture became turbid or protein precipitates forms.
3. Transfer of 1 ml of obtained mixture and add 3 ml of distilled water.
4. Swirl the turbid mixture and dissolving of protein precipitate could be observed.

Protein sample _____

Effect of $(NH_4)_2SO_4$ addition _____

Effect of H_2O addition _____

Discussion:

1. What are the reasons for stability loss of aqueous protein solutions and their precipitation?
2. Why is the precipitation of proteins by the salts of alkali metals, magnesium and ammonium reversible (the resulting protein precipitate can easily be dissolved again)?
3. Alkali, magnesium and ammonium salts are good deposition agents for proteins in weak acidic or sometimes neutral media. Why can some of them not precipitate proteins in the alkaline conditions?

Denaturation of Proteins by Heavy Metal Salts

Salts of heavy metals (e.g. Hg^{2+}, Pb^{2+}, Ag^{+}, Tl^{+}, Cd^{2+}, etc.) even at low concentrations initiate denaturation of proteins by reacting with carboxyl (–COOH) and thiol (-SH) groups. The result of this interaction is a formation of water insoluble compounds. That is why high protein food (milk, eggs, etc.) is used as an antidote to counteract the effect of poisons, like salts of heavy metals, particularly mercury.

Denaturation of proteins by heavy metal ions is irreversible. However, some protein precipitates formed by copper or lead salts are able to dissolve in the excess of such salts as a result of peptization effect.

Materials and apparatus:	***Reagents:***
Test tubes	Protein solutions
Plastic pipettes	Copper (II) sulfate, $CuSO_4$, 10% aqueous solution
	Lead acetate, $Pb(CH_3COO)_2$, 10% aqueous solution
	Distilled water

Procedure:

1. Fill two test tubes with 2 ml of protein solution.
2. Add dropwisely to the test tube #1 $CuSO_4$ solution at continuous mixing till the mixture became turbid or precipitate forms.
3. Add dropwisely to the test tube #4 $Pb(CH_3COO)_2$ solution at continuous mixing till the mixture became turbid or precipitate forms.
4. Share the mixture of both test tubes as it shown below.

Test tube #1		Test tube #4	
Test tube #2	Test tube #3	Test tube #5	Test tube #6

5. Add 3 ml of water to the to the test tube #2 and test tube #5. Fix the changes occurred in the test tubes.
6. Additionally add dropwisely of appropriate salt solutions to the test tube #3 and test tube #6 at continuous mixing. What changes are observed in the test tubes?

Protein sample _____

T.t.#1. Addition of $CuSO_4$ _____

T.t.#2. Addition of H_2O _____

T.t.#3. Addition of excess $CuSO_4$ _____

T.t.#4. Addition of $Pb(CH_3COO)_2$ _____

T.t.#5. Addition of H_2O _____

T.t.#6. Addition of excess $Pb(CH_3COO)_2$ _____

Discussion:

1. What chemical features of protein structure tends to precipitate proteins in a solutions of heavy metals salts?
2. Could you explain why the heavy metal ions in a living organism cause severe poisoning?
3. Heavy metal ions easily precipitate proteins from solutions. Their excess results in peptization and re-dissolution of protein precipitates. Give your idea why solutions of heavy metal salts cannot be an antidote to heavy metal poisoning of a living organism?

Experiment 5

Determination of Proteins Assay by Photometry

General Information

All substances are capable to absorb and reflect electromagnetic radiation (infrared, visible, ultraviolet, etc.). The ability of substances to absorb radiation is determined by the chemical structure of their molecules. As a result, each compound has its own absorbance spectrum. Under appropriate conditions, the content of this compound can be easily determined even in the presence of other compounds.

The method of quantitative spectrophotometry is ground on the ability of molecules to absorb electromagnetic radiation of different energy in a quantity proportional to their number, in other words, to the content of defined substance.

This characteristic is in the Bouguer-Lambert-Beer Law (The Lambert-Beer Law), which was discovered and studied in detail by the French scientist P. Bouguer and the German scientists J. Lambert and A. Beer in the 18th and 19th centuries. In its modern form, this law is applied in quantitative spectrophotometric analysis to determine the content of the compound in a solution. Mathematically, this law

connects the absorbance of electromagnetic radiation (light) by a substance and its concentration in solution:

$$A = \varepsilon \cdot l \cdot C$$

A – the absorbance;

ε[4] – the molar absorption coefficient or molar absorptivity (l mol^{-1} cm^{-1});

l – the optical path length of the light (thickness of the solution layer interacting with the light; cm).

To understand physical meaning A value, it can be presented as follows:

$$A = -\lg T = -\lg \frac{I}{I_0}$$

T – transmission (dimensionless quantity; it represents the radiation proportion passing through a solution of unknown compound);

I – radiation intensity which have passed through a solution layer;

I_0 – initial radiation intensity (irradiated by a source and do not transfer through a solution yet).

Generally, the molar absorptivity ε is unknown or may differ from theoretical value due to the difficulty to provide similar experimental conditions. So, a calibration experiment is carried out before analysing unknown sample. To do so, the solutions of the same substance with defined known concentrations (standard solutions) are used.

The measurement of absorption of the standard solutions is absolutely necessary to build the curve of concentration-absorption dependence for defined substance (*fig. 28*).

The Bouguer-Lambert-Beer Law states that there is a linear relationship between the concentration and the absorption of the solution. This dependence can be transforms to mathematical equation, fixing solution absorption and concentration of dissolved substance.

Both the absorption-concentration plot and the equation, which is derived from it, can help determine the concentration of a substance during the experiments with solutions of unknown concentration using the least square approach (*fig. 28*).

Discussion:

1. What phenomenon is fit in the principle of photometric analysis?
2. What two approaches are used to perform quantitative photometric analysis of substances?

[4] For each substance the molar absorption factor ε under the same experimental conditions (solvent type, additional constituents, temperature, etc.) is constant. There are tables with ε values that you can use. But this is the exception rather than the rule.

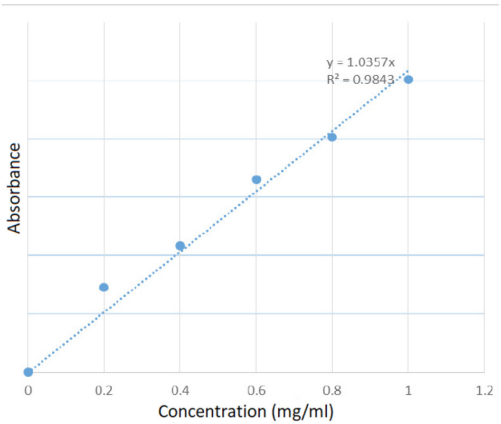


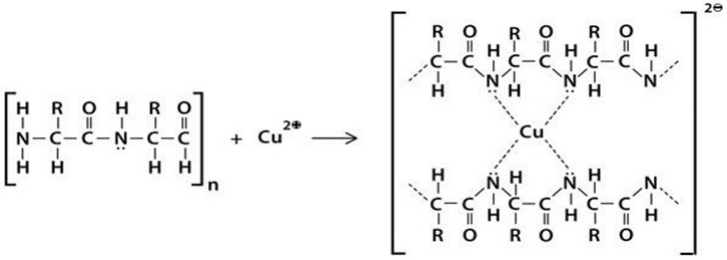
Fig. 28.
How to build
a calibration curve
for photometric
analysis

3. Take a close look at the main patterns of photometry. Could you identify the two most important properties that the sample should meet?

Biuret Method

The Biuret method is the simplest and most common method of photometric determination of total protein assay in solution. The chemical fundamentals of this method is based on the ability of Cu^{2+} ions to form coordination compounds with amide (peptide) -NH-CO- functions in the alkaline medium. According to the coordination activity of copper ions, at least two -NH-CO- groups are required to interact with each Cu^{2+} ion.

As a result of this interaction, a purple copper-protein complex is formed. The highly intensive absorption of visible light in a wavelength range of 540–575 nm is characteristic for this compound. The complex formation scheme is given below:



protein | Copper ions bluish | Cu^{2+}-protein complex purple

Materials and apparatus:

Beakers 50 ml, 100 ml, 400 ml
Measuring flasks 250 ml and 500 ml
Test tubes with stoppers
Test tube holder
Bottles 10 ml
Measuring pipettes 5 ml
Automatic pipette 0.1–1 ml
Glass cuvettes 5 ml
Spectrophotometer or colorimeter with 572 nm filter
Filter paper

Reagents:

Sodium hydroxide, NaOH, 6 M
Copper sulfate hydrate, $CuSO_4 \times 5H_2O$
Potassium-sodium tartrate hydrate, $KNaC_4H_4O_6 \times 4H_2O$
Potassium iodide, KI
Sodium azide, NaN_3 0.0015 M
Albumin
Distilled water

Procedure:

Preparing the solutions

a) *6 M NaOH*. Dissolve 60 g of NaOH in 100 ml of distilled water in 250 ml measuring flask and add a distilled water to 250 ml. Storage the solution in closed plastic bottle.
b) *Biuret reagent*. Dissolve 1.50 g $CuSO_4 \times 5H_2O$ in 250 ml of distilled water, add 4.5 d $KNaC_4H_4O_6 \times 4H_2O$ and 2.5 g KI and swirl the mixture. After salts dissolution add 50 ml of 6 M NaOH and carefully swirl the mixture for homogenization of the solution. Adjust the volume of solution to 500 ml with distilled water. Store the solution in well-closed plastic bottle for 6 months.
c) *Reference solution*. Prepare the solution in accordance with recipe (b) without addition of $CuSO_4 \times 5H_2O$.
d) *0.0015 M NaN_3*. Dissolve 0.05 g of sodium azide in 250 ml of distilled water and adjust the volume to 500 ml with distilled water.
e) *Protein solution*. Dissolve 3.0 g of albumin in 20 ml of 0.0015 M NaN_3 and adjust the volume to 30 ml with sodium azide solution.
f) *Standard protein solutions*. Prepare albumin solutions with different protein content by diluting approach from protein solution (e). Recommended volume for each solution is 10 ml. Recommended protein content should be taken as follow (gram per liter): 20, 40, 60, 80 and 100. The recipes are summarized in *Table 9*.

Table 9

Protein content, g/l	***Volume of Solution (e) added, ml***	***Volume of distilled water added, ml***
20	2	8
40	4	6

Protein content, g/l	*Volume of Solution (e) added, ml*	*Volume of distilled water added, ml*
60	6	4
80	8	2
100	10	0

Preparation of standard solutions, reference solutions and experimental measurements

Standard solutions

1. To number seven test tubes from #1 to #7.
2. Fill each one with 5 ml of biuret reagent (b).
3. Add 0.1 ml of distilled water to test tube #1 (***zero standard solution***).
4. Add 0.1 ml of standard protein solutions with different protein content (*see Table 9*) to each test tube from #2 to #6. Mark the test tubes appropriately.
5. To prepare unknown sample by addition of 0.1 ml of unknown protein solution to the test tube #7.
6. Swirl all test tubes carefully to homogenize the mixtures obtained.

Reference solutions

1. To number seven test tubes from #11 to #17.
2. Fill each one with 5 ml of reference reagent (c).
3. Add 0.1 ml of distilled water to test tube #11 (***zero reference solution***).
4. Add 0.1 ml of standard protein solutions with different protein content (*see Table 9*) to each test tube from #12 to #16. Mark the test tubes appropriately.
5. To prepare ***unknown reference sample*** by addition of 0.1 ml of unknown protein solution to the test tube #17.
6. Swirl all test tubes carefully to homogenize the mixtures obtained.

Experimental measurements

1. Switch on the spectrophotometer and wait for 20–30 min before measurements.
2. Make ready the glass cuvettes.
3. Fit the measuring wavelength at 560 nm (or use a light filter 572 nm).
4. Swirl ***standard protein solutions*** (test tubes #1 – #7) again before starting the measurements.
5. Measure an absorbance value of ***zero standard solution*** (test tube #1) and fit to zero the initial absorbance (A = 0.000 at λ = 560/572 nm).

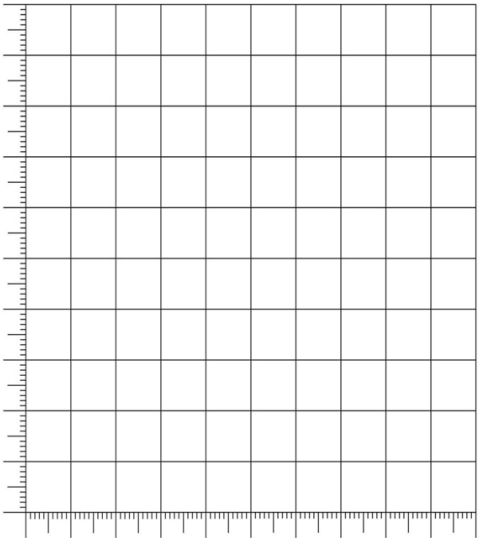
6. Measure absorbance values of ***standard protein solutions*** and unknown sample (test tubes #2 – #6 and #7) and write the results to A1 column of *Table 10*.
7. Swirl ***reference protein solutions*** (test tubes #11 – #17) again before starting the measurements.
8. Measure an absorbance value of ***zero reference solution*** (test tube #11) and fit to zero the initial absorbance (A = 0.000 at λ = 560/572 nm).
9. Measure absorbance values of ***reference protein solutions*** and unknown sample (test tubes #12 – #16 and #17) and write the results in A_0 column of *Table 10*.
10. Calculate real absorbance values A of copper-protein complexes using equation $A = A_1 - A_0$ and write the results to corresponding column of *Table 10*.

Table 10

Standard solutions			***Reference solutions***			***Absorbance***
Test tube	***Protein content, g/L***	***Absorbance A_1***	***Test tube***	***Protein content, g/L***	***Absorbance A_0***	***$A = A_1 - A_0$***
1	0	0.000	11	0	0.000	0.000
2	20		12	20		
3	40		13	40		
4	60		14	60		
5	80		15	80		
6	100		16	100		
7	—		17	—		

11. To plot the dependence of real absorbance value A from protein content in the standard solutions in all concentration range.
12. Using the least squares approach calculate the protein content in unknown sample.

Calibration Curve (Biuret Method)



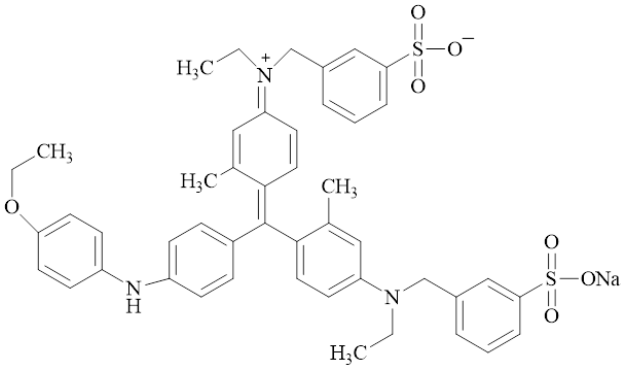
Discussion:

1. Is it possible to determine the content of irreversibly denatured proteins in unknown solution with biuret method and without additional processing?
2. Does the biuret method help to determine protein content in highly acidic medium? Why?
3. What is the purpose of drawing the calibration curve for quantitative photometric analysis?

Bradford Method

The quantitative analysis of proteins by the Bradford method is very simple, fast and accurate. It requires a minimal amount of sample and it sometimes becomes a critical success factor for analysis of cellular proteins, enzymes and for determination of protein content for gel electrophoresis, etc. This method provides precise determination of proteins in concentration range of 5–200 μg, but the results are highly depended on reagent quality and content.

Chemical background of Bradford method is the interaction of protein molecules with Coomassie Brilliant Blue G-250 dye solution in an acidic medium. The resulted protein complex dye has blue colour with a absorption maximum at 595 nm (a neat dye solution has an absorption maximum at 465 nm).



Coomassie Diamond Blue G-250

Due to the specific chemical structure of dye molecule, its interaction with protein molecules occurs on two parallel mechanisms:

(i) hydrophobic interactions realize as joing aromatic rings of dye with aromatic tyrosine, tryptophan and phenylalanine fragments;

(ii) ionic interactions: negatively charged sulfonate $-SO_3^-$ groups of dye molecules connect to positively charged protein molecules in acidic medium.

As a result, the formation of a protein-dye complex is responsible for changes in absorption spectrum and, hence, a colour of the solution. The high accuracy of this method[5] in measuring protein content is explained by high value and stability of molar absorption coefficient of protein-dye complex in 10-fold range of concentrations.

Materials and apparatus:

Beakers 50 ml, 100 ml
Measuring flask 200 ml, 1000 ml
Filtration funnel
Bottles 10 ml
Measuring pipettes 5 ml
Automatic pipette 0.1–1 ml
Measuring cylinder 50/100 ml
Glass or plastic cuvettes 1 ml
Spectrophotometer (colorimeter with glass filter of 572/595 nm)
Filter paper

Reagents:

Sodium hydroxide, NaOH, 1 M
Coomassie Brilliant Blue G-250 (dry or ready to use solution)
Ethanol
Phosphoric acid, H_3PO_4, 85% solution
Albumin
Distilled water

[5] Coomassie diamond blue G-250 reacts mainly with positively charged (arginine, histidine, lysine) or aromatic (tyrosine, tryptophan and phenylalanine) reactive species in protein molecules. Bradford method is highly sensitive to albumins and globulins. When a protein mixture is used, the accuracy of the method is reduced. Accuracy is also reduced for basic and acidic proteins.

Procedure:

Preparation of Bradford reagent solution

Dissolve 0.1 g of Coomassie Brilliant Blue G-250 dye in 50 ml of 95% ethyl alcohol and add to the mixture 100 ml of 85% H_3PO_4 solution. When the dye dissolved adjust a volume of solution to 1 L. Filter the solution before use.

Measurements

1. Switch on the spectrophotometer and wait for 20–30 min before measurements.
2. Make ready the unknown protein solution with protein content within a range of 0.05–1 mg/ml.
3. For specific requirements only![6] Add to each protein solution the equal volume of 1 M NaOH and gently swirl the mixture.
4. Prepare 1 ml of the standard protein solutions (albumin or ***γ***-globulin) with protein content in the concentration range of 0–1 mg/ml. If unknown protein solution were prepared using 1 M NaOH, use the same recipe for preparation of standard protein solutions.
5. Add 5 ml of Bradford reagent to each protein solution, gently swirl and keep for 5 min before measurements.
6. Measure an absorbance value of ***zero protein solution*** (protein content of 0 mg/ml) and fit to zero the initial absorbance (A = 0.000 at λ = 572/595 nm).
7. Measure an absorbance value of ***standard protein solution*** at wave length of 572 or 595 nm. Write the results to *Table 11*.
8. Wash carefully and dry the cuvette.
9. Plot Calibration curve as an absorbance-protein content dependence.
10. Measure of unknown protein sample solution. If measured absorbance A value lays within A of standard protein solutions use Calibration curve for determination of protein content or use the least squares method as an alternative approach.
11. If A value of unknown sample is out of measured range dissolve appropriately the solution of unknown protein and repeat the measurements.

> ***Note! For some unknown protein solution samples, the measurement procedure should be repeated several times to obtain an appropriate and precise results.***[7]

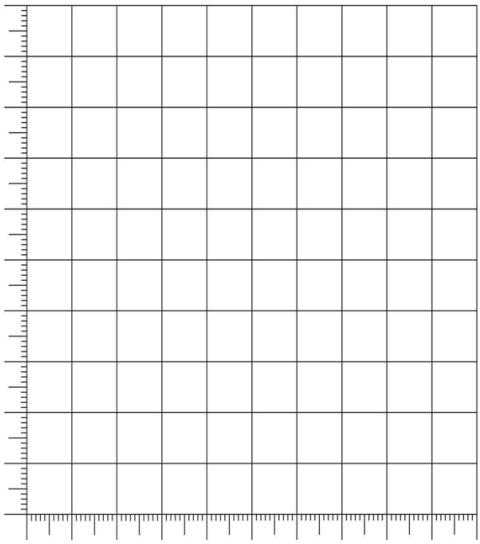
[6] In 1990, Stoscheck proposed adding 1 M NaOH solution to stabilize cell membrane proteins and reduce the effect of protein-protein interactions on the absorbance of the solutions examined.

[7] For some protein samples a dissolution and measuring procedures should be repeated few times to obtain correct and reproducible results.

Table 11

Sample	*Protein content, mg/ml*	*Absorbance A*	*Sample*	*Protein content, mg/ml*	*Absorbance A*
1	0	0.000	8		
2	0.2		9		
3	0.4		10		
4	0.6		11		
5	0.8		12		
6	1		13		
7	—		14		

Calibration Curve (Bradford Method)



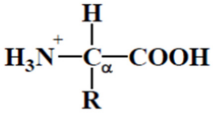
Discussion:

1. Remember the chemical structure of proteins and consider carefully the structure of G-250 dye molecule. Try to suggest possible interactions between dye and protein molecules allow this method to be used for quantitative analysis of proteins.
2. What is Coomassie dye structural feature provides it to be used for analysis in acidic conditions (in excess of phosphoric acid)?

Experiment 6

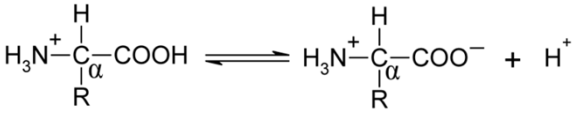
Determination of Dissociation Constants and Isoelectric Point of Amino Acids and Proteins by Titrimetry

Each amino acid contains at least two groups having the capability to be ionized: amino- and carboxyl groups. In acidic media, amino groups are protonated and amino acid became a follow structure:

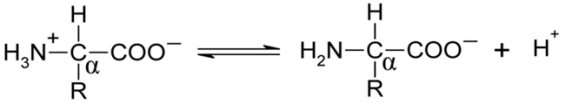


When protonated form of amino acid undergo the action of strong base, it demonstrates a behaviour of dibasic acids. That is, this form of acid can loss both H^+ of protonated amino group and carboxyl group. Acidic behaviour of amino acid can be more complex if side R substituent have additional ionizing functional groups.

We're going to focus on amino acids as dibasic acids. The dibasic acid ionization occurs in two stages. During the stages (I), when the pH is increased, the carboxyl group of the cationic form of amino acid is deprotonated to electroneutral molecule with two opposite charge sites:



At the stage (II), this double ionized amino acid is deprotonated and turned into an anion:



The proton joined to C atom is unable to dissociate due to non-ionic nature of C–H bond.

The sequence of dissociation processes depends on the acidity of protons capable to be ionized. The first to dissociate a more "acidic" proton by the reaction with lower pK_a value. Hence, in the molecule of amino acid, the proton of α-COOH group (pK_{a1}) dissociates before the H^+ of α-NH_3 group (pK_{a2}) does.

Furthermore, the titration curve (dependence of the pH of the amino acid solution on the degree of its neutralization by a strong base) has a characteristic form with two plateaus. The plateaus demonstrate the parts of the of the curve with relatively slow pH change while increasing the amount of added titrant (*fig. 29*).

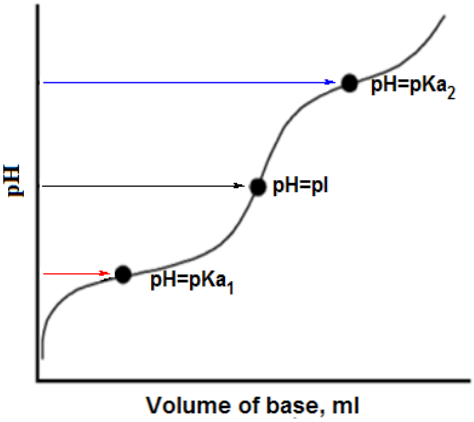


Fig. 29. Acid-base titration curve

Additional ionized functional groups in the structure of the amino acid (–COOH and/or $–NH_2$) increase the number of plateaus.

The plateaus near the pK_{a1} and pK_{a2} indicate the buffer properties of amino acids at corresponding pH range.

The titration curve, in addition to determination of pK_{a1} and pK_{a2}, defines another important characteristic of the amino acid. It is the pH at which the proton dissociation of the carboxyl groups is almost completed but the deprotonation of the amino group has not begun yet. This state is described as the amino acid molecule is still ionized, however the total molecule charge is zero. This ionized form of amino acid molecules is called "zwitterion", and the pH at double-ionized molecular form is called the isoelectric point (*pI*). The pI value can be identified by the experimental curve as the inflection point of the curve between pK_{a1} and pK_{a2}. Its theoretical value can also be calculated as the mean of pK_{a1} and pK_{a2} values.

Materials and apparatus:		*Reagents:*
Burette 25 ml	2	Amino acid, 0.2 M
Pipette 25 ml	1	Hydrochloric acid, HCl, 1 M
Conical flask 150–250 ml	2	Sodium hydroxide, NaOH, 1 M
pH-meter		Distilled water
Magnetic stirrer		
Laboratory holder		

Note! This technique can also be used for titration of proteins and determination of their acid-base characteristics. More diluted NaOH solution with a concentration of 0.1 M are used for this purpose. After adding each portion of titrant, the reaction medium should be carefully mixed. The interval between the addition of each new titrant portion must be at least 30 seconds.

Titration Curve

Procedure:

a) ***Titration of amino acid with 1 M HCl***

1. Fill the burette with 1 M HCl.
2. Measure 25 ml of 0.2 M amino acid solution and transfer to conical flask.
3. Put the magnetic stir bar to the flask and switch on stirring process with magnetic stirrer.
4. Carefully put pH measuring electrode to solution and fit it by the holder.
5. Measure the initial pH value of amino acid and write the result to *Table 12*.
6. Add HCl solution by portions of 0.5 ml by the burette.
7. Measure pH value of the mixture when new portion of titrant added at continuous stirring. Write a volume of HCl added and corresponding pH value to *Table 12*.
8. Repeat addition of titrant and measuring the pH till the pH reaches 1.5.

Note! The pH values change very sharply at the beginning the titration procedure. Near the pK_a region the pH values change much slowly. So, it is allowed to add larger portions of titrant (1 ml).

b) ***Titration of amino acid with 1 M NaOH***

1. Repeat the procedure well-described above using fresh portion of amino acid solution and 1 M NaOH solution as a titrant. The titration should be stopped when pH value reaches 13.

Table 12

1 M HCl				*1 M NaOH*			
V, ml	*pH*	*V, ml*	*pH*	*V, ml*	*pH*	*V, ml*	*pH*

Determination of pK_a and *pI*

Procedure:

1. Using Microsoft Excel or another software plot the pH vs volume of titrants curve as it is shown in *fig. 30*.
2. Mark the buffer zones as well as pK_a and pI points of the amino acid on the curve plotted. Write the values of pK_a of ionic groups and pI of amino acid.

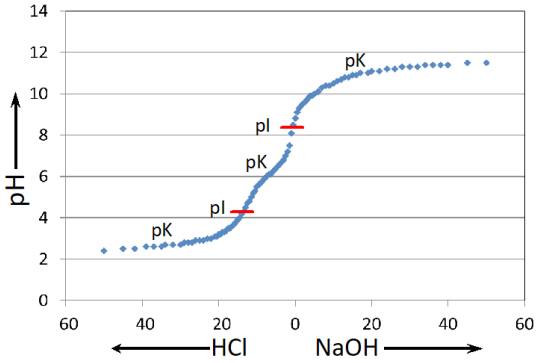


Fig. 30. Acid-base titration curves of zwitterionic form of amino acid

Discussion:

1. Do titration curves of strong base/strong base and strong acid/amino acid differ each other? What does this have to do with?
2. Could you explain the chemical transformations occur with the amino acid while the pH is changed? Do these changes correlate with pK and pI of amino acid?
3. Is the pH(V) curves for glycine and glutamic acid differ each other? What is the matter of the difference?

ENZYME PROTEINS PROPERTIES

Proteins are an important class of polymer biomolecules that perform various functions in living organisms. ***Enzymes*** are specific proteins. The main role of enzymes is to accelerate various chemical reactions in living organisms. Therefore, enzymes playing a role of biocatalysts.

Enzymes have all the physical and chemical properties of proteins. They have a high molecular mass, low resistance to elevated temperatures, to form colloidal solutions, to be precipitated by heavy metals salts, the ability to split into amino acids during hydrolysis, etc.

Depending on the chemical composition, the enzymes fall into two general groups: (i) simple proteins that contain only amino-acid residues and (ii) conjugated proteins that typically are the large molecules with complex structure in which polypeptide chain binds to other nonprotein molecules called ***coenzymes***.

The coenzyme binds to the protein part (***apoenzyme***) by weak non-covalent bonds that can easily be broken. Sometimes they are joined by a strong covalent bond and form ***prosthetic groups*** that remain attached to the protein molecule while the catalytic reaction occurs. Vitamins often act as coenzymes. The acceleration of chemical transformations activated by enzymes is due to a reduction in activation energy (the amount of energy that molecules require to initiate the reaction).

For example, in convenient chemical process about 210 kJ of energy should be used to break down 1 mol of C–N bonds. However, in nature, only 42–50 kJ are required for the similar enzyme-activated process. Man-made inorganic catalysts and natural biocatalysts (enzymes) have some common features and the differences.

The similarities are the follows:

- both catalysts can catalyse only thermodynamically allowed reactions and accelerate only those reactions that occur themselves, but at a much slower rate;
- they do not spend during a reaction and are not included to the final product composition;
- they do not shift the chemical equilibrium, but only accelerate the chemical transformation leading to the equilibrium state.

Enzymes have certain specific properties that inorganic catalysts do not have. Specifically, the enzymes are characterized by:

- high efficacy. They are much more effective than synthetic catalysts. Each enzyme molecule can catalyze from 10^3 to 10^7 reactions per 1 second and accelerate the reactions 10^5 to 10^{10} times;
- temperature sensitivity. The maximum efficacy of most enzymes observes at temperature range of 37–40 °C. When the temperature increases, the enzyme activity slows down and gradually stops. At 80 °C the enzymes irreversibly lose their activity due to damage the molecular structure. When the temperature drops from 37 °C to 0 °C and below, the enzymes lose their activity, but do not damage and can become active if they are carefully heated to the optimal temperature;
- pH sensitivity. The catalytic reactivity of enzymes is highly depend on concentration of H^+ ions. The optimal activity of most enzymes observed at near-neutral pH (from 6 to 8);
- high selectivity. Each enzyme acts only with a specific substance (substrate) and catalyses only one "own" reaction. The enzyme selectivity is determined by the chemical structure of protein;
- regulated activity. Catalytic efficacy of enzymes can be significantly changed by certain chemical compounds accelerating or supressing their activity. Some metal cations, anions and organic molecules act as accelerators. Heavy metal cations, some organic compounds can play a role of inhibitors.

All enzymatic reactions are reversible. This is due to the enzyme ability to catalyse both direct and reverse reactions. For example, under certain conditions the lipase enzyme breaks down fat into glycerol and fatty acids. This enzyme can also catalyse the synthesis of fats from these products.

Enzymatic reactions are divided into anabolic (synthesis) and catabolic (decay) ones. All these actions in a living organism is combined in one term "metabolism"

By enzymatic reactions, all enzymes are divided into seven major classes:

- ***oxidoreductases*** catalyse oxidation-reduction reactions, namely transferring electrons and hydrogen atoms from one substrate molecule to another one;
- ***transferases*** accelerate transfer reactions, like transfer of chemical groups from one substrate to another;
- ***hydrolases*** accelerate hydrolysis reactions which involve H_2O molecules;
- ***lyases*** catalyse non-hydrolytic and non-oxidative scission of C–C, C–O, C–N, C–S and other bonds; breaking and formation of double bonds in different substrates without participating H_2O and energy support of ATP;
- ***isomerases*** accelerate the isomerization reactions, e.g. intramolecular movement of different chemical functionalities;

- ***ligases*** catalyse the synthesis reactions, namely combining the molecules with a formation of new bonds using ATP energy source;
- ***translocases*** catalyse the transfer of ions and neutral molecules across the cell membrane or activate a separation process within the membrane.

Enzymes are essential in all life activities because they organise and regulate the metabolic processes in living organisms. Industrial importance of enzymes is also well-known. They are widely used in food processing (winemaking, cheesemaking, fast-food producing, etc.), textile treatment (leather goods production), chemical industry (biochemical technologies, detergents producing), pharmaceutical and cosmetic areas (enzymatic drugs, vaccine producing, cosmetology), agriculture (fodder production) and advanced technologies (molecular and genetic engineering).

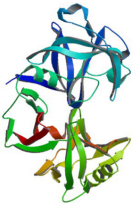
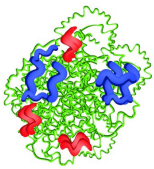
Experiment 1

Enzymatic Coagulation of Casein

One of the most important characteristics of enzymes as catalysts is their high activity and specificity. Among a great number of protolithic enzymes, which are involved in protein degradation, some enzymes are able to catalyse the scission of peptide (–NH–CO–) bonds only between certain amino acid residues (*Table 13*). It concerns some basic enzymes produced by the digestive glands in a human body and some animal species.

These enzymes, namely pepsin and chymosin, are responsible for the protein digestion. Despite of the "mission" and the "working place" similarity of these enzymes, they differ in their functionality and specificity. For example, pepsin, which catalyses the hydrolysis of peptide bonds, is mostly effective for scission of –NH–CO– formed by the aromatic amino acid residues of phenylalanine and tyrosine.

Table 13

Name	***Enzyme type***		
	Gastric enzyme		***Pancreas gland enzyme***
	Pepsin	***Chymosin (Rennin)***	***Trypsin***
Structure			

Name	*Enzyme type*		
	Gastric enzyme		*Pancreas gland enzyme*
	Pepsin	*Chymosin (Rennin)*	*Trypsin*
Molecular weight[8]	~34500	~36000	~24000
Number of amino acid residues	340	323	223
Isoelectric point and loss of activity	> 6.5	> 7	10.8
pH of maximum activity	1.9–3.9	3–5	7.8–8

Pepsin breaks down the most of natural proteins except keratin and collagen. This enzyme has protolithic activity at pH < 6 reaching a maximum efficacy at pH range of 1.5–2. Pepsin is highly active. For example, a one gram of pepsin can break down about 50 kg of egg albumin for 60 minutes.

Gastric juice of infants, as well as the secret of the fourth stomach section of calves and other ruminant animals, have another enzyme, i.e. rennin (chymosin), which differs from the pepsin. Rennin is the first chemically isolated enzyme. The Danish scientist Christian Hansen isolated it from the calf dried stomach by extracting it with a saline solution. "Setchug" (abomasum) is its household name in Ukraine, which was got from the name of the ruminate rennet stomach. Unlike pepsin, rennin is a more selective enzyme. Its main substrate is caseionogen that is one of the milk proteins. At the same time, rennin has weaker proteolytic characteristics than pepsin, as it is able to break only a small amount of peptide bonds of casein.

The optimal acidity for high efficacy of rennin is pH value from 3 to 5. The soluble caseinogen turns into insoluble casein by this enzyme. As a result, a coagulation of milk occurs (liquid milk is converted into a jelly-like solid).

Interestingly, but this widely useful industrial process has not been studied in details yet. It is considered that the enzyme reacts with the soluble caseinogen to form paracasein. Paracasein and the Ca^{2+} ions in the milk forms an insoluble salt and precipitates as a gel.

The rennin in the gastrointestinal juice of infants has a certain physiological significance. After milk coagulation (milk is the main infant food), its passing

[8] Enzyme molecular weight depends on the source of its origin.

slowly through the gastrointestinal tract. As a result, it is longer undergone by the enzymes (in other words, digested more effectively).

In addition to their physiological needs, rennin is very important for humans. In particular, rennin is widely used for cheese production. The main source of rennin is a young calf stomach (under 10 days old). This enzyme is called microbial rennin. Rennin can also be derived from some fungal strains. More recently, some advances in biotechnology and genetic engineering have led to a producing recombinant rennin synthesized by bacteria with the genome of calf rennin transferred to bacteria.

Enzymatic coagulation of milk casein during cheese production requires optimal conditions for providing rennin efficacy. There are optimal milk acidity and Ca^{2+} ions content and appropriate temperature regime for precipitation of casein gel (denaturation of milk protein). A role of calcium ions in a formation of casein precipitate (gel) during cheese-making process is obvious.

The protein gel cannot be formed from a milk with low Ca^{2+} content. It is occurring when cows being fed poor calcium food, or inactivation (chemical bonding) of Ca^{2+} cations due to pasteurization.

Part 1. Milk Fermentation

Materials and apparatus:

Beaker 100 ml	3
Petri dish 50 mm	3
Plastic pipettes	
Glass rods	
Knife	
Oven	
Electric heater	
Water bath	
Thermometer	

Reagents:

Fresh milk (non-pasteurized)
Citric acid, 5% solution
Calcium chloride, $CaCl_2$, 0.5% solution
Microbial rennin, 0.05% solution
Ethanol
Distilled water

Procedure:

1. Fill a beaker with 60 ml of cold fresh milk (less 15 °C) and add 1.5 ml of citric acid solution. Stir the mixture well.
2. Heat two other beakers in an oven to 34–35 °C.
3. Heat a beaker with milk carefully at continuous stirring to 32 °C.
4. Share the milk to 3 equal portions (20 ml) using as-heated beakers.

Note! Subsequent operations must be performed quickly so that the temperature of the milk does not fall down. To prevent cooling the beakers can be placed into a water bath heated to 35–37 °C or kept in warm place.

5. The beaker #1 covers with Petri dish and to place in an oven (34–35 °C).
6. Add 0.5 ml of $CaCl_2$ solution to the beaker #2, stir and add 1 ml of rennin solution. Stir the mixture quickly, cover the beaker with Petri dish and to place in an oven.
7. Add 1 ml of rennin solution to the beaker #3, stir quickly and place in an oven.
8. Leave all beakers with milk sample in an oven for 10–15 min at 34–35 °C. Check a consistence of milk samples, compare the quantities of protein gel and explain the difference.
9. Obtained casein gels cut carefully in the beaker on rectangular portions and heat in water bath to 70–80 °C for 30–40 min to dehydrate casein gel.
10. Separate a liquid phase (lactoserum) from casein precipitate and compare visually the protein precipitates in all beakers (quantities of dehydrated casein precipitate, volume of separated lactoserum).
11. The most strong and dense casein precipitate use for follow experiments.
12. To separate the casein from fats that coprecipitated with proteins, cover as-prepared casein with 10–15 ml of ethyl alcohol for 10 min.
13. Decant the alcohol from protein and repeat the extraction procedure once again.
14. Obtained fat-free casein dry carefully in warm place or in an oven for at 50–60 °C to constant weight.
15. Weight dry casein and calculate the protein content w (%) in milk sample:

$$w = \frac{m_{cas}}{m_{milk}} \times 100\%$$

where m_{cas} – weight of dry casein (g);
m_{milk} – weight of milk sample in which casein was deposited (g; use density of a milk as $\rho = 1.028$ g/cm^3).

16. All results of experiment write in *Table 14*.

Table 14

Characteristics	*Beaker #1*	*Beaker #2*	*Beaker #3*
Is a gel obtained?			
Comparative gel strength			

Characteristics	*Beaker #1*	*Beaker #2*	*Beaker #3*
The result of heating a gel			
Approx. quantity of casein precipitate			
Approx. volume of lactoserum			
Weight of dry casein, g			
Casein content in milk, %			

Discussion:

1. What compounds do enzymes belong to by their chemical structure? What is the functions of enzymes in living organisms?
2. What type of enzymes do pepsin, trypsin and chymosin belong to? What kind of reaction do they accelerate and what function in living organisms do they perform?
3. What are the general conditions for protein enzymatic coagulation? Could you explain how these conditions affect the process of casein precipitation?
4. Do you explain an extreme importance of enzyme rennin in the stomach juice of infants?

Part 1. Acidic Hydrolysis of Casein[9]

Materials and apparatus:

Test tube
Plastic pipettes
Spatula
Electric heater
Water bath

Reagents:

Casein sample
Hydrochloric acid, HCl, conc.
Sodium hydroxide, NaOH, 20% solution
Universal indicator paper
Distilled water

Procedure:

1. Weight 0.2 g of dry protein in a test tube equipped with stopper and gas-transfer tube.
2. Add to the test tube 2 ml of concentrated HCl and 1 ml of distilled water.

[9] You can choose any protein for the study.

3. Stir well the mixture for soaking the protein sample with acid solution.
4. Heat the test tube at 90–95 °C using water bath till full dissolution of the protein (do not swirl the mixture during the heating). It requires approximately 24 hrs. Periodic heating is allowed.
5. The protein hydrolysate neutralizes with NaOH solution to pH = 7 (control pH value using indicator paper.

Discussion:

1. What is a hydrolysis reaction?
2. What kinds of hydrolysis reactions do you know? What compounds are involved in this reaction?
3. Could you provide the examples of compounds that can be hydrolysed?
4. What are the structural characteristics of casein molecules that involve them in hydrolysis reactions?
5. What compounds are produced during the reaction of partial and complete casein hydrolysis?

Part 2. Determination of Casein Composition by Paper Thin Layer Chromatography (paper TLC)

Materials and apparatus:

Beaker 100 ml, 500 ml
Measuring cylinder 50 ml
Plastic pipettes
Capillary pipettes
Chromatographic paper
Pencil and Ruler
White paper A4
Stapler
Office binder
Electric oven
Aluminum foil
Gloves
Hair drier

Reagents:

Amino acids, 5% solution
Hydrolyzed protein sample
Butanol-1
Glacial acetic acid, CH_3COOH
Ninhydrin, 0.5% ethanol solution
Distilled water

Procedure:

a) Preparation of mobile phase

1. Mix 40 ml of Butanol-1 and 10 ml CH_3COOH.

2. Add dropwisele a distilled water to 45 ml of solvents mixture till slightly opaque solution forms.
3. Add dropwisely the initial solvent mixture to the opaque one till transparent solution obtained. The mobile phase is ready.

b) Experimental technique

1. All procedures with chromatographic paper perform in the gloves. Why is this requirement obligatory?
2. Take a piece of chromatographic paper of 13 × 18.5 cm in size.

Note! Keep the paper from one of the longer sides of paper (18.5 cm). Mark this side of a paper as "top".

3. Place the paper on a clear white A4 paper.
4. Draw carefully with a pencil a "start" line (1.5 cm from "bottom" side of chromatographic paper).
5. Draw carefully with a pencil a "finish" line (1.5 cm from "top" side of chromatographic paper).
6. Make the short marks along "start" line as it seen in *fig. 31*. For identification of the sample use "top" side of chromatographic paper and a pencil.
7. Use capillary pipettes to put the sample solutions on chromatographic paper.
8. Hydrolyzed protein solution and few reference amino acid solution are very recommended for TLC experiment to conclude the tentative composition of protein sample.
9. In accordance the mark lines and identification put the sample solution on chromatographic paper using capillary pipettes. Sizes of sample solution drops should be less than 5 mm.
10. Note! Wait for complete drying the sample before applying a new portion of the sample solution. An hairdryer can speed up the drying process.
11. Repeat the procedure at least 10 times waiting when a previous drop of sample solution dried.
12. When all samples were applied on corresponding labeled points leave the chromatographic paper on an open air for 10 min to complete the drying.
13. Roll up the chromatographic paper into cylinder-like construction and strap the opposite sides together with staples as it is shown in *fig. 32*. The stapled sides of the paper should not touch each other!
14. Fill developing chamber (e.g., large 500 ml beaker) with 20 ml of mobile phase consists of water-saturated solvent mixture butanol-1/acetic acid = 4:1 (vol.).

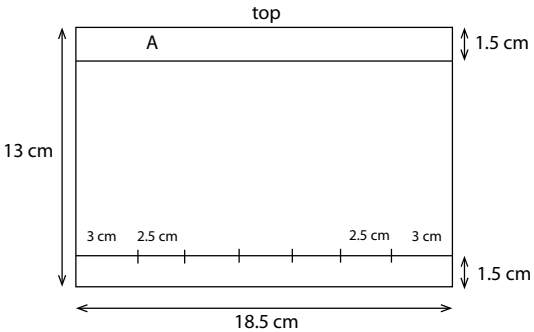


Fig. 31.
Sample preparation procedure for determination of amino acid composition of casein by paper TLC

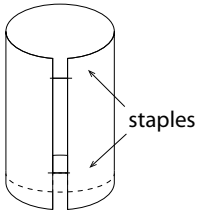


Fig. 32.
Preparation of paper sheet with the samples for chromatography developing

15. Fit cylindric chromatogram paper into developing chamber. The bottom side of cylinder should be deep into mobile phase onto a depth of 3–5 mm.
16. Cylinder wall should not touch to chamber wall.
17. Close up developing chamber.
18. The developing process continues 60–75 min. ***Do not move developing chamber during chromatography developing. Observing for mobile phase moving should be done through transparent glass side walls***.
19. When developing is finished take off the paper cylinder and mark the end line of mobile phase (if it differs from earlier drawn "finish" line).
20. Fit the chromatographic paper on white A4 paper and wait while solvent mixture evaporates.
21. Leave the staples from chromatographic paper and hang it using binders in fume hood.
22. Using hand sprayer to spray the chromatogram with ninhydrin solution avoiding an excess of the reagent.
23. Heat the chromatogram in an oven at 80 °C for 2 min.
24. Draw out the spots of all amino acids and measure the length between "start" line and a center of each spot (*l* value) and a length between "start" line and solvents front (*L* value). Calculate the retention factor R_f for each amino acid:

$$R_f = \frac{l}{L}$$

25. According to the results identify amino acid composition of casein and write all the data to *Table 15*.

Table 15

Retention factor R_f			*Amino acid*
Hydrolyzed protein 1	**Hydrolyzed protein 2**	**Hydrolyzed protein 3**	

Discussion:

1. Why are the paper and other TLC methods the most commonly used for analysis of the mixtures?
2. What chemical reagent determines the spot position of amino acids on a chromatogram? Explain it from a chemical point of view.
3. Is it possible to identify the presence of individual amino acids in casein by the reactions with ninhydrin or other chemicals without hydrolysis of protein molecules?

Experiment 2

Enzymatic Degradation of Proteins by Plant Enzymes

Proteins are an important building blocks and reactive species in our body, from cell components to biologically active molecules. Our body synthesizes all proteins it needs from the amino acids.

The main source for these blocks is food proteins. It's an important source of essential amino acids that our bodies cannot synthesize on their own. In order to use the amino acids "hidden" in the structure of protein molecules, the living

human body needs to "disassemble" of polypeptide chains of food proteins on separate components. It is very necessary to transform the "raw material" to amino acids required for synthesis of new important protein molecules.

Enzymes as highly specific biocatalysts belonging to the group of protolithic enzymes (protease) come to aid of the body. They break the peptide -NH-CO- bonds between amino acid residues in polypeptide chains and convert them into free amino acids or larger oligopeptide molecules (small polypeptides containing several amino acid residues). The proteases may differ in the structure of the catalytically active part of the molecule and are able to "work" in different ways. However, they are belong to the one protease group.

Depending on operating principles, the protolithic enzymes are divided into peptidases (exopeptidases) and proteinases (endopeptidases). Exopeptidases break down mostly the outer peptide bonds. Otherwise, endopeptidases break down the inner -NH-CO- bonds in the protein molecule.

Some proteinases are also characterized by so-called substrate selectivity. It means that they are able to hydrolyse the linkage between certain amino acids residues.

Commercial protolithic enzymes are used in the laboratories to establish the structure of proteins and polypeptides, in the food industry (in meat softening and cheese making technologies) and other industries (removing wool residues from a skin, softening the skin), medicine (clotting, cataract removal, treatment of digestive system disorder).

The raw materials for producing enzymes are such diverse biological material, like microorganisms, plant and animals' tissues. Some protolithic enzymes, which are capable to hydrolyse -NH-CO- bonds of collagen, can be found in well-known plants.

Plant-based enzymatic processe are widely used in the food industry to soften a meat. Food with high protease content is also a part of various marinade recipes for meat treatment for home uses.

The best-known plant proteases available for home use are:

- *bromelain* contained in the plants of pineapple families;
- *papain* contained in papaya melon fruit;
- *actinidine*, found in plants of the Actinidiaceae family (kiwi and actinidia are well-known members of this family).

Materials and apparatus:			***Reagents:***
Beaker 50 ml	3	Magnetic stirres	Gelatin
Measuring flask 200 ml		Filter paper	Agar
Measuring cylinder 10 ml		Grater	White egg
Test tubes with stoppers	5	Ruler	Fresh meat
Filtration funnel		Water bath	Kiwi
Plastic pipettes		Electric heater	Distilled water

Procedure:

A) Preparation of 5% gelatin gel (a day before the experiment)

1. Weight 0.25 g of gelatin in a beaker.
2. Add slowly 5 ml of water to the beaker with gelatin powder by few portions (1–2 ml per one time). Wait for gelatin swelling before addition of new portion of water.
3. When full volume of water (5 ml) added heat slowly the gelatin gel till a transparent solution form. Do not overheat the gelatin-water mixture (>70 °C).
4. Share the gelatin solution on two test tubes (test tube #1 and #2) and leave in cold place for gel solidification.

B) Preparation of 1.5% agar gel

1. Weight 0.15 g of agar in a beaker.
2. Add 10 ml of water and wait for 20–30 min till agar swell.
3. Heat the mixture to 90–95 °C at continuous stirring for dissolution of agar and formation of clear solution.
4. Fill a test tube with 2 ml of hot agar solution and leave in cold place for gel solidification (test tube #3).

C) Preparation of denatured white egg

1. Separate carefully white egg from yolk.
2. Small portion of white egg (2 ml) put into a test tube heat in boiled water bath for 7 min for denaturation of egg proteins and appearing white rubber-like solid (test tube #4).

D) Preparation of enzyme solutions (Enz and Enz^T reagents)

1. A half of peeled kiwi fruit grind carefully.
2. Add 15 ml of water to the mixture, mix well and filter a juice (***Enz*** reagent).

Note! If you take ready-to-use enzymes (bromelain, actinidine, papain or specific protease enzymes isolated from bacteria or fungi), prepare their solution with a concentration of 1–5 mg/ml by prolonged stirring the portion of enzyme in distilled water at ambient conditions. To improve dissolution and increase activity the enzyme you can add diluted CH_3COOH (pH = 5–6). To test the enzyme activity, you need 20 ml of enzyme solution.

3. To evaluate an effect of temperature on enzyme activity treat 10 ml of kiwi juice at 60 °C for 10 min (***Enz^T*** reagent). Cool the solution before use.

E) Studying the enzyme activity

1. Mark a top front of gelatin gel or denatured white egg by thin permanent marker on each test tube.

2. A piece of fresh meat (1 cm^3) weight and place to another test tube. The same piece of meat weight and store as a control sample in closed bottle in a freezer till the end of experiment.
3. Test tubes #1 (gelatin), #3 (agar) and #4 (denatured white egg) fill with 2 ml of fresh (***Enz***) kiwi juice.
4. Put a piece of meat to the test tube #5 and add 2 ml of fresh (***Enz***) kiwi juice.
5. Test tube #2 (gelatin) fill with thermally treated (***Enz^T***) kiwi juice.
6. Observe the changes in front of gels in the test tubes through defined time intervals.
7. An effect of juice enzymes on a meat could be observed by comparing fresh meat from a freezer (control sample) and enzymatically treated meat (visual observing, weight of both meat samples).
8. All results write to *Table 16*.

Table 16

Test tube	***Observation at defined time intervals***				
	1 hr	***2 hrs***	***4 hrs***	***6 hrs***	***24 hrs***
1					
2					
3					
4					
5					

Discussion:

1. What are similar and different characteristics for the two enzyme groups: (i) pepsin, trypsin and (ii) bromelain, actinidin?
2. What is the use of a large group of protolithic enzymes? Try to answer from the scientific and practical perspectives.
3. Based on your own theoretical findings, try to define the function of protolithic enzymes in plants.
4. Why do some protolithic enzymes not affect protein molecules? Why are protolithic enzymes capable to leave protein molecules in their neat form?

Experiment 3[10]

Properties of Proteins as Catalysts. Fermentation Process

Fermentation is the general name for the biochemical processes of converting of an organic compound into another one by high-activity natural catalysts, enzymes. Not all proteins have catalytic activity. But those having catalytic activity are an extremely effective catalysts that overcome all artificial man-made catalysts.

Humans have been using protein catalytic activity for their own needs for thousands years. Until recently, we didn't know what acts effectively in such processes as baking bread, some drinks, etc. Over the last 100–150 years, humans have gained knowledge that can provide studying of some usual processes. We have learned to apply the knowledge to create new technologies for our life improving.

Among the best known processes where proteins act as biological catalysts, enzymatic starch, $(C_6H_{10}O_5)_n$, hydrolysis, (equation 1) and alcohol fermentation of glucose, $C_6H_{12}O_6$ (equation 2) are presented below:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2\uparrow \quad (2)$$

The catalyst in reaction (1) is amylase enzyme (*fig. 33*). Several types of amylase are known, for example, *α*-, *β*- and γ-amylases. They differ by their ability to accelerate breaking down of some chemical bonds in in polymerized form of glucose, starch. This enzyme is found in human saliva, some animals, many plants and their seeds, fungi.

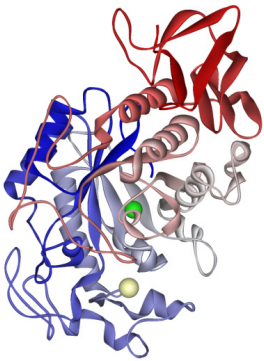


Fig. 33[11].
3D structure of amylase

[10] The contest entries of the 11th International Youth Science Olympiad IJSO-2014, 2–11 December, 2014, Mendoza, Argentina.
[11] Yellow ball is Ca^{2+} cation; green ball is Cl^- anion.

The reaction (2) is catalysed by the zymase. It is a mixture of various protein enzymes that help transform glucose $C_6H_{12}O_6$ and other carbohydrates into ethyl alcohol and CO_2. This process is called ***alcoholic fermentation***. It has been known since ancient times. Many peoples of the world used it to make bread and drink for religious ceremonies using catalytic properties of zymase.

Alcoholic fermentation is a series of biochemical reactions of subsequent transformation of glucose (as well as fructose known as grape sugar and sucrose, which is commonly known white sugar) to ethyl alcohol and carbon dioxide. Remarkably, both C_2H_5OH and CO_2 are the reaction by-products, because the energy is the main product of this process that occurs in living organisms.

In addition to the practical significance, the important features of the process as series of chemical reaction can easily be studied. Indeed, CO_2 is generated by the catalytic destruction of $C_6H_{12}O_6$ and can be measured with the basic laboratory equipment. It is difficult to isolate zymase from natural raw materials, but it is possible to use its carrier ***Saccharomyces cerevisiae*** (*fig. 34*), known as yeast[12]. This natural product is involved in the enzymatic transformation of carbohydrates under anaerobic conditions (i.e. in the absence of oxygen).

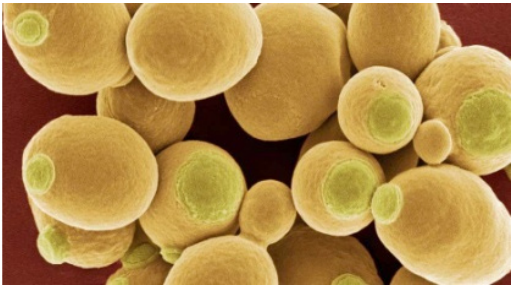


Fig. 34.
Microphotograph of yeast cells (Saccharomyces cerevisiae)

The alcoholic fermentation is one of the most important processes in modern biotechnology and it is used in:

- producing ethanol, from which biofuels are derived;
- producing baked goods in food industry;
- producing alcoholic beverages (beer, wine, strong alcohols).

So, in the simulated process instead of pure zymase of yeast, living storages of the protein enzymes can be used.

[12] Yeast are single-celled microorganisms from the Fungi family that have lost mycelial structure due to their predominant existence in liquid and semi-liquid media enriched by organic compounds. The cell size is about 5–10 μm and have a spherical or elliptical shape.

The fermentation process efficiency (and thus the efficacy of enzymes in which they participate) can be determined by measuring the amount of carbon dioxide emitted during the chemical transformation of glucose. Based on the measured amount of CO_2, it is possible to calculate the amount of C_2H_5OH generated by the fermentation of glucose and the amount of $C_6H_{12}O_6$ involved in this process.

Materials and apparatus:

Crystallizing dish 1000 ml
Measuring cylinder 1000 ml
Beaker 250 ml 2
Beaker 1000 ml
Buhner flask 500 ml with a stopper
Rubber tube
Glass rod
Laboratory stand with flask holder
Thin rubber carpet 15 cm × 15 cm
Thermometer
Timer

Reagents:

Glucose, $C_6H_{12}O_6$
Yeast
Bromothymol blue indicator, solution
Calcium hydroxide, $Ca(OH)_2$, saturated
Distilled water

Procedure:

A) Fit the measuring equipment *(fig. 35)*

1. Pour a water to crystallizing dish to a full volume.
2. Pour a water to measuring cylinder.
3. Close a neck of cylinder with rubber carpet and turn upside down keeping it closed by hand. Put a neck of cylinder in a water of crystallizing dish below a water level and take off a rubber carpet. ***Do not allow filling a cylinder with air***.
4. Fix a cylinder in laboratory stand. A neck of cylinder should be dep into a water as shown in *fig. 35*.

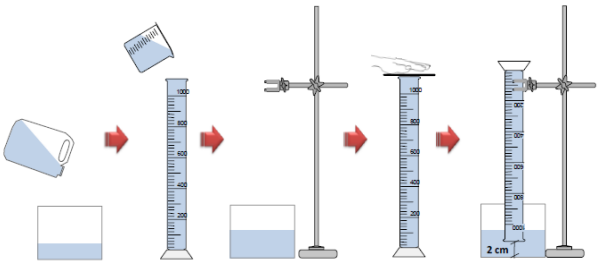


Fig. 35. Preparation of gas measuring cylinder

***B) Fit the reactor flask** (fig. 36)*

1. Heat 700–750 ml of water in large beaker to a temperature of 35–40 °C. Measure the temperature with thermometer adr write the result to *Table 17*.
2. Mark both 250 ml beakers as "A" and "B"? respectively.
3. Prepare the *Mixture "A"* and *Solution "B"* using warm water.

Mixture "A": In the beaker "A" mix 50 g of yeast and 150 ml warm (35–40 °C) water using glass rod. Adjust a volume of solution to 250 ml with warm water.

Solution "B": In the beaker "B" dissolve 4 g of glucose $C_6H_{12}O_6$ in 150 ml of warm (35–40 °C) water. Adjust a volume of solution to 250 ml with warm water.

4. Transfer Mixture "A" to the reactor (Buhner flask). If some quantity of Mixture "A" leaves on beaker walls wash it with small portion of Solution "B" and transfer to the reactor flask.

> ***Mixture "A" should be transferred quantitatively! Do not allow the leave some portions of Mixture "A" or Solution "B" in beakers or wash it with additional portions of warm water!***

5. Swirl carefully of reactive mixture and place the reactor onto rubber carpet.
6. Measure a temperature of reactive mixture and write to *Table 17*.
7. Close the reactive flask with a stopper tightly.

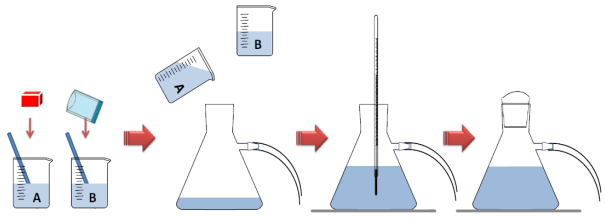


Fig. 36. Preparation of reactive mixture

***C) Glucose fermentation** (fig. 37)*

1. Connect a one end of rubber tube with reactor and put another one to upper side of water-filled cylinder.
2. Swirl the reactive mixture to free the rubber tube from air.
3. Write the ***zero*** volume of water in the cylinder to *Table 17*. This value requires to measure a volume of gaseous products which produced during fermentation process.
4. Start a timer (star point of reaction).
5. During observation time write every minute swirl the reactive mixture for 5 s. Do not allow the end of rubber tube to take away from cylinder.

6. During fermentation a gaseous product going through the tube to measuring cylinder. A water level should continuously fall down.
7. Every 2 min write a water level value to *Table 17* within time interval of 40 min.

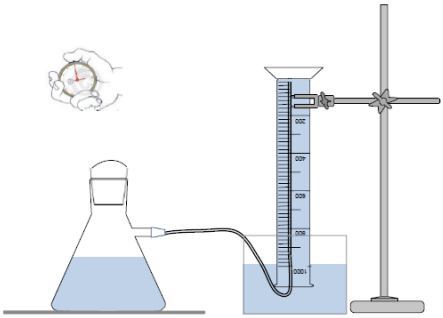


Fig. 37.
Scheme of measuring procedure

Table 17

Temperature of water before preparation of Mixture A and Solution B: _____°C		
Temperature of Mixture A and Solution B in reactor _____°C		
Time, min	***Volume of gaseous product, V, ml***	***Gas producing rate, F(t), ml/min***
0		
2		
4		
6		
8		
10		
12		
14		
16		
18		
20		
22		
25		
26		
28		

Time, min	*Volume of gaseous product, V, ml*	*Gas producing rate, F(t), ml/min*
30		
32		
34		
36		
38		
40		

8. After 40 min from the start of experiment close the rubber tube by clamp to prevent transfer of gases to measuring cylinder.
9. Remove a stopper from reactor flask. End of rubber tube should be in upper part of measuring cylinder.
10. Add another portion (4 g) of $C_6H_{12}O_6$ powder to reaction mixture and close it by stopper tightly.
11. Remove the clamp from rubber tube.
12. Swirl reactive mixture for 30 s for dissolution of glucose powder.

If the measuring cylinder is filled with gas less than half of a volume, the experiment can be continued. If the measuring cylinder is filled with gas more than half of the gas, the cylinder must be completely filled with water, as recommended at the beginning of the experiment.

13. Continue the experiment and measurements according to recommendations above.
14. Measure a volume of gaseous products produced every 2 min within time interval of 10 min. Write the results to *Table 18*.

Table 18

Time, min	*Volume of gaseous product, V, ml*	*Gas producing rate, F(t), ml/min*
42		
44		
46		
48		
50		

D) Identification of gaseous products with $Ca(OH)_2$

1. When the measurements are finished take off the rubber tube and put it into a beaker with 10–15 ml of saturated $Ca(OH)_2$ solution.
2. Swirl the reactor for 60 s for transferring the gases through $Ca(OH)_2$ solution. Observe the changes in a solution of reagent.

E) Reaction with bromothymol blue indicator

1. Take off the rubber tube from $Ca(OH)_2$ solution and transfer the gases through indicator solution.
2. Swirl the reactor for 60 s for transferring the gases through indicator solution to observe the color changes in a solution of reagent.
3. Calculate gas producing rate, *F(t) (ml/min)*, according to equation:

$$F = \frac{V_i - V_{i-1}}{\Delta t}$$

where V_i – volume of gas produced within time *t (ml)*;
V_{i-1} – volume of gas fixed at previous measurement *(ml)*;
Δt – time interval between the measurements *(2 min)*.

4. Plot the curve using data from *Tables 17* and *18*:

Plot A: dependence of gas produced *V (ml)* from reaction time *t (min)*.
Plot B: dependence of gas producing rate *F (t) (ml/min)* from reaction time *t (min)*.

5. Using the results of experiment and chemical reaction of fermentation process, calculate a mass of $C_6H_{12}O_6$ which converts to CO_2 and ethanol during first 40 min of reaction. For calculations use the follow data: $Ar_C = 12.00$; $Ar_H = 1.01$; $Ar_O = 16.00$; molar volume of gases 22.4 l/mol.
6. Calculate a quantity of ***unreacted glucose*** that do not convert to the products during first 40 min period of measurements. It can be done as follow:
   6.1. Select on the *Plot B* the time intervals of different fermentation stages within time range of 0–40 min:
   - *stage a* – increasing gas producing rate;
   - *stage b* – decreasing gas producing rate;
   - *stage c* – constant rate of gas producing.

At this way, put the marks for appropriate stages on the curve of *Plot B* and write each stage.

6.2. Calculate a natural logarithm of gas producing rate *(ln F)* at stage b, where gas producing rate decreases and write the results to *Table 19*.

Table 19

Time from the beginning the fermentation, min	*Gas producing rate F, ml/min*	*ln (F)*

6.3. Using *Table 19* build the dependence *ln F* from reaction time *t (min) (Plot C)*.
6.4. Draw a straight line that best fits the data points of *Plot C* (orange trend line).
6.5. Calculate the slope (A) and obtain the y-intercept (B) of the fitted line on the *Plot C*. Write the values in *Table 20*.
6.6. Linear equation of calibration curve (*fig. 38*) is:

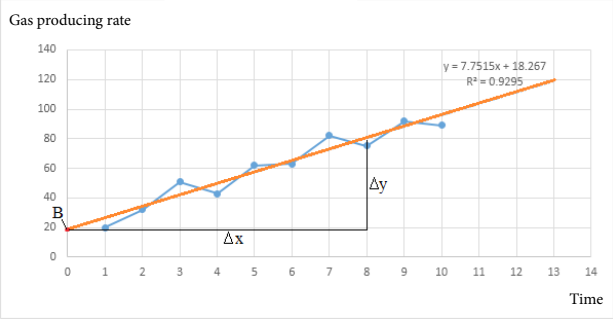


Fig. 38. Schematic curve of gas producing rate versus reaction time dependence

Table 20

Slope(A)	$A = \Delta y \div \Delta x$	$A =$
y-intercept (B)	B	$B =$
Linear equation	$ln\ F = A \times t + B$	$ln\ F =$

6.7. Theoretical volume of CO_2 V_m, that correspond to quantity of unreacted $C_6H_{12}O_6$ within 40 min time interval:

$$V_m = \frac{e^{(A \times t + B)}}{A},$$

where V_m – theoretical volume of CO_2 (ml);
A – slope of the fitting line obtained from *Plot C*;
B – intercept of the fitting line obtained from *Plot C*.

7. Calculate the amount of glucose that corresponds to V_m volume of CO_2. The mass of residual glucose in the reaction flask: $m_{gluc\ res}$ = _____ g.
8. Calculate the mass of $C_6H_{12}O_6$ that undergoes of fermentation process during first 40 min reaction interval. $m_{gluc\ ferm\ 40\ min}$ = _____ g.
9. Calculate the mass of CO_2 that produces from glucose during full period of measurements. $m_{CO_2\ full}$ = _____ g.
10. Calculate the mass of CO_2 that collects in the cylinder within first 40 min of reaction. $m_{CO_2\ ferm\ 40\ min}$ = _____ g.
11. Calculate the mass of CO_2 dissolved in reaction flask (solubility of CO_2 in a water of the cylinder equal to 0). $m_{CO_2\ sol}$ = _____ g.
12. Calculate a solubility of CO_2 in reaction flask. x_{CO_2} = _____ g/l.
13. Calculate the amount of C_2H_5OH produced during first 40 min of experiment. $n_{C_2H_5OH\ ferm\ 40\ min}$ = _____ mol.
14. Calculate a concentration of C_2H_5OH in reactive flask produced during first 40 min of experiment. $C_{C_2H_5OH\ ferm\ 40\ min}$ = _____ g/100 ml.
15. Gas producing rate decreases at stage b (*Plot B*) that explained by few reasons. The one of them – poisoning effect of ethanol on yeast. Compare calculated concentration of C_2H_5OH with toxic concentration threshold for yeast (14 g/100 ml) and evaluate the its effect on rate of fermentation reaction.

Discussion:

1. What is the main purpose of enzymatic alcohol fermentation reaction for living organism?
2. Why is this reaction used more commonly in the laboratory to study enzymatic reaction patterns?
3. Why does the enzymatic fermentation stop at defined time from the beginning even if the excess of initial reagents use?
4. Could you explain why starch can be used for alcohol fermentation reactions instead of glucose? How can you identify whether glucose or starch was used as the initial reagent without knowing the reaction medium composition?

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NOTES

Навчальне видання

ОСНОВИ БІОХІМІЇ. ПРОТЕЇНИ

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Навчально-методичне видання призначене для поглиблення знань і проведення лабораторних робіт за темою «Основи біохімії. Протеїни». У ньому узагальнено сучасні теоретичні знання про будову та функції амінокислот і їх полімерів — протеїнів у живих організмах. Надано практичні рекомендації щодо виділення, ідентифікації і дослідження протеїнів у вигляді лабораторних робіт.

Матеріали навчального видання можуть бути використані педагогами під час проведення лабораторних робіт, розроблення дослідницьких проєктів у закладах загальної середньої і позашкільної освіти, організації роботи з дітьми та учнівською молоддю в академічних установах і наукових центрах, а також вчителями хімії і біології як допоміжне видання для організації освітньої діяльності.