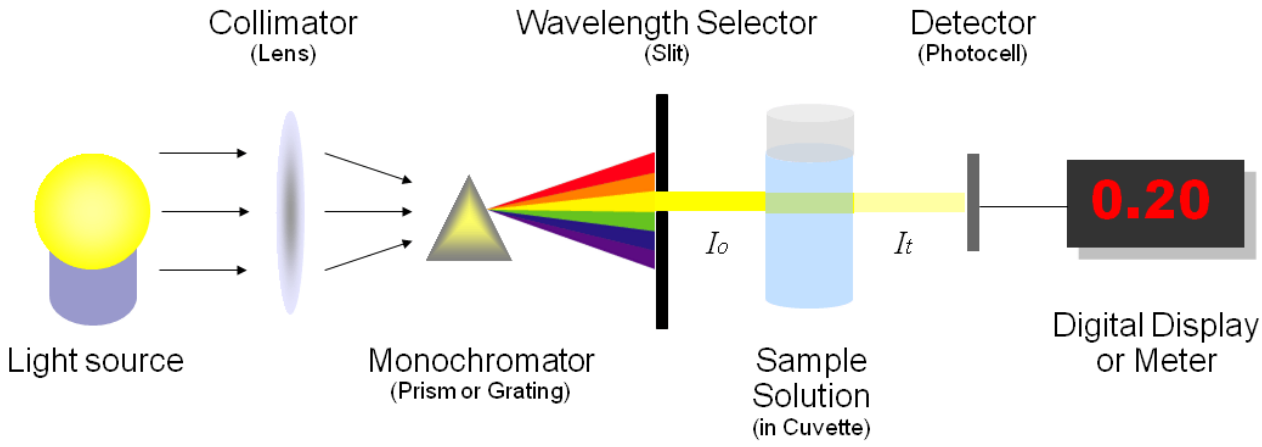
1. SPECTROMETRY

A spectrophotometer is a high-tech device necessary for measuring the spectral dependence of the degree of absorption or transmission, optical density in order to determine the concentration of various substances in solutions using electromagnetic radiation: visible, infrared, ultraviolet.

Principle of operation

Spectrometry methods involve analyzing the spectral composition of various biological materials using reflected or absorbed (passed through) electromagnetic radiation in the optical range based on their ability to reflect (absorb) different wavelengths. To do this, a comparison is made of two photostreams of optical radiation: incident on the sample and transmitted or reflected from/through the sample, see Fig. 1.1



The effectiveness of this analysis lies in the fact that all substances absorb light differently at different wavelengths. By the amount of absorbed light, you can determine the concentration of a substance and study the composition of its elements. The analysis can be carried out quantitatively and qualitatively.

Spectrophotometer device

Spectrum analyzers of different types consist of the following main elements:

• light source in the form of different types of lamps - tungsten (visible and infrared spectrum), deuterium (UV range), a combination of halogen-deuterium (ultraviolet and infrared);

• monochromator – prisms, diffraction gratings for highlighting narrow sections of the optical radiation spectrum;

• refractive, reflective, diffractive optical elements - to direct the light flux (glass, prisms, mirrors, light guides);

• compartment or cuvette for placing the test substance, solid or liquid;

• photodetector – to record the level of light radiation that passes through the sample under study;

• signal amplifier – for transmitting signals after a certain conversion for processing to a computer.

Spectrophotometers have a wide range of capabilities. They are used to measure the concentration of substances, their density, the presence of various inclusions, and identify impurities. They also determine the possibilities and rate of change in indicators when modifying the composition. Often used for accurate color classification and spectral analysis.

Analytical methods in spectrometry

Construction of calibration curves

In the process of constructing calibration curves, the student is taught techniques for dilution, measurement, and plotting. The student begins to understand the importance of standard solutions and calibration curves, which is classically constructed as a dependence of the optical densities of the colorimeter and the concentration of the analyzed compound (see Fig. 1.2) has 3 zones:

1. Non-sensitive zone - rarely present in real experiments

2. Directly proportional zone (first order reaction)

3. Zone off scale (zero order reaction)

1 zone, when there is not enough sensitivity for a particular compound and with increasing concentration of the compound, the optical density either does not increase or increases disproportionately.

Zone 2 is a directly proportional relationship, mathematically expressed as y=ax+b. This zone, the so-called working zone-2, is a first-order reaction. That is, with increasing concentration of a particular compound, the optical density value of OD increases proportionally. It is explained to the student that both optical densities and concentrations of various compounds should not exceed the parameters of zone-2. The figure shows that during a particular experiment, OD values can vary from 0.6 to 1.7 times the absorption rate, and from 1.1 to 2.15 times the concentration or amount of the compound being tested. All values obtained above or below zone 2 are incorrect for calculations.

Zone 3, if the indicators (of the analyzed compound) are higher than those in zone 2, the values are in zone 3. In this zone, as in zone 1 (due to the fact that this section is not directly proportional), it is not correct to calculate the values . In case of increased experimental indicators, the sample is diluted until the optical density indicators are within zone 2.

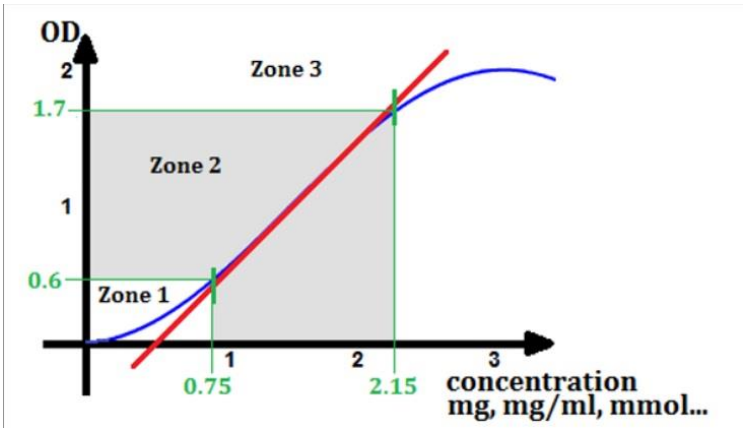


Figure 1

Determination of the concentration of biologically active compounds

The workshop includes methods for determining the concentration of protein and glucose in a biological extract (in this case, milk hydrolyzate, and hydrolyzate of (condensed) milk concentrate). The values of protein and glucose in milk hydrolyzate should be in the second zone. Considering that in this zone the dependence is directly proportional, calculations can be carried out using the following methods: Graphical determination (used when the calibration curve is stable and there is no danger of its shift, also when reagent is being saved). Measurements with a standard solution (used if there is a risk of a curve shift or change in slope). According to the data of the standard solution, if the OD value of the sample or standard solution is in zone-2, then the calculation is carried out by the chemical proportion:

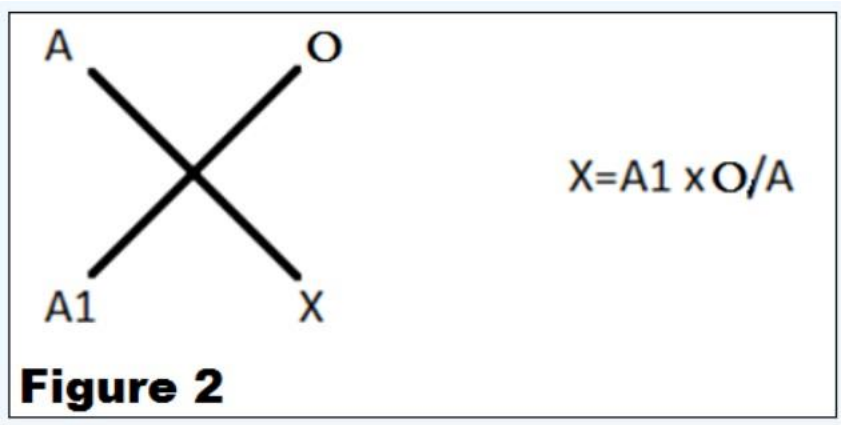


Figure 2

where A is the optical indicator of the standard solution, O is the concentration of the corresponding standard, A1 is the optical indicator of the desired solution, X is the desired value, concentration, of the desired solution. Example. The optical density of the standard solution should correspond to 1.7 OD (see Fig. 1.3). Let's assume that the OD of the test solution is 0.6, then according to the proportion, X=0.6 X2.15/1.7. The resulting value X=0.75 corresponds to the graphical indicator. Calculation using factor F (used in cases where the reagent is very stable). Calculation using a factor is a simplified calculation method, with a standard since the final formula in the calculation, with the standard is X = A1 * O/A where O/A are the parameters of a known standard solution, and dividing one by the other we get the factor -F t. e. the standard solution is measured once per series of sample measurements. Factor (F) O/A=F is calculated. The resulting sample OD value is simply multiplied by the vial (F). For an example, see fig. 2. The OD of a standard solution is measured, for example 2.15 mg/ml. The optical density of the standard solution should correspond to 1.7 OD (see Fig. 1.3). The factor F=2.15/1.7, F=1.26 is calculated. Let us assume that the OD of the test solution is 0.6. Then according to the proportion X=0.6*F i.e. X=0.6*1.26 i.e. X=0.75. The resulting value of 0.75 corresponds to the graphical indicator and calculated with the standard. All three calculation options are valid if the experimental data are in zone-2, see Fig. 2. Indicators of concentrated condensed serum will be in zone 3. Therefore, this sample is diluted four (4) times with saline solution and the measurement is performed again.

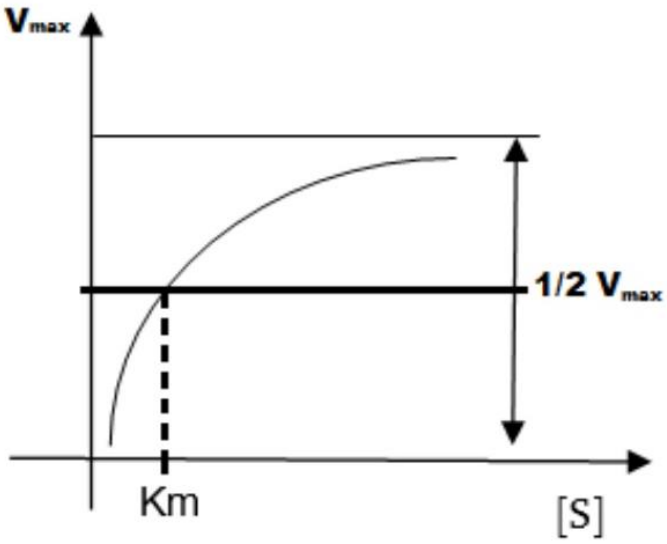
Due to the fact that the sample is diluted 4 times, after calculations the resulting value is multiplied by the number of dilutions, i.e. 4. Calculations can be carried out by any of the three methods described above.3

Enzymology

Determination of enzyme-substrate dependence and Michaelis-Menten constant (Km)

A student, at this stage of development, can measure glucose, which is a product of the hydrolysis of sucrose, and measure protein, that is, the enzyme that catalyzes the reaction. Accordingly, the concept of an enzyme is introduced. In this case, invertase, the substrate of which is sucrose. But the student is not yet ready to discuss activity, since the main problem of the relationship between the enzyme [E] and the substrate [S] is not yet clear to him. The main postulate is the relationship [S]=10Km. Therefore, before we measure activity, the student must determine Km, calculate the exact concentration of the substrate, time, temperature of reaction, i.e. must simulate a method for determining activity and only then a measurement called activity is carried out. An excess concentration of sucrose is taken, an enzyme is added, and in a minimum period of time (the time to achieve maximum detection of the product), glucose is measured as a product. In subsequent experiments, sucrose is diluted (the enzyme concentration and time do not change) until the reaction rate begins to decrease. Having reached the indicators of reducing the rate of the invertase reaction due to a decrease in the concentration of the substrate, a graph of the dependence of the reaction rate on the concentration of the substrate is plotted. The resulting dependence is depicted by the Michaelis-Menten equation (Km), (See Fig. 1.5).

Fig 1.5



The Michaelis-Menten equation arises from the general equation for the enzymatic reaction: E + S ↔ ES ↔ E + P, where E is the enzyme, S is the substrate, ES is the enzyme–substrate complex, and P is the product. Thus, the enzyme combines with the substrate to form an ES complex, which in turn is converted into a product, preserving the enzyme. Km is the Michaelis-Menten constant, which shows the concentration of the substrate when the reaction rate is equal to half the maximum rate for the reaction. It can also be thought of as a measure of how strong a substrate complex is for a given enzyme concentration, otherwise known as its binding affinity. An equation with a low Km value indicates greater binding affinity, since the reaction will approach Vmax faster. An equation with a high Km means that the enzyme does not bind as efficiently and Vmax will only be reached when the substrate concentration is high enough to saturate the enzyme.

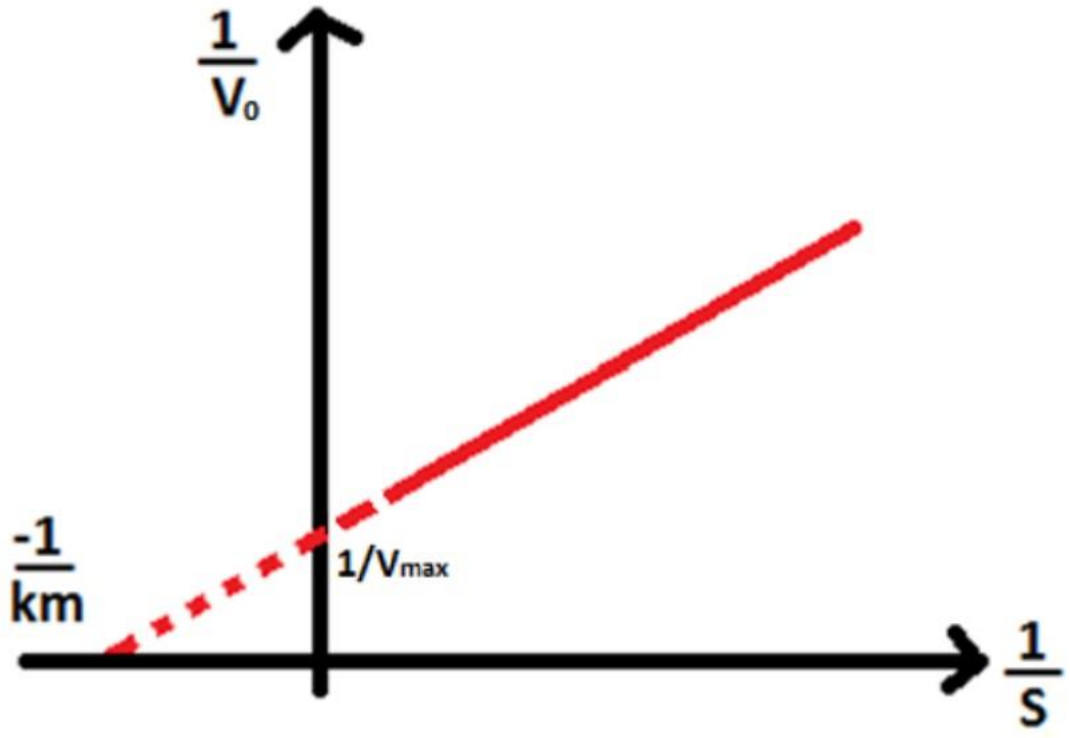
The graph of the Michaelis-Menten equation infinitely approaches the value of Vmax but never crosses it. Therefore, it is not always legal to determine Km from the dependence of Vmax on [S].

Taking the reciprocals of both sides of the Michaelis-Menten equation, we get:

$$\frac{1}{V_0} = \frac{K_M}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

To determine the values of Km and Vmax, the Michaelis-Menten equation can be converted into the Lineweaver-Burk equation (Fig. 1.6).

Fig. 1.6

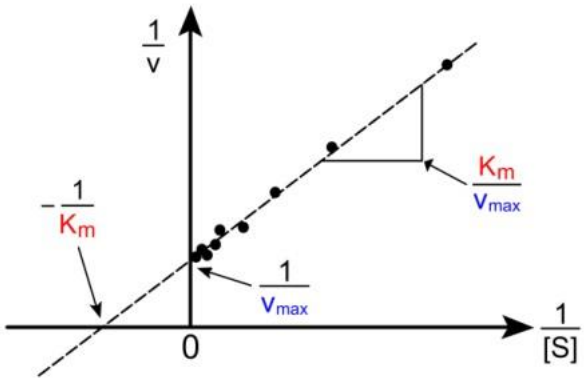


Lineweaver-Burk graph

The graph of the equation of inverse values on the abscissa and ordinate axes, also called the Lineweaver–Burk equation (Figure 1.6 and Figure 1.7): 1/Vo of 1/[S]intercept with the axis. 1/Vo - gives the value 1/Vmax; and the intersection with the X axis - gives the value -1/Km; and the slope is Km/Vmax. The Lineweaver–Burk plot is particularly useful for analyzing how enzyme kinematics change in the presence of inhibitors, competitive, noncompetitive, or a mixture of both. The value of Km is graphically calculated from the Lineweaver–Burk relationship. Extrapolation to the 1/[S] axis at the intersection point gives the exact value 1/Km. From the condition that the substrate concentration [S] must be equal to 10Km, that is, [S]=10*Km. We calculate the substrate concentration and simulate an experiment to determine the enzymatic activity of invertase.

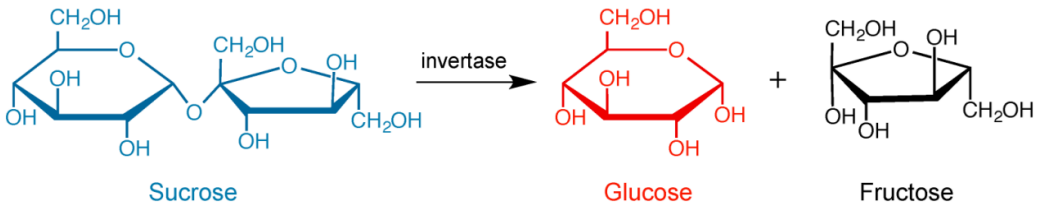
To calculate Km, we will use the Lineweaver-Burk relation (see Fig. 1.7), from which we will find the value 1/Km. Then we calculate the value of Km itself.

Figure 1.7 The Lineweaver-Burk Graph



$$\frac{1}{V} = \frac{Km}{Vmax} * \frac{1}{S} + \frac{1}{Vmax}$$

Determination of enzymatic activity of invertase



In the proposed kits, Km obtained experimentally should vary within 15-20. Accordingly, the maximum substrate concentration [S] = 10*Km for determining activity will be 200 mg/ml. A calculated substrate concentration of 200 mg/ml is taken, and the time for determining activity is selected so that the resulting product (glucose) is in zone 2 (in this case, 10 minutes) with the maximum value.

Calculation of volumetric enzymatic activity (enzyme activity in volumetric terms):

1.1 in the case of measuring changes in an indicator characterizing enzymatic activity,

according to the point principle (measurement before and after a certain time interval):

Units/ml = (Δ*V*Df*10000)/(t*ε*l*v)

cat/ml = (Δ*V*Df*)/(t*ε*l*v*1000),

Where:

Δ – measured value characterizing enzymatic activity (change

optical density, fluorescence intensity, etc.)

V – volume of the reaction mixture in which the enzymatic reaction occurs, ml

Df – Coefficient (dilution factor) of the test solution (sample) of the enzyme

t – Time after which the change in the measured parameter is recorded (min. in the case of expressing activity in U/ml and s. in the case of expressing activity in cat/ml)

ε – Molar absorption coefficient, l*mol-1*cm-1 (M-1*cm-1)

l – Optical path length, cm

v – Volume of enzyme solution, ml

MEASUREMENT - ε – Molar absorption coefficient, l*mol-1*cm-1 (M-1*cm-1)

BIERE-LAMBERT law: ε=A/C *I

Where :

C – prepared or known MOLAR solution of product or substrate

enzymatic reaction (conventionally 0.01 M – product solution).

A is the absorption of solution C at a specific wavelength. (relatively 0.6)

I - Optical path length, cm (conditionally 1 cm)

ε – Molar absorption coefficient, l*mol-1*cm-1 (M-1*cm-1)

a solution of the enzymatic reaction product of a certain MOLARITY is prepared (conventionally 0.01 M - solution of the product), solution C. The absorption (A) of solution C is measured at a certain wavelength. Conventionally, we obtain the values - 0.6. Considering that the length of the optical path is conditionally 1.

• Specific activity of a protein is calculated by dividing the U/ml value by mg/ml of protein.

The final value after division is U/mg – protein – specific activity.

• Total activity is calculated by multiplying the specific activity by the volume of enzyme solution.

Training in the method of protein precipitation and salting out

Comparison of total and specific activities

Most often in enzymology, in the first stages of enzyme purification, salting out or precipitation is used. This is most often due to fractionation and purification problems. The purpose of the laboratory workshop is to familiarize yourself with salting out and precipitation methods.

Hydrolysis of sucrose

One of the most common tasks in biotechnology is the hydrolysis of a particular substrate to the final product. In this case, the question can be posed as follows: remove sucrose from the solution. From the point of view of an industrial problem, the question may be as follows: to obtain fructose, glucose or glucose-fructose syrup (inversion, sugar); problems encountered in the analytical field are to conduct a quantitative analysis of sucrose. All of the above problems are associated with the hydrolysis of sucrose. The problem may be enzyme inhibition, inactivation, etc. The very formulation of this question includes the optimal selection of optimal conditions for the hydrolysis process, that is, determining the time, pH and temperature of hydrolysis, determining the concentration of the enzyme and substrate, and most importantly, determining the maximum depth of hydrolysis. That is, under what conditions does the maximum transformation of sucrose into invert sugar occur? The task in the proposed workshop is set as follows: to convert sucrose into hydrolysis products as much as possible, with minimal enzyme consumption, to achieve maximum transformation of sucrose into hydrolysis products, and also to achieve hydrolysis of sucrose in the minimum time. In this experiment, we a priori assume that parameters such as pH temperature, enzyme concentration, and substrate are optimally selected. For variable parameters, we leave the question of time, i.e., during what time the

selected enzyme concentration and substrate at pH - 4.5, temperature 55 °, will carry out maximum hydrolysis of sucrose.

Chromatography

Thin layer chromatography

Thin layer chromatography (TLC) is a simple, fast and inexpensive procedure that allows you to quickly obtain an answer to the content of a particular component in an unknown solution. TLC is also used to determine the identity of a compound, in which case the movement of the compound from the initial position to the point of final movement is expressed in centimeters and denoted by Rf. The Rf of the unknown substance is compared with the Rf of the known compound (marker); equality indicates that it is identical to the test sample and the marker. Preferably, both samples are analyzed on the same TLC plate. The TLC plate may be a glass, metal or plastic material coated with a thin layer of solid adsorbent (usually silica).

In thin layer chromatography, a small amount of the mixture to be tested is placed on the bottom of a plate. The TLC plate is then placed in the solvent pool chamber so that only the bottom of the plate is immersed in the liquid. This liquid or eluent is the mobile phase and slowly wicks up the TLC plate by capillary action. As the solvent moves, for each component of the mixture, an equilibrium is established between the molecules of this component adsorbed on the solid surface and the molecules in the solution. The components differ in solubility and strength of adsorption on the adsorbent. Some components will travel further on the plate than others. When the solvent reaches the top of the wafer, the wafer is removed from the chamber, dried, and stained to visualize the components. If the connections are colored, visualization is easy. The compounds are usually not stained, so an ultraviolet lamp is used to visualize the plates. Often the plate itself contains a fluorescent dye that glows everywhere except where the organic compound is on the plate.

The Rf value is determined by the retention factor, or Rf is defined as the distance traveled by the compound divided by the distance traveled by the solvent.

Rice. 2.1.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

Figure 7

For example, if the distance of movement of the compound is 2.1 cm and the solvent is 2.8 cm, Rf will be equal to 0.75: (see Fig. 2.2)

Rice. 2.2.

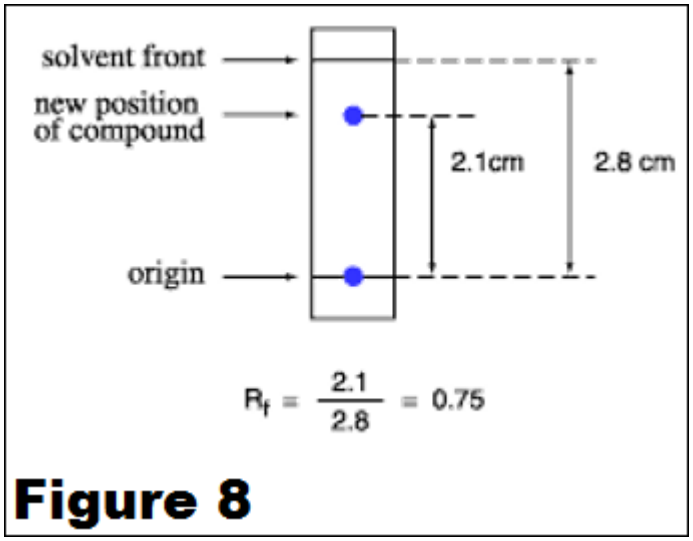


Figure 8

The Rf of a compound is constant from one experiment to the next only if the following chromatographic conditions are also constant:

• Solvent system

• Adsorbent

• Adsorbent thickness

• Entered sample amount

• temperature

Because these factors are difficult to maintain from experiment to experiment, relative Rf values are usually assumed. "Relative Rf" means that the values are relative to a standard, or it means that you are comparing Rf values on the same wafer at the same time. The higher the Rf of the compound, the greater the distance the sample travels on the TLC plate. When comparing two different compounds studied under the same chromatographic conditions, the compound with a larger Rf is less polar because it reacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structure of the compounds in a mixture, you can predict that a compound with low polarity will have a higher Rf value than a polar compound on the same plate. Rf can provide supporting evidence regarding the identity of a compound. The samples and the reference standard are placed side by side on a TLC plate. If the test substance has the same Rf value as the standard, then it is most likely (but not necessarily) the same compound. If they have different Rf values, they are definitely different compounds. Note that this identity test must be performed on a single plate since it is difficult to accurately reproduce all factors affecting Rf from experiment to experiment.

Problems with using TLC and how to solve them

Examples of common problems in TLC:

• The joint rises as a streak rather than a patch: the sample may have been overfed. This requires diluting the sample. Or the pattern may simply contain many components, creating many spots that rise together and look like a stripe. In such cases, the experiment may not go as expected.

• The sample moves in a smear or upper crescent shape: Compounds with strong acidic or basic groups (amines or carboxylic acids) sometimes cause these shapes on the TLC plate. In such cases, a few drops of ammonium hydroxide (amines) or acetic acid (carboxylic acids) are added to the solvent.

• The sample descends in a descending crescent shape: The adsorbent has probably broken down at the point of introduction of the sample, resulting in the formation of a crescent shape.

• The front of the plate dissolver lands unevenly: either the adsorbent has fallen on the sides of the plate, or the sides of the plate are touching the walls of the container (or the paper used to impregnate the container). Sloping areas make it difficult to accurately measure Rf values.

• There are a lot of random stains visible on the plate: it is important not to accidentally drop organic compounds onto the plate.

• Blue spots appear on the plate during the test: an ink pen may have been used to mark the starting point instead of a pencil.

2.2 Gel filtration chromatography

Gel filtration chromatography is a molecular weight separation method. It is also called molecular sieve or gel permeation chromatography. In gel filtration chromatography, the stationary phase consists of porous beads with a well-defined range of pore sizes. Stationary phase gel filtration has a fractionation range, meaning that molecules within that molecular weight range can be separated. Proteins small enough can fit into all the pores, in which case the proteins have access to both the mobile phase inside the beads and the mobile phase between the beads. Proteins that are too large to fit into any pore are eluted with a buffer solution. They only have access to the mobile phase between the beads and therefore elute first. The medium-sized proteins are partially embedded in the pores, meaning they can fit into some but not all of the pores of the beads. These proteins are then separated into large ("washed out") and small ("fully incorporated") proteins.

Consider the corresponding column fractions for glutamate dehydrogenase (MW 290,000), lactate dehydrogenase (MW 140,000), serum albumin (MW 67,000), ov albumin (MW 43,000) and cytochrome (MW 24,001). Bio-Gel P-150 (fraction range 15000 – 150000). When a protein mixture is applied to a column, glutamate dehydrogenase elutes first because it exceeds the upper

fractionation limit. It is therefore completely washed out from the inside by the porous stationary phase and follows the so-called dead volume (V0). Cytochrome c is below the lower fractionation threshold, completely penetrates the pores and elutes at the end. Other proteins partially penetrate the pores of the gel and elute in accordance with the decrease in molecular weight. These separations can be described by the equation where Vr is the volume of protein retained, V0 is the volume of the mobile phase within the column, between the stationary phase beads (sometimes called dead volume), Vi is the volume of the mobile phase inside the porous beads (also called total volume), and K is distribution coefficient (determines the ability of the protein to penetrate the stationary phase, values vary from 0 to 1). In the above mixture of proteins, the partition coefficient (K) for glutamate dehydrogenase will be 0 (as completely permeable), K = 1 for cytochrome c (as completely permeable), and K will range from 0 to 1 for other proteins falling within the fractionation range columns.

In practice, gel filtration can be used to separate proteins by molecular weight. It can also be used to replace the protein buffer system.

2.3 Ion exchange chromatography

Ion exchange chromatography is the reversible adsorption of charged molecules by immobilized ionic groups on a matrix of opposite charge. Separation can be achieved by adsorption of samples and subsequent replacement of them on the matrix with anions or cations.

In equilibrium, exchange groups are associated with counterions. After reaching equilibrium, a sample is added, the molecules of which undergo a charge exchange, replacing counterions reversibly, i.e. connect to the matrix. The unbound sample will pass through the dead volume column. Substances are removed from the column by increasing the ionic strength of the buffer. The ion exchange material is an insoluble matrix to which groups with a specific charge are covalently attached. Negatively charged carriers bind positively charged ions (cations). When a second type of ion exchange carrier is present, it can replace and/or exchange the negative charge. These resins are called anion exchangers.

Anion exchange resins are positively charged and bind and/or exchange negatively charged ions (anions). Some groups of side chain amino acid residues in proteins are ionizable (for example, lysine or glutamic acid), as are the N-terminal amino and C-terminal carboxyl groups, so proteins are charged molecules. This function can be used to separate various proteins using ion exchange chromatography.

To separate proteins, two ionic groups can be used covalently attached to a polymer such as cellulose: carboxymethylcellulose (CM-cellulose) and diethylaminoethylcellulose (DEAE-cellulose). CM cellulose has a carboxymethyl functional group - CH2OCH2COOH. At neutral pH, the carboxymethyl group ionizes as - CH2OCH2COO, so cellulose CM has a negative charge,

so it is a weak cation exchanger. DEAE cellulose contains a diethylaminoethyl group. It is positively charged at neutral pH, so DEAE - cellulose is a weak anion exchanger.

The separation of proteins using ion exchange chromatography depends on the difference in charge of different proteins. The charge of a protein depends on the number and type of ionized amino acid side chain groups. Lysine residues, when ionized, have a positively charged side chain group, whereas glutamic acid residues have a negatively charged side chain group. The group has a different pKa; that is, the pH at which it dissociates halfway. Thus, the total number of charges on a particular protein at a particular pH will depend on the number and type of ionized amino acid side chain groups. Since by definition different proteins have different amino acid compositions, they will have different charges at a given pH and can therefore be separated based on this principle. Every protein has an isoelectric point (pI) where, at a certain pH, the total number of negative charges is equal to the number of positive charges, and so under these conditions it has no charge. pI is the isoelectric point of proteins. When the pH of the protein in the buffer is below the pI, the protein does not bind to the ion exchange resin. Below this pH value, the protein will have a net positive charge and bind to the cation exchanger, and above this pH value it will have a net negative charge and bind to the anion exchanger. In principle, either a cation exchanger or an anion exchanger is used to bind the protein. In practice, proteins are stable and functionally active over a fairly narrow pH range, so the choice of ion exchanger is often dictated by the pH stability of the desired protein. For CM cellulose, the counterion is usually Na+, and for DEAE cellulose, the counterion is usually Cl.

Once a suitable resin (gel) has been selected, it is mixed with a buffer to form a suspension that can be loaded onto a suitable chromatography column. The pH of this starting buffer is critical because it determines the charge of the proteins released. The initial pH of the buffer must be at least one pH unit above or below the pI of the protein that will bind to the resin to ensure adequate binding. However, it should be noted that CM cellulose and DEAE cellulose are examples of weak ion exchangers. A weak ion exchanger is one that ionizes only within a limited pH range. Thus, DEAE cellulose begins to lose charge above pH 9, and CM cellulose begins to lose charge below pH 5. The term "weak" does not refer to the strength of the binding of ions to the resin, nor does it refer to physical strength. The effective starting pH range when using DEAE cellulose or CM cellulose is only pH 5-9. In addition to choosing the correct pH of the starting buffer, you must consider its ionic strength. The affinity of proteins for ion exchange resins decreases with increasing ionic strength. Indeed, this property is exploited by one of the methods for eluting bound proteins.

3. Electrophoresis

The electrophoresis method, proposed at the beginning of the twentieth century, is now widely used in biology and medicine to separate proteins for research and clinical purposes. Using electrophoresis, a protein mixture can be separated into individual components, which makes it possible to determine the molecular weight of the protein or its subunits and confirm the purity of the isolated protein.

The result of electrophoresis is an electropherogram-pattern obtained after separation of a complex mixture using electrophoresis and specific manifestation. An electropherogram of proteins in human biological fluids (blood serum, urine, cerebrospinal fluid, etc.) allows doctors to obtain significant diagnostic information.

For example, in a healthy person, the relative content of protein fractions when determined in blood serum by paper electrophoresis is as follows:

Albumin - 55-65%,

$\alpha 1$ – globulins - 3-6%,

$\alpha 2$ – globulins - 7-10%,

β – globulins - 7-12%,

γ – globulins - 13-19%.

In many diseases, a change in the ratio of fractions is observed, while the total amount of protein usually changes little. Detection of these changes using electrophoresis is widely used for diagnostic purposes.

Electropherograms of enzyme proteins (zymograms) make it possible to study changes in the activity and isoenzyme spectrum of such proteins under the influence of external and internal factors in both humans and other organisms. The obtained data is used in medicine, biotechnology, various sectors of agriculture and the food industry.

BASIC TERMS AND DEFINITIONS

Electrophoresis is the movement of charged particles in a solution under the influence of an electric field.

An electropherogram is a picture obtained after the separation of a complex mixture using electrophoresis and specific manifestation.

The electrophoretic method in biochemistry is a method of spatial separation of molecules having different charges and sizes by placing them in an electric field.

The electrophoretic mobility (u) of a given molecule is the speed of movement of a charged molecule (expressed in cm/h) in an electric field of 1 V/cm.

The isoelectric point is the pH value of the medium (denoted as pI) at which the positive and negative charges of ionized groups are compensated, so the charge of the entire protein molecule is zero.

Zonal electrophoresis - electrophoresis is carried out at a constant (not changing) pH value of the buffer solution filling the given medium (paper, gel, etc.). The sample under study is applied as a spot or a thin layer onto the carrier, along which it moves in an electric field.

Polyacrylamide gel (PAGE) is a product of copolymerization of acrylamide (creating a linear "base") and N, N′ - methylene bisacrylamide (serving for cross-linking linear chains).

3.1 The principle of the protein electrophoresis method.

Electrophoretic mobility

In solution, proteins are in the form of charged particles. The charge on the surface of proteins arises as a result of the dissociation of groups located in the side radicals of amino acids (carboxyl, amino, imidazole, etc. groups), as well as during the binding of ions. Since the degree of dissociation of groups depends on the pH of the solution, the magnitude and sign of the total charge of the protein molecule depend on the pH of the medium, as well as on the ionic strength (the intensity of the electric field created by the ions in the solution). To calculate ionic strength, you need to find the product of the concentration of each ion and the square of its charge, add all the resulting values and divide the total in half. If two or more electrolytes are present in a solution, the total total ionic strength of the solution is calculated.

For each protein, there is a pH value of the medium (denoted as the pI isoelectric point) at which the positive and negative charges of the ionized groups are compensated, so the charge of the entire protein molecule is zero. In a buffer with a pH equal to the pI of the protein being studied, the lack of charge on the protein molecule makes it impossible for it to move in an electric field. Due to differences in amino acid composition, different proteins have different pI values.

At pH≠pI, protein molecules acquire a charge and, under the influence of an electric field, move to an oppositely charged electrode - cathode (-) or anode (+). For example, acidic proteins rich in monoaminodicarboxylic amino acids (asp, glu) in a weakly alkaline buffer will acquire a negative net charge due to the dissociation of COOH groups to COO- and H+ and will move towards the anode. For electrophoretic separation, the optimal pH value of the working buffer is one that determines the maximum difference in the charges of the different proteins that make up the initial mixture, and not their maximum charge. Typically, electrophoresis is carried out in a medium (buffer) with a pH value that differs by 3 - 4 units from the average pI value for proteins of a given type. This makes it possible to achieve good electrophoretic mobility (see below) and at the same time maintain noticeable differences in charge between the molecules. It is preferable to use a buffer of known and constant ionic strength based on singly charged ions. The working buffer also requires a significant capacity, since the local concentration of protein in the areas of molecular accumulation formed during the separation of the mixture can be significant. Therefore, buffers with a concentration of at least 0.1-0.2 M are used.

When carrying out electrophoresis, an electric field is created using a power source - a stabilized rectifier capable of producing an adjustable voltage of up to 500 - 1000 V at a current of several tens of milliamps (mA). It is preferable to use a current-stabilized rectifier.

Classification of electrophoretic methods

The main types of electrophoresis are:

- Zonal electrophoresis,

- Isotachophoresis

- Isoelectric focusing

- Immunoelectrophoresis

Zonal electrophoresis is carried out at a constant (not changing) pH value of the buffer solution filling the given carrier (paper, gel, etc.). The sample under study is applied as a spot or a thin layer onto the carrier, along which it moves in an electric field. A more complicated version of zonal electrophoresis is disk electrophoresis (multiphase zonal electrophoresis), in which pH and other characteristics that are constant within one "phase" change abruptly when moving to another "phase." The principles of disk electrophoresis are discussed in section 1.3.

Isoelectric focusing creates a smooth pH gradient in the electrophoresis medium. The protein stops at a zone where the pH value is equal to its isoelectric point (pI). To create a pH gradient, a solution of polyamino-polycarboxylic acids is usually used, with which the carrier is saturated. In the absence of an electric field, this mixture usually has a pH of 6.5. When an electric field is applied, these acids provide a linear pH gradient from 3 to 10.

In the case of isotachophoresis, charged ions are initially separated according to their charge and mobility, and then move in an electric field at equal and constant speeds.

Immunoelectrophoresis combines electrophoretic separation of proteins with immunoprecipitation based on the antigen-antibody reaction. This type of electrophoresis is superior to others in sensitivity and resolution.

By purpose they distinguish:

- analytical (for analyzing the composition of a mixture, less often - for obtaining small quantities of separated substances) electrophoresis,

- preparative (for obtaining drugs - significant quantities of pure substances) electrophoresis.

Based on the degree of denaturation of the separated proteins, they are distinguished

- native electrophoresis,

- electrophoresis under denaturing conditions.

Unlike native electrophoresis, electrophoresis under denaturing conditions involves the use of chemical reagents that destroy the spatial structure of the separated proteins.

According to the direction of fractionation, electrophoresis is distinguished, in which proteins move in one direction, and two-dimensional electrophoresis, in which separation is first carried out in one direction, and then in a direction perpendicular to the first. Two-dimensional electrophoresis can dramatically increase resolution when separating mixtures consisting of a large number of different proteins.

Depending on the orientation of the carrier (gel, paper, etc.), electrophoresis can be vertical or horizontal.

The classification according to the type of liquid phase carrier is presented in Table 1. It also reflects the development of electrophoretic methods in a historical aspect. Electrophoresis was the first to be developed without any carrier: the electrical circuit between the electrodes was closed through a buffer solution, in which the proteins were separated. Later, in electrophoresis, liquid phase carriers—polymers—that served as a "framework" for the buffer began to be used. The use of carriers made it possible to significantly reduce convection (mixing) and, consequently, improve the quality of protein separation. The carrier can be in the form of a powder, film, gel, etc. Subsequent developments were devoted to improving the properties of the carriers.

The ideal carrier should:

a) sharply reduce convection;

b) be easy to prepare;

c) have high thermal conductivity (with low thermal conductivity it is difficult to cool the system);

d) have a low adsorption capacity and chemical inertness towards substances subjected to electrophoresis;

e) have no charge on the surface of the particles, so as not to cause endo-electroosmosis. If the separated proteins are negatively charged, then during electrophoresis they should move towards the anode (+), but endo-electroosmosis "pulls" them in the other direction, towards the cathode (-), interfering with electrophoretic separation.

Gels easily take on different geometric shapes, so the name of the electrophoretic method using them indicates the configuration of the working space. Electrophoresis gel can be polymerized:

- in tubes,

- in capillaries,

- in plates ("slabs" – from the English slab),

The main disadvantage of tube electrophoresis is the lack of heat transfer: the temperature in the center of the gel cylinder is higher than that of its surface adjacent to the glass. This leads to bending of the protein zones. One test sample is applied to one tube.

The heat outflow can be increased by using very thin tubes - capillaries. Thin plates also achieve much more efficient heat removal than tubes. Thus, with air cooling, effective heat removal is possible with a current of 50 - 100 mA on a vertically located plate (that is, the power dissipated in the form of heat does not exceed 20 W). In addition, the plate configuration allows, under absolutely identical conditions, to separate several (10 - 13) protein samples at once. The plates are easy to scan and convenient to cut. Compared to cylindrical gels, gel sheets can significantly reduce the protein concentration in the applied sample.

Advantages and disadvantages of using different media in electrophoresis

Method name, medium

Advantages of the method and/or medium

Flaws

Electrophoresis with a moving boundary (in free solution). There is no carrier. The first electrophoretic method that made it possible to separate proteins. It is difficult to avoid convection-mixing of the separated zones; the study requires a sample of tens of mg of protein; resolution is low (no more than 8 components in a sample).

On filter or chromatography paper (50s of the XX century) Reduced convection, separated zones can be fixed and colored. The equipment is simpler. Opacity. Contamination and unevenness of the paper interfere with separation. "Tails" in electropherograms due to high adsorption capacity. The background is colored, making it difficult

recognition of protein zones.

On cellulose acetate films (known since 1957) Fast, requires less sample for analysis. Low adsorption capacity helps to avoid the appearance of "tails" on the electropherogram. After staining, the background remains colorless. Suitable for

immunoelectrophoresis. Opaque in aqueous solutions (can be achieved by immersing in mineral oil). More expensive than using paper. Not very suitable for preparative electrophoresis.

In starch gel

(suggested by O. Smith) The first carrier with molecular sieve properties. Actively prevents convection. Increases resolution. Low transparency, fragility, pore size can be changed only within small limits. Preparing a high-quality gel is labor-intensive.

In agar and agarose gels Satisfactory transparency, high plasticity (easier to cut, more convenient to paint and determine enzymatic activity directly in the gel), ease of manufacture. Due to the

negative charge on the sulfate and COOH groups of the agar network, electroosmosis occurs, leading to an uneven distribution of the electric field, and sometimes hydrostatic

pressure. Chemical interaction of substances with agar is possible.

In polyacrylamide (PAGE) gel (proposed by L. Ornstein and D.

Davis) Chemically inert, can be boiled. You can set the required pore size and ensure the properties of the molecular sieve. High transparency. Easily

prepare. Elastic, durable. Today it is the best carrier, but it is prepared from acrylamide, a toxic substance.

3.2 Features of electrophoresis in polyacrylamide gel

Polyacrylamide gel (PAGE) has many qualities of an ideal carrier (Table 1). Having the properties of a molecular sieve, it provides electrophoretic separation of protein mixtures not only by charge, but also by particle size and shape. During PAGE electrophoresis, large molecules whose sizes are comparable to the diameter of the gel pores move more slowly, while small molecules pass freely and quickly through the pores of the gel.

PAGE is formed by copolymerizing acrylamide (creating a linear "backbone") and N,N'-methylenebisacrylamide (serving for cross-linking linear chains).

CH2= CH-CO-NH2 CH2= CH-CO-NH-CH2-NH-CO-CH= CH2

Acrylamide N,N'-methylenebisacrylamide

By changing the concentration of acrylamide from 2 to 50%, you can set a certain porosity of the gel. For example, the pore diameter in a gel containing 7.5% acrylamide is 5 nm, and 30% acrylamide is 2 nm. When choosing the gel concentration, take into account the average molecular weight (Mr) of the substances being separated and the shape of their molecules (see Table 2).

table 2

Selection of acrylamide concentration

With different ranges of molecular masses of the studied proteins

As a result of copolymerization, a three-dimensional gel network is formed, the structure of a fragment of which is shown in Fig. 1. Every second carbon atom of the linear chain contains an acid amide group, which ensures the hydrophilicity of the polymer. At the same time, PAGE does not contain ionized groups.

Fig. 1 Structure of polyacrylamide gel

Copolymerization requires initiators and catalysts (redox systems that are sources of free radicals). The most commonly used system is of two components, presented below:

$(NH_4)_2S_2O_8$ $(CH_3)_2N\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)_2$

Ammonium persulfate (PSA). Synonym - ammonium persulphate Function: polymerization initiator

N,N,N',N'-tetramethylethylenediamine (TEMED) Function: catalyst for the formation of PAAG

The mechanism of action of persulfate is illustrated in Fig. 2: when the O-O bond is broken, two free radicals are formed, each of which stimulates the breaking of the double bond in the acrylamide molecule and attaches to it, also forming free radicals. Each such radical, in turn, causes the cleavage of the double bond and the addition of the next acrylamide molecule to form a new radical, etc. The polymerization chain reaction continues until two radicals, having met each other, form a regular covalent bond. By the same mechanism, methylene bisacrylamide can be incorporated into the growing chain of a linear polymer by one of its terminal vinyl groups. If its second end is integrated into another linear polymer chain, then a "cross-link" is formed (shown in Fig. 1). Without a cross-linking agent, only long, thin, longitudinal fibers are formed in the gel [1].

Rice. 2 The mechanism of action of ammonium persulfate as an initiator of acrylamide polymerization

ATTENTION!

a) Ammonium persulfate gradually decomposes, in aqueous solutions - very quickly. When dissolved, persulfate produces a characteristic clicking sound. If a 1% solution of persulfate in water has a pH < 2, or no clicks are heard during dissolution, then the persulfate is not suitable.

b) Persulfate is capable of oxidizing the substances being separated, which can lead to the appearance of additional bands or inactivation of enzymes. Therefore, before the experiment, it is recommended to carry out electrophoresis without applying the sample in order to remove excess persulfate.

To formulate the gel, "master" solutions of high concentration are usually used. It is convenient, for example, to prepare a 40% aqueous solution of a mixture of monomers (T=40). Let us recall that T expresses the percentage ratio of the mass of both monomers to the final volume of their solution, and C is the ratio of the mass of NN' - methylene bisacrylamide to the sum of the masses of the two monomers. It follows that C does not change when the mother solution is diluted. So, if it is assumed that the parameters for the working gel are T = 10 and C = 2.6, then when preparing the mother solution, you can take T = 40 and C = 2.6. In practice, this means that you

need to weigh out (40X2.6)/100 = 1.04 g of methylene bisacrylamine in a 100 ml volume. The buffer stock solution can have two or five times the concentration. In the latter case, it is useful to check that the pH of the buffer is maintained at appropriate dilution. The mixture of the calculated volumes of stock solutions of monomers and buffer is adjusted to the required volume with water[2].

It is better to add ammonium persulfate in a minimal amount, which can be ignored. To do this, prepare a concentrated, usually 10%, persulfate stock solution, the remains of which must soon be thrown away, since it is not stored. The volume of TEMED added to the mixture can also always be neglected.

Native and SDS-PAGE electrophoresis

Both native electrophoresis and electrophoresis under denaturing conditions can be carried out in polyacrylamide gels.

Native PAGE electrophoresis is used to separate proteins that have not been denatured (that is, proteins in their native state), including in cases where it is necessary to preserve the enzymatic or any other functional activity of proteins. The electrophoretic mobility of a protein in its native state depends simultaneously on its total charge, molecular weight, and spatial configuration of the polypeptide chain. To establish a strict quantitative correlation between one of these parameters and the electrophoretic mobility of the protein, it is necessary to exclude the influence of all others.

In cases where it is necessary to fractionate proteins solely by molecular weight, PAGE electrophoresis is used under denaturing conditions. Such a system was developed by W.K. Laemmli. This method allows you to estimate the number of polypeptides in a protein mixture; it achieves a very clear separation of zones, but the activity of enzymes can be completely or significantly lost due to their denaturation. Denaturing conditions are achieved by treating the sample with a threefold excess of sodium dodecyl sulfate (synonym: sodium lauryl sulfate), abbreviated as SDS (Fig. 3). More often this substance is designated SDS - from the English “sodium dodecyl sulfate”. The SDS anion carries a negative charge.

Due to hydrophobic interactions, SDS anions are sorbed on proteins in proportion to their volume, turning any polypeptide into an unbranched rod with a negative charge that significantly exceeds the intrinsic charge of the protein molecule. SDS binds to most proteins approximately equally - in a ratio of 1.4 mg of SDS per 1 mg of protein. Since in the presence of SDS the size/charge ratio becomes almost the same for any protein, division occurs solely by molecular weight.

Note that for complete denaturation, proteins with S-S bonds are treated with β-mercaptoethanol, which has a strong unpleasant odor, before using SDS, so the work is carried out under traction. As an alternative to β-mercaptoethanol, dithiothreitol (C4H10O2S2, Mr =

154.25) is used; it requires 2 times less, it is less volatile and does not have such a specific odor, however, it is much more expensive.

Disc electrophoresis

Disc electrophoresis gets its name from two English words: discontinuity (heterogeneous, intermittent) and discoid (disc-shaped). The first emphasizes the heterogeneity of the electrophoretic medium used in this method. The second describes a random feature - the disc-shaped shape of the zones of separated proteins under the conditions chosen by the discoverers of the method. During disk electrophoresis, abrupt changes in the concentration (and, consequently, porosity) of the gel, pH, and voltage gradient are created.

Vertical disk electrophoresis involves the use of two and sometimes three gel layers layered on top of each other:

1) Starting gel (sample gel) – not always present. It is located on top, contains a sample and a "witness" dye, which will show the movement of the electrophoretic front. The starting gel prevents mixing of the sample solution with the electrode buffer.

2) Concentrating gel is a large porous gel. The size of its pores limits diffusion, but does not provide the gel with the properties of a molecular sieve in relation to the separated proteins. This gel is needed for electrochemical concentration of sample proteins, i.e. the concentrating gel collects the mixture of proteins before moving into the separating gel into one narrow strip.

3) Separating (separating, resolving, "running" gel) – lower fine-porous gel, in which, in fact, electrophoretic and molecular sieve separation of sample components occurs.

Disc electrophoresis is carried out in a combined buffer system - with different pH values and different component compositions in the electrode parts of the upper and lower gel (Fig. 4).

The presence of an intermittent buffer system increases the resolution of the method due to the resulting electrochemical effect, which ensures the concentration of sample components and the formation of a thin starting zone at the boundary of the concentrating and separating gels.

Buffer mixtures are selected so that the electrophoretic mobility of ions in one of them is higher than in the other. For example, in the electrophoretic system shown in Fig. 4, the electrode buffer with pH 8.3 has slower glycinate ions, and the concentrating gel buffer has faster chloride ions. Under the influence of an electric field, "fast" chloride ions rush towards the anode (+), leaving behind a space that is lean and onium, as a result of which a sharp drop in voltage is observed. As a result of the resulting voltage drop in this space, the "slow" glycinate ion begins to accelerate, catches up with the "fast" chloride ion and subsequently moves at the same speed as the fast one.

Since the mobility of the separated components of the mixture under study in an electric field is lower than the mobility of "fast" ions and higher

"slow" ions, then in space with a sharp drop in voltage they are also accelerated and at the boundary of two ion fronts they are concentrated into a thin strip.

At the interface between the concentrating large-porous gel and the fine-porous separating gel, ions are inhibited as a result of the molecular sieve effect: the higher the molecular weight, the slower the substance passes through the small pores of the separating gel. In this case, the "slow" glycinate ion is ahead of the ions of the protein sample, since the mass of glycinate is relatively small, and creates a space behind itself with a voltage drop. The "delayed" (compared to glycinate) ions of the sample are forced to move forward in this space formed by the "slow" ions. In addition, in the separating gel buffer, which has a pH of 8.8, the acidic groups of proteins dissociate and proteins of different structures acquire different negative charges, which causes them to move towards the anode at a speed proportional to the charge. As a result, due to the difference in the separated proteins both in mass and in charge, the narrow starting zone is destroyed, forming zones of individual protein components of the separated mixture in the separating gel.

THEORETICAL BASIS OF ELISA

Immunochemical methods of analysis, based on the specific binding of the compound being determined by the corresponding antibodies, have widely entered analytical practice and are used in various fields of medicine, agriculture, microbiological and food industries, and for environmental protection purposes. Indication of the resulting antigen-antibody complex can be carried out if a label is introduced into one of the initial components of the reaction system, which is easily detected by an appropriate highly sensitive physicochemical method. Isotopic, enzymatic, fluorescent, paramagnetic and other labels turned out to be very convenient for this purpose, the use of which made it possible to increase the sensitivity of classical immunochemical methods of analysis millions of times, and reduce the analysis time to several minutes.

The most widely used are heterogeneous methods of enzyme immunoassay, based on the use of polystyrene plates for the immobilization of antibodies or antigens, the specific binding of the analyte to the walls of the wells of the plate and the subsequent detection of the formed immunocomplexes using enzyme-labeled components.

5.1 Structure and properties of antigens and antibodies. Genetically foreign substances, entering the body of higher animals and humans, are capable of causing a number of specific processes in them aimed at their removal from the body. The body system that performs this function is called the immune system, and the processes themselves are called immunological. The most important of them is the formation of specific blood proteins - antibodies (immunoglobulins). Substances that can cause specific immunological reactions in the body are called antigens. The ability of antigens to cause an immune response is called immunogenicity, and the ability to form complexes with antibodies is called antigenicity. Antigens include proteins, polysaccharides, nucleic acids, both in purified form and in the form of components of various biological structures (cells, tissues, viruses, etc.).

On the surface of a complex antigen molecule, functional groups or residues that determine antigenic specificity, called antigenic determinants or epitopes, can be identified. The number of epitopes on the surface of a complex molecule determines the valency of the antigen. The concept of antigenic determinant includes the sequence of chemical functional groups that form it and their spatial arrangement. In protein molecules, the antigenic determinant is formed by a set of amino acid residues (can vary from 5 to 20). Antigenic determinants of proteins are of two types - sequential, i.e. representing a sequence of amino acid residues in a polypeptide chain, and conformational, formed by amino acid residues from various parts of the protein globule. In many cases, a single amino acid substitution in the structure of the antigenic determinant or a change in the conformation of the protein globule is sufficient to change the antigenic specificity of the macromolecule. If two antigens have only part of the same antigenic determinants, they are called cross-reacting antigens.

Low molecular weight substances that are not capable of inducing the formation of antibodies themselves, but acquire immunogenic properties after conjugation with high molecular weight

carriers, for example, bovine serum albumin, are called haptens. Haptens include a wide range of natural compounds: peptide and steroid hormones, various drugs, antibiotics, vitamins, oligosaccharides, etc.

The biological function of antibodies is to protect the body from the penetration of foreign substances by forming strong specific immune complexes with the corresponding antigens and their subsequent removal from the body. The ability of antibodies to form highly specific, strong immunocomplexes with various substances and the ability to obtain antibodies in the required quantities are the basis of immunochemical methods of analysis.

In the body, antibodies are produced by specific blood cells - B-lymphocytes, each of which has on its surface up to 100,000 receptors of the same specificity, capable of recognizing any foreign antigen. An antigen, encountering a receptor complementary to it in the bloodstream, selects (selects) the corresponding B-lymphocyte, which then, transforming into a plasma cell and dividing repeatedly, forms a clone of cells. Each clone of plasma cells secretes antibodies that are structurally homogeneous. However, since the antigen immediately activates a large number of types of B lymphocytes in the blood, which contain receptors of varying degrees of specificity in relation to the original antigen, such an immune response and antibodies are called polyclonal. Animal serum containing antibodies specific to a given antigen is called antiserum, and it is usually indicated against what antigen and what animal it was produced (for example, rabbit antiserum against human red blood cells). It is fundamentally important that polyclonal antibodies, even against a single antigenic determinant, are heterogeneous both in the structure of the active center and in physicochemical properties. If the antigen is polyvalent, for example, a protein, then antibodies are formed in the blood serum directed against each individual antigenic determinant, which further complicates the composition of the antibodies.

In the mid-70s, a fundamentally new way of producing antibodies was developed, based on the fusion (hybridization) of lymphocytes of an immunized animal with myeloma cells to form new cells - hybridomas. A special feature of such cells is their ability to multiply and produce antibodies under artificial conditions outside the body. Using special cloning methods, it is possible to isolate one hybrid cell, which, when multiplying, will secrete in unlimited quantities antibodies of only one type - monoclonal antibodies, which are homogeneous both in specificity and in physicochemical properties.

5.2 Structure of antibodies. According to their chemical structure, immunoglobulins belong to a large class of natural compounds - glycoproteins, i.e. proteins containing oligosaccharides in their structure. Despite the huge variety of antibodies and their heterogeneity, they all have some common structural elements that ensure the performance of their basic functions.

According to their antigenic, effector properties and structural features, immunoglobulins are divided into five main classes: IgA, IgD, IgE, IgG and IgM (Ig stands for immunoglobulin).

The common structural unit of all immunoglobulins is a complex of four polypeptide chains - two identical light chains with a molecular weight of 23 kDa each (L-chains, from the English word light - light) and heavy chains with a molecular weight of 53,000 each (H-chains, from the English heavy - heavy). Each of the light chains is tightly connected to the NH2-terminal regions of the heavy chains due to the presence of interchain disulfide bonds and a variety of weak hydrophobic, electrostatic and other interatomic interactions. Similar connections exist between free sections of heavy chains. In general, the structure of such a complex resembles the Latin letter Y (or T) and is characteristic of immunoglobulins of the IgG, IgD, and IgE classes (Fig. 1).

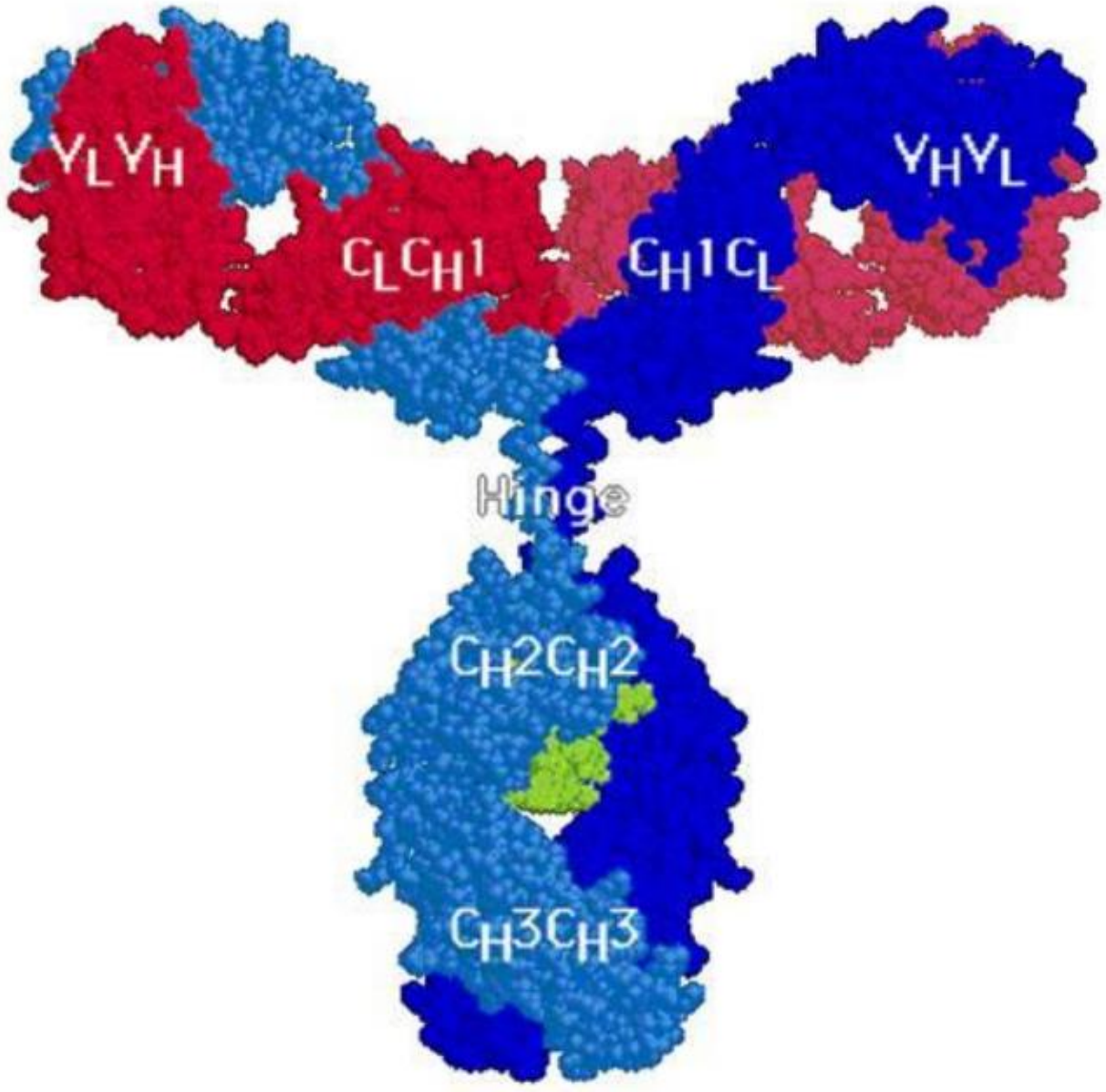


Fig. 5.1. Spatial structure of the IgG molecule

Under the action of the proteolytic enzyme papain, the IgG molecule breaks down into three fragments, two of which are identical and retain the ability to bind antigens (the so-called Fab fragments) and the third, capable of crystallization (Fc fragment), which is responsible for the

effector function of antibodies (Fig. 2) . Another proteolytic enzyme, pepsin, breaks the peptide bond located closer to the COOH end of the chain from the S-S bond between the H chains in the Fc fragment. As a result, the so-called pFc'-fragment is formed, which represents the residues of heavy chains and two Fab-fragments connected by disulfide bonds, designated as the F(ab')2-fragment.

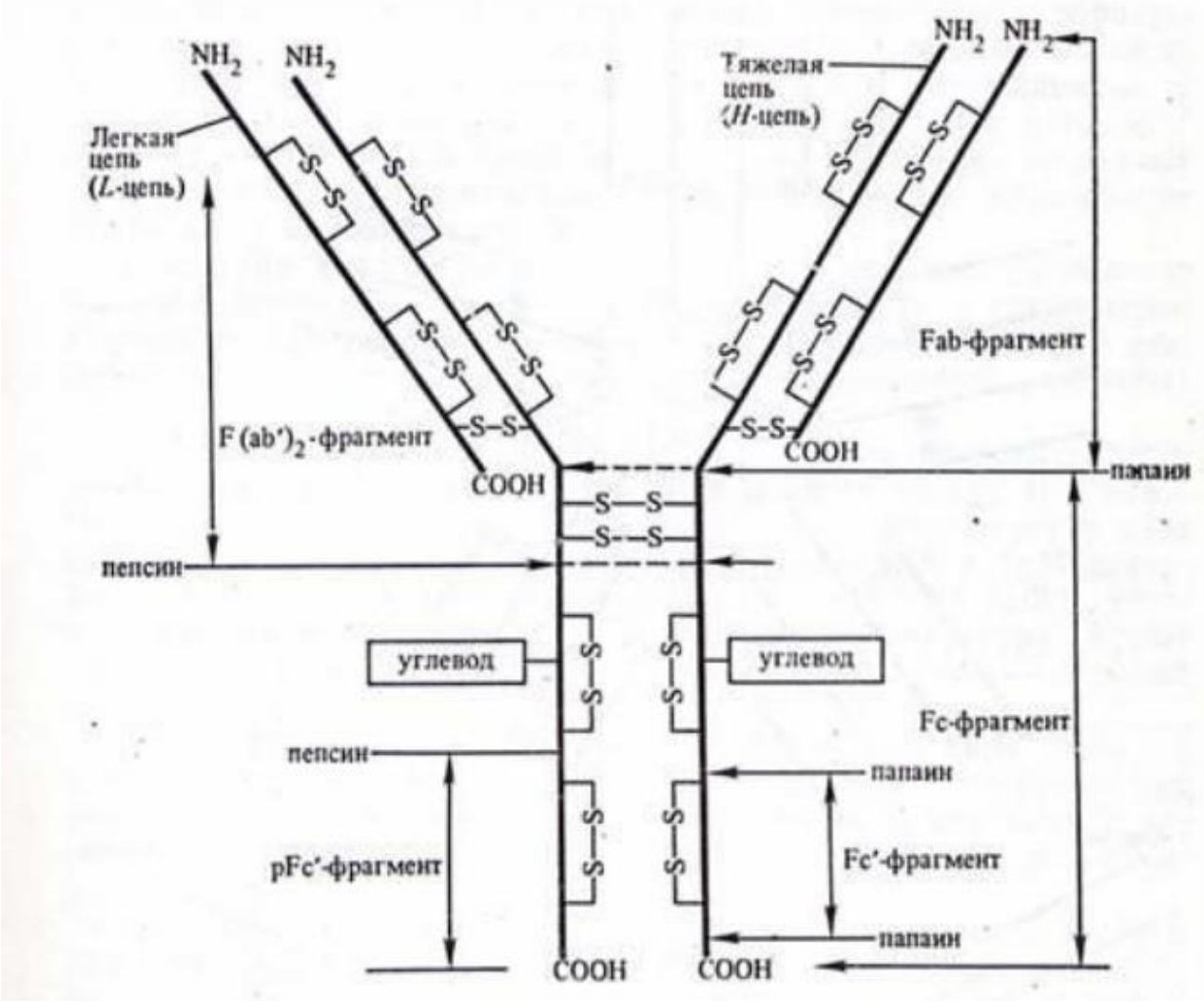


Fig. 5.2. Schematic representation of the structure of the IgG molecule.

The antigen-binding center is located in the NH2-terminal parts of the H- and L-chains. Thus, each IgG molecule, as well as F(ab')2 fragments, contains two identical antigen-binding centers, and the Fab fragment contains one.

Antibody molecules have a large number of S-S bonds, which can be divided into 3 categories - interchain, intrachain and bonds between the H-chains of individual four-chain complexes, which determine the formation of polymer molecules - IgM and IgA. The structure of immunoglobulins of various classes is determined by the number and location of S-S bonds in the molecules, as well as the number of four-chain elements. IgM is present in serum as a pentamer of four-chain complexes connected by S-S bonds between H-chains. Some serum IgA is also present in dimeric and tetrameric forms (Fig. 3).

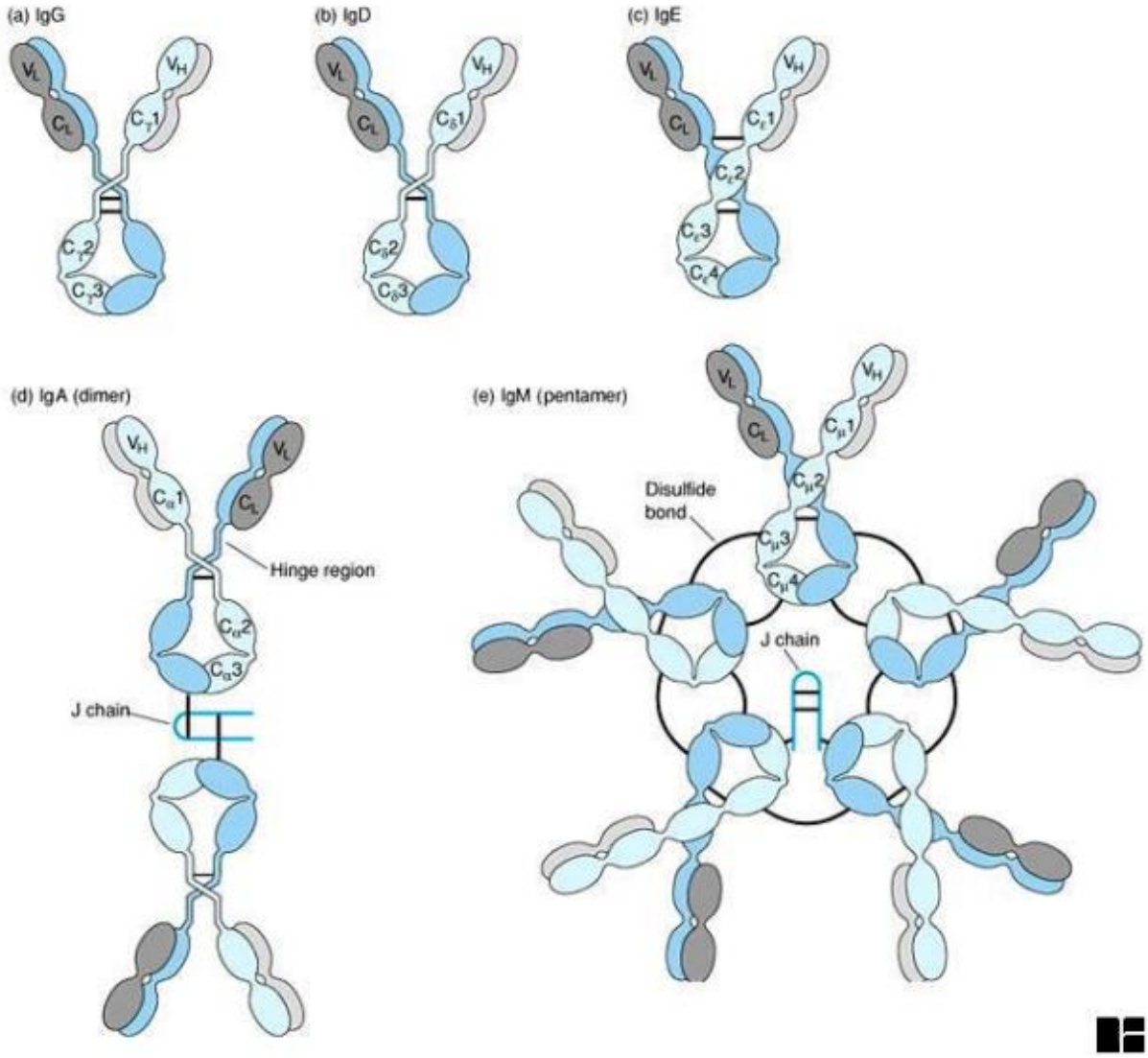


Fig. 5.3. Schematic representation of the structure of immunoglobulin molecules of various classes

The light chains of immunoglobulins are of only two types - □ or □, and are common to all five classes, while the heavy chains have structural, immunological and chemical features characteristic of each class of immunoglobulins. When studying the amino acid sequence, it was discovered that all light and heavy chains have one fundamental structural feature: they consist of two parts - variable (V) and constant (C) (Fig. 4).

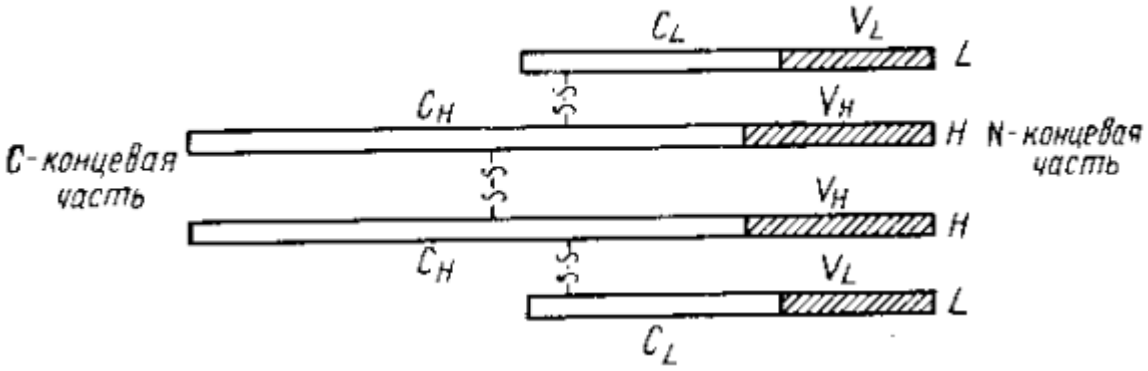


Fig. 5.4. Schematic representation of the arrangement of constant and variable regions in the IgG molecule.

The constant or constant part of the light chains (CL) includes 107 amino acid residues of the COOH-terminal region, the constant part of the heavy chain is approximately three times longer (or four times in the case of IgM and IgA) longer than the variable part.

According to X-ray diffraction analysis, sections of the peptide chains near the loop form a globular structure, which includes about 110 amino acid residues (Fig. 5). Such globules in the structure of antibody molecules are called domains. The NH2-terminal domain of the heavy chain is designated as VH, and the three subsequent ones in the constant region of the heavy chain are designated as CH1, CH2 and CH3 (for the light chain, VL and CL, respectively).

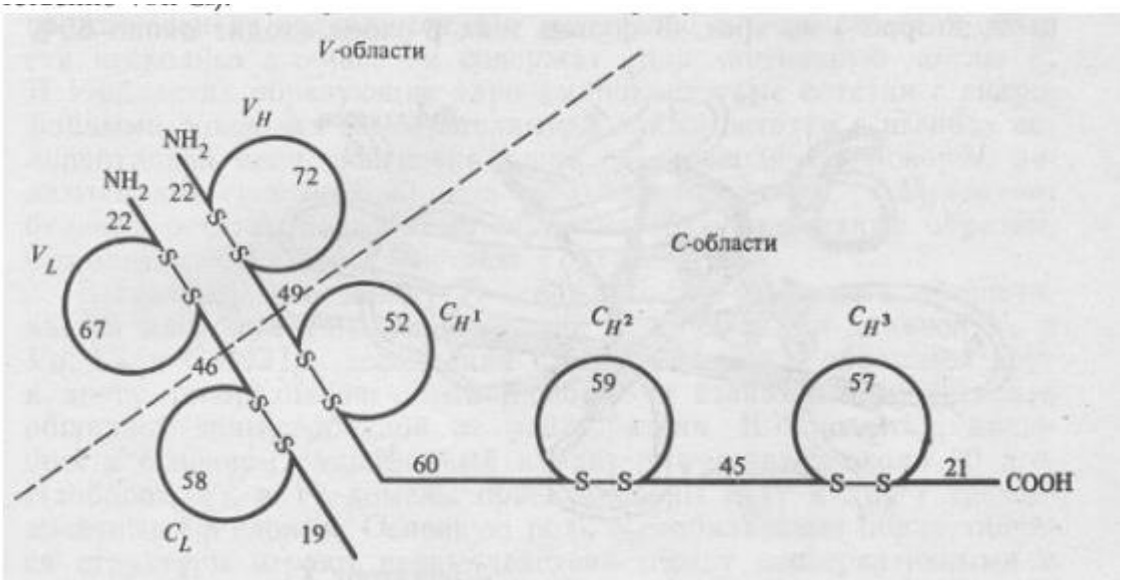


Fig. 5.5. Schematic representation of the localization of domain regions in the light and heavy chains of immunoglobulins.

Antigen binding occurs in the solvent-accessible cleft of the active site, formed by the variable domains in the NH2-terminal part of the light and heavy chains. The Fab and F(ab')2 fragments of immunoglobulins have the ability to bind antigens with the same efficiency as native antibody molecules. The basic principle of organization of antigen-binding centers of immunoglobulins is

the polycentric structure. Small antigenic determinants bind to a limited region of the active center, complementary to this determinant. Large determinants can occupy almost the entire binding region.

Antibodies formed in response to the injected antigens into the body specifically interact with these antigens. The primary interaction is based on the general principles of any bimolecular reaction. Since in this case the reaction product is an antigen-antibody complex, the immune reaction is reversible and is described by the same kinetic and thermodynamic parameters as any complex formation process.

5.3 Enzymes as tags in immunoassays. The fundamental possibility of using enzymes as labels in enzyme immunoassay is due to the extremely high sensitivity of detecting enzymes in solution. There are known amplification systems that make it possible to detect the presence of only a few hundred enzyme molecules in 1 ml of solution. The main requirements for enzyme molecules to be able to use them as tags are the following: high specific catalytic activity, accessibility, the possibility of obtaining the enzyme in a highly purified state, preservation of catalytic activity after chemical modification when obtaining enzyme-antibody (antigen) conjugates, stability, simplicity and sensitivity of the method for determining the concentration (activity) of the enzyme.

The most widely used enzymes at present are horseradish peroxidase and alkaline phosphatase. For photometric recording of peroxidase activity, the currently preferred substrate is tetramethylbenzidine. After stopping the enzymatic reaction with sulfuric acid, the optical density of the solution is measured at a wavelength of 450 nm.

5.4 Enzyme immunoassay methods. The primary process in enzyme immunoassay (or immunochemical) analysis is the stage of "recognition" of the analyzed compound by an antibody specific to it. Since the process of formation of immunochemical complexes occurs in a strictly quantitative relationship, determined by the affinity, concentrations of components and reaction conditions, a quantitative assessment of the formed immune complexes is sufficient to determine the initial concentration of the analyzed compound. For such an assessment, it is possible either to directly determine the concentration of the formed immunocomplexes (type 1), or to quantify the remaining free specific binding sites (type 2). The second general stage of any enzyme immunoassay method is the formation of a bond between the enzyme-labeled compound and a specific complex or free binding sites. And finally, the final mandatory process in an enzyme immunoassay is the transformation of an enzyme label into a corresponding signal, measured by some physicochemical method (spectrophotometric, fluorimetric, luminescent, etc.), which is achieved by measuring the rate of conversion of the substrate or the amount of product formed for a fixed period of time.

Taking into account the above-described approaches for determining specific complexes, further classification of enzyme immunoassay methods can be carried out according to the type of reagents used in the first stage of analysis. If at the first stage only the analyzed compound and its corresponding binding centers (antigen and specific antibodies) are present in the system, then

the method is non-competitive. For a Type 1 non-competitive assay, the optimal ratio of components is one in which the concentration of binding sites significantly exceeds the concentration of the compound being determined. A necessary condition for non-competitive analysis type 2 is compliance with the ratio of excess or comparable concentration of the test compound (antigen) and specific binding sites, since in this case the difference in the total number of binding sites and the number of formed immune complexes is determined. If, at the first stage of the analysis, the system simultaneously contains the analyte and its analogue (an enzyme-labeled analyte or an analyte immobilized on a solid phase), competing for specific binding centers that are in relative deficiency, then the method is competitive. A necessary condition for the competitive method is the lack of specific binding sites in relation to the total concentration of the analyzed compound and its analogue.

The next principle of classification of enzyme immunoassay methods is their division according to the type of reactions carried out at each of the immunochemical stages. In accordance with this, all methods can be divided into two groups - homogeneous and heterogeneous.

5.5 Heterogeneous enzyme immunoassay methods. Heterogeneous enzyme immunoassay combines methods in which the analysis is carried out in a two-phase system, and phase separation can occur at any stage of determination. For the sake of convenience of classification, it is advisable to separate heterogeneous methods according to the nature of the first stage of "recognition," which is decisive for the entire analysis. If at the first stage the antigen or antibody is used in an immobilized state and the formation of a specific immunocomplex takes place on the solid phase, then the method is classified as a solid phase assay. If, at the first stage of the analysis, the formation of specific immune complexes occurs in solution, and only then a solid phase with an immobilized reagent is used for separation purposes, then it is advisable to classify such methods as homogeneous-heterogeneous.

The variety of heterogeneous enzyme immunoassay methods, related to types 1 and 2, is due to the possibility of introducing an enzyme label into both the antigen molecule and the antibody molecule. In addition, for a specific analysis scheme, it is decisive which of the reagents - antibody or antigen - is used in an immobilized form to separate immunochemical complexes from unbound components.

As an example of a heterogeneous non-competitive method of performing an enzyme-linked immunosorbent assay, we present one of the most common schemes for the enzyme-linked immunosorbent assay of proteins (multivalent antigens), based on the use of a pair of antibodies of different antigenic specificity, one of which is immobilized on the surface of a solid carrier, and the second is conjugated with an enzyme label (for example, horseradish peroxidase). The analysis is carried out as follows. The test sample is added to the wells of a polystyrene plate with sorbed antibodies and incubated for 1 hour, during which the analyzed antigen reacts with the antibodies and forms an immunocomplex on the surface of the wells. The plate is washed from

unbound components and enzyme-labeled antibodies are added. After secondary incubation and removal of excess antibody-enzyme conjugate, the enzymatic activity of the carrier is determined, which is proportional to the initial concentration of the antigen under study. At the stage of identifying a specific immunocomplex, the antigen appears to be sandwiched between molecules of immobilized and labeled antibodies, which gave rise to the widespread use in the literature of the name "sandwich" method. Another name often found in the literature is the two-site assay. The scheme can be used to analyze only those antigens on the surface of which there are at least two antigenic determinants located far from each other, and the method is not suitable for determining a large number of monovalent antigens (for example, low molecular weight hormones, medicinal compounds, pesticides).

Competitive solid-phase analysis of low-molecular-weight antigens can be implemented according to the following scheme. A solution containing the antigen being analyzed and a fixed concentration of the antigen-enzyme conjugate is added to the antibodies immobilized on the carrier. After incubation, the carrier is washed from unbound free and labeled antigen and the enzymatic activity on the carrier is recorded, which is inversely proportional to the concentration of the antigen being determined.

Competitive solid-phase methods are less sensitive than non-competitive methods. The detection limit of various compounds for them is limited by both the sensitivity of enzyme label registration and the affinity of antibodies, while for non-competitive methods, in the absence of nonspecific interactions, it is only by the sensitivity of enzyme determination. Therefore, to achieve high sensitivity of the competitive method, it is necessary to use high-affinity antibodies.

5.6 Homogeneous enzyme immunoassay methods. Homogeneous methods include those that are carried out in a single-phase system and do not require the stage of mechanical separation of the formed complexes. In all schemes for conducting a homogeneous enzyme-linked immunosorbent assay, register concentration non formed specific antibody-antigen complex, get free centers of specific bounded, is recorded. However, in contrast to heterogeneous patterns, the observed enzymatic activity corresponding to the concentration of unoccupied specific binding sites can either decrease or increase, due to the different nature of the effect of ligand binding on enzymatic activity. The entering of a label into the antigen molecule is one of the most common approaches in homogeneous enzyme immunoassay methods. All homogeneous methods are competitive and are based on the simultaneous interaction of the analyzed and labeled antigens with antibodies. After the formation of the corresponding immunochemical complex in the solution, the enzymatic activity is measured, which is proportional to the concentration of free or bound labeled ligand.

One of the common methods is EMIT analysis (enzyme multiplied immunoassay technique), based on a change in the activity of the enzyme tag in the enzyme-antigen conjugate during the formation of a complex with antibodies, which occurs as a result of conformational rearrangements in the enzyme molecule or steric exclusion of the accessibility of the substrate

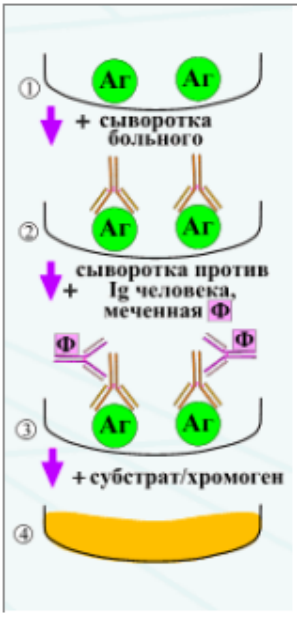
molecule to the active center of the enzyme during complex formation of the conjugate with antibodies. The advantages of homogeneous methods are a significant reduction in analysis time (several minutes); the disadvantages are lower sensitivity and the possibility of the composition of the analyzed sample influencing the analysis results.

Promising directions for the further development of enzyme immunoassays are the creation of express methods based on the use of membrane and immunochromatographic systems, flow injection methods, kinetic analysis, immunobiosensory devices that allow express analysis, including the simultaneous determination of several antigens in one sample, in real life. time.

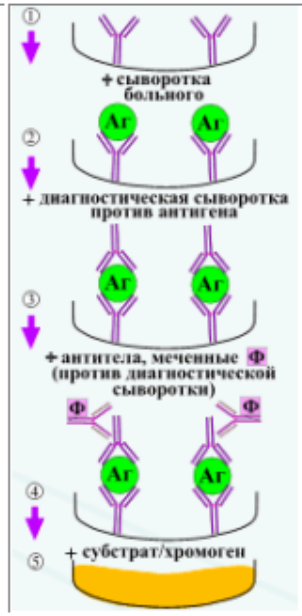
5.7 Enzyme-linked immunosorbent assay, or method (ELISA) - detection of antigens using corresponding antibodies conjugated to a tag enzyme (horseradish peroxidase, beta-galactosidase and or alkaline phosphatase). After combining the antigen with the enzyme-labeled immune serum, the substrate/chromogen is added to the mixture. The substrate is cleaved by the enzyme and the color of the reaction product changes - the intensity of the color is directly proportional to the number of bound antigen and antibody molecules. ELISA is used for the diagnosis of viral, bacterial and parasitic diseases, in particular for the diagnosis of salmonellosis, mycoplasmosis, etc., as well as for the determination of hormones, enzymes, medications and other biologically active substances contained in the test material in minor concentrations - 1010 - 1012 g/ l (See Fig. 5.6).

Твердофазный ИФА - вариант теста, когда один из компонентов иммунной -реакции (антиген Ag или антитело) сорбирован на твердом носителе, напр., в лунках планшеток из полистирола. Компоненты выявляют добавлением меченых антител или антигенов. При положительном результате изменяется цвет хромогена. Каждый раз после добавления очередного компонента из лунок удаляют не связавшиеся реагенты путем промывания.
I. При определении антител в лунки планшеток с сорбированным антигеном последовательно добавляют сыворотку крови больного, антиглобулиновую сыворотку, меченную ферментом Ф и субстрат/хромоген для фермента.
II. При определении антигена в лунки с сорбированными антителами вносят антиген (напр., сыворотку крови с искомым антигеном), добавляют диагностическую сыворотку против него и вторичные антитела (против диагностической сыворотки), меченные ферментом, а затем субстрат/хромоген для фермента.

I. Определение антител в сыворотке больного (в лунках планшеток с сорбированным антигеном Ag)



II. Определение антигена в сыворотке больного (в лунках планшеток с сорбированными диагностическими антителами)



Конкурентный ИФА для определения антигенов: искомый антиген(1) и меченый ферментом антиген(2) конкурируют друг с другом за антитела (3), сорбированные на твердой фазе.

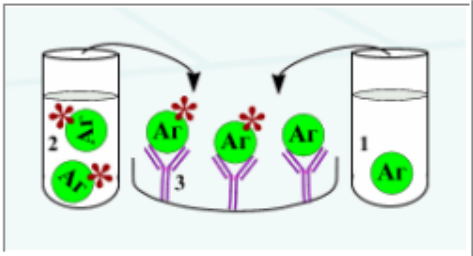
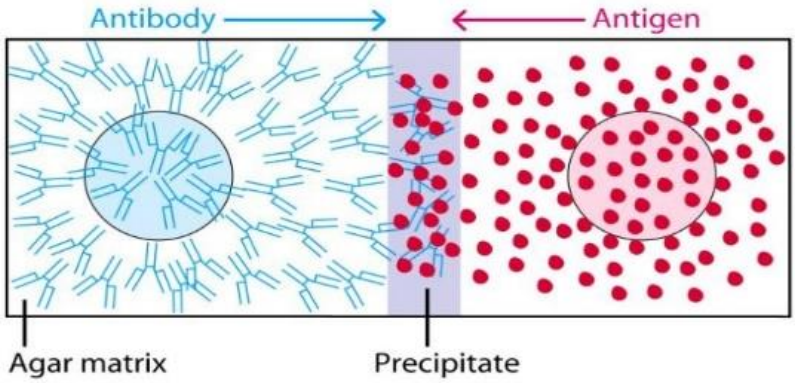


Fig 5.6

Competitive ELISA for the determination of antibodies: the desired antibodies and enzyme-labeled antibodies compete with each other for antigens adsorbed on the solid phase

Method for determining antibody antigen interactions by precipitation

This method is used in the first step of testing immune serum. After immunization, blood is taken from animals and the presence of AT antibodies in the blood of the immunized animal is checked using the method of precipitation in Agarose gel. If the answer is positive, the serum is subjected to further processing.



Method – DOT and titer determination

DOT in English means "dot", respectively, the DOT method is a method of point application of a sample onto a nitrocellulose membrane and its specific coloring, where diaminobenzidine DAB or 4-chloro-1-naphthol is used as a substrate. These chromogens, when oxidized, form an insoluble complex and settle at the site of oxidation, i.e., where oxidase is present, respectively coloring the antigen or antibody applied to the nitrocellulose membrane. (See diagram)

The chromogen from a soluble compound, after oxidation by HR-peroxidase, passes into an insoluble complex that is sorbed by peroxidase. Accordingly, indicating the presence of AG.

The DOT method is widely used in immunochemistry and medical diagnostics, and is especially widely used as a qualitative analysis to determine the types of various viruses. Recently, quantitative analysis has often been carried out using the DOT titration method. The advantage of the DOT method is its ease of implementation and high specificity.

In the process of performing the DOT method, the student masters methods of high specificity and sensitivity, acquiring the skills of a high-level specialist.