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Department of Fundamental Medical Disciplines

Molecular Biology Practicals

by

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CONTENT

Introduction

The understanding of how living organisms function in healthy and diseased states includes an appreciation of how they operate at the molecular level. Molecular Biology is becoming an important basis for modern medicine not only for explanation of the diseases, but for diagnostics and treatment of them also. For example, it is clear today that cancer diseases have molecular mechanisms, which are regulated on a genetic level. Today the molecular methods in medicine are expanding rapidly and it is now possible to take into account for medical treatment the genetic profile of individual patients built on the results of nucleic acid microarrays diagnostics. Some successful gene therapy experiments with use of corrected DNA fragments introduced to the genome of humans suggest that soon this approach will become viable for medical practice. We believe that teaching molecular biology is necessary for future doctors.

This book is composed of twenty practicals, which consist of the brief theoretical background, principle of the methods used and procedures. These include three laboratory works on cytochemical detection of DNA and RNA in the cell, the rest of practicals are devoted to different basic methods of isolation and studies of the nucleic acids, lipids, carbohydrates, amino acids and proteins, particularly human hemoglobines. The multi-choice questions examples in four classes of biological molecules (nucleic acids, proteins, carbohydrates and lipids) were added to some of the practicals corresponding to these topics.

We hope that this book can help foreign medical students to learn and understand Molecular Biology.

Authors

Practical 1. Hoechst or DAPI staining of DNA in eukaryotic and bacterial cells.

Theoretical background. 4′,6-Diamidino-2-Phenylindole (DAPI) and Hoechst dyes (HDs) are blue fluorescent, nucleus-specific dyes for live or fixed cells staining. The dyes have little fluorescence in solution, but become brightly fluorescent upon double strand DNA(dsDNA) binding. The cells can be stained without a wash step. The dyes are stable and allow live cells to survive few days or longer in specimens and because of this property these stains are called *supravital*.

DAPI was first synthesised in 1971 in O.Dann's lab as anti-trypanosomiasis compound. It was ineffective as a drug, however, able to attach to dsDNA and became more strongly fluorescent, when dsDNA bound. This findings led to its use as mitochondrial DNA identifying agent in *ultracentrifugation.* Later the DAPI was adopted as dsDNA stain in *fluorescence microscopy* (FM). Since late 1970s it is used in flow cytometry (FC) and for quantification of dsDNA in cells and viruses [7].

When bound to dsDNA the DAPI has maximums in absorbance at $\lambda_{\mathbf{Abs}}$ =358 nm (UV light) and in emission at λ_{Em}= 461 nm (blue light). So, in FM the DAPI is excited by UV light and is detected through a blue/cyan filter. The emission peak is enough broad. When joined to RNA, it is less fluorescent (λ_{Em} ≈500 nm) [12, 16].

Hoechst 33258, 33342 and 34580 (HDs) belong to a family of bisbenzimide blue fluorescent dyes used to stain dsDNA. They were invented by the German company Hoechst AG in the early 1970s [4, 10]. First 2 are most commonly used. In contact with dsDNA the HDs 33258 and 33342 are excited by UV light at 352 and 350 nm, respectively, and fluoresce with maximum at λ_{Em} = 461 nm. Unbound dyes emit light with maximums in the 510–540 nm range. Upon dsDNA binding, their fluorescence increases ~30-fold due to suppression of rotational relaxation and hydratation [1, 5, 13].

HDs stain dsDNA in methods: FM, *imunocytochemistry*, often with other fluorophores [12]; FC, e.g., for cell sorting by phase of the cell cycle [8]; dsDNA determination and quantification in the presence of RNA [11]; automated dsDNA detection [19]*;* chromosomes (chrs) sorting [11].

Stem cells (hematopoietic or embryonic) are effectively efflux the HDs and can be detected via FC in the *side population*. This is done by passing the HDs-emitted fluorescence through both red and blue filters, and plotting HD red data against blue.

Live or fixed cell membranes are HDs-permeable. Cells with specific ATP-binding cassette transporter proteins can also actively efflux these stains. Comparing to DAPI, the HDs have additional C_2H_5 group that renders them more cell-permeable. HD 33342 exhibits a 10-fold greater cell permeability than HD 33258. HDs are less toxic, than DAPI and HDs-stained cells survive better. DAPI is somewhat less cell membrane permeant than HDs, and applyed at a higher concentration, usually at concentration 10 μg/mL, for live cells and at 1 μg/mL for fixed (dead) cells. Because of more high concentration of DAPI than HDs needed for live cell staining, the DAPI is often substituted by HDs for live cells [20]. DAPI dilactate is more soluble DAPI salt, good for making stock and ready-to-use aqueous solutions of DAPI [17].

HDs are soluble in water or organic solvents such as dimethyl sulfoxide and dimethyl formamide up to 10 mg/mL. HD 33258 is more water soluble than HD 33342. The aqueous solutions are kept at 2–6°C for ≤ 6 months in light-proof containers or at −20°C or below for longterm storage.

DAPI and HDs are used also, when multiple fluorescent stains are applied in a single sample. Partial fluorescence overlap between DAPI or HDs and green-fluorescent molecules like *fluorescein* and *green fluorescent protein* (GFP) has little effect on study results. If extremely precise image analysis is necessary, spectral unmixing approach can be applied.

Both dyes penetrate cell membranes and used at 1 μg/mL as nuclear counterstains for live or fixed cells [17]. HDs 33342 and 33258 are quenched by thymidine substitutive, the bromodeoxyuridine (BrdU), which is commonly used to detect dividing cells. When BrdU is integrated into dsDNA, it is supposed that the bromine deforms the minor groove so that HDs can't reach their optimal binding site. The HDs bind even stronger to BrdU-substituted dsDNA, however, no fluorescence observed. BrdU can be used together with HDs to monitor cell cycle progress [3,9].

High specificity towards dsDNA make HDs dyes excellent targeting moieties, which can be conjugated to various other molecules in order to tether them to dsDNA for studies of cells and medicinal treatment [4].

The DAPI and HDs bind to the minor groove of double-stranded dsDNA with affinity to A-T rich sequences and significant increase in fluorescence *[16] after these dyes binding*.

Joining of DAPI and HD stains to dsDNA, may interfere with DNA replication. Thus, they are potentially *mutagenic* and *carcinogenic*. HD stain 33342 as less cytotoxic is used to sperm sex sorting in animal or human. Safety of this method has been questioned [2, 14].

DAPI is used to check for contaminant *Mycoplasma* or virus dsDNAs in cell cultures or growth media [18].

In bacterial or eukaryotic cells dsDNAs are stained with DAPI solutions 0.1–12 μg/ml for 1-30 minutes at 18-37°C, then, washed to remove unbound dye. Green fluorescence may be observed on samples overloaded by dye or washed only partially. HDs are often used instead of DAPI. HDs or DAPI at concentration 12-15 μg/mL in phosphate buffer saline (PBS) or 150 mM NaCl solution stain live or killed bacteria during 30 minutes at 18-37°C more dimly than mammal cells. Dead cells tend to stain more brightly than live cells. In *Saccharomyces cerevisiae* at 12-15 μg/mL, DAPI and HDs preferentially stain nuclear and cytoplasmic dsDNA of dead cells. In live yeast, HDs shows dim nuclear and cytoplasmic staining, while DAPI shows dim mitochondrial staining. The fluorescence intensity of HDs is pH dependant [12].

Experiment 1. Staining of live cells

Materials and equipment. Laboratory gloves, fluorescence microscope, centrifuge, thermostatic chamber, pipette with disposable sterile noses, marker for glass, fresh bakery yeast *Saccharomyces cerevisiae* from supermarket; HeLa cells culture grown on medium DMEM (Dulbecco's Modified Eagle Medium)

(Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1.375 mg penicillin/streptomycin antibiotics (Gibco/Invitrogen) in 10-cm culture dish; 125 mL of the same sterile medium for HeLa cells; 0.1% trypsin - ethylenediamine tetraacetic acid (EDTA) solution; phosphate buffer saline (PBS); Antifade mounting medium (Boster); Hoechst 33258 and DAPI dyes (ThermoFisher Scientific).

Procedure.

Medium exchange method provides the uniform exposure of live cells to stain. They must be collected by centrifugation step to exchange the medium. However, for some cell types, morphology and/or viability may be affected by this procedure. Floating dead cells may be lost during medium removal.

1. **Wear gloves!** Dyes are potentially *mutagenic* and *carcinogenic*. Take few milliliters of fresh medium and add to it Hoechst 33258 to 1 μg/mL or DAPI to 10 μg/mL content.
2. Heat 0.1% trypsin - EDTA to room temperature and incubate sterile medium to 37℃.
3. Check grown HeLa culture under microscope for cell confluency and for cell death. If the cells reached ≈ 90% confluency, remove out spent medium using pipette and wash cells 2 times with PBS.
4. Cover completely cells area of the culture dish with 1 ml of 0.1% trypsin-EDTA solution and keep the cells at 37ºC for 2 minutes
5. Observe cells under the microscope. If they are fully detached, remove out the 0.1% trypsin-EDTA using pipette.
6. Wash cells 2 times with PBS.
7. Add 6 ml of fresh medium to the dish, gently pipet the cells, centrifugate at 1,000 rpm for 5 minutes.
7. Remove the supernatant. Do not disturb the cell pellet.
8. Gently resuspend the cells from the pellet in 2 ml of fresh medium containing Hoechst 33258 at 1 μg/mL or DAPI at 10 μg/mL. **Note**: DAPI or Hoechst 33258 can be combined with other fluorescent dyes.
9. Incubate cells at room temperature or 37°C for 5-15 minutes.
10. Apply 20 μL drop of the stained suspension onto each of 2 glass slides, place a cover slip on one of them and allow another to air dry completely without cover slip.
11. Observe covered preparation at objectives 4×, 10× and 40×. Once good view has been located, rotate the high-dry objective out of the light pass and drop the immersion oil on the light spot. Rotate the oil-immersion objective and place its lens in contact with the oil drop.
12. Place 2 dots of antifade mounting medium on the air-dried drop of culture and cover them with cover slip. Squeeze the excess of antifade mounting medium out by gentle pressing against cover slip surface with blunt end of the pencil eraser. Allow the mounting medium to dry during 1 hour before using an oil-immersion microscopy.

    Note: Washing is not needed for specific staining, but nuclear fluorescence is stable after washing. If protected from light, mounted slides can be stored at 4˚C to -20˚C for 2-3 weeks [17].

13. Observe the HeLa cells with blue stained nuclei and nuclei with bright blue chrs within them (Fig.1). The culture of HeLa cells originate from cervical cancer of *He*nrietta *La*cks, the patient who died in 1951 at age of 31. This tumor cells often contain more or less than 46 chrs (2n number in human). Although 50 to 70 per nucleus most frequently found, HeLa cells containing 200 chrs have been observed [15]. Count chrs in your preparations.

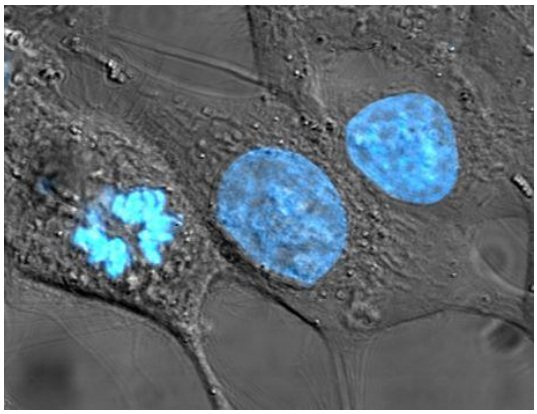

Fig. 1. The HeLa cells: Hoechst 33258 stains nuclei are blue. The leftmost cell is in mitotic prometaphase. Its chrs fluoresce brightly as they consist of highly compacted DNA. Magnification 1000 ×. *By Masur, 2007* [6].

Live HeLa cells staining by direct addition of 10× dye

Direct addition of 10× dye is a suitable method that doesn't require medium exchange, but suspension must be mix immediately yet gently to avoid high transient stain concentration or disruption of cells by pipetting. Adding highly concentrated dye directly to cells in culture is not good, as this will result in local areas of high dye exposure [17].

1. The dye is included into culture medium at 10 times the final staining concentration. Dilute DAPI to 100 µg/mL, or Hoechst 33258 to 10 µg/mL. **Note:** DAPI or Hoechst 33258 can be combined with other fluorescent dyes.
2. Add 1/10 volume of 10× dye directly to the well without removing the medium from the cells.
3. Provide thorough mixing the medium with dye by immediate gentle pipetting up and down. The plates with larger well sizes (e.g., 24-well to 6-well plates), can be gently swirled to mix.
4. Cells are incubated at 18-37°C for 5-15 minutes, than prepared onto slides as described above. **Note:** Washing can be omitted for specific staining, but nuclear staining is stable after washing [17].

Experiment 2. Staining of fixed tissues and fixed or non-fixed microbes.

Materials and equipment. Fluorescent microscope, centrifuge, marker for glass, fresh bakery yeasts *Saccharomyces cerevisiae* from the supermarket, liquid cultures of *Eschericha coli* and *Bacillus subtilis* on defined media; phosphate buffer saline (PBS);

antifade mounting medium (Boster); 150 mM NaCl solution; Antifade mounting medium (Boster); Hoechst 33258 and DAPI dyes (ThermoFisher Scientific); formaldehyde fixed tissue sections.

Procedure.

1. Add the dye to PBS at 1 μg/mL.**Note:** DAPI or Hoechst 33258 can be included together with labeled antibodies or other fluorescent stains; the dyes also can be diluted in buffers with detergent or blocking agents if convenient.
2. Prepare suspension from bakery yeast in PBS. Spin this suspension and cultures of *E.coli* and *B. subtilis* in centrifuge and suspend the pellets in PBS for washing twice.
3. Place 10 μL of bacterial suspensions (*Escherichia coli* or *Bacillus subtilis*) or yeast suspension (*Saccharomyces cerevisiae*) onto each of 2 glass slides per one culture.
4. Allow drop of suspension on each slide to dry near the flame. To fix microbes on 1 slide of 2 prepared for each culture, move the slide quickly through the flame upper part 2-3 times. **Note:** Do not burn the cells! Control temperature of the slides by keeping them in your fingers. Mark fixed slides "F" with marker. As an alternative, yeast suspension (1 mL) can be fixed with 0.1 mL 36% formaldehyde.
5. Stain all the glass slides with 12-15 μg/mL Hoechst 33258 or DAPI in phosphate buffer saline (PBS) or 150 mM NaCl solution for 30 minutes at 18-37°C.
6. Prepare slides for microscopy as described above.
7. Add the PBS with dye to fixed tissue sections, incubate at 18-37°C for at least 5 min.
8. Prepare slides for microscopy; washing is optional but not required.
   Note: Samples can be stored at 4°C after staining and before imaging.
   DAPI can be included directly in antifade mounting medium for 1-step mounting and staining. When use DAPI or Hoechst 33258 in mounting medium, longer incubation times may be required for DAPI to penetrate the cell nuclei well [17].
9. Observe preparations in microscope at 10×; 40× and in immersion objectives. Compare fixed and non-fixed preparations.
   It is known, that dead bacterial cells tend to stain more brightly, than live cells. In dead yeast cells, DAPI and Hoechst 33258 stain the dsDNA within nucleus and cytoplasm. In live yeast cells, Hoechst 33258 shows dim nuclear and cytoplasmic staining, while DAPI shows dim mitochondrial staining [17]. What are your results?

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Practical 2. Methyl green - pyronin method for DNA and RNA detection in cells.

Theoretical background. Methyl green-pyronin (MGP) staining is a classical technique utilizing two basic dyes for the detection and differentiation of DNA and RNA. This cytochemical procedure allows to study nucleic acids without their extraction from the cells [24].

Methyl green (MG) or CI 42585 is a stain with positive charge (Fig. 1, A). Friedrich Miescher, a Swiss physician and biologist discovered the DNA in 1869 and was first who used MG for staining of this macromolecule preparations [*2*]. Currently, the fluorescent DNA staining of nuclei by MG is used for far-red visualization of live cell nuclei and in malignant tumor prognosis [11, *19, 20*]. MG excited by 244 or 388 nm light in aqueous solution with pH7, emits fluorescent light at 488 or 633 nm, respectively. In DNA bound state at neutral pH in water, MG becomes fluorescent in the far red region with an Ex_{max} = 633 nm and an Em_{max} = 677 nm also [*19*]. It is flat molecule (Fig.1, A) able to insert into DNA major groove. Its interaction with the DNA is proved to be electrostatic [*10*]. MG has affinity to A-T reach regions of the major groove [20] and is specific to negatively charged phosphate radicals in the double strand DNA (dsDNA). It stains the dsDNA blue-green.

MG is an effective and cheap dye for DNA staining in polyacrylamide or agarose gels with detection limits for faint bands as well as relative densitometric quantitation equivalent to another fluorescent dye, the ethidium bromide [17].

MG is used in *fluorometric assays*, flow cytometry (FC), fluorescent embryo labeling [*19, 20*] and as an exclusion *viability stain* for cells.

MG may be used as a pH indicator. Its solution is a suitable counterstain for chloroacetate esterase, nonspecific esterase, alkaline phosphatase, peroxidase, naphthol AS acetate esterase, and acid phosphatase. A typical working concentration of MG as a counter stain is 0.1- 0.5% [14]. It is used in screening of nuclease producers in microbiological studies [16].

MG powders often contain crystal violet. Its presence in MG solution makes interferes with staining of DNA. Thus, crystal violet must be removed by extraction several times with chloroform until no traces of violet stain could be seen in extracts.

Pyronin Y or CI 45005 (syn. = pyronin G, pyronin J, pyronin) (P) is a cationic dye also (Fig. 1, B). However, it has no specific affinity to dsDNA and its attachment to the negatively charged RNA results in pink-red color. P intercalates RNA. In combination with Hoechst 33342 pyronin Y (PY) can be used in method of differential staining of RNA at the DNA presence [4].

The P dye accumulates in the mitochondria of living cells. At low concentrations the *cytostatic* effect is presented as cell arrest in the G1 phase, whereas at high concentrations the *cytotoxic* effect is manifested as cell arrest in the G2 and S phase of the cell cycle [3]. In combination with Alcian blue P is used in studies of lungs diseases [15].

P is a fluorescent compound with an excitation peak at 547 nm and an emission peak at 566 nm [9]. P stains the cytoplasm and most nucleoli, as RNA containing

materials, in pink-red. MG stains DNA blue-green [13]. Thus, combined use of P with MG in the MGP staining allows DNA and RNA differentiation [12, 24].

MGP staining distinguishes proliferating from differentiated non-proliferating cell nuclei after acid denaturation of DNA [22]. The MGP method is useful in identifying the distribution of Nissl substance in bodies of neurons cell [23].

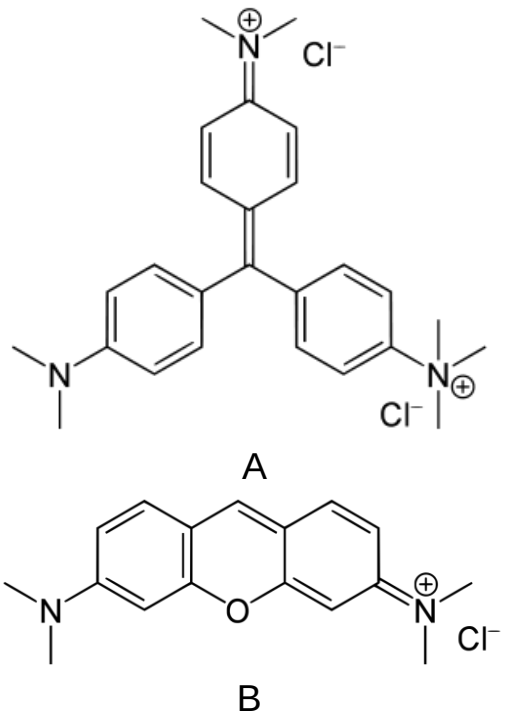

Fig. 1. Skeletal formulae of MG (A) and P (B). *By Yikrazuul,* 2008 [6, 7].

MGP method utilizes formalin fixed tissue or alcohol-ether fixed smears [*18*, *19*]. The technique is widely used in diagnostic cytology of human and animal diseases [5]. An example of MGP stained preparation is presented in Fig. 2. *Theileria annulata*, a tickborne intracellular protozoan parasite of cattle, present in Southern Europe, North Africa, the Near and Middle East, Central Asia, India, China. It causes both: mortality and reduced production in animals [8].

Experiment 1. Demonstration of DNA and RNA nucleic acids in tissues

Materials and equipment. Racks, glass slides, cover slips, tubes, flasks, pipettes and fluorescence microscope. Stains MG (CI 42585) and P Y (CI 45005) (Sigma-Aldrich).

Fixative: 10% buffered neutral formalin is used as fixative [18] . Other fixative which may be used are Carnoy's, formol saline and Zenker's. Any well fixed paraffin embedded tissue can be used. Paraffin tissue sections cut at 5-6 μm [1,10], xylene or xylene substitute for deparaffinization, fresh reagent alcohol.

0.2M acetic acid - Na acetate, pH 4.2. Prepare 0.2 M sodium acetate (Na acetate anhydrous, 2.05 gm + distilled water to total of 125 mL), 0.2M acetic acid (acetic acid, glacial, 1.5 mL + distilled water, 123.5 mL), than mix 0.2M acetic acid, 35 mL + 0.2M sodium acetate 12.5 mL. Adjust, if necessary, pH to pH 4.2 with 0.2M acetic acid or 0.2M Na acetate solutions. Store at 4ºC.

0.5% MG Solution (0.2M acetate buffer, pH 4.2, 5 ml + MG, 0.025 gm)

MGP solution (0.5% MG solution, 5 ml + P, CI 45005, 0.0025 gm)

The P content in commercial preparations falls between 50% and 90%. For good staining, it is necessary for some samples of P to increase its amount up to 0.005 gm.

Sigma-Aldrich, Inc. sells the ready to use MGP solution (Cat. No. HT7016-500) made of certified MG, 0.012% and P (certified), 0.01% methanol, 0.75%, in deionized water. MGP solution should be stored at room temperature (18–26°C).

Procedure.

1. Put laboratory gloves on clean hands.

2. Deparaffinize tissues using xylene or xylene substitute and hydrate through alcohols to distilled water.
3. Rinse slide thoroughly in distilled water.
4. Flood sections with MGP solution of room temperature. Let stand for 2 to 7 minutes.
5. Note: Increasing stain time intensifies P staining (red color); decreasing time intensifies MG staining (blue-green to green color) [12].
6. Dip quickly 1-2 times in distilled water of room temperature.
7. Blot with blotting paper.
8. Dehydrate slide through 3 changes of fresh reagent alcohol.
9. Place in acetone for 10 seconds.

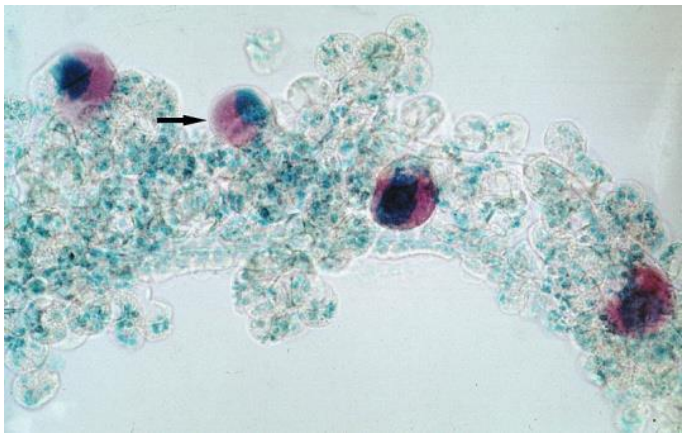

Fig.2. *Hyalomma anatolicum anatolicum* salivary gland with sporoblast stage (arrow) of *T. annulata*. *By A.R. Walker* 2012 [8].

10. Dip 3 times in equal parts acetone /xylene.
11. Clear in fresh xylene (not in a xylene substitute), 3 changes.
12. Mount with permanent mounting medium.
13. Observe the stained tissue slides under fluorescence microscope.

Staining Results: The cells nuclei are blue to blue-green due to DNA presence. The nucleoli and cytoplasm are pale pink to red due to RNA staining by P.

Experiment 2. Detection of DNA in protozoan cells and avian blood cells

In contrast to the discoid red blood cells of human and other mammals, the erythrocytes of birds are ellipsoid and contain the nuclei [21]. Thus, they are good for DNA staining in the cell nuclei.

Materials and equipment. Laboratory gloves, racks, glass slides and cover slips, tubes, flasks, pipettes and fluorescence microscope.

Protozoa culture, chiken blood, formo-saline, acetone, distilled water, glycerine, heparin 10 000 IU, MGP solution as above, acetic acid, xylene, laboratory gloves, tubes, racks, glass slides and cover slips, flasks, pipettes with sterile noses, syringes, and fluorescence microscope.

Solution of heparinized saline: Add sodium heparin to physiological saline (0.9%) to make a final concentration of 200 IU/ml.

Solution of formo-saline: Dissolve 10 mL formalin (40% solution of formaldehyde) in 90 mL of normal (0.9%) saline.

Ready to use MGP solution (Cat. No. HT7016-500) made of certified MG, 0.012% and P (certified), 0.01% methanol, 0.75%, in deionized water (Sigma-Aldrich, Inc.).

Procedure.

Protozoan Culture Preparation Collect soft plant remnants from the ground into dark jar half-filled with pond water. Incubate the jar without cover at room temperature for development of protozoa culture.

1. Put laboratory gloves on clean hands.
2. Place a drop of protozoa culture onto washed dry glass slide.
3. Add a drop of acetic acid (for 2 minutes) to fix the microbes.
4. Rinse the slide with distilled water.
5. Apply a drop of MGP stain for 15 minutes.
6. Again wash with distilled water and then dehydrate it with acetone.
7. Finally, wash it with xylene.
8. Mount the slide with glycerine.
9. Observe the green blue color of DNA in nuclei under the fluorescence microscope.

Preparation avian blood smear

1. Take 1 mL of chicken blood in a syringe containing 1 mL of heparinized saline. Blood can be taken from jugular vein, wing or brachial vein and medial metatarsal vein.
2. Place a drop of chicken blood on slide and make a thin smear as shown in Fig. 3.
3. Dry the smear in sterile air flow.
4. Fix the blood cells onto surface of glass slide by addition of few drops of formal saline (for 7 minutes).
5. Cover smear with several drops of MGP stain for 15 minutes.
6. Dip quickly 1-2 times in distilled water of room temperature.
7. Blot with blotting paper.
8. Dehydrate preparation in acetone.
9. Wash glass slide smear with xylene.
10. Place 15 μL drop of glycerine onto smear and cover slide with cover slip.
11. Observe smears under fluorescence microscope.

Staining Results: The nuclei in the chicken RBC are green-blue due to stained DNA and cytoplasm is pink colored due to RNA presence.

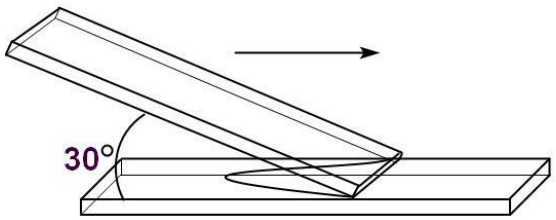

Fig. 3. Preparation of blood smear.
By D.B. Pylypiv.

Literature

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Practical 3. Feulgen staining of nuclear DNA in eukaryotic cells.

Theoretical background. The Feulgen procedure was invented by German physician and chemist Joachim Wilhelm Robert Feulgen during 1914-1924 [*6, 7*]. He also discovered animal and plant DNA (*"thymonucleic acid"*) congeniality and estimated the nature of nucleic acids as a polymers of nucleotides with four kinds of nitrogenous bases [11]. It is, probably, the most simple cytochemical method allowing indication and evaluation of DNA levels into cells. Schiff's reagent is the stain used in this technique. It specifically stains the DNA due to reaction of Schiff's (or Schiff's-like) reagent with aldehyde groups exposed at C1 as the result of the nitrogen bases cleavage from deoxyribose by 1N HCl hydrolysis at 60°C within about 10 minutes.

Usually, a sulfite rinse followed the hydrolysis, but this is now considered worthless [4]. Optionally, the sample can be counterstained with Light Green SF yellowish [5]. DNA should be stained red. The background, if counterstained, is green. To avoid the bacground fading, Light Green SF can be replaced with Fast Green FCF [3], which also has more brilliant color. Then, it is dehydrated with alcohol, cleared with xylene, and mounted in a resinous medium [4].

The pink color intensity correlates with DNA content in cell. However, Feulgen technique is a semi-quantitative. It is becoming quantitative for DNA, if the only aldehydes remaining in the cell are those produced in hydrolysis of DNA. It is used for DNA quantification in nuclei by image cytometry for ploidy evaluation in oncology [1, 2, 8].

The DNA content cannot be measured directly from microscopy. It is not easy to discern visually differences in the intensity of the Feulgen stain reaction within the nuclei. The microdensitometer or microscpectrophotometer are used to measure the intensity of the pink color for a given organelle [10]. Using this method, it was early determined that interphase cells were composed of two populations, those with diploid and those with tetraploid levels of DNA. The nuclei looked identical, but one contained twice as much DNA within it. This gave rise to the division of the interphase period of the cell cycle to a G1, an S, and a G2 based on the synthesis of that extra DNA [*4*].

After quantitative DNA-staining, the nuclear Integrated Optical Density (IOD) is the cytometric equivalent of its DNA content. The quantitation of nuclear DNA requires a rescaling of the IOD values by comparison with those from cells with known DNA content. Therefore the DNA content is expressed in a "c" scale in which 1c is half the mean nuclear DNA content of cells from a normal (non-pathological) diploid population in G0/G1 cell cyle phase [1].

Because the intensity color in the Feulgen reaction depends from many variables in its conduction, several attempts were made in standardization of the method [1, 9, 10].

The application of the Feulgen staining in electron microscopy allowed to study of the structural organization of DNA *in situ* [2].

As DNA from any of organisms has the same nature, we can use the cells from any source (humam, animals or plants) to demonstrate its presence in nuclei. We are using the onion roots for teaching purposes in this practical.

Materials

Schiff's reagent, 1N HCl, sodium or potassium metabisulphite, freshly prepared bleaching solution (5 ml of 10% sodium metabisulphite + 5 ml of 1N HCl + 90 ml of distilled water), 45% acetic acid, mixture glycerol:water (1:1, v/v), hydroponically growing onion.

Preparation of Schiff's reagent

1. Dissolve 0,5g of basic fuchsin in 90 mL of boiling distilled H_2O
2. When cooled to approximately 45°C, add slowly 10 mL of 1N HCl
3. Cool to room temperature and add 1g of $K_2S_2O_5$ or $Na_2S_2O_5$ (metabisulfite can be substituted on sulfite, hydrosulfite or sulfurous acid).
4. Shake for 3 minutes and leave it the dark at room temperature overnight or until a light straw or faint pink color develops.
5. Add 0,5 g of fine activated charcoal and shake for 3 minutes.
6. Filter solution (should be transparent).
7. Store at 4°C in a tightly-stoppered bottle in the dark.

Procedure.

1. Fresh onion root tips are transfered to distilled water through alcohol:
Absolute ethanol 5 min, than 90%, 70%, 50% and 30% ethanol 5 min each
2. Distilled H_2O 5 min
3. Cool 1N HCl 2 min, then 1N HCl at 60°C in water bath, 6 min
4. Cool 1N HCl quick rinsing. Then rinse in distilled water once

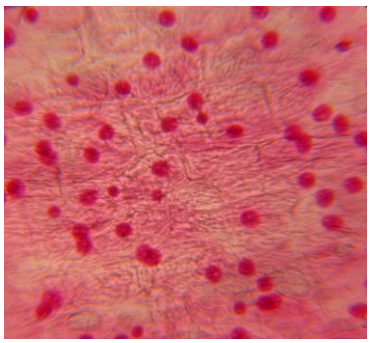

Fig. 1. Feulgen staining of nuclei in onion tip cells.
The tissue consists of more than one layer of cells. This results in numerous dark pink nuclei on the field of view of light microscope. Magnification 400 ×. *By B.M.Sharga.*

5. Schiff's reagent 5 min
6. Bleaching solution, 1 min. Then rinse in distilled water once
7. 45% acetic acid, 5 min.
8. Place the root tip in a drop of 45% acetic acid onto glass slide (or in a drop of 50% glycerol (distilled H_2O + glycerol 1:1), cover it with cover slip and make a pressured preparation by gently pressing between finger and thumb.
9. Detect cells bright pink cytoplasm and dark pink nuclei at objective 40× (Fig.1).
10. Compare pink color intensity in tissue of tip end and its middle part. It is directly proportional to the amount of DNA present per unit area.

Answer the MCQs

1. DNA content in Feulgen stained nuclei can be measured by____mounted on microscope **a)** microdensitometer **b)** microscpectrophotometer **c)** microcalorimeter **d)** a & b
2. Feulgen staining **can't** be used for **a)** LM **b)** EM **c)** LM and EM **d)**vital cell staining

3. The Feulgen reaction **a)**consists of: acid hydrolysis; DNA nitrogen bases cleavage and aldehyde groups formation on deoxyriboses; Schiff's base reaction with aldehyde groups with production of red-violet product **b)** consists of all mentioned in a), but for RNA and ribose **c)** is DNA-specific **d)** is RNA-specific
4. Methyl green is used in **a)** far-red imaging of live cell nuclei **b)** fluorescent DNA staining in cancer prognosis **c)** *flow cytometry* **d)** exclusion *vability stain* **e)** a-d
5. The methyl green binds DNA by **a)** intercalation **b)** affinity to minor groove AT rich regions **c)** electrostatic action with the DNA major groove **d)** bis-intercalation
6. Pyronin stains RNA **a)** red **b)** blue **c)** green **d)** yellow
7. Hoechst dyes stain DNA to fluoresce **a)** blue t **b)** red **c)** green **d)** none of the above
8. DAPI stain DNA fluorescing **a)** blue **b)** red **c)** green **d)** yellow
9. Hoechst stain DNA fluorescing **a)** blue **b)** red **c)** green **d)** yellow
10. Which not binds to minor groove? **a)** DAPI **b)** HD33258 **c)** HD33342 **d)** MG
11. Match: Acridin orange fluoresces **i)** red with **ii)** green with **a)** RNA **b)** ssDNA **c)** dsDNA.
12. Find untrue for HD 33258, 33342 **a)** permeant **b)** AT-selective **c)** major groove–binding **d)** dsDNA-selective binding **e)** cell-cycle studies **f)** chromosome and nuclear counterstain
13. Ethidium bromide is used in **a)** flow cytometry **b)** electrophoresis **c)** dead-cell staining **d)** as a chr counterstain **e)** all of the above

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Practical 4. Extraction of nucleoproteins, their hydrolysis and reactions on hydrolysis products.

Theoretical background. Many proteins biside recidues of amino acids contain other substances. Such proteins are called *conjugated proteins*. Conjugated proteins that are covalently or noncovalently linked to nucleic acids are called *nucleoproteins*, those, joined to lipids are referred as *lipoproteins*. *Glycoproteins* are proteins bond to carbohydrates. Proteins can also be combined with various low molecular weight organic groups, metal-containing groups and metals.

The DNA of the nucleus is bound with about an equal mass of a group of histone proteins and forms with them a highly condensed nucleoprotein complex called *chromatin* [4]. Typical nucleoproteins include nucleosomes, ribosomes, viral nucleocapsid proteins. Depending on type of joining nucleic acids, the nucleoproteins are categorized into deoxyribonucleoproteins (associated with DNA) and ribonucleoproteins (associated with RNA).

The positive charge of most of nucleoproteins, facilitates interaction with the negatively charged nucleic acids. The tertiary structures of many of them were studied with X-ray crystallography, nuclear magnetic resonance and cryo-electron microscopy [2, 5]. The deoxyribonucleoproteins participate in regulating DNA replication transcription and site-specific recombination [1]. The mRNA always associate with ribonucleoproteins and function as ribonucleoprotein complexes [4].

The studies of nucleoproteins are important for estimation of disease-related genes regulation and for search of new proteomic tags for diseases. The extraction of nucleoprotein is necessary for the studies of spatial structure and biological functions. The nucleoproteins can be extracted from cultured cells or tissues, particularly by the methods of Lee et al. [3], Zerivitz, Akusjärvi [6], as presented below.

Experiment 1. Extraction of nucleoproteins

Reagents, solutions.

Phosphate solution buffer: pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4.

Buffer A: pH 7.9, 10 mM HEPES, 1.5 mM $MgCI_2$, 10 mM KCI, 1 mM DTT, with 1× protease inhibitor cocktail and phosphatase inhibitor cocktail.

Buffer B: pH 7.9, 20 mM HEPES, 1.5 mM $MgCI_2$, 25% glycerol, 420 mM NaCI, 0.2 mM EDTA, 1 mM DTT, with 1× protease inhibitor cocktail and phosphatase inhibitor cocktail.

Buffer C: pH 7.9, 20 mM HEPES, 20% glycerol, 100 mM KCI, 0.2 mM EDTA, 1 mM DTT, with 1× protease inhibitor cocktail and phosphatase inhibitor cocktail.

Equipment: Homogenizer, centrifuge, pipettes, flasks, water bath

Procedure

Cultured cells preparation. Note. Baker's yeast if no cultured cells.

1. Centrifuge at 500 × g at 4°C for 5 min to harvest the cells
2. Wash the cells with cold PBS, centrifuge at 500 × g at 4°C for 2 to 3 min, and discard the supernatant.

3. Repeat Step 2, carefully discard the supernatant, leaving the cell pellet as dry as possible.
4. Add 1 mL cold Buffer A for 1×10^7 cells, vortex for 15 sec to fully suspend the cell pellet.
Skip to Step 10

Tissue preparation:

5. Weigh the tissue, cut the tissue into small pieces and then collect the fragment into a fresh 1.5 mL tube.
6. Wash the tissue with cold PBS, centrifuge at 500 × g at 4°C for 5 min
7. Discard the supernatant carefully to leave the pellet as dry as possible.
8. Add 1 mL cold Buffer A for 20~100 mg tissue, homogenize tissue using a homogenizer.
9. Vortex the tube vigorously for 15 sec to fully suspend the cell pellet.

Protein extraction:

10. Incubate the tube on ice for 10 min, and vortex again for 5 sec.
11. Centrifuge at 16,000 × g at 4 °C for 5 min, and immediately transfer the supernatant into a fresh 1.5 mL tube for cytoplasmic extraction.
12. Suspend the pellet with 0.75 mL cold Buffer B and vortex the tube for 15 sec.
13. Incubate on ice for 40 min, and vortex the tube for 15 sec very 10 min.
14. Centrifuge at 16,000 × g at 4 °C for 10 min, and immediately transfer the supernatant into a fresh 1.5 mL tube.
15. Reextract the residual pellet with 0.25 mL cold Buffer B as the same procedure describes at Step 14.

Dialysis (optional)

16. Following extraction, the nuclear extract is dialyzed for 2 hr against 100 volumes of Buffer C.
17. Centrifuge at 2000 × g at 4 °C for 10 min to remove precipitate.
Note: The short dialysis period results in less precipitation of denatured protein and is sufficient to adjust the extract to the desired ionic conditions.
18. Store at -80 °C until use.

Experiment 2. Hydrolysis of nucleoproteins and reactions on hydrolysis products.

Reagents 1% copper sulfate solution, 10% sulfuric acid solution, concentrated sulfuric acid, 2.5 g of baker's yeast, 10% sodium hydroxide solution, concentrated ammonia solution, 2% ammoniacal silver nitrate solution, thymol, diphenylamine reagent, 0.2% α- naphthol solution, ammonium molybdate,
Ammonium molybdate. This reagent is prepared as follows. 20 ml of 10% ammonium molybdate solution is mixed with 3 ml of HCl, then 5 g of NH_4Cl are added.

Equipment. Litmus papers, a 200 ml round bottom flask with a refrigerator for hydrolysis, a stopper for this flask with a long glass tube, a water bath, 16 mL fresh glass test tubes.

Procedure.

1. Transfer 5 mL of the extract obtained in experiment 1 to the 200 ml round bottom flask and add 10 mL of 10% sulfuric acid solution.
2. Close the flask with a stopper with a long glass tube.
3. Boil gently for 10 min. If very turbid, filtrate.
4. Qualitative reactions with nucleoprotein components of the hydrolysate are carried out with the filtrate as described below.

Biuret reaction for protein component. To 10 drops of hydrolyzate add 20 drops of 10% sodium hydroxide solution to the alkaline reaction (check by litmus paper), then add 4 drops of 1% copper sulfate solution. A pink or pink-purple color appears.

Silver test on purine bases. To 10 drops of hydrolysate add dropwise a concentrated solution of ammonia (approximately 10 drops) to the alkaline reaction on litmus paper, then add 10 drops of 2% ammonia solution of silver nitrate. Leave the tube to stand for 3-5 min. A light brown silver salts of purine bases precipitate.

Reactions to pentose. To 10 drops of hydrolyzate add 3 drops of 1% alcohol solution of thymol, mix and VERY CAREFULLY AND SLOWLY pour 20-30 drops of concentrated sulfuric acid on the wall of the test tube. When mixed at the bottom of the tube, the red product of furfural reaction with thymol is formed.

To 10 drops of hydrolyzate in another test tube pour 6 drops of 0.2% alcohol solution of α-naphthol and 40 drops of concentrated sulfuric acid. A pink-purple color appears.

Reaction to ribose and deoxyribose. Diphenylamine reagent with ribose gives a green color, and with a solution of deoxyribose - blue. To 10 drops of hydrolyzate pour 40 drops of 1% solution of diphenylamine reagent and boil in a water bath for 10 minutes. A blue-green color appears.

Molybdenum test for phosphoric acid residues. To 10 drops of hydrolyzate add 40 drops of ammonium molybdate and heat in a water bath at 75 ° C for 15 min. The solution turns lemon yellow.

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Practical 5. Protein precipitation reactions.

Theoretical background. Protein precipitation is the process in which protein is separated from any extra contaminants. Precipitation of proteins with salts of alkali and alkaline earth metals is called "salting". Different proteins are precipitated by different concentrations of salts, so when salting out, proteins can be divided into fractions. Thus, *serum albumin* is precipitated by a saturated solution of ammonium sulfate in a neutral medium, and globulins are precipitated by semi-saturated solutions in such a medium.

To "salt out" a protein, different neutral salts can be utilized, however, ammonium sulfate is most used. At high salt concentration the protein interacts with the salt as opposed to the water, which leads to less interaction between the water and the protein's solvation layer, which in turn leads to more hydrophobic patches being exposed to interact with one another. This results in protein precipitation.

Ammonium sulfate is the preferred, because it is high on the Hofmeister series and highly soluble. The process takes during 30 min to an hour before the sulfate ammonium/protein mixture is sent to the centrifuge.

Proteins are easily precipitated by salts of heavy metals (lead, copper, silver, mercury, etc.). High concentrations of heavy metal salts are required for precipitation.

Salting of proteins is their precipitation with salts of alkali and alkaline earth metals, which depends on the radii of ions and their ability to hydrate. The ability of salt to precipitate proteins increases in the Hofmeister series, for anions: $SCN^- < ClO_4^- < NO_3^- < I^- < ClO_3^- < Br^- < NO_3^- < Cl^- < CH_3COO^- < HPO_4^{2-} < SO_4^{2-} < F^- < PO_4^{3-}$, and for cations: guanidinium $< Ca^{2+} < Mg^{2+} < Li^+ < Na^+ < K^+ < NH^{4+} < Rb^+ < Sc^+ < Mo^{2+} < Sr^{2+} < Ba^{2+}$ [1, 2, 4, 7, 9, 10].

To precipitate hydrophilic proteins, their water shell is removed with alcohol and the charge is removed with electrolyte. The water shell is the protective layer of proteins, that prevents them from coagulating. The same electric charge of the protein helps to repel ions. If salts of alkali and alkaline earth metals are added to protein solutions, their ions are adsorbed to protein molecules and neutralize them. Therefore, colloidal particles are enlarged, which causes the formation of a protein precipitate. Alkali metal salts, when dissolved, bind large amounts of water, which dehydrates protein molecules. At high concentrations of salts, the ions of which are also hydrated, the water shells of protein molecules are destroyed due to competition for water with salt ions. After losing charge and hydrate shell, proteins precipitate.

In a neutral medium, serum albumin is precipitated with saturated and globulins with semi-saturated $(NH_4)_2SO_4$ solutions. Ammonium sulfate has a good salting ability and precipitates proteins at pH7, and even better - in a weakly acidic environment [5]. Salts, such as NaCl, precipitate proteins only at pH <7. NaCl, KCl, $MgSO_4$ precipitate globulins at full saturation, and at weak acidification (at the isoelectric point) these salts precipitate albumins.

Proteins from solutions are easily (at low concentrations of additives) precipitated by salts of heavy metals (Pb, Cu, Ag, Hg), which are adsorbed on the surface of protein

molecules, form strong salt-like complex compounds and change the electric charge and structure of macromolecules [6, 8]. Excess salts of Pb acetate and Cu sulfate cause the dissolution of the precipitate formed by them due to the adsorption of excess metal ions and recharging of the protein complex. Deposition of heavy metals with salts leads to denaturation of the protein.

The complete deposition of proteins is at the *isoelectric point* (pI). In strongly acidic and alkaline solutions, proteins have a charge and do not precipitate when heated. There are 2 main factors of stabilization of proteins in solutions: charge and hydrate shell, which is formed due to the orientation of water dipoles around the hydrophilic residues of amino acids [3, 6, 8]. The -OH group attracts two, the COOH attracts three, and the peptide bond attracts four H_2O molecules.

The charge of a protein molecule arises from the ionization of functional groups of side radicals of amino acid residues. The solubility of proteins is determined by the amino acid composition, features of their structure and properties of solvents. Neutral salts increase the solubility of a protein due to the interaction of their ions with the polar groups of proteins.

The basis of protein precipitation is the destruction of the hydrate shell, which is observed when adding to protein solutions substances such as alcohol, acetone, concentrated solutions of neutral salts or when changing the electric charge of protein molecules to isoelectric state due to changes in pH.

The precipitation reactions are reversible and irreversible. Conversely, the precipitated proteins are not subject to profound structural changes and they can be returned into solution. Protein molecules retain their original properties. Most reactions of salting out, precipitation of proteins with alcohol or acetone with their short-term action at low temperatures are reversed. By carefully precipitating proteins with water-withdrawing compounds, *protein crystals* are obtained that still contain some of the water. Further drying converts these crystals into an amorphous mass.

In irreversible precipitation reactions, proteins undergo profound structural changes and cannot re-enter solution. The biological and enzymatic activity of the protein, the ability to crystallize, changes its shape and size, increases the reactivity and the number of free chemical groups, especially sulfhydryl. The protein becomes less hydrophilic.

Protein, interacting with mineral acids (except phosphoric), is denatured and precipitated due to dehydration and neutralization of charges of colloidal particles and the formation of complex salts. With prolonged action and an excess of all mineral acids, except HNO_3, there is a partial hydrolysis and recharging of the protein, its precipitate dissolves. The HNO_3 protein precipitation reaction is highly specific. It is used in clinical analysis for protein.

Precipitation of proteins can be done by medium pH changing. At low pH, proteins have an overall positive charge because the amino groups gain an extra proton. At high pH, they have a net negative charge due to the carboxyl on the protein backbone losing its proton. At pH value of isoelectric point (pI), a protein has no net charge. This leads

to reduced solubility because the protein is unable to interact with the watery medium and will then fall out of solution.

When heated to 40°C, most proteins coagulate. Some proteins coagulate at 40-55°C, while others can withstand boiling (protamines, histones, gelatin, casein, DNA polymerase of thermophilic microbes). With increasing temperature, thermal coagulation accelerates. The fullest and fastest protein precipitation when heated is at the isoelectric point (pI). For most proteins, pI is present in a weakly acidic medium (pH≈5). Exceptions are histones and protamines from pI in alkaline medium (pH ≈ 8).

In strongly acidic (with the exception of nitric, trichloroacetic, sulfosalicylic acids) and alkaline solutions denatured protein does not precipitate when heated, because protein molecules are recharged. This increases their stability in solution as a result of electrostatic repulsion forces. But in strongly acidic solutions, proteins can coagulate when heated whith a sufficient amount of neutral salt, the adsorption of ions of these salts leads to the neutralization of the charge.

Trichloroacetic (CCl_3COOH) (TCA) and sulfosalicylic ($HO_3SC_6H_5(OH)COOH$) (SSA) acids are very sensitive specific reagents for protein. Trichloroacetic acid precipitates only proteins and does not precipitate the products of their decomposition - peptides, urea, amides, amino acids. It is used in the determination of protein and non-protein nitrogen of tissues. Sulfosalicylic acid precipitates both proteins and peptides. The mechanism of protein precipitation by organic acids is dehydration of the protein molecule and removal of electric charge.

Trichloroacetic Acid (TCA) is commonly used at low concentration (usually, ~15%). It exposes even more of the proteins hydrophobic structure, resulting in increased precipitation, but have a very high risk of leaving the rest of protein nonfunctional. Thus, TCA is mainly, used when an active protein is not needed. In addition, because you are using an acid, extra steps need to be taken once the precipitation process has been completed, namely adding a base or washing off the protein with acetone. The acetone decrease the dielectric constant of an organic solvent resulting in a loss of solubility and therefore precipitation.

Most organic solvents (alcohol, acetone, ether, chloroform, phenol) precipitate proteins from neutral and weakly acidic solutions. Their action is to dehydrate protein particles and reduce the dielectric constant of aqueous solutions and ionization of proteins, which reduces their stability in solutions. For cold precipitation, when the resulting precipitate is quickly separated from the alcohol, the protein may dissolve again in water. Prolonged action of alcohol denatures the protein. Protein precipitation by phenol and chloroform or chloroform-phenol mixture (deproteinization) is used in the isolation and purification of nucleic acids.

Precipitation of proteins by reagents on alkaloids (tannin, picric acid, yellow blood salt, etc.) is irreversible and is due to the fact that proteins, like alkaloids, have nitrogenous heterocyclic groups. The mechanism of precipitation is the formation of insoluble salt-like compounds of alkaloid reagents with basic nitrogen groups. In this complex, the protein is a cation and the alkaloid reagent is an anion. Protein precipitation is carried out in an acidic environment, as protein particles are recharged and converted into cations. At pH> 7 the protein precipitate dissolves. Protamines and

histones have a positive charge and are well precipitated by alkaloid reagents in a neutral medium.

The hydrogen bonds of the secondary protein structure are broken down in 8M urea solution. There is a so-called melting of the secondary structure of the protein, which leads to changes in its physicochemical properties such as light scattering, viscosity, optical rotation and denaturation. RNAase restores its hydrogen bonds, -S-S- bonds and conformation by removing urea and mercaptoethanol from solution.

Precipitation reactions are used for the isolation of proteins from tissue, various biological fluids, separation of their mixture, and many other studies.

Reagents and equipment.

Reagents: saturated solution of ammonium sulfate and its salt $(NH_4)_2SO_4$; about 4 ml of serum; chicken egg white -1% and 10%; copper sulfate -5%; lead acetate - 5%; 1%, 10% and concentrated CH_3COOH; 10% NaOH; 10% NaCl and NaCl powder; concentrated HCl, H_2SO_4, HNO_3; 80% and 10% solutions of TCA (CCl_3COOH), 10% solution SSA ($HO_3SC_6H_5(OH)COOH$); ethyl alcohol; 5% solution of $K_4[Fe(CN)_6]$; 5% solution of $AgNO_3$, 100% and 80% acetone, chloroform, phenole, 8M urea.

Test tubes, pipettes, microcentrifuge Eppendorf with tubes, mixer Vortex, water bath, ethyl alcohol burners, lighters, racks, funnels, paper filters.

Experiments
Procedures.

1. Salting of proteins. Pour 4 ml of serum into a test tube and add an equal volume of saturated $(NH_4)_2SO_4$ solution. Stir, precipitate *globulins*. The precipitate is filtered off and $(NH_4)_2SO_4$ powder is added to the filtrate until fully saturated. Precipitates *albumin*. Filtrate. Carry out a biuret reaction with the filtrate and make sure that there are no proteins in the filtrate.

To precipitate egg white to 10 ml of its solution in a beaker, add $(NH_4)_2SO_4$ very slowly (in saturated liquid form or as a salt). To obtain the desired saturation, you can use Table 1. Stir on a magnetic stirrer for about 1 hour. Precipitated at 10,000 g, 30 min in a centrifuge.

To precipitate with sodium chloride, pour 2 ml of egg white solution into a test tube, add NaCl powder to full saturation (until a new portion of powder remains undissolved). After a few minutes, a precipitate of *globulins* appears. Filter the contents of the test tube. *Albumins* remain in the filtrate, which do not precipitate in neutral solutions, even when sodium chloride is added to full saturation. Add 1 ml of 1% acetate solution to the filtrate and bring to the boil. In a weakly acidic environment, albumin precipitates. After 2 min, filter the albumin and check the filtrate for the absence of protein by boiling and biuret reaction.

2. Precipitation of proteins with salts of heavy metals. In 3 test tubes pour 10-20 drops of protein solution. 5% lead acetic acid is added dropwise to the first tube, a 5% solution of copper sulfate is added to the second, and a 5% solution of silver nitrate is added to the third. A precipitate forms in all test tubes.

Table 1. Preparation of different concentrations of ammonium sulfate in protein solution

Desired saturation, %	The amount (mL) of saturated $(NH_4)_2SO_4$ to be added to 10 ml of egg proteins solution	Desired saturation, %	The amount (mL) of saturated $(NH_4)_2SO_4$ to be added to 10 ml of egg proteins solution
10	1,11	65	18,57
20	2,5	70	23,33
30	4,29	75	30
40	6,67	80	40
50	10	85	56,66
55	12,22	90	90
60	15	95	190

3. Precipitation of proteins with mineral acids. Give 10 drops of concentrated HNO_3 to the test tube, tilt the test tube and add 10 drops of protein solution to the wall. A white precipitate forms a protein precipitate in the interface.

4. Termal precipitation of proteins. In 4 test tubes pour 20-25 drops of protein. The content of the 1st tube is heated, a protein precipitate is formed. In the 2nd test tube add 1 drop of 1% acetic acid solution, heat, a white complete precipitate forms. In the 3rd tube add 0.5 ml of 10% acetic acid solution and heat, no precipitate is formed. In the 4th test tube pour 0.5 ml of 10% sodium hydroxide solution - the precipitate does not fall off when heated.

5. Precipitation of proteins with organic solvents. Deposition of proteins with acetone is a simple method that gives a precipitate suitable for further study. To a 1.5 ml Eppendorf microcentrifuge tube compatible with acetone (not polycarbonate), place 0.2 ml of diluted protein and 0.8 ml of 100% acetone, close the tube and mix vigorously. Incubate for 1 h at - 20 ° C in a freezer with explosion protection.

The protein was precipitated by centrifugation for 20 min at 15,000 g, 4°C. **Warning!** Acetone is a flammable compound, so the total volume of the mixture during precipitation should not exceed 1.5 ml. Carefully drain the supernatant, dry the tube at room temperature on a paper towel for 20-30 minutes. If further studies of the protein are required, it is resuspended in buffer suitable for the appropriate experiment by Vortex mixing.

Precipitation with ethanol or phenol is by adding it to the protein solution in 2 times the volume of the protein solution, precipitation with chloroform can be carried out by adding it to the test tube in the same volume as the protein solution.

6. Precipitation of proteins by organic acids. *Protein precipitation by trichloroacetic acid.* Precipitation of proteins with acetone is a simple method that gives a precipitate suitable for further research, but when working with a large sample of protein, such precipitation requires the creation of a large volume of acetone to bring its concentration to 80%, which is impractical and dangerous. In this case, it is better to use TCA for precipitation. **Warning!** TCA can cause burns if it gets on the skin. Put on gloves, place 2 ml of protein solution in 3 ml of Eppendorf microcentrifuge tube compatible with acetone (not polycarbonate), add 0.125 ml of TCA, bringing its

content in the mixture to 4%. Incubate on ice for 30 min. Centrifuge for 10 min at 15,000 g, 4°C. Carefully drain the supernatant. Add 80% cold acetone to the precipitate to remove TCA residues. Resuspend the protein precipitate on a Vortex mixer. Centrifuge for 10 min at 15,000 g, 4°C, carefully drain the supernatant. Re-wash in 80% cold acetone 4 more times. Carefully drain the supernatant, dry the precipitate at room temperature on the surface of a paper towel for 30 min. If further studies of the protein are required, it is resuspended in buffer suitable for the appropriate experiment by stirring on a Vortex mixer.

Precipitation with sulfosalicylic acid is carried out similarly. In both experiments, a white precipitate of protein produced.

7. Precipitation of proteins by urea. Pour 1 ml of protein solution into a test tube, add 1 ml of 8M urea solution. A protein precipitate is formed.

8. Precipitation of proteins with reagents on alkaloids. Pour 1 ml of 1% egg white solution into three test tubes and add 1% acetate solution dropwise. Then pour in the first test tube 2-3 drops of 10% solution of picric acid, in the second - 5% tannin solution, in the third - 5% solution $K_4[Fe(CN)_6]$. Sludge formation is observed in all test tubes. Present the results of observations in the form of figures or tables.

Answer the MCQs

1. Which amino acid have no stereoisomers? a) glycin b) cysteine c) proline d) alanine
2. Which produces the disulfide bridges? a) glycin b) cysteine c) proline d) alanine

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Practical 6. Qualitative reactions on aminoacids and proteins.

Theoretical background. Amino acids of the plant organism belong to 2 groups:
1) proteinogenic amino acids (20 compounds), which are part of protein molecules;
2) free amino acids (more than 200 compounds), that are in the free state in the cytoplasm, among them both: proteinogenic and non-proteinogenic.

Free amino acids involved in biosynthesis of all nitrogenous compounds - proteins, peptides, amines, amides, alkaloids, many other substances of secondary origin - chlorophyll, cyanogenic glycosides, amino sugars, betaines, phenolic compounds, vitamins and coenzymes, purine, pyrimidines and pyrimids. Some free amino acids (aspartate, glutamate and arginine) are the main transport and reserve form of nitrogen in the plant. "Stress" amino acids, *i.e.*, proline, alanine, dicarboxylic and their amides are involved in non-specific adaptive reactions of the plant organism under the action of stress factors of any nature.

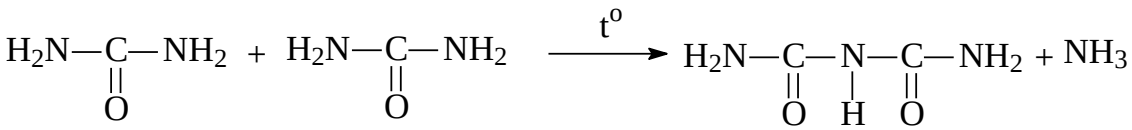
Free amino acids are important components of human brest milk. Particularly, free glutamine and glutamate have effects on immune parameters relevant for allergic sensitization and infections in early life [18]. The increase of the free amino acid content in human body is observed in row plasma<muscle tissue<skeletal tissue [1]. Difference in patterns of free amino acids in plasma in people of different geographical origin and infection-related alterations of free amino acids in plasma has been observed [8]. Decrease in almost all free amino acids concentrations is observed during acute infection, the exception being concentrations of phenylalanine [19].

Proteins are the most important natural organic compounds that determine the structure and function of the body. To be healthy we must consume enough of proteins. The proteins content varies in different food. Thus, the leaves contain up to 10-15% by weight of raw material. The protein content in cereal seeds up to 15-20% of dry weight, in legumes up to 25-35% (in soybean seeds up to 36,5% [17].

The classical division of proteins into groups, which is based on the ability to dissolve in certain solvents is a result of the study of plant proteins. There are the following groups of proteins: *albumins* - proteins soluble in water; *globulins* - insoluble in water, but well soluble in saline aqueous solutions (globulins are the bulk of the protein of legumes and oilseeds); *glutelins* - insoluble in neutral solvents, soluble in dilute acids and alkalis (contained in cereal seeds); *prolamines* are insoluble in water, but well soluble in 70-80% aqueous ethanol (characteristic only for cereal seeds).

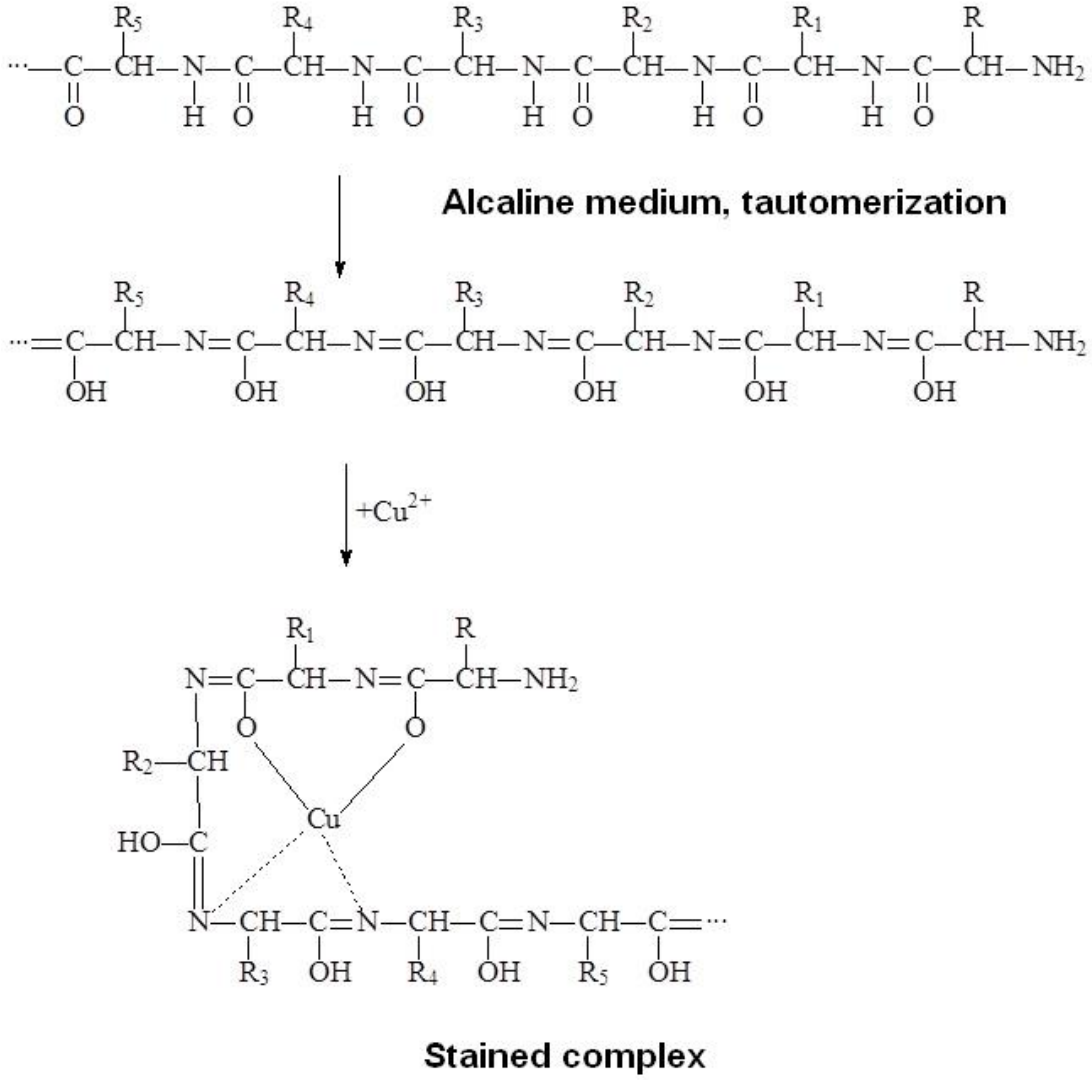
Qualitative reactions to proteins, peptides and amino acids are of practical importance [2-16].

1. Biuret reaction. The reaction is named after a derivative of urea - biuret, which also gives this reaction. Biuret is formed by heating urea with cleavage of ammonia.



2 molecules of urea **biuret**

In an alkaline medium in the presence of Cu^{2+} ions, proteins and peptides give a purple color with red or blue hues, depending on the number of peptide bonds. Peptones - products of incomplete hydrolysis of proteins - give a red tint. The reaction requires the compound to have at least 2 peptide bonds [5]. Amino acids, like histidine, also give a positive biuret reaction. This color has a complex compound of copper with groups that have formed a peptide bond or its analogue (-CO-NH-).



Stained complex

In an alkaline medium, the peptide group is tautomerized to the enol form. With an excess of alkali, OH groups dissociate, a negative charge is formed, and as a result, oxygen interacts with Cu^{2+}, forming salt-like bonds. Copper forms salt-like bonds with the nitrogen atoms in the peptide bond. The complex compound is stable.

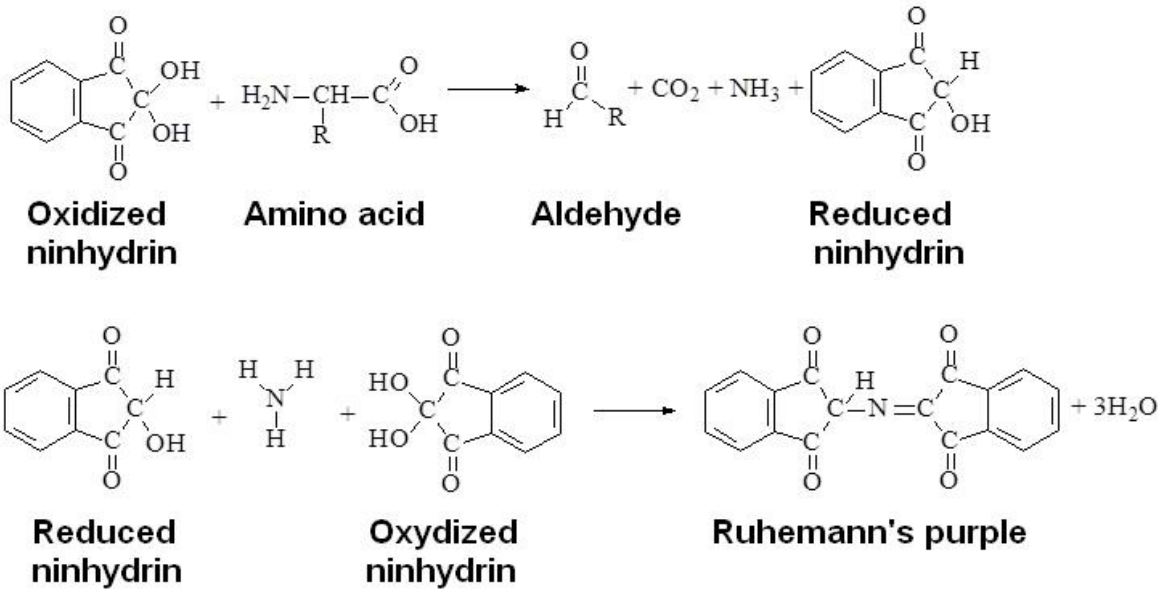
The biuret reaction is evidence of peptide bonds. The intensity of the color depends on the concentration of protein and copper sulfate in solution.

Compounds that give color in the biuret reaction are also bile pigments, biguanide, methylbiuret, oxybiuret, tetruret. Ammonium salts, tris, sucrose and glycerin affect the color of proteins. Lipids and detergents give turbidity.

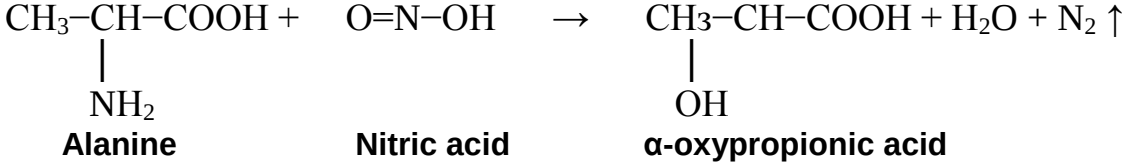
The biuret reaction was first discovered by F.Rose (1833) [14] and re-discovered by the Polish physiologist G.Piotrowski (1857) [13]. Later, researchers proposed a number of modifications [2, 3, 7, 15,].

2. Ninhydrin reaction. Proteins, as well as peptides and amino acids give a blue or purple color with ninhydrin. This reaction was discovered by Siegfried Ruhemann in 1910 and is characteristic only of amino groups in the α-position. It is used to detect proteins, peptides and amino acids in biological solutions, chromatograms [6].

When heated to 100-105°C in solution with free amino acids or peptides or proteins, ninhydrin is reduced and amino acids are oxidized to form ammonia, aldehyde and carbon dioxide. The reduced ninhydrin condenses with ammonia and the oxidized ninhydrin molecule to form the Ruhemann's purple complex. Ninhydrin (0.05-0.25% solution in acetone or ethanol, m/v) is used as a group reagent for localization of amino acids in chromatography [3], α-nitrogen of amino acids in soil hydrolysates [11] and for colorimetric determination of protein concentration. Detection of fingerprints with ninhydrin in forensics is possible, because on the skin are always present amino acids, peptides, amines [12].

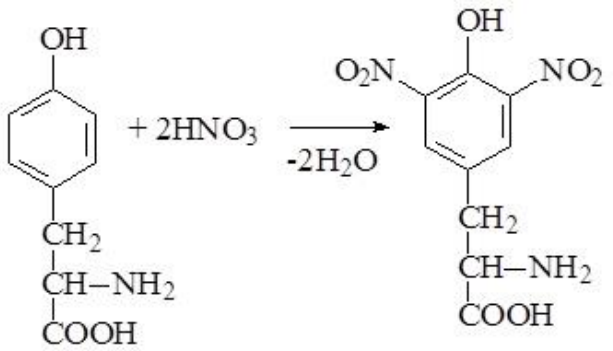


3. Reaction of amino acids with nitric acid. The presence of α-amino groups in amino acids can be proved by its ability to react with nitric acid:



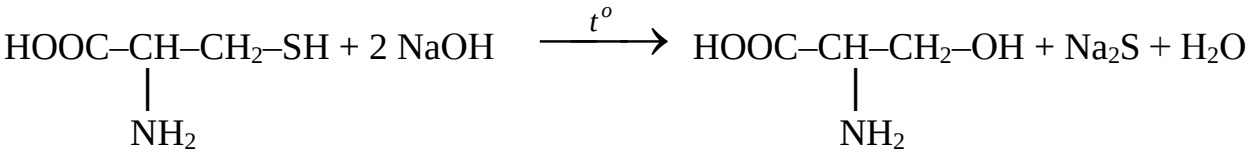
But nitric acid in the free state is unstable, so instead take a mixture of sodium nitrate and any strong acid. Nitric acid is displaced from the salt of this acid and reacts with the amino acid [3].

4. Xanthoproteic reaction. Most proteins when heated with concentrated nitric acid give a yellow color, that when added alkali or ammonia turns orange. This reaction is characteristic of tryptophan, phenylalanine and tyrosine, which have a benzene ring in their structure and are present in almost all proteins [2]. When acids are exposed to these amino acids, they are nitrated to form a yellow nitro compound. In this case, the protein precipitates a yellow precipitate, which becomes orange when alkali is added. Typically, a 20% solution of HNO_3 is used for this reaction.

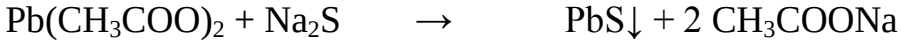


Tyrosine **3,5-di-nitrotyrosine**

5. Fol's reaction. Most proteins contain sulfur-containing amino acids - cysteine and cystine. Methionine contains strongly bound sulfur and does not react. The reaction is that when the protein is boiled with caustic soda and lead acetate (Fol's reagent), the solution begins to darken. Alkali destroys cystine, cysteine and methionine, resulting in the decomposition of sulfur in the form of sodium sulfide, which, interacting with lead salt, gives a black precipitate of lead sulfide [3].

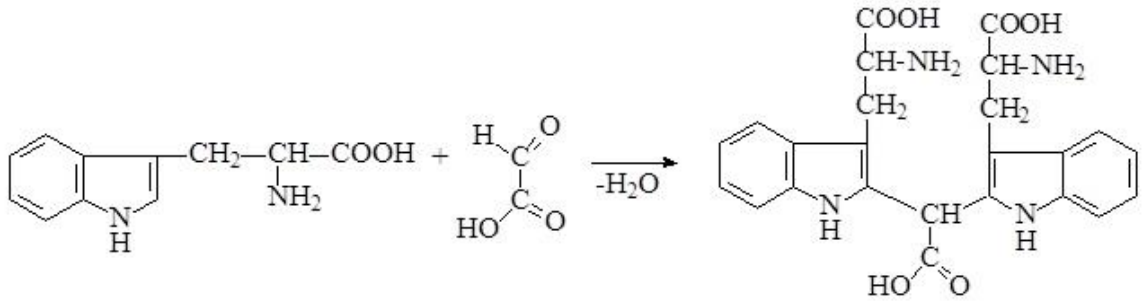


Cysteine **Serine**



Lead acetate **Lead sulfide**

6. Adamkiewicz's reaction. By adding a small amount of acetate to the protein solution and at the presence of concentrated sulfuric acid, a red-purple ring is formed at the liquids boundary. This reaction is due to the presence of tryptophan in the protein molecule, which reacts with acetate to give colored compounds. The intensity of the color depends on the amount of tryptophan in the proteins. Concentrated sulfuric acid is involved in the reaction as a substance that removes water [4, 10].



Tryptophane Glyoxylic acid Di-(2-(3-(2-amino-2-carboxy-ethyl))indolyl)acetic acid

Reagents and equipment. Egg white solution 10%, NaOH solution 10%, copper sulfate solution 1%, distilled water, pure 16 mL tubes, pipettes for 2 and 5 mL, spectrophotometer or photoelectrocolorimeter. Ninhydrin (0.25% solution in acetone or ethanol, m/v); 10% NaOH solution, 20% HNO_3 solution; 5% solution of lead acetate, glacial acetic acid; concentrated H_2SO4; 5% $NaNO_2$ solution; 5% alanine solution; clean 16 mL test tubes, pipettes for 2 and 5 ml with bulbs.

Biuret reagent. According to the method [7], 1.5 g of $CuSO_4 \cdot 5H_2O$ and 6 g of mixed sodium-potassium tartrate (Roche salt - $NaKC_4H_4O_6 \cdot 4H_2O$) are dissolved in 500 mL of water. With vigorous stirring, add 300 mL of 10% (w/v) NaOH (carbonate-free). Potassium iodide (1 g) can also be added to prevent the formation of copper (I) precipitates. The volume of the mixture is adjusted to 1 L with water. The mixture is stored in a dark plastic flask to protect from light.

Procedure.

1. Biuret reaction. Qualitative definition. To 10 drops of 1% egg white solution add 10 drops of 10% NaOH solution and 4 drops of 1% copper sulfate solution. Stir the mixture. It turns purple.

Quantitative determination. Mix 4 ml of biuret reagent and 1 mL of protein solution (diluted if necessary) containing 1-10 mg of protein. Incubate the mixture at room temperature for 30 minutes Determine the absorption of light at a wavelength in the range of 540-560 nm.

Protein concentration is determined using a calibration graph (usually the linear dependence of absorbance on protein concentration), which is constructed using a protein standard (1-10 mg). Absorption values for different proteins are slightly different (but not more than 2 times). The error of determination by this method is smaller than by others.

2. Reaction with ninhydrin. To 10 drops of protein solution add 10 drops of 0.5% alcohol solution of ninhydrin. The mixture is boiled for 1-2 minutes. The solution in the test tube becomes pink, and then crimson-purple. If the tube is left alone, the solution becomes dark purple after a while.

3. Reaction of amino acids with nitric acid. In one test tube make 6 drops of 5% alanine solution and 1 drop of nitric acid, in another (for control), transfer 6 drops of

water and 1 drop of nitric acid. Add 3 drops of sodium nitrite solution to each test tube and shake. Where is gas formed and why?

4. Xanthoproteic reaction. Add 10 drops of egg white solution and 6 drops of concentrated HNO_3 to a clean test tube and boil gently. A precipitate of protein is formed, which turns yellow when heated. Cool the tube and carefully add the excess ammonia or NaOH. The yellow color will turn orange.

5. Fol's reaction. Put 10 drops of protein solution, 10 drops of sodium hydroxide and 1 drop of lead acetate solution in a test tube. Boil for 3 min and let the test tubes stand for 1-2 min. A black or brown precipitate of lead sulfide appears.

6. Adamkiewicz's reaction. Pour 0.5 mL of egg white into one test tube and 0.5 mL of gelatin into the other. Give 1 mL of glacial acetic acid in each test tube. Heat the mixture slightly, cool and carefully add 1 mL of concentrated H_2SO_4 along the wall. A red-purple ring is formed in the interface. Place the tubes in a boiling water bath for 10 minutes. Does the color of the contents of the tubes change?

Answer the MCQs

1. How many proteinogenic amino acids do you know? **a)** 5 **b)** 20 **c)** 22 **d)** 25 **c)**50
2. Find an aromatic amino acid: **a)** alanine **b)** asparagine **c)** arginine d) phenylalanine
3. Find the heterocyclic amino acid: **a)** alanine **b)** asparagine **c)** arginine **d)** histidine
4. Find the hydrophobic amino acids: **a)** alanine **b)** leucine **c)** arginine **d)** histidine
5. Find the hydrophilic amino acids: **a)** alanine **b)** leucine **c)** arginine **d)** arginine
6. The polar amino acids are: **a)** alanine, arginine, glycine **b)** glycine, histidine, serine **c)** tryptophan, valine, phenylalanine **d)** lysine, threonine, ptoline
7. Which are the non-polar amino acids? **a)** alanine, arginine, glycine **b)** glycine, histidine, serine **c**) tryptophan, valine, phenylalanine **d)** tyrosine, methionine, serine
8. The examples of essential amino acids are: **a)** glycine, tyrosine **b)** alanine, cysteine **c)** methionine, phenylalanine **d)** serine, arginine
9. In amino acids solutions with low pH: **a)** hydrogen ions bind to COO^- anion **b)** hydrogen ions cleave from NH_3^+ group **c)** amino acids react as cations and perform basic properties **d)** amino acids react as anions and perform acidic properties
10. What maintains structure of protein molecules? **a)** hydrophobic interaction **b)** van der Waals forces **c)** electrostatic attractions **d)** hydrogen bonds **e)** disulfide bridges **f)** all of these
11. Wich is the secondary structure of proteins? **a)** subunits **b)** α-helix and β-sheet **c)** domens **d)** polypeptide chain

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Practical 7. Separation of amino acids by paper chromatography.

Theoretical background. Chromatography covers a wide range of methods for separating molecules based on the differences in their distribution between 2 phases - mobile and stationary. In these methods, the investigated substances dissolved in the mobile phase - gas or liquid - pass through the material of the stationary phase. Depending on the affinity for the mobile and stationary phases, molecules of substances move at different speeds, and therefore the compounds are separated from each other. The following types of chromatography are often used in molecular biology:

1. Ion exchange chromatography. In column chromatography, the solution containing the molecules to be separated passes through a column filled with a matrix material that is able to bind the molecules as they pass through it. Interaction with the matrix slows down the passage of different molecules to varying degrees, which allows them to collect in the form of individual fractions as they leave the column.

To separate charged molecules, the matrix contains positively or negatively charged side chains. For example, if the molecules to be separated are positively charged, use a matrix with negatively charged side chains. As it passes through the matrix, positively charged molecules will bind to the negatively charged matrix. The bound molecules are then washed with a solution having a different pH and/or ionic strength, in several steps, or using a continuous gradient of these properties in this solution. Molecules of different sizes come out of the column at different speeds and can be collected as separate fractions. For example, a sulfonated polystyrene matrix can be used to attach amino acids from a solution. Next, a buffer with increasing pH is passed through the column. Amino acids are separated and leached from the column when the pH reaches the values of their isoelectric points [8, 9, 12].

2. Adsorption chromatography. In this column chromatography, the separating molecules are adsorbed to the matrix by forces other than the interaction of electric charges. An example of such a matrix is hydroxylapatite, a hydrogenated form of calcium phosphate that is often used to fractionate proteins and nucleic acids. Hydroxyl apatite is particularly suitable for the fractionation of double-stranded DNA, which binds to it much more strongly than single-stranded DNA [8, 9,].

3. Gel filtration chromatography. This type of column chromatography that separates molecules by size rather than by the ability to bind to the matrix. The column matrix in such chromatography consists of small beads of polymer or glass with many micropores. Large molecules do not penetrate the pores, but pass around the balls and leave the column faster than molecules that penetrate and temporarily linger in them.

Therefore, the speed of passage of the molecule through the column is a direct function of its size. Commercially available beads for the separation of compounds with a wide range of molecular weights [8, 9].

4. Affinity chromatography is one of the most selective types of column chromatography because it uses a matrix with chemical groups that specifically bind to molecules that must be purified. It allows you to isolate individual proteins from a complex mixture. For example, a substrate for a specific enzymatic reaction covalently

bound to a matrix is used to purify the corresponding enzyme. If a crude mixture of enzymes passes through the column, each molecule that acts on the substrate will selectively bind in the column. In such immunoaffinity chromatography, antibodies to a specific protein bind to the matrix. When a mixture of proteins passes through the column, only the protein corresponding to antibody reacts and remains. This protein is then washed out of the column by changing the pH, which weakens the binding between the antibody and the protein [8, 9, 11].

5. High performance liquid chromatography (HPLC). Most types of column chromatography are slow because it takes time for the liquid to pass through the matrix in the column. HPLC uses high pressure pumps that accelerate the passage of the solution through the column. A steel column and matrix in the form of spherical granules that cannot be compressed are used. In this system, the separation, which should last for hours, takes place in minutes and with better resolution [1, 7, 8].

6. Gas-liquid chromatography is used to separate molecules in the gas phase. This type of chromatography is limited to the range of molecules that are easily converted to a gaseous state (eg, fatty acids, steroids, other lipids). The molecules that separate must pass through a matrix to which they have different affinities. However, in gas chromatography, the matrix is not in a column, but in a long spiral through which the gas phase containing the sample under study passes. The matrix itself is inert, but it joins a heavy liquid with which molecules that must be separated can interact. The molecules of the sample pass at different speeds, depending on their relative affinities to the phase of the heavy liquid [4].

7. Thin layer and paper chromatography. The principle of the method is that the mobile solvent (phenol, pyridine, dichloroethane, butanol, isobutane, acetonitrile, ethyl acetate, *etc.*) is absorbed by a strip of chromatographic paper (or a thin layer of stationary phase on glass) and carries an amino acid solution. The thinner the layer, the better its resolution. By changing the chemical nature of the stationary phase, molecules can be separated on the principles of ion exchange, adsorption, gel filtration. Thin layer and paper chromatography are used to separate small molecules, because large molecules are not capable of significant migration and are not separated enough in such conditions [3, 5, 10].

Paper chromatography to separate amino acids. The study of free amino acids is important for studying the metabolism of proteins in the body. Normally, serum contains about 21-42 mol/L (30%) of amino acids. Up to 0.5 g of amino acids per day is excreted in the urine. Changes in the concentration of amino acids in the serum and urine are observed in hepatic insufficiency, increased protein breakdown, impaired amino acid breakdown, as well as impaired renal excretory function. Amino acid deficiency primarily affects the heart muscle, and can indicate digestive disorders in the gastrointestinal tract.

This method is widely used in research to separate amino acids in various biological materials and to study the structure of proteins. Individual amino acids are differently retained in the stationary phase and lag behind the movement of the solvent front. If the chromatography paper is dried and spots of amino acids painted with a solution of ninhydrin, the locations of individual amino acids are revealed. The ratio of

the distance from the point of application of the mixture at the start to the middle of the spot to the distance travelled by the "front" of the solvent gives the retention coefficient Rf, which is characteristic of this amino acid under specific conditions. The speed of movement depends on the affinity of the amino acid to the solvent and paper.

When use as a solvent of water-saturated phenol, the position of the amino acid spot and, accordingly, the coefficient Rf of this amino acid depends on the ratio of its solubility in phenol to solubility in water. This means that hydrophobic amino acids will have higher Rf values than hydrophilic ones (Table 1).

Table 1. Rf values for amino acids separated in paper chromatography [1]

Amino acids	Rf values in mobile phase			
	n-butanol + acetic acid + H_2O (12:3:5)	Phenole saturated with H_2O	Phenole saturated with H_2O + ethanol + H_2O (15:4:1)	n-butanol + piridin + H_2O (1:1:1)
Alanine	0,30	0,58	0,41	0,37
γ-aminobutyric	0,40	0,78	0,56	0,26
Arginine	0,15	0,83	0,66	0,15
Asparagine	0,12	0,40	0,22	0,20
Aspartic acid	0,23	0,20	0,06	0,20
Valine	0,51	0,78	0,62	0,48
Hydroxyproline	0,22	0,67	0,44	0,30
Histidine	0,11	0,65	0,50	0,24
Glycine	0,23	0,42	0,26	0,29
Glutamine	0,17	0,27	0,29	0,23
Glutamic acid	0,28	0,33	0,12	0,20
Isoleucine	0,67	0,85	0,71	0,56
Leucine	0,70	0,85	0,73	0,60
Lysine	0,12	0,42	0,57	0,13
Methionine	0,50	0,82	0,62	0,53
Methionine sulfone	0,22	0,60	0,55	0,31
Proline	0,34	0,90	0,75	0,34
Serin	0,22	0,35	0,22	0,33
Taurine	0,20	0,35	0,28	0,38
Tyrosine	0,45	0,60	0,43	0,60
Threonine	0,26	0,48	0,33	0,36
Tryptophan	0,50	0,77	0,58	0,62
Phenylalanine	0,60	0,84	0,72	0,63
Cysteine	0,08	0,22	0,05	0,14
Cystine	0,05	0,15	0,16	0,15
Ethanolamine	0,38	0,72	0,83	0,43

Experiment

Reagents and equipment. A mixture of amino acids in distilled water, chromatographic paper in the form of wide ribbons, chambers for chromatography or large tubes; a mixture of phenol: water (2:1, v/v); spray attachment with bulb, 0.5% alcohol solution of ninhydrin, hair dryer or hands dryer.

Procedure.

1. At the lower end of the chromatographic paper strip, ≈ 2 cm from the edge, draw a pencil circle with a diameter of 2-4 mm.
2. Approximately 5 μL of a mixture of several amino acids in distilled water is applied to the center of the circle by means of a Pasteur pipette with fine nose and dried with a hair dryer. Application and drying is carried out 3 times.
3. Pour 1-2 ml of phenol saturated with water into a test tube. Insert the strip of chromatog paper strip it into the test tube until the lower edge of the paper is immersed in phenol by 1 cm. Close the tube with a stopper.
   Note: For a group of students, you can also use a sheet of filter paper for various samples and a bath for chromatography or mason jar.
4. Chromatography takes place for 40-60 min at room temperature. Then the tape is removed and dried in an oven for 10-15 min at 90-95°C to remove phenol vapors or in a fume hood with a hair dryer.
5. Next, a strip of paper is sprayed with a solution of ninhydrin or is placed onto surface of this liquid in wide Petri dish for a cesond, then removed
6. Heat a strip under a hair dryer at 100 - 105ºC in a fume hood for 5-6 min. The colored spots of amino acids that reacted with ninhydrin appear.
   Note. Not all of amino acids produce purple spots with ninhydrin: histidine and glycine form red-gray, phenylalanine, tyrosine and aspartic acid - blue, tryptophan - brown, asparagine - dirty yellow, proline - yellow.
   Therefore, often a mixture of 50 ml of 0.1% solution of ninhydrin in ethanol + 2 ml of collidine is used to identify amino acids by color. In some studies, it is recommended to add 10 ml of CH_3COOH. The lower the purity of collidine, the better the color. The chromatogram is heated also to observe the development of color. The color formed by each amino acid is best determined experimentally. Tyrosine changes color a few seconds after its appearance, and spots of many other amino acids in a few min form a purple and blue color. If keep the chromatograms after spray at room temperature, lighter spots appear and more slowly than when heated.
7. Measured in mm the distance from the middle of the spots and the "front" of the solvent to the "start line" (Fig. 1).
8. Calculate the retention factor Rf for each amino acid from the ratio of the height of the rise of the spot to the height of the rise of the solvent, record the results.
9. The value of Rf in the Table 1 determines which amino acids were in the test solution.
9. Make notes about the colors of spots and about other your observations during the experiment.
10. Stick the strip with measurements in a laboratory notebook. Discuss experiment and make conclusion.

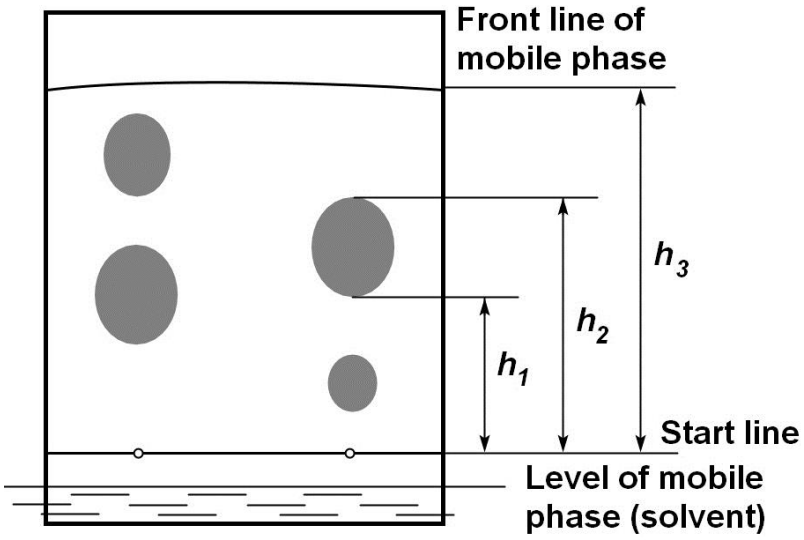

Fig. 1. Paper chromatography. The h_1 and h_2 - distances from srart line to amino acid spot's bottom and top edges, respectively; h_3 – distance, to which front line travelled from the srart line. To determine the retention factor value (Rf), the height of the rise of the spot is divided by the height of the rise of the mobile phase (solvent): *Rf = $(h_1+h_2):2:h_3$. By D.B.Pylypiv.*

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Practical 8. Determiation of isoelectric point of the protein

Theoretical background. The isoelectric point of the protein (pI) is the pH value of the medium at which the protein molecule has the same number of positive and negative charges, and therefore is neutral. At pH above or below this value, negative or positive charges will dominate in the protein, respectively. Proteins in pI precipitate from solution. The most common proteins with a pI value close to 7. To determine the pI use solutions of protein with different pH, which is achieved by using buffers with different pH. Particularly, protein casein is easily precipitated in pI.

Proteins made up of chains of amino acids, each of which has a different pH value. The total pH of the protein consists of a mixture of pH values of individual amino acids, because they form ions in a particular solution in which they are dissolved. The isoelectric point (pI) of a protein is the pH at which the protein has no charge. This property can be used to separate a protein with known pI in a heterogeneous mixture with other proteins, for example, in bidirectional gel electrophoresis, in which isoelectrofocusing of proteins is used.

Amino acids have a terminal amino group, which is basic, has a high pH value. The other end of the amino acid is a carboxyl group that is acidic, low pH. At different pH values, protein amino acids have different charges (Fig. 1).

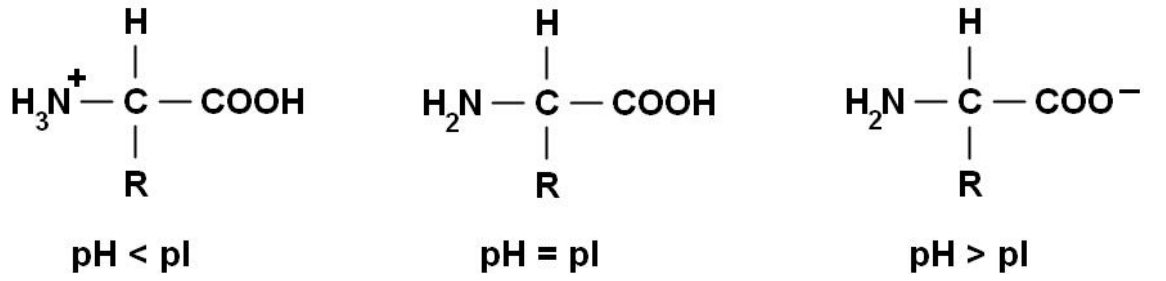

Fig.1. Charging of aminoacids at different pH. *By D.B. Pylypiv.*

Proteins consist of polar, nonpolar, positively and negatively charged amino acids, which together give it a total positive or negative charge, which can change under the influence of changes in pH. Therefore, proteins below their isoelectric point have a positive charge, and above - a negative charge. At the isoelectric point, the proteins become neutral and stop moving in the electromagnetic field. The separation of proteins in the column of polyacrylamide gel with a pH gradient, the *isoelectrofocusing*, is done according to the value of pI (actually - by charge), which is the first step for the preparation of bidirectional gel electrophoresis.

Casein is a globular colloidal protein. Globular proteins are hydrophobic, but under certain conditions they are soluble in ether. Casein becomes less and less soluble as the pH of the medium approaches pI and easily precipitates at the isoelectric point, where it becomes neutral.

Flegr J. (1990) proposed a model of intracellular electrical sorting of enzymes and organelles in the cytosol, based on isoelectrofocusing. Focusing can occur in a centrally symmetric gradient pH7.2-6.4 in the cytosol of the yeast *Saccharomyces cerevisiae* with a negatively charged nucleus. From the published data on energy capacity and calculation of electrical resistance of *S. cerevisiae* cells, the maximum value of the electric field that can be in the cytosol was estimated. The results showed that the intensity of the centrally symmetric intracytosolic electric field can reach 90 mV/cm, which is sufficient for sorting cytosolic proteins according to their isoelectric points (Fig. 2). It is hypothesized that positively charged ions are transported from the cell to its periphery through the endoplasmic reticulum (ER) tanks that form the nuclear envelope, creating an electric field that provides pH gradient formation and ampholyte electrofocusing.

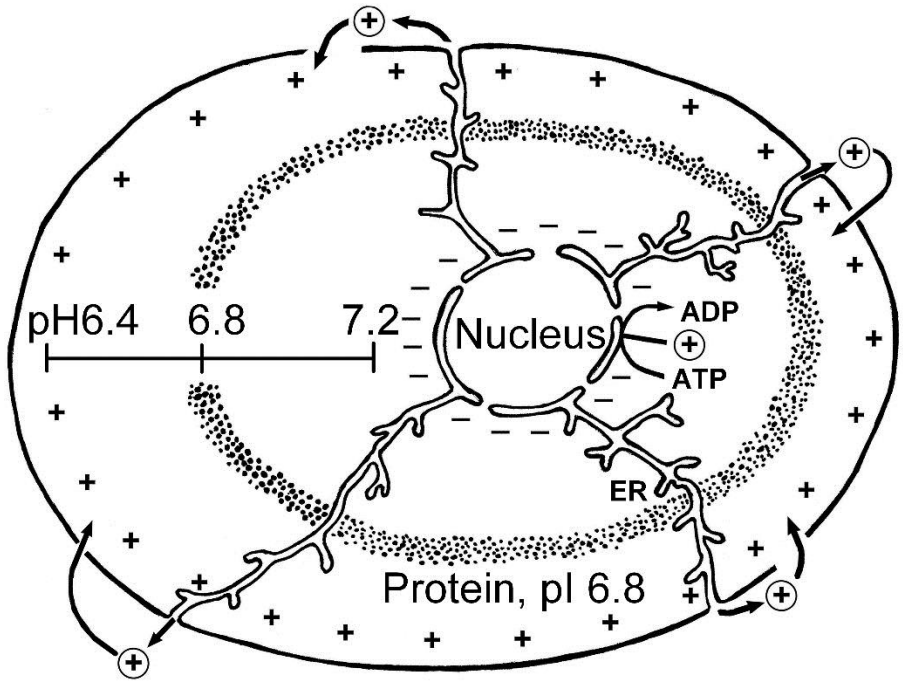

Fig. 2. Model of generation of an electrical field into cell and isoelectric focusing of protein at pH 6.8. *Modified from J. Flegr [2]*

Although direct experimental confirmation of intracellular isoelectrofocusing is still lacking, a number of phenomena of important physiological significance can be understood only on the basis of its actual existence [1, 2].

Reagents and equipment.

CH_3COOH 0.2M; 0.4% solution of casein in 0.2M Na acetate; 7 tubes, pipettes.

Procedure.

To determine the pI of casein, give reagents in volumes in 7 tubes, according to Table.1. Stir. After 5-10 min, the greatest turbidity will be in the test tube with pI.

Table 1. Preparation of solutions to determine the isoelectric point of casein

Distillated water	0,2 M CH_3COOH, mL	0,4% solution of caseine in 0.2 M of sodium acetate, mL	pH of mixture	Turbidity level „0-6”
0.4	1.6	0.2 to all of the tubes	3.8	
1.2	0.8		4.1	
1.6	0.4		4.4	
1.8	0.2		4.7	
1.9	0.1		5.0	
1.94	0.06		5.3	
1.97	0.03		5.6	

Answer the MCQs.

1. A protein that provides all nine essential amino acids is considered **a)** complete **b)** complementary **c)** normal **d)** non-essential
2. The neutral amino acid with equal number of ionized groups of opposite charge is **a)** zwitterion **b)** cation-anionic **c)** acidic-basic **d)** amphoteric
3. The proteins fully composed of α-helix structure are: **a)** fibroin **b)** α-keratin, collagen **c)** myoglobin, hemoglobin **d)** serum albumins
4. The example of proteins having only β-sheets are: **a)** elastin **b**) collagen **c)** pathological prions **d)** antifreeze proteins
5. The structural motifs in protein molecules are: **a)** domens **b)** subunits **c)** α-helix, β-sheet **d)** coiled coil, α/β-barrel, β-α-β motif, **e)** hairpin loop, helix-loop-helix **f)** helix-turn-helix, zinc finger, Greek key.
6. The quaternary structure of proteins is associated with **a)** domens **b)** subunits **c)** α-helix **d)** β-sheet.
7. Subunits (protomers) in multisubunit proteins join by: **a)** disulfide bridges **b)** peptide bonds **c)** hydrogen bonds **d)** hydrophobic interactions
8. A protein possessing four-level structure: **a)** hemoglobin **b)** myoglobin **c)** keratin **d)** collagen
9. Which is NOT a metaloprotein **a)** hemoglobin **b)** myoglobin **c)** keratin **d)** catecholoxydase
10. In which disease the misfolded protein Prion Protein Scrapie, (a modified Prion Protein Cellular) behave like an infectious agent? **a)** Alzheimer’s disease **b)** Creutzfeld-Jakob disease **c)** Fabry’s disease **d)** sicle cell anemia

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Practical 9. Nucleic acids studies in *Halobacterium halobium*.

Theoretical background. The cells of *Halobacterium halobium* grow and multiply under extreme environmental conditions at NaCl concentrations up to 30%. When in distilled water, they first lose their shape, and then become spherical and burst due to osmotic effects. This releases of cellular contents, particularly, nucleic acids (NA) . Let us study *H.halobium* NA by the method of Perry J.W., Morton D. [1].

Reagents and equipment. *For a couple of students*: test tube with culture of *H. halobium*, sticks with cotton wool wound on the end, 10 mL of 95% ethanol, a glass wand, inoculation loop, sterile pipette for 5-10 mL, 10 mL graduated cylinder, test tubes for 16 mL.

For a group of 5 students: 500 mL glass, electric bath, 6 test tubes with screw cap, permanent marker. To the laboratory: Vortex mixer, 4% NaCl solution, diphenylamine and orcinol solutions, liquid standards of DNA and RNA, paper towels, distilled water.

Experiment 1. Isolation of nucleic acids.
Procedure.

Growing a culture of bacteria to isolate nucleic acids. Cultivate *Halobacterium halobium* in Petri dishes in a thermostate at 37 ° C for a week on a medium of the following composition, g/L : NaCl - 250, KCl - 2, $MgSO4 \cdot 7H_2O$ - 20, $Na_3C_6H_5O_7$ -3, yeast extract -10, peptone -10, agar-agar -20.

Isolation of nucleic acids is performed by students in pairs.

1. Using a 10 mL cylinder, measure 1.5 mL of distilled water into a clean test tube.
2. Using a stick with a cotton swab at the end, carefully remove the bacterial mass of the colonies from the agar plate in the Petri dish as much as possible by twisting the swab on the stick over the colonies.
3. Transfer the bacterial mass into a test tube with distilled water. For the best separation, first apply biomass from the cotton end to the wall surface of the test tube, and then rinse it with distilled water. Bacterial cells in distilled water swell and burst, releasing their contents.
4. Wipe the glass rod with a napkin soaked in 95% alcohol and mix well the contents of the tube for complete lysis of all *Halobacterium halobium* cells.
5. Using a sterile pipette, carefully add dropwise to the wall of the tube 3 mL of 95% ethanol. The alcohol should form a layer above the aqueous phase. This should be done carefully so as not to mix the aqueous and alcoholic phases.
6. Wipe the inoculation loop with a tissue dampened with 95% alcohol and immerse it in the test tube so that the loop is at the interface between the two phases. In this position, the loop should be rotated in one direction so that mixing occurs only at the interface of the alcohol layer and the suspension of destroyed cells. Nucleic acids are deposited at the interface between the two phases. The strands of nucleic acids will be wound on a loop in the form of a cotton-like viscose cloud around the loop, which can be removed from the solution. Nucleic acids wound on a loop contain fragments of plasma membranes attached to them, other cell remnants, proteins. The precipitate can already be analyzed for its main components.

Experiment 2. Test for DNA.

Procedure.

1. Turn on the water bath to bring it to a boil.
2. Place 2 mL of 4% NaCl solution in a clean test tube and mark it with a marker "1 Precipitate" (experimental precipitate)
3. Place the nucleic acids from the loop in this tube and dissolve them. A Vortex mixer can be used for better dissolution.
4. Place 2 mL of DNA standard in another tube and label it as "2 StandardDNA".
5. Pour 2 mL of distilled water into the third test tube and mark it as "3 Control H_2O".
6. Add 2 mL of diphenylamine solution to each tube.
   Note. ***Warning!*** *Diphenylamine contains concentrated sulfuric acid and glacial acetic acid. If it gets on the skin, it should be immediately washed off with plenty of tap water and immediately inform the teacher.*
7. Put open test tubes in a water bath for 15 min.
8. Enter the color of the solution of each tube in table 1.

Experiment 3. Test for RNA.

Procedure.

1. Place 2 mL of 4% NaCl in a clean test tube and mark it with the marker "4 Precipitate RNA" (experimental RNA precipitate)
2. Place the nucleic acids from the loop in this tube and dissolve them. A Vortex mixer can be used for better dissolution.
3. Place 2 mL of RNA standard in another tube and label it as "5 Standard RNA ".
4. Pour 2 mL of distilled water into test tube 3 and label it as "6 Control H_2O ".
5. Add 2 mL of orcinol to each tube.
   Note. ***Warning!*** *Orcinol contains HCl. If it gets on the skin, it should be immediately rinsed with plenty of water under the tap and immediately notify the teacher.*
6. Put the test tubes open in a water bath for 20 min.
7. Write the color of the solutions in table.1.Conclude about substances in the tubes.

Table 1. Determination of nucleic acids of *Halobacterium halobium.*

Test tube	Test tube contents	Color after boiling
1 Precipitate DNA	Precipitate + diphenylamine	
2 Standard DNA	Standard DNA + diphenylamine	
3 Control H_2O	H_2O + diphenylamine	
4 Precipitate RNA	Precipitate + orcinol	
5 Standard RNA	Standard RNA + orcinol	
6 Control H_2O	Control H_2O + orcinol	

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Practical 10. Isolation of bacterial genomic DNA.

Theoretical background. If to stretch the DNA in one human body cell way out, it would be about 2 m long [1]. The DNA in human somatic cells is presented as DNA of 46 chromosomes (chrs) consisting of 3 billion base pairs (bp) plus mitochondrial chrs DNA build of 16.600 bp [*8]*. Human DNA in chrs is arranged as a 5 hierarchy coil. This allows the 3 billion base pairs in each cell to fit into a space just 6 μm across [1]. DNA of mitochondrial chr in most eukaryotes is circular, like in bacteria [*8]*. In general, the chrs of bacteria, such as *Salmonella, Shigella,* and *E. coli* comprise a single circular DNA molecule consisting of about 4×10^6 bp, with a relative molecular mass of 4×10^9 and a total length of about 1.4 mm. [4]. Different bacteria can harbor *plasmids*, smaller circles of double stranded DNA molecules containing several genes, e.g., for resistance to antibiotic also [*8*]. Plasmids as circles are present in mitochondria [Griffiths] and in circle or linear shape in yeast also *[5].*

While most of eukaryotic DNA is tightly wrapped around histones when packaged as chrs in nuclei, the DNA of chrs in most of the bacteria lacks histones as partners to condence. The chr DNA of bacteria is *coiling* and *supercoiling* without histones. The supercoiling was described by J.Vinograd and his colleagues in 1963, when they discovered that 2 closed, circular DNA molecules of the same molecular mass could exhibit different rates of sedimentation through density gradient. Because supercoiled molecule occupies less volume, it moves more rapidly, than its relaxed counterpart in response to a centrifugal force or electrical field and produces separate band in tube or electrophoresis gel [7]. Bacterial chr can be positively supercoiled (secondary twisting is in the same direction as the double helix) or negatively supercoiled in opposite to DNA helix direction. Most of bacterial genomes as well as circular viral and mitochondrial DNA are negatively supercoiled.

The enzymes called ***topoisomerases*** (Ts) were discovered by J. Wang in 1971. They change the *topology* of the DNA duplex from relaxed to supercoiled state and back to relaxed (T II). They are able to knot and unknot single stranded DNA or carry out the reaction of catenation-decatenation in double stranded DNA circles (T II) or cut and seal DNA strands (T I). Ts II also play crucial role in separation of duplicated pairs of chrs during mitosis. Human T II is a target for a number of anticancer drugs (e.g., etoposide and doxorubicin), which kill the malignant tumor cells..

The supercoiling plays a key role in linear eukaryotic chrs compaction also, allowing them to fit in small volume of nucleus [7]. The chrs of bacteria are located in area of nucleoid, a dence region of cytoplasm without membrane boundary.

The isolation of DNA from bacteria is easier than from eukaryotic cells [10]. Starting with the method of Marmur [9] as prototype, several protocols have been designed for the isolation of DNA from bacteria. Usually, the methods include 3 basic and few optional steps [6, 11, 12].

First, grown cells are disrupted to release their content. It is performed by *enzyme-detergent cell lysis*, in which lipid bi-layer of plasma membranes is broken by detergents, e.g., sodium dodecyl sulfate (SDS), Triton X-100. Proteins and RNA,

optionaly, are destroyed by adding of protease and RNase, respectively. After that, concentrated salt solution is added to clump the proteins and RNA together with remnants of plasma membrane. This cell debris is removed by centrifugation.

Second is *phenol-chloroform extraction* in which phenol further denaturates and extracts the proteins and chloroform extracts the lipids. After centrifugation organic phase contains proteins and lipids and aqueous phase contains nucleic acids (DNA and RNA or small RNA fragments, if RNA-se was applied). The aqueous phase is removed and mixed with chloroform to remove phenol residues containing proteins.

Third is *DNA precipitation and purification* from remaining proteins, detergents, salts, reagents used during the cell lysis. This most commonly done by following:

a) *precipitation of DNA* by ethanol or isopropanol, in which DNA is insoluble and precipitate as 'cloud', which forms a pellet during centrifugation. To improve the DNA precipitation, the ionic strength increased, usually, by adding of Na acetate.

b) *minicolumn purification* in which nucleic acids bind to solid absorbent particles depending on pH and salt concentrations.

Following isolation, the DNA is dissolved in a slightly alkaline buffer, usually in a Tris-EDTA (TE) buffer, or in ultra-pure water.

In our laboratory work, we use the modification of Marmur (1961) method also, as follows. The cell membranes of bacteria *E. coli* are destroyed by the SDS ion detergent. Most proteins and lipids from the destroyed suspension are removed by extraction with a phenol/chloroform mixture. By carefully shaking such a mixture, the proteins are denatured and precipitated from solution by centrifugation. Lipids will dissolve in the chloroform part of this mixture. DNA is sedimented by ethanol at the presence of sodium acetate ions. The RNA impurities are destroyed by ribonuclease, and the protein residues are hydrolyzed by proteinase K. After that, the DNA solution is again purified by phenol and chloroform, and then the purified DNA is precipitated with ethanol in the presence of Na cations from the sodium acetate.

The output of this method is less than half of the DNA contained in the cell, but this method is easy to perform.

Reagents and equipment. Laboratory gloves, racks, pipettes, flasks, tubes, spectrophotometer with UV-Vis measurement range, centrifuge, *E. coli* 18h-culture.

Distillation and equilibration of phenol. **Note.** This should be handled under a fume hood before lesson. Commercial phenol is crystalline and should be distilled at 160°C to remove oxidation products which cause the breakdown of phosphodiester bonds in nucleic acids. It must be equilibrated to pH 7.8, because the DNA partitions in organic phase, if pH is acidic. Addition of antioxidant hydroxyquinoline provides yellow color for organic phase. Equilibration of phenol includes next steps [2]:
a) Melt distilled phenol at 70°C and add the hydroxyquinoline to concentration 0,1%.
b) Then, cool the solution to room temperature and add equal volume of 0.5 M TrisCl, pH 8.0 and stir on magnetic stirrer for 20 min. After separation of two phases turn off the stirrer and remove the upper aqueous phase using glass pipette.
c) Do step b) again, however, this time add 0.1 M TrisCl, pH 8.0.
d) Check the pH of the lower organic layer with a pH paper. Repeat the step c) till the pH of phenol phase reaches 7.8. **e)** After attaining the pH 7.8 and removal of final

aqueous phase, add 0.1% 0.1M Tris (pH 8.0) and 0,2% β-mercaptoethanol. Store in a brown bottle at 4°C.

Note. The β-mercaptoethanol is toxic and carcinogenic compound.

Chloroform : isoamyl alcohol (49:1, vol/vol): Mix 49 ml of chloroform with 1 ml. Isoamyl alcohol decreases the foam formation during extraction and prevents phosgene (very toxic gas) production, when chloroform comes in contact with air oxygen and UV light. Prepare mixture just before use.

Note. This should be handled under a fume hood.

Tris-EDTA (TE) Buffer solution: comprised of 10 mM Tris (pH 8.0), 1 mM EDTA; *1M TrisHCl*: 30,28 g Trizma-Base per 250 mL pH8; 3M *sodium acetate*: (246 g/L), pH=5,2; sodium dodecyl sulfate (SDS), 96% ethanol.

RNase: 0,878 мг NaCl (pure for analysis grade) dissolve in 1 ml of 10 mM TrisHCl buffer, then add 1.2 mg of RNase. Heat the solution for 15 min at 100°C and slowly cool to room temperature, store at -20°C.

Proteinase K: Dissolve 1.2 mg of proteinase and 120 mg SDS in 1.2 ml TE buffer. Store at -20°C.

Procedure.

1. **Note.** Keep the Biosafety Level 2 guidelines for handling microorganisms [3].
2. Transfer 10 mL of 18 h *E. coli* culture in each of centrifuge tubes and pellet the cells at 7000 rpm during 10 minutes. Discard the supernatant.
3. Resuspend pellet with 6 mL of TE-buffer and transfer to a 10-mL centrifuge tubes: 2 mL in each.
4. Add 0.1 mg of SDS per 10 mL of suspension. Mix gently (the cells are lysed and the DNA is released into solution, the solution becomes viscous).
5. Add an equal volume (1:1, vol/vol) of phenol to chloroform: isoamyl alcohol mixture (49:1, vol/vol).
6. Add 2.5 mL of this phenol : chloroform : isoamyl alcohol mix to each of 2 mL suspension. Gently mix and centrifuge for 10 min at 5000 rpm. The mix is divided into 2 phases: aqueous - above and organic - below. Between them is the interphase - a layer of precipitated proteins.
7. Carefully transfer the upper phase with the dissolved DNA into fresh tubes, trying not to disturb the interphase, and repeat the step 6 two times (cut off the pipette tip to avoid damage of long DNA chains during aqueous phase collection).
8. Transfer the upper phase after centrifugation to clean tubes and add an equal volume of chloroform : isoamyl alcohol mixture (to remove phenol residues). Gently mix and centrifuge at 15,000 rpm for 10 min.
9. Transfer the upper phase to fresh tubes and add 2.5 volumes of 96% ethanol and 3M sodium acetate solution, 1:10 of the volume of DNA solution. Carefully mix until the formation of "cloud" of DNA. Centrifuge at 15,000 rpm for 10 min.
10. Discard the supernatant, add 2 ml of 70% ethanol to the DNA pellet for DNA purification from salts and centrifuge at 15,000 rpm for 5 min.
11. Rinse the pellet with 0.5 ml of 96% ethanol and dry for ≈ 20 min at room temperature. Do not overdry DNA pellet, this results in bad dissolving of DNA.

12. Dissolve the DNA residue in 2 ml of TE-buffer. Add 80 µl of RNase solution to the DNA solution and incubate for 1 hour at 37°C.
13. Add 100 µl of a proteinase K solution to the same DNA solution and incubate under the same conditions for another hour.
14. Repeat steps 6-10 (DNA purification and precipitation) and dissolve the precipitate DNA in TE buffer. Store in fridge at + 5°C.
15. To estimate the concentration of pure DNA (*C, µg/ml*) in solution, measure the absorbance of resulted DNA- TE buffer solution in spectrophotometer against TE buffer blank at 260 nm (A_{260}), adjust the A_{260} measurement for turbidity (measured by absorbance at 320nm (A_{320}), multiply by the dilution factor, and take into account that an A_{260} of 1.0 = 50 µg/mL of pure dsDNA:

$$C, \mu g/ml = (A_{260} - A_{320}) \times dilution\ factor \times 50\ \mu g/mL.$$

Answer the MCQ

1. PCR is NOT used in diagnostics of: **a)** Lyme borreliosis **b)** rachitis **c)** muscular dystrophy; phenylketonuria **d)** sickle cell anemia **e)** chlamidia; viral diseases

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Practical 11. Guanine and cytosine content determination in DNA.

Theoretical background. Guanine and cytosine content (GC or G+C-content) is the percentage of guanine (G) and cytosine (C) in a DNA or RNA molecule out of 4 total bases (guanine (G), cytosine (C), adenine (A), thymine (T) in DNA or uracil (U) in RNA [4]. GC content can be accurately calculated for properly sequenced DNA or RNA molecules by simple arithmetic or by software tools, for example, OligoCalc [13].

The method of Wallace is suitable for T_m calculation in DNA oligonucleotides with known composition (14-20 bp) and used in polymerase chain reaction (PCR) and in blotting experiments design. The method takes into account that the 3 hydrogen bonds between G and C (G≡C) in sum are stronger than 2 hydrogen bonds together between A and T (A=T). It assigns 2°C to each A-T pair and 4°C to each G-C pair. Thus, the T_m is the sum of these values for all individual base **pairs** in double strand:

$$T_m = 2\ °C(A + T) + 4\ °C(G + C) \qquad (1)$$

For longer strands other formulas must be used [12].

There are a number of ways for determining of the molar percentages of G and C (mol% G+C) in experimental DNA samples. It can be done by methods of: 1) hydrolysis and subsequent nucleotide or purine and pyrimidine bases separation 2) *buoyant density* 3) melting point 4) bromination 5) apurination 6) liquid chromatography and others.

RNA impurity interferes with determination by all these methods, except for techniques based on the measurement of *buoyant* density and melting point.

The flow cytometry is usually used, when large numbers of samples need to be analyzed [17].

Among the simplest methods for estimation of mol% G+C is to measure a *melting point* or *melting temperature* (T_m) of the double stranded DNA using spectrophotometer. The melting of double helix is the process of its strands separation by gradual raising the temperature in buffer solution of DNA, which causes the hydrogen bounds that hold the double helix structure to collapse. This denaturation process is described by a melting curve, that allows estimation of a T_m for DNA sample (Fig.1). The UV-light absorbance of DNA at a λ = 260 nm increases highly when the double-helix DNA molecule separates into two single strands during incubation at sufficient temperatures [18]. The point of absorbance increase by half corresponds to the state, when half of total DNA molecules number are unwound.

Double helices constructed only with AT- and only with GC-pairs may differ in T_m by 40°C. Therefore, the nature of the melting curves of natural DNA strongly depends on the distribution of sites rich in AT- and GC-pairs along its molecule. When the solution of DNA is slowly heated, the molecules are denatured at well-defined temperatures.

The transition from the negative DNA duplex to the untangled denatured form can be found by observation of increasing in light absorption in the ultraviolet region. The higher the DNA content of G+C pairs, the higher the T_m of this DNA. This is because the G+C (triple hydrogen bond) pairs are more stable and their dissociation requires more energy than the destruction of the A+T (double hydrogen bond).

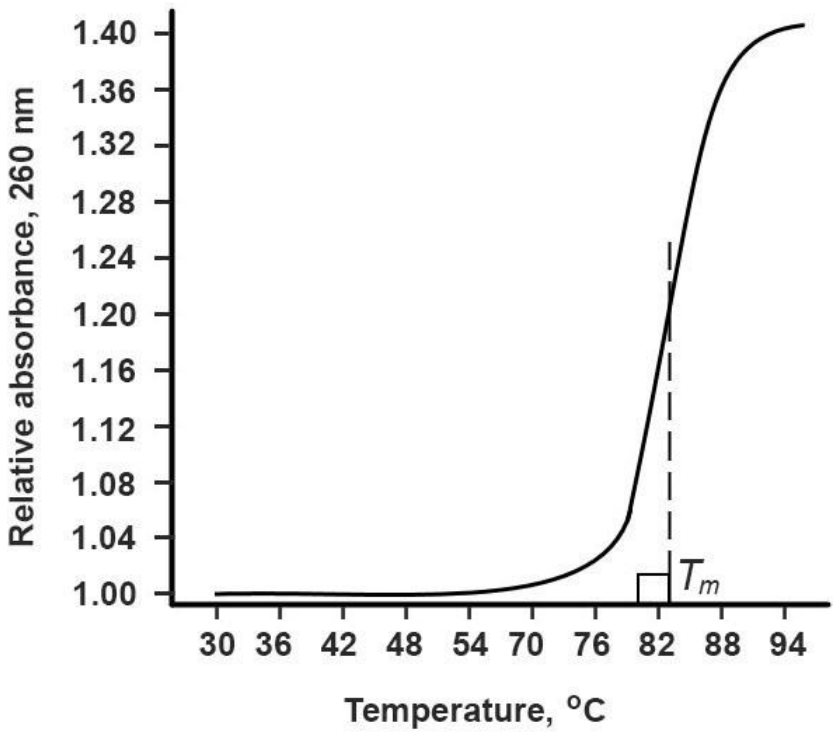

Fig.1. Thermal denaturation of DNA. The melting temperature (T_m) is determined by drawing a perpendicular line through the midpoint of the sigmoid curve to the temperature axis. *By Diana B. Pylypiv.*

The total number of GC pairs is a constant characteristic for species and it is used in systematics. The GC-content in human genomes ranges from 35% to 60% across 100-Kb fragments [9]. The GC-content of the *Saccharomyces cerevisiae*, the bakery yeast, is 38% [6], and in malaria parasite, the *Plasmodium falciparum*, it is 20% [7]. The *Actinobacteria* are rich in GC pairs [8], for example, in *Streptomyces coelicolor* A3(2), the GC-content is 72% [5].

The distribution of GC reach regions, the *isochores*, is mosaic within complex genomes [2], and results in different staining intensity in chromosomes [3]. As a rule, the isochore region includes many protein-coding genes [1, 15] and the length of the coding sequence is directly proportional to higher GC-content [14]. The higher AT content, the shorter gene sequence [19].

The correlation between the T_m and the composition of the DNA bases was described by J. Marmur and P. Doty, who where first in discovering the ability of thermally *denaturated* DNA to *renaturate* (to *reanneal*), when slowly cooled, and restore its function as genetic material in transformation of bacterial cells [11].

Further studies on reannealing had led to the development of different methods that utilize *nucleic acid hybridization*, in which complementary strands of different origin can pair to produce hybrid molecules in such techniques as DNA sequencing, DNA cloning, DNA amplification, FISH, etc.

The rate at which genomes from different organisms hybridize has provided information concerning the types of sequences within these genomes.

The *reannealling* of prokaryotic DNA occurs along symmetrical curves suggesting that these genomes primarily consist of genes arranged in a linear array. However, eukaryotic DNA fragments reanneal typically with demonstration of 3 steps on the curve, that correspond to the reannealling of 3 broad classes of DNA sequences. The 3 classes reanneal at different rates because they differ as to the number of times their nucleotide sequence is repeated within the population of fragments. These classes are called the ***highly repeated fraction***, the ***moderately repeated fraction***, and the ***non-repeated fraction*** [10].

Non-repeated sequences are very slow in complementary pairing, when reanneal, as they are present in a single copy in the genome and localize to a particular site on a particular chromosome. They have Mendelian inheritance. The genes for virtually all proteins other than histones are included in this fraction. These genes comprise less than 1.5% of the human genome. They usually belong to families of related genes of globins, actins, myosins, collagens, tubulins, integrins and most others.

Moderately repeated fraction is presented by sequences repeated within the genome anywhere from a few to tens of thousands of times. It includs some sequences that code for known gene products, either RNAs (e.g., rRNAs) or proteins (including histones), but the bulk of this DNA fraction lacks a coding function. These noncoding elements are scattered (i.e., *interspersed*) throughout the genome. Most of these repeated sequences can be grouped into two classes that are referred to as **SINE**s (**S**hort **In**terspersed **E**lements) or **LINE**s (**L**ong **In**terspersed **E**lements).

Highly repeated fraction (or ***tandem repeats***) constitutes from ≈ 1 to 10% of the total DNA. These sequences are typically short (a few hundred nucleotides at their longest) and present in clusters in which the given sequence repeats itself over and over again without interruption (in end to end manner, i.e., *in tandem*). They are categorized on *satellite DNAs*, *minisatellite DNAs*, and *microsatellite DNAs* [10].

Satellite DNAs are short sequences (≈ five to a few hundred bp in length) that form very large linear arrays, each containing up to several million bp of DNA. In many species, the composition of these DNAs is different from the bulk of the DNA, thus satellite DNA can be separated into a distinct "satellite" band in density gradient centrifugation. Satellite DNAs evolve very rapidly, causing the sequences to vary even between closely related species. The satellite DNA is located mainly in heterochromatin of chromosomes in centromeres, telomeres, and sometimes even in the euchromatin region. Satellite DNA has been known to be 'non-coding' for proteins, but recent studies suggests that some of the satellite DNA does undergo transcription [10, 16]**.**

Minisatellite DNAs (≈ 10-100 bp in length) are found in sizeable clusters containing as many as 3000 repeats. They occupy shorter stretches of the genome than do satellite sequences. They are unstable, with variable number of copies from one generation to the next, most likely due to unequal crossing over. Thus, the length of a particular minisatellite locus is highly variable, even among members of the same family. Great variability (*polymorphism*) in length of minisatellite DNAs is used in *DNA fingerprinting* for individuals identification.

Microsatellite DNAs are the shortest sequences (1- 9 bp) usually present in small clusters (≈ 10-40 bp), scattered quite evenly through the genome. DNA replicating enzymes have trouble copying regions of the genome containing these sequences, thus, microsatellites change in length through the generations. Due to their variable lengths in population, the micro satellite DNAs were used in analyzes of the different populations relationships to prove the African origin of human [10].

Experiment. Determination of melting temperature in isolated DNA sample

Materials and Equipment. Laboratory gloves, racks, pipettes, flasks, tubes, spectrophotometer with UV-Vis measurement range and thermo-adjustable cuvette, 1x SSC buffer: 0.15 M NaCl; 0.015 M trisodium citrate, pH = 7.0; standard DNA preparation with known G+C content and isolated DNA preparation.

Procedure.

1. Put laboratory gloves on clean hands.
2. Dissolve the isolated and standard DNA in 0.5 x SSC buffer until final concentrations of 50 μg/mL. To estimate the concentration of pure DNA (*C*) in solution, measure the absorbance at 260 nm (A_{260}), adjust the A_{260} measurement for turbidity (measured by absorbance at 320nm (A_{320}), multiply by the dilution factor, and take into account that an A_{260} of 1.0 = 50 μg/mL of pure dsDNA:

$$C\ (\mu g/ml) = (A_{260} - A_{320}) \times dilution\ factor \times 50 \mu g/mL \qquad (2)$$

If necessary, dilute or add more of DNA preparation and control the obtained DNA concentration using equation (2) again.

Note. The value of T_m is strongly influenced by the ionic strength of the buffer used, therefore, it is necessary to use a standard DNA preparation having the same ionic strength as the tested DNA samples. Thus, dissolve all DNA preparations, including standard DNA, in the same buffer batch.

3. Place 2 ml of standard DNA solution in a thermo-adjustable cuvette of the spectrophotometer. Turn on the heating current to heat the ethylene glycol in the thermostat tank in which the cuvette with DNA solution is placed. Next to the DNA solution cuvette, place another cuvette with a blank (SSC buffer without DNA) and a mercury thermometer. The change in temperature in the cuvette with DNA solution is determined by the mercury thermometer in cuvette with blank.
4. Turn on the thermostat for heating, while constantly measuring the readings of A_{260} in a cuvette with DNA solution. The measurement of A_{260} intervals should be consistent with the temperature lift by 0.5° (or every 2°).
5. Construct the melting curves A_{260} versus t°C for the test and standard DNA samples using software tools, such as Excell or Origin. Find the inflection point (midpoint) of the sigmoid curve. Lower the perpendicular from this point to the temperature axis (Fig.1) and thus, determine the value of T_m.
6. Determine the molar percentage of G+C using the formula:

$$\%(G+C)_x = \%(G+C)_{st} + 1{,}99\,(T_{mx} - T_{mst}), \qquad (3)$$

where T_{mx} and T_{mst} - the melting points of the experimental and standard DNA solutions, and $\%(G + C)_x$ and $\%(G + C)_{st}$ - the guanine and cytosine content of the experimental and standard DNA, respectively.

Answer the MCQs

1. The monomer of nucleic acids is **a)** nucleoside **b)** nucleotide **c)** pentoses **d)** phosphate group **e)** nitrogenous bases
2. The correct structue of **nucleoside** is: **a)** pentose-nitrogenous base **b)** two phosphates-pentose-nitrogenous base **c)** phosphate-pentose-nitrogenous base **d)** three phosphates-pentose-nitrogenous base
3. The correct structue of **nucleotide** are: **a)** pentose-nitrogenous base **b)** two phosphates-pentose-nitrogenous base **c)** phosphate-pentose-nitrogenous base **d)** three phosphates-pentose-nitrogenous base
4. The thymidine is **a)** nucleoside containing thymine and ribose **b)** nucleoside containing thymine and deoxyribose **c)** nucleotide containing thymine and ribose **d)** nucleotide without pentose
5. In living things, including human, the main "energetic currency" is **a)** ATP **b)** ADP **c)** AMP **d)** acetyl-CoA
6. The purines are: **a)** adenine, guanine **b)** adenine, thymine **c)** guanine, cytosine **d)** cytosine, thymine, uracil
7. The purimidines are: **a)** adenine, guanine **b)** adenine, thymine **c)** guanine, cytosine **d)** cytosine, thymine, uracil
8. What is commonly NOT present in DNA? **a)** adenine **b)**guanine **c)** thymine **d)** cytosine **e)** uracil
9. Not thermostable polymerase is **a)** Pfu polymerase **b)** Tth polymerase **c)** Vent polymerase **d)** DNA polymerase III **e)** Taq polymerase

Solved problem

Problem 1. Calculate the melting temperature for the sequence:

GGTGCGGTCAGG
CCACGCCAGTCC

Solution: There are 1 A, 2 Ts, 7 G and 2 Cs to insert into the formula (1):

T_m **= 2°C(A+T)+4°C(G+C)=** 2°C(1+2)+ 4°C(7+2) =2°C(3)+ 4°C(9) =42°C.

Problems for individual solution by students

Problem 1. Calculate the melting temperature of the DNA fragment:

AGTCTGGGA
TCAGACCCT

Problem 2. T_m of DNA fragment depends from **a)** the length of fragment **b)** the GC content in the fragment **c)** the buffer solution **d)** all of the above

Problem 3. Using the equation (3), calculate the $\%(G+C)_x$ of DNA you have isolated if it differs in T_m from standard by 10ºC and GC content for standard DNA is 40%.

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Practical 12. RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction method of P. Chomczynski & N. Sacchi.

Theoretical background. A chaotropic agent is a substance in solution that can disrupt the hydrogen bonding network between water molecules (i.e. exerts chaotropic activity). Among common chaotropic agents are: phenol, n-butanol, ethanol, 2-propanol, lithium acetate, lithium perchlorate, magnesium chloride, sodium dodecyl sulfate, urea, thiourea, guanidinium chloride, guanidinium thiocyanate.

For example, guanidinium thiocyanate or guanidinium isothiocyanate (GITC), (Fig.1) as a chaotropic agent, is able to disrupt the hydrogen bonding network between water molecules. This affects the stability of the native state of macromolecules (e.g., nucleic acids and proteins) in the solution, by decreasing the hydrophobic interactions effect. Particularly, GITC reduces the order conferred to protein structure by hydration shell and may cause the protein denaturation. Thus, the GITC is often applied as a protein denaturant. However, the substance is most frequently used as a protector of DNA or RNA in nucleic acid isolation from cells. During this process it acts as a denaturing agent onto RNase and DNase enzymes [5].

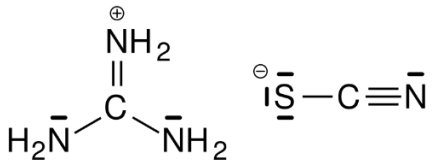

Fig. 1. Chemical structure of guanidinium thiocyanate. *By Dirk Dschanz* (2007) [4]

The structure and functions of biological macromolecules are dependent on the net effect of hydrophobic interactions, van der Waals forces and hydrogen bonds. By interfering with these forces the GITC denatures protein macromolecules, reduces enzymatic activity and induces stress on a cell. At critical concentration in the hydrophobic region of plasma membrane it causes lipid bi-layer destruction and lysis of the cell [1].

Guanidinium thiocyanate can denature the viruses as they principally consist of nucleic acid enclosed in a variable protein shell. Then, deactivated viruses can be safely studied.

Two prominent scientists, Piotr Chomczynski and Nicoletta Sacchi developed a very useful and simple single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction [3]. This method is widely used for isolating total RNA from biological samples of different sources. The basis of the method is that RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, phenol, chloroform and sodium acetate, followed by centrifugation. When pH is acidic, total RNA remains in the upper aqueous phase, while most of DNA and proteins go either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol (Fig.2).

The method allows to isolate RNA from cells or tissues in less than 4 hours. The technique is good in retrieval of small and large, low-abundance and high-abundance RNA isoforms. The protocol of the method was re-edited by authors in 2006 [2] and to our knowledge, presenting the best way of total RNA isolation from different sources, such as human, animal and plant tissues or fungi and bacteria.

Reagents and Equipment. ***Reagents*** Guanidinium thiocyanate, N-laurosylsarcosine (Sarkosyl), Na citrate, Na acetate (anhydrous), 2-mercaptoethanol, phenol (nucleic acid grade), glacial acetic acid, chloroform, isoamyl alcohol, isopropanol, ethanol, diethylpyrocarbonate (DEPC), sodium dodecyl sulfate (SDS).

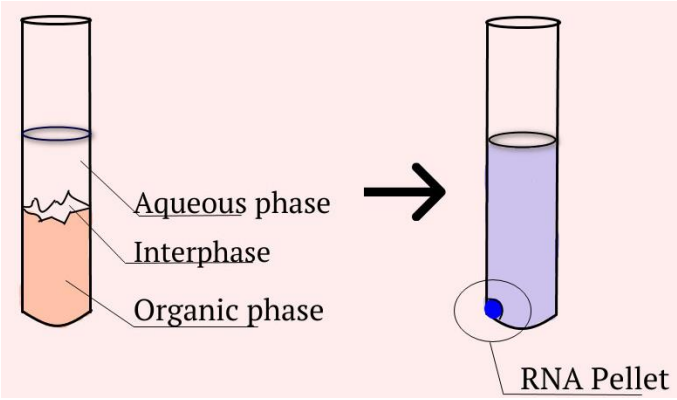

Fig. 2. Phase separation (left), RNA precipitation from aqueous phase with isopropanol (right).
By Diana B. Pylypiv.

Water-saturated phenol. Note. This reagent should be prepared under a fume hood. Use distilled water heated to 65°C to dissolve 100 g of phenol crystals (nucleic acid grade). Remove the upper water phase and store up to 1 month at 4°C.

2 M sodium acetate, pH 4.0. Note. This reagent should be prepared under a fume hood. Mix 40 ml of water and 35 ml of glacial acetic acid. Dissolve 16.42 g of sodium acetate (anhydrous) in this mixture. Adjust pH to 4.0 with glacial acetic acid and bring to a final volume of 100 ml with DEPC-treated water. The solution will be 2 M with respect to Na ions. This reagent can be stored for up to 1 year in a room. Denaturing solution (DS). Note. To minimize handling of guanidinium thiocyanate, dissolve it directly in the manufacturer's bottle. The 2-mercaptoethanol should be handled under a fume hood.

Denaturing solution (DS) is 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) N laurosylsarcosine (Sarkosyl) and 0.1 M 2-mercaptoethanol. To prepare a stock solution, dissolve 250 g guanidinium thiocyanate in 293 ml water at 65 °C. Then add 17.6 ml of 0.75 M sodium citrate, pH 7.0, and 26.4 ml of 10% (wt/vol) Sarkosyl. Store up to 3 months at 25 °C or room temperature. To prepare the working DS, add 0.36 ml of 98% 2-mercaptoethanol to 50 ml of stock solution. Working DS can be stored up to 1 month at room temperature.

Chloroform:isoamyl alcohol (49:1, vol/vol). Note. Prepare just before use. This should be handled under a fume hood. Mix 49 ml of chloroform with 1 ml of isoamyl alcohol.

Isopropanol. Use directly as sold.

Ethanol, 75%. Add 75 ml absolute ethanol to 25 ml DEPC-treated water.

DEPC-treated water. Note. DEPC should be handled in a fume hood. Add 0.2 ml DEPC to 100 ml water. Shake intensively to dissolve the DEPC. Autoclave the solution to inactivate DEPC.

Sodium dodecyl sulfate (SDS) 0.5%. Note. Do use a mask while weighing SDS. Dilute 1 ml of 10% SDS in 19 ml of DEPC treated water. Store at room temperature. Muscle tissue. The tissue removed from the inside of fresh meat without fat on ice.

Equipment. An autoclave, fume hood, spectrophotometer with UV-Vis

measurement range, centrifuge up to 12, 000 g, glass-Teflon homogenizer, pH meter, vortex, automatic pipettes, polypropylene tubes round-bottomed, disposable, sterile 4–15 ml centrifuge tubes with caps or glass centrifuge tubes (any alternative plastic tubes must withstand centrifugation at 10,000g with the mixture of DS and phenol-chloroform), racks, flasks, jars, tubes, automatic pipettes. Do use either disposable, sterile plasticware or non- disposable glassware or plasticware that must be RNase-free. For this, glassware can be baked at 150°C for 4 h and plasticware can be soaked for 10 min in 0.5 M NaOH, rinsed with water and autoclaved [2].

Procedure.

1. Wear disposable gloves, as skin epidermis cells or bacteria and molds from hands can contaminate your samples and can be sources of RNA and RNases.
2. Add 1 ml DS per 100 mg fresh tissue, minced on ice using sterile scalpels and sterile scissors and homogenize with a few strokes in a glass-Teflon homogenizer. **Note.** Avoid keeping samples in solution D for more than 30 min.
3. To fragment the DNA and minimize its presence in the aqueous phase, resuspend the lysate at least 10 times with a sterile, disposable 1-ml pipette tip.
4. Transfer the tissue to a 4-ml polypropylene tube.
5. Add the following sequentially to 1 ml of lysate: 0.1 ml of 2 M Na acetate, pH 4.0, mix thoroughly by inversion; 1 ml water-saturated phenol, mix thoroughly by inversion; 0.2 ml of chloroform/isoamyl alcohol (49:1), shake vigorously by hand for 10 s with caps tightly closed.
6. Cool the samples on ice for 15 min.
7. Centrifuge for 20 min at 10,000 g at 4 °C.
8. Transfer carefully using a pipette the upper aqueous phase, which contains mostly RNA, to a clean tube.
9. Add to the aqueous phase 1 ml isopropanol to precipitate the RNA.
10. Incubate the samples for at least 1 h at –20°C.
11. Centrifuge for 20 min at 10,000 g at 4°C and discard the supernatant. The RNA precipitate, often invisible before centrifugation, should form a gel-like pellet.
12. Dissolve the RNA pellet in 0.3 ml DS.
13. Transfer to a 1.5-ml microcentrifuge tube.
14. Add 0.3 ml isopropanol.
15. Incubate the samples for at least 30 min at –20°C.
16. Centrifuge for 10 min at 10,000g at 4°C and discard the supernatant. This second RNA precipitation improves the removal of DNA and proteins from RNA, however, slightly decreases the overall RNA yield.
17. Resuspend the RNA pellet with 0.5–1 ml of 75% ethanol and vortex for a few seconds.
18. Incubate samples for 10–15 min at room temperature to dissolve possible residual traces of guanidinium.
19. Centrifuge for 5 min at 10,000 g at 4 °C and discard the supernatant.
20. Air-dry the RNA pellet for 5–10 min at room temperature. Never let the RNA pellet air-dry completely, as this will greatly decrease its solubility.

21. Dissolve the RNA pellet in 100–200 μl of either DEPC-treated water or 0.5% SDS. SDS is a weak RNase inhibitor.
22. Incubate RNA 10–15 min at 60°C to ensure complete solubilization.
23. To evaluate the quantity and purity of the extracted RNA, it is necessary to measure sample absorbance at $\lambda = 260$ nm and $\lambda = 280$ nm. The reading at 260 nm allows calculation of the RNA concentration. An OD = 1 corresponds to ≈ 40 μg/ml of single-stranded RNA. Pure preparations of RNA have an absorbance ratio A260/A280 between 1.8 and 2.0. If there is contamination with proteins or phenol, the A260/A280 ratio will be much lower, it decreases the accuracy of RNA quantification [6].
24. For spectrophotometric quantification, an aliquot of the RNA should be dissolved in 1 mM Na2HPO4, with a pH > 7.5. A more acidic pH will affect the UV absorption spectrum of RNA and would significantly decrease the A260/A280 ratio [7].

Answer the MCQs

1. What is commonly NOT present in DNA? **a)** adenine **b)**guanine **c)** thymine **d)** cytosine **e)** uracil
2. The highest amount of minor nucleobases (approximately 20%) is present in **a)** DNA **b)** rRNA **c)** mRNA **d**) tRNA
3. How many hydrogen bonds are between A-U in RNA structure? **a)** 2 **b)** 3 **c)** 4 **d)** 1
4. What is commonly NOT present in RNA? **a)** adenine **b)**guanine **c)** thymine **d)** cytosine **e)** thimin

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Practical 13. Determination of RNA/DNA ratio in mammalian liver tissue

Theoretical background. In 1957 W.C. Schneider suggested a method for the determination of intracellular nucleic acids (NAs) [1]. By this method the chilled tissue sample is homogenized with a chilled blender. Next, the nucleic acids are extracted from the homogenate using trichloroacetic acid (TCA) and ethanol. Cold TCA precipitates the nucleic acids, proteins and lipids from the rest of the homogenized sample. This precipitate can be pelleted from the TCA-ethanol mix by centrifugation [1]. In purification, ethanol as polar solvent is used to dissolve the polar fats. Finally, Schneider's method uses hot TCA to dissolve out and partially degrade the DNA and RNA into solution.

The *diphenylamine reagent* contains CH_3COOH and H_2SO_4 to cleave the phosphodiester bonds and hydrolyze the glycosidic bonds between the pentose and purines when heated with DNA. Deoxyriboses are then converted by the acid to ω-hydroxylevulinyl aldehyde and react with the diphenylamine to produce the blue compounds. The more intense blue, the higher DNA concentrations.

The *orcinol reagent* contains HCl to cleave some of the phosphodiester bonds and hydrolyze the glycosidic linkages between the sugars and purines of RNA. The acid also converts ribose to furfural that can react with the orcinol in the presence of Fe (III) ions to produce green compounds. The major difference between the 2 reactions is that the orcinol reaction colors all five membered sugar rings including the RNA rings and some of the DNA rings. 10% of the intensity of the green colored solution is caused by the DNA concentration [1].

The color reactions were performed with increasing concentrations of pure DNA or RNA to set up a colorimetric assay. The isolated, purified and colored DNA or RNA samples' absorbance can be measured in an spectrophotometer on *light wavelengths* (λ) range 340 - 1000 nm due to a diffraction grating. The sample absorbs particular λ and all not absorbed light strikes the phototube, which converts the transmitted light energy to an electric measurable current [1].

The *Beer-Lambert law* states that absorbance is directly proportional to solute concentration and to the length of the light path (Bregman, 2002). The graph 'absorbance *versus* concentration' built for a series of known concentrations of pure DNA or pure RNA is called standard graph (or curve) or calibration graph.

Then, extracted DNA or RNA sample's absorption value can be superimposed onto a corresponding standard graph to determine the unknown concentrations of these samples or these concentrations can be calculated from the corresponding equations of the calibration graphs.

Experiment 1. Extraction of the nucleic acids

Reagents and materials. Frozen calf liver as the source of NAs; distilled H_2O; 10% TCA; standard of DNA from Sigma-Aldrich or any other firm.

Equipment. Waring Blender, Vortex, centrifuge, water bath, buret, test tubes with metallic racks, pipettes, Pasteur pipettes.

Procedure.

1. Homogenize 30 g of frozen calf liver by mixing the cubed sample in a chilled Waring Blender with 120 mL of distilled H_2O. Each lab group received 2.0 mL of the liver homogenate. **Note.** Cold is necessary to avoid tissue decay [2].
2. Add 5.0 mL of cold 10% TCA to the chilled homogenate, mix.
3. Centrifuge at high speed for 2 min. Remove supernatant using a Pasteur pipet.
4. Resuspend the pellet in 5.0 mL of chilled 10% TCA and centrifuge for another 2 min. Again remove and discard the supernatant.
5. Then add 10mL of 95% ethanol and resuspend the pellet.
6. Centrifuge for 2 min, and decant the supernatant to obtain the pellet. This ethanol wash must be performed twice for further purification.
7. Add 5.0 mL of 5% TCA to the tube to disperse the purified pellet and place the tube in a 90°C water bath for 15 min agitate 3 times during heating.
8. Centrifuge the tube for 2 min and decant the desired supernatant into a test tube.
9. Suspend the pellet with 5.0 mL of 5% TCA, centrifuge and decant, to ensure all of the nucleic acids are decanted into the test tube.

Experiment 2. Diphenylamine Reaction.

Procedure.

1. Prepare the 5-sample experiment in six marked test tubes as stated in Table 1.

Table 1. Test tubes arrangement for diphenylamine reaction

Test tube	Nucleic acid extract, mL	DNA standard, µg/mL	Volume (mL) of DNA standard	5% TCA, mL	Diphenylamine reagent, mL
Blank	-	0	-	2	4
1	-	100	2	-	4
2	-	200	2	-	4
3	-	300	2	-	4
4	-	400	2	-	4
5	2	-	-	-	4

2. Mix properly the content in all 6 tubes after diphenylamine reagent added.
3. Place the tubes in a boiling water bath for 10 min, cool quickly, and then transfer to 6 identical cuvettes (or measure against blank in one cuvette with increase of DNA concentration, and wash with 5% TCA before measuring the extract sample DNA).
4. Set the spectrophotometer to a wavelength of 600 nm, place the blank cuvette in the sample holder and adjust the absorbance *A* to zero/100% Transmittance.
5. Measure the absorbance values of tubes 1-5 and recorded as shown in Table 2.

Table 2. Example of readings from spectrophotometer for diphenylamine reaction

Tube	DNA, µg/mL	A_{600}
1	100	0.189
2	200	0.350
3	300	0.529
4	400	0.639
5 (NA extract)	unknown	0.135

Experiment 2. Orcinol reaction.

Procedure.

1. Prepare the six marked test tubes for experiment according to Table 3.

Table 3. Test tubes arrangement for orcinol reaction

Test tube	Nucleic acid extract, mL	RNA standard, μg/mL	Volume (mL) of RNA standard	5% TCA, mL	Orcinol reagent, mL
Blank	-	0	-	3	3
1	-	100	0.4	2.6	3
2	-	200	0.4	2.6	3
3	-	300	0.4	2.6	3
4	-	400	0.4	2.6	3
5	0.4	-	-	2.6	3

2. After all 6 tubes received the orcinol reagent dispensed from a buret, mix properly the content in each of them.
3. Place the tubes in a boiling water bath for 20 min, cool quickly, and then transfer to six identical cuvettes (or measure against blank in one cuvette with increase of RNA concentration, and wash 3 times with 5% TCA before measuring the extracted RNA sample).
4. Set the spectrophotometer to a wavelength of 660 nm, place the blank cuvette in the sample holder and adjust the absorbance A to zero/100% Transmittance.
5. Measure the absorbance values of tubes 1-5 and record as shown in Table 4.

Table 4. Example of readings from spectrophotometer for orcinol reaction

Tube	RNA, μg/mL	A_{660}
1	100	0.115
2	200	0.211
3	300	0.301
4	400	0.43
5 (NA extract)	unknown	0.150

Results

All the standard solutions (tubes 1-4) were of known concentrations and their corresponding absorbance (*A*) values were measured for both: diphenylamine and orcinol reaction. These graphs abide by the Beer-Lambert law and manifest a direct correlation between the concentration and absorbance: the higher concentration, the higher the values of absorbance. The diphenylamine reaction measurements, results in a positively sloped graph with equation y = 0.0016x + 0.0178. The point of zero DNA concentration and zero *A* for the blank cuvette's absorbance were plotted also. It is a line of best fit starting at the origin. It corresponds to the Beer-Lambert law, as absorbance is directly proportional to the concentration of the solute, i.e., dissolved DNA standard (Fig.1).

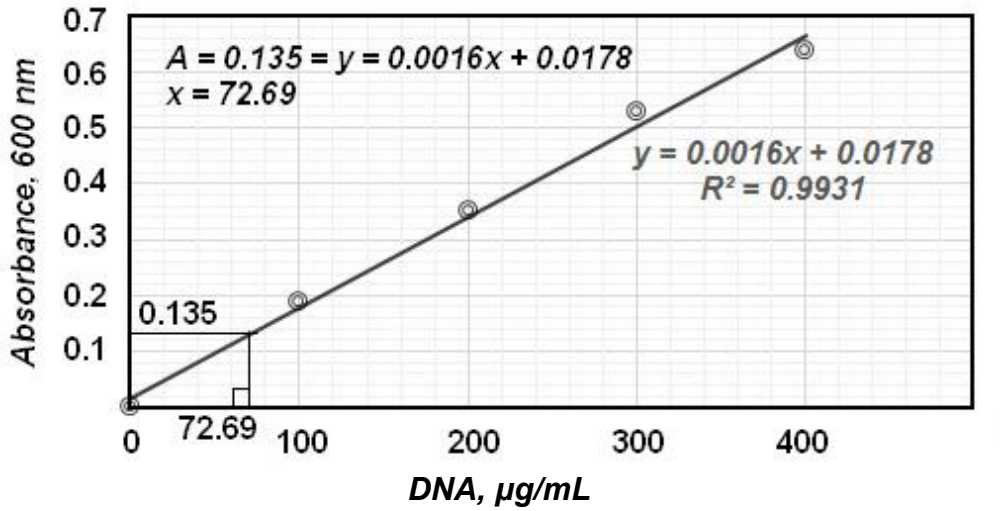

Fig.1 Standard graph for diphenylamine reaction

From this graph equation $y = 0.0016x + 0.0178$ the concentration x of the unknown DNA extract can be calculated as $0.135 = 0.0016x + 0.0178$, $x = 72.69$ µg/mL. Here 0.135 is absorbance of the unknown DNA extract. To find the concentration graphically, it is necessary to locate the absorbance value of 0.135 on the *y*-axis and then move horizontally from that point until the line of best fit is crossed. From the cross point a vertical line drawn downward to cross the DNA concentration axis shows the extracted DNA concentration, 72.69 µg/mL. If 30 g of bovine liver were homogenized in 120 mL of distilled water, the concentration of DNA in the original bovine liver is: (120×72.69)/30 = 290,76 µg per gram ≈ 0.29 µg of DNA per milligram of bovine liver.

The standard graph for the orcinol reaction demonstrates the direct positive correlation relationship of two variables, the RNA concentration and reaction products color intensity, according to the Beer-Lambert law. The point of zero absorbance *versus* zero RNA concentration in blank is plotted and a line of best fit goes through the origin too. The estimation of the unknown RNA concentration in extract for the orcinol reaction graph differs from that of the diphenylamine reaction experiment, because there is a some color contribution from the DNA concentration of the extract (≈10%) that is measured in the unknown RNA sample absorbance (Bregman, 2002). Thus, to determine the absorbance of the unknown RNA in the extract, 10% or 0.015, the absorbance at the concentration of DNA in the extract, is subtracted from the NA extract absorbance value 0.150 in the orcinol reaction.

The calculated absorbance of the unknown RNA is: $A = y = 0.150 - 0.015 = 0.135$. Using the slope equation on the graph the unknown RNA concentration is found as: $0.135 = 0.001x + 0.0022$, $x = 132.8$ µg/mL.

The same can be estimated graphically by location the absorbance value of 0.135 on the *y*-axis and then move horizontally from that point until the line of best fit is crossed. From the cross point a vertical line drawn downward to cross the DNA concentration axis shows the extracted DNA concentration, 132.8 µg/mL.

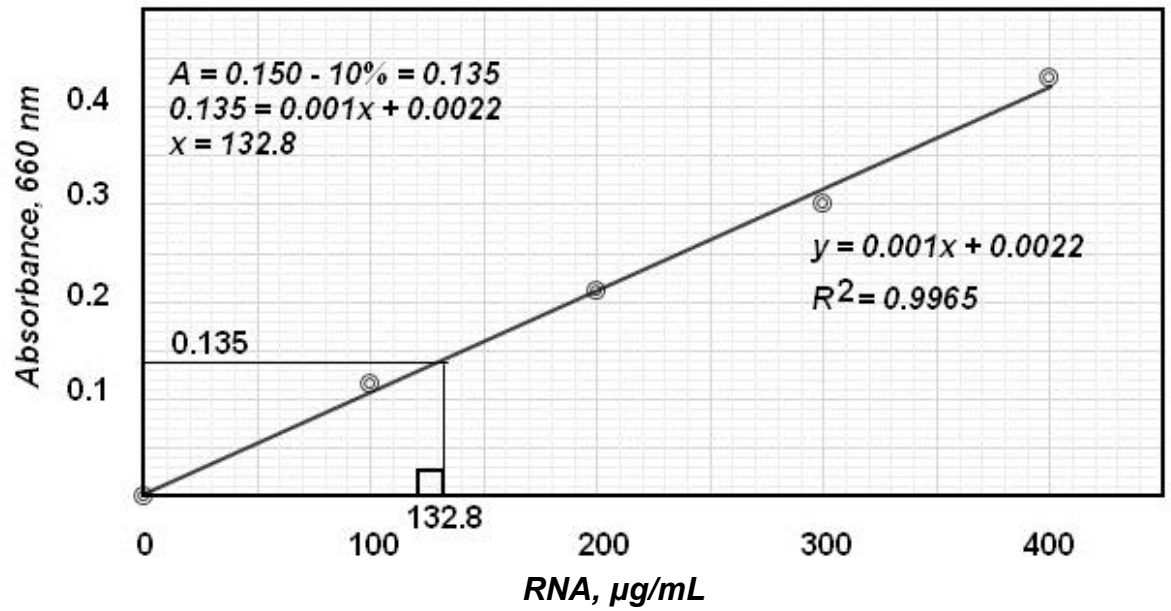

Fig.2 Standard graph for orcinol reaction

The concentration ratio RNA/DNA in the extract is: 132.8/72.69 = 1.83. Initial RNA concentration: (120×132.8)/30 = 531.2 µg/g ≈ 0.53 µg/mg of bovine liver.

Using NAs standard solutions the unknown NAs' concentrations were determined by measuring the intensity of the color produced when diphenylamine or orcinol was added [1]. To set the spectrophotometer to compensate for the *A* of the solvent, we adjusted the instrument to *A* = 0 with a blank cuvette containing the same substances as all the other cuvettes, except the NAs. Against this blank, the standard solutions and extracted samples were read. Therefore, the recorded *A* values are the true amount of light the NAs absorb. The results ebaid with the Beer-Lambert law as they show a direct correlation between the *A* and the levels of NAs: more concentrated solutions of NA demonstrated more strong light absorbance. A positive slope graph was produced in experiments 1 and 2. The unknown concentrations of DNA and RNA were measured as 72.69 and 132.8 µg/mL, respectively. The RNA/DNA ratio 1.83 suggests high synthetic activities in the liver tissue, needed for translation [3]. Similar results were obtained for the fish liver [4].

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Practical 14. Denaturing gel electrophoresis of RNA.

Theoretical background. *Electrophoretic separation* (*ES*) is widely used in science, forensics, medical and veterinary practice. Modern *ES* is not only analytical. Combined with PCR or mass spectrometry it is preparative method also [17].

ES is based on that in electrical field charged particles migrate to the opposite charge electrodes. Varying in charge and size, molecules migrate in aqueous media at different velocities and form separate fractions. The electrophoretic mobility depends on the charged groups' pK and the molecule or ion size. It is influenced by the type, concentration and buffer pH, by the temperature, support material, field voltage. The electrophoretic mobility of substance is calculated relative to the migration distance of an internal standard, e.g., bromophenol blue. *ES* is carried out in free solutions as in capillary and free flow systems, or in media such as thin-layer plates, films or gels [2, 10]. Three basic methods of *ES* are: *electrophoresis (E)* or *zone electrophoresis (ZE)*; *isotachophoresis (ITP)*; *isoelectric focusing (IEF)* (Fig.1).

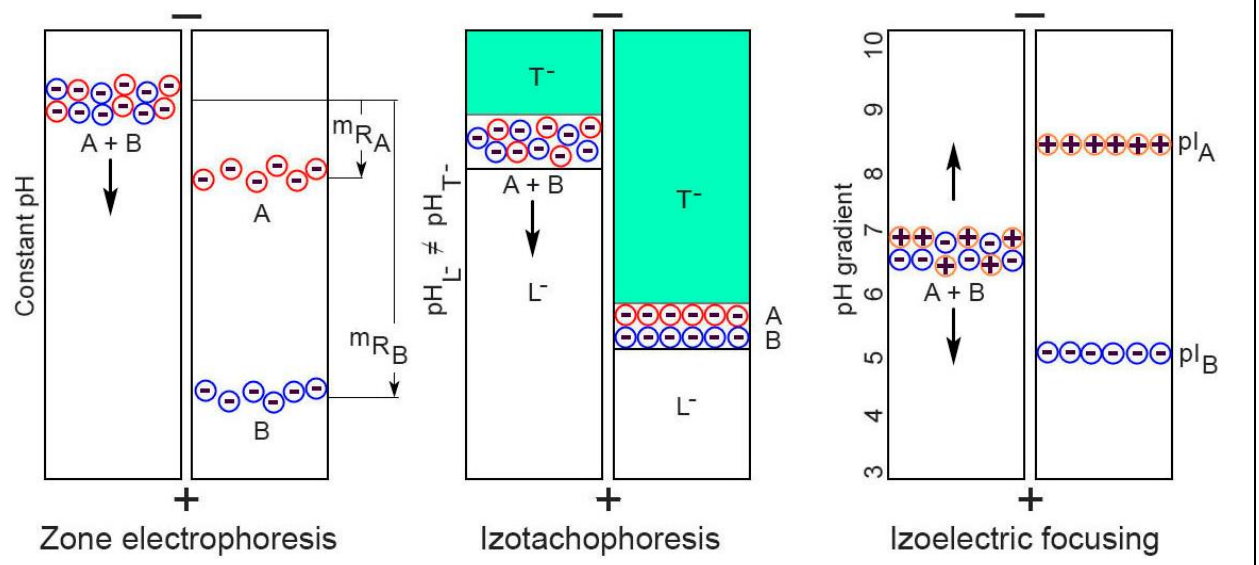

Fig 1. Three basic electrophoretic separation principles. *By Diana B. Pylypiv.*

ES separates proteins, peptides, sugars, nucleic acids (NA) and all charged cells, amino and organic acids or bases, drugs, pesticides, inorganic anions and cations.

The *E* is applied to non-amphoteric or amphoteric molecules. A constant pH is ensured by a buffer system. The migration distances during a defined time is a measure of electrophoretic mobilities of the substances. Diffusion during the process of separation can lead to blurred zones, reducing the *E* sensitivity and resolution.

ITP is the 2 buffer system. Two electrolytes with different pH are used: *leading electrolyte (L)* with a high mobility and a *terminating (trailing) electrolyte (T)* with a low mobility. The components are separated according to their electrophoretic mobilities in stacks: the substance with the highest mobility directly follows the *L*, the one with the lowest mobility migrates directly in front of the *T*. There is a concentration

regulating effect which works against diffusion in *ITP*. *ITP* is mostly used for the samples stacking in disc-*E*.

IEF is used for amphoteric substances, e.g., peptides or proteins. The molecules move towards the electrodes until they reach a position in the pH gradient where their net charges are zero. This pH value is called "isoelectric point" (pI) of the substance, which is now uncharged and electric field has no effect on it. If substance diffuses away, it will gain a net charge, and the applied electric field will direct it back to its pI. This effect is named *focusing*. If some substances are unstable at certain pH, the sample must be applied in correct place of the pH gradient.

The sample should not contain solids or fatty components, able to block the matrix pores. Usually, sample solution is centrifuged, sometimes desalted, then, mixed with tracing dye and glycerol or sucrose to increase its density before loading with syringe into immersed gel wells or into glass tubes. In SDS *E*, the sample is denatured, i.e., converted into molecule-detergent micelles. Charged substances (DNA, RNA, dyes, phenols, bases or organic acids) are easy to run. Amphoteric molecules (amino acids, peptides, proteins) possess acidic as well as basic groups. They have net charges depending on the pH of the buffer.

In *proteome* analysis complex mixtures of few thousand proteins have to be separated in one gel, so the sample preparation greatly influences the result. Proteins are often sensitive to pH or buffer substances: changes in conformation, denaturation, complex formation, intermolecular interactions occur. The concentration of the sample also plays a role, e.g., when the sample enters the pores of the gel, overloading effects of protein molecules can occur.

Selective sample extraction, particularly, the extraction of not easily soluble substances determines the buffer nature. Stabilizing medium nature, e.g., gel, depends on the size of the analyzed molecule.

The sample *ES* is done in a buffer with a constant pH and ionic strength. The ionic strength should be minimum, so that both the contribution of the sample ions to the total current and their speed will be high enough. Minimum buffering capacity is required so that the pH of the samples does not have any effect on *ES*.

In *E*, the buffer ions move through the gel to electrodes as the sample ions do. This should be with minimum energy to avoid too much of Joule heat development.

For constant pH, the supplies of electrode buffers must be sufficient. The use of buffer gel strips or wicks instead of tanks is feasible only in horizontal *E*. In vertical or capillary systems, the used pH is often high (or low), so maximum sample molecules are charged, and thus migrate in the same direction. If a gel matrix is free of any ions from polymerization, amphoteric buffers is applied, which do not migrate during *E*. Such a buffer substance must possess a high buffering capacity at its pI. For some samples, no buffer reservoirs are necessary.

Electroendosmosis: Static support, stabilizing medium, e.g., the gel and/or the surfaces of the separation equipment can carry charged groups: e.g., carboxylic groups in starch or agarose, sulfonic groups in agarose, silicium oxide on glass surfaces. These groups become ionized in basic or neutral buffers: in the electric field they will be attracted to anode. But they are fixed, and can't migrate. To compensate this, the

counterflow of H_3O+ ions to the cathode occurs: *electroendosmosis*. In gels, this effect is observed as a water flow which carries the solubilized substances towards the cathode. The electrophoretic and electroosmotic migrations are then additive. This results in blurred zones and drying of the gel near anode. If fixed groups are positively charged, the electro-osmotic flow is directed to anode.

This practical is devoted to the *agarose gel electrophoresis (AGE)* of RNA sample prepared on previous lesson. Total isolated RNA of the cell mainly consists of ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA).

All kinds of RNA molecules (and DNA molecules also) are charged negatively due to phosphates groups of their nucleotides. Thus, RNA molecules are attracted towards the positive electrode and we can separate them on the basis of size. RNA comes from isolation procedure in different 3D shapes caused by intramolecular bonding. For accurate sizing of RNA in the gel, these bonds must be broken, i.e. RNA must be denatured. This allows to compare the sizes of RNA molecules more accurately, because all of them now into same shape. The *glyoxal* and *formaldehyde* are the most common RNA denaturants [11].

To separate NA, horizontal agarose gel is commonly used. A buffer, an aqueous solution of organic and inorganic salts, conducts the electrical current through the gel between electrodes. We will utilize the fluorescence of Ethidium Bromide (EtBr) and SYBR green II stain bound to RNA for visualization of the RNA bands into gel.

Early EtBr usage was as a veterinary trypanocide [16]. It is regarded as a mutagenic as it intercalates double-stranded (ds) DNA and RNA [4, 14]. The fluorescence of EtBr increases 21-fold upon binding to dsRNA, 25-fold on binding ds DNA (although histones interfere binding of EtBr to DNA). EtBr is used in fluorimetry of NA [7, 9, 13]. It is able to bind to single-stranded DNA (although not as strongly)[4] and triple-stranded DNA [12]. Excitation/emission (Ex/Em) wavelengths reported for the EtBr-nucleic acid complex are: Ex 526, Em 605 nm (aqueous)[4] Ex 360, Em 590 nm (in PBS) [1]. Ex 525, Em 600 nm (10 mM TBE, pH 8.0) [14], Ex 510, Em 590 nm [3] Ex 482 nm (blue-green), Em at 616 nm(red-orange) [5]. EtBr fluorescence increases as solvent polarity decreases [4].

SYBR green II is a stain that causes the RNA bands to fluoresce at 520 nm when excited with UV light of 497 nm or 254 nm. The detection limit is 500 pg of RNA per band in non-denaturing gels with 300 nm transillumination (down to 100 pg with 254 nm epi-illumination) [8]. On denaturing agarose/formaldehyde gels and polyacrylamide/urea gels, the sensitivity of SYBR Green II is reduced, though still superior to that of EtBr. Without any washing or destaining steps, SYBR Green II can detect as little as 1 ng of RNA per band in agarose/formaldehyde gels or polyacrylamide/urea gels using 254 nm epi-illumination, and ≈ 4 ng of RNA per band using 300 nm transillumination [6].

Experiment. Denaturing agarose gel electrophoresis (*DAGE*) of RNA

Materials. Northern Max-Gly gel prep-running buffer, 10X, (Cat. no. AM8678) and Northern Max-Gly sample loading dye with premixed EtBr and bromophenol blue into the solution (Cat. No AM8551) both are from Life Technologies; MOPS (3-(*N*-

morpholino)propanesulfonic acid) buffer 1X (Cat. No **69947**), RNA Sample Loading Buffer for NA electrophoresis, without EtBr (contains 62.5% deionized formamide, 1.14M formaldehyde, 200 μg/ml bromphenol blue, 200 μg/ml xylene cyanole, in MOPS-EDTA-sodium acetate at 1.25x working concentration) (Cat. No R1386) and SYBR Green II stain (Cat. No S9305) all are from Sigma-Aldrich; DEPC-treated water, agarose, total RNA sample; Millennium Markers™ (ThermoFisher Scientific, Cat. No AM7150), RNA samples, TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8), 100% ethanol, ice.

Equipment. Thermostat or water bath set at 65-70°C, microcentrifuge, horizontal *GE* apparatus; bath; UV transilluminator; micropipettes and filter tips; microcentrifuge with pestle, microcentrifuge tubes, laboratory gloves, racks, pipettes, flasks, tubes.

Note. Deactivate RNAses on gloves, lab bench, tube racks, micropipettes, etc. using RNase*Zap*™ RNase decontamination solution (Sigma-Aldrich, R2020).

NA has decreased stability and degrades during laboratory procedures. This may result in blared banding and smears in gel. To minimize these, work quickly.

Procedure 1. *DAGE* of RNA with glyoxal as denaturing agent.

1. To reduce RNases in the solution, dilute 100 mL of 10X solution of the buffer NorthernMax-Gly gel prep-running buffer in 900 mL of DEPC-treated water.
2. Prepare 1% agarose gel by mixing 1 g of agarose powder with 100 mL of 1X Northern Max-Gly gel prep-running buffer in a microwavable flask. Boil 1-3 min with care to dissolve all agarose powder and cool to ≈ 50°C.
3. Pour the agarose into a gel tray containing a comb for 25-30 μL wells. Allow to set at room temperature for 20 min. **Note.** Pour slowly to avoid bubbles formation. They can be pushed towards the edges of the gel with a pipette tip. To enhance polymerization, the casting tray may be cooled in advance into fridge.
4. Using micropipette place a drop of RNA sample into a microcentrifuge tube. Add the same volume drop of Northern Max-Gly Sample Loading Dye (contains EtBr, glyoxal and bromophenol blue). Pipette up and down to mix liquids. Treat the Millennium Markers™ similarly. **Note.** Don't exceed the well volume.
5. Incubate samples for 10 min at 65°C to *denaturate* and *glyoxylate* the RNA. This prevents formation of secondary structure. If heating in a water bath, use a floating tube holder to prevent contamination of the samples.
6. Spin tubes briefly and place on ice (or store at –20°C for several days before *E*).
7. Remove comb and bumpers from the agarose gel. Place the casting tray with the gel in the electrophoresis box. Fill electrophoresis tank with 1X MOPS buffer until the gel is covered by few millimeters above its surface.
8. Load the samples into the agarose gel wells using fine pipette tips. Make sure there is no air bubbles in the end of the tip. Insert the tip just inside the top of the well and release the sample slowly. Then carefully remove the tip. Note each sample's location. The gel must also contain at least 1 lane with Millennium Markers™, loaded in the same way as isolated RNA samples.

9. Run the gel at 5 V/cm, measured between electrodes. Check the gel after 10 min to ensure that it does not run too slowly. Allow to run until the bromophenol blue dye front (corresponding to ≈ 500 nt) has migrated ≈ 3/4 of the gel length.
10. Visualize RNA bands by placing gels on a transilluminator UV light.

Procedure 2. *DAGE* of RNA with formaldehyde as denaturing agent.

1. Mix and boil gently 1 g of agarose powder in 72 mL DEPC-treated water until complete dissolving, then cool to 60°C.
2. Add 10 mL 10X MOPS running buffer and 18 mL 37% formaldehyde.
3. Pour the agarose solution into a casting tray containing a comb with 25-30 μL wells. Allow to set at room temperature for 20 min.
4. Add 1 volume sample to 2-5 volume of RNA sample loading buffer. Mix well.
5. Heat the samples at 65°C for 10 min, and after that chilled on ice immediately. Treat in the same way the RNA ladder.
6. Remove comb and bumpers from the gel and place it into electrophoresis box. Add 1X MOPS buffer to the tank and cover the gel with few millimeters of it.
7. Load RNA samples into the agarose gel wells. Note each sample‘s location. The gel must also contain the lane with Ambion™, Millennium Markers™.
8. Run the gel as in procedure 1.
9. Dilute (1:5000) the stock SYBR Green II RNA gel stain in TBE. The pH of the staining solution must be between 7.5 and 8.0 (preferably pH 8.0).
10. Place the gel into the top of a pipet-tip box and cover the gel by staining solution. Do not use glass (it adsorbs the dye) or non-polypropylene plastic containers. Place it in the dark or envelope it with aluminum foil. The washing of formaldehyde out of gels prior to staining is not necessary.
11. Shake gently at room temperature. The optimum staining time is 20–40 min, depending on the gel thickness and the agarose percentage. No destaining is required. Store staining solution in the dark (fridge). Reuse 3 to 4 times.
12. Illuminate the stained gel in 300 nm UV transillumination, or for better sensitivity, 254 nm epi-illumination.
13. Polaroid 667 black and white film is used to photograph the gel with a SYBR Green gel stain photographic filter. For 300 nm transillumination, typically a 1-2 sec exposure using an f-stop of 4.5 is adequate. For 254 nm epiillumination (especially, with a hand held lamp), exposures on the order of 1-1.5 min may be required for maximum sensitivity.
14. **Note**. SYBR Green II can be included to the loading buffer at a concentration of 1:1,000. First prepare a 1:100 dilution of SYBR Green II Nucleic Acid Gel Stain in high quality anhydrous DMSO. Store dilution 1:100 in the freezer and reuse. Add 1 μl of this dilution to 9 μl -10 μl of your sample before loading [6].

    EtBr stained gels can subsequently be stained with SYBR Green II stain. There will be some decrease in sensitivity when compared to a gel stained only with SYBR Green II. Dilute the stock solution in a polypropylene container. Nucleic acid bound SYBR Green II Nucleic Acid Gel Stain fluoresces green under

UV transillumination. It is not recommended to photograph gels with a 254 nm transilluminator. An outline of the UV light source can appear in photographs.

A filter that will allow a 525 nm transmission and exclude other wavelengths (e.g., those in the infrared) is required [6, 15].

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Practical 15. Lipids extraction from tissues or food stuffs and lipids separation by thin layer chromatography.

Theoretical background. ***Total lipids extraction method*** from various tissues, particularly, from brain gray and white matter, liver, and muscle was invented by Jordi Folch, one of the founders of structural chemistry of complex lipids and neurochemistry together with his co-workers [1, 2, 3, 4].

This method remains one of the most widely used protocols, a standard against which all other lipid extraction techniques are evaluated. The method consists of homogenizing the tissue with a 2: 1 chloroform-methanol mixture and washing the extract by addition to it of 0.8 its volume of either water or an appropriate salt solution. The resulting mixture separates into two phases. The lower phase is the total pure lipide extract. Without mineral salts, substantial amounts of acidic lipids that are present in the upper water phase would be lost during washing. These salts alter the distribution of lipids and practically eliminate them from the water phase [].

The moderately polar chloroform:methanol mixture (2:1, v/v) is applied, as the tissue cells are of high water content. At this stage of the extraction, many of the lipids are still associated with non-lipid components such as carbohydrates (polysaccharides) and proteins, as they were in the cells. These can be separated from lipids by the addition of an aqueous solution of salt (*e.g.*, KCl or NaCl), which helps the mixture to separate into 2 phases. The upper portion (methanol and water phase) contains mostly the non-lipid contaminants (residual amount of the more polar lipids might be present also). The lipids are extracted into the chloroform phase (bottom of the tube), which is the least polar and most dense, filling about 60% of the total working volume.

Table 1. The solvents used in J.Folch method.

Reagents	Polarity index	Density	Phase position in tube
0.73% NaCl solution in water	10.2	1.01. g/cm^3	Upper
Methanol	5.1	0.79 g/cm^3	
Chloroform	4.1	1.49 g/cm^3	Bottom

Polarity index is a relative measure of the ability of the solvent to interact with various polar test solutes. The higher the polarity index, the more polar the solvent (Table 1).

Chloroform is relatively nonpolar while methanol and water are more polar. Methanol and water are similar enough in polarity that they can mix. However, the added charges from the dissolved salt shift this dynamic in favor of separate phases, where the polar and charged molecules are drawn into the polar solvent layer.
Nonpolar layer is on the bottom, since chlorophorm has more high density than water.

Cells cotain different molecules closely packed or bonded together. If these bonds and intermolecular interactions are not sufficiently disrupted, they stay in large clumps during the procedure. Some large molecules with complex behaviors also reside here (*e.g.* DNA). All of these are locate in interface and reffered as “cellular debris” (Fig.1).

Thin layer chromatography *(TLC)* is a widely used and simple method for the separation of components of the solution due to difference in their polarity [5]. When

a standard is included into the procedure, this technique allows the identification and quantification of each component of the mixture.

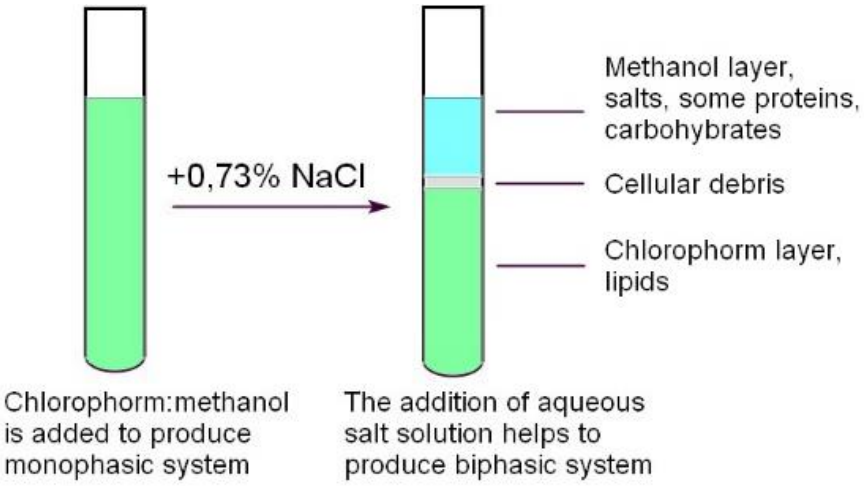

Fig. 1. Total lipids extraction. *By D.B.Pylypiv*.

The TLC technique utilizes: 1) stationary phase, i.e., matrix, and a 2) mobile phase, a mixture of solvents. Precise selection for the 2 phases depends on the type of mixture, that is necessary to separate.

The stationary phase for TLC is an absorbent material, e.g., silica gel (SiO_2), prepared as a thin layer attached onto a plate of glass or metal. Few microliters drops of extracted mixture (e.g., lipid extract from cells) is applied at the bottom of the solid phase plate onto the start line often with standard. This is done several times allowing each drop to be completely absorbed before the next drop application at the same place. The plated sample is then "developed" by placing the TLC plate bottom end in the mobile phase solvent system into a closed chamber. The solvent system is selected based on its ability to dissolve the components that need to be separated. To saturate the chamber with solvent vapors, a sheet of Whatman filter paper is stuck to the chamber walls, while treated by mobile phase.

The solvent (mobile phase) migrates up the plate and carries with it the mixture components. The more soluble the components in the solvent, the faster it will migrate up the plate. The absorbent material on the plate has an effect on component migration. For instance, silica, which is polar, interacts strongly with the polar components. As a result, polar components will "stick" to the silica, and will migrate more slowly than weakly polar or nonpolar components. Usually, as the polarity of a component (molecule) decreases, the ability for it to move up the plate increases.

Experiment 1. Extraction of lipids.

Materials Any animal or plant tissues and lipid-rich foods are suitable for this experiment, *e.g.*, eggs, wallnuts and avocados, mayonnaise. We have choosen the eggs and avocado.

Solutions and solvents Chloroform, methanol, 0.73% NaCl solution. **Note:** Chloroform and methanol are toxic compounds and should be used only in a fume hood, and must be handled with the personal protective equipment, including safety goggles and gloves.

Chlorophorm-methanole solution. Using a glass bottle in a fume hoof, prepare the chloroform:methanol solution such that it is 2:1 v/v. Store in tightly capped dark bottle as per lab safety instructions.

Equipment 15mL conical tubes and/or glass tubes, paper cups for manipulating food samples, mortar and pestle to grind solid foods, spoon or spatula, glass Pasteur pipettes, rubber pipette bulbs, rack for the test tubes, centrifuge (if you do not have a centrifuge, see note under step 10 of protocol), micropipettes and tips (if no micropipettes, use glass Pasteur pipettes), benchtop centrifuge, marker.

Note: Lipid preparations are best done in glass since plastic materials can affect lipid extractions — glass or Teflon-coated plastics are preferred. This is critical for analytical assays or assays that have a high sensitivity only.

Procedure.

1. Use the mortar and pestle to gomogenize avocado flesh as paste.
2. Crack the egg and separate the white and yolk into different paper cups.
3. Label tubes.
4. Transfer ~ 2 g of egg or avocado in marked tubes.
5. To each tube with eggs or avocado, add 1 mL of chloroform:methanol (2:1, v/v) mixture.
6. Secure the cap on the tube and vigorously shake the samples for 30 sec.
7. Place the tubes back into a rack, and remove the caps. **Note.** At this point, a monophasic system is produced whereby components are dissolving in the solution.
8. Add 267 µL of the 0.73% NaCl solution to each tube to make a chloroform: methanol: water mixture (2:1:0.8, v/v/v).
9. Secure the caps back onto the tubes and again shake vigorously for 30 sec.The addition of the aqueous salt solution transforms the mixture into a biphasic system, and helps to separate the hydrophobic lipid components from water-soluble (hydrophilic) components. Because chloroform is more dense than methanol and water, the chloroform-lipid phase will move toward the bottom of the tube, and on top of this will sit the less dense methanol-salt phase (thereby creating a biphasic system).
10. Spin conical tubes in a benchtop centrifuge at 2,500 rpm for 2 min. **Note.** Do not to disturb the phase separation while remove the tubes from the centrifuge. If no a benchtop centrifuge, allow the tubes to sit, undisturbed, for 30-60 min to completely separate phases.
11. Transfer the top, methanol-water phase, to a waste container labeled for methanol disposal. **Note.** This phase can be used to assess non-lipid components.
12. Label a fresh glass tube with the appropriate label. Using a glass pipette, transfer the bottom, chloroform-lipid phase to this freshly labeled glass tube. **Note.** There

will still be a small amount of biomass in the tube, above the chloroform-lipid phase — try to get to the chloroform-lipid phase without disturbing the biomass too much. You can just quickly "push" your glass pipette tip through the biomass to get to the bottom.

These lipids can then be separated by TLC, providing us insights into the lipids composition in each sample.

Experiment 2. Separation of lipids by thin layer chromatography (TLC)

Materials. Extract of the lipids from the above experiment. Lipid standards: Triglyceride mix #17811-1AMP (Merk or other scientific supplier).

Solvents and reagents Petroleum Ether #32299-M, Diethyl Ether #309966, Acetic Acid # 33209-M, Resublimed Iodine #451045 (All are from Sigma-Aldrich or other scientific supplier). **Note.** *These items should be used in a fume hood, and must be handled with the appropriate personal protective equipment, including gloves and safety goggles*.

Mixture of the solvents. petroleum ether: diethyl ether: acetic acid, 84:15:1 (v/v/v) To separate the lipids, particularly for neutral lipids such as triglycerides, a silica coated plate (stationary phase), *e.g.*, 'Silufol' and an organic, largely nonpolar solvent mobile phase petroleum ether: diethyl ether: acetic acid, 84:15:1 (v/v/v) can be used. For the lipids visualization resublimed iodine can be used, which binds to double bonds in lipid hydrocarbon chains and aromatic compounds.

The solvent mixture prepared in proportion as stated in Table 2 is able to capture both nonpolar and polar species in the lipid extract. The petroleum ether (polarity index 0.1) is extremely nonpolar, and will allow the most nonpolar lipid in the mixture to be "dissolved." The acetic acid, which has a high polarity index (6.2), is much more polar and is able to ionize, and serves as a solvent for the more polar lipid. The components in this solvent are not in equal quantities: the polarity index and the relative amount of each solvent dictate how it will carry specific lipid species up the stationary phase. The less polar a component in a mixture is, the faster it will move up in the silica layer on the TLC plate.

Table 1. Mobile phase characteristics.

Mobile phase component	Polarity Index	Ratio mobile phase
Acetic Acid	6.2	1
Diethyl Ether	2.8	15
Petroleum Ether	0.1	84

Equipment. Silica-coated TLC plates #100390 Supelco from Sigma-Aldrich (or other scientific supplier); TLC Chamber (if absent, use ball jars); Pipette tips or glass capillary tubes; Whatman paper, micropipette (can use glass Pasteur pipette), hotplate, fume hood, marker, pencil, lab tape.

Procedure.

Note. *Use a fume hood, and appropriate personal protective equipment, including gloves and safety goggles*.

1. Prepare the TLC chamber, or a ball jar, with the solvent system.
2. Pre-cut the wick such that it is a little bigger than the TLC plate and insert into the solvent. Dump paper will stick to the wall of the TLC chamber and provide the saturation of the air in the chamber with vapours of the solvent.
3. Close the lid to the TLC chamber and allow it to equilibrate for about 10 min or more.
4. Turn on the hot plate to a minimum setting.
5. Prepare the visualization chamber by placing some resublimed iodine crystals into a mason jar and secure the lid.
6. Using a ruler draw the start line at the distance of 2 cm from the bottom end of TLC plate
7. Mark the origin points for standard and total lipids extracts with a pencil onto line.
8. Keeping the TLC plate on its edges between the tomb and fingers above the hot plate, spot 5 μL of the standard in the first lane point. Allow the spot to dry. Repeat 2 times for a total of 15 μL standard.
9. Next, slowly spot the lipid extract in the designated lanes in increments of 5 μL, allowing the spots to dry. Repeat 5 times for a total of 25 μL of sample. ***Note.*** *If no micropipettes, a glass Pasteur pipette can be used instead. Make sure that you allow the spot to dry in between each drop application.*
10. Place the bottom of the TLC plate into the solvents mix in TLC chamber, in front of the humid wick. Cover the chamber with lid and allow the plate to develop.
13. When the solvent front nears the top of the plate, remove the plate from the TLC chamber, quickly mark the solvent front with a pencil, and allow it to dry. ***Note.*** *It typically takes 30-60 minutes for the solvent front to reach near the top of the TLC plate.*
14. Place the plate into the iodine chamber for visualization and secure the lid for about 3-5 min.
15. Remove and lightly outline the developed spots with pencil and/or take a picture with the phone.

Results

13. Detect the triglycerides plaque at the same level as plaque of triglycerides standard, cholesteryl ester above it and free fatty acids, diacylglycerol/FC and least moved phospholipids spots below.
14. Locate the position of the lipid spots centers on the TLC plate and measure the distance travelled by the individual lipid component. Calculate their retention factors (Rf values) by dividing these distances onto distance travelled by solvent and compare them with those of standards.

Answer the MCQs

1. The phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine are **a)** phosphoglycerides **b)** glycolipids **c)** sphingolipids **d)** waxes
2. The specific gangliosides are important, particularly **a)** as cholera toxin receptors **b)** as inductors of neuronal tumor cells differentiation **c)** as pituitary glycoprotein

hormones receptors **d)** as deregulator of neuronal leptin and insulin signaling in obesity **e)** all of these

3. What is NOT true about gangliosides? **a)** Guillain–Barré syndrome is linked to the production of anti-ganglioside antibodies **b)** Tay–Sachs disease is gangliosidosis **c)** A ganglioside is a molecule composed of glycosphingolipid with one or more sialic acids linked on the sugar chain **d)** More than 60 gangliosides are known, which differ from each other mainly in the position and number of NANA residues **e)** NeuNAc, an acetylated derivative of the carbohydrate sialic acid, makes the head groups of gangliosides cationic at pH 7
4. What is NOT true? **a)** galactosylceramide is the principal glycosphingolipid in brain tissue **b)** galactosylceramides are major constituents of oligodendrocytes **c)** galactocerebrosides are typically found in neural tissue, while glucocerebrosides **d)** are found in other tissues **e)** glucocerebrosides are typically found in neural tissue, while galactocerebrosides are found in other tissues **f)** glucosylceramide, a major constituent of skin lipids, is essential for lamellar body formation in the stratum corneum and to maintain the skin water permeability barrier.
5. A defect in the degradation of glucocerebrosides is **a)** Gaucher's disease **b)** Fabry's disease **c)** Krabbe disease **d)** all of these
6. *Bacteria* usually do not require cholesterol for growth. Which microbe is an exception? **a)** *Mycoplasma* **b)** *Staphylococcus* **c)** *Streptococcus* **d)** *Enterobacter*
7. What is NOT true about chylomicrons? **a)** They are the largest lipoproteins, 75–600 nm (being ≈ 90 % lipid, 10% protein) and therefore the lowest density **b)** They function as containers to carry triglycerides, cholesterol, and apolipoproteins from the intestine, through the lymphatic system and bloodstream to tissues, primarily to heart muscle, skeletal muscle, adipose tissue, and lactating mammary tissue **c)** Chylomicrons leave the enterocytes by exocytosis and enter the lymphatic system via lacteals in the villi of the intestine **d)** Chylomicron is a ball-like container build of double layer of phospholipids with embedded apolipoproteins in it **e)** The transfer of triglycerides and cholesteryl esters to the tissues depletes the lipid-protein aggregates of chylomicrons and leaves remnant chylomicrons, which are eventually taken up by the liver

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Practical 16. Separation and analysis of some sugars by thin layer chromatography method of Souly Farag.

Theoretical background. This practical lesson experiment is based on method described by Souly Farag [1].

Carbohydrates defined as aldehydes or ketones of polyhydric alcohols, which also include those biopolymers, yield these compounds on hydrolysis. They occur in all kingdoms of living things and serve diverse structural and metabolic roles. Sugars such as glucose are among the major sources of energy. Starch and glycogen act as storage polysaccharides in plants and animals, respectively. In addition, carbohydrates are structural components of cell walls, connective tissues in animals and exoskeletons of invertebrates. They are also parts of biomolecules like nucleic acids, coenzymes such as NAD(P), FAD, etc. [3]

It is accepted generaly, that quality of food, particularly, characterized by composition and levels of carbohydrates. Today, thin-layer chromatography (TLC) is the method of choice for separating sugars. Earlier, Souly Farag suggested to use the TLC for the detection of many carbohydrates present in beets and in factory juices [2]. Direct visual comparison with known standards provided reliable semi-quantitative information. If greater accuracy is required on quantitative analyses, the intensity of the spots may be measured by transmission densitometry.

Bernardi et al. [1] used more advanced technique, the High-Performance Thin Layer Chromatography (HPTLC) amog other planar chromatography methods for detection of complex sugar mixtures in submerged microbial cultures.

Samples. Canned birch sap or sugar beet thick juice from factory or similar high in sugars items.

Solvents and reagents. Acetic Acid #33209-M; chloroform 99,5% #45ZP78; diphenylamine #820528; aniline #101261 Supelco; acetone #179124, ortho-phosphoric acid #7664-38-2 Supelco all are from Sigma-Aldrich (or other scientific supplier). **Note.** *These items should be used in a fume hood, and must be handled with the appropriate personal protective equipment, including gloves and safety goggles*. Glucose 5% solution (Yuriya-Farm, Ukraine); fructose #F0127 Sigma-Aldrich (or other scientific supplier); D-(+)-raffinose pentahydrate, #050068 Supelco, Merk; sucrose #84097 Sigma-Aldrich.

Solvent. Prepare solvent system which consists of a mixture of chloroform, acetic acid, and water 3 : 3.5 : 0.5 by volume, respectlvely.

Standards 0.5% solutions were prepared by dilution of glucose, fructose, raffinose pentahydrate or sucrose in distilled water.

Spraying agent made from 1 gram diphenylamine and 1 ml of aniline in 100 ml acetone. This mixture is further mixed with 85% ortho-phosphoric acid prior to use (10 : 1, v/v, respectively) .

Equipment. Silica-coated TLC plates #100390 Supelco, from Sigma-Aldrich (or other scientific supplier); TLC Chamber; Pipette tips or glass capillary tubes; Whatman paper, micropipette (can use glass Pasteur pipette), hotplate, fume hood, marker, pencil, lab tape.

Procedure.

Note. *Use a fume hood, and appropriate personal protective equipment, including gloves and safety goggles.*

1. Dilute sugar beet thick juice (1:4, v/v) and the canned birch sap (1:2, v/v) in distilled water.
2. Using a ruler draw with pencil the start line at the distance of 2 cm from the bottom end of TLC plate. Mark points for all standarts and for sugar beet thick juice and the canned birch sap 1.5-2 cm apart.
3. Turn on the hot plate to a minimum setting.
4. Keeping the TLC plate on its edges between the tomb and fingers above the hot plate, spot 1 μl of the 0,5% standards and diluted shugar beet juice and canned birch sap to the corresponding points on start line.
5. Dry the plate in warm air for approximately 20 minutes.
6. Insert thr TLC plate in solvent system to have the level of solvent on half way to the start line from the TLC plate bottom end. Cover container with lid.
7. Allow the solvent to move upward about 12 cm. Mark the level of the solvent on developed TLC plate.
8. Remove the plate from the tank. Leave to dry it in air flow till TLC plate become white.
9. Place the plate back in the same developing solvent and let the solvent move upword about 12 cm again (to the marked level).
10. Then, TLC plates should then be dried in air flow again.
11. To detect the sugars location, spray spraying agent onto TLC plates into fume cab.
12. Identify the kind of sugar in particular regions of the TLC plate, comparing with stained spots of standards.
13. The expected results from the bottom to the top of developed TLC plate: raffinose, sucrose, glucose, fructose.
14. Locate the position of the sugars spots centers on the TLC plate and measure the distance travelled by the individual sugar component from the start line. Calculate their retention factors (Rf values) by dividing these distances onto distance travelled by solvent and compare them with those of standards.

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Practical 17. Evaluation of sucrose level in soybeans using thin-layer chromatography by method of J. Robyt and M.Zeller.

Theoretical background. This practical lesson experiment is based on method of of sucrose level level evaluation in soybeans described by J. Robyt and M.Zeller [4].

The Leguminosae family of plants, particularly, French beans, garden pea, soybeans and other beans, when consumed by human, can produce embarrassing digestive gas. This is due to incomplete sugars digestion by human body. The digestive gas produced by soybeans is largely associated with 2 sugars, stachyose and raffinose, that are not completely digested by enzymes in the small intestine. Instead, these sugars pass into the large intestine where *Escherichia coli* and other bacteria utilize these sugars, with production of non-smelling gasses like hydrogen, nitrogen, and carbon dioxide. The bacteria also produce sulfur compounds, particularly, hydrogen sulfide (H_2S) and the gas methane (CH_4) that have offensive odors. On average, humans release about 1 L of gas a day (≈14 expulsions). The levels of raffinose and stachyose are reduced by 90% and the sucrose content is increased by 40% in some modern cultivars of soybeans. This greatly decreases the digestive gas problem and gives to the soybeans a sweeter taste [3, 4].

Sucrose is easily digestible. It is disaccharide, i.e., it's molecule composed of 2 sugars, the glucose and fructose (Glc-Fru) which are easily utilized by human organisms. *Raffinose* is a trisaccharide (Gal-Glc-Fru). It is created when a sucrose molecule adds galactose. Because raffinose is difficult to digest, it passes into the large intestine where gut bacteria digest raffinose, producing gasses that have an objectionable odors.

Stachyose is difficult to digest also. When one more galactose bonds to a raffinose molecule, stachyose is formed (Gal-Gal-Glc-Fru)**.** As a stachyose assembled of 4 sugars, it is classified as a tetrasaccharide or oligosaccharide.

Thus, carbohydrates vary in the number of carbon atoms and in the size of their molecules. If two carbohydrates have similar characteristics, they can be difficult to distinguish. Methods for separating carbohydrates were among the earliest chromatographic procedures.

TLC is widely used in medical and biological laboratories for separation, purification, identification and quantitative determination of different compounds, particularly, sugars [1, 2, 4]. It is low cost, ease, and rapid method that allows a small quantities of compound to be detected in mixtures. In this practical TLC uses a support material or stationary phase (silica gel), a solvent or mobile phase (isopropanol), and a detection method (treatment of separated sugars with solution of N-(1-naphthyl) ethylenediamine dihydrochloride in methanol acidified by sulfuric acid).

Samples. Soybeans with high sucrose content, soybeans with normal sucrose content.

Reagents. N-(-1-naphthyl) ethylenediamine dihydrochloride #N9125; carbohydrate standards in distilled water – 10 mg per mL- mixture of sucrose #47289 (Supelco); from Sigma-Aldrich: stachyose #S4001, raffinose #R0250 70% isopropyl alchohol #563935.

Solvent. 100 ml of 70% isopropyl alchohol for one chromatograpy jar.

Dipping reagent (for sugars detection). 3 grams N-(1-naphthyl) ethylenediamine dihydrochloride per 1 L of methanol + 50 mL of concentrated sulfuric acid (H_2SO_4). It can detect most carbohydrates in the nanogram range of 50-2,000 ng directly on the TLC plate [4].

Equipment. Silica-coated TLC plates 5 cm ×10 cm size (e.g., Whatman K5 thin-layer chromatography plates, measuring cup, kitchen strainer, 1-liter glass container, standard laboratory gloves and goggles, carbohydrate standards, three 10-µl micropipettes, 2 glass beakers, mixing bowls, or other containers large enough to contain 1/2 cup of soybeans plus enough water to keep them covered overnight, two 1.5 ml microcentrifuge tubes, blender, 3-4 paper towels, 2 wide-mouth (> 5 cm wide) glass jars with lids, wide-mouth mason canning jars, hairdryer that has low/high heat settings. Plastic wrap large enough to enclose the chromatography plate, one pencil, one ruler, masking tape and marker pen.

Procedure.

Note. *Use a fume hood, and appropriate personal protective equipment, including gloves and safety goggles*.

Preparation of chromatography chamber. Pour the 70% isopropyl alcohol into wide-mouth (>5 cm wide) glass jars to a depth of 7 to 8 mm. Tightly cap the jars.

Preparation of dipping reagent.

1. In a glass container that can hold at least 1 liter, dissolve 3 g N-(1-naphthyl) ethylenediamine in 1 L of methanol.
2. **Note.** VERY CAREFULLY AND SLOWLY add 50 ml H_2SO_4 (sulfuric acid). Follow all safety precautions on the chemical containers and teacher's recommendations.
3. **Note.** Mix by stirring with a **glass** rod. DO NOT stir with a metallic utensil.
4. The reagent may be stored indefinitely in a closed glass container at room temperature. Do not use a metal lid.

1. *Preparation of samples*

1. Measure one-half cup (75 g) of normal soybeans into a container and add enough tap water (two cups) to keep the soybeans covered as they soak for 18-24 hours. The soybeans should stay covered with water for this entire time, so check the containers periodically. Label the container "Normal."
2. Do the same with the high sucrose soybeans. Label the container "High Sucrose."
3. Use a pencil and ruler to make a **very light** start line and three dots along the length of the horizontal line 1.5 cm apart. **Note.** do not make any dots closer than 1 cm to the edge of the plate.
4. Label the dots by very lightly writing "N" for normal, "S" for sugar standards, "H" for high sucrose soybean extract. **Note.** Write the labels under each dot.
5. Drain the water from the high sucrose and normal soybeans.
6. Measure the normal soybeans into a measuring cup.
7. Put the soybeans and an equal amount of distilled water in a blender and blend on high for 1 min.

8. While the mixture is blending, place a double layer of coffee filters in a kitchen strainer.
9. Filter the mixture, now a whitish liquid, through the double layer of coffee filters into a glass beaker or container.
10. Alternatively, centrifuge the filtrate with either a clinical or microcentrifuge for 10 min.
11. Prepare a 1/5 dilution by transferring one drop of the filtrate to a 1.5 ml microcentrifuge tube. Add 4 drops of distilled water to make the 1/5 dilution.
12. Repeat the steps 7 through 12 for high sucrose soybeans.
13. Using a new 10 µl micropipettes, transfer the drop of filtrates onto points "N" for normal and "H" for high sucrose content.
14. Using a new 10 µl micropipette drop of standard should be placed on the pencil dot labeled "S".
15. When all drops are on the plate and have been dried, put on lab gloves. Place a plate in the solvent jar with the pencil line at the bottom. Cap the jar tightly. The level of solvent should be about halfway between the bottom of the plate and the horizontal line.
16. The solvent will rise up the plate by capillary action, turning the plate a gray, damp color. The solvent will rise to the top of the plate in about 1.4 hrs. Mark with pencil the front level of the solvent.
17. TLC plates can be removed from solvent to dry in air flow. The experiment can be continued the next class time, so plates should be covered with plastic wrap to prevent dusting. Only dry TLC plates can be wrapped.
18. Pour dipping reagent into a mason jar to a depth equal to the height of the TLC plate.
19. Wearing lab gloves, dip the TLC plate into the reagent for only a second.
20. Use the hairdryer on high setting to dry the TLC plate. Leaning the plates upright is the best way to dry them. The plates are dry when the white color returns.
21. Continue to dry/heat the plates until the dark spots appear. **Note.** *Handle a hot plate with caution. The dried plates should be handled with gloves to avoid sulfuric acid residue contact.*
22. Check the sizes and densities of blue-black spots that appear in a lane above each labeled pencil dot on the plate. Using Fig.1 as a guide, locate the spots of sucrose, raffinose, and stachyose in the "S" lane. Compare the spots that appear in the lanes above the "N" (normal soybeans) and "H" (high sucrose soybeans) to the positions of the spots for pure sucrose, raffinose, and stachyose in the "S" lane. Use a pencil to mark the spots with an "Suc" for sucrose, "Raf" for raffinose, and "Stach" for stachyose. **Note:** Very often a glucose spot develops above sucrose.
23. Compare the size and blackness of the "Suc", "Raf" and "Stach" spots that appear in the normal soybean lane with those in the high sucrose soybean lane. The "Suc" spot should be blacker and/or larger in the high sucrose soybean lane, reflecting the 40% increase in sucrose. The "Raf" and "Stach" spots should be lighter and/or smaller in the high sucrose soybean lane, reflecting the 90% reduction in raffinose and stachyose.

24. The plates can be kept with spots for up to 2 years, if wrapped in plastic wrap and kept in a cabinet or cupboard away from light [4].

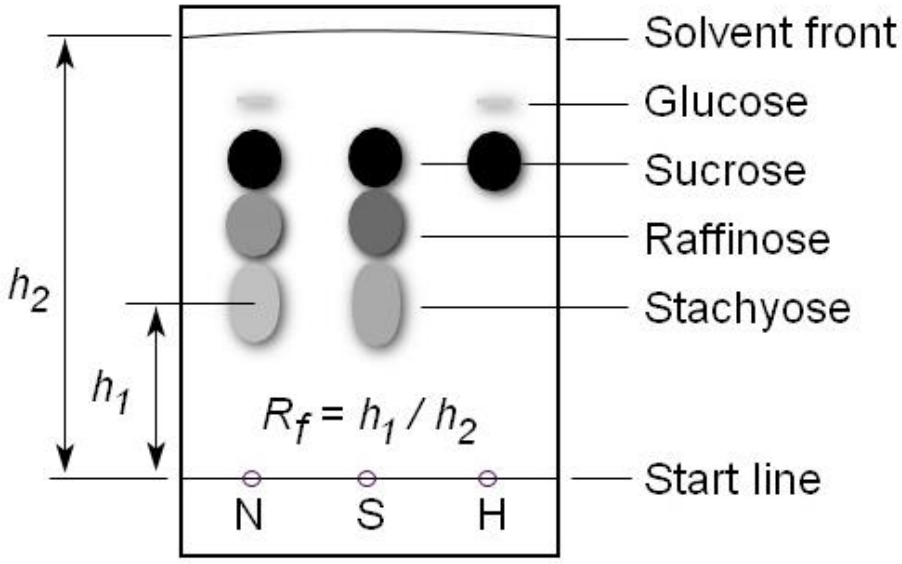

Fig. 1. Developed TLC plate illustrating separated sugars and calculation of the retention factor, *Rf* for stachyose. N – normal soybean extract, S – standard, H – high sucrose soybean extract. *By D.B.Pylypiv.*

Answer the MCQs

1. Chitin is a polymer of **a)** sucrose **b)** maltose **c)** fructose **d)** N-acetyl-glucose amine
2. Chitin is an important structural component of human parasites: **a)** fungi **b)** insects **c)** bacteria **d)** fungi and insects
3. The sugars $(CH_2O)_n$ important in cell metabolism have *n* values in range from **a)** 3 to 7 **b)** 10 to 15 **c)** 11 to 16 **d)** 16 to 20
4. What is NOT a disaccharide? **a)** glycogen **b)** lactose **c)** sucrose **d)** maltose

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Practical 18. Solubility test and peripheral blood smear for hemoglobin S and sickled red blood cells detection.

Theoretical background. Haemoglobin (Hb) is a protein of red blood cells (RBC) with the main duty of oxygen transport. It combines with O_2 to form unstable oxyhaemoglobin Hb.O_2 at lungs. Then, through the circulatory system it passes the O_2 to tissues and returns carbon dioxide (CO_2) from body to the lungs. Hb consists of 4 polypeptide chains of globins, with heme group each and 141 or 146 amino acid residues arranged in 7 or 8 helical regions joined by non-helical segments. Haemoglobinopathies is a group of important hereditary disorders, resulting from mutations in the genes coding for the Hb synthesis. On the basis of genetic defect(s) involved, the haemoglobinopathies are categorized on thalassaemias (Ts) and haemoglobin variants (Hb Vs). Usually, the Ts are developed because of low production of normal globins due to mutations in regulatory genes and Hb Vs resulting from abnormal structure of the globins due to defects in their coding DNA sequences. Both of these conditions may be present in one patient or separately. When Ts defect or deletion happens, it results in low/no production of one of the globin chains. This can cause the formation of abnormal Hb and anemia. There are 2 major forms of the disease, α - and β - Ts.

The genes for Hb A1 and Hb A2 are involved in α-Ts. This leads to low α-globin synthesis and a subsequent excess of β chains in adults and γ chains in newborns. The β chains excess form unstable tetramers Hb H, which have abnormal oxygen dissociation curves [8].

The mutations in the HBB gene on chromosome 11 cause β-Ts. Their severity depends on the nature of the mutations. They can prevent β chains formation causing the most severe form, β thalassemia major (βo) or may allow low β chain production in case of β thalassemia intermediate (β+). In both matters there is an excess of α chains although, unlike in α-thalassaemia, the tetramers are not formed. The α chains bind to the membranes of erythrocytes, damaging and aggregating them.

The normal haemoglobin types are:

- Hb A – the main form in adults (95-98% of total Hb) build of 2 α and 2 β protein chains (α2β2);
- Hb A2 – typically 2-3% of Hb in adults, it has 2 α and 2 δ protein chains (α2δ2);
- Hb F – the primary Hb produced by the fetus, decreasing drastically soon after birth and comprising up to 2% of Hb of adults; it has 2 α and 2 γ protein chains (α2γ2).

The most spread of Hb Vs include: Hb S, Hb C, Hb E, Hb D-Punjab, Hb O-Arab, Hb G-Philadelphia. Many Hb Vs do not cause pathology, particularly, in heterozygotes. However, those affecting RBC function usually cause anemia [8].

The sickle cell disease (SCD) is the most widespread and most severe form of anemia, comprising >70% of anemias worldwide [10]. It is caused by the homozygous inheritance of the βS-mutation and abbreviated to as SCD, SCD SS or SCA. The Hb with this mutation is able to transport O_2, but once the O_2 is released, the wrong molecules stick to one another and form rod-shaped structures in the RBCs [2]. The

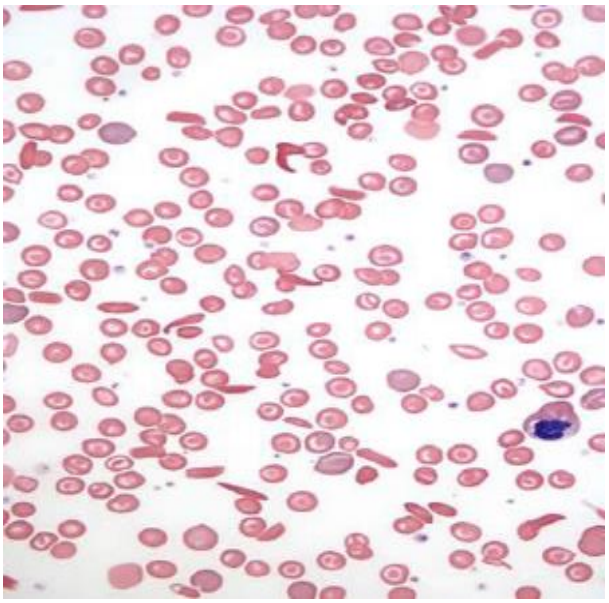
RBCs become abnormal, fragile, rigid, sickle, star, holly leaf and tailed in shape (Fig.1). This decreases their flexibility and ability to move within small vessels. This leads to aggregation of the RBCs and vascular occlusions resulting in pain throughout the body, painful episodes termed for 5-6 days [6]; cerebrovascular accidents (strokes) and acute chest syndrome with inflammation of lungs, pulmonary hypertension; spleen necrosis [14]; hand and foot syndrome with possible numbness and tingling. Jaundice appears, since the liver is unable to process the increased number of dead RBCs, leading to a buildup of bilirubin [5]. Fatigue, pallor, irritability may also occur. Complications of SCD include: neurological (seizures, cerebrovascular accident, meningitis), pulmonary (acute pulmonary infarction, pneumonia, atelectasis, acute chest syndrome), musculoskeletal (avascular necrosis, osteomyelitis, hand and foot syndrome), visual (blindness, retinopathy), genitourinary (eclampsia, nocturia, hematuria), dermatological (stasis ulcers of hands, ankles, feet), other organs (splenomegaly, acute hepatomegaly, gallstones) [5].

In heterozygotes, with one sickle gene and one normal adult Hb gene (Hb AS or 'sickle cell trait') partial erythrocytes sickling may appear at low oxygen pressure. Hb S is an abnormal Hb produced from a point mutation in the HBB gene that results in replacement of glutamic acid by valine at the position 6 on the β-globin chain of subunit (βS) of the haemoglobin molecule [11]. SCD is inherited as autosomal recessive trait with 25% chance of offspring with disease in family, where both parents are carriers of one sickle cell allele:

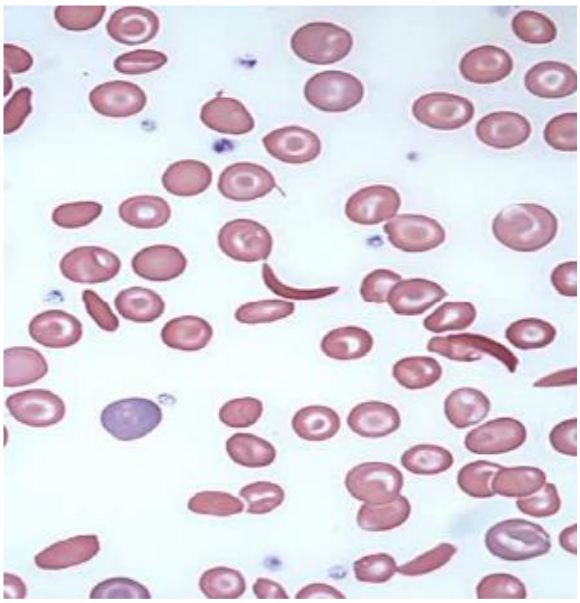
A- allele for normal HbA, healthy condition;
S – allele for HbS, disease in homozygous SS individuals
Couple genotypes, P: mother ♀AS × ♂AS father
Gametes, G: A, S and A, S
Children, F: AA 25% (healthy) AS 50% (carriers) and SS 25% (diseased)

However, SCD can also result from the inheritance of βS in combination with other HBB mutations, the two most common being a second structural β-globin variant βC (SCD SC) [12] and one of the many β-Ts mutations that lead to the insufficient production of normal β-globin (SCD S/β-thalassaemia) [13]. The Hb D-Punjab and Hb O-Arab can result in SCA when combined with Hb S in offspring also [15, 16]. Both: SCD and Ts genotypes evolved in people living in humid Tropical and Mediterranean climates with endemic malaria. These provide a degree of resistance to *Plasmodium* parasites, the causal agents of the disease.

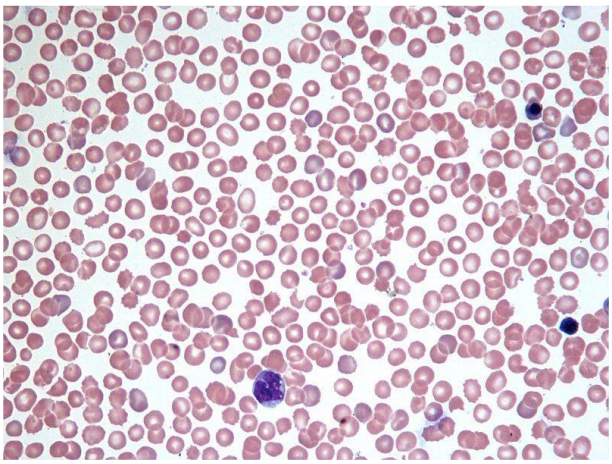
There are several methods in use to diagnose SCA. They are based on estimation of the presence of Hb S and/or abnormally-shaped RBC. These tests include: Hb solubility test, Hb electrophoresis, isoelectric focusing, high-performance liquid chromatography (HPLC), density-based separation by centrifugation in multiphase systems [2], and/or a peripheral blood smear. It is possible to diagnose SCA in molecular genetic tests searching for 2 copies of the Hb S gene. However, this technique is expensive and is used mainly in prenatal diagnosis to identify Hb Vs and determine the probability of SCD child birth.



A



B



C

Fig. 1. Blood smears from sickle cell disease patients:
a, b - adults, c, - neonate.
Modified from Keith Chambers, Leicester Royal Infirmary. Creative Commons BY-SA [7].

The Hb solubility test is based on the relative insolubility of HbS when it interacts with a strong reducing agent in a patient's blood sample mixed with a reagent containing saponin, 2.3M potassium phosphate buffer and sodium hydrosulfite. Saponin acts as detergent, destroying the membranes of RBC to release Hb. Sodium hydrosulfite is a reducing agent and detaches the O2 from Hb. The Hb S, if present, will form liquid crystals providing turbidity to the solution. Deoxygenated Hb S is insoluble in concentrated phosphate buffer and precipitates. Other Hbs are more soluble in the reducing agent. Particularly, deoxygenated Hb A is soluble at these conditions and the tube remains transparent. We can see through the tube the black lines on a card placed behind the sample (negative result of the test). In a positive screen the lines are not visible because of turbidity. This indicates the Hb S or another sickling Hb presence.

At least 20% of wrong Hb is necessary for this result. Either homozygotic for this mutation individual ("sickle cell anemia") or heterozygotic one ("sickle cell trait", in which Hb S is usually 30-45%) may have positive results. It can also be observed for Hb C Harlem and Hb C Georgetown.

The test is prone to error in cases of blood with hyperlipidemia; extreme leukocytosis; erythrocytosis; multiple myeloma; high plasma protein levels at hyperglobulinemia. These may mislead to false positive results.

False negative results are observed in anemic individuals with Hb < 7.0 g/dL. To overcome this problem, packed erythrocytes (0.01 mL) may be used. False negative screens may occur, if Hb S concentration is less than 20%; at high fetal Hb or after recent blood transfusion also. The blood samples from infants younger than 6 months (they have high levels of Hb F and low levels of other Hbs, particularly, Hb S) and normal Hb specimens from people with recent blood transfusion have false negative results in this test also.

The Hb D-Punjab and Hb O-Arab are not detectable by this test. Reference tubes show negative results. Often 2 tubes are used: one for positive and another for negative result standard.

Experiment 1. Solubility Test for Hemoglobin S

Principle: The Hb S is not soluble when combined with sodium dithionite, a reducing agent. The saponin in the whole blood sample lyses the RBC and Hb is released. Hb S, if present, will form liquid crystals and give a turbidity to the solution. The transparent solution is seen when Hb S is absent or present at the amount less than 20%. Turbidity can be observed for Hb C Harlem, Hb C Georgetown, Hb C Ziguinchor, and Hb S Travis in this test also. Other Hbs are more soluble at these conditions.

Reagents and equipment

Note. *Avoid contact of blood and reagents with eyes, mouth and skin. Do use gloves!*

Calibrated test tubes, 12 × 75 mm in diameter; micropipet, 20 -100 μL; disposable micropipette tips; pipette, 2.0 mL; paper-board and test tube holder (tubes should be held on distance 2.5 cm from the reading card; reading card should have 16-18-font straight black lines printed 0.5 cm apart on white carton); day light lamp; gloves. Stock solution: Saponin 5 g; KH_2PO_4, crystals 84,5 g; K_2HPO_4, anhydrous 108 g; Add distilled water to 500 mL. (The reagent should be stored in the fridge at 4°C not more than 1 month).

Working solution: Add 5 mg sodium dithionite ($Na_2S_2O_4$) to 1 mL of stock solution.

Specimen: The whole blood treated by EDTA, heparin or sodium citrate to prevent coagulation in the tube is suitable for this experiment. It is acceptable to store the specimen at 4ºC for up to 3 weeks before use in the test.

Reference tubes: The specimen from a patient should be compared with "sickle cell trait" (30-45% Hb S) and a negative "healthy" sample from adults with known Hb AA as controls.

Procedure.

1. Take the specimen and reagents from the fridge and allow them to attain room temperature before the test.
2. Pipette 2 mL of working solution into the test tube.
3. Add 20 μL of whole blood specimens to the test tube.

4. Mix well and place the test tube with reference tubes for 6 minutes in the tube holder at room temperature.
5. Observe the turbidity or its absence by looking onto a reading card through the test tube.

Results

1. Negative result: suspension is transparent, the lines on the reading card are clearly visible.
2. Positive result: suspension is turbid, the lines on the reading card are not visible through it.
3. The test does not differentiate between the genotypes Hb S ("sickle cell disease", S/S) and Hb S "sickle cell trait", A/S). To distinguish between these 2 genotypes, a Hb electrophoresis at alkaline pH should be performed. The electrophoresis at alkaline pH (and, occasionally, at acidic pH) allows to separate the Hb S from Hb C Ziguinchor, Hb C Harlem, Hb C Georgetown and Hb S Travis, which are resulting in turbidity in this test also.

Experiment 2. Differentiation between the genotypes Hb S "sickle cell disease"(S/S) and Hb S "sickle cell trait"(A/S)

Principle: The method is based on a modification of the sodium dithionite hemoglobin solubility test by Louderback et al. (1974) [9], allowing to differentiate the Hbs "sickle cell disease" (genotype SS), "sickle cell trait" (genotype AS) and normal *Hb* individuals (genotype *Hb AA*) by results of *Hb* dissolving followed by centrifugation.

Reagents and equipment

Cautions! Avoid contact of blood and reagents with eyes, mouth and skin. **Do use gloves!**

Calibrated test tubes, 12 × 75 mm in diameter; micropipet, 20 -100 μL; disposable micropipet tips; pipets, 2.0 mL; paper-board and test tube holder (tubes should be held on distance 2.5 cm from the reading card; reading card should have 16-18-font straight black lines printed 0.5 cm apart on white carton); day light lamp; clinical or serological centrifuge, gloves.

Buffer solution is a 280 g/L $(NH_4)_2SO_4$ solution, the pH of which is adjusted to 7.1 ± 0.1 with K_2HPO_4 (1 mol/L).

Working solution Add 1 g of Na dithionite, the reducing agent ($Na_2S_2O_4$), plus 1 g of detergent saponin to 100 ml of buffer solution. Dissolve the reagents by vigorous shaking. The working solution can be stored at refrigerator temperature (2 to 8ºC) and is stable for as long as 1 month in tightly stoppered bottle.

Specimen: 0.1 ml of whole blood sample containing anticoagulant (heparin, EDTA, ACD, etc.)

Reference tubes: Blood sample obtained from an individual with known normal adult *Hb* (genotype *AA*) should be used as control throughout both steps of the experiment.

Procedure.

1. Brought the working solution to room temperature.

2. Add the working solution to the 2-ml line of a calibrated 12 × 75 mm test tube.
3. Add to this tube 100 μL of the wholeblood testspecimen. Blood sample containing anticoagulant (heparin, EDTA, ACD, etc.) may be used.
4. Mix the test sample thoroughly with the working solution by inverting the tube several times.
5. Allow to stand at room temperature for 5 min.
6. The tube is then examined visually in the tube holder near to a light source and on a distance 2.5 cm from the reading card.

Results

1. If the solution became turbid, the lines cannot be seen through the tube, then the specimen is either heterozygous or homozygous for *Hb S*.
2. Differentiation of heterozygosity is determined by centrifuging the tube in a clinical or serological centrifuge for 3 mm at 3400 to 5000 rpm.
3. Examine the specimen with the same light source to indentify the following possibilities in centrifuged tubes:
a) the supernatant is *clear, pink*. There is a small amount of *red* precipitate, then the sample is *heterozygous* for *Hb S*.
b) the supernatant solution is *clear, yellow*. There is a large amount of *red* precipitate, then the sample is *homozygous* for *Hb S*.
c) the uniform *red* supernatant with only a small amount of *white* precipitate then the sample is *homozygous* for *Hb A*.

Experiment 3. Presence of sickling phenomenon in blood sample

***Principle*:** The *RBC* containing sickle hemoglobin (*Hb S*) become sickle shaped, when deoxygenated by strong reducing agent, as sodium metabisulfite. RBC with normal *Hb A* will not sickle [4].

Reagents and equipment

Cautions! Avoid contact of blood and reagents with eyes, mouth and skin. **Do use gloves!**
Blood collected in K2 EDTA tube; 2% Sodium metabisulfite solution in distilled water; DPX Mountant; clean glass slide; cover slip; glass rods; light microscope.

Procedure.

1. Place a drop of whole EDTA anticoagulated blood on the glass slide. **Cautions**! Handle all samples as infectious.
2. Add 2 drops of 2% sodium metabisulfite.
3. Mix well with a glass rod.
4. Place a cover slip on a mix and press it gently to remove any of air bubbles from the blood.
5. Using the glass rod seal the preparation on perimeter of cover slip with DPX Mountant.
6. Check the preparation for the presence of sickle cells - "holly oak leaf" form of RBCs after 30 min under magnification 100-400 ×. Count sickled cells in 10 microscope fields and calculate the sickle cell index (SCI) [1], the percent of sickle cells from total

red cell population. Percent of sickle cells is increased with age and correlated with disease severity, especially at hemolytic complications.
7. Re-examine the same preparation for sickling phenomenon after 24 hours.
8. Make the preparation variant as steps 1-5, but without adding of 2% Na metabisulfite and use this to compare with blood preparation, treated by this salt solution.
9. Test is “positive” if sickle cells present or as “negative”, if they are absent.
10. Is the difference in results between the 30 min and 24 hrs exposition experiments?

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Practical 19. SDS-discontinuous polyacrylamide gel electrophoresis of proteins by U.K. Laemmli.

Shapiro A.L. with co-workers were among first who used sodium-dodecyl-sulfate (SDS) as additive to protein samples and to the polyacrylamide gels (PAAGs) [6]. Prof. Ulrich K. Laemmli (University of Geneva) made significant improvements to the method of electrophoresis. Particularly, he used a buffer containing SDS to help in fractionation of phage T4 proteins [3]. This buffer is now often utilized for treatment of proteins in SDS-PAGE. Weber K, Osborn M. (1969) showed the reliability of molecular weight determinations by dodecyl sulfate-PAGE [8].

After the papers of Laemmli [3], Shapiro et al. [6] and Weber, Osborn [8] the SDS-PAGE gels became popular and appeared in many variations. Finally, 2D gel techniques, with isoelectric focusing of proteins in one direction followed by regular SDS-PAGE in the other direction were developed. The first inseminative paper in this field was published by O'Farrell [5]. Then people invented the way to transfer the proteins separated in SDS-PAGE to the nitrocellulose membrane to stain them with antibodies [7] and nylon-like polyvinylidene difluoride (PVDF) membranes, where they can be treated with antibodies or stained with Coomassie Blue, and sequenced directly [4]. Now SDS-PAGE discontinuous electrophoretic system, developed by U. K.Laemmli, is widely used to separate proteins with molecular masses between 5 and 250 KDa.

SDS is a detergent whith hydrophobic dodecyl end and highly charged part (the sulfate group). The dodecyl part acts on hydrophobic amino acids in proteins. Since the 3D structure of most proteins depends largely on hydrophobic interactions in their core, the hot SDS treatment transforms globular proteins into linear negatively charged molecules. Now they will move towards anode. The β-mercaptoethanol is usually added to cleave disulfide bonds within or between protein chains, allowing molecules to adopt an extended monomeric form. As a rule, the largest linear molecules are retarded the most by PAAG, and the smallest ones the least. Some proteins have few or no hydrophobic residues, thus run on SDS page in a fashion which inaccurately reflects their molecular weight (M_w). Phosphorylation and especially glycosylation can also cause proteins to run more slowly. The cross-linked proteins don't run as their molecular weight would predict, generally running slower, particularly, on higher percentage gels with smaller pores. However, a particular protein runs on a particular position on a particular percentage PAAG in a characteristic manner.

When the proteins are heated in the presence of SDS, the detergent binds to the polypeptide chain and gives it a homogeneous negative charge. Most proteins per 1 g of their weight bind 1.4 g of SDS regardless of amino acid composition and sequence. The peptide chain takes the form of a rigid ellipsoid, its small axis has a constant length, and the size of the large axis is linearly related to the molecular weight of the protein, resulting in a constant-to-mass ratio. Formed SDS polypeptide complexes, moving in acrylic-bottom gel, are separated according to their masses, small molecules move faster. M_w of the unknown protein can be determined with a deviation of 10% from its real value by comparing it with the mobility of standard proteins.

SDS-PAGE resolution has been significantly improved by the use of a concentrating (stacking) gel that uses the principle of isotachophoresis - the concentration of a relatively large sample volume into narrow and concentrated bands (or zones). In the separating gel, the negatively charged SDS protein complex migrates through a sieve-like polyacrylamide matrix, where the components of the protein mixture are separated by M_w. The electrophoretic separation of a hypothetical mixture of 4 proteins (*P1-4*) through a concentrating gel of pH 6.8 and a separating gel of pH 8.8 is shown in Fig. 1.

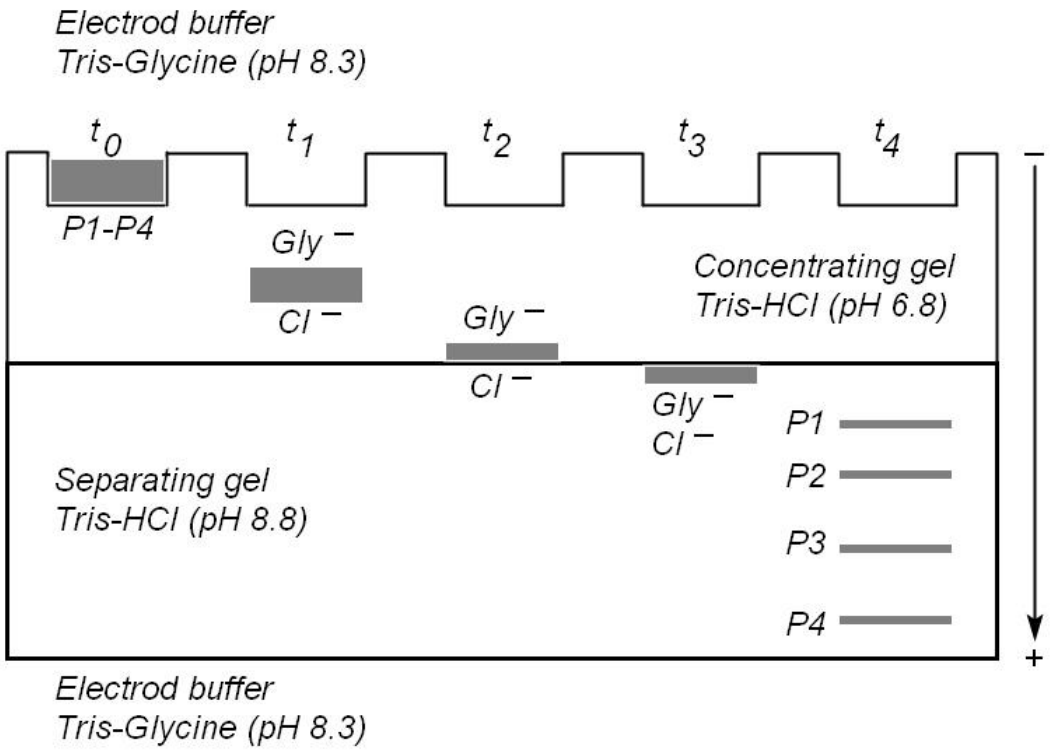

Fig. 1. Scheme of electrophoretic separation in PAGE.
t_0 is the beginning of the separation, when a high volume protein mixture is applied to a concentrating gel well with a pH of 6.8. After turning on the voltage, the sample volume begins to decrease, creating a disk - a narrow concentrated zone between the leading chlorine ion of the separating buffer and the closing glycine ion of the electrode buffer with pH 8.3 (t_1), decreasing in size as it moves to the separating gel. Upon completion of the concentrating (t_2) process, the formed disc begins to penetrate the pores of the separating acrylamide gel with much smaller pores and other pH values - 8.8 (t_3), thus changing the mobility of protein ions. The proteins under study, which have different molecular weights and diffusion coefficients, will migrate with different velocity and will separate (t_4). *By Diana B.Pylypiv.*

The polyacrylamide gel is formed by polymerization of acrylamide molecules and the crosslinking bifunctional monomer N, N-methylenebisacrylamide. The polymerization process can be induced: **1)** chemically – by a combination of TEMED (N,N,N′, N′-tetramethylethane-1,2-diamine) and ammonium persulfate

2) photochemically – by a combination of TEMED and riboflavin-5-phosphate or using methylene blue as a source of free radicals.

The pore size of acrylamide gels is determined mainly by the total amount of acrylamide per unit volume (*T*) and the relative % of bis-acrylamide (*C*) - crosslinking component.

T is the total concentration in % of acrylamide and bis-acrylamide monomers in grams per 100 ml. *C* - indicates the percentage (by weight) of bis-acrylamide (cross-linker) relative to the total number of acrylamide monomers (acrylamide + bis).

$$T = (A + B / 100ml) \times 100\%; C = (B / A + B) \times 100\%,$$

were A - weight of acrylamide, B - weight of bis-acrylamide in grams.

The pore size is directly proportional to the rate of polymerization. Therefore, all factors affecting the rate of polymerization are also determining the pore size of the gel matrix. For example, a decrease in pores can be caused by an increase in the polymerization temperature, and *vice versa*. The use of urea induces an acceleration of the activation of acrylamide monomers by ammonium persulfate, which leads to the formation of small pores. Addition of polyethylene glycol results in a macro-porous gel matrix.

Gels with an acrylamide concentration of less than 3% are too soft and require 0.5% agarose to work with them. Gels with an acrylamide concentration of >35% are too brittle. Usually, for homogeneous gels, the concentration of acrylamide is in the range of 5-20%, for gradient gels - 3-30%.

Proteins separated in gels can be visualized by use of immunoblots and autoradiography, by use of fluorochromes, silver staining, or such organic stains, as Fast green FCF, Amido Black, Coomassie blue. For example, Coomassie blue can detect 30-100 ng of protein. Its sensitivity can be increased at elevated temperatures or when colloidal Coomassie is used [2].

The solutions are made up as below [1]; All except the ammonium persulfate can be stored at room temperature for few months. Acrylamide/bisacrylamide solutions may be light sensitive, store these in dark or aluminium foil covered bottles. The ammonium persulfate should be made up each week, and stored at 4°C. Ammonium persulfate is rather unstable and decays to produce free radical SO_4^- ions, which react with the acrylamide molecules and initiate their polymerization. The acrylamide (CH_2=$CHCONH_2$) polymerization occurs by opening the double bond. Acrylamide molecules react with each other to produce a linear polyacrylamide molecule, and the incorporation every now and then of a bis-acrylamide generates cross links between such linear molecules. Bisacrylamide is basically two acrylamides bound together, the formula being $(CH_2$=$CHCONH)_2CH_2$. We can vary the amount of this, the usual range being 1 part in 20 to 1 part in 50. The TEMED is $(CH_3)_2NCH_2CH_2N(CH_3)_2$ and acts as a catalyst, speeding up the decay of the ammonium persulfate. Molecular oxygen inhibits polymerization by reacting with the free radical SO_4^- ions, which is the reason why PAGE gels are poured in tubes or between plates and not in open top horizontal apparatuses, as can be done with

agarose. It's a good practice to layer some isopropanol on top of the gel as this prevents oxygen getting in and inhibiting polymerization. If your gel doesn't polymerize it's most likely because the ammonium persulfate has gone off. This is only stable for a few days at 4°C.

Experiment

Equipment. Apparatus for gel electrophoresis, *e.g.*, 'Biorad', pipettes with fresh tips, cylinders to measure liquids, flasks and tubes with lids, vacuum apparatus, balances, paper towel.

Stock solutions. Follow the Tables 1-10 to prepare all necessary solutions [1].

Table 1. Buffer for concentrating gel (4 ×) 0.5 M Tris-HCl pH 6.8

Tris	60.5 g	6.05 g	3 g	Bring the pH to 6.8 with a solution of 6 M HCl, allow to stand at room temperature, check and bring the pH to 6.8. Bring deionized water to final volume. Filter. Store at 4°C in a fridge.
	850 mL	85 mL	40 mL	
Bring to	1000 mL	100 mL	50 mL	

Table 2. Buffer for stacking gel (4 ×) 1.5 M Tris-HCl pH 8.8

Tris	181.5	18.15	9.1	Bring the pH to 8.8 with a solution of 6 M HCl, allow to stand at room temperature, check and bring the pH to 8.8. Bring deionized water to final volume. Filter. Store at 4°C in a fridge.
	850 mL	85 mL	40 mL	
Bring to	1000 mL	100 mL	50 mL	

Table 3. Electrod buffer (10×) Tris-glycine buffer pH 8.3, 0,1% SDS

Tris	30 g	3 g	The buffer is not titrated with hydrochloric acid. After cooking, leave to stand for the pH adjustment, re-measure pH. Stored at room temperature. Before use, dilute 10 times and filter. pH 8.3
Glycine	144 g	14.4 g	
SDS	10 g	1.0 g	
H_20	1000 mL	100 mL	

Table 4. Stock solution, 10% SDS

SDS	10 g	1 g	Note. Store at room temperature. If cooled to 4° C, it becomes solid.
Bring to volume with distilled H_20	100 ml	10 ml	

Table 5. Buffer for samples (2×)

(4×) conc. buffer	2 mL	1 mL	Mixed with the sample solution in a ratio 1:1. It is important to maintain a ratio of SDS to protein 1.4 g / 1 g. The concentration of protein in the final solution should not exceed 10 µg/µl. Heat for 2 min at 95°C. Heat the sample to 60°C (and above) and mixing initiates the process of SDS binding to the protein. Store at 4°C.
Glycerol	1.6 mL	0.8 mL	
10% SDS	3.2 mL	1.6 mL	
2-mercaptoethanol	0.8 mL	0.4 mL	
1% brom phenol blue	0.4 mL	0.2 mL	
Total volume	8 mL	4 mL	

Table 6. 1% solution of agarose

agarose	1 g	0.5 g	Used to create agarose tube before pouring the separating gel. Store at 4 ° C.
Total volume	10 mL	5 mL	

Table 7. Solutions of the chemical polymerization agents

Ammonium persulfate,10%	1 g	0.1 g	Ammonium persulfate is a very hygroscopic compound and is immediately destroyed when dissolved in water. The solution should be prepared immediately prior to the polymerization (each time new). In the normal state it is a dry salt, the efficiency of the reagent drops with the accumulation of moisture. When dissolved in water, the noise of gas bubbles should be observed.
H_2O	10 mL	1 mL	
TEMED (N, N, N′, N′-tetramethylethane-1,2-diamine)	Without dilution		Hygroscopic compound, easily oxidizable. Store in a dark glass, tightly closed container at 4 ° C, not more than 6 months after opening. Intense yellow may be an indicator of the inability of the reagent.

Table 8. Solution of acrylamide and bis-acrylamide 30% T / 2.6%C.

Acrylamide	29.22 g	14.6 g	The solution should be filtered through a membrane filter and degassed with a vacuum pump and Bunsen flask (15-20 min before the adding of the polymerization initiators). Purification and degassing can be carried out by the addition of crushed activated charcoal followed by filtration. The solution should be prepared weekly. Store at 4°C in a dark place.
Bis-acrylamide	0,78 g	0.4 g	
H_2O	100 mL	50 mL	

Table 9. Separating (T-12%) and stacking (T-5%) gels.

Ingredients	Separating		Stacking gell	
H_2O	32.9 mL	4.94 mL	65.3 mL	5.63 mL
1.5M Tris-HCl, pH 8.8	25 mL	3.75 mL	-	-
0.5M Tris-HCl, pH 6.8	-	-	25 mL	2.5 mL
10% SDS	1 mL	0.15 mL	1 mL	1 mL
Acryl/Bic T30% C2.6%	40 mL	6 mL	16.6 mL	1.66 mL
10% Ammonium persulfate	1 mL	0.15 mL	1 mL	1 mL
TEMED	0.1 mL	0.015 mL	0.1 mL	0.01 mL
Total volume	100 mL	15 mL	100 mL	10 mL

Table 10. Staining of proteins in developed gels

Staining ingredients		The dye is dissolved in alcohol and then water is added. The mixture is settled during 4 hours or night, then filtered. Store at 4°C in a dark place.
Acetic acid	100 mL	
Metanol	500 mL	
H_2O	400 mL	
Coomassie Brilliant Blue	1 g	
Total volume	1000 mL	
Washing ingredients		The background is washed with constant stirring. Change the solution when stained with the dye to a fresh one.
Acetic acid	70 mL	
Metanol	120 mL	
H_2O	810 mL	
Total volume	1000 mL	

Procedure.

1. To prepare glass plates of an electrophoretic chamber:
   1.1. Wash glass plates of gel-electrophoresis apparatus with detergent.
   1.2. Rinse with tap water.
   1.3. Rinse with distilled water.
   1.4. Dry in oven 60-120 ° C, allow to cool.
2. Assamble an electrophoretic camera.
3. Outline the height of the gel filling with marker.
4. Pour the agarose to seal.
5. Fill separating gel.
   5.1. Prepare the mixture according to the prescription in the required volume. Stir but not shake. Add polymerizers, mix.
   5.2. Using an automatic dispenser (5 mL), carefully pour the mixture of separating gel into the chamber without creating bubbles.
   5.3. Carefully layer 0.3 - 0.5 mL of water (0.1% solution SDS, saturated solution of n-butanol or isopropanol). As the degree of polymerization increases, the boundary between gel and water will become more and more clear.
   5.4. The degree of polymerization is controlled by the residue of the separation gel solution in a tube. For 7-10 min the gel will begin to polymerize. Leave it for 30 min.
   5.5. Remove the water before applying the concentrating gel mixture.
6. Pour concentrating (stacking) gel:
   6.1. Prepare the mixture according to the prescription in the required volume. Stir but not shake. Add polymerizers, mix.
   6.2. Carefully apply a mixture of concentrating gel on top of separating gel to the upper edge of the inner glass.
   6.3. Immerse the Teflon comb into the stacking gel solution so that 1.0-1.5 cm is left between the upper edge of the separating gel and the lower edge of the comb teeth.

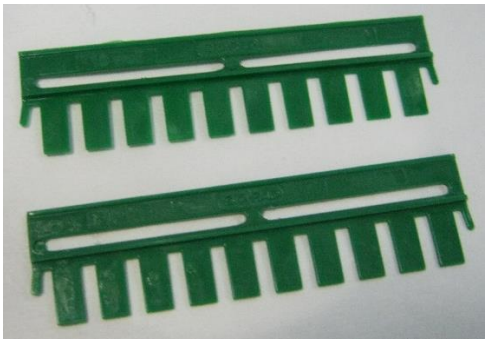

Fig. 1. Teflon combs used to make the wells into stacking gel plate. *By B.M.Sharga.*

To prevent the bubbles formation, the comb should be inserted under the angle to the liquid gel surface.

6.4. Polymerization takes about 2 hours. Changing the refractive index around the comb teeth will be an indicator of the degree of polymerization. For convenience, mark the position of the wells with a marker.

6.5. Carefully remove the combs from the concentrating gel, assemble the electrophoretic camera according to the manufacturer's instructions.

6.6. Fill the camera with electrode buffer. Remove the air from the wells formed in the concentrating gel, wash with electrode buffer using an automatic dispenser to remove the acrylic acid formed in the wells as a result of polymerization (Fig.2)

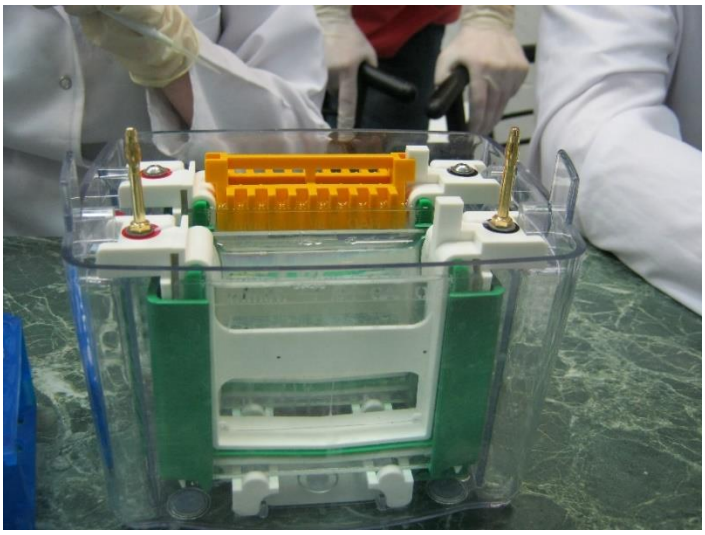

Fig. 2. Wash the wells with electrode buffer using an automatic dispenser. *By B.M.Sharga.*

7. Sample preparation:
   7.1. Mix the protein sample with the sample buffer (x2) in a 1: 1 ratio. Ideal are the conditions where 1.4 g of SDS is per 1 g of protein. In any case, the final protein concentration should not exceed 10 μg/μl.
   7.2. Heat the sample in a water bath or heating block to 95°C for 2-5 min to denaturate the protein and bind it to SDS. Cool to room temperature. Centrifuge.
   7.3. Apply the samples to the wells of concentrating gel as it is shown on Fig.
8. Start electrophoretic separation:
   8.1. Do not exceed the voltage, current and power characteristics of the type of camera used.
   8.2. The cathode (-) must be attached to the upper tank and the Anode (+) to the lower.
   8.3. A constant 90V voltage at room temperature will take 16-18 hours to cross the finish line with the bromophenol blue front. If the camera is equipped with a cooling system (8°C) - a constant voltage of 200 V will provide a total time 6-8 hours of the electrophoresis.

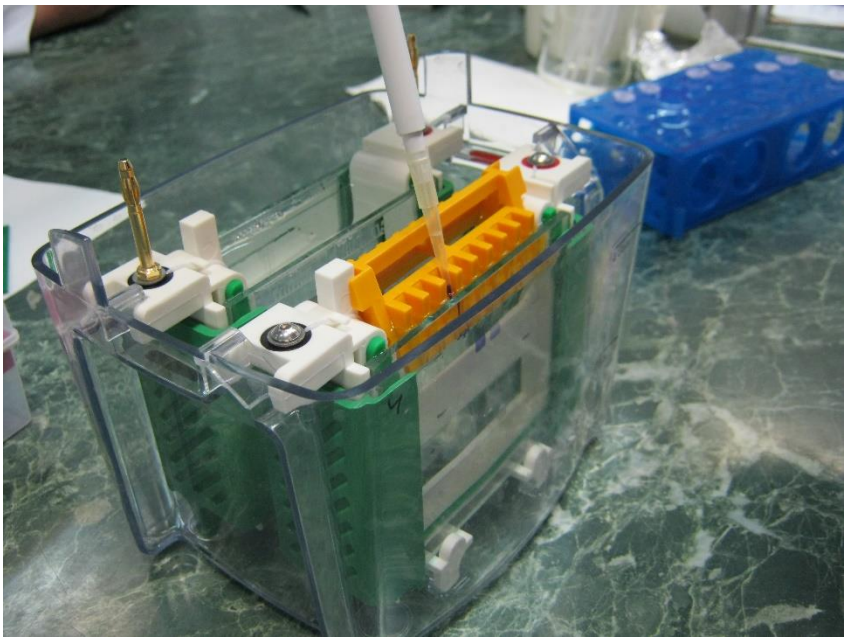

Fig. 3. Loading of samples into the wells.
By B.M.Sharga.

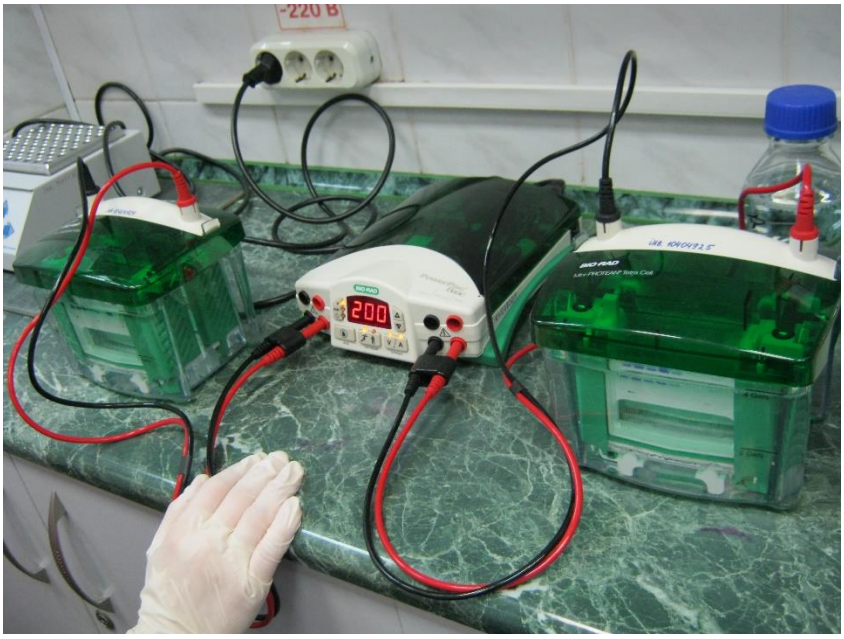

Fig. 4. Electrophoresis. Note. High voltage! Do not touch the working apparatus. *By B.M.Sharga.*

The bubbles formation on electrode is evidence of electrophoresis start (Fig

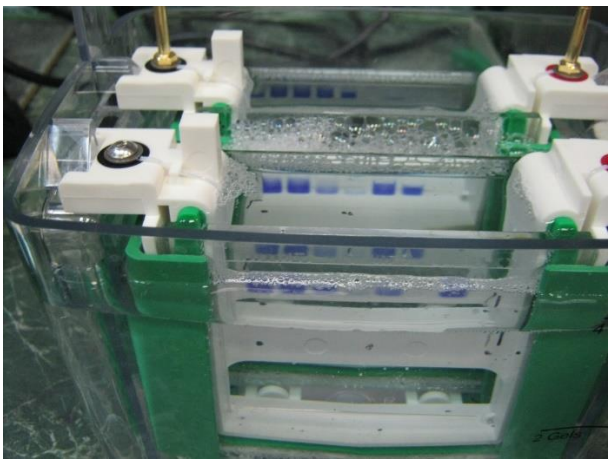

Fig. 5. This electrophoresis stopped at the start to show, how loaded protein samples start to move through the stacking gel. Note the bobbles formed by current in a buffer. *By B.M.Sharga.*

8.4. Switch off electrophoretic apparatus, prepare gel for visualization.

9. Visualization of gels (Fig. 6):

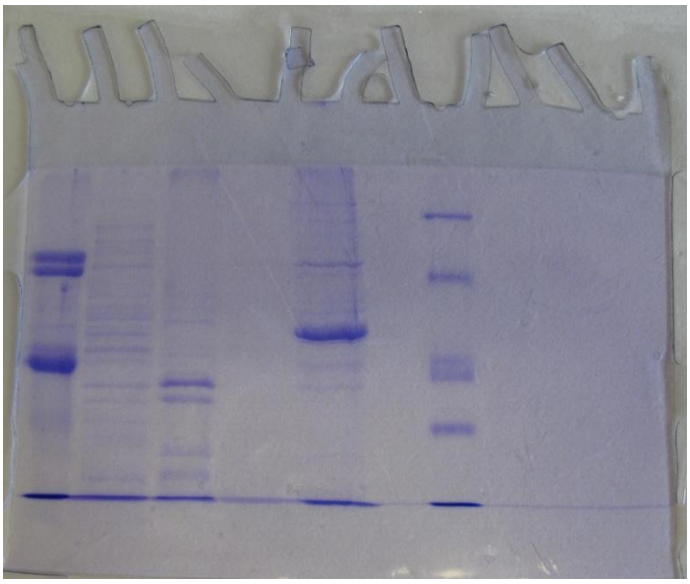

Fig. 6. Gel-electrophoresis plate stained with Coomassie blue. Extreme right: The ladder with known M_w of proteins. All the rest are experimental lanes with many of proteins. The M_w of them are estimated from comparison with ladder. *By B.M.Sharga.*

9.1 Put the gel in a plastic container and pour the mixture with the dye at the rate of 100 mL per gel 8x10 cm.

9.2 Leave the gel under constant stirring overnight at room temperature.

9.3 Drain the dye solution next day.

9.4 Add the required volume of washing detergent. Leave the gel with constant stirring until sufficient background discoloration. 9.5 Store the gel in 5% acetic acid solution or air dry. Place between two stretched layers of cellophane. Leave to dry. Air-dried gels can be stored long time.

Answer the MSQs

1. What is NOT true about two dimensional gel electrophoresis (2D-GE)? **a)** In the first dimension, proteins are separated by the pI value and in the second dimension by the relative molecular weight. **b)** Although it was described in 1975 by O'Farrell **c)** It utilizes pH gradient strips to separate prteins by pI value in first demention **d)** SDS PAGE layer is used to separate the all proteins of cell or tissue in second dimension **e)** It is usually used to separate nucleic acids
2. Among following gel electrophoreses the method that utilizes isoelectric focusing is **a)** pulsed field gel electrophoresis **b)** SDS gel electrophoresis **c)** agarose gel electrophoresis **d)** polyacrylamide gel electrophoresis **e)** 2D gel electrophoresis

3. Appearance of a turbidity or white precipitate in sulphosalicilic acid test in the sample solution evidencing the presence of **a)** carbohydrate **b)** lipid **c)** DNA **d)** protein **e)** RNA
4. Characteristic features of proteins with four-leveled structure: **a)** self-assembly ability **b)** they usually contain even number of subunits (2, 4, 6) **c)** high specificity of interaction between subunits **d)** they usually possess new biological properties that are not manifested by their precursors with tertiary structure
5. Gel pore size in polyacrylamide gel electrophoresis or Agarose gel electrophoresis is controlled by modulating the **a)** current strength **b)** concentrations of acrylamide and bis-acrylamide or agarose powder used in creating a gel **c)** buffer ratio **d)** voltage
6. Immuno-gel-electrophoresis is a method in which **a)** antibodies to several diseases agents can be detected in one gel **b)** several diseases agents can be detected in one gel **c)** the antibodies and antigens interaction take place due to their diffusion into gel after the antigens electrophoresis **d)** the proteins-antigens are electrophoretically separated and then detected by the mix of specific antibodies into the gel
7. In sodium dodecyl sulfate gel electrophoresis separation is based on **a)** molecular weight **b)** charge **c)** shape **d)** size
8. Ninghydrin detects the fingerprints due to its reaction with skin **a)** RNA **b)** DNA **c)** lipids **d)** amino acids, peptides, amines **e)** carbohydrates

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Practical 20. Hemoglobin electrophoresis.

Theoretical background. Electrophoresis is the movement of the charged molecules in an applied electric field. Molecular migration in an electric field is influenced by the size, shape, charge and chemical composition of the molecule and also by pores size in hydrogel.

Identification of abnormal hemoglobins in human blood sample can be performed by electrophoresis technique. The congenital disorder of hemoglobins (hemoglobinopathies) that originate from a defect in the globin chain, are identified by this method [1, 2, 3].

Hemoglobin, obtained from hemolyzed red blood cells, is placed on a supporting medium (buffer-saturated gel type matrix) and allowed to migrate towards the anode at different rates in an electrophoretic apparatus, which enables the hemoglobin identification.

Samples. The hemoglobin content in the hemolysate should be 10.0 g%. If it is more than 10.0 g%, add a few drops of distilled water to get the hemoglobin content of 10 g%. Use venous blood as specimen. 2 mL of venous blood in a tube containing 3.6 mg of K2 EDTA vacutainer.

Note. *Handle all samples and reagents with care and avoid contact with eye, mouth and skin. Handle all samples as potentially infectious. Discard used reagents and sample as per disposal procedure.*

Reagents. EDTA vacutainer, cathode buffer (pH 8.6), anode buffer (pH 9.1), carbon tetrachloride (CCl_4), normal saline (NS), 1% agarose, 3% acetic add, amidoblack, Drabkin's solution (potassium cyanide and potassium ferricyanide).

Equipment

Electrophoretic apparatus, centrifuge, pipettes, tubes.

Procedure.

Preparation of the hemolysate

1. Collect 2 mL venous blood in K2 EDTA vacutainer.
2. Transfer 1 mL of blood into the test tube -12 x 100 mm and add 4 mL of NS, mix gently and centrifuge the sample.
3. Discard the supernatant and repeat the washing of RBCs with saline thrice.
4. Then remove all the saline and add 2 to 3 drops of distilled water in the deposit.
5. Close the tube with a rubber stopper and shake it vigorously for 10 min. This will lyse the RBCs.
6. Then add 1 mL of carbon tetrachloride and shake the tube again for another 10 minutes.
7. After this centrifuge the tube for 30 minutes. On centrifugation 3 layers are separated.
8. The top layer, the hemolysate, the middle layer that is the stroma and the bottom layer is of carbon tetrachloride.
9. Pipette out the hemolysate using a pasteur pipette and check its hemoglobin content by cyanmethemoglobin method. To do this, add 20 μL of hemolysate to 5 mL of Drabkin's solution in the test tube and mix. Allow to stand for 4-5 min to develop

color. Within the next 8-10 min measure the intensity of the color at 540 nm in a hemoglobinometer.
10. If the hemoglobin content is < 10.0 g % repeat the procedure using another sample.

Electrophoresis

1. Prepare hemolysate like the above mentioned procedure
2. Prepare 1% agarose. Take four clean microscopic slides and pour 2 mL of that onto the slide, making sure to avoid any air bubbles. Allow it to solidify without drying.
3. Write the sample identification mark on the back of the slide with a marker pencil.
4. The adjusted hemolysate is applied at the cathode end of the slide using a cover slip.
5. Place the slide inside the tank, cut Whatman No: 1 filter paper into 25 x 25 mm and use it to bridge the slide and buffer.
6. The current (2 mA/sample and a voltage of 250 V) is passed for all four slides from cathode to anode.
7. Run the electrophoresis for 4 hours.
8. Switch off the gel electrophoresis apparatus. Take the slides out, fix them in methanol for 30 min.
9. Then dry the slides by incubating at 37°C for 12 - 18 hours
10. Stain the dried slides in 1% amido black for 8 min and then destain in the mixture of 90% methanol containing 10% acetic acid until the background is clear.
11. Destain again with pure methanol for 5 min air. Dry the slides and label them.
12. Interpret results in comparison of hemoglobins electrophoretic migration with those of normal hemoglobin A found in healthy adults [2].

Answer the MSQs

1. Proteins in GE may be visualized by **a)** labeled antibodies **b)** silver stain **c)** Coomassie Brilliant Blue **d)** autoradiography if protein is labeled with radioisotope
2. SDS GE **a)** denaturates protein and imparts net "-" charge **b)** imparts to proteins net "+" charge **c)** imparts to proteins net "-" charge **d)** decharges proteins
3. The most common gel electrophoresis (GE) for total cell proteins separation is **a)** PAGE **b)** agarose GE **c)** SDS GE **d)** pulsed field GE **e)** 2D GE
4. Sicle cell anemia carrier is indicated in GE by **a)** fast and slow band **b)** slow band **c)** fast band **d)** all of these

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