FRACTIONATION AND QUANTITATIVE DETERMINATION OF SERUM PROTEINS

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THEORETICAL BACKGROUND

1. Plasma and serum

The blood plasma is the blood's liquid extracellular matrix which by itself is straw-yellow in color. It is containing 91% water, 7% blood plasma proteins and 2% other dissolved material (e.g. nutrients, ions, waste products). Protein components are mostly albumin, globular proteins (globulins) and fibrinogen. Globulins can be further classified to α1 -, α2 -, β and γ globulins. The major protein components of the plasma are detailed in **Figure 1**.The **blood plasma** always contains fibrinogen and other blood clotting components playing important roles in blood clotting. If plasma is needed, during blood collection anticoagulant containing tube is used, and then centrifuged. The supernatant is the plasma. The term **serum** refers to plasma without the fibrinogen and blood clotting proteins, and it can be obtained after clotting above the clot. If serum is needed, native (untreated) tube is used for collection and allowed to clot the blood. . Nowadays special vacuum devices with special tubes are used for blood collection (**Figure 2.**).

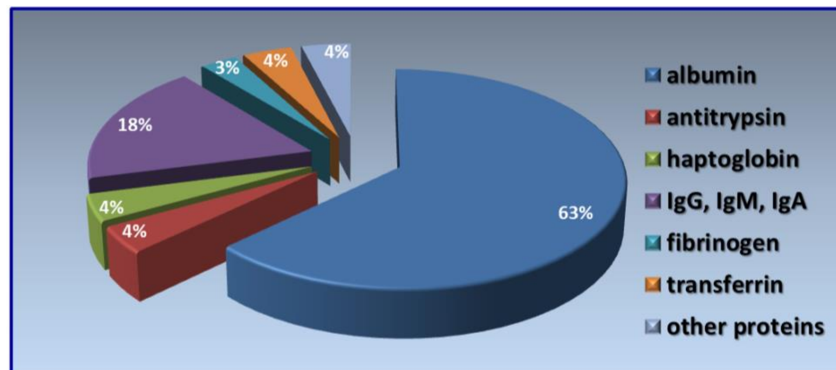


Figure 1. Major protein components of the blood plasma

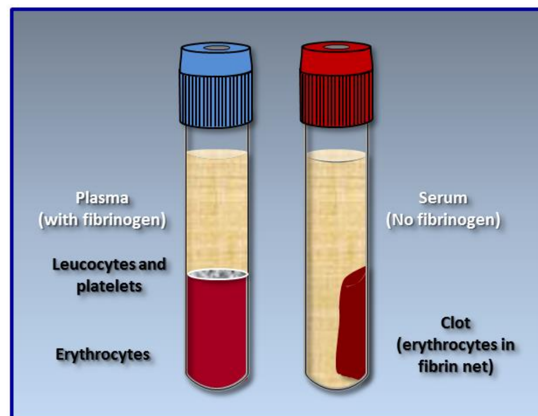


Figure 2. Vacuum tubes for blood collection after centrifugation.
Blue top: Citrate tube; Red top: Native blood tube

2. Salting out of globulins from human serum – Ammonium Sulfate Precipitation

If blood serum is semi-saturated with **ammonium sulfate** at room temperature, serum globulins are precipitated while albumin remains soluble. During the experiment globulins are salted out and the precipitate is separated by centrifugation. The supernatant contains the albumin, while the precipitate contains globulins. After separation the precipitate can be dissolved in 0.9% NaCl solution.

3. Desalting of albumin by gel filtration chromatography

After ammonium sulfate precipitation serum albumin and globulin fractions are separated. The supernatant containing albumin is desalted by chromatography on **Sephadex G25 gel filtration** gravity **column**. Sephadex is a trademark for cross-linked dextran gel used for gel filtration, produced by Pharmacia. The name is acronym derived from „**se**paration **Pha**rmacia **dex**tran". "**25**" means that 10 grams of gel particles (or beads) can bind 25grams of water, while "**G**" refers to gel filtration. The number of crosslinks between dextran molecules affects the water binding capacity; higher number of crosslink provides a more solid product, which reduces the amount of bound water. Sephadex G-25 has a fractionation range for proteins of molecular weights 1000 to 5000 Da, with a size - exclusion limit of approximately 5000 Da (5 kDa). Molecular weight of the Albumin is approximately 65 kDa. It means that the albumin cannot penetrate into the beads, so it is excluded and elutes sooner.

During gel filtration, NaCl solution is flowed through the column continuously and fractions are collected. Later the protein content of fractions will be determined with **Biuret** reaction and **turbidity assay** is used for salt concentration measurement. Equipment and materials used on practical are shown in **Figure 3**.

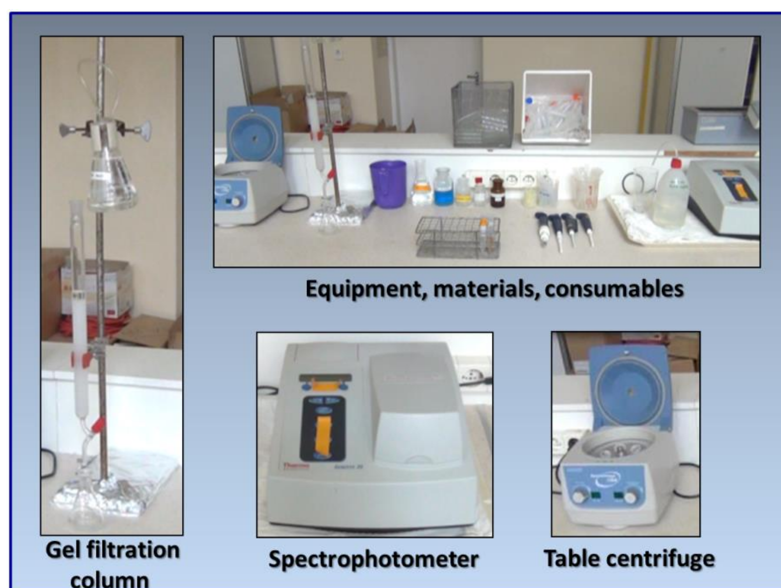


Figure 3. Equipment, materials and consumables of gel filtration column separation of serum proteins

4. Protein concentration determination with different methods

Biuret reaction

The **biuret reaction** is a chemical test used for detecting the presence of peptide bonds. In the presence of peptide bonds, a copper (Cu II) ion forms violet-colored complexes with the protein molecule through its nitrogen atom in alkaline solution. The intensity of the color, and the absorption at 540 nm (using spectrophotometer), is directly proportional to the protein concentration, according to the Beer-Lambert law. The biuret reaction can be used to assess the concentration of proteins because peptide bonds occur with the same frequency per amino acid in the peptide. A peptide of at least three amino acids in length is necessary for a positive, measurable color shift. Several types of tests within this method have been developed, such as the BCA test or the modified Lowry test.

During practical, this method is used for determining the protein concentration of serum sample; fractions are collected during gel filtration and dissolved globulin fraction.

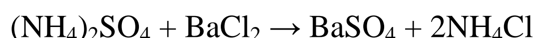
Determination of albumin content using bromcresol purple

Bromcresol purple (BCP) or 5',5"-dibromo-o-cresolsulfophthalein, is a special pH indicator. Besides its primary function, bromcresol purple is used in diagnostic laboratories to measure albumin levels in serum samples. At acidic pH BCP binds specifically with human serum albumin, and changes its color to green. The BCP albumin assay kit is designed to measure albumin directly in biological samples. The method utilizes the ability of bromcresol purple to form complexes with albumin. The absorbance of the colorful, stable complex can be measured at 610 nm using a spectrophotometer, and its intensity is directly proportional to the albumin concentration in the sample.

In our experiment, the BCP reagent will be used for specific determination of albumin levels in human serum sample.

5. Determination of the sulfate salt of the chromatography fractions with turbidimetry

For determination the approximate concentration of the sulfate ion (salt) of the collected fractions a barium-chloride ($BaCl_2$) based **turbidity assay** is used. Turbidity is the cloudiness of a fluid caused by large numbers of individual particles that are generally invisible. In this case particles are barium sulfate salt precipitates. In the fractions under examination the ammonium-sulfate and barium-chloride react, barium sulfate is produced and its amount (and opalescence) depends on the number of barium sulfate particles:



$$(NH_4)_2SO_4 + BaCl_2 \rightarrow BaSO_4 + 2NH_4Cl$$

Turbidity of fractions will be estimated by eye inspection on a five grade scale using one to five crosses (where “+” is the least turbid and “+++++:”the most turbid fraction).

6. Comparison of protein and salt amount of different fractions – effectivity of desalting

Protein concentrations determined by Biuret reaction and turbidity results related to the same fractions are plotted against the number of fractions on the same graph as shown in **Figure 4,** and then the total amount of protein collected in fractions is calculated.

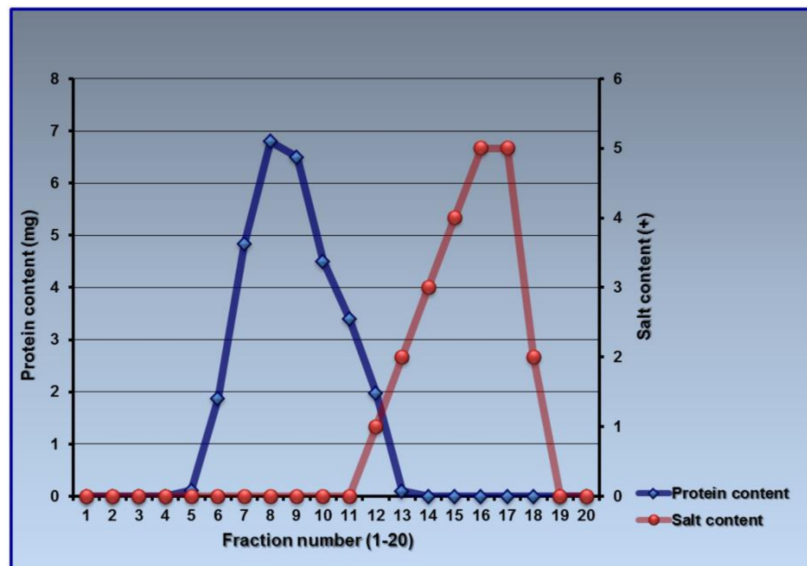


Figure 4. Result of desalting of the albumin fraction on gel filtration column, protein and ammonium – sulfate salt content as a function of fraction number

7. Electrophoretic separation of serum proteins on cellulose acetate membrane

Purified albumin, globulin and serum samples are applied onto cellulose acetate membrane.. Equipment used on practical is shown in **Figure 5**.

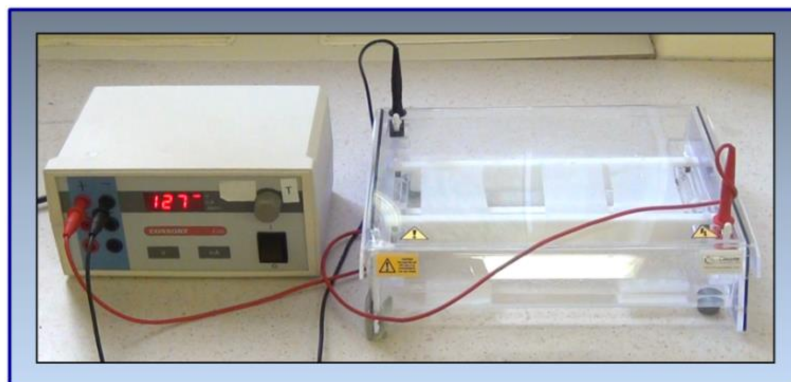


Figure 5. Electrophoretic device used in the practical

Cellulose acetate membrane separation of the plasma proteins are mainly based on their overall charge, therefore proteins occurring in the same fractions may have very different molecular weight. In the experiment, the pH of the running buffer is 8.6, so net charges of the separated proteins are negative, and loaded onto the membrane closer to the cathode. During separation proteins migrate towards the positive pole with different speed according to their net charge.

After separation, the membrane is stained with Amido black stain, and the result is evaluated. **Figure 6**. illustrates an electrophoresis result. Amido black is an amino acid specific dye used to stain for total protein on transferred membrane blots.

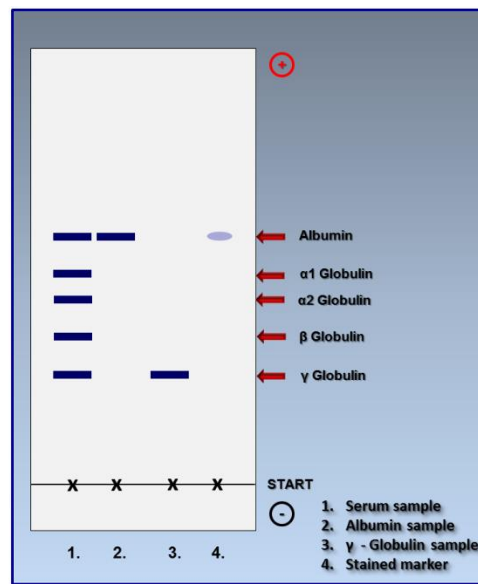


Figure 6. Result of the separation of serum proteins by membrane electrophoresis

8. Clinical correlations

Albumin is the most abundant plasma protein in human blood. Albumin has important physiological roles, including maintenance of colloid osmotic pressure and pH, binding of key substances (e.g. free fatty acids, bile acids, bilirubin, calcium, magnesium, etc.), and has anti-oxidant and anticoagulant effects. From a diagnostics point of view the albumin is a reliable prognostic indicator for liver disease, nephritic syndrome, malnutrition and protein-losing enteropathies. High levels are associated with dehydration.

Examination of globulins is also very important for diagnostic purposes, for example, for diagnosis of multiple myeloma; lymphoma, immune disorders, immune deficiencies and anemia. Groups of serum proteins and their diagnostic significances are summarized in **Table 1**. For more information, see the following article: O'Connell T.X. et al.: Am Fam Physician.; 2005, 71:105-12. (http://www.aafp.org/afp/2005/0101/p105.pdf).

Protein (group)	Normal values	Main function(s)	Higher than the normal value	Lower than the normal value
Albumin	3,8 - 5,0	Transports substances; tissue growth, tissue repair.	Dehydration.	Kidney or liver disease; inflammation; poor nutrition.
α1 - Globulins	0,1 - 0,3	Main element: α - 1 – antitrypsin, indicator of inflammatory diseases.	Disease leading inflammation (chronic or acute).	Liver disease; congenital emphysema (rare).
α2 - Globulins	0,6 – 1,0	Indicator of inflammatory diseases.	Kidney disease, disease leading to inflammation (chronic or acute).	Liver disease, poor nutrition; breakdown of RBCs
β - Globulins	0,7 – 1,4	Transfer processes; immunoreactions.	Anemia; multiple myeloma; high cholesterol level.	Poor nutrition; liver cirrhosis.
γ - Globulins	0,7 – 1,6	Immunoreactions; indicator of autoimmune diseases.	Rheumatoid arthritis; infection; liver cirrhosis; inflammatory disease; multiple myeloma; lymphoma.	Immune disorders and immune deficiencies.

Table 1. Groups of serum proteins, normal values, interpretations of deviations

ADDITIONAL THEORETICAL KNOWLEDGE

The human blood

The blood is a specialized tissue of the human body that delivers necessary substances such as nutrients and oxygen to the cells and responsible for the transportation of metabolic waste products and removal of carbon dioxide. The amount of the blood circulating in the body is about 8 percent of the total body weight.

Composition of the human blood

Histologically the blood is a fluid connective tissue, that consist of about 45% formed elements (cells and cell-like formations) and 55% blood plasma. Formed elements are platelets, white blood cells (leukocytes) and red blood cells (erythrocytes) (**Figure 7.**). These elements are mostly specialized for special functions, not able to divide and do not have a long life time. Platelets are important elements of the blood clotting process; white blood cells are part of immune system; hemoglobin containing red blood cells are responsible for the oxygen transport between the lung and peripheral tissues.

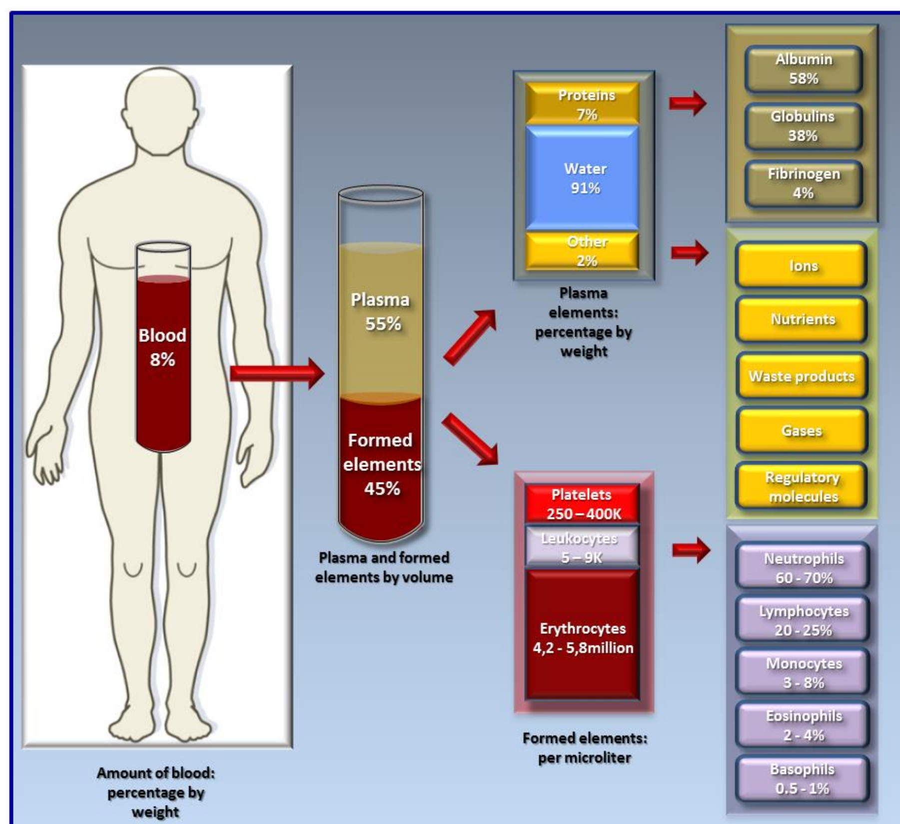


Figure 7. Composition of the human blood and approximate proportions of its components

Methods for protein purification and separation

The **protein purification or separation** is an essential step for the characterization of the function, structure and interactions of the protein of interest. This process may separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Usually the purification process is a series of different separation methods where the goal is to either obtain an isolate of a target protein from a complex mixture (e.g. cells, tissues, whole organs or organisms), or to isolate the target from other contaminants. The separation process usually exploit differences in the size, physico-chemical properties, charge, binding affinity and biological activity of the proteins.

Protein purification is either preparative or analytical. In case of preparative separation the aim is to produce a relatively large quantity of purified proteins for subsequent use. Proteins can be purified for analytical purposes, and in this case the quantification of different proteins and visualization of different isoforms can be relevant.

Desalting and different solubility

In large scale protein purification, one main method and usually the first common step to isolate proteins is the reversible **desalting (precipitation)** with **ammonium - sulfate** ($(NH_4)_2SO_4$), a simple, cheap and effective way of protein fractionation. This is performed by adding increasing amounts of ammonium - sulfate and fractionating the different fractions of precipitate protein with centrifugation. This method is based on the common property of proteins that at high salt concentrations proteins are not aggregating, since the surface charges are neutralized, thus molecules will tend to bind together and, form large complexes. High amount of salt will decrease the size of the protein hydration shell, first the shells of globular proteins, which are start to precipitate earlier. The desalting is a reversible process, and fractionated proteins are re-soluble. Precipitation salt can be removed by a further purification step, such as dialysis or gel filtration chromatography.

Gel filtration chromatography

Gel filtration or size – exclusion (SEC) chromatography separates different molecules (e.g. oligonucleotides, peptides, proteins) on the basis of size. Molecules move through a bed of porous beads. Smaller molecules can diffuse further into the pores of the beads and therefore move through the bed more slowly. Larger molecules cannot enter into the pores or thus move through between the beads and move faster and can be eluted more quickly. Both molecular weight and three-dimensional shape contribute to the separation process (**Figure 8**.).

Macroscopic beads forming the gel bed are synthetically derived polysaccharide (dextran) molecules. The organic chains are cross-linked to give a three-dimensional network. Bounds between hidroxil groups of the glucose units are attached by epichlorohydrin.

Wet hydrophilic gel is used as a column loaded into a special (glass or plastic) vessel, which is considered the **stationary phase**, while the buffer (eluent) used for the separation is the **mobile phase**. Separated components occur in collected fractions of moving mobile phase.

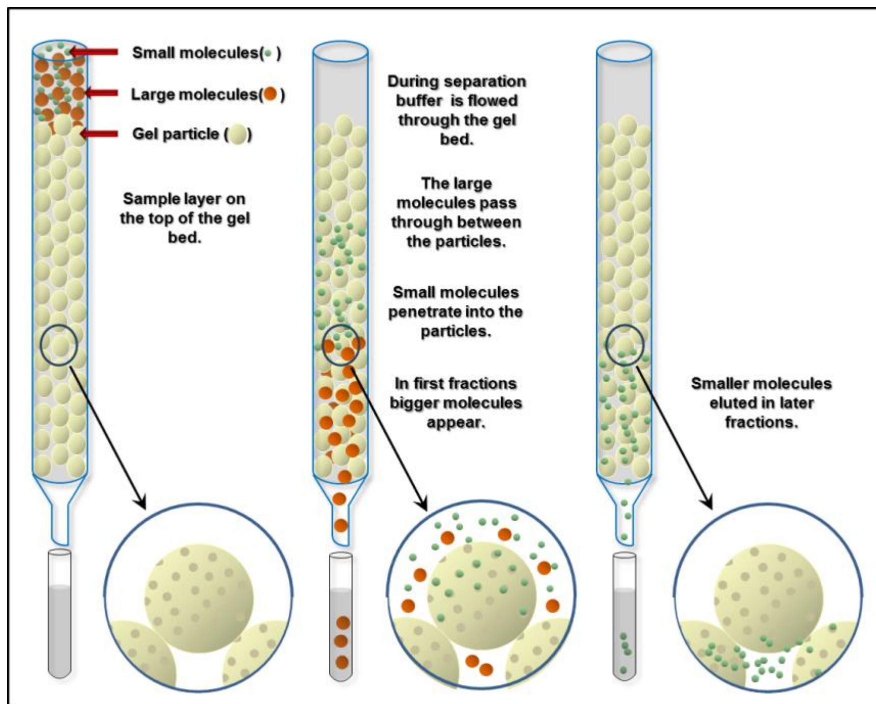


Figure 8. Principle of the gel filtration chromatography

Volume relation in the gel filtration column

The column can be characterized by different volumetric conditions. The **total volume (V_t)** is the whole column volume including spaces between the beads and volumes of pores. The **void volume (V_0)** is the liquid around the beads, equals the **exclusion** volume, or the **elution volume** of a molecule that is larger than the largest pore size of the separating gel, therefore explores only the mobile phase, and is entirely excluded from the gel. **Size exclusion molecular weight** – the weight, above which molecules are larger than the pore size, cannot penetrate into the pores.

Volume inside or **internal volume (V_s, V_i)** equals to the volume of the stationary phase, the actually volume of the liquid inside the gel particles that is fully accessible only to molecules small enough to travel smoothly even through the smallest pores of the gel. V_s itself is difficult to determine; therefore it is replaced by the **V_t-V_o** value, also accounting for the volume of the gel material itself **(Figure 9.).**

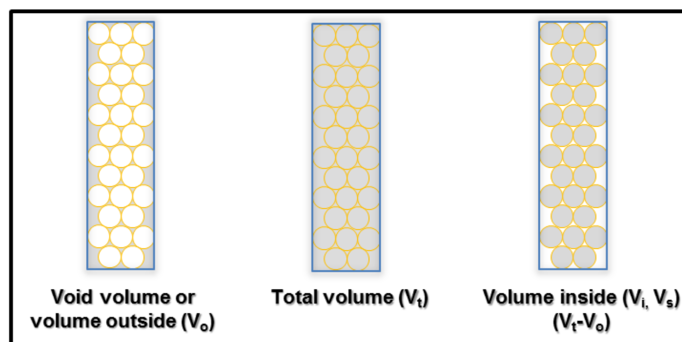


Figure 9. Liquid compartments of chromatography gel bed

The gel filtration chromatography can be used to determine of molecular size, for separation of different materials, or for salt removal and buffer exchange.

Dialysis

Dialysis is the process of separating molecules in solution by diffusion through a semipermeable membrane. Diffusion is a spontaneous, thermal movement of molecules in solution from an area of higher concentration to a lower concentration until equilibrium is reached. The movement is affected by the pore size of the membrane, causes differential diffusion patterns, permitting the separation of molecules.

Dialysis can be used to remove small molecules, contaminants and, salts from the sample or for buffer change.

The electrophoresis

The **electrophoresis** is the motion of dispersed particles under the influence of a spatially uniform electric field. It is caused by the presence of a charged interface between surface of the particle and the surrounding fluid. Movement of positively charged particles (cations) is called cataphoresis, while the flow of negatively charged particles (anions) is called anaphoresis. This differential movement of particles forms the basis of a technique used for separating molecules according to their size and charge. During this process negatively charged proteins move towards to the positive pole **(Figure 10.).**

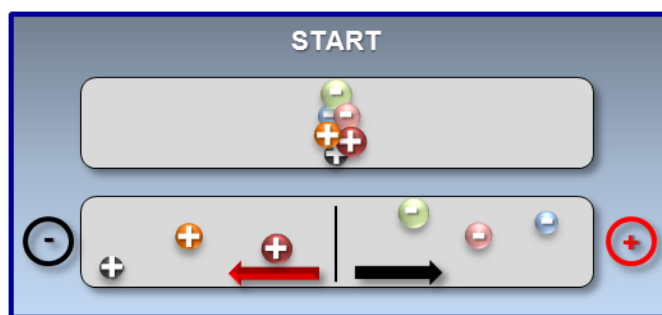


Figure 10. Principle of the electrophoresis

The separation can be performed in different gels or membrane surfaces according to the types of samples (RNA, DNA, and protein) or the aim of the process (i.e., analytical or preparative).

Usually polyacrylamide gel electrophoresis (PAGE) is used for quantitative analysis of proteins. This technique is providing a cleaner separation and a more accurate resolution.

Cellulose acetate membrane electrophoresis

Cellulose acetate electrophoresis first of all can be useful for separation of multimeric proteins formed by different isoforms since each isoforms will have a different brut charge

due to its unique amino acid sequence. During separation it utilizes the native protein charge based on the isoelectric point (pI) of the protein. The carrier will be a partially or fully O-acetylated absorbent; a water-insoluble cellulose membrane.

A protein sample is placed on the center of a cellulose acetate membrane and the membrane is placed in buffer of a desired pH then voltage is applied across the membrane. The proteins with an isoelectric point greater than the pH of the buffer migrate towards the positive pole, while proteins with lower isoelectric point than the pH of the buffer move towards the negative pole. Ultimately positively charged proteins migrate towards the cathode while negatively charged proteins migrate toward the anode.

Diagnostic methods

Serum protein electrophoresis

In medicine, protein electrophoresis is a method for analyzing the proteins mainly in blood serum samples.

Serum protein electrophoresis (SPEP, SPE) is a laboratory test for examining serum globulins specifically. After collection, the blood serum sample is placed onto a membrane (or into a gel or capillary tube) and - as previously described - exposed to an electric current to separate the serum proteins according to their size and electrical charge. Then the membrane is stained. The five groups of proteins usually analyzed during an SPEP test are: serum albumin; α -1 globulins; α -2 globulins; β globulins and finally γ globulins.

After separation, albumin and globulin groups are generally equal in proportion, but **albumin** as a molecule is smaller and lightly, negatively-charged, leading to an accumulation on the electrophoretic membrane. A small band which sometimes appears in front of albumin band represents transthyretin (also named prealbumin). Under the albumin band globulins are classified by their sub-fractions (bands). The **alpha (α) band** consists of two parts, **α1** and **α2**. Main representatives of **α1 band:** α1-antitrypsin, α1-acidic glycoprotein. Main elements **α2 band** are haptoglobin, α2-macroglobulin, α2-antiplasmin and ceruloplasmin. The **beta (β) band** mainly contains the transferrin, plasminogen, fibrinogen, representatives of clotting factors. The **gamma (γ) band** usually contains immunoglobulins (IgA, IgD, IgE, IgG and IgM), but paraproteins (abnormal immunoglobulin products related to different diseases) usually appear in this band (e.g. paraprotein of multiple myeloma).

Some forms of medication, biologicals, pharmaceutical derivatives, products released by cells may appear in their own bands, but those are usually small. Abnormal bands (spikes) are seen in „monoclonal gammopathy of undetermined significance" due to the high amount of monoclonal antibodies produced by abnormal but not tumorous plasma cells. Diagnostically, too high or too small protein amounts on the membrane may indicate problems.

After the separation and staining the bands are analyzed by densitometry, so quantities of protein related to a given band will be precisely quantifiable and comparable **(Figure 11.)**.

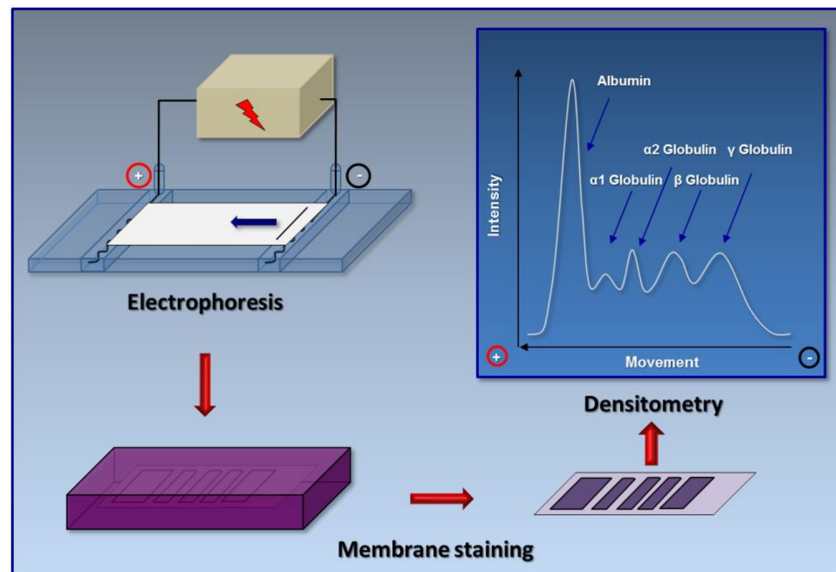


Figure 11. Electrophoretic separation process of serum proteins

General medical procedure involves the determination of numerous proteins in plasma (e.g. enzymes, hormones), some of them also determined by electrophoresis. However, gel electrophoresis is mainly a research tool.

Total Protein measurement and Albumin - Globulin (A/G) Ratio

Serum total protein, also known as **total protein test**, is a method for measuring the total amount of protein in serum in the clinical biochemistry. The protein fraction of the plasma is basically made up of albumin and globulin. These fractions can be separated and analyzed using protein electrophoresis, but the total protein test is faster and cheaper, and it estimates the total of all fractions together. The traditional method for measuring total protein uses the biuret reagent or refractometry. Nowadays this measurement is a part of automatized blood analysis.

Basically the **albumin-globulin ratio (60% / 40%)** is the amount of albumin fraction compared to globulins in blood serum, plasma, or in urine. This test can help to diagnose diseases connected to kidney or liver dysfunction or a problem that causes protein loses in digestive tract. Total protein content of the serum is between 60 and 80 mg/ml (g/L), where albumin concentration is 38-50 mg/ml and globulin concentration is 25-30 mg/ml.

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