MINISTRY OF HEALTH CARE OF UKRAINE Kharkiv National Medical University D.P. Grynyov department of microbiology, virology and immunology

STANDARD PROTOCOLS

TO LABORATORY CLASSES IN MICROBIOLOGY, VIROLOGY AND IMMUNOLOGY

for the II year English media students of medical and dentistry faculty

PART 1

student _____ year _____group

Surname_____

_____

Teacher_____

_____

Kharkiv 2018

Standard protocols to laboratory classes in microbiology, virology and immunology for the II year English media students of medical and dentistry faculty (Part 1) / M.M. Mishyna, Yu.A. Mozgova, N.I. Kovalenko, T.M. Zamaziy, O.O. Vovk. – Kharkiv: KNMU, 2018. – 104 p.

Surname _____ Date _____

Morphology and physiology of microorganisms.

Protocol № 1

Theme: Regulations during working and techniques of safety working in the bacteriological laboratory. Immersion microscope. Cocci. Simple methods of staining.

Safety Rules:
Safety in the microbiology lab is essential. We will be using potential pathogens and precautions will be strictly followed.

1. GOOD LAB PRACTICES:

- Table tops must be disinfected before and after each lab.
- Hands should be washed before and after every experiment.
- Dress appropriately, protective eyewear, gloves and labcoats are important barrier precautions.

- Contaminated material must be disposed of properly in the biohazard waste area! Plates are disposed of in the biohazard can. Never pour a broth culture down the sink, place the used tube in the disposal rack. Everything must be autoclaved (sterilized) before it is ultimately discarded.
- Incinerators and Bunsen burners will be used to sterilize microbiological tools and tube tops. Many of the stains we use contain flammable substances; orient flammable material away from the burners. If Bunsen burners are used long hair must be pulled back in a ponytail.

- Food and drink are **NEVER , No, NOT EVER,** allowed in the lab.
- Microscopes and slides will always be returned to the properly labeled area. Use only lens paper to thoroughly clean prepared slides before putting them away.
- Read all reagents before using them and pay attention to any warning precautions on the label.
- Carefully label and date **all** cultures.
- The safest and smartest way to conduct a lab experiment is to read it before you begin and prepare a flow sheet.

2. IN THE EVENT OF AN ACCIDENT:

- Culture spills require **immediate** notification of the instructor. Paper towels soaked with disinfectant are used to prevent aerosol formation.
- If you burn yourself notify the instructor immediately.
- Body fluids must be considered as potentially hazardous. If you or your lab partner bleed for any reason notify the instructor immediately.

3. SPECIAL PRECAUTIONS:

- **A special precaution with the hood is important. Never turn on the ultraviolet light. It is damaging to skin and eyes.** The UV light is used by the instructor and technician only.
- Locate the fire extinguisher, shower, eye wash station, sinks, and disinfectant before leaving the lab the first day of class.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

_ STRUCTURE OF IMMERSION MICROSCOPE

I. Review the parts of the microscope indicated below. Write the function of each part below. Identify the structures on your assigned scope and draw a line to the correct part on the picture.

Mechanical part: arm, base, mechanical stage, mechanical stage control, condenser, coarse adjustment focus, fine adjustment focus, slide holder.

Optical part: ocular lens, oil immersion objective.

Resolving power or a microscope's resolution is defined as *the distance between two objects at the point at which they still appear to be two distinct objects. Resolution refers to the ability to view two closely spaced points as two points rather than one. The resolution of a standard light microscope is 0.2 micrometers (i.e., 0.0002mm=200nm).*

Resolution is not magnification. **Magnification** is a microscope's ability to increase size -- it does not improve clarity.

Total magnification of immersion microscope – *is the product of the magnifying power of the two individual lenses (ocular and objective).*

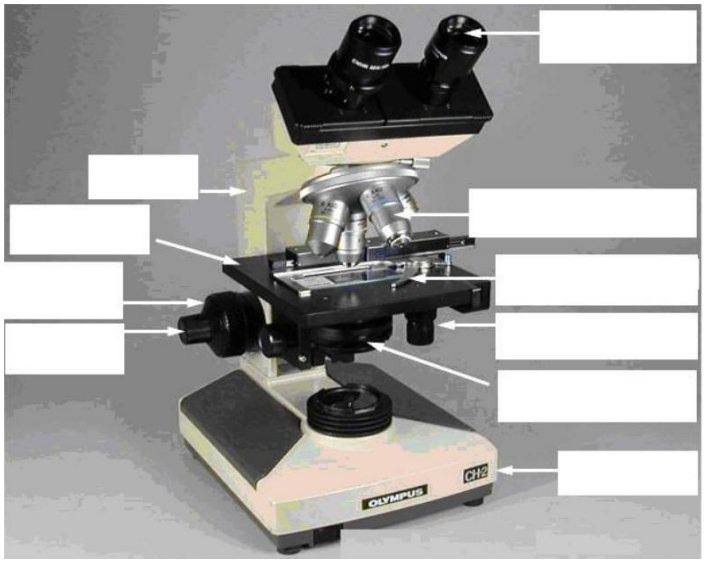


Fig. 1. Structure of immersion microscope.

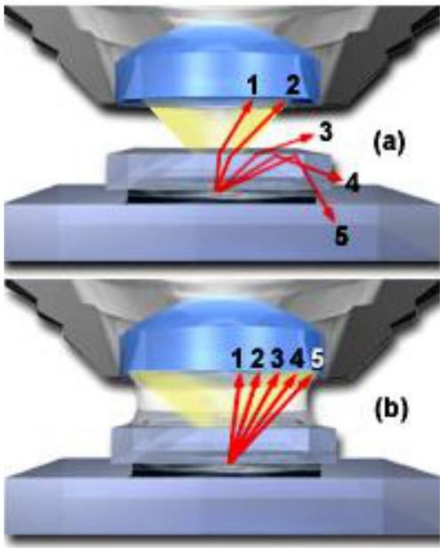
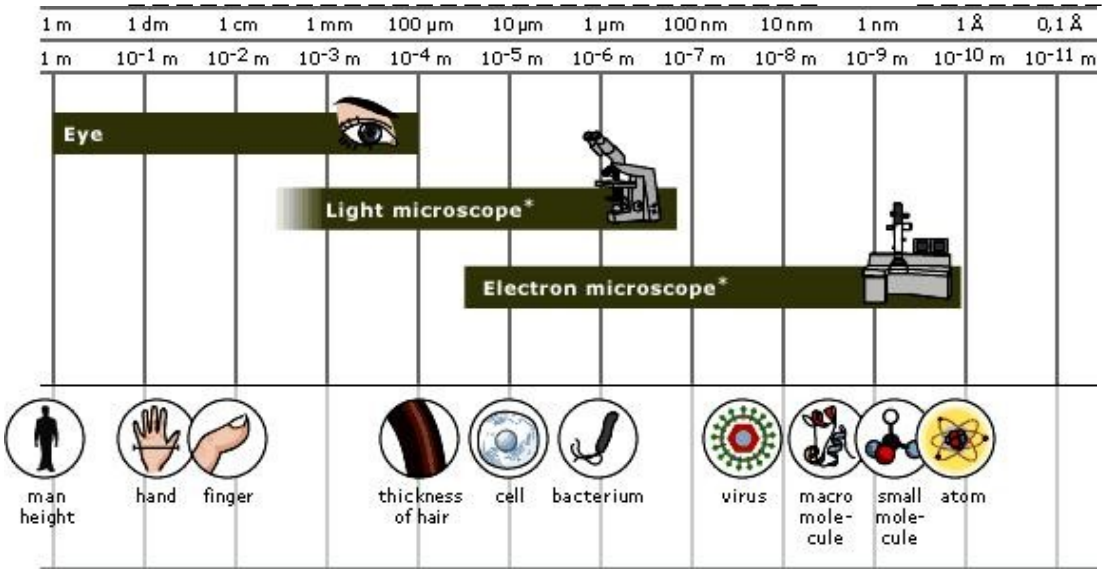


Fig. 2. Principle of dry objective (a) and oil immersion (b).

The principle of oil immersion is demonstrated in Figure 2 where individual light rays are traced through the specimen and either pass into the objective or are refracted in other directions. Figure 2(a) illustrates the case of a dry objective with five rays (labeled 1 through 5) shown passing through a sample that is covered with a coverslip. These rays are refracted at the coverslip-air interface and only the two rays closest to the optical axis (rays 1 and 2) of the microscope have the appropriate angle to enter the objective front lens. The third ray is refracted at an angle of about 30 degrees to the coverslip and does not enter the objective. The last two rays (4 and 5) are internally reflected back through the coverslip and, along with the third ray, contribute to internal reflections of light at glass surfaces that tend degrade image resolution. When air is replaced by oil of the same refractive index as glass, shown in Figure 2(b), the light rays now pass straight through the glass-oil interface without deviation due to refraction. The numerical aperture is thus increased by the refractive index of oil.

What can you see with the different types of microscopes? The human eye is capable of distinguishing objects down to a fraction of a millimeter. With the use of light and electron microscopes it is possible to see down to an angstrom and study everything from different cells and bacteria to single molecules or even atoms.

Surname _____ Date _____



MICROSCOPE USE

1. Always use the microscope on a hard stable surface.

2. Place the slide in the slide holder, center the slide using the two mechanical stage control knobs under the stage on the right-hand side of the microscope, and place a rounded drop of **immersion oil** on the area to be observed.

3. Rotate the **oil immersion objective** until it is locked into place.

4. Make sure the **iris diaphragm lever** in front under the stage is **almost wide open**, (toward the left side of the stage; and the knob under the stage on the left-hand side of the stage controlling the height of the condenser is turned so the **condenser is all the way up**.

5. Watching the slide and objective lens carefully from the front of the microscope, lower the oil immersion objective into the oil by raising the stage until the lens **just touches** the slide. Do this by turning the **coarse focus away from you** until the spring-loaded objective lens **just begins to spring upward**.

6. While looking through the eyepieces, turn the **fine focus towards you at a slow steady speed** until the specimen comes into focus. (If the specimen does not come into focus within a few complete turns of the fine focus control and the lens is starting to come out of the oil, you missed the specimen when it went through focus. Simply reverse direction and start turning the fine focus away from you.)

7. Using the **iris diaphragm lever, adjust the light** to obtain optimum contrast.

8. When finished, **wipe the oil off of the oil immersion objective** with lens paper, unplug the power cord, and wrap the cord around the base of the microscope.

II. Observe the smears below. Describe cell shape and arrangement of the bacteria. Using appropriately colored pencils draw the following cells.

Staphylococcus aureus	**Streptococcus pyogenes**	**Streptococcus pneumoniae**	**Erythrocytes and cocci**

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

III. You will produce a smear of the pure cultures (*S. epidermidis*).

There are 3 steps in preparing a smear for staining. Remember to use aseptic technique and flame the loop before and after each use.

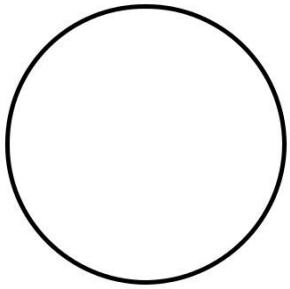
1. Preparation of the slide - Clean and dry the slide thoroughly to remove oils.

2. Preparation of the smear – When making a smear from solid media cultures, start by putting a very small drop of water in the center of the slide and then mix a loopful of bacteria from the surface of solid media in the water, spreading it out to the size of a nickel. **Burn the remaining bacteria off of the loop.** Allow the smear to air dry.

3. Fixation - The point of fixation is to attach the organisms and cells to the slide without disrupting them. Pass the slide (film-side up) through the flame of the bunsen burner 3 or 4 times to heat-fix.

Simple Method of Staining the Smear

1. Place the smear on the staining rack over the sink.
2. Cover the smear with the methylene blue or carbol fuchsin stains and leave it for 1 minute.
3. Pick up the slide by one end and hold it at an angle over the staining tray. Using the wash bottle on the bench top, gently **wash off the excess stains** from the slide.
4. Allow the smear to air dry or blote with blotting paper.



Staphylococcus epidermidis (methylene blue stain)

IV. Write the examples of pathogenic cocci:

a. Division in **one plane** produces either a **diplococcus** or **streptococcus** arrangement:

diplococcus (pair of cocci):_____;

streptococcus (chain of cocci): _____.

b. Division in **two planes** produces a **tetrad** arrangement (square of 4 cocc).

c. Division in **three planes** produces a **sarcina** arrangement (cube of 8 cocci).

d. Division in **random planes** produces a **staphylococcus** arrangement (grape-like clusters): _____.

ADDING THEORETICAL MATERIAL

Medical microbiology (Gr. *micros* – small, *bios* – life, *logos* – science) – is the study of causative agents of infectious diseases of humans and their reactions to such infections. In other words, it deals with etiology, pathogenesis, laboratory diagnosis, treatment, epidemiology and control of infection.

Bacteria are unicellular free-living organisms without chlorophyll having both DNA and RNA. Their biological properties and predominant reproduction by binary fission (a process by which one bacterium splits into two) relates them to prokaryotes.

On the basis of shape, bacteria are classified as sphericaly shaped (cocci), rod-shaped (bacteria, bacilli, and clostridia), spiral-shaped (vibrios, spirilla, spirochetes) and filamentous forms.

Cocci (Gr. *kokkos* – berry). These forms of bacteria are: spherical, oval, elongated, flattened on one side, lanceolate.

Cocci are approximately **0.5 micrometer (µm)** in diameter and may be seen in one of the following:

a) **diplococcus** arrangement is a result of division in one plane. It is a pair of cocci (*Streptococcus pneumoniae*, *Neisseria gonorrhoeae, Neisseria meningitis).*

b) **streptococcus** arrangement also is a result of division in one plane, but it is a chain of cocci (*Streptococcus pyogenes*).

c) **tetrad** arrangement is a result of division in two planes. It is a square or group of 4 cocci (*Tetracocci*).

d) **sarcina** arrangement is a result of division in three planes. It is a cube or packet of 8, 16, 32, etc. cocci.

e) **staphylococcus** arrangement is a result of division in random planes. It is cocci in irregular, often grape-like clusters (*Staphylococcus aureus*).

Bacterial morphology (form and structure) may be examined in two ways:

1 – by observing living unstained organisms, or

2 – by observing killed stained organisms.

Since bacteria are almost colorless and therefore show little contrast with the broth in which they are suspended, they are difficult to observe when unstained.

Staining microorganisms enables one to:

- see greater contrast between the organism and the background,
- differentiate various morphological types (by shape, arrangement, gram reaction, etc.),
- observe certain structures (flagella, capsules, endospores, etc.).

Before staining bacteria, it is necessary to **"fix" the organisms to the glass slide**. If the preparation is not fixed, the organisms will be washed off from slide during staining.

The **procedure for heat fixation** is as follows:

1. If the culture is taken from an **agar medium**:

a. Using the dropper bottle of distilled water found in your staining rack, place **1/2 a drop of water** on a clean slide by touching the dropper to the slide.

b. Aseptically remove a **small amount** of the culture from the agar surface and **just touch it several times** to the drop of water until it just turns cloudy.

c. **Burn the remaining bacteria off of the loop.** (If too much culture is added to the water, you will not see stained individual bacteria.)

d. Using the loop **spread the suspension over the entire slide** to form a thin film.

e. Allow this thin suspension to **completely air dry**.

f. Pass the slide (film-side up) through the flame of the bunsen burner 3 or 4 times to heat-fix. *Caution: Too much heat might distort the organism and, in the case of the gram stain, may cause gram-positive organisms to stain gram-negatively. The slide should feel very warm but not too hot to hold.*

2. If the organism is taken from a **broth culture:**

a. Aseptically place 2 or 3 loops of the culture on a clean slide. **Do not use water**.

b. Using the loop **spread the suspension over the entire slide** to form a thin film.

c. Allow this thin suspension to **completely air dry**.

d. Pass the slide (film-side up) through the flame of the bunsen burner 3 or 4 times to heat-fix.

After a procedure of fixation staining of smear canbe provided. Methods of staining are divided into **simple** (using of only one dye – fuchsin or methylene blue) and **complex** (differential, special).

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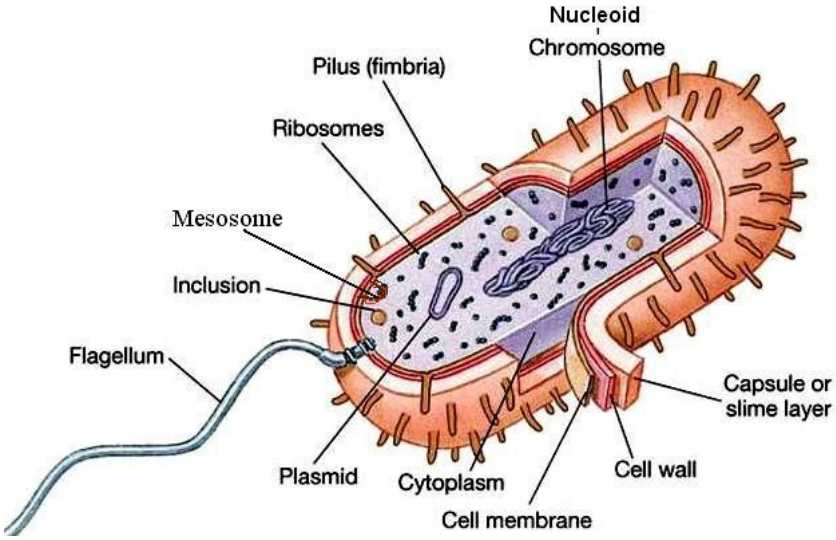
Protocol № 2

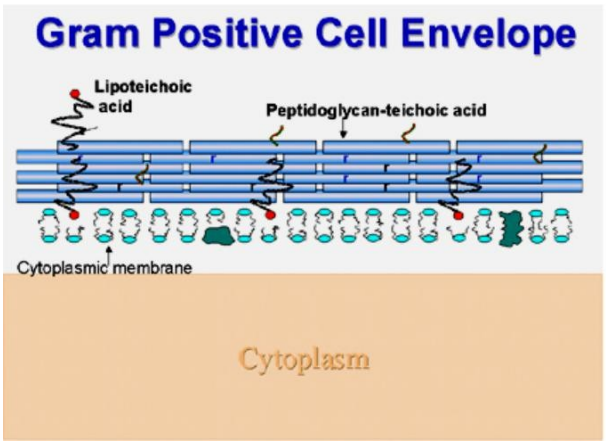
Theme: **Rod-shaped bacteria. Bacterial anatomy. Gram's staining.**

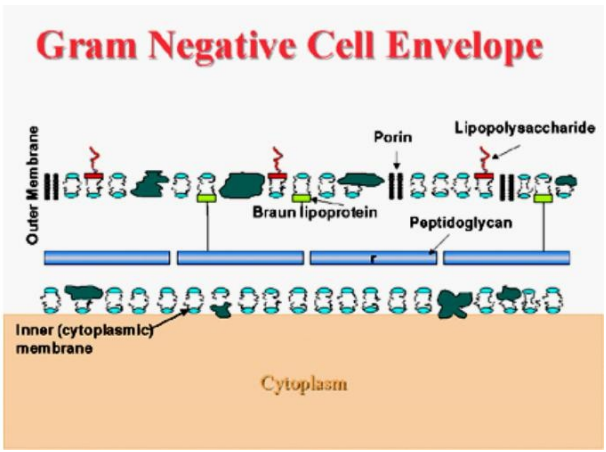


The bacterial cell structure

Comparison of eukaryotic and prokaryotic cell

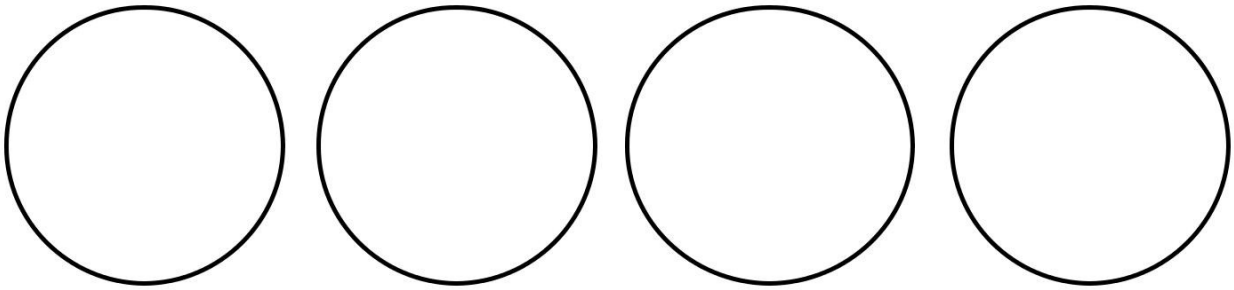
Organells	Eukaryotic cell	Prokaryotic cell
Nucleus		
Nuclear membrane		
Nucleoid		
Chromosome		
DNA		
Plasmid		
Mitochondria		
Mesosome		
Ribosomes		
Lysosomes		
Golgi apparatus		
Endoplasmic reticulum		
Cell wall		





S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

I. Observe the smears below. Describe cell shape and arrangement of the bacteria. Using appropriately colored pencils draw the following cells.



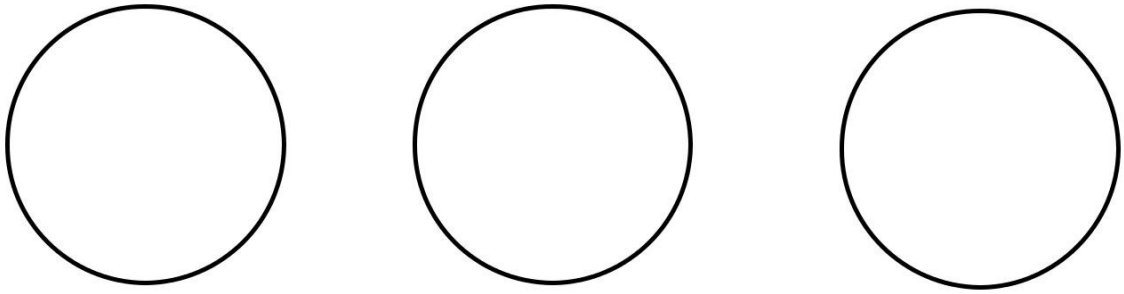
Escherichia coli
small rods

Bacillus
anthracoides
large rods

Mixture of
bacteria

Erythrocytes
and
rods

II. You will produce a smear of pure culture (*E. coli or Bacillus anthracoides)* and the mixture of bacteria (*S. epidermidis*, *E. coli and B. anthracoides)*. Using appropriately colored pencils draw the following cells.



Escherihia coli
(Gram stain)

Staphylococcus epidermidis
(Gram stain)

Mixture of bacteria
(Gram stain)

Gram Staining of the Smear

1. Place the smear on the staining rack over the sink.
2. Cover the smear area with the crystal violet (gentian violet) stain and leave it for 1 minute and then shake off the excess stain.
3. Apply Gram's iodine covering the smear completely for 1 minute and then shake off the excess stain.
4. Using the Gram's decolorizer (ethyl ancochol), apply it a drop at a time to the smear area until no more color leaves the area (for 30 seconds). Quickly rinse with water to stop the decolorizing process.
5. Apply fuchsin to the smear for 1 minute, then wash the slide with water.
6. Blot dry and observe using oil immersion microscopy.

III. Describe cell shape and arrangement of the bacteria. Write the examples of pathogenic rod-shaped bacteria:
a single bacillus: _____,
streptobacillus: bacilli in chains _____,
coccobacillus: oval and similar to a coccus _____,
arranged at angles to each other (like Chinese or Latin letters):_____

_ ADDING THEORETICAL MATERIAL

Rod-shaped forms. Rod-shaped forms are subdivided into bacteria, bacilli and clostridia.

Bacteria are hotdog-shaped bacteria including those microorganisms, which, as rule, do not produce spores (*E. coli, Salmonella, Shigella*).

Bacilli (*B. anthracis*) and clostridia (*C. tetani, C. botulinum*) include microbs the majority of which produce spores.

All rod-shaped bacteria exhibit differences in form and can be subdivided into three groups: a single bacillus; streptobacillus (bacilli in chains); diplobacillus (bacilli in pairs). Some other bacilli are arranged at angles to each other, presenting Latin or Chinese letters (*Corynebacterium diphtheriae*).

Size of rods varies from 2 to 10 μm: small rods are 2-4 μm and long rods 5-10 μm.

Bacterial anatomy.

Cell wall. In addition to conferring rigidity upon bacteria, the cell wall protects against osmotic damage. It is porous and permeable to substances of low molecular weight.

Chemically, the rigid part of the cell wall is peptidoglycan: this is a mucopeptide composed of strands of alternating N-acetylglucosamine and N-acetylmuramic acid residues. Structural rigidity is conferred by interstrand peptide cross-links between N-acetylmuramic acid molecules.

Structure of the cell wall differs in Gram-positive and Gram-negative bacteria. **Gram-negative cell wall:** This differs from that of Gram-positive bacteria by the presence of an outer membrane, which contains lipopolysaccharide (LPS). It also contains specific proteins (outer membrane proteins), which include pore-forming proteins through which hydrophilic molecules are transported. Other proteins are receptor sites for phages and bacteriocins. The lipid is embedded in the outer membrane, whereas the polysaccharide which is anchored to the lipid projects from the cell surface. The periplasm or periplasmic space separates the peptidoglycan layer from the cytoplasmic membrane.

Gram-positive cell wall: The peptidoglycan layer of the cell wall of Gram-positive bacteria is much thicker than in Gram-negative bacteria. There is no periplasm and the peptidoglycan is closely associated with the cytoplasmic membrane.

Teichoic or teichuronic acids are part of the cell wall of Gram-positive bacteria: the maintain the level of divalent cations outside the cytoplasmic membraine.

Antigens: the cell wall may contain antigens, such as the polysaccharide (Lancefield) and protein (Griffith) antigens of streptococci.

Bacteria with deficient cell walls: Bacteria can survive with deficient cell walls: these can be induced in the laboratory by growth in the presence of some antibiotics and a hyperosmotic environment to prevent lysis.

Bacteria without cell walls are of four types:

1. **Mycoplasma:** a genus of naturally occurring bacteria which lack cell walls; stable and do not require hypertonic conditions for maintenance.
2. **L-forms**: cell-wall-deficient forms of bacteria, usually produced in the laboratory but sometimes spontaneously formed in the body of patients treated with penicillin; more stable then protoplasts or spheroplasts; they can replicate on ordinary media.
3. **Spheroplasts:** derived from Gram-negative bacteria; retain some residual but non-functional cell-wall material; osmotically fragile; produced artificially by lysozyme or by growth with penicillin or any other agent capable of breaking down the peptidoglycan layer: must be maintained in hypertonic medium.
4. **Protoplasts:** derived from Gram-positive bacteria and totally lacking cell walls; unstable and osmotically fragile; produced artificially by lysozyme and hypertonic medium; require hypertonic conditions for maintenance.

Cytoplasmic membrane. The cytoplasmic membrane is a trilaminar structure formed of proteins buried in a phospholipid bilayer. It acts as a semipermeable membrane though which there is uptake of nutrients by passive diffusion. It is also the site of numerous enzymes involved

in the active transport of nutrients and in various other cell metabolic processes. Chemically, bacterial cytoplasmic membranes lack the sterols usually found in their eukaryotic cell equivalents.

Mesosomes. These are convoluted invaginations of cytoplasmic membrane, often at sites of septum formation, and are involved in DNA segregation during cell division. They are the site of respiratory enzyme activity, and may perform a function similar to that of mitochondria in eukaryotic cells.

Nuclear material. The single circular chromosome, which is the bacterial genome or DNA undergoes semiconservative replication bidirectionally from a fixed point – the origin. Chromosomal DNA is condensed into about 50 supercoiled domains associated with an RNA core. DNA-binding proteins (histon-like) regulate supercoiling and influence expression.

Ribosomes are distributed throughout the cytoplasm and are the sites of protein synthesis. Composed of RNA and proteins; organized in two sub-units: 30s and 50s.

Cytoplasmic inclusions. Sources of stored energy, e.g. polymetaphosphate (volutine), poly-hydroxybutirate (lipid), polysaccharide (starch or glycogen).

Gram's staining.

First described by Gram in 1884. It is' used to study morphologic appearance of bacteria. Gram's stain differentiates all bacteria into two distinct groups:

a. Gram-positive organisms.

b. Gram-negative organisms.

Principle: Some organisms are not decolorized and retain color of basic stain, e.g. gentian violet (Gram-positive organisms) while the others lose all gentian violet when treated with decolorizing agent and take up the counter stain, e.g. dilute carbol fuchsin or sufranin (Gram-negative organisms).

Mechanism: There are two major theories to explain the reaction.

I. ***Chemical Theory:*** In Gram-positive organisms the iodine combines with chemical substance in cell (or cell wall) and helps in holding firmly the gentian violet. The substance is thought to be magnesium ribonucleate or unsaturated fatty acid. Gentian violet iodine complex is attached to the protoplast of Gram stained bacterium. The integrity of cell wall is a must for a positive stain.

II. ***Physical Theory:*** Gentian violet and iodine enters inside the cell and combines to form large molecule. In Gram-positive bacteria cell acts as barrier so that iodine and gentian violet complex is retained in the cell even in the presence of acetone (or alcohol). This complex is soluble in acetone and or alcohol.

Procedure: Bacterial suspension is spread in the form of thin films on the surface of clean glass slide and allowed to dry in air. It is called smear. Smear is fixed by passing the slide over the flame two or three times. Now proceed as follows:

1. The bacteria are first stained with the basic dye **gentian violet**. Both gram-positive and gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with **gram's iodine solution**. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both gram-positive and gram-negative bacteria remain purple after this step.

3. **Gram's decolorizer**, a mixture of **ethyl alcohol and acetone**, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative are decolorized.

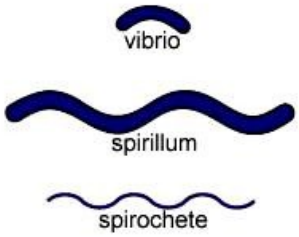
4. Finally, the counterstain **fuchsin** is applied.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

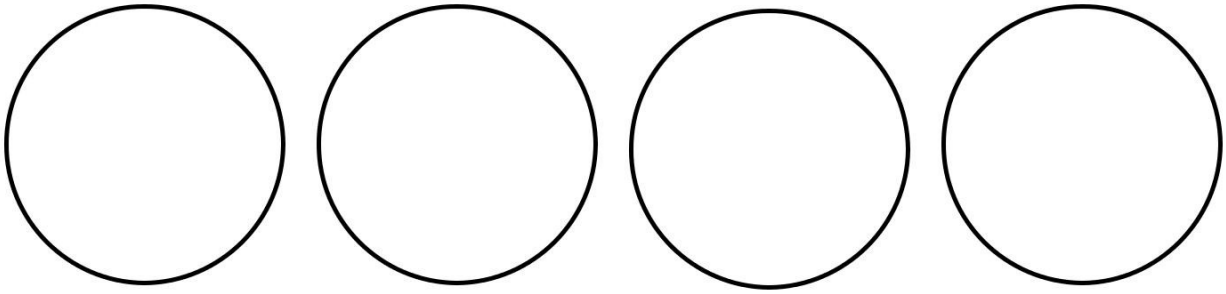
Protocol № 3

Theme: Vibrios. Spirochetes. Bacterial flagella. Studying of motility.

Spiral-shaped bacteria occur in one of three forms:

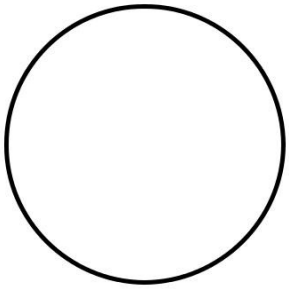


I. Observe the smears below. Describe cell shape and arrangement of the bacteria. Using appropriately colored pencils draw the following cells.



Vibrio cholera (Gram stain)	**Treponema pallidum** (Burry stain)	**Leptospira** (Morosov stain)	**Borrelia recurrentis** (Giemsa stain)

II. You will produce a smear of the pure cultures (*Vibrio).* Using appropriately colored pencils draw the following cells.



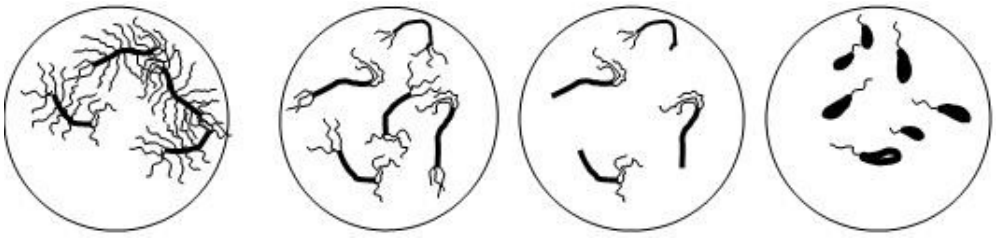
Gram stain

III. Describe cell shape of the bacteria. Write the examples of pathogenic spiral bacteria.

Vibrio cholera: an incomplete spiral or _____ shaped. Disease:_____.
Spirillum: a thick, rigid spiral _____. _____.
Helycobacter pylori. Disease: _____.
Spirochete: a thin, flexible spiral:
Treponema pallidum (_____). Disease: _____
Borrelia rucurrentis (_____).Disease:__ _____
Borrelia persica, Borrelia caucasica, Borrelia duttoni _____
Borrelia burgdorferi
(_____).Disease:__ _____
Leptospira interrogans (_____). Disease:
_____

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

IV. The arrangement of the flagella about the bacterium. Write the examples of pathogenic species:



1 2 3 4

Examples:

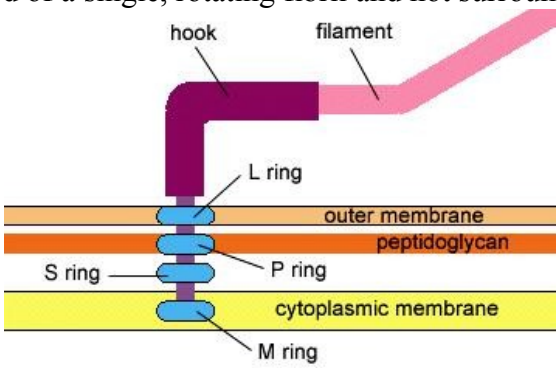
1. **peritrichous** - completely surrounded by flagella: _____

2. **amphitrichous** - single flagella at both poles: _____

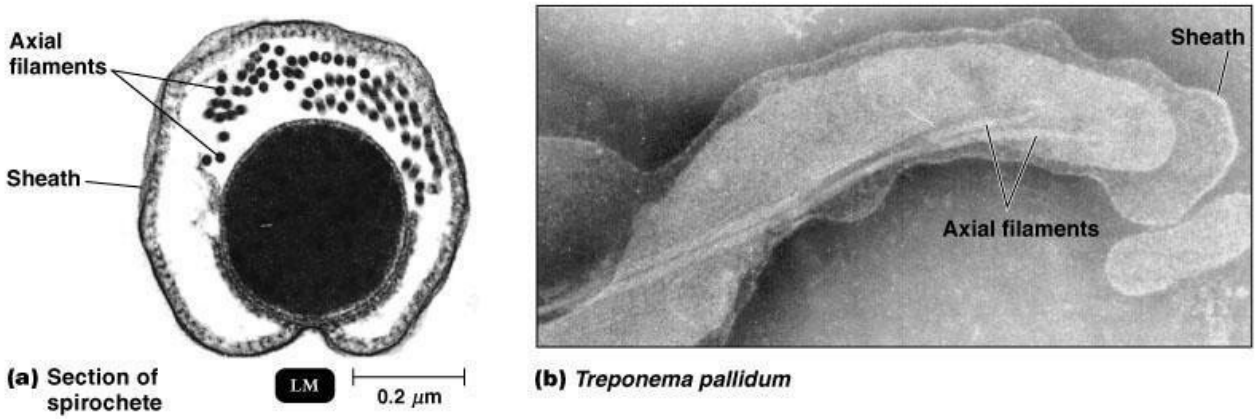
3. **lophotrichous** - two or more flagella at one pole of the cell: _____

4. **monotrichous** - a single flagellum at one pole: _____

Each flagellum is composed of a single, rotating fibril and not surrounded by a membrane:



Cross-section of spirochetal cell shows endoflagella – axial filaments inside of cytoplasm of cell.



ADDING THEORETICAL MATERIAL

The spirals range from 5-40 μm long but some are over 100 μm in length.

Spiral-shaped bacteria occur in one of three forms:

1) *vibriones:* an incomplete spiral or comma-shaped cells (curved rods), typical representative of this group is *V. cholerae*;

2) *spirilla* – are coiled forms of bacteria exhibiting twists with one or more turns. Only one pathogenic species is known – *Spirillum minus* – which is responsible for a disease in humans transmitted though the bite of rats – ratbite fever – sodoku;

3) *spirochetes* – are flexible spiral forms, which are the thinnest of the bacteria, often having a width of only 0.25-0.5 μm.

Pathogenic to humans spirochetes belongs to following genera:

Treponema – thin, flexible cells with 6-14 **regular twists**. The size of Treponema varies from 0.05-0.2 μm in width and 10-18 μm in length. A typical representative is the causative agent of syphilis *T. pallidum*.

Leptospira – are characterized by very thin cell structure. The leptospirae form 12-18 coils wound close to each other, shaping small primary spirals with **hooked ends** resembling "C" or "S" letters. *L. interrogans* is a causative agent of leptospirosis.

Borrelia – have **large irregular spirals**, the number of which varies from 3 to 10. Pathogenic for man are the causative agents of epidemic and endemic relapsing fever (*B. recurrntis, B. duttonii, etc.*).

Flagella

Many bacteria are able to move under their own power. Most motile bacteria have special organelles termed flagella. The bacterial flagellum is a semi-rigid, helical tube composed of protein (including flagellin) and anchors to the bacterial cytoplasmic membrane and cell wall by means of disk-like structures. The rotation of the inner disk causes the flagellum to act much like a propeller.

Each flagellum consists of hook and basal body. It originates in a spherical body (basal granule) located just inside cell wall.

Bacterial motility constitutes unicellular behavior. In other words, motile bacteria are capable of a behavior called taxis. Taxis is a motile response to an environmental stimulus and functions to keep bacteria in an optimum environment. Some bacterial species are able to taste their environment and respond to specific chemical foodstuffs or toxic materials and move towards or away from them (chemotaxis).

One group of bacteria, the spirochetes, has internally-located axial filaments or endoflagella. Axial filaments wrap around the spirochete towards the middle from both ends. They are located above the peptidoglycan cell wall but underneath the outer membrane and have a similar function to flagella.

The number and arrangement of flagella are characteristics of each bacterium. Flagella may be arranged on bacterial body in following manner:

1. Monotrichous – a single flagellum at one end of the microorganism (vibrio).
2. Amphitrichous – single flagella at both poles (*Alcaligenes feacalis*).
3. Lophotrichous – two or more flagella at the pole of the cell (*Pseudomonas*)
4. Peritrichous – flagella distributed over the entire cell (*Proteus vulgaris*).

Pilli or **fimbriae.** The types of pili (or whether they are produced at all) vary both among and between species. Some are involved in sexual conjugation and others allow adhesion to host cells. Pili are hair-like projections of the cell, pili are short, thin and straight. They are composed of protein known as pillin. There are three forms of fimbriae: 1) Common pili; 2) F [fertility] pili; 3) Col I (colicin) pili.

Motility may be observed:

a) by using special-purpose microscopes (phase-contrast microscopy, when special optics convert slight variations in specimen thickness into corresponding visible variation in brightness, and the bacterium and its structures appear darker than the background; and dark-field microscopy, that uses a special condenser to direct light away from the objective lens, the organism will appear bright against the dark background);

b) by detecting the spreading growth in semisolid agar medium;

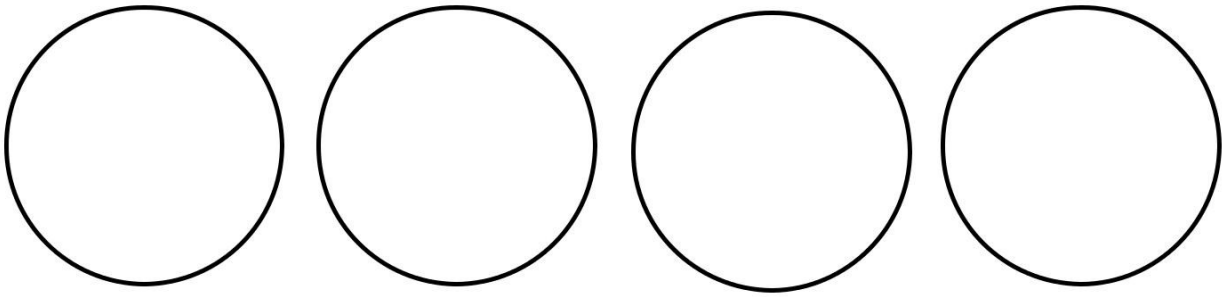
c) by flagella staining.

Surname_____Date_____

Protocol № 4

Theme: Spores. Sporulation. Spore staining.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



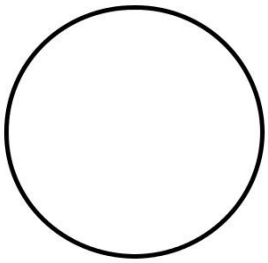
Clostridium botulinum (Gram stain)

Clostridium tetani (Gram stain)

Bacillus anthracis (Gram stain)

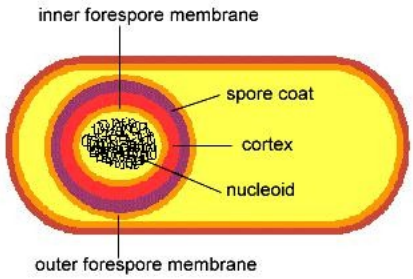
Bacillus anthracis (Gansen stain)

II. You will produce a smear of the pure cultures of Bacillus anthracoides. Using appropriately colored pencils draw the following cells.

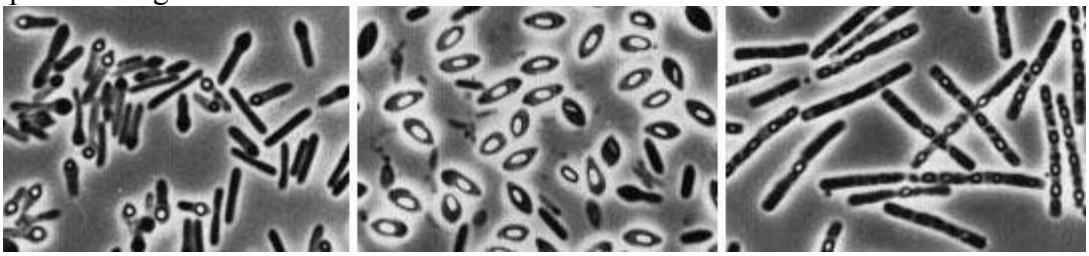


Gansen stain

III. Study the structure of bacterial spore.



IV. Study shape and arrangement of spores. Write the examples of pathogenic sporeforming bacteria.



1. Spherical central: _____

2. Oval subterminal bulging:_____

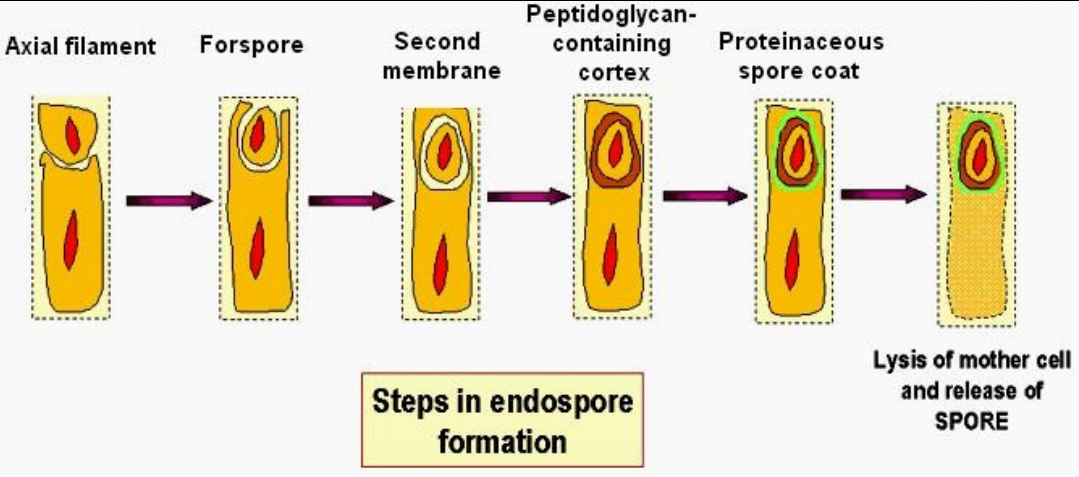
S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

3. Terminal spherical bulging: _____

Y. Describe stages of sporulation.

1._____

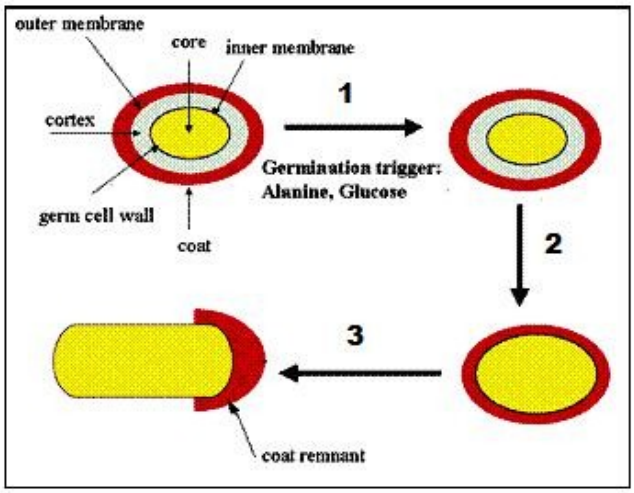
2. _____
3. _____
4. _____
5. _____
6. _____



YI. Describe stages of spore germination.

1. _____**:** Cation release; Ca^{2+}-dipicolinic acid release; partial core hydration; some lose of resistance.
2. _____**:** cortex hydrolysis; futher core hydrolysis; start to metabolic activity; core expansion; futher loss of resistance.
3. _____**:** increase of metabolic activity; RNA synthesis; protein synthesis; escape from the spore coat.

Events during spore germination



Gansen (Ozheshko) stain

1. Place a piece of blotting paper over heat-fix a smear of *Bacillus anthracoides* and saturate with **carbol fuchsin**.

2. Holding the slide with forceps, carefully heat the slide in the flame of a bunsen burner until the stain **just begins to steam**. Remove the slide from the heat until steaming stops; then gently reheat. Continue steaming the smear in this manner for **five minutes**. **As the carbol fuchsin evaporates, continually add more. Do not let the paper dry out.**

3. After five minutes of steaming, **wash** the excess stain and blotting paper off the slide **with water**. Don't forget to wash of any dye that got onto the **bottom** of the slide.

4. Cover the slide with 0,5 % H_2SO_4 .

5. Wash the slide with water.

6. Now flood the smear with **methylene blue** and stain for **one minute**.

7. Wash off the excess methylene blue with water.

8. Blot the slide dry and observe using oil immersion microscopy.

With this endospore staining procedure, endospores will stain **red** while vegetative bacteria will stain **blue.**

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

ADDING THEORETICAL MATERIAL

Spores are specialized cell structures that may allow survival in extreme environments. They are resting cells, highly resistant to desiccation, heat, and chemical agents. Only a temperature of 121 °C with a steam under pressure (autoclaving) for 150 minutes is utilized to kill spores.

The two most common gram-positive rods that form spores are:

- the obligatory aerobic genus Bacillus. Bacilli produce spores in diameter equal or smaller then the diameter of cell;
- the obligatory anaerobic genus Clostridium. Clostridia spores in diameter are bigger then cell diameter.

When nutritional conditions become unfavorable the sporulation begins. Different morphologic and chemical events occur at sequential stages of the process. Six different stages have been identified. Morphologically, sporulation begins with the formation of an axial filament (the I stage, when DNA is aligned into a long filament). The process continues with the II stage – forespore septum formation (DNA splits into individual chromosomes and cytoplasmic membrane begins to invaginate), then begins the third stage – when the cortex has formed; the fourth stage – formation of spore coats; the fifth – maturation of spore, and the last stage – when the mother cell lyses and free endospores is released.

Properties of endospores:

1. The core is the spore protoplasm. It contains a complete nucleoid, all of the components of the protein-synthesizing apparatus, and an energy-generating system based on glycolysis.
2. The spore wall is the innermost layer surrounding the inner spore membrane. It contains normal peptidoglycan and becomes the cell wall of the germinating vegetative cell.
3. The cortex is the thickest layer of the spore envelope. It contains an unusual type of peptidoglycan which is extremely sensitive to lysozyme, and an autolysis of which plays a role in spore germination.
4. The coat is composed of a keratin-like protein containing many intramolecular disulfide bonds. The impermeability of this layer confers on spores their relative resistance to antibacterial chemical agents.
5. The exosporium is a lipoprotein membrane containing some carbohydrate.

Arrangement of spores in the vegetative cell may be central, subterminal and terminal.

The germination is the process of conversion of spore into a vegetative cell under favorable nutritional conditions, and occurs in three stages: activation, initiation, and outgrowth.

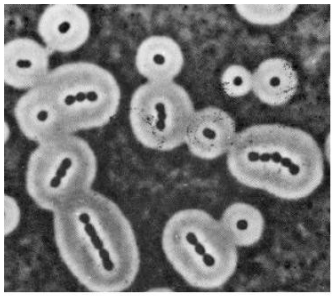
1. Activation – most endospores cannot germinate immediately after they have formed. But they can germinate after they have rested for several days or are first activated, in a nutritionally rich medium, by one or another agent that damages the spore coat. Among the agents may be heat, abrasion, acidity, and compounds containing free sulfhydryl groups.
2. Initiation – once activated, a spore will initiate germination if the environmental conditions are favorable. Initiation is triggered by L-alanine or adenosine (it depends on species). Then a variety of spore constituents are degraded by hydrolytic enzymes.
3. Outgrowth – it is a period of active biosynthesis which terminates in cell division. Outgrowth requires a supply of all nutrients essential for cell growth.

Surname _____ Date _____

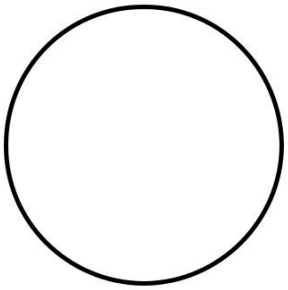
Protocol № 5

Theme: Bacterial capcules. Methods of detection of capsules.

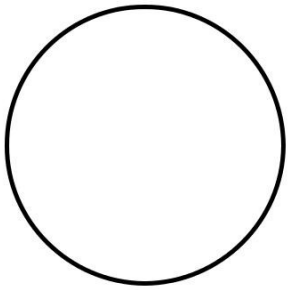
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Capsulles (SEM)

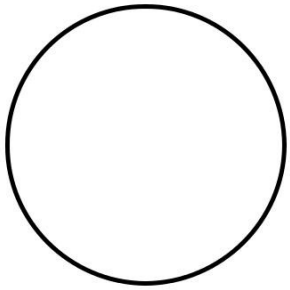


Klebsiella pneumoniae (Burry-Gins stain)



Capsule in a smear-imprint (Methylene blue stain)

II. You will produce a smear of the pure cultures of Klebsiella pneumoniae. Using appropriately colored pencils draw the following cells.



Burry-Gins stain

III. Study the examples of pathogenic capsule-producing bacteria.

1. _____
2. _____.
3. _____
4. _____.
5. _____
6. _____.
7. _____
8. _____.

Burry-Gins stain.

1. Emulsify small amount of culture in a drop of Indian ink on a slide.
2. Using another slide, spread it out over the entire slide to form a thin film.
3. Let it completely air dry and fix by passing in over flame three times.
4. Stain with dilute carbol fuchsin for 1 minute.
5. Wash the smear with water.
6. Blot dry and observe using oil immersion microscopy.

The organism will pick up the red dye, background – black while the capsule will remain colorless.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

ADDING THEORETICAL MATERIAL

Most bacteria have two structures, the cell wall and the cytoplasmic membrane, that surround the cell's cytoplasm, which has the consistency of jelly. Some bacteria have a third layer, the glycocalyx. Collectively, these structures are referred to as the cell's envelope.

If glycocalyx appears as an extensive, tightly bound accumulation of gelatinous material adhering to the cell wall, it is called a capsule. If glycocalyx appears unorganized and more loosely attached, it is referred to as a slime layer. The slime layer is a loose structure that easily washed off. The capsule is a thick, structured layer that is not readily removed.

Bacterial colonies with capsules often appear moist, glistening, and slimy. Capsules are produced only by certain bacteria and often only when they are grown with nutrients that can be converted into these capsules.

Capsules vary in their chemical composition depending on the species of bacteria. Most are composed of polysaccharides, which are given the general name exopolysaccharide (exo means "outside"), but some consists of polypeptides made up of repeating subunits of only one or two amino acids. Of special interest is the fact that these animo acids are generally of the D-, or unnatural, form.

Nature of Capsular Substances Formed by Various Bacteria

Genus and Species	Capsular Substances
Gram-positive bacteria	
S. pneumoniae	Polysaccharides: type 111, glucose, glucoronic acid; other types, various sugars and amino sugars
Streptococcus spp.	Polysaccharides: hyaluronic acid (group A); others containing amino sugars, uronic acids
B. anthracis	γ–Glutamyl polypeptide
Gram-negative bacteria	
H. influenzae	Polyribosephosphate
Klebsiella spp.	Polysaccharides: sugars such as hexoses, fucose, uronic acids
N. meningitidis	Polysaccharides: N-acetylmannosamine phosphate polymer (group A); sialic acid polymers (group B and C)

Capsules have several functions. Encapsulated bacterial cell generally have greater pathogenicity because capsules protect the bacteria against white blood cells called phagocytes. The phagocytes of the body, which engulf and then killed the bacteria, have a great difficulty in surrounding the encapsulated organisms, whereas the unencapsulated organisms are quickly engulfed and destroyed.

Capsules also serve to attach bacteria to a wide variety of surfaces, including human teeth, plant roots, the small intestine, rocks, and even to other bacteria.

In soil and water, capsules help prevent bacteria from being engulfed by protozoans. The glycocalyx plays a role in the adherence of bacteria to surfaces in their environment, including the cells of plant and animal hosts.

Capsular material is antigenic and may be demonstrated by serological methods. When a suspension of encapsulated bacterium is mixed with its specific anticapsular serum and examined under the microscope, the capsule becomes very prominent and appears "swollen" due to an increase in its refractivity.

Surname_____Date_____

Protocol № 6

Theme: Morphology of rickettsia, chlamydia and mycoplasma.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.

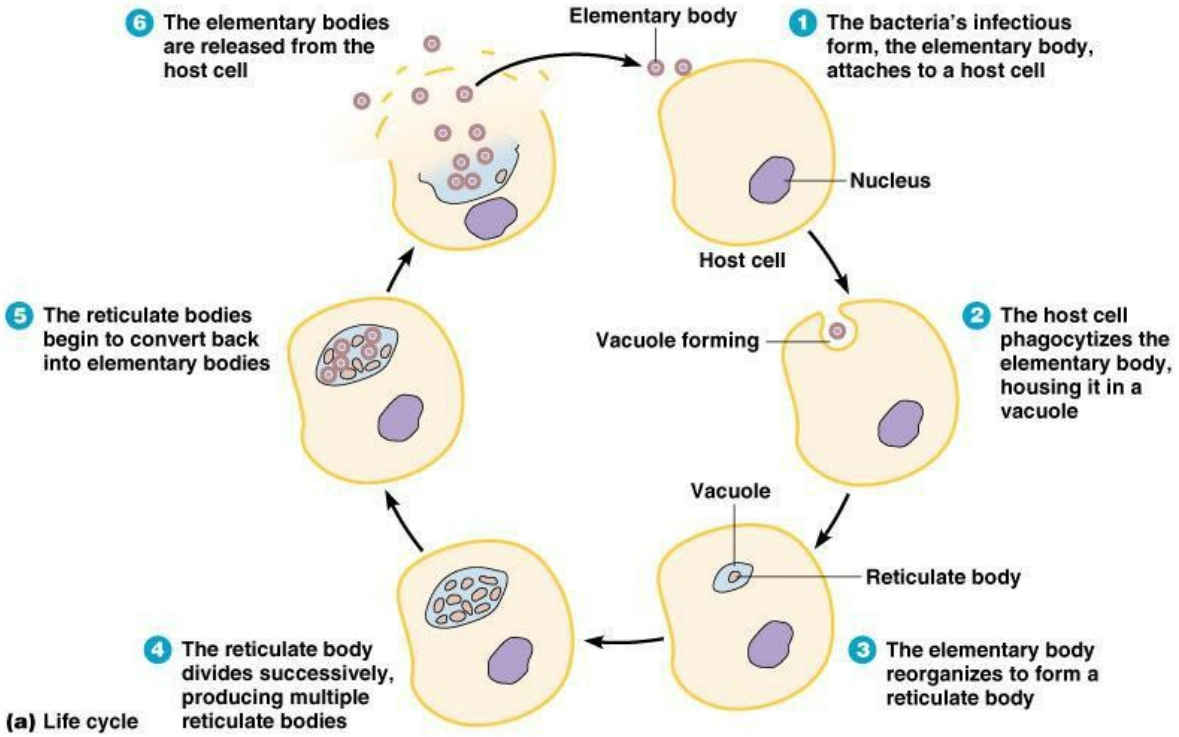
Rickettsia prowazeckii (Zdrodovsky stain)

Rickettsia prowazeckii (Giemsa stain)

Mycoplasma (Giemsa stain)

Chlamydia trachomatis (Giemsa stain)

II. Study the infectious life cycle of Chlamydia.



S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _
III. Examples of pathogenic bacteria.

Chlamydia

1. _____
2. _____.
3. _____

Methods of staining of Chlamydia:

I. _____

II. _____

Rickettsia

1. _____
2. _____.
3. _____
4. _____.
5. _____
6. _____.

Methods of staining of rickettsia:

I. _____

II. _____

III. _____

Mycoplasma

1. _____
2. _____.
3. _____
4. _____.
5. _____
6. _____.

Methods of staining of mycoplasma:

I. _____

II. _____

III. _____

ADDING THEORETICAL MATERIAL

Rickettsia are small microorganisms that are obligate intracellular parasites transmitted to humans by arthropods. Rickettsial diseases typically exhibit fever, rashes, and vasculitis. They are grouped on the basis of clinical features, epidemiologic aspects, and immunologic characteristics.

Properties of Rickettsiae: they are pleomorphic, appearing either as short rods, or as cocci, and they occur singly, in pairs, in short chains, or in filaments. When stained, they are readily visible under the light microscope. With Giemsa's stain, they appear blue; with Macchiavello's stain they appear red, in contrast with blue-staining cytoplasm around them. Rickettsiae contain both RNA and DNA, have cell walls that are made up of peptidoglycans, contain various enzymes. Rickettsiae grow in different parts of the cell (cytoplasm, cytoplasmic vacuoles, and nucleus). Rickettsiae are quickly destroyed by heat, drying, and bacterial chemicals. But remember that rickettsial growth is enhanced in the presence of sulfonamides, and rickettsial diseases are made more severe by these drugs.

Chlamydia general properties:

- obligate intracellular parasites
- cannot synthesize ATP; rely on cellular ATP for many of their metabolic reactions
- will not grow in artificial media
- possess ribosomes and synthesize their own proteins
- cell wall contains LPS but does not have a peptidoglycan layer
- all three species share a common LPS antigen
- are not affected by β-lactam antibiotics
- have a common reproductive cycle that takes 24-48 hours

The **life cycle** of Chlamydia is fairly complex:

- the elementary body is the infectious, extracellular form of the organism. It is the most differential form responsible for both spreading of the infection within a host and host-to-host transmission.

- the reticulate (initial) body is the replicative, intracellular form that evolves from the elementary body 5-6 hours after penetration of the target cell. Once differentiated, the reticulate body begins to divide by binary fission and continues to divide for 18-24 hours

- the intermediate body results from the condensation of the reticulate bodies and looks like a bull's eye when the infected cell is viewed under microscope.

All Chlamydia are highly infectious, but species differ in their degree of virulence. *C. trachomatis* causes ocular, genital and respiratory infections. *C. psittaci* causes psittacosis [also known as ornithosis (from ornithos, birds) or parrot fever] in humans.

Mycoplasmas have extremely small size. They are pleomorphic; several different forms exist, varying from small spherical shapes to longer branching filaments. They are Gram-negative, but stain poorly with Gram's stain. They can grow on complex but cell-free media (colonies may have a "fried egg" appearance on agar).

Mycoplasmas are bacteria, which lack cell walls. They are bounded by the cytoplasmic membrane and resemble L-forms of bacteria but, unlike them, are independent naturally-occurring microorganisms.

Mycoplasma and related organisms

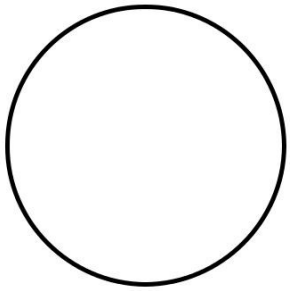
Genus, species	Habitat
Mycoplasma pneumonia	Human respiratory tract
Mycoplasma orale Mycoplasma salivarium	Human mouth
Mycoplasma hominis Mycoplasma genitalium	Human genital, and possibly respiratory, tracts
Ureaplasma urealyticum	Human genitourinary tract

Surname_____Date_____

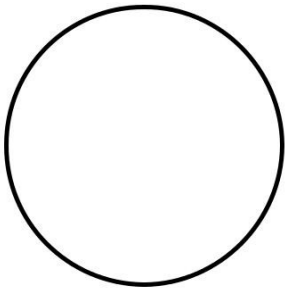
Protocol № 7

Theme: Morphology of pathogenic protozoa.

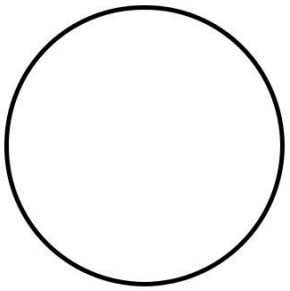
I. Observe the smears below. Using appropriately colored pencils draw the following cells.



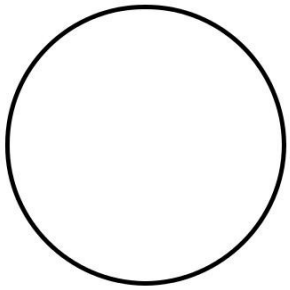
Leishmania in cell culture (Giemsa stain)



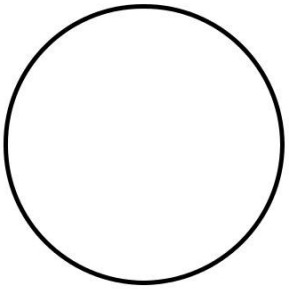
Leishmania in cell culture (Giemsa stain)



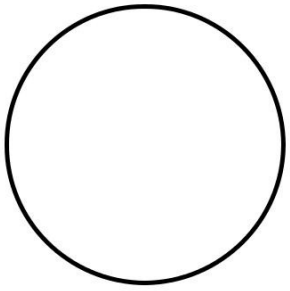
Toxoplasma (Giemsa stain)



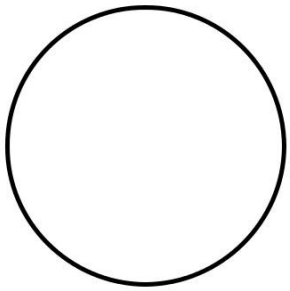
Trichomonas vaginalis (Methylene blue stain)



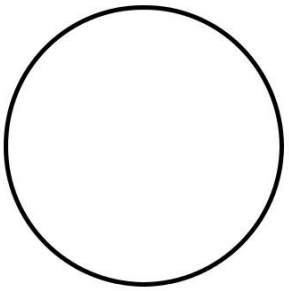
Giardia lamblia (Giemsa stain)



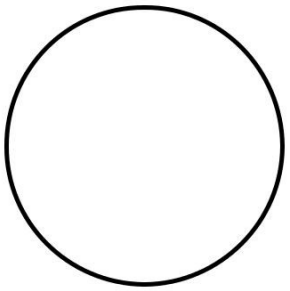
Entamoeba hystolytica (Ferriferous hematoxilyne stain)



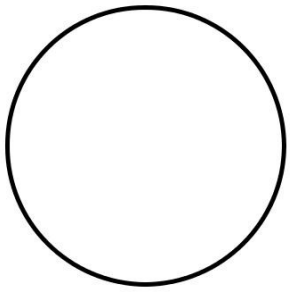
Trypanosoma cruzi (Giemsa stain)



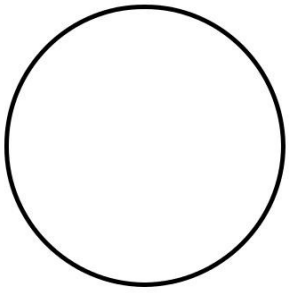
Balantidium coli (Ferriferous hematoxilyne stain)



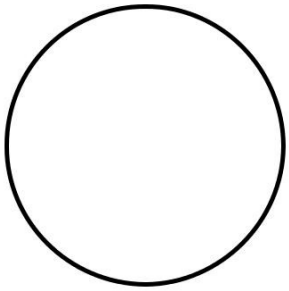
lasmodium malariae schisont (Giemsa stain)



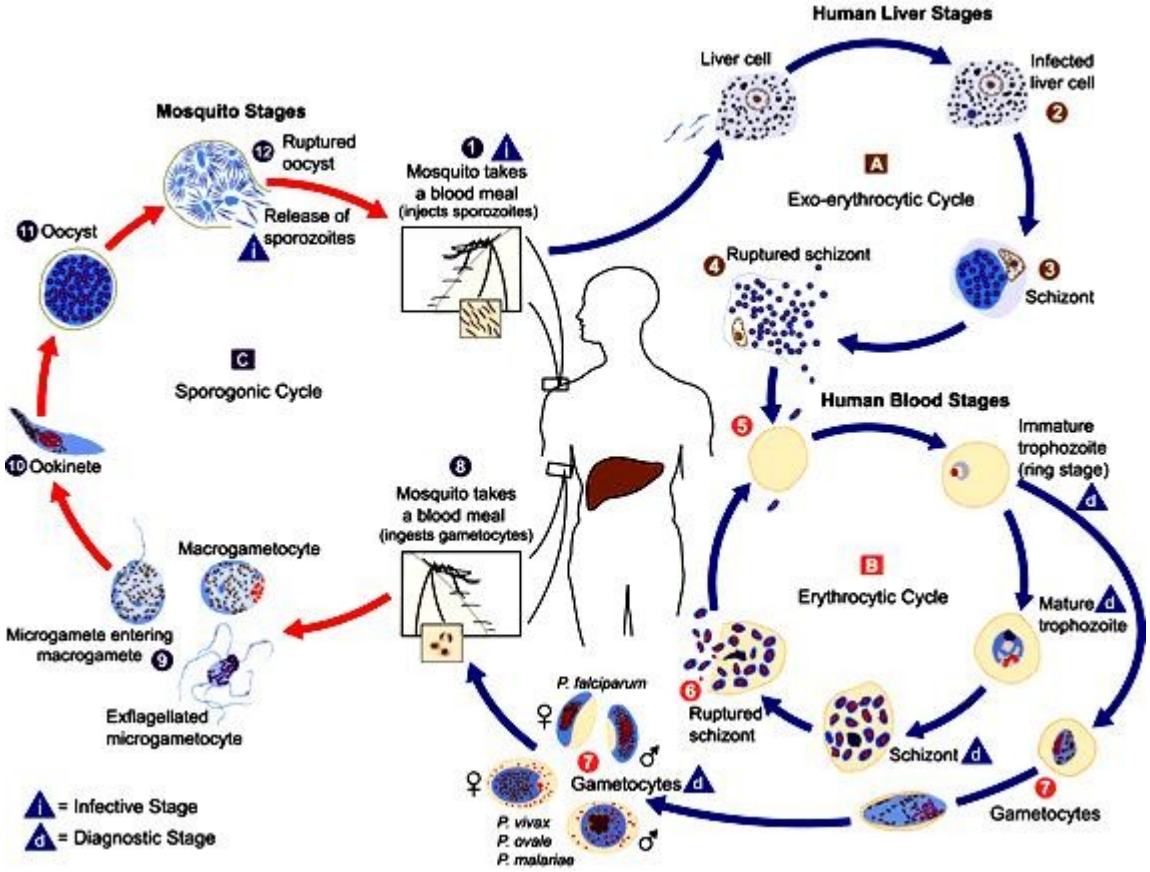
Plasmodium falciparum ring stage (Giemsa stain)



Plasmodium vivax schisont (Giemsa stain)



Plasmodium vivax gametocytes (Giemsa stain)



The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼.Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

ADDING THEORETICAL MATERIAL

The Protozoa are considered to be a subkingdom of the kingdom Protista, although in the classical system they were placed in the kingdom Animalia.

Protozoa are one-celled animals found worldwide in the most habitats. Most species are free living, but all higher animals are infected with one or more species of protozoa.

Protozoa are microscopic unicellular eukaryotes that have a relatively complex internal structure and carry out complex metabolic activities. Some protozoa have structures for propulsion or other types of movement.

Entamoeba histolytica is a common parasite in the large intestine of humans, the causative agent of amebiasis. Prevalent geographic distribution – tropical and subtropical regions.

Morphology. Three stages are encountered: the active ameba, the inactive cyst and the intermediate precyst. The amoeboid trophozoite is the only form present in tissues. It is also found in fluid feces during amebic dysentery. Cysts are present only in the lumen of the colon and in mushy or formed feces. ***Trophozoites*** – the cytoplasm is glassy and contains only red blood cells and spherical vacuoles; nucleus is spherical in shape and placed eccentrically; movement results from long finger-like pseudopodia extension of ectoplasm into which the endoplasm flows; trophozoites multiply by binary fission. ***Pre-cystic stage*** – The parasite is colorless, round or oval, smaller than trophozoite but larger than cyst, endoplasm is free from RBC, etc. with sluggish pseudopodia activity; nucleus remain intact. ***Cysts*** –Cyst begins as a uninucleated body but divides by binary fission and develops into binucleated (immature cyst) and quadrinucleated (mature cyst) structures.

Lifecycle: Cysts of Entamoeba are formed in bowel of human and are passed with stools. Cysts are swallowed with contaminated food and drinks by man. They pass through stomach and reach intestine. Cyst wall is weakened because of alkaline pH and cytoplasmic mass containing 4 nuclei, (metacyst) comes out. The nuclei divide by binary fission giving 8 daughter trophozoites. Trophozoites which are actively motile move towards ileocaecal region.

Giardia lamblia, a flagellate, is the only common protozoan found in the duodenum and jejunum of humans. It is the cause of giardiasis. This parasite occurs all over the world.

Morphology. Giardia lamblia is found in the following two forms: ***Trophozoites*** – are heart-shaped, bilateral symmetric organisms with the same sizes: 10 to 20 μm in length and 6-15 μm in width and 1 to 3 μm in thickness; a large concave sucking disk in the ventral portion occupies much of the ventral surface; the dorsal surface area is meant for diffusion of nutrients; the anterior end is rounded and posterior end is pointed; there are two axostyles, two nuclei with prominent central karyosomes and 4 pairs of flagella; it multiplies by binary fission. ***Cysts*** – Trophozoites are transformed into cysts under unfavorable conditions; the cyst is thick-walled, highly resistant, 8 to 14 μm in length, ellipsoid, contains 2 nuclei as immature and 4 nuclei as mature cysts.

Lifecycle: Cyst is the infective form. Cysts are ingested through water and edibles. Acidic pH of stomach initiates excystation which is completed in duodenum thus releasing trophozoites (2 trophozoites from each cyst). The trophozoites establish themselves in the bowel wall and start multiplying by binary fission. It can also localize itself in biliary tract.

Balantidium coli, the cause of balantidiasis or balantidial dysentery, is largest intestinal protozoan parasite of humans. Also found in pigs and monkeys. Geographical distribution is worldwide.

Morphology. ***Trophozoites*** are ciliated, oval organisms with pointed anterior end and have cytostome; its posterior end is round; cytoplasm contains kidney-shaped large macronucleus and small micronucleus. ***Cysts*** – are oval with thick outer wall, cilia are absent.

Lifecycle: No intermediate host is required. The cysts are passed in stool. Infection occurs by ingestion of cyst with contaminated food or drinks. In the wall of intestine excystation occurs and developed trophozoites live and subsequently multiply by binary fission on the mucosa of large intestine.

Trichomonas vaginalis is a flagellate protozoan and causes vaginal trichomoniasis in humans (In female it is found mainly in vagina and in male it is in urethra.). Geographical distribution is worldwide that means in all social groups and in all climates.

Morphology. It is found only in trophozoitic form which bears following characteristics: it is pear-shaped, with short undulating membrane which comes up to the middle of the body, 4 anterior flagella (the organism moves with a characteristic wobbling and rotating motion), a prominent axostyle which bifurcates the body into two parts; it multiplies by binary fission in longitudinal axis.

Trypanosoma brucei is a hemoflagellate, causes African trypanosomiasis (sleeping sickness) and have two variants: T. brucei gambiense (is transmitted by the streamside tsetse fly *Glossina palpalis* and extends from west to central Africa; produces a relatively chronic infection) and T. brucei rhodesiense (is transmitted by the woodland-savanna tsetse fly *Glossina*

palidipes, Glossina morsitans and occurs in east and southeast savannahs of Africa; produces a smaller number of cases but is more virulent).

Morphology. It occurs in trypomastigote form in the vertebrate host as spindle-shaped elongated organism with pointed anterior and blunted posterior ends. The nucleus is central in position and the kinetoplast is situated at the posterior end. The flagellum starts just adjacent to the kinetoplast, curves around the body to form an undulating membrane and continues beyond the anterior end as free flagellum. The trypomastigotes of *T. brucei* are highly polymorphic, varying in shape and size in different stages. The size may vary from 10 μm x 3 μm to 20 μm x 3 μm. Many antigenic variants of the organism occur.

Lifecycle: Bush-buck and other antelopes may serve as reservoirs of *T. brucei rhodesiense*, whereas humans are the principal reservoir of *T. brucei gambiense*. A multiplying epimastigote stage precedes the formation of infective trypomastigotes in the intermediate hosts (several species of Tsetse fly). The infective metacyclic form (trypomastigotes) of the organism is introduced into man by the bite of the insect vector (tsetse fly). After multiplication at the site of inoculation, the parasite attacks the blood of the definite host. The trypomastigote forms are picked up by the tsetse fly during its blood meal from the infective definitive host to continue cycle.

Trypanosoma cruzi is a hemoflagellate, causes American trypanosomiasis (Chagas' disease), is transmitted by the triatomine bugs and extends from South to Central America. It is a parasite of the muscular and nervous tissues and also of reticulo-endothelial system.

Morphology. It is same as *Trypanosoma brucei* except that it is C or U shaped in stained films. It does not multiply in peripheral blood. Amastigotes are seen in cells of striated muscle (heart and skeletal muscle), neurocytes and reticulo-endothelial cells as round or oval bodies with nucleus and kinetoplast. Multiplication occurs only at this stage.

Lifecycle: It is similar to that of *T. brucei* except: 1) it is transmitted by the triatomine bug; 2) passes to humans when infected bug feces are rubbed into the conjunctiva, the bite site, or a break in the skin; and 3) does not multiply in peripheral blood.

The genus ***Leishmania,*** widely distributed in nature, has a number of species that are nearly identical morphologically. Differentiation is based on a number of biochemical and epidemiologic criteria. *Leishmania donovani* causes visceral leishmaniasis (Kala-azar, black disease, dumdum fever); *Leishmania tropica* (*L. t. major, L. t. minor* and *L. ethiopica*) cause cutaneous leishmaniasis (oriental sore, Delhi ulcer, Alepo, Delhi or Bagdad boil); *L. braziliensis* (also, *L. mexicana* and *L. peruviana*) are etiologic agents of monocutaneous leishmaniasis (espundia, Uta, Chiclero ulcer); *Leishmania infantum* causes child Kala-azar. The New World forms are all carried by sandflies of the genera *Lutzomyia* and *Psychodopygus*. Old World leishmaniasis is transmitted by sandflies of the genera *Phlebotomus*. Leishmania donovani complex is distributed in South East Asia and South America. L. braziliensis – Central and South America.

The natural habitat of *Leishmania donovani* in humans is reticuloendothelial system especially spleen, liver, bone marrow, intestinal mucosa. It may be found in endothelial cells of kidneys, suprarenal capsules, lungs, meninges, cerebrospinal fluid and also in the macrophages of intestinal wall.

Morphology. There are two forms: amastigote form (also called nonflagellated form or leishmanial form) and promastigote form (also called flagellated form or leptomonad form).
Amastigotes are found in the cells of reticuloendothelial system of vertebrate hosts. They are rounded or ovoid with very delicate cell membrane, large nucleus, kinetoplast, parabasal body, blepharoplast, flagellum is absent.
Promastigotes are found in sandflies and in cultures. They are spindle-shaped cells with centrally placed nucleus, kinetoplast, parabasal body and blepharoplast, flagellum projects from the front and may be of the same length as the body of parasite. Undulating membrane is absent.

Lifecycle: Leishnamia is transmitted by the bite of sandfly, which carry the promastigote in the anterior gut and pharynx. The parasites gain access to mononuclear phagocytes where they transform into amastigotes and multiply until the infected cell ruptures. The released parasites

infect other cells and the process is repeated, producing a cutaneous lesion or visceral infection depending upon the species of parasite and the host response.

Toxoplasma gondii is a protozoan of worldwide distribution that infects a wide range of animals and birds but does not appear to cause disease in them. In humans it produces either congenital or postnatal toxoplasmosis. The parasite habitats endothelial cells, leucocytes, body fluid and tissue cells.

Morphology. It occurs in following three forms: ***Trophozoites*** – boat-shaped or crescentric-shaped, thin-walled cells without flagella, cilia and pseudopodia; stain lightly with Giemsa's technique; nucleus lies near blunt end; multiplies by endogeny which means internal budding. Rapid proliferation of trophozoites in acute infection (invade mammalian cells) is called tachyzoites. Trophozoites may be found extracellular too. ***Tissue Cysts*** – contain thousands of bradyzoites; act as source of infection and thus are responsible for transmission of infection; they are formed during the chronic stage of infection and may involve any organ of the body predominantly skeletal muscles, heart muscles and brain; exhibit asexual multiplication, i.e. schizogony. ***Oocysts*** – are spherical or oval-shaped, contain sporoblast. Freshly passed oocysts are not infective; they attain infectiveness only after development in soil or water. Infective form contains 8 sporozoites. Oocysts are formed as a result of sexual reproduction, i.e. gametogony or sporogony and only in the definitive host, i.e. cat and members of feline family.

Lifecycle: is completed as under: 1) Enteric Cycle in Cat and 2) exoenteric cycle in humans.

1) Ingested oocyst releases sporozoites. These sporozoites penetrate the epithelial cells of the intestine. They become rounded and grow within host cells. Thereafter asexual division occurs resulting in the formation of merozoites. Some merozoites manage to seek entry in extra intestinal tissues and thus tissue cysts formation occurs in other organs of body. Other merozoites are changed into sexual forms and thus start sexual reproduction named gametogony. A motile microgamete fertilizes a macrogamete which results in the formation of oocysts which undergo development through various stages before it becomes infective.

2) Ingestion of oocysts contaminated improperly cooked meat results in infection. Alternatively, oocysts may be ingested from cat. Only asexual form of reproduction occurs in humans with the formation of merozoites only. Merozoites enter lymphatic and blood. They develop into tissue cysts in different organs of the body. Human to human infection is possible through placenta giving rise to congenital toxoplasmosis. It is worthwhile to point out that oocysts are not formed in the intestine of man.

The sporozoa of the genus ***Plasmodium*** are pigment-producing amoeboid intracellular parasites of vertebrates, with one habitat in red cells and another in cells of other tissues. Transmission to humans is by the bloodsucking bite of female *Anopheles* mosquitoes of various species. Four species of plasmodia typically infect humans: *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium falciparum.* They occur in all countries in the tropics and subtropics. *Plasmodium ovale* is not reported in India whereas *Plasmodium vivax, Plasmodium falciparum* and *Plasmodium malariae* exist in India.

Lifecycle: All species complete lifecycle in man and female anopheles mosquito.

Lifecycle in Man (Schizogony). Female anopheles mosquito bites man and this results in the injection of sporozoites which circulate in blood (about 1 hour) when some sporozoites attack liver cells and start pre-erythrocytic cycle. In 6-15 days' time release of thousands of merozoites occurs after completion of pre-erythrocytic cycle. Some of these merozoites are phagocytized whereas others start erythrocytic schizogony.

In *Plasmodium vivax* and *Plasmodium ovale* hepatic form known as hypnozoites persist and remain active in hepatocytes for considerable time before they grow and undergo pre-erythrocytic schizogony with liberation of merozoites in bloodstream causing lapse of the infection.

During erythrocytic schizogony, parasite assumes a form of ring and then of trophozoite.

In trophozoitic form malarial pigments start appearing. Trophozoites are transformed to schizonts with division of chromatin surrounded by pieces of cytoplasm. On attaining maturity schizont ruptures with the liberation of merozoites. They in turn invade fresh erythrocytes.
After undergoing erythrocytic schizogony some merozoites are transformed into gametocytes. Gametogony occurs in the erythrocytes of capillaries of internal organs. Mature gametocytes seek entry to peripheral blood from where they are carried to vector (mosquito) when it bites the patient.

Lifecycle in Mosquito (Sporogony): The female anopheles mosquito during blood meals from malarial patient sucks blood containing plasmodia in various forms and stages. The presence of male and female mature gametocytes should be there for continuing sexual cycle (sporogony) in the mosquito. At least 12 gametocytes per cu mm of blood and macrogametocytes must be in excess. The other forms of plasmodia, i.e. asexual forms are destroyed in the stomach of mosquito.

First of all, gametocytes become rounded and exflagellation occurs with the formation of 4 to 8 filamentous structure (microgametocyte). These filaments are detached and form microgametes. One of these microgametes penetrates the macrogamete. This follows the transformation of fertilized macrogamete into zygote. It happens from 20 minutes to 2 hours after ingestion of blood by mosquito.

Zygote becomes very active and now called ookinete which penetrates the muscle wall of stomach and comes to lie below the outer limiting membrane of stomach wall in the form of oocyst. Oocyst further increases in size, containing many sickle-shaped structures called sporozoites. Oocyst containing sporozoites is called sporocyst.

Sporocyst ruptures with the liberation of sporozoites in body cavity of mosquito. Sporozoites are disseminated in all parts of body except ovaries. Sporozoite has special affinity for salivary glands. They pass through the glands and reach the lumen of salivary duct with maximum concentration of sporozoites. The mosquito is now infective.

Surname_____ Date_____

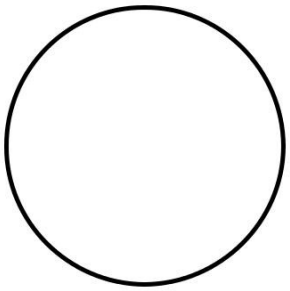
CLASSIFICATION OF PROTOZOA

TYPES	**SARCOMASTIGOPHORA**		**APICOPLEXA**	**CILIOPHORA**
SUBTYPES	***SARCODINA***	***MASTIGOPHORA***	***SPOROZOA (класс)***	-
SPECIES	1. ***Entamoeba histolitica*** (amebiasis).	***1. Leishmania tropica*** (cutaneous leishmaniasis). ***2. Leishmania donovani*** (Kala-azar). ***3. Tripanosoma brucei*** (sleeping sickness). **4**. ***Tripanosoma cruzi*** (Chagas' disease). **5. *Giardia lamblia*** (lambliosis). **6. *Trichomonas vaginalis*** (vaginitis, urethritis, prostatitis).	**1**.***Plasmodium vivax,*** ***2. Plasmodium ovale*** (bening tertian malaria). **3. *Plasmodium malariae*** (quartan malaria). **4. *Plasmodium falciparum*** (malignant tirtian malaria). **5. *Toxoplasma gondii*** (toxoplasmosis). **6. Babesia species** (babesiosis)	**1. *Balantidium coli*** (balantidiasis)

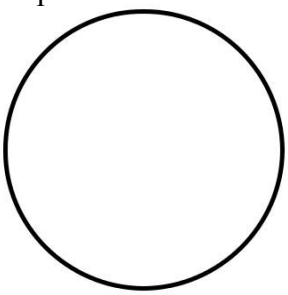
Protocol № 8

Theme: Morphology of human viruses and bacteriophages.

I. Observe the smears below. Using appropriately colored pencils draw the following cells.



Babesh bodies
(Lenz stain)



Guarniery bodies
(Giemsa stain)

II. Determine:

Viruses – _____

_____

Major viral characteristics:

1. _____
2. _____.
3. _____
4. _____.
5. _____
6. _____.

Main Families of Human Viruses

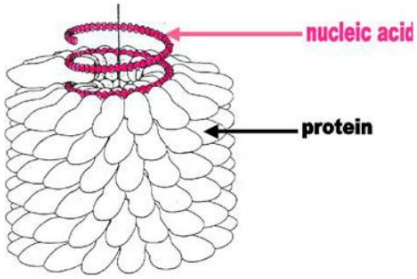
Families	Viruses	Nucleic Acid	Symmetry	Envelope
RNA Viruses				
Picornaviridae	Polyo-, hepatitis A	SS+	Icosaedral	No
Togaviridae	Rubella	SS+	Icosaedral	Yes
Flaviiviridae	Hepatitis C, G	SS+	Icosaedral	No
Coronaviridae	Coronaviruses	SS+	Helical	Yes
Retroviridae	HIV	SS+	Helical/Icosaedral**	Yes
Bunyaviridae	Congo-Crimian hemorrhagic fever	SS-	Helical	Yes
Arenaviridae	Lassa	SS-	Helical	Yes
Orthomyxoviridae	Influenza	SS-	Helical	Yes
Paramyxoviridae	Parainfluenza, measles, mumps	SS-	Helical	Yes
Rhabdoviridae	Rabiers	SS-	Helical	Yes
Reoviridae	Reoviruses	DS-	Icosaedral	No
Flaviviridae	Yellow fever	SS+	Icosaedral	Yes
DNA Viruses				
Parvoviridae	Parvoviruses	SS	Icosaedral	No
Adenoviridae	Adenoviruses	DS	Icosaedral	No
Herpesviridae	Herpes simplex, chicken pox	DS	Icosaedral	Yes
Poxviridae	Small pox	DS	Complex	Yes
Hepadnaviridae	Hepatitis B	DS (partial)	Icosaedral	Yes

DS – double-stranded; SS – single-stranded; —+ll - positive-stranded RNA; —— — -negative-stranded DNA * - Circular, single-stranded, RNA-defective genome (viroid); ** - Depending on the subfamily.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

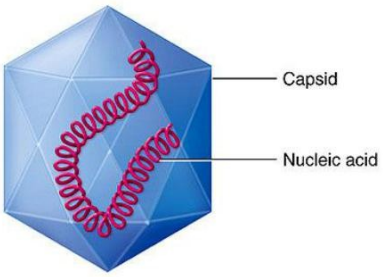
Morphology of viruses:

Helical symmetry

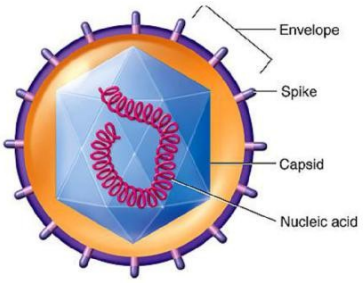


Icosahedral symmetry

Naked virus

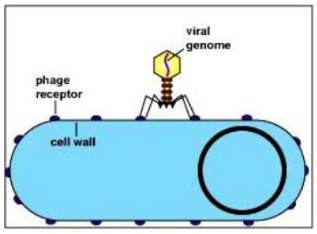


Enveloped virus

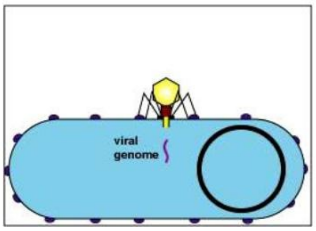


III. Lytic or virulent phages are phages, which can only multiply on bacteria and kill the cell by lysis at the end of the life cycle.

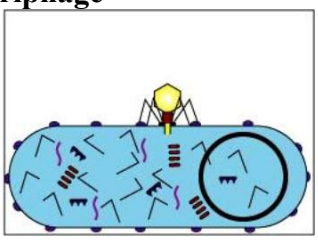
The lytic life cycle of bacteriphage



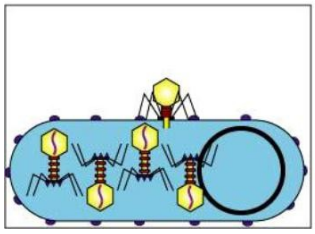
1. _____



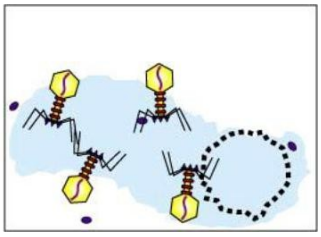
2. _____



3. _____



4. _____

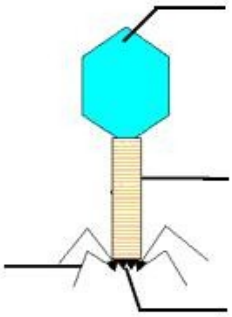


5. _____

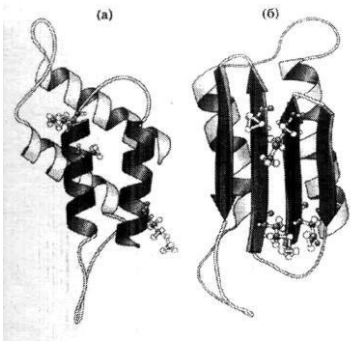
Temperate bacteriophage infects a bacterium, it can either 1) replicate by the lytic life cycle and cause lysis of the host bacterium, or it can 2) incorporate its DNA into the bacterium's DNA and assume a noninfectious state. The cell harboring a prophage is termed a **lysogen**.

Lysogenic conversion - when a cell becomes lysogenized, occasionally extra genes carried by the phage get expressed in the cell. These genes can change the properties of the bacterial cell. This process is called lysogenic or phage conversion. This can be of significance clinically. e.g. Toxin production by Corynebacterium diphtheriae is mediated by a gene carried by a phage. Only those strains that have been converted by lysogeny are pathogenic.

***Morphology of a bacteriophage*:**



Structure of prions:



a) cellular – PrPc

b) scrapie – PrPsc

A prion has been defined as **"small proteinaceous infectious particles which resist inactivation by procedures that modify nucleic acids"**. Prions contain protein only.

ADDING THEORETICAL MATERIAL

Viruses are small obligate intracellular parasites, which by definition contain either a RNA or DNA genome surrounded by a protective, virus-coded protein coat. Viruses may be viewed as mobile genetic elements, most probably of cellular origin and characterized by a long co-evolution of virus and host. For propagation viruses depend on specialized host cells supplying the complex metabolic and biosynthetic machinery of eukaryotic or prokaryotic cells. A complete virus particle is called a virion. The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be expressed (transcribed and translated) by the host cell. The viral genome, often with associated basic proteins, is packaged inside a symmetric protein capsid. The nucleic acid-associated protein, called nucleoprotein, together with the genome, forms the nucleocapsid. In enveloped viruses, the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane and studded with an outer layer of virus envelope glycoproteins.

Viral morphology provides the basis for grouping viruses into families. A virus family may consist of members that replicate only in vertebrates, only in invertebrates, only in plants, or only in bacteria. Certain families contain viruses that replicate in more than one of these hosts.

Besides physical properties, several factors pertaining to the mode of replication play a role in classification: the configuration of the nucleic acid (ss or ds, linear or circular), whether the genome consists of one molecule of nucleic acid or is segmented, and whether the strand of ss RNA is sense or antisense. Also considered in classification is the site of viral capsid assembly and, in enveloped viruses, the site of nucleocapsid envelopment. Lists the major chemical and morphologic properties of the families of viruses that cause disease in humans.

The use of Latinized names ending in -viridae for virus families and ending in -virus for viral genera has gained wide acceptance. The names of subfamilies end in -virinae. Vernacular names continue to be used to describe the viruses within a genus. In this text, Latinized endings for families and subfamilies usually are not used.

Viruses are distinct among microorganisms in their extreme dependence on the host cell. Since a virus must grow within a host cell, the virus must be viewed together with its host in any consideration of pathogenesis, epidemiology, host defenses, or therapy. The bilateral association between the virus and its host imposes specific conditions for pathogenesis. For example, rhinoviruses require a temperature not exceeding 34 °C; this requirement restricts their growth to only those cells in the cool outer layer of the nasal mucosa, thereby preventing spread to deeper cells where temperatures are higher.

The intracellular location of the virus often protects the virus against some of the host's immune mechanisms; at the same time, this location makes the virus vulnerable because of its dependence on the host cell's synthetic machinery, which may be altered by even subtle physical and chemical changes produced by the viral infection (inflammation, fever, circulatory alterations, and interferon).

Viruses are difficult targets for chemotherapy because they replicate only within host cells, mainly utilizing many of the host cell's biosynthetic processes. The similarity of host-directed and virus-directed processes makes it difficult to find antiviral agents specific enough to exert a greater effect on viral replication in infected cells than on functions in uninfected host cells. It is becoming increasingly apparent, however, that each virus may have a few specific steps of replication that may be used as targets for highly selective, carefully aimed chemotherapeutic agents. Therefore, proper use of such drugs requires a thorough knowledge of the suitable targets, based on a correct diagnosis and a precise understanding of the replicative mechanisms for the offending virus.

The Bacteriophages

Viruses that attack bacteria were observed by Twort and d'Herelle in 1915 and 1917. Probably every known bacterium is subject to infection by one or more viruses or "bacteriophages" as they are known ("phage" for short, from Gr. "phagein" meaning "to eat" or "to nibble").

Like most viruses, bacteriophages typically carry only the genetic information needed for replication of their nucleic acid and synthesis of their protein coats. When phages infect their host cell, the order of business is to replicate their nucleic acid and to produce the protective protein coat. But they cannot do this alone. They require precursors, energy generation and ribosomes supplied by their bacterial host cell.

Bacterial cells can undergo one of two types of infections by viruses termed **lytic infections** and **lysogenic (temperate)** infections.

The first step in the replication of the phage in its host cell is called **adsorption**. The phage particle undergoes a chance collision at a chemically complementary site on the bacterial surface, then adheres to that site by means of its tail fibers.

Following adsorption, the phage injects its DNA into the bacterial cell. This process is called **penetration** and it may be both mechanical and enzymatic.

Immediately after injection of the viral DNA there is a process initiated called **synthesis of early proteins**. This refers to the transcription and translation of a section of the phage DNA to make a set of proteins that are needed to replicate the phage DNA. During this period the cell's energy-generating and protein-synthesizing abilities are maintained, but they have been subverted by the virus. The result is the **synthesis of several copies of the phage DNA**.

The next step is the synthesis of late proteins. Each of the several replicated copies of the phage DNA can now be used for transcription and translation of a second set of proteins called the **late proteins**. The late proteins are mainly structural proteins that make up the capsomeres and the various components of the tail assembly. Lysozyme is also a late protein that will be packaged in the tail of the phage and be used to escape from the host cell during the last step of the replication process.

Having replicated all of their parts, there follows an **assembly** process. The proteins that make up the capsomeres assemble themselves into the heads and "reel in" a copy of the phage DNA. The tail and accessory structures assemble and incorporate a bit of lysozyme in the tail plate.

While the viruses are assembling, lysozyme is being produced as a late viral protein. Part of this lysozyme is used to escape from the host cell by lysing the cell wall peptidoglycan from the inside. This accomplishes the **lysis of the host cell** and the **release of the mature viruses**, which spread to nearby cells, infect them, and complete the cycle.

Lysogenic or temperate infection rarely results in lysis of the bacterial host cell. Lysogenic viruses, such as lambda which infects *E. coli*, have a different strategy than lytic viruses for their replication. After penetration, the virus DNA integrates into the bacterial chromosome and it becomes replicated every time the cell duplicates its chromosomal DNA during normal cell division. The life cycle of a lysogenic bacteriophage is illustrated below.

Temperate viruses usually do not kill the host bacterial cells they infect. Their chromosome becomes integrated into a specific section of the host cell chromosome. Such phage DNA is called **prophage** and the host bacteria are said to be **lysogenized**. In the prophage state all the phage genes except one are repressed.

The prophage supplies genetic information such that the lysogenic bacteria exhibit a new characteristic (new phenotype), not displayed by the non-lysogenic cell, a phenomenon called **lysogenic conversion**. Hence, *Corynebacterium diphtheria* can only produce the toxin responsible for the disease if it carries a temperate virus called phage beta. Only lysogenized streptococci produce the erythrogenic toxin (pyrogenic exotoxin) which causes the skin rash of scarlet fever; and some botulinum toxins are synthesized only by lysogenized strains of *C. botulinum*.

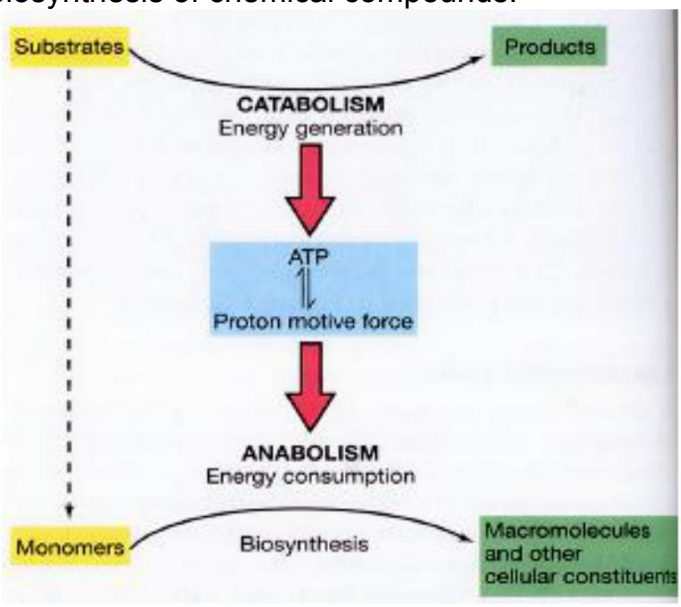
Surname_____ Date_____

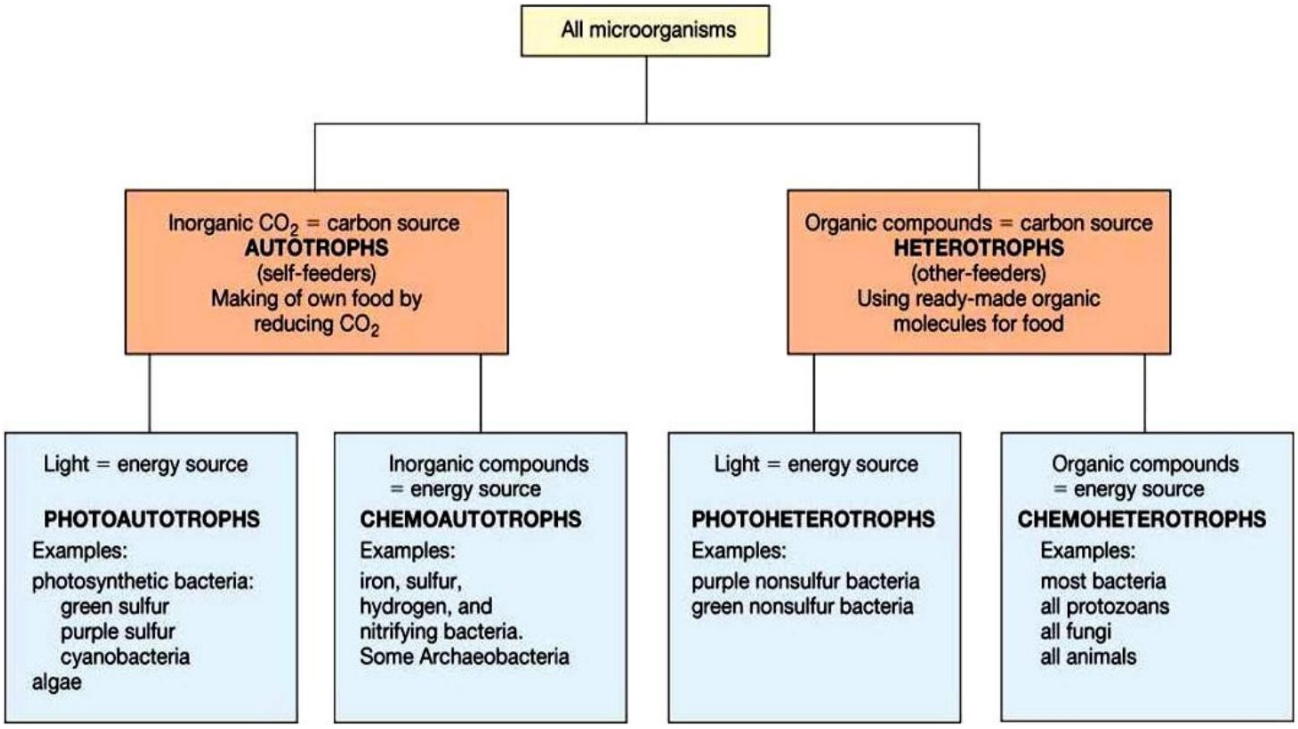
Protocol № 9

Theme: Bacterial metabolism. Nutritional requirements of bacteria. MPB, MPA. Sterilization and disinfection.

Catabolism - breakdown of chemical compounds. Glucose is metabolized in discrete steps so that energy is released in usable forms - harnassed as ATP (metabolic currency).

Anabolism - biosynthesis of chemical compounds.





Autotrophs _____.

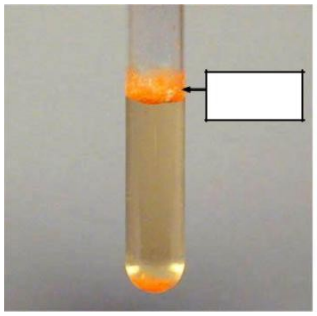
Heterotrophs _____

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _

I. Simple nutrient media:

meat peptone broth (MPB) – meat broth + 0.1 % NaCl + 1 % peptone; meat peptone agar (MPA) – meat peptone broth + agar (solidifing agent)

II. Growth characteristics of bacteria in MPB: pellicle, turbidity, sediment (deposit); on plate agar: colonies; on stroke (slant) agar – pellicle.



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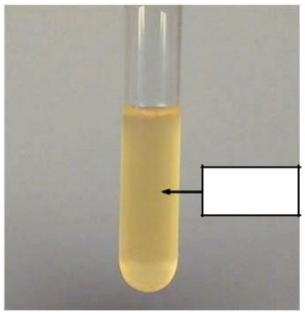

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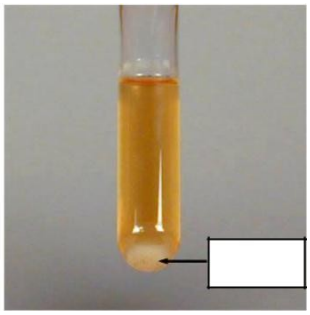

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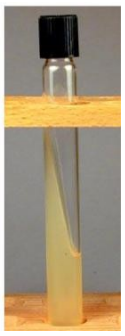

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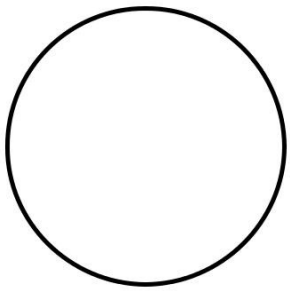
III. Prepare the smear from pure culture of E.coli. Stain the smears after Gram. Microscopy the prepared smears under oil immersion microscope.



Escherichia coli
(Gram stain)

IV. Seed pure cultures of S. epidermidis, E.coli, B.anthracoides on either MPB, or MPA, using stroke culture method.

V. Study methods of sterilization and disinfection:

Sterilization - the process of **inactivation (or removal) of all life forms)** in a material or on an object. A sterile object is one free of all life forms, including endospores.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _ _

Methods of Sterilization

Method	Treatment	Mode of Action*	Appropriate Materials for Treatment
Incineration	1 sec at more than 1000 ^{0}C	Denatures proteins	Inoculating loops
Autoclaving	Stream at 121°C under a pressure of 15-17 psi for 15 min to several hours	Coagulate proteins	Heat-stable items such as most culture components, glass and metal, but few plastics
Hot air oven	160^0 C for 2 hours 170^0 C for 1 hour	Coagulate proteins	Glass and metal, but not liquids or plastics
Membrane filtration	0.22 µm to 0.45 µm pore filters. (viruses are not stopped by this treatment)	Filter traps microbes	Solutions of heat-sensitive compounds (amino acids, vitamins, antibiotics, sugars, etc.)
Ionizing radiation	Exposure to ultraviolet or X- or gamma-rays	Damages nucleic acids	Heat-sensitive solids (plastics). Effective on surfaces only
Gas	Exposure to reactive gases (ethylene oxide)	Inactivates enzymes	Heat-sensitive solids such as some plastics

Disinfection - reduction in the number of pathogenic microorganisms to the point where they no longer pose a threat of disease.

Disinfectants - chemicals used on inanimate objects to control microbes.

Antiseptics - chemicals used on living tissue to control microbes.

Sterilants - chemicals that kill all life.

Typical Chemical Controls

Chemical	Mode of action	Example
Phenol	coagulates proteins & disrupts membranes	(hexaclorophene, triclosan) Found in lysol, listerine
Alcohols (70-90%)	denature proteins, dissolve lipids in membranes	Isopropanol Ethanol Tincture
Halogens	create free radicals, are strong oxidizing agents destroying essential metabolic compounds	Cl^- ,Br^- , Fl^- & I^- (cleaning, drinking water, wounds)
Oxidizing Agents	create hydoxyl radicals	Peroxide (H_2O_2) Paracetic acid Ozone O_3
Heavy Metals	combine with and inactivate enzymes at Sulfur atoms	silver nitrate ($AgNO_3$), silver sulfadiazine
Surfactants (QUATS)	denature cell proteins, disrupts membranes, interfere with metabolic processes	Quaternary ammonium compounds benzalkonium chloride
Aldehydes	alkylating agent, alter protein & nucleic acid	(formaldehyde & gluteraldehyde)

ADDING THEORETICAL MATERIAL

Nutrition and Growth of Bacteria. Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as **nutrients** or **nutritional requirements**. Many bacteria can be grown the laboratory in **culture media** which are designed to provide all the essential nutrients in solution for bacterial growth. Bacteria that are symbionts or obligate intracellular parasites of other cells, usually eukaryotic cells, are (not unexpectedly) difficult to grow outside of their natural host cells. Whether the microbe is a mutualist or parasite, the host cell must ultimately provide the nutritional requirements of its resident.

Many bacteria can be identified in the environment by inspection or using genetic techniques, but attempts to isolate and grow them in artificial culture has been unsuccessful. This, in part, is the basis of the estimate that we may know less than one percent of all prokaryotes that exist.

The Major Elements

At an elementary level, the nutritional requirements of a bacterium such as *E. coli* are revealed by the cell's elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and traces of Zn, Co, Cu, and Mo. These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells.

Major elements, their sources and functions in bacterial cells.

Element	% of dry weight	Source	Function
Carbon	50	organic compounds or CO_2	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores
Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

Carbon and Energy Sources for Bacterial Growth

In order to grow in nature or in the laboratory, a bacterium must have an energy source, a source of carbon and other required nutrients, and a permissive range of physical conditions such as O_2 concentration, temperature, and pH. Sometimes bacteria are referred to as individuals or groups based on their patterns of growth under various chemical (nutritional) or physical conditions. For example, phototrophs are organisms that use light as an energy source; anaerobes are organisms that grow without oxygen; thermophiles are organisms that grow at high

temperatures.

All living organisms require a source of energy. Organisms that use radiant energy (light) are called phototrophs. Organisms that use (oxidize) an organic form of carbon are called heterotrophs or (chemo)heterotrophs. Organisms that oxidize inorganic compounds are called lithotrophs.

The carbon requirements of organisms must be met by organic carbon (a chemical compound with a carbon-hydrogen bond) or by CO2. Organisms that use organic carbon are heterotrophs and organisms that use CO2 as a sole source of carbon for growth are called autotrophs.

Thus, on the basis of carbon and energy sources for growth four major nutritional types of prokaryotes may be defined.

Major nutritional types of procaryotes

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

Almost all eukaryotes are either photoautotrophic (e.g. plants and algae) or heterotrophic (e.g. animals, protozoa, fungi). Lithotrophy is unique to prokaryotes and photoheterotrophy, common in the Purple and Green Bacteria, occurs only in a very few eukaryotic algae. Phototrophy has not been found in the Archaea, except for nonphotosynthetic light-driven ATP synthesis in the extreme halophiles.

Growth factors are required in small amounts by cells because they fulfill specific roles in biosynthesis. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells. Growth factors are organized into three categories.

1. **purines and pyrimidines**: required for synthesis of nucleic acids (DNA and RNA)
2. **amino acids**: required for the synthesis of proteins
3. **vitamins**: needed as coenzymes and functional groups of certain enzymes.

Culture Media for the Growth of Bacteria

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a **culture medium**, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties.

The manner in which bacteria are cultivated, and the purpose of culture media, varies widely. **Liquid media** are used for growth of pure batch cultures, while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes. The usual gelling agent for solid or **semisolid medium** is **agar**, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at 100 ^{o}C and remains liquid until cooled to 40 ^{o}C, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution.

Types of Culture Media

Culture media may be classified into several categories depending on their composition or use. A **chemically-defined (synthetic) medium** is one in which the exact chemical

composition is known. A **complex (undefined) medium** is one in which the exact chemical constitution of the medium is not known. **Defined media** are usually composed of pure biochemicals off the shelf; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. A defined medium is a **minimal medium** if it provides only the exact nutrients (including any growth factors) needed by the organism for growth. The use of defined minimal media requires the investigator to know the exact nutritional requirements of the organisms in question. Chemically-defined media are of value in studying the minimal nutritional requirements of microorganisms, for enrichment cultures, and for a wide variety of physiological studies. Complex media usually provide the full range of growth factors that may be required by an organism so they may be more handily used to cultivate unknown bacteria or bacteria whose nutritional requirement are complex (i.e., organisms that require a lot of growth factors, known or unknown). Complex media are usually used for cultivation of bacterial pathogens and other fastidious bacteria.

Most pathogenic bacteria of animals, which have adapted themselves to growth in animal tissues, require complex media for their growth. Blood, serum and tissue extracts are frequently added to culture media for the cultivation of pathogens. Even so, for a few fastidious pathogens such as *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the cause of leprosy, artificial culture media and conditions have not been established. This fact thwarts the ability to do basic research on these pathogens and the diseases that they cause.

Other concepts employed in the construction of culture media are the principles of selection and enrichment. A **selective medium** is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. One can also adjust the physical conditions of a culture medium, such as pH and temperature, to render it selective for organisms that are able to grow under these certain conditions.

A culture medium may also be a **differential medium** if allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium. Thus a **selective, differential medium** for the isolation of *Staphylococcus aureus,* the most common bacterial pathogen of humans, contains a very high concentration of salt (which the staph will tolerate) that inhibits most other bacteria, mannitol as a source of fermentable sugar, and a pH indicator dye. From clinical specimens, only staph will grow. *S. aureus* is differentiated from *S. epidermidis* (a nonpathogenic component of the normal flora) on the basis of its ability to ferment mannitol. Mannitol-fermenting colonies *(S. aureus)* produce acid which reacts with the indicator dye forming a colored halo around the colonies; mannitol non-fermenters (*S. epidermidis*) use other non-fermentative substrates in the medium for growth and do not form a halo around their colonies.

An enrichment medium employs a slightly different twist. An **enrichment medium** contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment.

Surname _____ Date _____

Protocol № 10

Theme: Isolation of aerobic bacteria in pure culture. Selective media for bacterial growth.

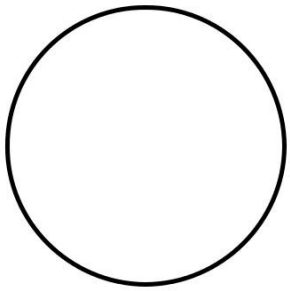
Pure culture – is one in which all organisms are descendants of the same organism.

I. Selective media have agents added which will **inhibit the growth of one group of organisms while permitting the growth of another**.

Examples of selective nutrient media:

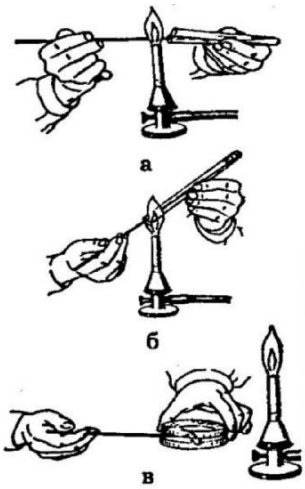
a) 1% peptone basic agar and basic water (_____),
b) bile broth (_____),
c) Loeffler serum slope (_____),
d) yolk-salt agar (_____),
e) Cabouroud agar (_____).

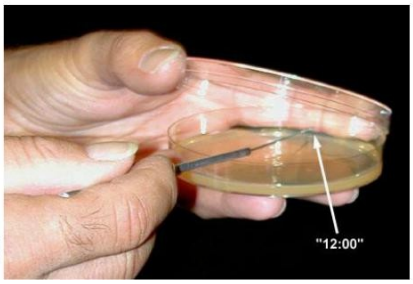
II. Prepare a smear from a mixed culture containing S. epidermidis, E. coli, B.anthracoides. Stain the smear after Gram. Microscopy, recognize and identify S. epidermidis, E.coli, B.anthracoides in the smear of mixed culture using oil immersion microscope.

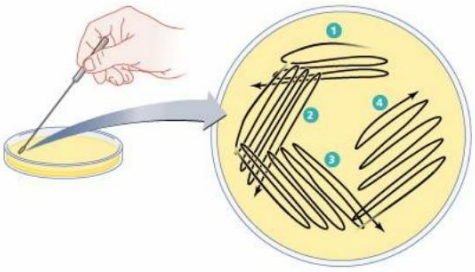


Mixture of bacteria (Gram stain)

III. Seed mixed culture using streak plate method of isolation of bacteria.







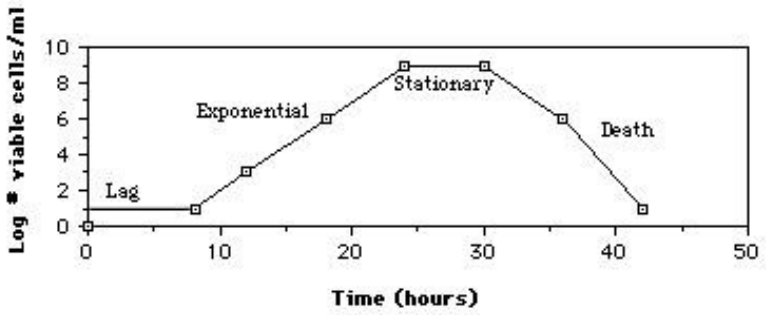
IV. Describe growth of bacteria:

a) in MPB: _____;
on MPA: _____.

V. Prepare the smear from broth or slant culture. Stain the smears after Gram. Microscopy the prepared smears under oil immersion microscope.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

VI. The typical bacterial growth curve.



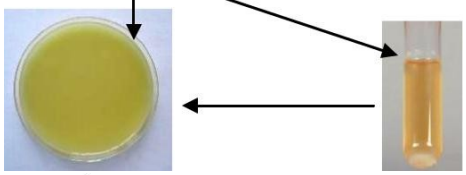
When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Time measurements are in hours for bacteria with short generation times.

VII. Study the cheme of isolation of pure culture:

SCHEME OF ISOLATION OF BACTERIA IN PURE CULTURE

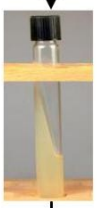
Specimen for examination: blood, pus, stool, sputum, etc.

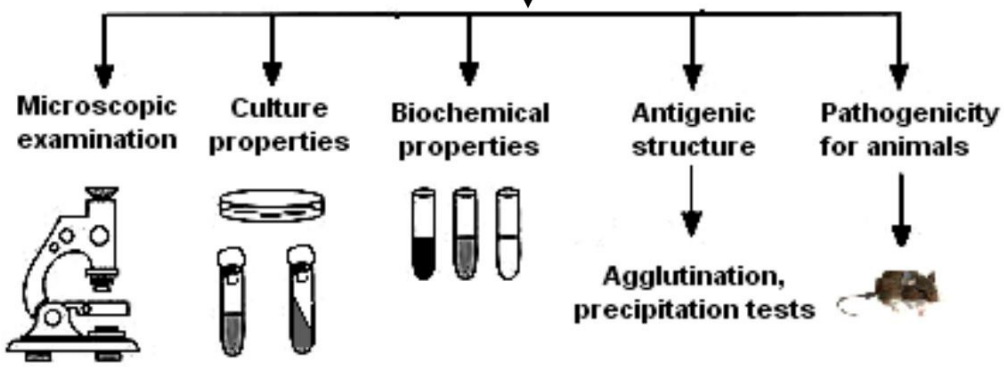
1.



2.

3.





ADDING THEORETICAL MATERIAL

The prokaryotes exist in nature under an enormous range of physical conditions.

The Effect of pH on Growth. The pH, or hydrogen ion concentration, $[H^+]$, of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Appreciating that pH is measured on a logarithmic scale, the $[H^+]$ of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes! Most free-living procaryotes can grow over a range of 3 pH units, about a thousand-fold change in $[H^+]$. The range of pH over which an organism grows is defined by **three cardinal points**: the **minimum pH**, below which the organism cannot grow, the **maximum pH**, above which the organism cannot grow, and the **optimum pH**, at which the organism grows best. For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing $[H^+]$ on the rates of enzymatic reaction.

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called **acidophiles**. Those which grow best at neutral pH are called **neutrophils** and those that grow best under alkaline conditions are called **alkaliphiles**. Obligate acidophiles, such as some *Thiobacillus* species, actually require a low pH for growth since their membranes dissolve and the cells lyse at neutrality. Several genera of Archaea, including *Sulfolobus* and *Thermoplasma*, are obligate acidophiles. Among eukaryotes, many fungi are acidophiles, but the champion of growth at low pH is the eukaryotic alga *Cyanidium* which can grow at a pH of 0.

In the construction and use of culture media, one must always consider the optimum pH for growth of a desired organism and incorporate **buffers** in order to maintain the pH of the medium in the changing milieu of bacterial waste products that accumulate during growth. Many pathogenic bacteria exhibit a relatively narrow range of pH over which they will grow. Most diagnostic media for the growth and identification of human pathogens have a pH near 7.

The Effect of Temperature on Growth. Microorganisms have been found growing in virtually all environments where there is liquid water, regardless of its temperature. In 1966, Professor Thomas D. Brock, then at Indiana University, made the amazing discovery in boiling hot springs of Yellowstone National Park that bacteria were not just surviving there, they were growing and flourishing. Brock's discovery of thermophilic bacteria, archaea and other "extremophiles" in Yellowstone is summarized for the general public in an article at this web site.

Subsequently, prokaryotes have been detected growing around black smokers and hydrothermal vents in the deep sea at temperatures at least as high as 120 degrees. Microorganisms have been found growing at very low temperatures as well. In super cooled solutions of H_2O as low as -20 degrees, certain organisms can extract water for growth, and many forms of life flourish in the icy waters of the Antarctic, as well as household refrigerators, near 0 degrees.

A particular microorganism will exhibit a range of temperature over which it can grow, defined by three cardinal points in the same manner as pH. Considering the total span of temperature where liquid water exists, the prokaryotes may be subdivided into several subclasses on the basis of one or another of their cardinal points for growth. For example, organisms with an optimum temperature near 37 degrees (the body temperature of warm-blooded animals) are called **mesophiles**. Organisms with an optimum T between about 45 degrees and 70 degrees are **thermophiles**. Some Archaea with an optimum T of 80 degrees or higher and a maximum T as high as 115 degrees, are now referred to as **extreme thermophiles** or **hyperthermophiles**. The cold-loving organisms are **psychrophiles** defined by their ability to grow at 0 degrees. A variant of a psychrophile (which usually has an optimum T of 10-15 degrees) is a **psychrotroph**, which grows at 0 degrees but displays an optimum T in the mesophile range, nearer room temperature. Psychrotrophs are the scourge of food storage in refrigerators since they are invariably brought in from their mesophilic habitats and continue to grow in the refrigerated environment where they spoil the food. Of course, they grow slower at 2 degrees than at 25 degrees.

Psychrophilic bacteria are adapted to their cool environment by having largely unsaturated fatty acids in their plasma membranes. Some psychrophiles, particularly those from the Antarctic have been found to contain polyunsaturated fatty acids, which generally do not occur in prokaryotes. The degree of unsaturation of a fatty acid correlates with its solidification T or thermal transition stage (i.e., the temperature at which the lipid melts or solidifies); unsaturated fatty acids remain liquid at low T but are also denatured at moderate T; saturated fatty acids, as in the membranes of thermophilic bacteria, are stable at high temperatures, but they also solidify at relatively high T. Thus, saturated fatty acids (like butter) are solid at room temperature while unsaturated fatty acids (like safflower oil) remain liquid in the refrigerator. Whether fatty acids in a membrane are in a liquid or a solid phase affects the fluidity of the membrane, which directly affects its ability to function. Psychrophiles also have enzymes that continue to function, albeit at a reduced rate, at temperatures at or near 0 degrees. Usually, psychrophile proteins and/or membranes, which adapt them to low temperatures, do not function at the body temperatures of warm-blooded animals (37 degrees) so that they are unable to grow at even moderate temperatures.

Thermophiles are adapted to temperatures above 60 degrees in a variety of ways. Often thermophiles have a high G + C content in their DNA such that the melting point of the DNA (the temperature at which the strands of the double helix separate) is at least as high as the organism's maximum T for growth. But this is not always the case, and the correlation is far from perfect, so thermophile DNA must be stabilized in these cells by other means. The membrane fatty acids of thermophilic bacteria are highly saturated allowing their membranes to remain stable and functional at high temperatures. The membranes of hyperthermophiles, virtually all of which are Archaea, are not composed of fatty acids but of repeating subunits of the C5 compound, phytane, a branched, saturated, "isoprenoid" substance, which contributes heavily to the ability of these bacteria to live in superheated environments. The structural proteins (e.g. ribosomal proteins, transport proteins (permeases) and enzymes of thermophiles and hyperthermophiles are very heat stable compared with their mesophilic counterparts. The proteins are modified in a number of ways including dehydration and through slight changes in their primary structure, which accounts for their thermal stability.

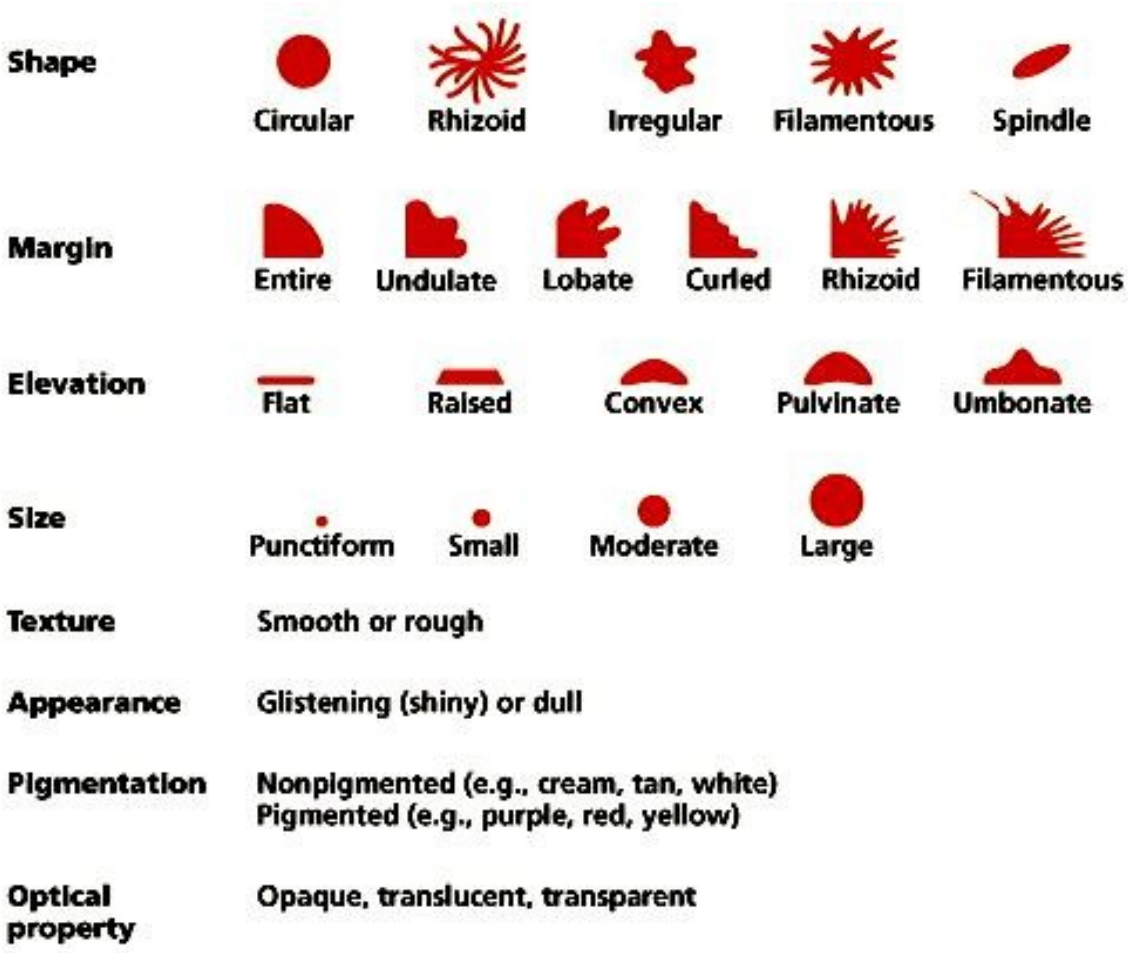
Surname _____ Date _____

Protocol № 11

Theme: Colony morphology. Pigments of bacteria.

A colony is a visible mass of microorganisms growing on an agar surface and usually originating from a single organism or arrangement of organisms.

Morphology of colonies



Pigments of bacteria

Water soluble: _____.
Water insoluble: _____.
Water and alcohol insoluble: _____

1.Microscopy of demonstration microslides of pure culture of E.coli, B.anthracoides, S.epidermidis.

2.Microscopy of demonstration colonies of each species of bacteria E.coli, B.anthracoides, S.epidermidis.

3.Examination of seeding of mixed culture (E.coli, B.anthracoides, S.epidermidis) on Petri dish with MPA. Description of isolated colonies of each species of bacteria.

4.Choose isolated colony of species wanted to obtain in pure culture.

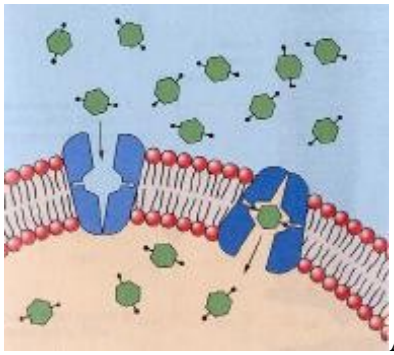
5.Prepare the smear from isolated colony of given species. Stain smear after Gram. Microscopy the smear.

6.Transfer isolated colony of given species on an agar slant tube.

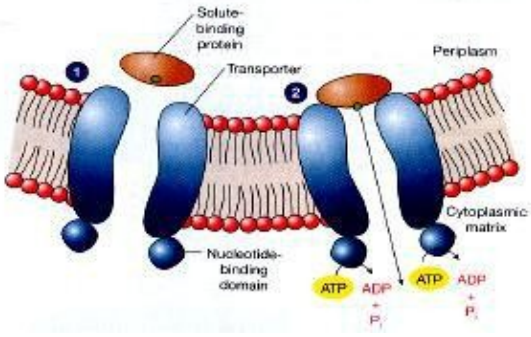
7.Characteristics of colony morphology of E.coli, B.anthracoides, S.epidermidis:

Characteristics	E.coli	S.epidermidis	B.anthracoides
shape			
size			
margin (edge)			
surface			
optical characteristics			
pigmentation			
consistency			
Gram's staining			

8. Pathways of penetranion of nutrients in a bacterial cell:



A



B

A. Facilitated diffusion. The membrane carrier can change conformation after binding an externall molecule and subsequently release the molecule on the cell interior. It then returns to the outward positin and is ready to bind another soluble molecule.

B. Active transport: (1) The soluble binding protein binds the substrate and approaches the transporter complex. (2) The soluble binding protein attaches to transporter and releases the substrate, which is moved across the membrane with the aid of ATP hydrolysis.

ADDING THEORETICAL MATERIAL

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell.

Colony morphology can sometimes be useful in bacterial identification. Colonies are described as to such properties as size, shape, texture, elevation, pigmentation, effect on growth medium. In this blog post you will find common criteria that are used to characterize the bacterial growth.

Colony Shape: It includes form, elevation and margin of the bacterial colony.

1. **Form of the bacterial colony:** – The form refers to the shape of the colony. These forms represent the most common colony shapes you are likely to encounter. e.g. circular, irregular, filamentous, rhizoid etc.
2. **Elevation of bacterial colony:** This describes the "side view" of a colony. These are the most common. e.g. **flat, raised, umbonate (having a knobby protuberance), crateriform, convex, pulvinate (cushion-shaped)**

3. **Margin of bacterial colony:** The margin or edge of a colony may be an important characteristic in identifying an organisms. Common examples are **entire (smooth), irregular, undulate (wavy), lobate, curled, filiform** etc. Colonies that are irregular in shape and/or have irregular margins are likely to be motile organisms. Highly motile organism swarmed over the culture media. Such as Proteus spp.

Size of the bacterial colony: The size of the colony can be a useful characteristic for identification. The diameter of a representative colony may be measured in millimeters or described in relative terms such as pin point, small, medium, large. Colonies larger than about 5 mm are likely to be motile organisms.

Appearance of the colony surface: Bacterial colonies are frequently shiny and smooth in appearance. Other surface descriptions might be: dull (opposite of glistening), veined, rough, wrinkled (or shriveled), glistening.

Consistency/Texture: Several terms that may be appropriate for describing the texture or consistency of bacterial growth are: dry, moist, viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like).

Color of the colonies (pigmentation): Some bacteria produce pigment when they grow in the medium e.g., green pigment produces by *Pseudomonas aeruginosa*, buff colored colonies of *Mycobacterium tuberculosis* in L.J medium, red colored colonies of Serratia marcescens.

Opacity of the bacterial colony: Is the colony transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass), iridescent (changing colors in reflected light).

Pigments of bacteria

Some produce which can be seen after they grow into colonies. Pigments can help identify bacteria. For example, some bacteria produce water soluble pigments which spread through the medium in which they grow. *P. aeruginosa* («*aeruginosa*», which derives from a Latin word denoting the color of copper rust) produces a green fluorescent pigment (fluorescein/pyoverdin) and a blue pigment (pyocyanin). *P. aeruginosa* is usually recognized by the pigments it produces and the distinctive smell of cultures. When the culture is left at room temperature, pigment colour becomes more intense.

Staphylococcus aureus (*aureus* - "golden", Latin) produces multiple carotenoid pigments, one being golden yellow pigment (Staphyloxanthin).

Others produce pigments that are soluble in fat. To determine this one can remove some of a pigmented colony and shake it in oil. If the oil becomes pigmented the pigment is fat soluble. Sometimes a species of bacteria will only produce their pigments under certain environmental circumstances. For example, *Serrattia marsescens* produces a brick red pigment when grown at room temperature, but no pigment when grown at body temperature (37 ^{0}C). Other species produce pigment as the colonies age or when a particular nutrient is present in the media.

Surname_____ Date_____

Protocol № 12

Theme: Identification of isolated pure culture. Enzymes of bacteria. Differential diagnostic media.

Exoenzymes _____
_____.

Endoenzymes _____.

Differential media contain additives **that cause an observable color change in the medium when a particular chemical reaction occurs**. They are useful in differentiating bacteria according to some biochemical characteristic. In other words, they indicate whether or not a certain organism can carry out a specific biochemical reaction during its normal metabolism.

Examples:

1. Media, which involve pH-related reactions (for the determination of carbohydrates fermentation)**:**

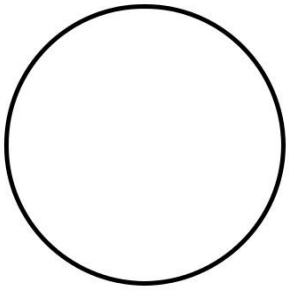
a. **Hiss media** are used in Durham tubes. Organisms, which ferment the sugar (glucose), will cause the pH indicator to change color upon production of acidic products. Additionally, if insoluble gas (H_2) is produced during fermentation, a bubble will be seen in the inverted Durham tube.

b. **MacConkey agar and Endo agar are** useful in differentiating the various gram-negative enteric bacilli belonging to the bacterial family *Enterobacteriaceae.* The color changes in the colonies are a result of bacterial fermentation of the sugar lactose while colorless colonies indicate lactose nonfermenters. The appearance of typical members of this bacterial family on Endo agar is as follows: *Escherichia coli*: large, red colonies with a metallic sheen; *Salmonella* and *Shigella*: large, colorless colonies.

2. Media, which do not involve pH-related reactions:

a. **Meat-peptone gelatin, MPB -** media for the determination of proteolytic action. b. **Blood agar** distinguishes between hemolytic and nonhemolytic bacteria. Hemolytic bacteria (e.g. many streptococci and staphylococci) produce clear zone around their colonies because of red blood cell destruction.

I. Prepare smears from isolated pure cultures of E.coli, B.anthracoides, S.epidermidis. Stain smears after Gram. Microscopy with immersion oil microscope.



Staphylococcus epidermidis
(Gram stain)

II. Transfer of isolated pure cultures of E.coli, B.anthracoides, S.epidermidis in Hiss media and MPB for testing on indole and hydrogen sulfide production.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

III. Study the tables "Biochemical properties (carbohydrate fermentation and protein hydrolysis with production of indole and hydrogen sulfide) of E.coli, B.anthracoides and S.epidermidis ".

Carbohydrate fermentation

Microorganisms	Maltose	Mannitol	Glucose	Sucrose	Lactose
Escherichia coli					
Acid	+	+	+	-	+
Gas	+	+	+	-	+
Fermentation	+	+	+	-	+
Staphylococcus epidermidis					
Acid	+	+	+	+	+
Gas	-	-	-	-	-
Fermentation	+	+	+	+	+
Bacillus anthracoides					
Acid	+	-	+	+	-
Gas	-	-	-	-	-
Fermentation	+	-	+	+	-

Protein hydrolysis

Microorganisms	Indole	Hydrogen Sulfide
Escherichia coli	+	±
Staphylococcus epidermidis	-	+
Bacillus anthracoides	-	+

ADDING THEORETICAL

MATERIAL Bacterial enzymes are subdivided into following classes. **Class 1. Oxidoreductases.**

To this class belong all enzymes catalysing oxidoreduction reactions. The substrate that is oxidized is regarded as hydrogen donor. The systematic name is based on *donor: acceptor oxidoreductase*. The common name will be *dehydrogenase*, wherever this is possible; as an alternative, *reductase* can be used. *Oxidase* is only used in cases where O_2 is the acceptor.

Class 2. Transferases.

Transferases are enzymes transferring a group, *e.g.* a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor). The systematic names are formed according to the scheme *donor: acceptor grouptransferase*.

Class 3. Hydrolases.

These enzymes catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Although the systematic name always includes *hydrolase*, the common name is, in many cases, formed by the name of the substrate with the

suffix *-ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

Class 4. Lyases.

Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. The systematic name is formed according to the pattern *substrate group-lyase*.

Class 5. Isomerases.

These enzymes catalyse geometric or structural changes within one molecule. According to the type of isomerism, they may be called *racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases* or *cycloisomerases*.

Class 6. Ligases.

Ligases are enzymes catalysing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The systematic names are formed on the system *X: Y ligase (ADP-forming)*.

The enzymes which are always present in the organism in constant amounts regardless of its metabolic state are called as **constitutive** enzymes. For example, the enzymes involved in central pathway of catabolism such as glycolysis are constitutive enzymes.

Sometimes, the enzymes appear in cells only when they are needed in the presence of their substrates or other agents which cause their de novo synthesis through DNA dependent synthesis of specific messenger RNA for the specific enzymic protein. Such enzymes are called as **inducible** or **inductive** enzymes or **induced** enzymes and this process of their synthesis is called as enzyme induction. The substrate or any other agent capable of inducing the synthesis of an enzyme is called as inducer or inducing agent. Enzyme induction is widespread in microorganisms but fewer examples are known in higher plants and animals. In bacterium Escherichia coli (E. coli) an example of the inducible enzyme is β-galactosidase which catalyses the hydrolysis of lactose to yield D-Glucose and D-Galactose.

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

Protocol № 13

Theme: Respiration of bacteria. Cultivation and isolation of anaerobic bacteria.

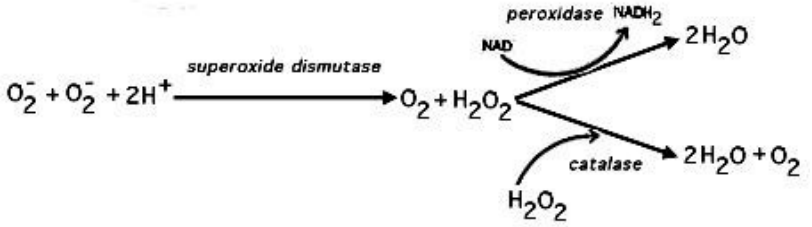
I. Describe the results of seeding of isolated pure cultures of E.coli, S.epidermidis, B.anthracoides in Hiss media and MPB and interpret them.

Respiration is the process cells use to convert the energy in the chemical bonds of nutrients to ATP energy. It is the oxidation of a source of energy by removal of electrons and donation to an inorganic terminal electron accepter. The path the electrons follow from source to accepter usually involves a membrane bound system that creates a proton gradient. This proton gradient can do work and is used to create ATP.

Aerobic Respiration is the catabolism of nutrients to carbon dioxide, water, and energy, in which molecular oxygen is the final electron acceptor.

Anaerobic Respiration is the catabolism of nutrients to carbon dioxide, water, and energy, in which an inorganic molecule other than oxygen (O_2) is the final electron acceptor or even an organic compound, such as fumarate. For example, some bacteria called sulfate reducers can transfer electrons to sulfate (SO_4^{2-}) reducing it to H_2S. Other bacteria, called nitrate reducers, can transfer electrons to nitrate (NO_3^-) reducing it to nitrite (NO_2^-). Other nitrate reducers can reduce nitrate even further to nitrous oxide (NO) or nitrogen gas (N_2).

During growth and metabolism, oxygen reduction products (superoxide radical, hydrogen peroxide, and hydroxyl radical) are generated within microorganisms and secreted into the surrounding medium. A microorganism must be able to protect itself against such oxygen products or it will be killed. Most facultative and aerobic organisms contain a high concentration of an enzyme called superoxide dismutase. This enzyme converts the superoxide anion into ground-state oxygen and hydrogen peroxide, thus ridding the cell of destructive superoxide anions (Fig.). Many organisms possess catalase or peroxidase or both to eliminate the H_2O_2. Catalase convers peroxide into water and ground-state oxygen.



The action of superoxide dismutase, catalase and peroxidase.

All strict anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2.

II. Classification of bacteria due to oxygen requirements. Study examples of pathogenic species:

Obligate aerobic bacteria: _____.

Microaerophylic bacteria: _____.

Facultative anaerobic bacteria: _____.

Obligate anaerobic bacteria: _____.

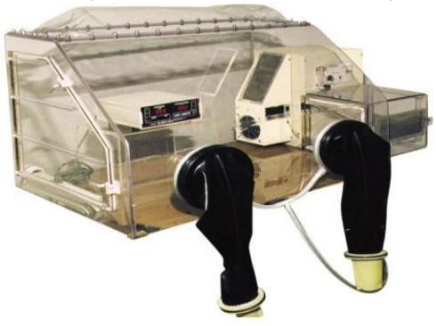
III. Microscopy of demonstration microslides of anaerobic bacteria stained after Gram.

Clostridium tetani (Gram stain)	**Clostridium botulinum (Gram stain)**	**Clostridium perfringens (Gram stain)**
Clostridium septicum (Gram stain)	**Clostridium hystolyticum (Gram stain)**	**Clostridium novyi (Gram stain)**

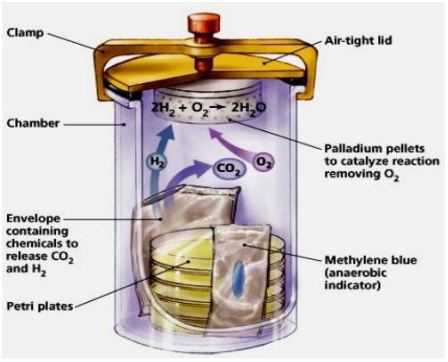
IV. Prepare smears from seeding of soil in Kitt-Tarozzi medium and milk under oil. Stain smears after Gram. Microscopy with immersion oil microscope.

V. Methods of cultivation of obligative anaerobic bacteria.

1. Adding chemicals to the medium that react with oxygen, such as sodium thioglycollate, cystein, and ascorbic acid.
2. Boiling of the medium to drive out the air before the bacteria are inoculated.
3. **The anaerobic glove box isolator (A)**. It is essentially a large clear-vinyl chamber, with attached gloves, containing a mixture of 80 % nitrogen, 10 % hydrogen, and 10 % carbon dioxide. A lock at one end of the chamber is fitted with two hatches, one leading to the outside and the other to the inside of the chamber. Specimens are placed in the lock, the outside hatch is closed, and the air in the lock is evacuated and replaced with the gas mixture. The inside hatch is then opened to introduce the specimen into the chamber.
4. The **anaerobic (or anaerobe) jar (B)** is a medium-sized glass or plastic jar with a tightly fitting lid containing palladium-coated alumina particles, which serve as a catalyst. It can be set up by two methods. The easiest uses a commercially available hydrogen and carbon dioxide generator envelope (**GasPak**) that is placed in the jar along with the culture plates. The Gas-Pak contains sodium borohydrate and sodium bicarbonate that react upon addition of water. Oxygen within the jar and the hydrogen that is generated are converted to water in the presence of the catalyst, thus producing anaerobic conditions. Carbon dioxide, which is also generated, is required for growth by some anaerobes and stimulates the growth of others. **Pack-Anaero** works as oxygen absorber-CO_2 generator. Only to open the aluminium pack and put the white sachet into the container. No need to add water and use catalyst. The sachet absorbs O_2 in the jar, generates about 16% of CO_2, and produces anaerobic atmosphere.



A



B

ADDING THEORETICAL MATERIAL

The prokaryotes exist in nature under an enormous range of physical conditions such as O_2 concentration, Hydrogen ion concentration (pH) and temperature. The exclusion limits of life on the planet, with regard to environmental parameters, are always set by some microorganism, most often a prokaryote, and frequently an Archaeon. Applied to all microorganisms is a vocabulary of terms used to describe their growth (ability to grow) within a range of physical conditions. A thermophile grows at high temperatures, an acidophile grows at low pH, an osmophile grows at high solute concentration, and so on. This nomenclature will be employed in this section to describe the response of the prokaryotes to a variety of physical conditions.

The Effect of Oxygen. Oxygen is a universal component of cells and is always provided in large amounts by H_2O. However, prokaryotes display a wide range of responses to molecular oxygen O_2.

Obligate aerobes require O_2 for growth; they use O_2 as a final electron acceptor in aerobic respiration.

Obligate anaerobes do not need or use O_2 as a nutrient. In fact, O_2 is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic prokaryotes may live by fermentation, anaerobic respiration, bacterial photosynthesis, or the novel process of methanogenesis.

Facultative anaerobes (or **facultative aerobes**) are organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions (no O_2) they grow by fermentation or anaerobic respiration, but in the presence of O_2 they switch to aerobic respiration.

Aerotolerant anaerobes are bacteria with an exclusively anaerobic (fermentative) type of metabolism but they are insensitive to the presence of O_2. They live by fermentation alone whether or not O_2 is present in their environment.

Terms used to describe O_2 Relations of Microorganisms.

Group	**Environment**		
	Aerobic	**Anaerobic**	**O_2 Effect**
Obligate Aerobe	Growth	No growth	Required (utilized for aerobic respiration)
Microaerophile	Growth if level not too high	No growth	Required but at levels below 0.2 atm
Obligate Anaerobe	No growth	Growth	Toxic
Facultative Anaerobe (Facultative Aerobe)	Growth	Growth	Not required for growth but utilized when available
Aerotolerant Anaerobe	Growth	Growth	Not required and not utilized

The response of an organism to O_2 in its environment depends upon the occurrence and distribution of various enzymes which react with O_2 and various oxygen radicals that are invariably generated by cells in the presence of O_2. All cells contain enzymes capable of reacting with O_2. For example, oxidations of flavoproteins by O_2 invariably result in the formation of H_2O_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or $O_2^{\cdot-}$. Also, chlorophyll and other pigments in cells can react with O_2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.

In aerobes and aerotolerant anaerobes the potential for lethal accumulation of superoxide is prevented by the enzyme superoxide dismutase. All organisms which can live in the presence of O_2 (whether or not they utilize it in their metabolism) contain superoxide dismutase. Nearly all organisms contain the enzyme catalase, which decomposes H_2O_2. Even though certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they decompose H_2O_2 by means of peroxidase enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O.

Obligate anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2.

All photosynthetic (and some non-photosynthetic) organisms are protected from lethal oxidations of singlet oxygen by their possession of carotenoid pigments which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state. Carotenoids are said to "quench" singlet oxygen radicals.

Distribution of superoxide dismutase, catalase and peroxidase in procaryotes with different O_2 tolerances.

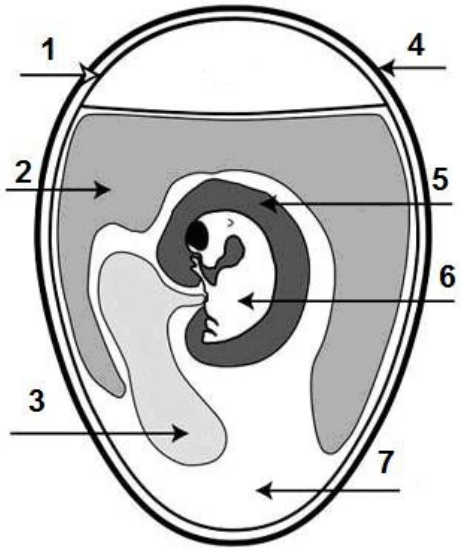
Group	**Superoxide dismutase**	**Catalase**	**Peroxidase**
Obligate aerobes and most facultative anaerobes (e.g. Enterics)	+	+	-
Most aerotolerant anaerobes (e.g. Streptococci)	+	-	+
Obligate anaerobes (e.g. Clostridia, Bacteroides)	-	-	-

Surname_____ Date_____

Protocol № 14

Theme: Cultivation of viruses, rickettsia and chlamydia.

I. Study the structure of chicken embryo:



II. Preparation the egg for virus cultivation.

1. Candling eggs is the process of holding a strong light above or below the egg to observe the embryo. A candling lamp consists of a strong electric bulb covered by a plastic or aluminium container that has a handle and an aperture. The egg is placed against this aperture and illuminated by the light.

Candling is done in a darkened room or in an area shielded by curtains. Under the candling lamp, the embryo appears as a dark shadow with the head as a dark spot. Healthy embryos will respond to the light by moving. The blood vessels are well defined in a healthy embryo. After an embryo has died, the blood vessels start to break down. They then appear as streaks under the shell when viewed under the candling lamp. Candling will also reveal cracks in the eggshells. Eggs with cracked shells should be discarded.

2. Marking the inoculation site: a line is drawn on the shell marking the edge of the air sac.

3. Inoculation of the allantoic cavity: the shell surface is first disinfected with iodine and penetrated with a small sterile drill. After inoculation, the drill hole is sealed with gelatin and the egg is incubated.

4. Incubation the chicken embryo in termostate.

5. Harvesting allantoic fluid to test for presence of haemagglutinin

Haemagglutination test.

Some viruses can agglutinate red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination. Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles.

III. Classification of cell cultures.

Cell/Tissue Culture. Tissue culture refers to the growth and maintenance of living tissue cells in vitro. There are two basic types: explant culture and cell cultures.

Explant Culture. These are cultures of small fragments of specific tissue removed from the host animal directly.

Cell cultures result from the breaking down of various host tissues into individual cells. Cell cultures can be further subdivided into primary, semi-continuous, and continuous cell cultures.

Primary Cell Cultures (Rhesus monkey kidney cell culture, Human amnion cell culture, Chick embryo fibroblast cell culture). These are derived from fresh tissues that have been enzymatically digested with trypsin or other protease to release individual cells. As a result, primary cultures are often composed of many different cell types. Under in vitro conditions, primary culture cells rarely divide or divide at a very low frequency. Primary cultures rarely survive beyond passage 20th in vitro.

Semi-continuous Cultures (WI-38 – Human embryonic lung cell strain, HL-8 - Rhesus embryo cell strain). Also known as diploid cell lines, as they contain the normal diploid chromosome characteristic of the species from which they were derived. Semi-continuous cultures tend to die out between the 30th and 50th passage in vitro. Semi-continuous cultures are usually fibroblastic.

Continuous Cell Cultures (HeLa – Human carcinoma of cervix cell line, HEP-2 – Human epitheliioma of larynx cell line, McCoy – Human synovial carcinoma cell line, KB – Human carcinoma of nasopharynx cell line, Vero – Vervet monkey kidney cell line, BHK-21 –Baby Hamster kidney cell line). Also known as heteroploid cell lines, as the cells have an abnormal number of chromosomes. These cell cultures are derived from normal or neoplastic tissue and are characterized by their ability to be propagated in vitro indefinitely.

IV. Culture media for cultivation of cell cultures (Henx media, 1999 medium).

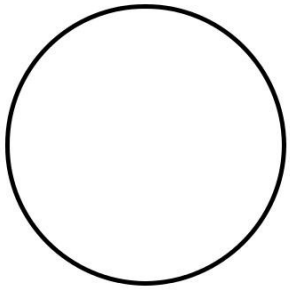
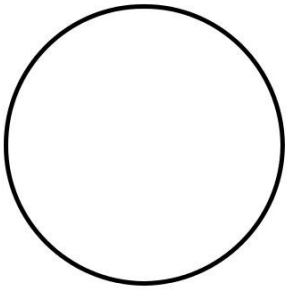
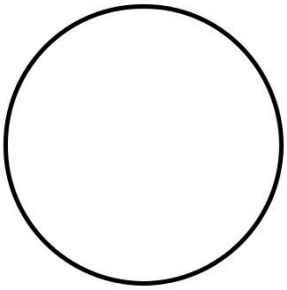
Besides meeting the basic nutritional requirement of the cells, the culture medium should also have any necessary growth factors, regulate the pH and osmolality, and provide essential gases (O_2 and CO_2). The —food‖ portion of the culture medium consists of amino acids, vitamins, minerals, and carbohydrates. These allow the cells to build new proteins and other components essential for growth and function as well as providing the energy necessary for metabolism. The growth factors and hormones help regulate and control the cells growth rate and functional characteristics. Instead of being added directly to the medium, they are often added in an undefined manner by adding 5 to 20 % of various animal sera to the medium.

V. Cytopathic effect of viruses.

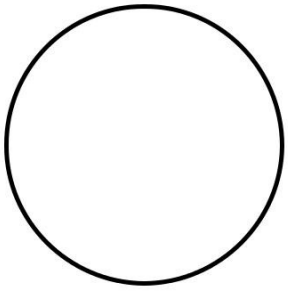
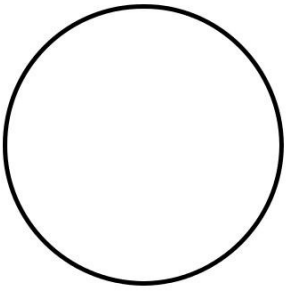
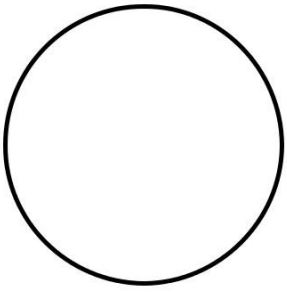
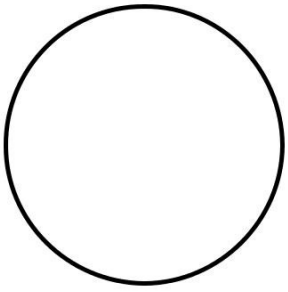
The virus-caused cell damage or destruction is referred to as the **cytopathic effect** (CPE). Observable cytopathic effects include:

a. Cells rounded up and aggregated in grape-like clusters, as with adenoviruses;
b. Cells round up, shrink, and lyse, leaving large amounts of cellular debris, as with enteroviruses;
c. Cells become swollen and round up in focal areas, as with herpesviruses; and
d. Cells fuse producing multinucleate cells (syncytia), as with paramyxoviruses (measles).
e. Inclusion bodies formation, as with influenza, small poa, rabies viruses.

VI. Observe the smears below.

		
Rickettsia	Chlamydia trachomatis	Control of tissue

Cytopathic effects of viruses:

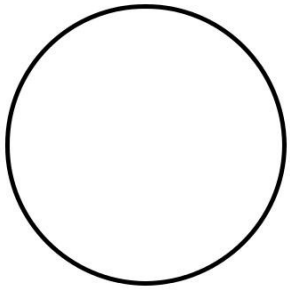
			
Lysis of monolayer	Rounded cell	Multinucleated cell	Inclusion body

Surname_____ Date_____

Protocol № 15

Theme: Infection and infectious process. Infecting of the experimental animals.

I. Microscopy demonstration the microslide of smear-imprint from the spleen of infected mice with B.anthracoides.



Capsule-producing bacteria in smear-imprint. Methylene blue stain

Infection. Upping the ante in terms of progression towards disease is infection, the actual growth of an organism, particularly a pathogenic one, on or within a host. Infection can lead to disease (particularly if we define infection as a property solely of pathogenic microorganisms) but does not necessarily do so since hosts actively attempt to prevent the progression of infection to disease.

Disease is a disturbance in the state of health (of the host) wherein the body cannot carry out all its normal functions. Disease, or illness, is characterized by changes in the host that interfere with normal function.

Contamination is the converse of sterility, **i.e.,** an environment that is not sterile is one that is contaminated with microorganisms. Contamination is the first step toward the occurrence of infectious disease, i.e., a organism must be present (in the wrong place and at the wrong time as far as the host is concerned) to start down a path leading to disease. Note, however, that just because something is contaminated with a microorganism (including contamination of yourself) does not mean that disease is occurring or will occur.

Pathogenicity is an organism's capacity to cause disease; **that** is, whether or not it can cause disease.

Note that this capacity is typically context dependent:

Pathogenicity is higher in some environments (e.g., infection of the blood) than others (e.g., presence in the lumen of the gastrointestinal tract), or

Pathogenicity is higher on some hosts but not on others depending on host susceptibility (in general or specifically towards a particular pathogen).

Pathogenicity can also depend on the number of organisms present, where many organisms have a greater potential of bypassing host defenses than fewer organisms of the same kind.

For an organism to be pathogenic it must be able to invade a host, multiply in the host, evade host defenses, and harm the host in some way.

Virulence is the degree of disease an organism has the potential to cause.

That is, a highly virulent pathogen can cause significant disease whereas an avirulent microorganism can cause little or no disease.

The terms pathogenicity and virulence are closely related with pathogenicity referring to an organism's binary ability to cause disease (or not) given specific circumstances and virulence referring to the degree of disease caused (also dependent on specific circumstances).

Attenuation is a decline in virulence imposed on a pathogen by growing the pathogen under conditions that decrease its adaptation to growth on a given host; this is often done by growth in tissue culture or in otherwise non-host species.

That is, by increasing a pathogen's adaptation to one condition (e.g., tissue culture), the pathogen's adaptation to another condition (e.g., us) may be reduced.

Attenuation is often employed in the development of live vaccines (e.g., Sabin oral polio vaccine).

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _ _

BACTERIAL VIRULENCE FACTORS

1. ADHERENCE FACTORS

a. Adhesins. Fimbriae. Common pili.
b. Capsule.
c. Lipopolysaccharides, teichoic and lipoteichoic acids.
d. Biofilm formation.

2. INVASINS

INVASIN	BACTERIA INVOLVED	ACTIVITY
Hyaluronidase	Streptococci, staphylococci and clostridia	Degrades hyaluronic of connective tissue
Collagenase	*Clostridium* species	Dissolves collagen framework of muscles
Neuraminidase	*Vibrio cholerae* and *Shigella dysenteriae*	Degrades neuraminic acid of intestinal mucosa
Coagulase	*Staphylococcus aureus*	Converts fibrinogen to fibrin which causes clotting
Leukocidin	*Staphylococcus aureus*	Disrupts neutrophil membranes
Streptolysin	*Streptococcus pyogenes*	Repels phagocytes and disrupts phagocyte membrane
Hemolysins	Streptococci, staphylococci and clostridia	Phospholipases or lecithinases that destroy red blood cells (and other cells) by lysis
Lecithinases	*Clostridium perfringens*	Destroy lecithin in cell membranes
Phospholipases	*Clostridium perfringens*	Destroy phospholipids in cell membrane

3. BACTERIAL ENDOTOXINS AND EXOTOXINS

PROPERTY	ENDOTOXIN	EXOTOXIN
SOURCE	Gram-negative bacteria	Gram-positive bacteria
CHEMICAL NATURE	Lipopolysaccharide	Protein
RELATIONSHIP TO CELL	Part of outer membrane	Extracellular, diffusible
DENATURED BY BOILING	No	Usually
ANTIGENICITY	Weakly	Highly
FORM TOXOID	No	Yes
POTENCY	Relatively low (>100ug)	Relatively high (1 ug)
SPECIFICITY	Low degree	High degree
ENZYMATIC ACTIVITY	No	Usually
PYROGENICITY	Yes	Occasionally

Biological effects of some bacterial exotoxins with enzymatic activity

TOXIN	ENZYMATIC ACTIVITY	BIOLOGICAL EFFECTS
Cholera toxin (A-5B)	ADP ribosylates eukaryotic adenylate cyclase Gs regulatory protein	Activates adenylate cyclase; increased level of intracellular cAMP promote secretion of fluid and electrolytes in intestinal epithelium leading to diarrhea
Diphtheria toxin (A/B)	ADP ribosylates elongation factor 2	Inhibits protein synthesis in animal cells resulting in death of the cells
Pertussis toxin (A-5B)	ADP ribosylates adenylate cyclase Gi regulatory protein	Blocks inhibition of adenylate cyclase; increased levels of cAMP affect hormone activity and reduce phagocytic activity
E. coli LT (A-5B)	ADP ribosylates adenylate cyclase Gs regulatory protein	Similar or identical to cholera toxin
Shiga toxin (A/5B)	Glycosidase cleavage of ribosomal RNA	Inactivates the mammalian 60S ribosomal subunit and leads to inhibition of protein synthesis and death of the susceptible cells; pathology is diarrhea, hemorrhagic colitis and/or hemolytic uremic syndrome
Pseudomonas Exotoxin A (A/B)	ADP ribosylates elongation factor-2 analogous to Dt	Inhibits protein synthesis in susceptible cells, resulting in death of the cells

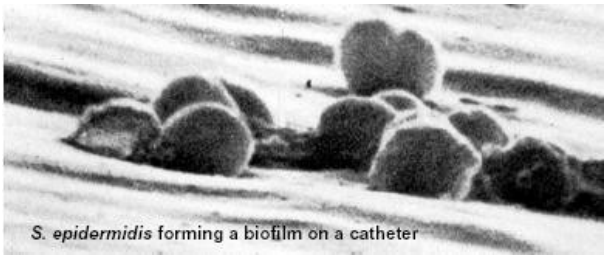
Botulinum toxin (A/B)	Zn^{2+} dependent protease acts on synaptobrevin at motor neuron ganglioside	Inhibits presynaptic acetylycholine release from peripheral cholinergic neurons resulting in flaccid paralysis
Tetanus toxin (A/B)	Zn^{2+} dependent protease acts on synaptobrevin in central nervous system	Inhibits neurotransmitter release from inhibitory neurons in the CNS resulting in spastic paralysis
Anthrax toxin LF (A2+B)	Metallo protease that cleaves MAPKK (mitogen-activated protein kinase kinase) enzymes	Combined with the B subunit (PA), LF induces cytokine release and death of target cells or experimental animals
Bordetella pertussis AC toxin (A/B) and Bacillus anthracis EF (A1+B)	Calmodulin-regulated adenylate cyclases that catalyze the formation of cyclic AMP from ATP in susceptible cells, as well as the formation of ion-permeable pores in cell membranes	Increases cAMP in phagocytes leading to inhibition of phagocytosis by neutrophils and macrophages; also causes hemolysis and leukolysis
*Staphylococcus aureus*Exfoliatin B	Cleaves desmoglein 1, a cadherin found in desmosomes in the epidermis (also a superantigen)	Separation of the stratum granulosum of the epidermis, between the living layers and the superficial dead layers.

* toxin subunit arrangements: A-B or A-5B indicates subunits synthesized separately and associated by noncovalent bonds; A/B denotes subunit domains of a single protein that may be separated by proteolytic cleavage; A+B indicates subunits synthesized and secreted as separate protein subunits that interact at the target cell surface; 5B indicates that the binding domain is composed of 5 identical subunits.

CLASSIFICATION OF INFECTIONS

CLINICAL MANIFESTATION

ACUTE	TYPICAL	ASYMPTOMATIC
CHRONIC	ATYPICAL	LATENT
IN NATURE		
EXOGENOUS	PRIMARY	SUPERINFECTION
ENDOGENOUS (AUTIONFECTION)	SECONDARY	REINFECTION
	MIXED	RELAPSE
INFECTION OF BLOOD		
BACTERAIMIA SEPTICAEMIA (SEPSIS) SEPTICOPYAEMIA	VIREMIA	TOXAEMIA (TOXINAEMIA)
IN LOCATION		
FOCAL		GENERALIZED (SYSTEMIC)
INTENSITY OF THE SPREAD OF INFECTION		
ENDEMIC	EPIDEMIC	PANDEMIC



S. epidermidis forming a biofilm on a catheter

Adherence mechanisms: Biofilm Formation. Biofilms - important adherence mechanism; involved in dental plaque and infections of heart valves, catheters and other foreign implants

II. Study desription of types of infectious diseases.

Acute infection develops rapidly but is soon over. For example, food poisoning.

Chronic infection develops slowly and is not soon over. For example, mycoses, tuberculosis, etc.

Subacute infection is the gray zone between acute and chronic.

Latent infection is sign-less or symptom-less for a long while before signs and symptoms appear (i.e., AIDS).

Inapparent (subclinical) infection that does not display signs or symptoms or, at least, all of the signs typically associated with a given syndrome. Minimally asymptomatic carriers of disease display inapparent infections.

Local infection is confined to a certain area (e.g., a pimple.)

Focal infection begins as a local infection but then spreads beyond the local area as a bacteremia, or toxemia. Note that the term focal infection is commonly employed in dentistry referring to the introduction of microorganisms, microbial waste products, or microbial toxins into the blood from the mouth, particularly associated with mouth infections (to some extent a controversial linkage) or following invasive dental procedures.

Systemic infection is spread throughout the body in the blood or lymph.

Septicemia is the growth of bacteria in the blood (a.k.a., blood poisoning).

Bacteremia is the presence, without multiplication, of bacteria in the blood.

Viremia is the presence of virus in the blood.

Toxemia is the presence of toxins in the blood (i.e., diphtheria).

Primary infection is the infection of a not-currently infected person.

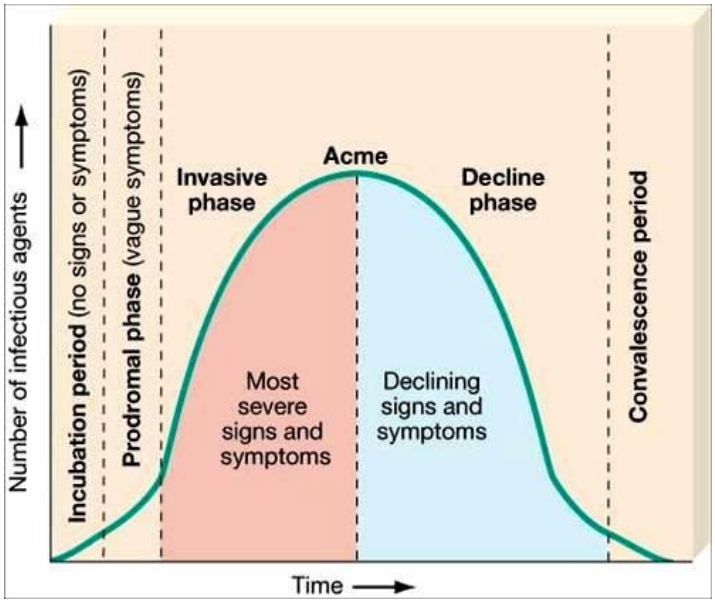
Secondary infection is an infection that quickly follows a primary infection. It may result from the treatments or from alterations in the immune system.

Superinfection is a secondary infection caused by the treatment of a primary infection (i.e., as in the superinfection by an antibiotic-resistant organism following antibiotic treatment; syphilis, gonorrhoea).

Reinfection is an infection caused by the same species of microorganism after recovery (syphilis, gonorrhoea).

Mixed infection is a syndrome that is caused by a combination of two or more infections (i.e., different pathogens).

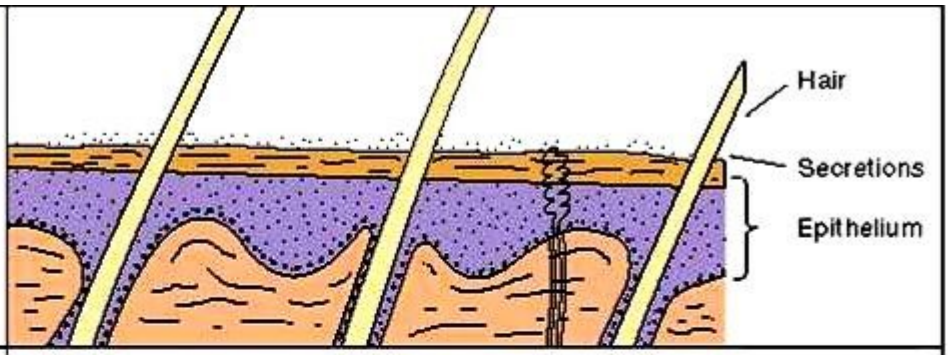
STAGES OF INFECTIOUS DISEASE



CONSTITUTIVE DEFENSES OF THE HOST

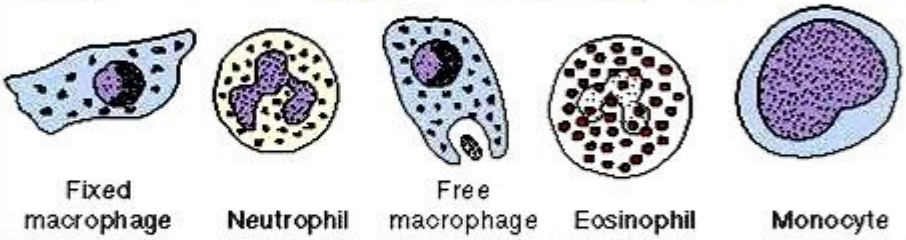
PHYSICAL BARRIERS

Prevent approach and deny access to pathogens



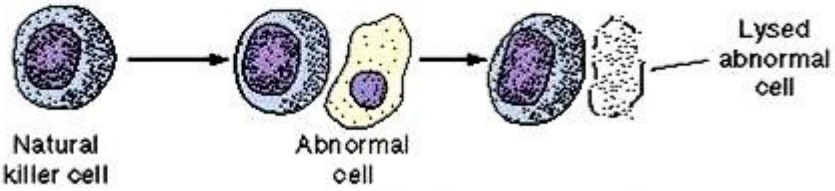
PHAGOCYTES

Remove debris and pathogens



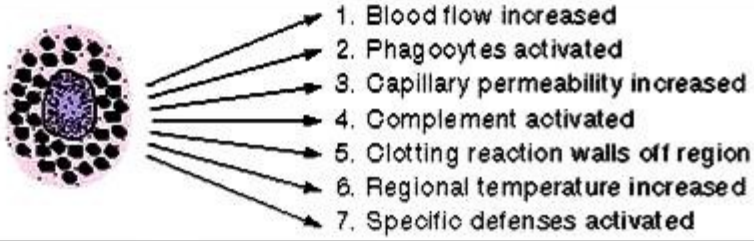
EXTRACELLULAR KILLING

Destroys abnormal cells



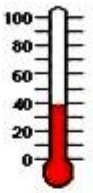
INFLAMMATORY RESPONSE

Multiple effects



FEVER

Mobilizes defenses, accelerates repairs, inhibits pathogens



Body temperature rises above 37°C in response to pyrogens

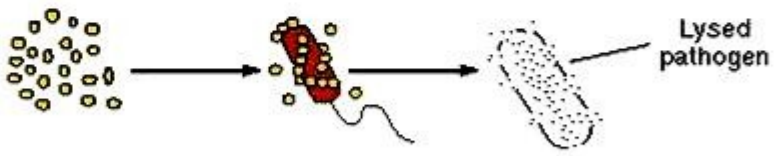
INTERFERONS

Increase resistance of cells to infection, slow the spread of disease

Released by activated lymphocytes and macrophages and by virus-infected cells

COMPLEMENT SYSTEM

Attacks and breaks down cell walls, attracts phagocytes, stimulates inflammation

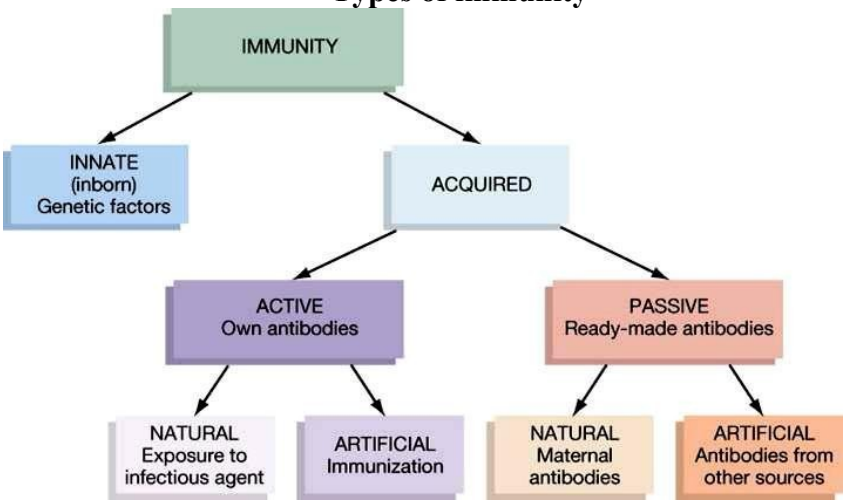


Surname_____ Date_____

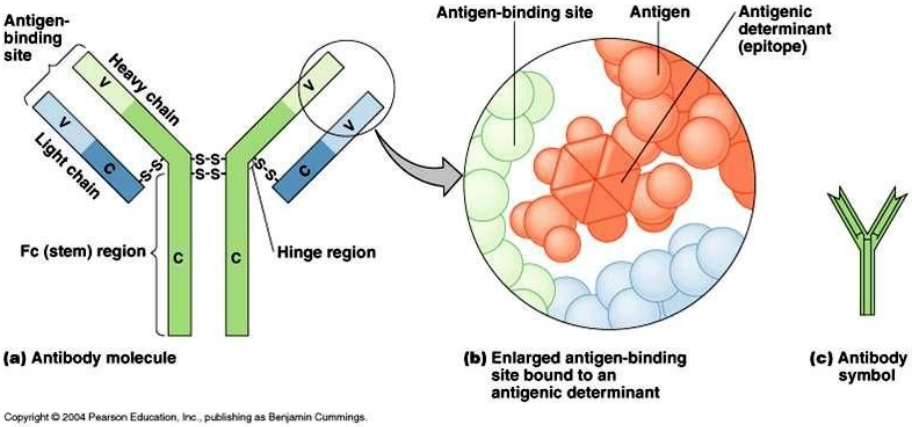
Protocol № 16

Theme: Studying of immunity. Agglutination and precipitation tests.

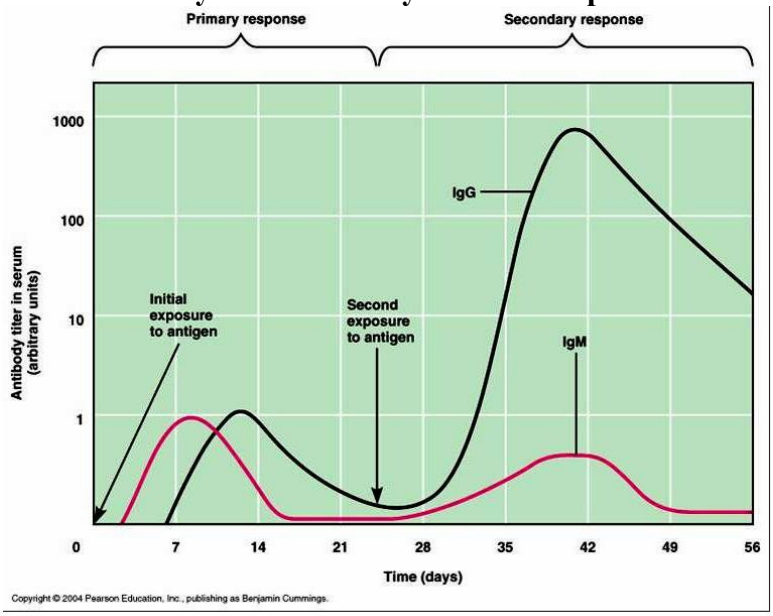
Types of immunity



Structure of antibody

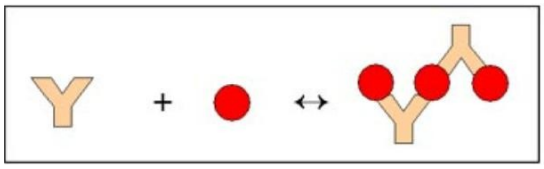


Primary and secondary immune responses



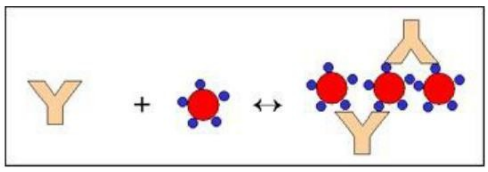
S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

Agglutination: When a particulate antigen is mixed with its antibody in presence of electrolytes at a suitable temperature and pH, then the particles are clumped or agglutinated.



Antibody Antigen Agglutination

Agglutination



Antibody Antigens on the red blood cell Passive hemagglutination

Passive hemagglutination

I. Perform the slide agglutination test to identify unknown antigens such as microorganisms from an isolated colony on the plate culture.

There are at least three genera of bacteria that can produce adequate colonies on meat peptone agar (MPA): Escherichia, Salmonella spp. and Shigella spp. Known antiserums are available for each of the 3 groups of bacteria listed above and contain antibodies against their antigenes.

Procedure:

a. Place different known antisera (E.coli, Salmonella or Shigella) on a clean glass slide.

b. Add the suspected bacteria (the unknown antigen) from an isolated colony to each drops.

c. Rotate the slide carefully for 30-60 seconds. A positive antigen-antibody reaction appears as a clumping or agglutination of the bacteria.

d. Agglutination of the bacteria indicates positive reaction. No agglutination is negative.

Salmonella antiserum E.coli antiserum Shigella antiserum

Result: _____

_____

II. Perform of the tube agglutination test to identify unknown antibodies in the patient's serum. Defin the titer of the serum.

Procedure:

a. Make a series of dilutions of the patient's serum. Include tubes with 1 ml saline for control.

b. Use perfectly clean and dry test tubes and prepare dilutions beginning with 1:2 and doubling through 1:32 or so: add 1 ml of serum to 1 ml of physiological saline and then dilute serially by mixing 1 ml diluted serum with 1 ml saline and discarding 1 ml from the last tube.

c. Add 0.1 ml of the known antigen to each serum dilution and to saline for controls.

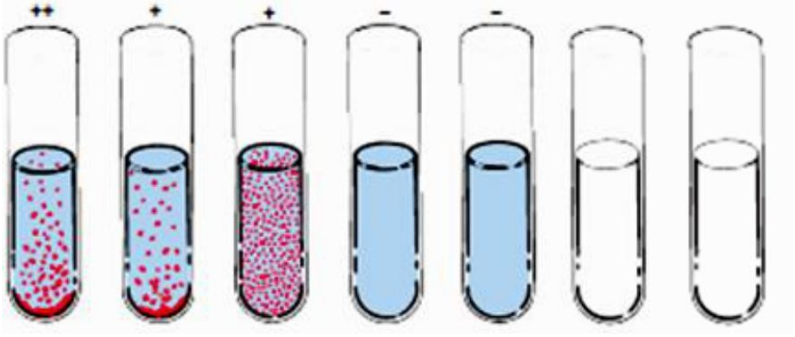
d. Incubate the test 1 hour at 37^{o}C and let tubes stand at room temperature for 15-20 minutes before reading.

e. Reading: look first at the control tubes and proceed only if they show satisfactory results. There should be no appreciable sedimentation of the bacteria.

Surname _____ Date _____

SCHEME OF TUBE AGGLUTINATION TEST

Ingredients	Tube						
	1	2	3	4	5	Control	
Physiological saline (ml)	1,0	1,0	1,0	1,0	1,0	1,0	1,0
Patient serum 1:50 (ml)	1,0 →	1,0 →	1,0 →	1,0 →	1,0	1,0	-
Diagnosticum – antigen (ml)	0,1	0,1	0,1	0,1	0,1	-	0,1
Serum dilution	1:100	1:200	1:400	1:800	1:1600	-	-



Pesult: _____.

III. Perform the ring precipitation test to identify unknown antigens.

Precipitation: When a soluble antigen combines with its antibody in presence of electrolytes (NaCl) at a suitable temperature and pH the antigen antibody complex forms insoluble precipitate.

Scheme of ring precipitation test

Ingredients	Tubes					
	1	**2**	**3**	**4**	**5**	**6**
Atniserum (precipitins)	0,5	0,5	0,5			
Normal serum				0,5	0,5	0,5
Extract under study		0,5			0,5	
Noraml extract			0,5			0,5
Extract with antigen	0,5			0,5		
Result	+	+ or -	-	-	-	-

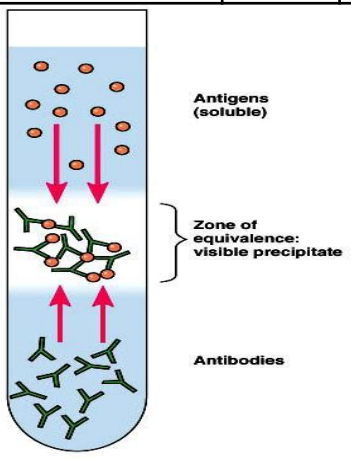


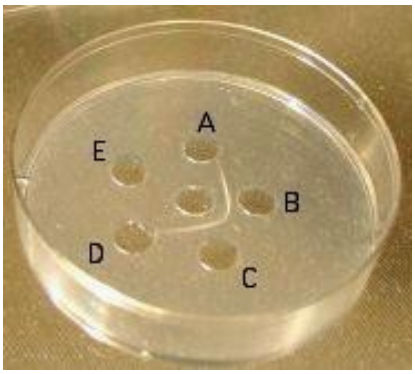
Diagramm of ring precipitation test

Positive test

S u r n a m e _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ D a t e _ _ _ _ _ _ _ _ _ _ _

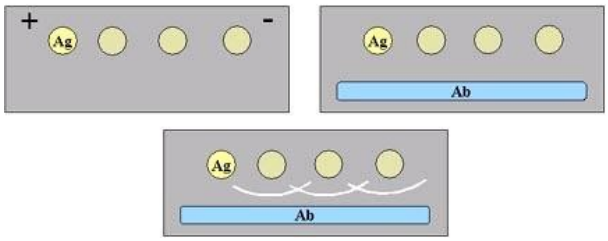
IV. Study the principle of the gel precipitation tests:

a. The double immunodiffusion assay (Ouchterlony test) is based on the principle that diffusion of the antibody and antigen (hence, double diffusion) through agar can form stable and easily observable immune complex.



Positive test: _____
Negative test: _____

b. Immunoelectrophoresis is a variation of the precipitation in gel technique. It combines precipitation with electrophoresis. A complex mixture of antigens is placed in a well punched out of an agar gel and the antigens are electrophoresed so that the antigens are separated according to their charge. Then antibodies are added. As the antibodies diffuse into the agar, precipitin lines are produced in the equivalence zone when an Ag/Ab reaction occurs. This test is used for the qualitative analysis of complex mixtures of antigens and components in a patient's serum.



ADDING THEORETICAL MATERIAL

The immune system consists of factors that provide innate and acquired immunity, and has evolved to become more specific, complex, efficient, and regulated. One of the principal functions of the human immune system is to defend against infecting and other foreign agents by distinguishing self from non-self (foreign antigens) and to marshal other protective responses from leukocytes. The immune system, if dysregulated, can react to self-antigens to cause autoimmune diseases or fail to defend against infections.

The immune system is organized into discrete compartments to provide the milieu for the development and maintenance of effective immunity. Those two overlapping compartments: the lymphoid and reticuloendothelial systems (RES) house the principal immunologic cells, the leukocytes. Leukocytes derived from pluripotent stem cells in the bone marrow during postnatal life include neutrophils, eosinophils, basophils, monocytes and macrophages, natural killer (NK) cells, and T and B lymphocytes. Hematopoietic and lymphoid precursor cells are derived from pluripotent stem cells.

Cells of the immune system intercommunicate by ligand-receptor interactions between cells and/or via secreted molecules called cytokines. Cytokines produced by lymphocytes are termed lymphokines (i.e., interleukins and interferon-γ) and those produced by monocytes and macrophages are termed monokines.

L Y M P H O I D S Y S T E M

Cells of the lymphoid system provide highly specific protection against foreign agents and also orchestrate the functions of other parts of the immune system by producing

immunoregulatory cytokines.

The lymphoid system is divided into 1) central lymphoid organs, the thymus and bone marrow, and 2) peripheral lymphoid organs, lymph nodes, the spleen, and mucosal and submucosal tissues of the alimentary and respiratory tracts.

The thymus instructs certain lymphocytes to differentiate into thymus-dependent (T) lymphocytes and selects most of them to die in the thymus (negative selection) and others to exit into the circulation (positive selection). T lymphocytes circulate through the blood, regulate antibody and cellular immunity and help defend against many types of infections.

The other classes of lymphocytes, B cells (antibody-forming cells) and natural killer (NK) cells, are thymic-independent and remain principally in peripheral lymphoid organs.

R E T I C U L O E N D O T H E L I A L S Y S T E M

Cells of the RES provide natural immunity against microorganisms by 1) a coupled process of phagocytosis and intracellular killing, 2) recruiting other inflammatory cells through the production of cytokines, and 3) presenting peptide antigens to lymphocytes for the production of antigen-specific immunity.

The RES consists of 1) circulating monocytes; 2) resident macrophages in the liver, spleen, lymph nodes, thymus, submucosal tissues of the respiratory and alimentary tracts, bone marrow, and connective tissues; and 3) macrophage-like cells including dendritic cells in lymph nodes, Langerhans cells in skin, and glial cells in the central nervous system.

M Y E L O I D C E L L S

Neutrophils: Neutrophils are the first circulating phagocytic cells recruited to the site of infection and inflammation to ingest, kill, and digest pathogens. These cells are produced from myeloid stem cells in the bone marrow. Neutrophils constitute the large number of leukocytes in the blood. After stimulation, mature neutrophils display more motility, adherence, phagocytic activity, and intracellular killing than any other type of cell. Neutrophils persist in the circulation for only several hours. Then, they are either removed by the RES or migrate into inflammatory sites.

Eosinophils: Eosinophils play a major role in the killing of parasites, particularly hemoflagellates, echinococcus, and enteric nematodes. This killing is due to a basic protein and a cationic protein contained in large cytoplasmic granules that are unique to eosinophils. These cells also play a prominent role in the pathogenesis of the allergic inflammation. These cells are induced to grow and differentiate by interleukin-5 and are recruited to inflammatory sites by agents such as platelet-activating factor from the lipoxygenase segment of the arachidonic acid pathway.

Basophils: Basophils and their tissue counterparts, the mast cells, play a major role in defense against parasites and in allergic inflammation. These cells are distinguished by many large cytoplasmic granules that contain heparin and histamine and by high affinity receptors for IgE antibodies. If these cell bound IgE antibodies are cross-linked by antigens, the cells degranulate and are activated to produce and secrete a group of low-molecular weight vasoactive mediators and certain proinflammatory cytokines, e.g. tumor necrosis factor α (TNF-α) and interleukin-5 (IL-5).

Monocytes/Macrophages: Monocytes and macrophages are involved in phagocytosis and intracellular killing of microorganisms. Macrophages process protein antigens and present peptides to T cells. These monocytes/macrophages are highly adherent, motile and phagocytic; they marshal and regulate other cells of the immune system, such as T lymphocytes; serve as antigen processing-presenting cells; and act as cytotoxic cells when armed with specific IgG antibodies.

Macrophages are differentiated monocytes, which are one of the principal cells found to reside for long periods in the RES. Macrophages may also be recruited to inflammatory sites, and be further activated by exposure to certain cytokines to become more effective in their biologic functions.

Monocytes and macrophages are activated by bacterial products such as endotoxin (lipopolysaccharides); autocrine agents, such as TNF-α, IL-1, and IL-8; cytokines such as

interferon-γ (IFN-γ) and a special group of mediators called chemokines. Activated macrophages play a prominent effector role in cellular immunity by 1) ingesting and killing pathogens, 2) clearing immune complexes, and 3) aiding in the genesis of specific immune responses by antigen presentation.

LYMPHOID CELLS

These cells provide efficient, specific and long-lasting immunity against microbes and are responsible for acquired immunity. Lymphocytes differentiate into three separate lines: thymic-dependent cells or T lymphocytes that operate in cellular and humoral immunity, B lymphocytes that differentiate into plasma cells to secrete antibodies, and natural killer (NK) cells. T and B lymphocytes are the only lymphoid cells that produce and express specific receptors for antigens.

T Lymphocytes: These cells are involved in the regulation of the immune response and in cell mediated immunity and help B cells to produce antibody (humoral immunity). Mature T cells express antigen-specific T cell receptors (TcR) that are clonally segregated (i.e., one cell lineage-one receptor specificity). Every mature T cell also expresses the CD3 molecule, which is associated with the TcR. In addition, mature T cells display one of two accessory molecules, CD4 or CD8.

T Helper Cells: These cells are the primary regulators of T cell- and B cell-mediated responses. They 1) aid antigen-stimulated subsets of B lymphocytes to proliferate and differentiate toward antibody-producing cells; 2) express the CD4 molecule; 3) recognize foreign antigen complexed with MHC class II molecules on B cells, macrophages or other antigen-presenting cells; and 4) aid effector T lymphocytes in cell-mediated immunity.

Cell-mediated immunity (delayed hypersensitivity) plays an important role in defense against many intracellular infections such as *Mycobacterium tuberculosis*. This inflammatory reaction is initiated by the recognition of specific antigens by Th1 cells. Consequently, lymphokines are generated which recruit activated macrophages to eliminate foreign antigens or altered host cells.

T Cytotoxic Cells: These cells are cytotoxic against tumor cells and host cells infected with intracellular pathogens. These cells 1) usually express CD8, 2) destroy infected cells in an antigen-specific manner that is dependent upon the expression of MHC class I molecules.

T Suppressor Cells: These cells suppress the T and B cell responses and express CD8 molecules.

Natural Killer Cells: NK cells are large granular lymphocytes that nonspecifically kill certain types of tumor cells and virus-infected cells. Killing by NK cells is enhanced by cytokines such as IL-2 and IFN-γ. NK cells are also activated by microorganisms to produce a number of cytokines [(IL-2, IFN-γ, IFN-α, and tumor necrosis factor-α (TNF-α)]. These circulating large granular lymphocytes do not express CD3, TcR or immunoglobulin, but display surface receptors (CD16) for the Fc fragment of IgG antibodies.

B Lymphocytes: These cells differentiate into plasma cells to secrete antibodies and are involved in processing proteins and presenting the resultant peptide antigen fragments in the context of MHC molecule to T cells. The genesis of μ and δ chain-positive, mature B cells from pre-B cells is antigen-independent. Pre-B cells in the bone marrow undergo gene rearrangement for IgM heavy (H) chains and consequently express those proteins in the cytoplasm (the μ chain), but no immunoglobulin light (L) chains. B cell development is characterized by recombinations of immunoglobulin H and L chain genes and expression of specific surface monomeric IgM molecules. At this stage of development, B cells are highly susceptible to the induction of tolerance. Once these cells acquire IgD molecules on their surface, they become mature B cells that are able to differentiate after exposure to antigen into antibody-producing plasma cells.

The activation of B cells into antibody producing/secreting cells (plasma cells) is antigen-dependent. Once specific antigen binds to surface Ig molecule, the B cells differentiate into plasma cells that produce and secrete antibodies of the same antigen-binding specificity.

Serology, in practice, usually refers to the diagnostic identification of antibodies or antigens in the serum.

Agglutination test

When the specific antibodies (agglutinins) bind to surface antigens of bacteria/virus or any antigens immobilized in particulate matter (such as latex particle) and cause the formation of a visible clumps, such test is called agglutination test. Agglutination test can be performed in:

- Surface of glass slides: Rapid reading/evaporation
- Test tubes: More sensitive because of longer incubation

Types of Agglutination.

- Identification of an organism with known antibodies
- Identification of serum antibodies with known antigens.

Bacterial agglutination test

1. Measure the antibody produced by the host against bacterial agglutinins
2. Best performance when used in sterile physiologic saline

Uses:

Disease diagnosis: bacterial agent is difficult to cultivate in vitro. Some examples of such diseases are tetanus (*Clostridium tetani*), yersiniosis (*Yersinia pestis*), leptospirosis (*Leptospira interrhogans*), brucellosis (*Brucella spp.)*, tularemia (*Francisella tularensis)*.

Slide Agglutination test

- Use of Antisera (Ab)
- to identify bacterial culture by causing agglutination of the organisms
- Diagnostic purpose
- Epidemiologic purpose.

Hemagglutination

1. Treated animal RBC is used as a carrier of antigen
2. Passive hemagglutination: Ag that are being bound by Ab are not the Ag of RBC but are passively bound Ag.

Examples

1. Microhemagglutination test for Syphilis (MHA-TP)
2. Hemagglutination treponemal test for Syphilis (HATTS)
3. Passive hemagglutination tests for antibody to extracellular antigen of Streptococci
4. Rubella indirect hemagglutination test
5. Hemagglutination Inhibition Test (HAI) for Avian Influenza
6. Quantitative Micro Hemagglutination Test (HA)

Latex agglutination test for Ag detection

1. Latex beads coated with specific antibody are agglutinated in the presence of homologous antigen (bacteria).
2. Used to determine the presence of capsular antigen of

1. *H. influenzae*

2. *N. meningitidis:* rapid detection of meningococcal capsular Ag in CSF.

3. *Streptococcus spp.*

Latex agglutination test for Ab detection

1. Latex particles coated with specific antigen
2. Commercially available for the accurate and sensitive detection of antibody to

1. Cytomegalovirus
2. Rubella virus
3. Varicella-zoster virus
4. Heterophile antibody of infectious mononucleosis
5. Teichoic acid antibodies of Staphylococci
6. Antistreptcoccal antibodies
7. Mycoplasma antibodies and others.

Coagglutination (COAG)

Specific antibody is bound to the surface protein A of staphylococci (Cowan type 1 strain of *Staphylococcus aureus*). Soluble microbial antigen in the specimen is mixed with the COAG reagent, resulting in the agglutination of the staphylococcal cells.

Antibody COAG Reagent + Antigen in specimen = Staphylococcal cells (Agglutinated)

Ring Precipitation Test

Precipitation reaction occurs when soluble antigen (serum protein, cell lysates or tissue lysates) binds its specific antibody and then visible precipitin appeared. The precipitation reaction can take place not only in solution, but also in semisolid media such as agar gels. The precipitation reactions include Ring precipitation, Single radial immunodiffusion, Double immunodiffusion, Rocket electrophoresis, Immunoelectrophoresis and Countercurrent electrophoresis.

Ring precipitation test is a kind of precipitation reaction, overlaying a solution of antibody with a solution of antigen, in such manner that a sharp liquid interface is formed.

Application. Identification of the specific antigen qualitatively (such as Ascoli Test bacteria typing and blood typing).

Materials:

1. Antibody: rabbit anti-human serum
2. Antigen: human serum
3. Saline
4. Micropipette, precipitate tube (inner diameter 3~4mm, one end sealed), etc.

Procedure.

1. Prepare two precipitate tubes, add about 30 μl antibody (about 1 cm high) in each tube.
2. Add 1:100 diluted equal amount of antigen on the top of the antibody slowly along the precipitate tube with another micropipette; Equal amount of saline id added on the top of the antibody in the another precipitate tube as control.
3. Place the two precipitate tubes at room temperature for 2~10 min.

Attention: Add antigen very carefully and slowly on the top of antibody along the precipitate tube.

Results. The appearance of a sharp liquid interface and white ring refers to positive result, while negative result or control tube has no white ring.

Elek Test.

Elek test is an **in vitro immunoprecipitation (immunodiffusion) test** to determine whether or not a strain of *Corynebacterium diphtheria* is toxigenic. A test strip of filter paper containing diphtheria antitoxin is placed in the center of the agar plate. Strains to be tested (patient's isolate), known positive and negative toxigenic strains are also streaked on the agar's surface in a line across the plate and at a right angle to the antitoxin paper strip.

Antitoxin diffuses away from the strip of filter paper, toxin produced by toxin-producing strains diffuse away from growth. At the zone of equivalence a precipitin line is formed.

Procedure:

1. Mix a tube of melted nutrient agar with 2 ml of sterile horse serum.
2. Rotate the tube to mix the serum and agar. Do not shake the tube.
3. Pour the mixture into a sterile petri dish.
4. Using lightly flamed forceps, lay the strip of anti-toxin impregnated filter paper across the centre of the petri dish allowing it to sink beneath the agar surface.
5. Allow the agar to set, then lift one corner of the lid and let the plate dry for 30-45 min in the incubator.
6. When dry inoculate with a toxinogenic strain of *C. diphtheriae* by streaking a single line of inoculum across the plate and paper strip at right angles to the strip.
7. Repeat this about 1 inch away from the C. *diphtheriae* inoculum with a test strain.
8. Incubate the plate for 24 hours and observe the results.

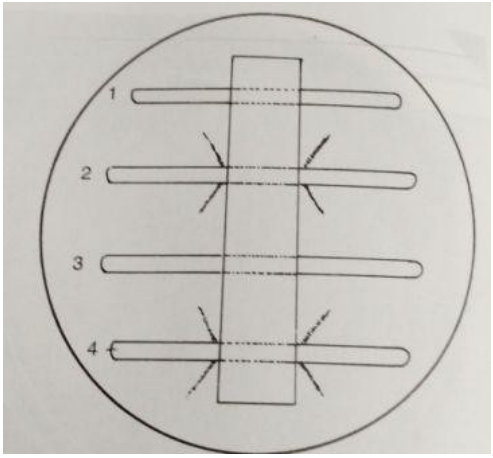
Result:

After 24 hours of incubation at 37 °C, plate is examined with transmitted light for the presence of fine precipitin lines **at 45 degree angle to the streaks.**

Positive Test: Precipitin lines form at zone of equivalence, test organism is toxigenic.

If toxin is produced by the test strain, it diffuses sideways from the streak. The antitoxin diffuses from the filter paper and where the toxin and the antitoxin meet (at zone of equivalence) a **precipitin line formed**.

The control strain also will cause a precipitate to form which will coalesce with the precipitate of the test strain to form a line of identity.



1. Line 1 is a negative control
2. Line 2 is the positive control
3. Line 3 is a test organism that is a nontoxigenic strain *C. diphtheriae*
4. Line 4 is a test organism that is a toxigenic strain of *C. diphtheriae*

Surname_____ Date_____

Protocol № 17

Theme: Studying of immunity. Complement fixation tests. Lysis test.

Bacterial lysis takes place with the participation of two components: a specific antibody contained in the immune serum and a nonspecific substance of any normal or immune serum – complement. Lysins are specific antibodies, which cause the dissolution of bacteria, plant and animal cells. It is called bacteriolysins, haemolysins, etc.

During immunization of rabbits with a suspension of sheep erythrocytes antibodies accumulate in the blood capable of altering the erythrocytes. If hemolytic serum (antibody), sheep erythrocytes (antigen) and complement are placed in a test tube in definite quantitative proportions, then in a few minutes a change will take place in the mixture. It changes from turbid red to pink (lacquered) as a result of haemolysis (hemoglobin goes out of the stroma of erythrocytes).

Complement can be considered as part of the constitutive host defense mechanisms (it is present at constitutive levels) because of its role in inflammation and phagocytosis. However, the antimicrobial activities of complement can be activated completely by reactions between antigens and antibodies and, therefore, it may play a role in the inducible defenses, as well.

Complement is an enzymatic system of serum proteins made up of 9 major components (C1 - C9) that can be stimulated in a cascading fashion (or many Ag-Ab reactions) to produce biologically active fragments that either directly attack foreign substances or enhance the functions of certain types of inflammatory leukocytes, resulting in disruption of membranes. Therefore, complement (C') may be involved in the lysis of certain bacteria, some viruses, and other microorganisms. In addition, some C' components play a part in phagocytic chemotaxis, opsonization and the inflammatory response.

The complement system consists of three recognition-stimulation pathways that are designated as the classical, alternative, and lectin pathways, either of which may lead to the formation of a cell membrane attack complex.

Complement is activated in the **classical pathway** by reactions between antibodies and antigens on the surface of a microbe by their binding to the C1q subunit of the first component of complement. The reaction between IgG and Ag activates the complement and initiates a "cascade reaction" on the surface of the microbe that results in **the principal effects of complement** which are: 1. Generation of inflammatory factors. 2. Attraction of phagocytes. 3. Enhancement of phagocytic engulfment. 4. Lysis of bacterial cells (lysozyme-mediated) or virus-infected cells.

An alternative pathway (sometimes called the "properdin pathway") of complement activation exists which is independent of immunoglobulins. Insoluble polysaccharides (including bacterial LPS, peptidoglycan and teichoic acids) can activate complement. This allows antibody-independent activation of the complement cascade that may be important in initial (pre-antibody) defense against various types of infections caused by bacteria.

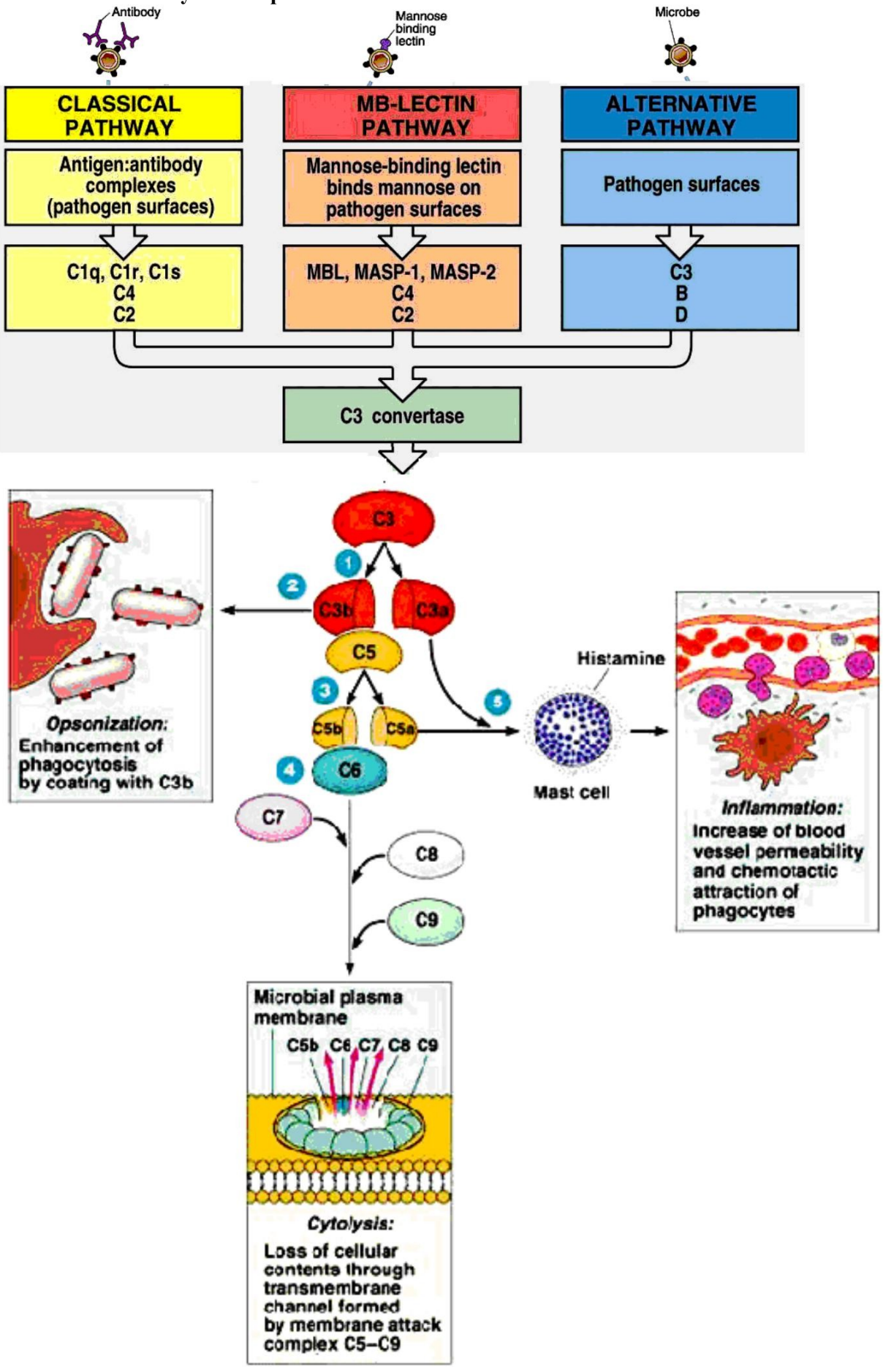
The lectin pathway is activated by lectin binding to sugars on the bacterial cell surface (mannan binding protein).

The activation of the complement system eventually leads to the formation of the **membrane attack complex** that consequently lyses cells. Once the membrane attack complex is formed, discrete holes are created in the surface membranes of the target cells. Consequently, extracellular fluid accumulates in the target cell, eventually leading to its lysis.

Antigen/Antibody complexes can also be measured by their ability to fix complement because an Ag/Ab complex will "consume" complement if it is present whereas free Ag's or Ab's do not. Tests for Ag/Ab complexes that rely on the consumption of complement are termed complement fixation tests and are used to quantitate Ag/Ab reactions.

Complement fixation tests are most commonly used to assay for antibody in a test sample but they can be modified to measure antigen.

Pathways of complement activation and role in host defense



S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

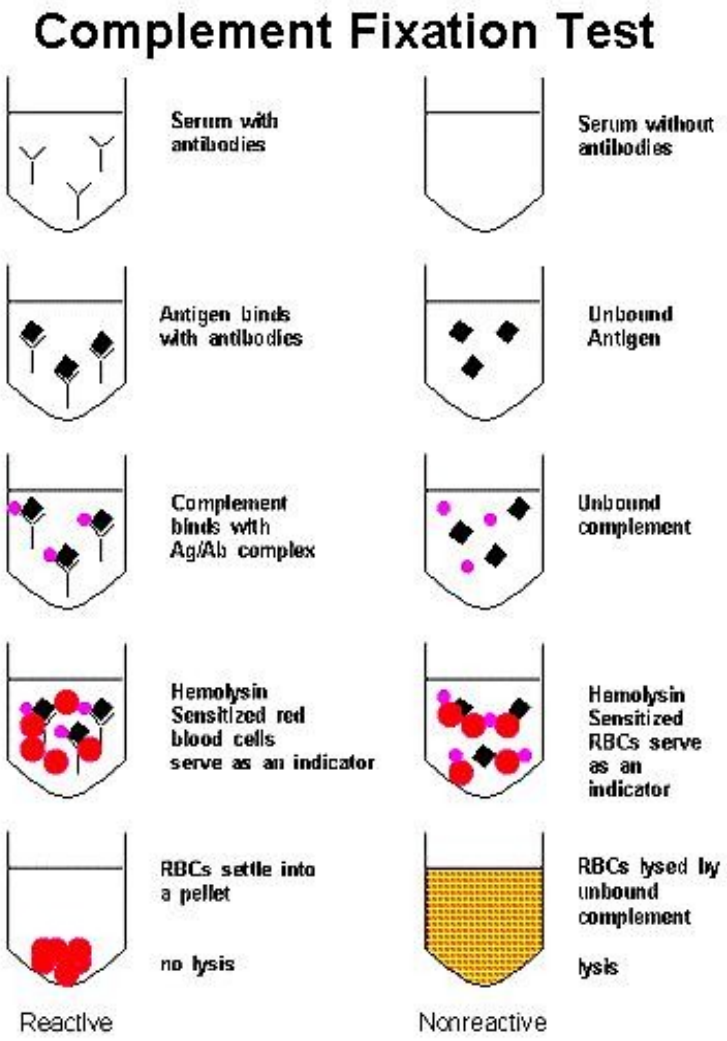
I. Perform the complement fixation test to identify unknown antibodies in a patient's serum.

Principle of complement fixation test:

a) Test serum is added to one test tube. A fixed amount of antigen is then added to both tubes. If antibody is present in the serum, immune complexes form.

b) When complement is added, if complexes are present, they fix complement and consume it.

c) Indicator cells and a small amount of antierythrocyte antibody is added to the two tubes. If there is complement present, the indictor cells will lyse (a negative test); if the complement is consumed, no lysis will occur (a positive test).



MATERIALS

1. Test serum from a patient diluted 1:10 (labelled **SERUM**); this has been heated at 56°C to inactivate complement.
2. Preparation of microbial antigen.
3. Red cells coated with antibody (Hemolytic system - HS).
4. Physiological solution (PhS).
5. Guinea pig complement diluted.

METHOD

1. Make a two-fold dilution series in PhS of the diluted test serum over seven tubes with a unit volume of 200µl. Remember to discard the residual 200µl from tube 7.

2. Add 200µl of 1:10 serum to tube 8. This will constitute the serum control, that is, the tube which contains antibody, but no antigen.

3. Add 200ml of complement to each tube.

4. Add 200µl of antigen to tubes 1 to 7 and tube 9.

5. Adjust the volumes of the control tubes (8-10) with PhS.

6. Mix and INCUBATE for 30 minutes at 37°C to allow fixation of complement.

7. Remove tubes from the waterbath and resuspend HS. Add 400µl HS to all 10 tubes.

8. Incubate at 37°C for a further 30 minutes.

Ingredients	1	2	3	4	5	6	7	8	9	10
200µl PhS	-	+	+	+	+	+	+	-	-	-
200µL serum mix and transfer discard from the 7th tube	+	+	-	-	-	-	-	+	-	-
200µl COMPLEMENT	+	+	+	+	+	+	+	+	+	+
200µl antigen	+	+	+	+	+	+	+	-	+	-

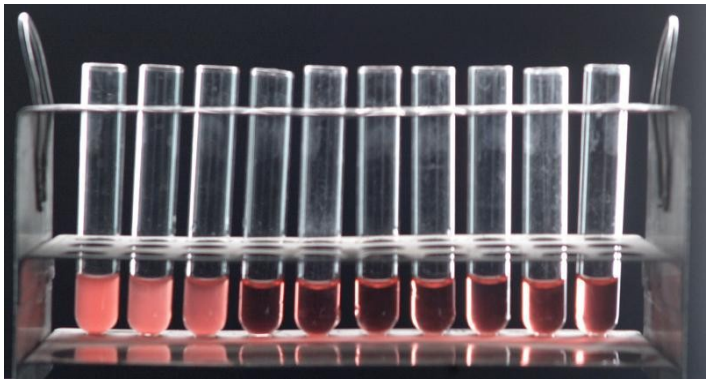
RESULTS

Record the pattern of haemolysis.

What is the complement fixation titre of antibody in the serum?

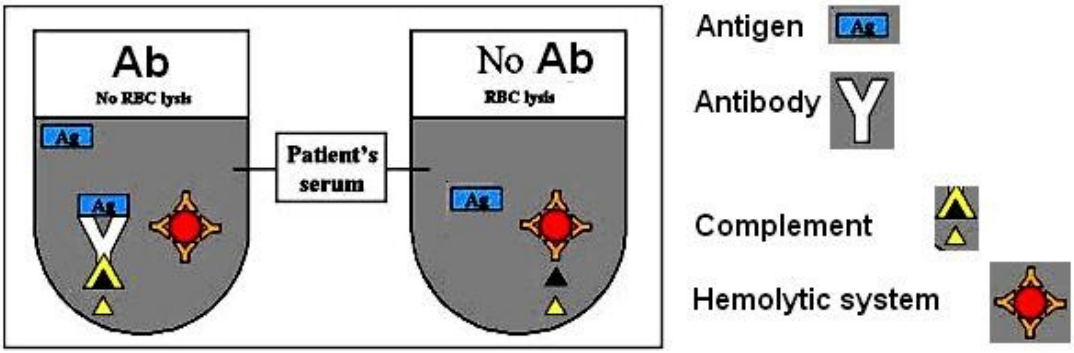
A series of control tubes have been included in your experiment (tubes 8-10). What do they control for and why?

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _ _
Do the controls indicate that the reaction was specific?
How could this test be used to diagnose whether this patient has contracted syphilis?



II. Interpret the results of the complement fixation test.

Antigen is mixed with the test serum to be assayed for antibody and Ag/Ab complexes are allowed to form. A control tube in which no Ag is added is also prepared. If no Ag/Ab complexes are present in the tube, none of the complement will be fixed. However, if Ag/Ab complexes are present, they will fix complement and thereby reduce the amount of complement in the tube. After allowing for complement fixation by any Ag/Ab complexes, a standard amount sensitized indicator cells, usually sheep red blood cells (RBC) previously coated with anti-erythrocyte complement-fixing antibodies, are added to the mixture. The amount of antibody-coated RBC is predetermined to be just enough to completely use up all the complement initially added if it were still there. If all the complement was still present (i.e. no Ag/Ab complexes formed between the Ag and Ab in question), all the RBC will be lysed. If Ag/Ab complexes are formed between that Ag and Ab in question, some of the complement will be consumed and thus when the antibody-coated RBC's are added not all of them will lyse. By simply measuring the amount of RBC lysis by measuring the release of hemoglobin into the medium, one can indirectly quantitate Ag/Ab complexes in the tube. On the other hand, if the specific antibodies are present in the test serum and complement is consumed by the immune complex, insufficient amounts of complement will be available to lyse the indicator cells. Absence of lysis shows that specific antibodies are present in the serum.



Positive test *Negative test*

ADDING THEORETICAL MATERIAL

The Complement System

The complement system consists of a group of glycoproteins in the extracellular space that can be stimulated in a cascading fashion to produce biologically active fragments that either directly attack foreign substances or enhance the functions of certain types of inflammatory leukocytes. The complement system consists of two recognition-stimulation pathways that are designated as the classical and alternative pathways, either of which may lead to the formation of a cell membrane attack complex.

The Classical Pathway

The classical pathway of the complement system may be activated by antigen-antibody complexes of the IgG, IgG3, or IgM isotypes by their binding to the C1q subunit of the first component of complement. Consequently, the C1qrs subunits of C1 form an esterase that cleaves the next component, C4, to two fragments, the larger of which, C4b, binds covalently to hydroxyl or amino groups on cellular membranes. The next component, C2, after binding to C4b is partially digested by C1s esterase to form C2b. The resultant membrane-bound complex, C4b2a, is an enzyme (C3 convertase) that cleaves C3 into two biologically active fragments, C3a and C3b.

The Alternative Pathway

The alternative pathway of the complement system is activated independently of antigen-antibody complexes. The major exogenous activators of the pathway are microbial agents and their products. The major components of the pathway are the serum protein factors B, D, and P (properdin). A small amount of C3 in the fluid phase, which normally is spontaneously activated, interacts with factor B to form C3Bb, which cleaves other C3 molecules to form C3b. C3b in turn attaches to surfaces and binds factor B. The resultant C3bB is then cleaved by factor D to form C3bBb, the C3 convertase of the alternative pathway. That enzyme is distinct from the one generated from the classical pathway but serves the same purpose. This complex then is stabilized by factor P.

The binding of C3 to factor B is prevented, particularly in the fluid phase, by a regulatory molecule, factor H. The more vigorous activation of this pathway occurs when the host is exposed to microorganisms that are poor in sialic acid. In those circumstances, the binding of factor B to C3 is favored, and the activation of the alternative pathway is not readily inhibited by factor H. Therefore, more C3b is generated and a positive amplification loop that generates more C3bBb (C3 convertase) is created. In contrast, sialic acid-rich encapsulated microorganisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Niesseria meningitidis* are incapable of activating the alternative pathway and require binding to specific IgG or IgM antibodies to activate the classical pathway and generate the C3b for phagocytosis and the formation of the membrane attack complex. The receptors for activated complement fragments are 1) CR1, principally on phagocytic cells for C3b; 2) CR2, principally on B cells for a fragment called C3d (receptor for EBV); and CR3 (Mac-1), on phagocytic and NK cells for inactivated C3b (C3bi) and C3d-g fragments.

The Membrane Attack Complex

The activation of the complement system eventually leads to the formation of the membrane attack complex that consequently lyses cells. The membrane attack complex is formed in the following manner. As a result of the formation of C3b, C5 is cleaved into two fragment, C5b and C5a. The larger fragment, C5b, combines with C6 and the complex attaches to the cell surface, where it forms the foundation for the sequential binding of C7, 8 and 9, e.g., the membrane attack complex. C3b and its degradation product, C3bi, are opsonins. C3a and C5a are chemotaxins and anaphylotoxins; C5a is the more potent of the two factors.

Once the membrane attack complex is formed, discrete holes are created in the surface membranes of the target cells. Consequently, extracellular fluid accumulates in the target cell, eventually leading to its lysis.

Complement Fixation Test.

The complement fixation test is one of the major traditional tests for the demonstration of presence of specific antigens or antibodies.

The complement fixation test (CFT) does not depend on hem-agglutinating activity of the virus, but the antibodies must fix the complement, and the sera must be free of anticomplementary activity.

The terminal components of the complement cascade, C789 (the membrane attack complex), can damage cell membranes in the presence of specific antibody, which fixes complement to the cell surface. In the CFT, erythrocytes are used as the target cell, because

complement-induced leakiness of the membrane can be visualized or measured calorimetrically as an increase in free hemoglobin. In the presence of specific antibodies to an infectious agent, any complement in the system is bound, **leaving no residual complement** for reaction with antibodies to the erythrocytes. Thus, the **presence of specific antibody is indicated by the absence of hemolysis.**

Materials and Reagents

1. Sheep erythrocytes suspension (5 % suspension of washed sheep RBCs)
2. Hemolysin (rabbit anti-sheep red-cell antibody)
3. Guinea pig complement, free of antibodies to the agent of interest

(**Note**: *Guinea pig is the commonest source of fresh complement)*

4. Barbital-buffered diluents
5. Plastic microtitre plate
6. Centrifuge adapter for microtitre plates
7. Water bath for incubation of plates
8. Color standards for judging hemolysis (prepared by lysing various concentrations of red cells)

Procedure of Complement Fixation Test

Complement Fixation Test (CFT) consists of two stage:

First step (Complement fixation stage): a known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement.

Note: patient's serum is heated at 56 °C for 30 minutes to inactivate endogenous complement which may disturb the test calibration.

1. If the **serum contains specific complement activating antibody, the complement will be activated or fixed** by the antigen-antibody complex.
2. However, if there is **no antibody** in the patient's serum, there will be no formation of antigen-antibody complex, thus complement will not be fixed but **will remain free** (In the indicator stage this complement will react with RBC coated with antibody to sheep RBC).

Second step (Indicator Stage): The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system.

1. If the complement is fixed in the first step owing to the **presence of antibody** there will be **no complement left to fix to the indicator system**. There won't be any lysis of RBCs.
2. However, if there is **no** specific antibody in the patient's serum, there will be no antigen-antibody complex, therefore, **complement will be present free or unfixed in the mixture**. This unfixed complement will now react with the antibody- coated sheep RBCs to bring about their lysis.

Results and Interpretation

• **No lysis** of sheep red blood cells (positive CFT) indicates the **presence of antibody** in the test serum,

• While **lysis** of sheep red blood cells (Negative CFT) indicates the **absence of antibody** in the serum

Notes: All reagents must be free of anticomplementary activity, the correct quantity of complement must be added, and the control specimens must react as expected.

Materials required for Quality Control

1. Known positive antibody or antigen
2. Known negative antibody or antigen
3. Serum control without antigen (to detect anticomplementary activity)
4. Antigen controls without serum (to detect anticomplementary activity)
5. Tissue control (the cells or tissue in which the antigen was prepared)
6. Buffer control without antigen or antibody
7. Back titration of complement to document the use of 5CH50 units

Controls should be used along with the test to ensure that

•Antigen and serum are not anticomplimentary

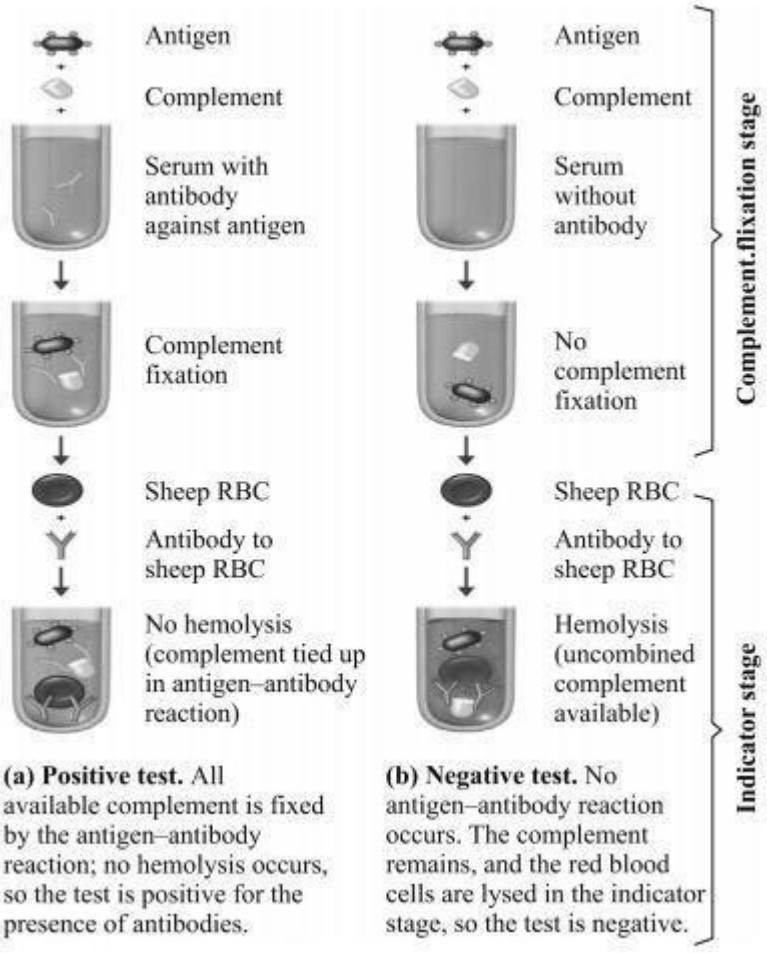
- The appropriate amount of complement is used and
- The sheep red blood cells do not undergo autolysis

Advantages of Complement Fixation Test

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical

Disadvantages of Complement Fixation Test

1. Not sensitive – cannot be used for immunity screening
2. Time consuming and labor intensive
3. Often non-specific e.g. cross-reactivity between HSV and VZV



(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

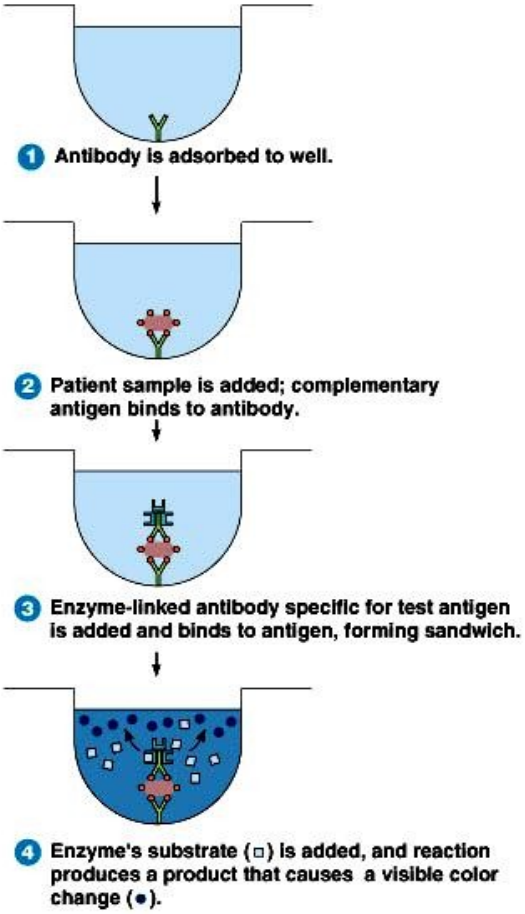
Surname_____ Date_____

Protocol № 18

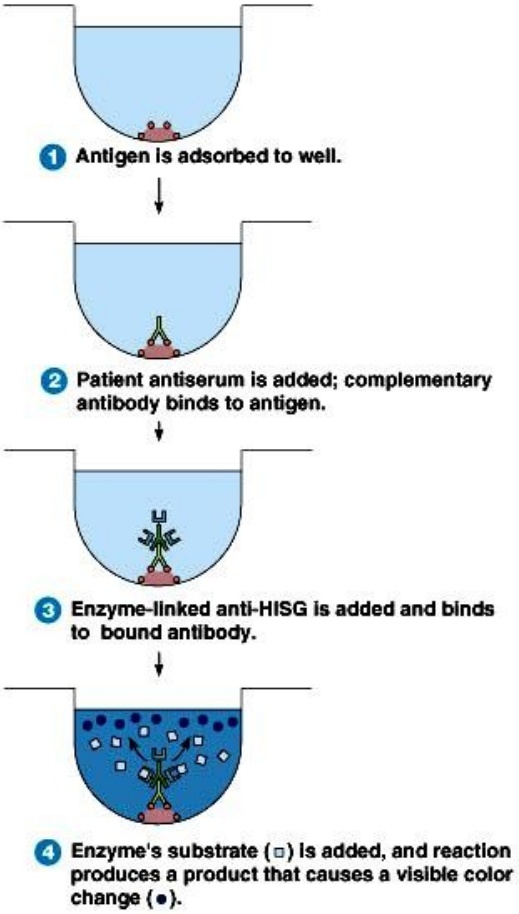
Theme: Studying of immunity. Serological "tagged antibody" tests. Polymerase chain reaction.

The direct enzyme-linked immunosorbent assay or the double antibody sandwich assay (ELISA) is used for the detection of antigens. In this assay, specific antibody is placed in wells of a microtiterplate (or it may be attached to a membrane). The antibody is absorbed onto the walls, sensitizing the plate. A test antigen is then added to each well. If the antigen reacts with the antibody, the antigen is retained when the well is washed to remove unbound antigen. An antibody-enzyme conjugate specific for the antigen is then added to each well. The final complex is formed of an outer antibody-enzyme, middle antigen, and inner antibody—that is, it is a layered (Ab-Ag-Ab) sandwich. A substrate that the enzyme will convert to a colored product is then added, and any resulting product is quantitatively measured by optical density scanning of the plate. If the antigen has reacted with the absorbed antibodies in the first step, the ELISA test is positive. If the antigen is not recognized by the absorbed antibody, the ELISA test is negative because the unattached antigen has been washed away, and no antibody-enzyme is bound.

The indirect enzyme-linked immunosorbent assay detects antibodies rather than antigens. In this assay, antigen in appropriate sensitizing buffer is incubated in the wells of a microtiter plate and is absorbed onto the walls of the wells. Free antigen is washed away. Test antiserum is added, and if specific antibody is present, it binds to the antigen. Unbound antibody is washed away. Alternatively the test sample can be incubated with a suspension of latex beads that have the desired antigen attached to their surface. After allowing time for antibody-antigen complex formation, the beads are trapped on a filter and unbound antibody is washed away. An anti-antibody that has been covalently coupled to an enzyme is added next.



A positive direct ELISA to detect antigens.



A positive indirect ELISA to detect antibodies

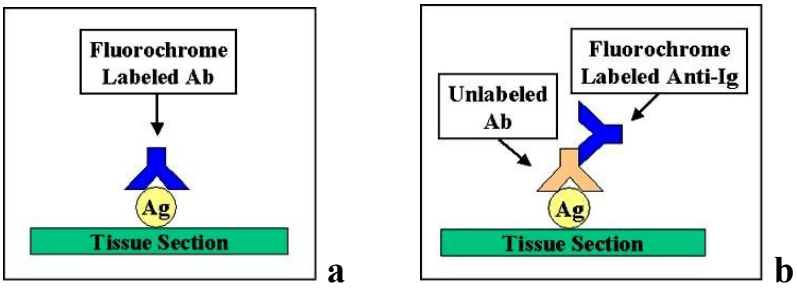
The antibody-enzyme complex (the conjugate) binds to the test antibody, and after unbound conjugate is washed away, the attached ligand is visualized by the addition of a chromogen and a substrate. The substrate for horseradish peroxidase is hydrogen peroxide. A chromogen is a colorless substrate acted on by the enzyme portion of the ligand to produce a colored product. The chromogene

for HRP is the tetramethylbenzidine (a colorless substance). If enzyme conjugate is present the enzyme HRP will reduce the substrate peroxide, which concurrently oxidizes the tetramethylbenzidine to a green color. Any resulting product is quantitatively measured by optical density scanning of the plate with a microtiter plate ELISA reader.

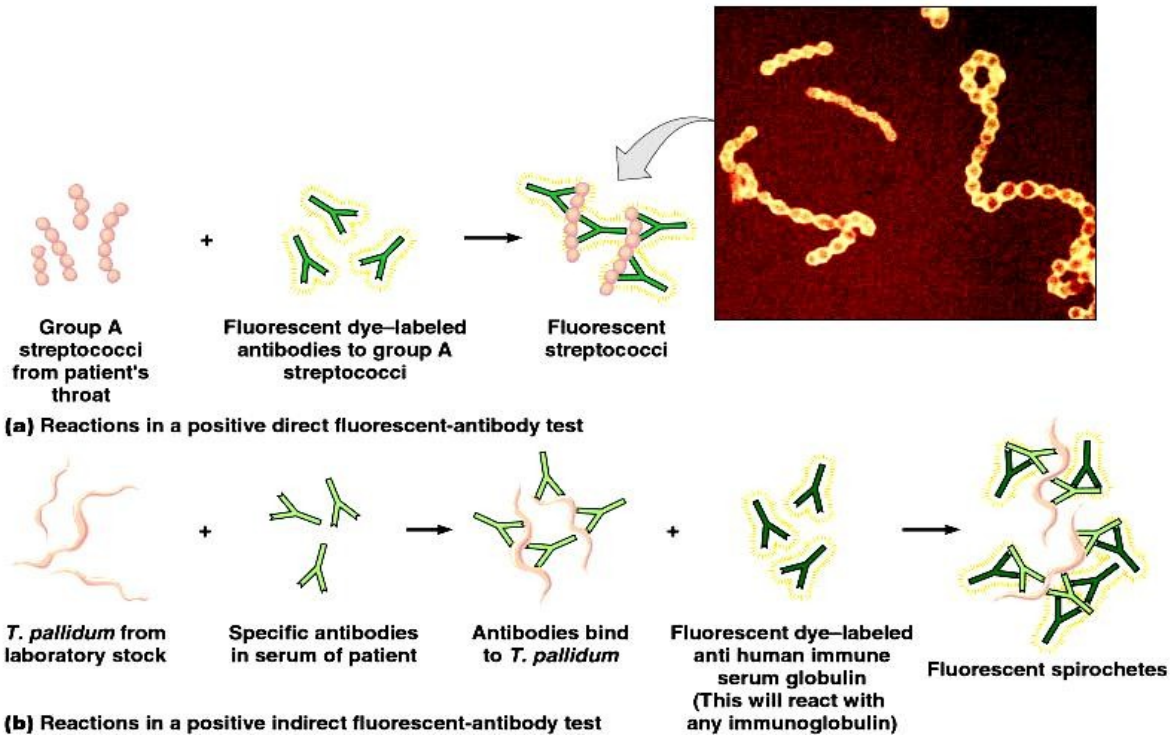
In Immunofluorescence dyes such as rhodamine B or fluorescein isothiocyanate can be coupled to antibody molecules without changing the antibody's capacity to bind to a specific antigen. Fluorochromes also can be attached to antigens. There are two main kinds of fluorescent antibody assays: direct and indirect.

Direct Immunofluorescence involves fixing the specimen (cell or microorganism) containing the antigen of interest onto a slide. Fluorescein-labeled antibodies are then added to the slide and incubated. The slide is washed to remove any unbound antibody and examined with the fluorescence microscope for a yellow-green fluorescence. The pattern of fluorescence reveals the antigen's location.

Indirect Immunofluorescence is used to detect the presence of antibodies in serum following an individual's exposure to microorganisms. In this technique a known antigen is fixed onto a slide. The test antiserum is then added, and if the specific antibody is present, it reacts with antigen to form a complex. When fluorescein-labeled anti-immunoglobulin is added, it reacts with the fixed antibody. After incubation and washing, the slide is examined with the fluorescence microscope. The occurrence of fluorescence shows that antibody specific to the test antigen is present in the serum.



Direct (a) ad indirect (b) immunofluorescence method



For **the radioimmunoassay** (RIA) radioactively labeled antibodies or antigens are used. The excess radioactive antigens or antibodies are washed away and the tubes are then counted using a gamma counter.

The **polymerase chain reaction** (**PCR**) is a biochemistry and molecular biology technique for exponentially amplifying a fragment of DNA, via enzymatic replication, without using a living

organism. PCR can be used for amplification of a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece.

PCR, as currently practiced, requires several basic components. These components are:

DNA template that contains the region of the DNA fragment to be amplified.

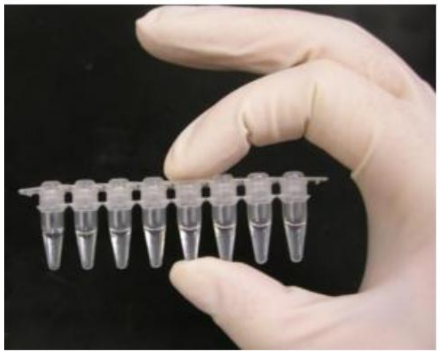
One or more *primers,* which are complementary to the DNA regions at the 5' and 3' ends of the DNA region that is to be amplified.

DNA polymerase (e.g. *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70°C), used to synthesize a DNA copy of the region to be amplified

Deoxynucleotide triphosphates, (dNTPs) from which the DNA polymerase builds the new DNA.

Buffer solution, which provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

The PCR is carried out in small reaction tubes (0.2-0.5 ml volumes), containing a reaction volume typically of 15-100 µl, that are inserted into a thermal cycler. This is a machine that heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. Most thermal cyclers have heated lids to prevent condensation on the inside of the reaction tube caps. Alternatively, a layer of oil may be placed on the reaction mixture to prevent evaporation.



A strip of eight PCR tubes

A thermal cycler for PCR

The polymerase chain reaction (PCR) technology is carried out in 3 stages.

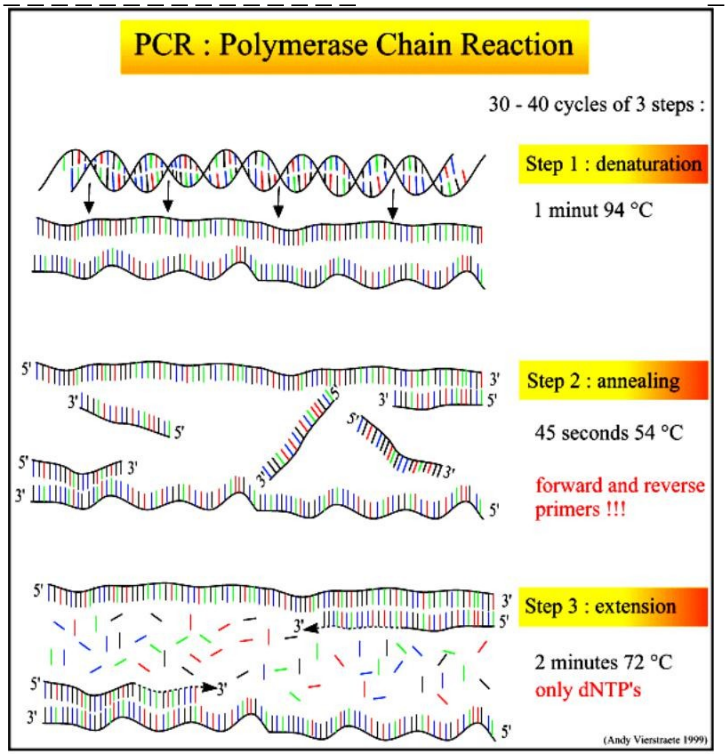
First, DNA is isolated from a cell and is heated to approximately 100 ^{0}C, causing the two strands to come apart as the hydrogen bonds between A-T and G-C break.

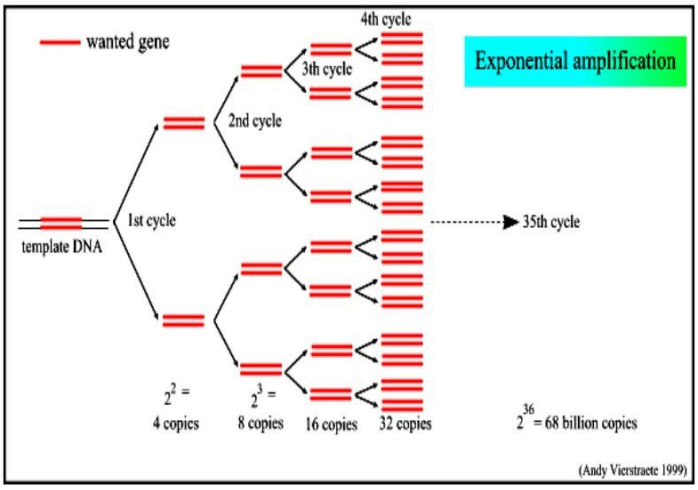
In the second step, the temperature is lowered and two short pieces of DNA, termed primers, are added. The primers are complementary to the two ends of the opposite strands of the DNA that are to be copied, termed the target DNA. The two primers renature by hydrogen bonding to their complementary nucleotide sequences.

The third step is the actual synthesis of a complementary strand of new DNA, which is an extension of each annealed primer. This synthesis requires a DNA polymerase that is not destroyed at high temperature and the nucleotide substrates for the reaction. For each primer that is annealed to a single-stranded DNA molecule is synthesized. Each new strand consists of added primer at one end, to which is joined a string of nucleotides complementary to the opposite strand of DNA, to which the primer is complementary.

After the first cycle, for 2 strands of original DNA, there will be a total of 4 strands; after the next cycle, there will be a total of 8.

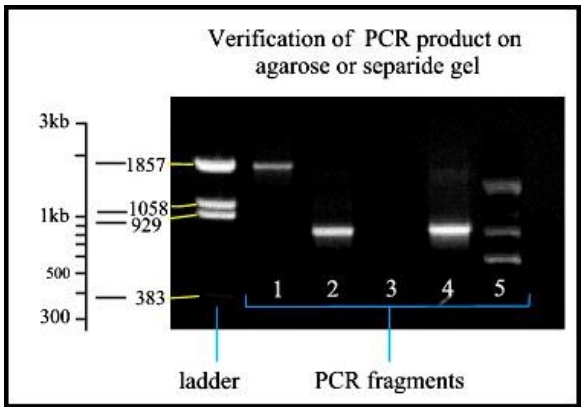
Another cycle is then initiated by raising the temperature to separate all of the DNA strands. An essential feature of the polymerase chain reaction is that the strands newly synthesized in the first cycle can serve as template strands foe the second cycle. The cycle is then repeated for the third time and so on. The sequences can be as short as 50 nucleotides or over 2.000 nucleotides in length. A single molecule of DNA can give rise to more than one billion molecules in an hour since each cycle of heating, cooling, and doubling the primed segment of DNA can occur in 15 seconds.





Further, the created copies of DNA are identified by gel electrophoresis with etidium bromide, dye which can combine with DNA at bright in ultraviolet light. Presence of brightening line in gel confirms the presence of DNA in material.

Ethidium bromide-stained PCR products after Gel electrophoresis.



To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is commonly employed for size separation of the PCR products. The size(s) of PCR products is thereby determined by comparison with a *DNA ladder*, which contains DNA fragments of known size, ran on the gel alongside the PCR products. In samples #1 and 3 the gene was not amplified by PCR, whereas bands for tissue #2 and #4 indicate successful amplification of the IGF gene. A positive control, and a DNA ladder containing DNA fragments of defined length (last lane to the left) to estimate fragment sizes in the experimental PCRs, were also ran on this gel. The ladder is a mixture of fragments with known size to compare with the PCR fragments

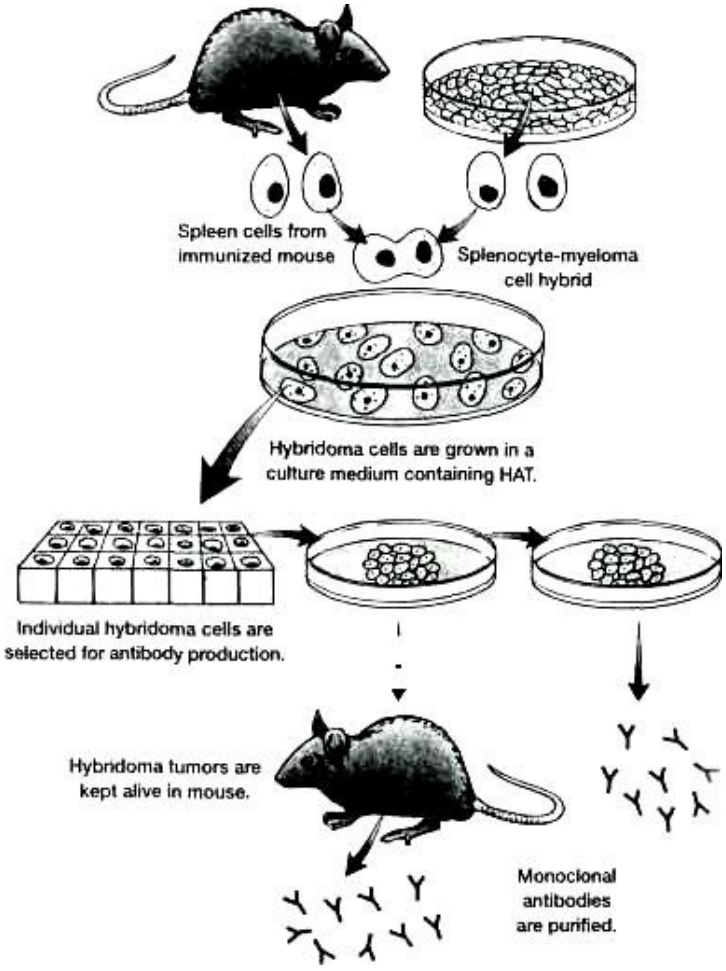
Surname_____ Date_____

Protocol № 19, part 1

Theme: Immune sera. Neutralization test. Titration of an antitoxic serum.

Immune sera are employed for treatment and prophylaxis of different infectious diseases and for diagnostic purposes.

1. Preparation of known antisera in animals involves inoculating animals with specific known antigens such as a specific strain of a bacterium. After the animals immune responses have had time to produce antibodies against that antigen, the animal is bled and the blood is allowed to clot. The resulting liquid portion of the blood is the serum and it will contain antibodies specific for the injected antigen. However, one of the problems of using antibodies prepared in animals (by injecting the animal with a specific antigen and collecting the serum after antibodies are produced) is that up to 90% of the antibodies in the animal's serum may be antibodies the animal has made "on its own" against environmental antigens, rather than those made against the injected antigen.



2. Preparation of known antibodies by monoclonal antibody technique. Monoclonal antibodies are antibodies of a single specific type. In this technique, an animal is injected with the specific antigen for the antibody desired. After appropriate time for antibody production, the animal's spleen is removed. The spleen is rich in plasma cells and each plasma cell produces only one specific type of antibody. However, plasma cells will not grow artificially in cell culture. Therefore, a plasma cell producing the desired antibody is fused with a myeloma cell, a cancer cell from bone marrow, which will grow rapidly in cell culture, to produce a hybridoma cell (it is hybrid of the two cells). The fusion mixture is then transferred to a culture medium containingacombinationof hypoxanthine, aminopterin, and thymidine (HAT). Aminopterin is a poison that blocks a specific metabolic pathway in cells. Myeloma cells lack an enzyme that allows their growth in the presence of aminopterin. However, the pathway is by-passed in spleen cells providedwiththeintermediate metabolites hypoxanthine and thymidine. As a result the hybridomas grow in the HAT medium but the myeloma cells die because they have a metabolic defect and cannot employ the bypass pathway.

When the culture is initially established using the HAT medium, it contains spleen cells, myeloma cells, and hybridomas. The unfused spleen cells die naturally in culture within a week or two, and the myeloma cells die in the HAT as just described. In contrast, the fused cells survive because they have the immortality of the myeloma and the metabolic bypass of the spleen sells. Some hybridomas that have the antibody-producing capacity of the original spleen cells are randomly placed in culture wells. The wells are individually tested for production of the desired antibody, and, if positive, the cells within the well are cloned. The clone is immortal and produces monoclonal antibody.

The hybridoma cell has the characteristics of both parent cells. It will produce the specific antibodies like the plasma cell and will also grow readily in cell culture like the myeloma cell. The hybridoma cells are grown artificially in huge vats where they produce large quantities of the specific antibody.

Monoclonal antibodies are now used routinely in medical research and diagnostic serology and are being used experimentally in treating certain cancers and a few other diseases.

3. Classification of immune sera.

Therapeutic and prophylactic sera are produced in a purified state. They are treated by precipitating globulins with ammonium sulphate, by fractionation, by the method of ultracentrifugation, electrophoresis and enzymatic hydrolysis, which allow the removal of up to 80 per cent of unrequired proteins. They have a less distinct toxic and allergic action.

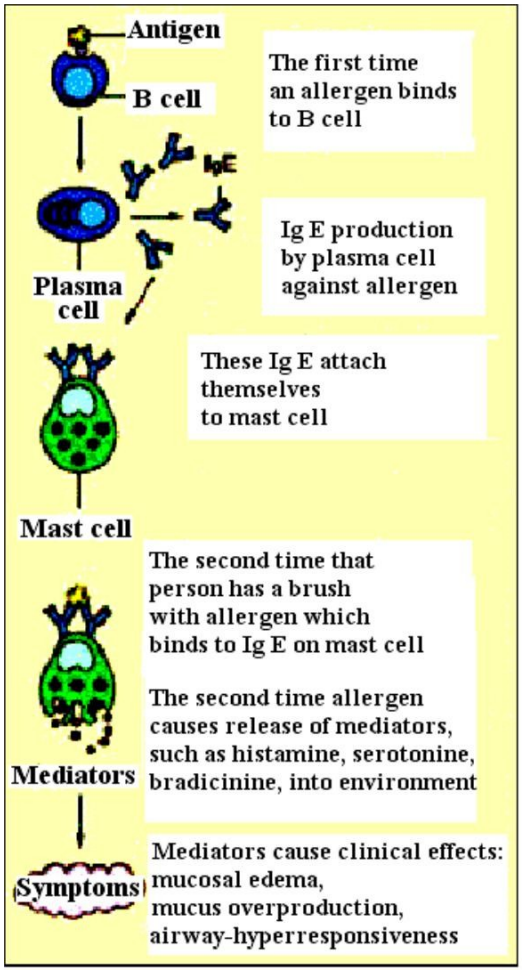
Sera thus produced are subdivided into antitoxic and antimicrobial sera. Antitoxic sera include antidiphtheritic, antitetanic sera and sera effective against botulism, anaerobic infections, and snake bite. Antimicrobial sera are used against anthrax, encephalitis, measles and influenza in the form of globulins and gamma globulins.

Gamma globulins are used for prophylactic purposes against measles, poliomyelitis, whooping cough, virus hepatitis, and smallpox. Gamma globulin is used together with vaccine against rabies. Specific gamma globulins with a directed effect are obtained from donors immunized against the given infection. Such gamma globulins contain a higher titre of antibodies.

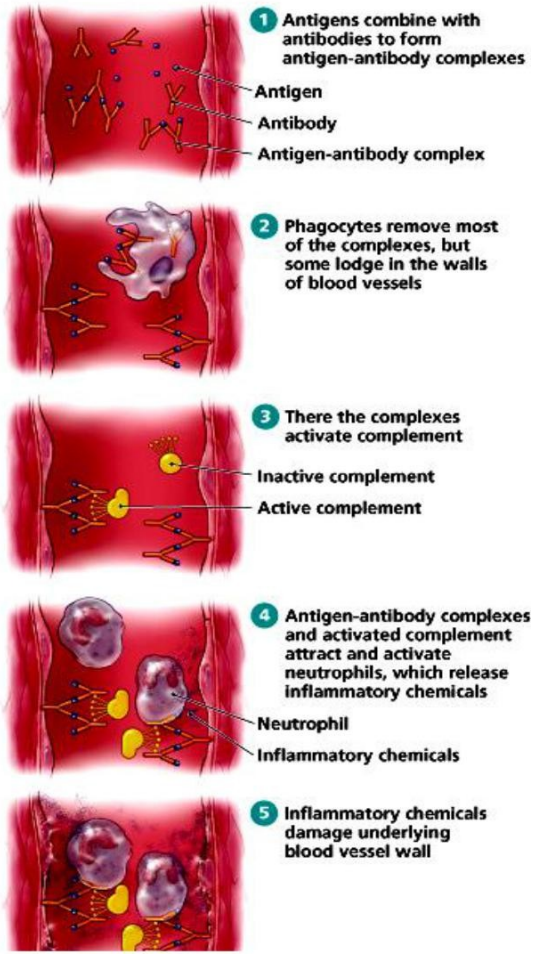
Diagnostic sera are used in serological reactions to determine unknown antigen or unknown antibodies in patient's serum.

4. Allergic reactions to the injection of immune sera.

Reaction type I



Reaction type III



Repeated injection of foreign proteins (sera) may induce allergic reaction of **immediate action (hypersensitivity type I) or anaphylaxis**. In the mechanism of anaphylaxis a definite role is played by the reaction of the antigen and antibody Ig E in tissues attended with the production of serotonin, heparin, bradykinin, etc. as a result of which a pathological process develops in the tissues and the smooth muscles contract. A preliminary desensitization according to Bezredka's method is necessary.

Serum sickness (immune complex reaction, type III) develops within 8-12 days after a single primary introduction usually of large doses of serum (from 10 ml and more). The mechanism of serum sickness is based on the interaction of the antigen and Ig G and formation immune complexes with absorb on vessels walls and cause inflammatory reaction. Bezredka's method does not avert serum sickness.

Surname_____ Date_____

Protocol № 19, part 2

Theme: Vaccines. Phagocytosis.

Immunization or vaccination is the means of providing specific protection against most common and damaging pathogens.

Artificially acquired active immunity: Immunization may be achieved by administering live or dead pathogens or their components.

Killed (inactivated) vaccines: These are preparations of the normal (wild type) infectious, pathogenic virus or bacterium that has been rendered non-pathogenic, usually by chemical treatment such as with formalin that cross-links viral or bacterial proteins.

Killed (heat, chemical or UVirradiation) viral vaccines include those for polio (Salk vaccine), influenza, rabies, hepatitis A, etc. Most bacterial vaccines are killed organisms (typhoid, cholera, plague, pertussis, *etc*.).

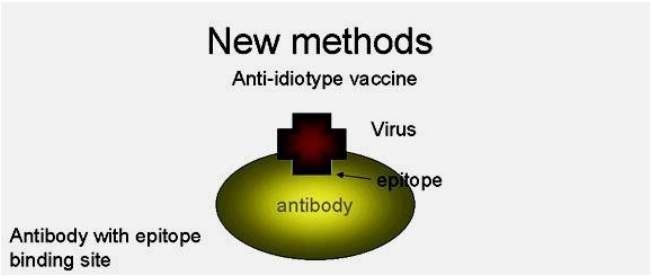
Attenuated (live) vaccines: These are live virus particles or bacterial cells that grow in the vaccine recipient but do not cause disease because the vaccine virus or bacterium has been altered (mutated) to a non-pathogenic form; for example, its tropism has been altered so that it no longer grows at a site that can cause disease.

Live vaccines are used against a number of viral infections (polio (Sabin vaccine), measles, mumps, rubella, chicken pox, yellow fever, *etc*.). The only example of live bacterial vaccine is one against tuberculosis (*Mycobacterium bovis*: BCG).

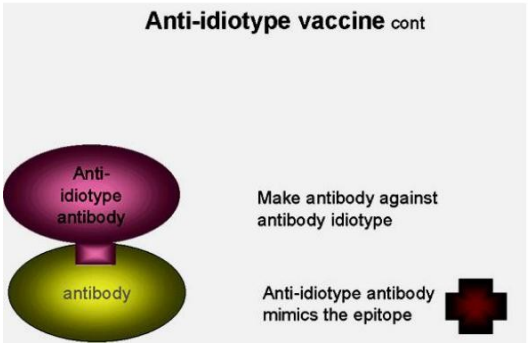
Sub-unit vaccines: These are purified components of the virus, such as a surface antigen, or bacterial cell wall components (influenza, haemophilus, pertussis, meningococcus, pneumococcus, etc.).

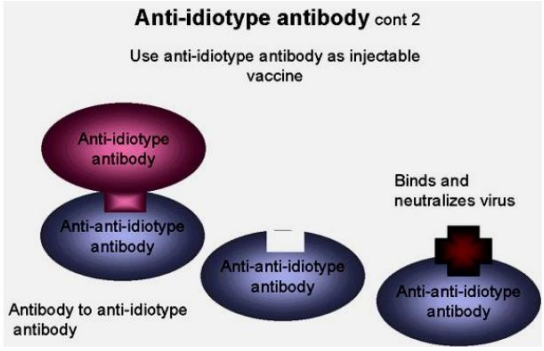
Toxoid: This is modified form of exotoxin of bacteria. When the pathogenic mechanism of an agent involves a toxin, a modified form of the toxin (toxoid) is used as a vaccine (*e.g.*, diphtheria, tetanus, cholera).

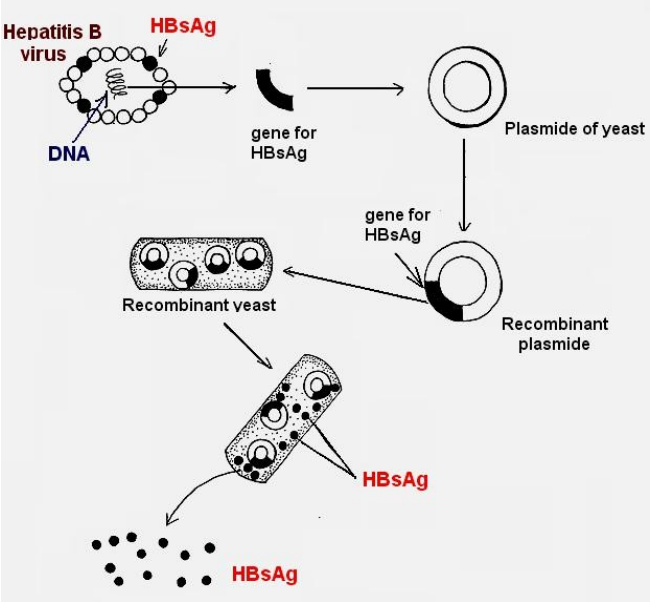
Recombinant vaccines: Some viral vaccines (hepatitis-B, rabies, *etc*.) consist of antigenic proteins cloned into a suitable vector (*e.g.*, yeast).



Anti-idiotype vaccines. An antigen binding site in an antibody is a reflection of the three-dimensional structure of part of the antigen, that is of a particular epitope. This unique amino acid structure in the antibody is known as the idiotype, which can be thought of as a mirror of the epitope in the antigen. Antibodies (anti-ids) can be raised against the idiotype by injecting the antibody into another animal. This gives us an anti-idiotype antibody and this, therefore, mimics part of the three dimensional structure of the antigen, that is, the epitope. This can be used as a vaccine. When the anti-idiotype antibody is injected into a vaccine, antibodies (anti-anti-idiotype antiobodies) are formed that recognize a structure similar to part of the virus and might potentially neutralize the virus. This happens: Anti-ids raised against antibodies to hepatitis B S antigen elicit anti-viral antibodies.







Recombinant or genetics-engeneering vaccines are based on the deliberate introduction of a DNA plasmid into the vaccine. The plasmid carries a protein-coding gene that transfects cells *in vivo* at very low efficiency and expresses an antigen that causes an immune response. These are often called DNA vaccines but would better be called DNA-mediated or DNA-based immunization since it is not the purpose to raise antibodies against the DNA molecules themselves but to get the protein expressed by cells of the vaccine. Usually, muscle cells do this since the plasmid is given intramuscularly. It should be noted that the plasmid does not replicate in the cells of the vaccine, only protein is produced.

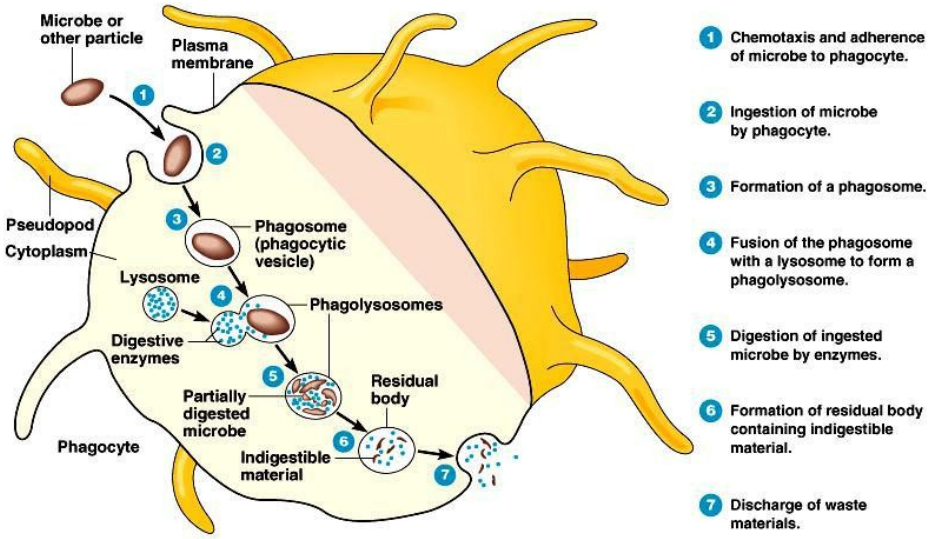
Phagocytosis

Phagocytosis is a defense adaptation which entails the seizure and digestion of foreign particles by phagocytes.

Phagocytes are a class of cells, which are capable of ingestion (engulfment) and destruction of microorganisms that are responsible for inciting the inflammatory response. First to accumulate around the invaders and initiate the phagocytic process are neutrophils. Later, local and blood-borne macrophages also migrate to the tissue site and initiate phagocytosis.

The total pool of macrophages is referred to as the system of mononuclear phagocytes. The system is scattered throughout connective tissue, basement membranes of small blood vessels, liver sinusoids, the spleen, lung, bone marrow and lymph nodes. Monocytes from the blood migrate into virtually every organ in the body where they mature into fixed macrophages. In the lymph nodes, they function as scavengers to remove foreign material from the circulation. Compared to neutrophils, macrophages are long-lived cells. As phagocytes, neutrophils play a more important role in the acute stages of an infection, while macrophages are principally involved in chronic types of infections. However, macrophages have another indispensable function in host defense: they "process" the antigenic components of infective agents and present them to lymphocytes, a process that is usually required for the initiation of the immune responses of the host. Macrophages are among an elite corps of antigen-presenting cells or APC's.

Steps involved in the phagocytic process in macrophages



Besides complete phagocytosis incomplete phagocytosis is observed in certain diseases (gonorrhoea, leishmaniasis, tuberculosis, leprosy) in which microorganisms are absorbed by phagocytes, but do not perish, are not digested, and in some cases reproduce.

Surname_____ Date_____

Protocol № 20

Theme: Antibiotics.

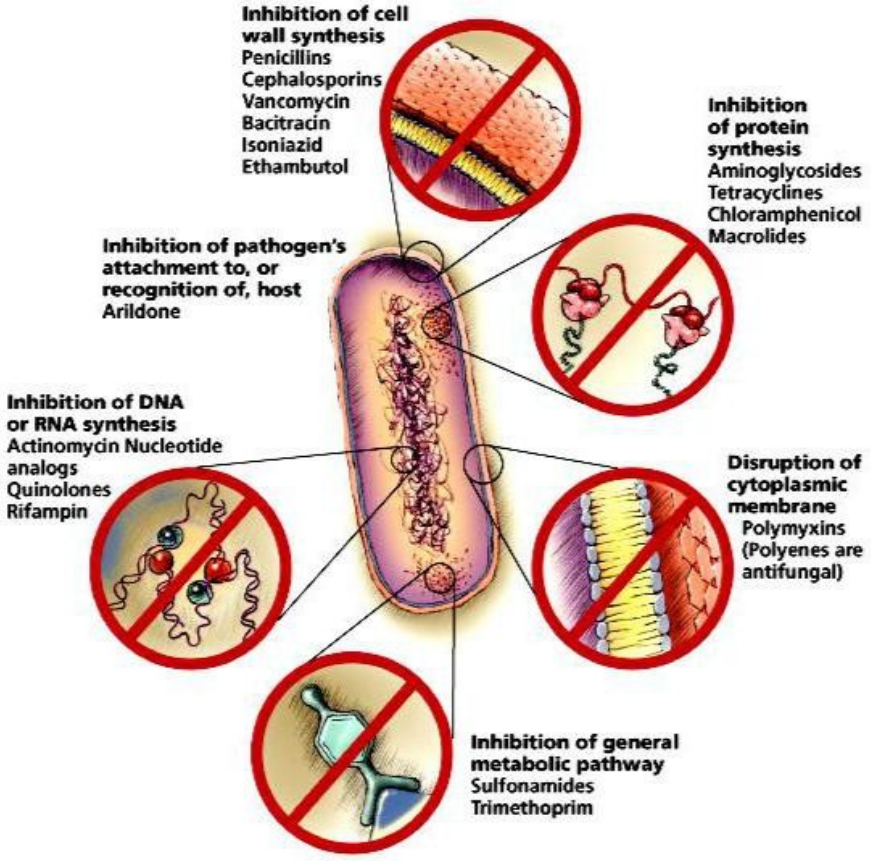
Antibiotics - substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms (in recent years many antibiotics have been obtained semisynthetically or synthetically). Antibiotics are classified according to origin, the chemical structure of the drug, the molecular mechanism of action, the spectrum of activity exerted on the cells.

In fact, only 3 major groups of microorganisms have yielded useful antibiotics. The **saprophytic molds *Penicillium* and *Cephalosporium*** produce Beta-lactam antibiotics: penicillin, cephalosporin, and their relatives. **Actinomycetes, mainly *Streptomyces* species** (filamentous, branching soil bacteria) produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, ivermectin, rifamycins, and most other clinically-useful antibiotics. Spore-forming, rod-shaped, Gram-positive bacteria of **the genus Bacillus**, such as *Bacillus polymyxa* and *Bacillus subtilis,* which produce polypeptide antibiotics (e.g. polymyxin and bacitracin.

According to the character of action some antimicrobial agents are **cidal in action**: they kill microorganisms (e.g., penicillins, cephalosporins, streptomycin, neomycin). Others are **static in action**: they inhibit microbial growth long enough for the body's own defenses to remove the organisms (e.g., tetracyclines, erythromycin, sulfonamides).

Antimicrobial agents also vary in their spectrum. Drugs that are effective against a variety of both gram-positive and gram-negative bacteria are said to be **broad spectrum** (e.g., tetracycline, streptomycin, cephalosporins, ampicillin, sulfonamides). Those effective against just gram-positive bacteria, just gram negative bacteria, or only a few species are termed **narrow spectrum** (e.g., penicillin G, erythromycin, clindamycin, gentamicin).

Antimicrobial medicines act by a number of different **mechanisms**, interfering with specific processes that are essential for growth and/or division ob bacterial cell. They can be separated into groups such as inhibitors of bacterial and fungal cell walls, inhibitors of cytoplasmic membranes, inhibitors of nucleic acid synthesis, and inhibitors of ribosome function.



For some microorganisms, susceptibility to chemotherapeutic agents is predictable. However, for many microorganisms (*Pseudomonas, S. aureus,* and gram-negative enteric bacilli such as *Escherichia coli, Proteus*, etc.) there is no reliable way of predicting which antimicrobial agent will be effective in a given case. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. Because of this, antibiotic susceptibility testing is often essential in order to determine which antibiotic to use against a specific strain of bacterium.

Tube dilution tests. In this test, a series of culture tubes are prepared, each containing a liquid medium and a different concentration of a chemotherapeutic agent. The tubes are then inoculated with the test organism and incubated for 16-20 hours at 35^0 C. After incubation, the tubes are examined for turbidity (growth). The lowest concentration of chemotherapeutic agent capable of preventing growth of the test organism is the minimum inhibitory concentration (MIC).

Subculturing of tubes showing no turbidity into medium lacking the chemotherapeutic agent can determine **the minimum bactericidal concentration** (MBC) - the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures.

Although often used in the quantitative sense, the word sensitive is also used in a qualitative sense to describe organisms susceptible to concentrations of antimicrobial drugs known to occur in the blood of patients under treatment. The word resistant is applied to microorganisms requiring substantially higher concentrations. Thus, an organism with an MIC of only 20 micrograms per ml of polymyxin would nevertheless be called resistant, since blood levels of this drug are usually lower than 5 micrograms per ml. Microorganisms requiring inhibitory concentrations on the borderline between sensitive and resistant are often called intermediate.

The agar diffusion test (Bauer-Kirby test) - a procedure commonly used in clinical labs to determine antimicrobial susceptibility. In the development of this method, a single high-potency disc of each chosen chemotherapeutic agent was used. Zones of growth inhibition surrounding each type of disc were correlated with the minimum inhibitory concentrations of each antimicrobial agent (as determined by the tube dilution test). The MIC for each agent was then compared to the usually-attained blood level in the patient with adequate dosage. Categories of "Resistant," "Intermediate," and "Sensitive" were then established.

Antibiotics may cause side effects or complication:

Side effects/Toxic effects	Examples
Overgrowth of pathogens (disbacteriosis)	Vaginal (*Candida*)
Pseudomembranous colitis	*Clostridium difficile*
Nephrotoxicity	Polypeptides, Aminoglycosides
Ototoxicity - 8th cranial nerve	Aminoglycosides
Ophthalmic toxicity	Ethambutol
Aplastic anemia (toxic effect on the haematopoietic organs)	Chloramphenicol (Levomycetin)
Anaphylactic reactions	Penicillin
Bone seeking	Tetracycline

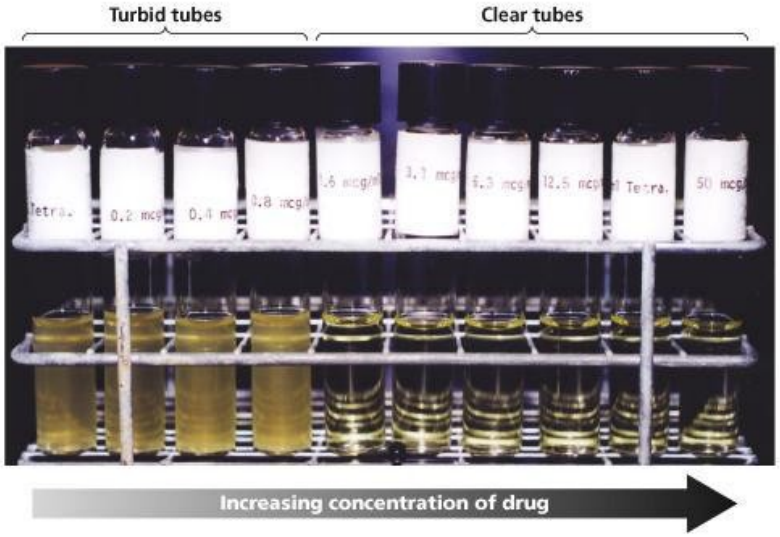
Of the great hazard is the formation of resistant bacteria to antibiotics, which lead to the formation of latent forms of infections marked by recurrences and exacerbations.

I. Performing of the tube dilution test to determine sensitivity of microorganisms to antibiotics. Defining of the minimum inhibitory and bactericidal concentrations.

Ingredients	Concentration of antibiotic (mcg/ml)						Control
	25	12,5	6,25	3,12	1,56	0,78	
MPB (ml)	1,0	1,0	1,0	1,0	1,0	1,0	1,0
Antibiotic (50 mcg/ml)	1,0	1,0	1,0	1,0	1,0	1,0	-
Bacteria, ml	0,1	0,1	0,1	0,1	0,1	0,1	0,1
MIC							Growth

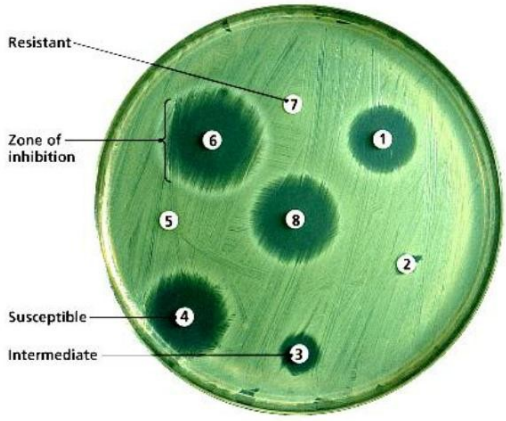
Surname _____ Date _____

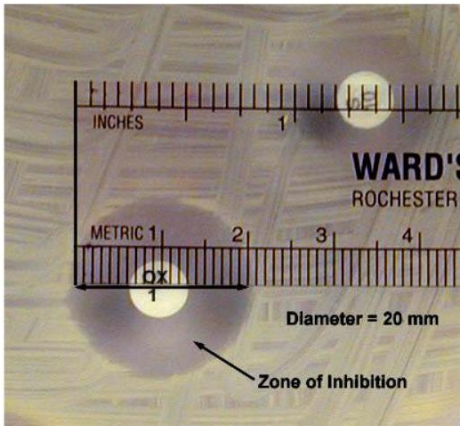
MBC							Growth



RESULT: MIC is _____.

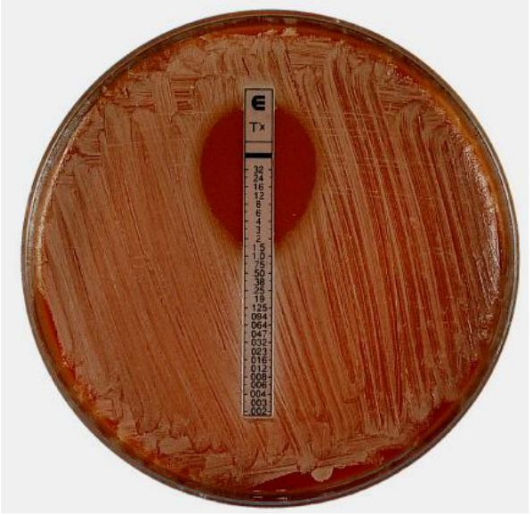
II. Performing of the agar diffusion test to determine sensitivity of microorganisms to antibiotics.

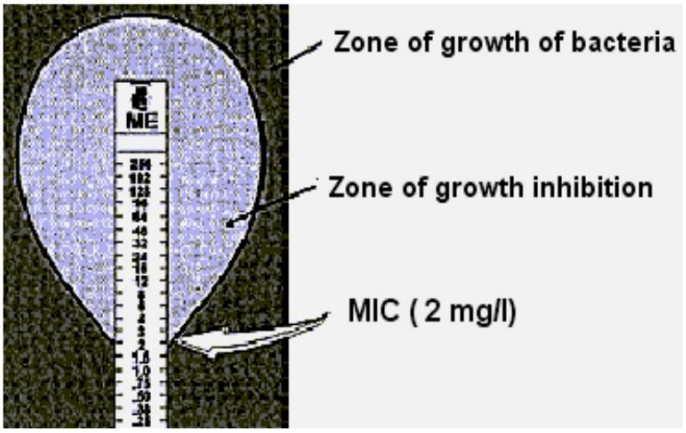




RESULT: isolated bacteria are susceptible to antibiotics _____, intermediate to antibiotic _____, and resistant to antibiotics _____.

III. Study the scheme and result of E test:





ADDING THEORETICAL MATERIAL

Antimicrobial Agents in the Treatment of Infectious Disease

Introduction

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: **antibiotics**, which are natural substances produced by certain groups of microorganisms, and **chemotherapeutic agents**, which are chemically synthesized. A hybrid substance is a **semisynthetic antibiotic**, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties.

The modern era of antimicrobial chemotherapy began in 1929, with Fleming's discovery of the powerful bactericidal substance, penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity.

Most of the natural antibiotics that are being used in agriculture and medicine are produced by three unrelated groups of microbes, including eukaryotic molds and two types of spore-forming bacteria.

Basis of Antimicrobial Action

Various antimicrobial agents act by interfering with (1) cell wall synthesis, (2) plasma membrane integrity, (3) nucleic acid synthesis, (4) ribosomal function, and (5) folate synthesis.

Cell wall synthesis is inhibited by ß-lactams, such as penicillins and cephalosporins, which inhibit peptidoglycan polymerization, and by vancomycin, which combines with cell wall substrates. Polymyxins disrupt the plasma membrane, causing leakage. The plasma membrane sterols of fungi are attacked by polyenes (amphotericin) and imidazoles. Quinolones bind to a bacterial complex of DNA and DNA gyrase, blocking DNA replication. Nitroimidazoles damage DNA. Rifampin blocks RNA synthesis by binding to DNA directed RNA polymerase. Aminoglycosides, tetracycline, chloramphenicol, erythromycin, and clindamycin all interfere with ribosome function. Sulfonamides and trimethoprim block the synthesis of the folate needed for DNA replication

Inhibition of Bacterial Cell Wall Synthesis

Peptidoglycan synthesis occurs in three stages. The first stage takes place in the cytoplasm, where the low-molecular-weight precursors are synthesized. A number of antimicrobial agents interfere with these early steps in cell wall biosynthesis. Cycloserine inhibits both alanine racemase and D-alanyl-D-alanine synthetase owing to the structural similarity of cycloserine and D-alanine and to the fact that cycloserine actually binds to the enzymes better than the D-alanine.

The second stage of cell wall synthesis is catalyzed by membrane-bound enzymes. The non-nucleotide portion of the precursor molecules previously made are transferred sequentially to a carrier in the cytoplasmic membrane. This carrier is a phosphorylated undecaprenyl alcohol. The lipid carrier functions as a point of attachment to the membrane for the precursors and allows for transport of the subunits across the hydrophobic interior of the cytoplasmic membrane to the outside surface. Bacitracin is a peptide antibiotic that specifically interacts with the pyrophosphate derivate of the undecaprenyl alcohol, preventing further transfer of the muramylpentapeptide from the precursor nucleotide to the nascent peptidoglycan.

The third stage of cell wall synthesis involves polymerization of the subunits and the attachment of nascent peptidoglycan to the cell wall. Polymerization occurs by transfer of the new peptidoglycan chain from its carrier in the membrane to the non-reducing *N*-acetylglucosamine of the new saccharide-peptide that is attached to the membrane. The new peptidoglycan is attached to preexisting cell wall peptidoglycan by a transpeptidase reaction that involves peptide chains in both polymers, one of which must possess a D-alanyl-D-alanine terminus. It is believed that the transpeptidase enzyme cleaves the peptide bond between two D-alanyl residues in the pentapeptide and become acylated via the carbonyl group of the penultimate D-alanine residue. This final reaction is inhibited by β-lactam antibiotics. These antibiotics contain a critical four-membered ring, which undergoes an acylation reaction with the transpeptidases that cross-link the polymers mentioned above. The β-lactam antibiotics are the penicillins (penams), cephalosporins (including oxacephems and cephamycins), penems,

thienamycins (carbapenems), and aztreonam (monobactams). The enzymes involved in this final process of cell wall formation are called penicillin-binding proteins since they were discovered by labeling with radioactive penicillin G. The enzymes are different in Gram-positive and Gram-negative bacteria and in anaerobic species. Differences in the penicillin-binding proteins explain, to some extent, differences in antibacterial activity of the β-lactam antibiotics. The penicillin-binding protein, to which a particular β-lactam antibiotic binds, affects the morphologic response of the bacterium to the agent. For example, some antibiotics bind to a penicillin-binding protein that is involved in forming the septum between dividing cells; as a result, the bacteria continue to grow into long filaments, which eventually die. Binding to another penicillin-binding protein results in rapid lysis of a bacterium because the wall bulges and the bacterium bursts. β-Lactams such as mecillinam (an amidino penicillin) do not bind to the penicillin-binding proteins of Gram-positive bacteria and therefore do not affect these bacteria. Aztreonam binds only to Gram-negative penicillin-binding proteins and does not inhibit Gram-positive or anaerobic species.

Vancomycin interrupts cell wall synthesis by forming a complex with the C-terminal D-alanine residues of peptidoglycan precursors. Complex formation at the outer surface of the cytoplasmic membrane prevents the transfer of the precursors from a lipid carrier to the growing peptidoglycan wall by transglycosidases. Biochemical reactions in the cell wall catalyzed by transpeptidases and D-carboxypeptidases are also inhibited by vancomycin and other glycopeptide antimicrobials. Because of its large size and complex structure, vancomycin does not penetrate the outer membrane of gram-negative organisms. With resistance to beta-lactams increasing in frequency among staphylococci and enterococci, glycopeptides such as vancomycin remain important therapeutic agents against such bacteria.

Antibiotics that Affect the Function of Cytoplasmic Membranes Bacterial Cytoplasmic Membranes

Biologic membranes are composed basically of lipid, protein, and lipoprotein. The cytoplasmic membrane acts as a diffusion barrier for water, ions, nutrients, and transport systems. Most workers now believe that membranes are a lipid matrix with globular proteins randomly distributed to penetrate through the lipid bilayer. A number of antimicrobial agents can cause disorganization of the membrane. These agents can be divided into cationic, anionic, and neutral agents. The best-known compounds are polymyxin B and colistemethate (polymyxin E). These high-molecular-weight octapeptides inhibit Gram-negative bacteria that have negatively charged lipids at the surface. Since the activity of the polymyxins is antagonized by Mg^{2+} and Ca^{2+}, they probably competitively displace Mg^{2+} or Ca^{2+} from the negatively charged phosphate groups on membrane lipids. Basically, polymyxins disorganize membrane permeability so that nucleic acids and cations leak out and the cell dies. The polymyxins are of virtually no use as systemic agents since they bind to various ligands in body tissues and are potent toxins for the kidney and nervous system. Gramicidins are also membrane-active antibiotics that appear to act by producing aqueous pores in the membranes. They also are used only topically.

Fungal Membranes

Fungal membranes contain sterols, whereas bacterial membranes do not. The polyene antibiotics, which apparently act by binding to membrane sterols, contain a rigid hydrophobic center and a flexible hydrophilic section. Structurally, polyenes are tightly packed rods held in rigid extension by the polyene portion. They interact with fungal cells to produce a membrane-polyene complex that alters the membrane permeability, resulting in internal acidification of the fungus with exchange of K^+ and sugars; loss of phosphate esters, organic acids, nucleotides; and eventual leakage of cell protein. In effect, the polyene makes a pore in the fungal membrane and the contents of the fungus leak out. Prokaryotic cells neither bind to nor are inhibited by polyenes. Although numerous polyene antibiotics have been isolated, only amphotericin B is used systemically. Nystatin is used as a topical agent and primaricin as an ophthalmic preparation.

A number of other agents interfere with the synthesis of fungal lipid membranes. These agents belong to a class of compounds referred to as imidazoles: miconazole, ketoconazole,

clotrimazole, and fluconazole. These compounds inhibit the incorporation of subunits into ergosterol and may also directly damage the membrane.

Antibiotics that Inhibit Nucleic Acid Synthesis

Antimicrobial agents can interfere with nucleic acid synthesis at several different levels. They can inhibit nucleotide synthesis or interconversion; they can prevent DNA from functioning as a proper template; and they can interfere with the polymerases involved in the replication and transcription of DNA.

Interference with Nucleotide Synthesis

A large number of agents interfere with purine and pyrimidine synthesis or with the interconversion or utilization of nucleotides. Other agents act as nucleotide analogs that are incorporated into polynucleotides.

Flucytosine (5-fluorocytosine) is an antifungal agent that inhibits yeast species. It is converted in the fungal cell to 5-fluorouracfl, which inhibits thymidylate synthetase resulting in a deficit of thymine nucleotides and impaired DNA synthesis. Adenosine arabinoside inhibits viruses. It is phosphorylated in virus-infected cells and acts as a competitive analog of DATP, inhibiting the incorporation of DATP into DNA. Acyclovir is a nucleoside analog that, after being converted to a triphosphate, inhibits the thymidine kinase and DNA polymerase of herpes viruses. Zidovudine (AZT) inhibits human immunodeficiency virus (HM replication by interfering with viral RNA-dependent DNA polymerase (reverse transcriptase).

Agents That Impair the Template Function of DNA

A number of substances bind to DNA by intercalation. None of them is useful as an antibacterial agent; however, chloroquine and miracil D (lucanthone) inhibit plasmodia and schistosomes, respectively. These agents are thought to intercalate into the DNA and thereby to inhibit further nucleic acid synthesis. Acridine dyes such as proflavine act by this intercalation mechanism, but because they are toxic and carcinogenic in mammals they cannot be used as antibacterial agents.

Inhibition of DNA-Directed RNA Polymerase

Rifamycins are a class of antibiotics that inhibit DNA-directed RNA polymerase. Polypeptide chains in RNA polymerase attach to a factor that confers specificity for the recognition of promoter sites that initiate transcription of the DNA. Rifampin binds non-covalently but strongly to a subunit of RNA polymerase and interferes specifically with the initiation process. However, it has no effect once polymerization has begun.

Inhibition of DNA Replication

DNA gyrase and topoisomerase I act in concert to maintain an optimum supercoiling state of DNA in the cell. In this capacity, DNA gyrase is essential for relieving torsional strain during replication of circular chromosomes in bacteria. The enzyme is a tetrameric protein composed of two A and two B subunits. A transient, covalent bond between the A subunit and DNA occurs during the double strand passage reaction catalyzed by gyrase. Quinolones such as nalidixic acid, bind to the cleavage complex composed of DNA and gyrase during this strand passage. This interaction of quinolone acts to stabilize the cleavage intermediate which has a detrimental effect on the normal DNA replication process. The effects of this inhibition result in the death of the bacterial cell. The newer fluoroquinolones such as ciprofloxacin, norfloxacin, and ofloxacin also interact with DNA gyrase and possess a broad spectrum of antimicrobial activity.

Nitroimidazoles such as metronidazole inhibit anaerobic bacteria and protozoa. The nitro group of the nitrosohydroxyl amino moiety is reduced by an electron transport protein in anaerobic bacteria. The reduced drug causes strand breaks in the DNA. Mammalian cells are unharmed because they lack enzymes to reduce the nitro group of these agents.

Antimicrobial Inhibitors of Ribosome Function

A number of antibacterial agents act by inhibiting ribosome function. Bacterial ribosomes contain two subunits, the 50S and 30S subunits, and it is possible to localize the action of antibiotics to one or both subunits. It is also possible to isolate the specific ribosomal proteins to

which an agent binds and to isolate bacterial mutants that lack a specific ribosomal protein and therefore show resistance to a particular agent.

Aminoglycosides act by binding to specific ribosomal subunits. Aminoglycosides are complex sugars connected in glycosidic linkage. They differ both in the molecular nucleus, which can be streptidine or 2-deoxystreptidine, and in the aminohexoses linked to the nucleus. Essential to the activity of these agents are free NH_4 and OH groups by which aminoglycosides bind to specific ribosomal proteins. Streptomycin, the first aminoglycoside studied, was a useful tool in elucidating protein synthesis. However, it is rarely used clinically today except to treat tuberculosis, and its mode of action differs to some extent from that of the other clinically useful aminoglycosides, which are 2-deoxystreptidine derivatives such as gentamicin, tobramycin, and amikacin. Streptomycin binds to a specific S12 protein in the 30S ribosomal subunit and causes the ribosome to misread the genetic code. Other aminoglycosides bind not only to the S12 protein of the 30S ribosome, but also to some extent to the L6 protein of the 50S ribosome. This latter binding is quite important in terms of the resistance of bacteria to aminoglycosides. Indeed, the aminoglycoside-type drugs can combine with other binding sites on 30S ribosomes, and they kill bacteria by inducing the formation of aberrant, nonfunctional complexes as well as by causing misreading.

Other agents that bind to 30S ribosomes are the tetracyclines. These agents appear to inhibit the binding of aminoacyl-tRNA into the A site of the bacterial ribosome. Tetracycline binding is transient, so these agents are bacteriostatic. Nonetheless, they inhibit a wide variety of bacteria, chlamydia, and mycoplasmas and are extremely useful antibiotics.

There are three important classes of drugs that inhibit the 50S ribosomal subunit. Chloramphenicol is a bacteriostatic agent that inhibits both Gram-positive and Gram-negative bacteria. It inhibits peptide bond formation by binding to a peptidyltransferase enzyme on the 50S ribosome. Macrolides are large lactone ring compounds that bind to 50S ribosomes and appear to impair a peptidyltransferase reaction or translocation, or both. The most important macrolide is erythromycin, which inhibits Gram-positive species and a few Gram-negative species such as *Haemophilus, Mycoplasma, Chlamydia,* and *Legionella*. New molecules such as azithromycin and clarithromycin have greater activity than erythromycin against many of these pathogens. Lincinoids, of which the most important is clindamycin, have a similar site of activity. Both macrolides and lincinoids are generally bacteriostatic, inhibiting only the formation of new peptide chains.

Drugs that Inhibit Other Biochemical Targets

Both trimethoprim and the sulfonamides interfere with folate metabolism in the bacterial cell by competitively blocking the biosynthesis of tetrahydrofolate, which acts as a carrier of one-carbon fragments and is necessary for the ultimate synthesis of DNA, RNA and bacterial cell wall proteins. Unlike mammals, bacteria and protozoan parasites usually lack a transport system to take up preformed folic acid from their environment. Most of these organisms must synthesize folates, although some are capable of using exogenous thymidine, circumventing the need for folate metabolism.

Sulfonamides competitively block the conversion of pteridine and *p*-aminobenzoic acid (PABA) to dihydrofolic acid by the enzyme pteridine synthetase. Sulfonamides have a greater affinity than *p*-aminobenzoic acid for pteridine synthetase. Trimethoprim has a tremendous affinity for bacterial dihydrofolate reductase; when bound to this enzyme, it inhibits the synthesis of tetrahydrofolate.

Antibacterial Agents that Affect Mycobacteria

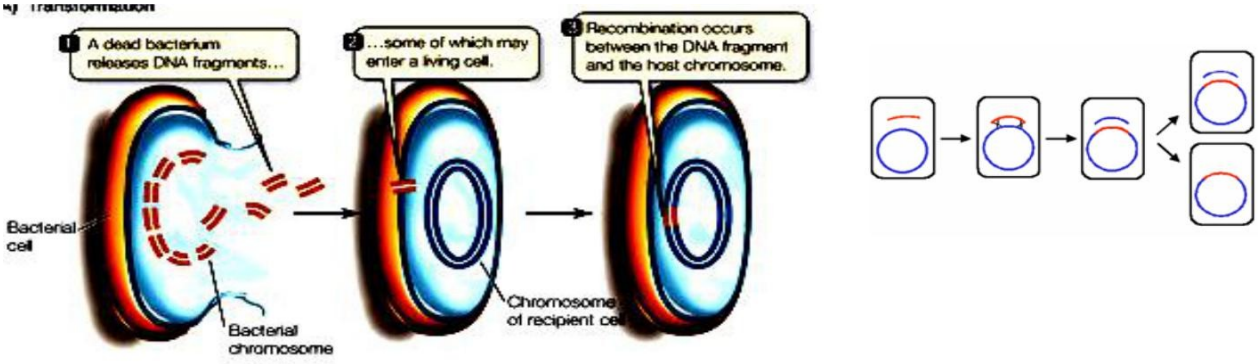
Isoniazid is a nicotinamide derivative that inhibits mycobacteria. It is mycocidal, affects the synthesis of lipids, nucleic acids, and the mycolic acid of the cell walls of these species. Ethambutol is mycostatic, whereas isoniazid. The other antituberculosis drugs, rifampin and streptomycin, affect mycobacteria in the same manner that they inhibit bacteria. Pyrazinamide is a synthetic analog of nicotinamide. It is bactericidal, but its exact mechanism is unknown.

Surname_____ Date_____

Protocol №21

Theme: Genetics of bacteria.

I. Transformation is gene transfer resulting from the uptake by a recipient cell of naked DNA from a donor cell



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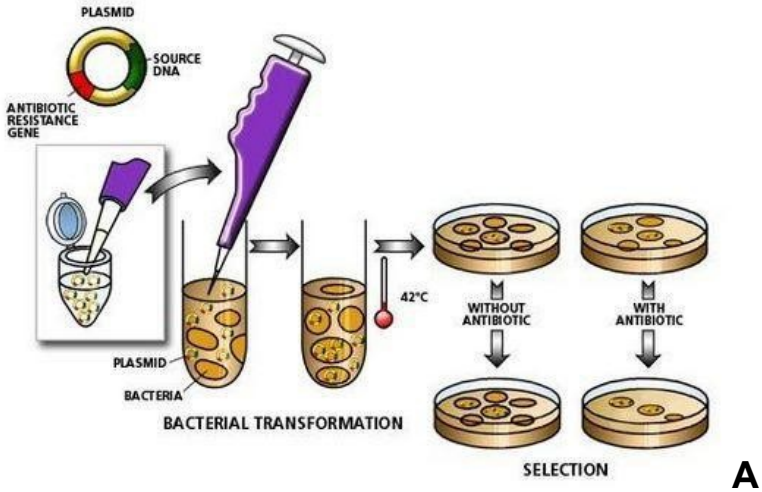
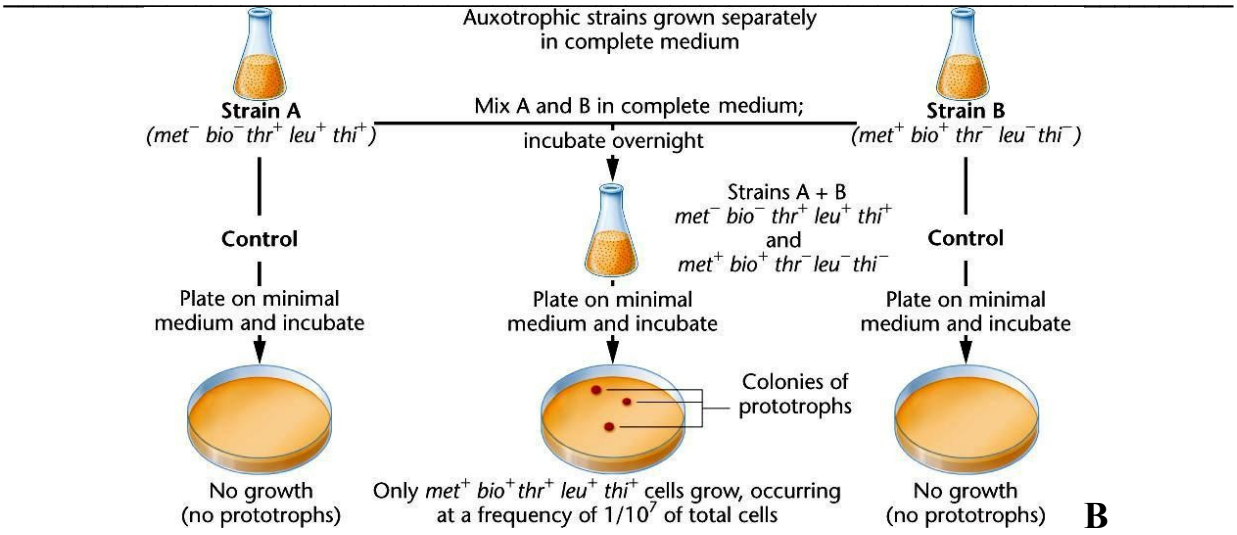


A

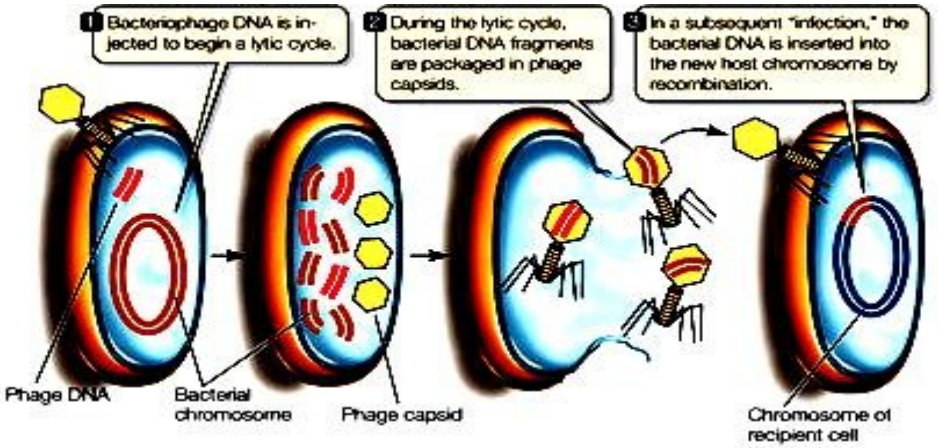


B

B. Autotroph A needs methionine (met) and biotin (bio) to grow; auxotroph B nedds threonine (thr), leucine (leu), and thiamine (thi); both require supplemented media. The two strains are mixed and plated on minimal medium and recovered wild-type prototrophs, indicating recombination (transformation) had occurred.

S u r n a m e _ D a t e _ _ _ _ _ _ _ _ _ _ _

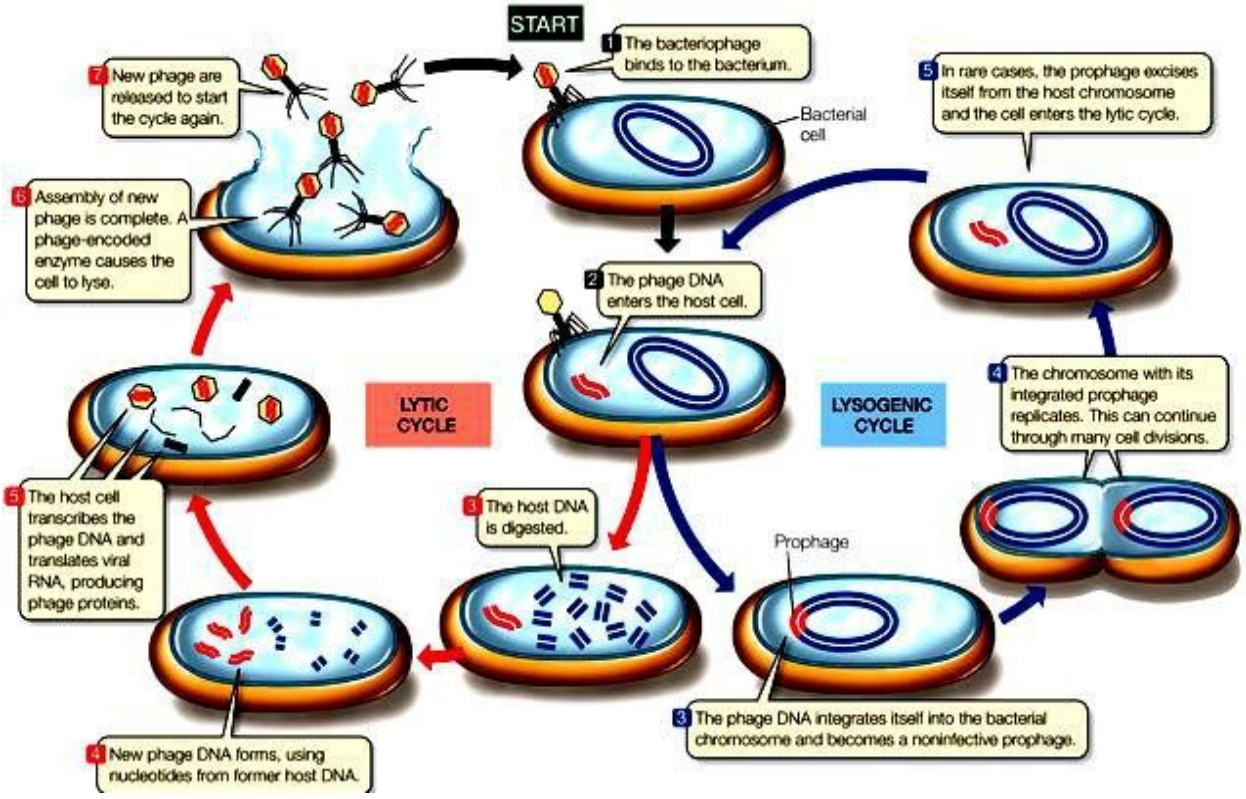
II. **Transduction** is the transfer of genetic information from a donor to a recipient by way of a bacteriophage.

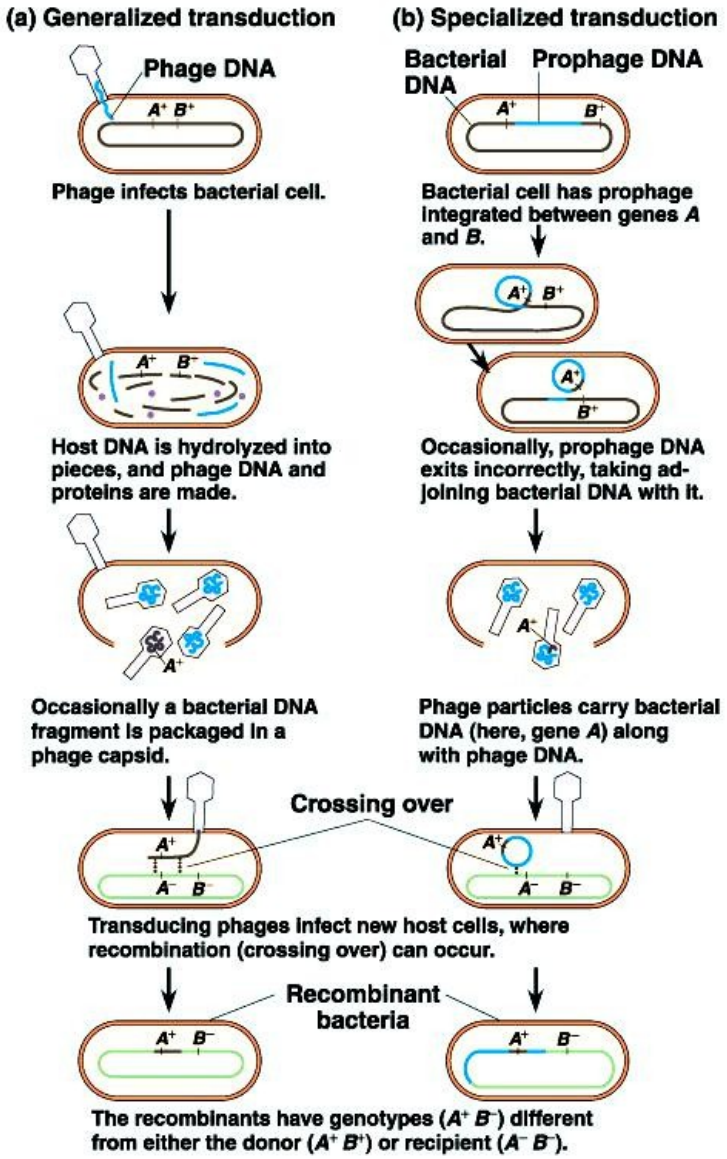


Bacteriophages are viruses that infect bacteria.

Virulent viruses undergo a lytic cycle, which causes the host cell to burst, releasing new virions.

Temperate viruses can also undergo a lysogenic cycle, in which a molecule of their DNA, called a prophage, is inserted into the host chromosome, where it replicates for generations. Such hosts are referred to as lysogenic bacteria.



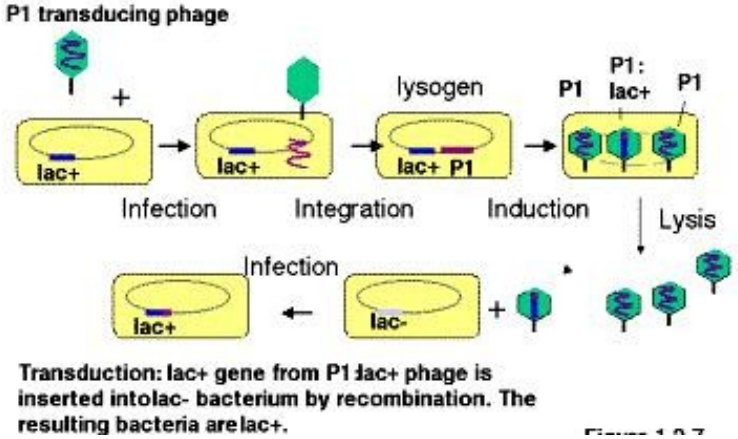


Note that the transducing phages are carrying one or a small number of bacterial genes.

A **generalized transducing phage** can integrate at many different locations on the bacterial chromosome. Imprecise excision from any of those locations generates a particular transducing phage, carrying a short section of the bacterial genome adjacent to the integration site. Thus a generalized transducing phage such as P1 can pick up many different parts of the *E. coli* genome.

A **specialized transducing phage** integrates into only one or very few sites in the host genome. Hence it can carryonly a few specific bacterial genes, e.g., λ *lac*.

The scheme of the experiment of trancduction:



Transfer of bacterial genes by transduction: A *lac*$^{+}$ transducing phage can convert a *lac*$^{-}$ strain to *lac*$^{+}$ by infection (and subsequent crossing over).

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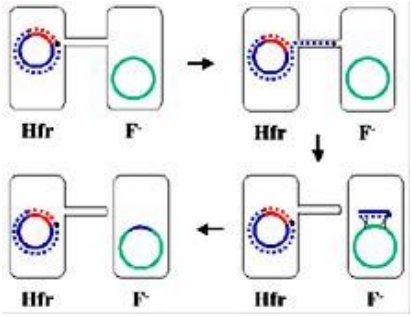


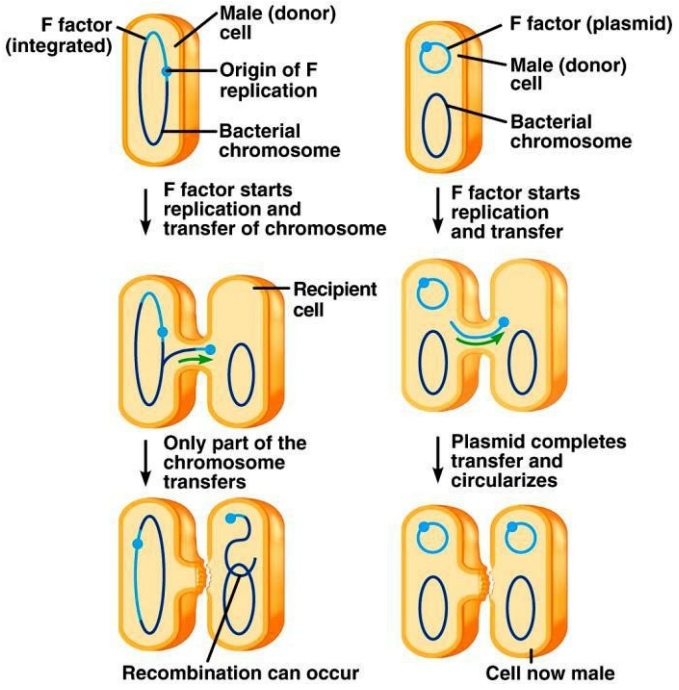
III. Conjugation is transfer of DNA from a donor to a recipient by direct physical contact between the cells.

Plasmids are extrachromosomal genetic elements capable of autonomous replication. An **episome** is a plasmid that can integrate into the bacterial chromosome.

Location of plasmids in bacterial cell:

Plasmide

Episome



Classification of Plasmids:

1. Transfer properties:

a. **Conjugative plasmids** are those that mediated conjugation. These plasmids are usually large and have all the genes necessary for autonomous replication and for transfer of DNA to a recipient (e.g. genes for sex pilus).

b. **Nonconjugative plasmids** are those that cannot mediate conjugation. They are usually smaller than conjugative plasmids and they lack one or more of the genes needed for transfer of DNA. A nonconjugative plasmid can be transferred by conjugation if the cell also harbors a conjugative plasmid.

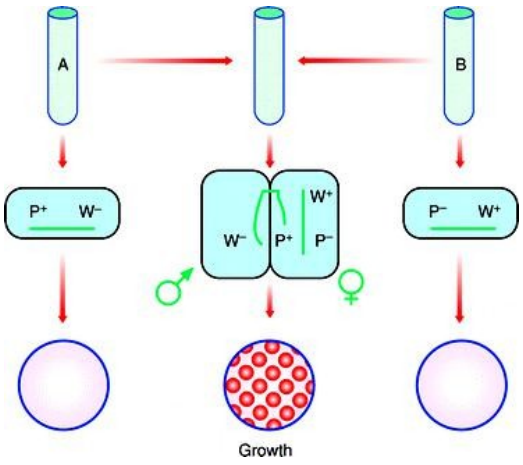
2. Phenotypic effects:

a. **Fertility plasmid (F factor).**

b. **Bacteriocinogenic plasmids** have genes which code for substances that kill other bacteria. These substances are called **bacteriocins or colicins**.

c. **Resistance plasmids (R plasmids)** carry antibiotic resistance genes.

<u>The scheme of the experiment of conjugation of bacteria:</u>



The strain in tube A requires tryptophan for growth (W^-), while that in tube B requires proline (P^-). Therefore, neither will grow on minimal medium. However, the W^- strain is an Hfr strain, so it transfers part of its chromosome to the P^- strain when the two are mixed together. Some of the progeny are now both W^+ and P^+, so they are able to grow on minimal medium.

Transposable genetic elements are segments of DNA that have the capacity to move from one location to another (*i.e.* jumping genes).

Transposons (Tn) are transposable genetic elements that carry one or more other genes in addition to those which are essential for transposition.

ADDING THEORETICAL MATERIAL

Genetic Information in Microbes

Genetic information in bacteria and many viruses is encoded in DNA, but some viruses use RNA. Replication of the genome is essential for inheritance of genetically determined traits. Gene expression usually involves transcription of DNA into messenger RNA and translation of mRNA into protein.

Genome Organization

The bacterial chromosome is a circular molecule of DNA that functions as a self-replicating genetic element (replicon). Extrachromosomal genetic elements such as plasmids and bacteriophages are nonessential replicons which often determine resistance to antimicrobial agents, production of virulence factors, or other functions. The chromosome replicates semiconservatively; each DNA strand serves as template for synthesis of its complementary strand.

DNA Replication

During replication of the bacterial genome, each strand in double-helical DNA serves as a template for synthesis of a new complementary strand. Each daughter double-stranded DNA molecule thus contains one old polynucleotide strand and one newly synthesized strand. This type of DNA replication is called semiconservative. Replication of chromosomal DNA in bacteria starts at a specific chromosomal site called the origin and proceeds bidirectionally until the process is completed. When bacteria divide by binary fission after completing DNA replication, the replicated chromosomes are partitioned into each of the daughter cells. The origin regions specifically and transiently associate with the cell membrane after DNA replication has been initiated, leading to a model whereby membrane attachment directs separation of daughter chromosomes (the replicon model). These characteristics of DNA replication during bacterial growth fulfill the requirements of the genetic material to be reproduced accurately and to be inherited by each daughter cell at the time of cell division.

Gene Expression

Genetic information encoded in DNA is expressed by synthesis of specific RNAs and proteins, and information flows from DNA to RNA to protein. The DNA-directed synthesis of RNA is called transcription. Because the strands of double-helical DNA are antiparallel and complementary, only one of the two DNA strands can serve as template for synthesis of a specific mRNA molecule. Messenger RNAs (mRNAs) transmit information from DNA, and each mRNA in bacteria functions as the template for synthesis of one or more specific proteins. The process by which the nucleotide sequence of an mRNA molecule determines the primary amino acid sequence of a protein is called translation. Ribosomes, complexes of ribosomal RNAs (rRNAs) and several ribosomal proteins, translate each mRNA into the corresponding polypeptide sequence with the aid of transfer RNAs (tRNAs), amino-acyl tRNA synthesases, initiation factors and elongation factors. All of these components of the apparatus for protein synthesis function in the production of many different proteins. A gene is a DNA sequence that encodes a protein, rRNA, or tRNA molecule (gene product).

Plasmids

Plasmids are replicons that are maintained as discrete, extrachromosomal genetic elements in bacteria. They are usually much smaller than the bacterial chromosome, varying from less than 5 to more than several hundred kbp, though plasmids as large as 2 Mbp occur in some bacteria. Plasmids usually encode traits that are not essential for bacterial viability, and replicate independently of the chromosome. Most plasmids are supercoiled, circular, double-stranded DNA molecules, but linear plasmids have also been demonstrated in *Borrelia* and *Streptomyces*. Closely related or identical plasmids demonstrate incompatibility; they cannot be stably maintained in the same bacterial host. Classification of plasmids is based on incompatibility or on use of specific DNA probes in hybridization tests to identify nucleotide sequences that are characteristic of specific plasmid replicons. Some hybrid plasmids contain more than one replicon. Conjugative plasmids code for functions that promote transfer of the plasmid from the donor bacterium to other recipient bacteria, but nonconjugative plasmids do

not. Conjugative plasmids that also promote transfer of the bacterial chromosome from the donor bacterium to other recipient bacteria are called fertility plasmids, and are discussed below. The average number of molecules of a given plasmid per bacterial chromosome is called its copy number. Large plasmids (>40 kilobase pairs) are often conjugative, have small copy numbers (1 to several per chromosome), code for all functions required for their replication, and partition themselves among daughter cells during cell division in a manner similar to the bacterial chromosome. Plasmids smaller than 7.5 kilobase pairs usually are nonconjugative, have high copy numbers (typically 10–20 per chromosome), rely on their bacterial host to provide some functions required for replication, and are distributed randomly between daughter cells at division.

Many plasmids control medically important properties of pathogenic bacteria, including resistance to one or several antibiotics, production of toxins, and synthesis of cell surface structures required for adherence or colonization. Plasmids that determine resistance to antibiotics are often called R plasmids (or R factors). Representative toxins encoded by plasmids include heat-labile and heat-stable enterotoxins of *E. coli*, exfoliative toxin of *Staphylococcus aureus*, and tetanus toxin of *Clostridium tetani*. Some plasmids are cryptic and have no recognizable effects on the bacterial cells that harbor them. Comparing plasmid profiles is a useful method for assessing possible relatedness of individual clinical isolates of a particular bacterial species for epidemiological studies. The role of plasmids in the evolution of resistance to antibiotics is discussed below.

Mutation

The complete set of genetic determinants of an organism constitutes its genotype, and the observable characteristics constitute its phenotype. Mutations are heritable changes in genotype that can occur spontaneously or be induced by chemical or physical treatments. Organisms selected as reference strains are called wild type, and their progeny with mutations are called mutants. Selective media distinguish between wild type and mutant strains based on growth; differential media distinguish between them based on other phenotypic properties.

Mutations are heritable changes in the genome. Spontaneous mutations in individual bacteria are rare. Some mutations cause changes in phenotypic characteristics; the occurrence of such mutations can be inferred from the effects they produce. In microbial genetics specific reference organisms are designated as wild-type strains, and descendants that have mutations in their genomes are called mutants. Thus, mutants are characterized by the inherited differences between them and their ancestral wild-type strains. Variant forms of a specific genetic determinant are called alleles. Genotypic symbols are lower case, italicized abbreviations that specify individual genes, with a (+) superscript indicating the wild type allele. Phenotypic symbols are capitalized and not italicized, to distinguish them from genotypic symbols. For example, the genotypic symbol for the ability to produce β-galactosidase, required to ferment lactose, is $lacZ^{+}$, and mutants that cannot produce β-galactosidase are $lacZ^{-}$. The lactose-fermenting phenotype is designated Lac^{+}, and inability to ferment lactose is Lac^{-}.

Spontaneous and Induced Mutations

The mutation rate in bacteria is determined by the accuracy of DNA replication, the occurrence of damage to DNA, and the effectiveness of mechanisms for repair of damaged DNA.

For a particular bacterial strain under defined growth conditions, the mutation rate for any specific gene is constant and is expressed as the probability of mutation per cell division. In a population of bacteria grown from a small inoculum, the proportion of mutants usually increases progressively as the size of the bacterial population increases.

Mutations in bacteria can occur spontaneously and independently of the experimental methods used to detect them. This principle was first demonstrated by the fluctuation test. The numbers of phage-resistant mutants of *E. coli* in replicate cultures grown from small inoculum were measured and compared with those in multiple samples taken from a single culture. If mutations to phage resistance occurred only after exposure to phage, the variability in numbers of mutants between cultures should be similar under both sets of conditions. In contrast, if

phage-resistant mutants occurred spontaneously before exposure of the bacteria to phage, the numbers of mutants should be more variable in the independently grown cultures, because differences in the size of the bacterial population when the first mutant appeared would contribute to the observed variability. The data indicated that the mutations to phage resistance in *E. coli* occurred spontaneously with constant probability per cell division.

Exchange of Genetic Information

Genetic exchanges among bacteria occur by several mechanisms. In transformation, the recipient bacterium takes up extracellular donor DNA. In transduction, donor DNA packaged in a bacteriophage infects the recipient bacterium. In conjugation, the donor bacterium transfers DNA to the recipient by mating. Recombination is the rearrangement of donor and recipient genomes to form new, hybrid genomes. Transposons are mobile DNA segments that move from place to place within or between genomes.

The biologic significance of sexuality in microorganisms is to increase the probability that rare, independent mutations will occur together in a single microbe and be subjected to natural selection. Genetic interactions between microbes enable their genomes to evolve much more rapidly than by mutation alone. Representative phenomena of medical importance that involve exchanges of genetic information or genomic rearrangements include the rapid emergence and dissemination of antibiotic resistance plasmids, flagellar phase variation in *Salmonella*, and antigenic variation of surface antigens in *Neisseria* and *Borrelia*.

Sexual processes in bacteria involve transfer of genetic information from a donor to a recipient and result either in substitution of donor alleles for recipient alleles or addition of donor genetic elements to the recipient genome. Transformation, transduction, and conjugation are sexual processes that use different mechanisms to introduce donor DNA into recipient bacteria

Transformation

In transformation, pieces of DNA released from donor bacteria are taken up directly from the extracellular environment by recipient bacteria. Recombination occurs between single molecules of transforming DNA and the chromosomes of recipient bacteria. To be active in transformation, DNA molecules must be at least 500 nucleotides in length, and transforming activity is destroyed rapidly by treating DNA with deoxyribonuclease. Molecules of transforming DNA correspond to very small fragments of the bacterial chromosome. Co-transformation of genes is unlikely, therefore, unless they are so closely linked that they can be encoded on a single DNA fragment. Transformation was discovered in *Streptococcus pneumonia* and occurs in other bacterial genera including *Haemophilus*, *Neisseria*, *Bacillus*, and *Staphylococcus*. The ability of bacteria to take up extracellular DNA and to become transformed, called competence, varies with the physiologic state of the bacteria. Many bacteria that are not usually competent can be made to take up DNA by laboratory manipulations, such as calcium shock or exposure to a high-voltage electrical pulse (electroporation). In some bacteria (including *Haemophilus* and *Neisseria*) DNA uptake depends on the presence of specific oligonucleotide sequences in the transforming DNA, but in others (including *Streptococcus pneumoniae*) DNA uptake is not sequence-specific. Competent bacteria may also take up intact bacteriophage DNA (transfection) or plasmid DNA, which can then replicate as extrachromosomal genetic elements in the recipient bacteria. In contrast, a piece of chromosomal DNA from a donor bacterium usually cannot replicate in the recipient bacterium unless it becomes part of a replicon by recombination. Historically, characterization of "transforming principle" from *S. pneumoniae* provided the first direct evidence DNA is genetic material.

Transduction

In transduction, bacteriophages function as vectors to introduce DNA from donor bacteria into recipient bacteria by infection. For some phages, called generalized transducing phages, a small fraction of the virions produced during lytic growth are aberrant and contain a random fragment of the bacterial genome instead of phage DNA. Each individual transducing phage carries a different set of closely linked genes, representing a small segment of the bacterial genome. Transduction mediated by populations of such phages is called generalized transduction, because each part of the bacterial genome has approximately the same probability

of being transferred from donor to recipient bacteria. When a generalized transducing phage infects a recipient cell, expression of the transferred donor genes occurs. Abortive transduction refers to the transient expression of one or more donor genes without formation of recombinant progeny, whereas complete transduction is characterized by production of stable recombinants that inherit donor genes and retain the ability to express them. In abortive transduction the donor DNA fragment does not replicate, and among the progeny of the original transductant only one bacterium contains the donor DNA fragment. In all other progeny the donor gene products become progressively diluted after each generation of bacterial growth until the donor phenotype can no longer be expressed. On selective medium upon which only bacteria with the donor phenotype can grow, abortive transductants produce minute colonies that can be distinguished easily from colonies of stable transductants. The frequency of abortive transduction is typically one to two orders of magnitude greater than the frequency of generalized transduction, indicating that most cells infected by generalized transducing phages do not produce recombinant progeny.

Specialized transduction differs from generalized transduction in several ways. It is mediated only by specific temperate phages, and only a few specific donor genes can be transferred to recipient bacteria. Specialized transducing phages are formed only when lysogenic donor bacteria enter the lytic cycle and release phage progeny. The specialized transducing phages are rare recombinants which lack part of the normal phage genome and contain part of the bacterial chromosome located adjacent to the prophage attachment site. Many specialized transducing phages are defective and cannot complete the lytic cycle of phage growth in infected cells unless helper phages are present to provide missing phage functions. Specialized transduction results from lysogenization of the recipient bacterium by the specialized transducing phage and expression of the donor genes. Phage conversion and specialized transduction have many similarities, but the origin of the converting genes in temperate converting phages is unknown.

Conjugation

In conjugation, direct contact between the donor and recipient bacteria leads to establishment of a cytoplasmic bridge between them and transfer of part or all of the donor genome to the recipient. Donor ability is determined by specific conjugative plasmids called fertility plasmids or sex plasmids.

The F plasmid (also called F factor) of *E. coli* is the prototype for fertility plasmids in Gram-negative bacteria. Strains of *E. coli* with an extrachromosomal F plasmid are called F^+ and function as donors, whereas strains that lack the F plasmid are F^- and behave as recipients. The conjugative functions of the F plasmid are specified by a cluster of at least 25 transfer (*tra*) genes which determine expression of F pili, synthesis and transfer of DNA during mating, interference with the ability of F^+ bacteria to serve as recipients, and other functions. Each F^+ bacterium has 1 to 3 F pili that bind to a specific outer membrane protein (the ompA gene product) on recipient bacteria to initiate mating. An intercellular cytoplasmic bridge is formed, and one strand of the F plasmid DNA is transferred from donor to recipient, beginning at a unique origin and progressing in the 5′ to 3′ direction. The transferred strand is converted to circular double-stranded F plasmid DNA in the recipient bacterium, and a new strand is synthesized in the donor to replace the transferred strand. Both of the exconjugant bacteria are F^+, and the F plasmid can therefore spread by infection among genetically compatible populations of bacteria. In addition to the role of the F pili in conjugation, they also function as receptors for donor-specific (male-specific) phages.

The F plasmid in *E. coli* can exist as an extrachromosomal genetic element or be integrated into the bacterial chromosome. Because the F plasmid and the bacterial chromosome are both circular DNA molecules, reciprocal recombination between them produces a larger DNA circle consisting of F plasmid DNA inserted linearly into the chromosome. *E. coli* contains multiple copies of several different genetic elements called insertion sequences (see section on transposons for more detail), at various locations in its chromosome and in the F plasmid. Homologous recombination between insertion sequences in the chromosome and the F plasmid leads to preferential integration of the F plasmid at chromosomal sites where insertion sequences

are located. The chromosomal sites where insertion sequences are found vary, however, among strains of *E. coli*.

Conjugation also occurs in Gram-positive bacteria. Gram-positive donor bacteria produce adhesins that cause them to aggregate with recipient cells, but sex pili are not involved. In some *Streptococcus* species, recipient bacteria produce extracellular sex pheromones that cause the donor phenotype to be expressed by bacteria that harbor an appropriate conjugative plasmid, and the conjugative plasmid prevents the donor cells from producing the corresponding pheromone.

Recombination

Recombination involves breakage and joining of parental DNA molecules to form hybrid, recombinant molecules. Several distinct kinds of recombination have been identified that depend on different features of the participating genomes and require the activities of different gene products. Specific enzymes that act on DNA (for example, exonucleases, endonucleases, polymerases, ligases) participate in recombination. Detailed discussion of the biochemical events in recombination is beyond the scope of this chapter.

Generalized recombination involves donor and recipient DNA molecules that have homologous nucleotide sequences. Reciprocal exchanges can occur between any homologous donor and recipient sites. In *E. coli*, the product of the *recA* gene is essential for generalized recombination, but other gene products also participate.

Site-specific recombination involves reciprocal exchanges only between specific sites in donor and recipient DNA molecules. The *recA* gene product is not required for site-specific recombination. Integration of the temperate bacteriophage l into the chromosome of *E. coli* is a well-studied example of site-specific recombination. The specific attachment (att) sites on the *E. coli* chromosome and λ phage DNA have a common core sequence of 15 nucleotides, within which reciprocal recombination occurs, flanked by adjacent sequences that are not homologous in the phage and bacterial genomes. In phage λ the product of the *int* gene (integrase) is required for the site-specific integration event in lysogenization; the products of the *int* and *xis* (excisionase) genes are both needed for the complementary site-specific excision event that occurs during induction of lytic phage development in lysogenic cells.

Surname _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ Date _ _ _ _ _ _ _ _ _ _ _

QUESTIONS TO EXAMINATION IN MICROBIOLOGY, IMMUNOLOGY AND VIROLOGY

Common microbiology

Morphology

1. State and describe arrangements of cocci and of bacilli. List of pathogenic representatives.

2. Define the following: simple stain, acidic dye, basic dye, direct stain, and indirect stain. State in chemical and physical terms the principle behind direct staining.

3. Gram's staining. State why the Gram's staining is said to be a differential stain. Describe the differences between a gram-positive and a gram-negative cell wall. Describe a theory as to why gram-positive bacteria retain the crystal violet-iodine complex while gram-negatives become decolorized.

4. State and describe three different spiral forms of bacteria. Name pathogenic representatives.

5. State the chemical nature and function of bacterial flagella. Describe methods and microscopes for testing for bacterial motility and indicate how to interpret the results.

6. Define arrangements of flagella. Name pathogenic representatives of motile bacteria. Methods of studying of motility.

7. State the chemical nature and major functions of bacterial capsules. Capsule stain. Name capsule-forming bacteria.

8. State the structure and function of bacterial endospores. Spore stain. Describe stages of sporulation. Name pathogenic representatives spore-producing bacteria.

9. Morphology of spirochetes. Methods of staining. Name of pathogenic representatives and diseases that they cause.

10. Morphology of mycoplasma. Name pathogenic representatives and diseases that they cause.

11. Morphology of Rickettsia. Pathogenic representatives and diseases that they cause. Methods of staining.

12. Morphology of Chlamidia. Pathogenic representatives and diseases that they cause. Life cycle.

13. Classification of pathogenic Protozoa. Methods of staining. Pathogenic representatives and diseases that they cause.

14. What is the virus particle or virion, and how is it different from living organisms?

15. Describe the lytic life cycle of bacteriophages.

16. Define the following terms: lytic or virulent phages, temperate phages, lysogen bacteria, lysogenic conversion.

17. Define the following terms: capsid, nucleocapsid, naked and enveloped viruses, types of symmetry.

18. Pathogenic representatives of DNA and RNA viruses and diseases that they cause.

19. Define the bacteriophage. Describe the structure of the bacteriophages.
20. Describe major types of relationships between virus and infected host cell.
21. Describe in detail each stage in animal virus reproduction. In what ways are animal viruses released from their host cells?
22. What is a prion? In what way does a prion appear to differ fundamentally from viruses?

Physiology

23. Classification of bacteria according to type of respiration and temperature requirements. Examples of bacteria.
24. Classification of obligate anaerobes. Examples of bacteria.
25. Sterilization and disinfection.
26. Chemical composition of bacterial cell.
27. Classification of bacteria based on nutritional requirements.
28. Classification of culture media.
29. Methods of isolation of pure culture.
30. Scheme of isolation of bacteria in pure culture.
31. Identification of bacteria. Selective media.
32. Special media for bacterial growth. Differential media.
33. Enzymes of bacteria. Classification of enzymes.
34. Methods of anaerobic culture.
35. Cultivation of rickettsiae and chlamydia.
36. Classification of cell cultures.
37. Cultivation of viruses. Cytopathic effects of viruses on host cell.

Immunity. Infection. Antibiotics. Genetics.

38. Immunity. Innate Immunity. Acquired Immunity.
39. Antigens. Properties of antigens.
40. Antibodies. Immunoglobulins. Structure of immunoglobulins. Immunoglobulin classes.
41. Antigen- Antibody reaction. Agglutination test.
42. Antigen- Antibody reaction. Precipitation test.
43. Antigen- Antibody reaction. Complement fixation test.
44. Antigen- Antibody reaction. Neutralization test.
45. Antigen- Antibody reaction. Immunofluorescence. ELISA.
46. The complement system. CFT.
47. Structure and functions of immune system.
48. Humoral immune response.
49. Cellular immune response.
50. Hypersensitivity. Immediate reaction.
51. Hypersensitivity. Delayed reaction.
52. Immune sera. Preparation.
53. Vaccines. Mechanism of action of vaccines.
54. Phagocytosis. Phagocytic cells.
55. Infection. Classification.

56. Bacterial virulence factors.
57. Antibiotics. Classification. Mechanisms, type and site of action.
58. Antibiotic sensitivity tests. Dilution test. Diffusion test.
59. Drug resistance.
60. Mutation. Types of mutation. Mutagenic agents.
61. Transmission of genetic material. Transformation. Transduction. Lysogenic conversion. Conjugation. Plasmids.
62. Plasmids. Features of bacterial plasmids.

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FOR NOTES

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