Contents

TOPIC 1. INTRODUCTION TO MICROBIOLOGY. THE SUBJECT AND TASKS OF MICROBIOLOGY. A SHORT HISTORICAL ESSAY.

Contents

Microbiology is a science, which study most shallow living creatures - microorganisms.

Before inventing of microscope humanity was in dark about their existence. But during the centuries people could make use of processes vital activity of microbes for its needs. They could prepare a koumiss, alcohol, wine, vinegar, bread, and other products. During many centuries the nature of fermentations remained incomprehensible.

Microbiology learns morphology, physiology, genetics and microorganisms systematization, their ecology and the other life forms.

Specific Classes of Microorganisms

- Algae
- Protozoa
- Fungi (yeasts and molds)
- Bacteria
- Rickettsiae
- Viruses
- Prions

The Microorganisms are extraordinarily widely spread in nature. They literally ubiquitous forward us from birth to our death. Daily, hourly we eat up thousands and thousands of microbes together with air, water, food. On our skin, in mouth and nasal cavities, on mucous membranes and in bowels enormous amount of microorganisms live and act. Many of them are found in earth cortex and in the air, and in the ocean’s, sea’s, river’s water, on all of latitudes, mainlands and continents.

For the first time term “microbe" was offered by French scientist Sh. Sedillot in 1878. It derives from Greek “microbe", that means briefly living, or most shallow living creature. Science, which learns the microorganisms, was named by E. Duclaux microbiology. For short development period this science accumulated great factual material. The separate microbiological branches such as bacteriology, mycology, protistology, virology quickly appeared.

Periods of microbiology development

- Morphologic
- Physiologic
- Prophylactic

Development of microbiological science was interlinked with art of glass and diamonds grinding. This brought to creation of the first microscope by Hans and Zacharian Jansen in Holland in 1590.

The discovery of microorganisms is associated with the name of Antony van Leeuwenhoek (1632-1723).

In 1683 Leeuwenhoek described the basic bacterium forms. His scientific supervisions Leeuwenhoek described in special letters and sent off them to the London Royal Scientific Society. He sent away about 300 letters. The Leeuwenhoek’s letters brought on enormous surprise among English scientists. They opened a fantastic world of invisible creatures. He named them “living animals" (animalcula viva) and in one of letter wrote: “In my mouth there are more animacula viva, than peoples in all United Kingdom".

These wonderful discovery of Dutch naturalist were the embryo, with which science of bacteria developed. Namely from these times starts the so-called morphological period in microbiology history (XVII middle of age). It is also called micrographycal period, as the study of microorganism came only to description of their dimensions and forms. Biological properties and their significances for man still a long time remained incomprehensible.

However, using the primitive microscopes of that time it was difficult to determine the difference between separate bacteria species. Even celebrated founder of scientific systematization of all of living organisms Karl Linney renounced to classify the bacteria. He gave them general name “chaos".

In the second half of XIX century microbiology strongly affirms as independent science. Namely these sciences were fruitful soil, on which Pasteur's talent evinced.

He studied wine "illness“, fermentation, made Pasteurization method, offered to grow microbes on artificial nutrient media, he proved, that on definite cultivation conditions the pathogenic bacteria lose its virulence, made vaccine against anthrax, rabies.

Physiological period has began

Not less important are scientific works of celebrated German scientist R. Koch.

He performed classic researches on etiology of anthrax, opened tuberculosis bacilli, cholera vibrio, proposed to isolate pure bacterial cultures on solid nutrient media (gelatin, potatoes), developed the preparations staining methods by aniline

dye-stuffs, method of hanging drop for examination of bacteria motility, offered apparatus for sterilization

The Patriarch of world and Ukrainian microbiology - I. Metchnikov

He studied inflammation pathology, phagocytosis, bases about antagonism of bacteria.

From all microbes-antagonists I.Metchnikov preferred the lactic bacteria. On their base he offered three medical preparations - sour clotted milk, yogurt and lactobacillin.

Now they are called by eubiotics. Classic Metchnikov's researches defined a prophylactic period in microbiology history.

In 1892 D. Ivanovskiy described an virus of mosaic tobacco illness – new class of infectious agents

Microorganisms constitute a very antique group of living organisms which appeared on the Earth's surface almost 3000 million years ago.

With the development of microbiology, came 4 important concepts:

1) discovery of microorganisms - refutes spontaneous generation

2) pure culture concept

3) germ theory of disease

4) role in chemical transformations

I. Discovery

- 1665 Robert Hooke - observed cells in sections of cork
~ (1680s) Leeuwenhoek - “animalcules” observed in rain water, other sources (Fig page

1) Spontaneous Generation = life from non-life, disproving this belief:
~ 1670 Redi - demonstrated larva present on meat was from eggs
~ 1765 Spallanzani and others used sealed flasks and treated air to prevent microbial growthhowever, others such as Needham claimed a vitalistic force in air was necessary forspontaneous generation to occur.
- 1860’s Louis Pasteur - “Father of Microbiology” used swan necked flasks

2) to disprove spontaneous generation. Also, observed that heat could kill these microbes. This led to the development of Pasteurization. (also see Tyndall)

II. Pure Culture Conceptpure culture = 1 type of organism grown in culture, once this was accomplished, microbes could be studied individually.

III. Germ Theory of Disease - proved role of microorganisms in causing disease.

A) - 1870’s Robert Koch - demonstrated 6 different infections in mice caused by 6 different bacteria; demonstrated anthrax was caused by bacteria
1884 - 4 postulates (Koch’s)

1) organism present in diseases cases, absent in healthy animals

2) can be isolated from diseased animal and grown in pure culture

3) disease can be reproduced by inoculation of healthy animal with culturedorganism

4) organism can be re-isolated from experimentally infected animal and returned to culture (pure)

B) Koch’s work and others led to the “Golden Age of Microbiology”

1) causes of many diseases were determined

2) improved culture methods

3) improved staining techniques

C) Lister in 1860s, developed aseptic surgical techniques, used carbolic acid (phenol) to treat dressings.

IV. Cell Theory

V. Role of Microorganisms in Chemical Transformations

A) 1837, demonstrated that Yeast generated alcoholglucose + H2O □□□□ ethanol + CO2

B) 1857 - 1876, Pasteur demonstrated such fermentations were microbial processes

C) 1897 Buchner discovered enzymes

VI. Areas developed from MicrobiologyDevelopment of microbiology led to the following areas:

1) Immunology

2) Chemotherapy

3) Virology

4) Molecular Biology

A) Immunology

1) 1796 Jenner - immunity to smallpox conferred by inoculation with cowpox

2) 1881 Pasteur - used “attenuated” virus for immunization against rabies

3) 1884 Metchnikoff - observed phagocytosis = cellular theory of immunity

4) 1890 Emil von Behring - immunity in cell free portions of blood = humoral theory

B) Chemotherapy

1) 1908 Ehrlich - treatment for syphilis (salvarsan = arsenic derivative)

2) 1929 Fleming – penicillin

3) 1944 Waksman – streptomycin

4) 1950’s - many antibiotics discovered

C) Virology

1) 1892 Iwanowski - showed an agent passing through a filter caused a disease in plants

2) 1931 - cultivation of viruses in chick embryos

3) 1949 - cultivation in cell cultures

D) Molecular Biology

1) Monoclonal antibodies - 1975 Kohler and Milstein fused myeloma and antibody producing cells = hybridoma which generates monoclonal antibodies

2) Genetic engineering - 1973 Chakrabarty transferred genes from one organism to another

Table 1. MICROBIOLOGY TODAY
Leading causes of death in the U.S.:

1870	1970
tuberculosis	heart disease
pneumonia and influenza	cancer
accidents	strokes
enteritis-diarrhea	accidents
scarlet fever	pneumonia and influenza

VII.

Table 2. Major microbial groups

Bacteria (variable shape)	0.5 - 1.5 μm by 1.0 - 3.0 μm	procaryotes
Viruses	0.015 - 0.2 μm	obligate intracellular parasites
Fungi (yeasts)	5 - 10 μm	eucaryotes, unicellular
Fungi (molds)	5 - 10 μm by several mm	eucaryotes, multicellular
Protozoans	2 - 200 μm	eucaryotes, unicellular
Algae	1.0 μm to feet	eucaryotes, unicellularand multicellular, photosynthetic

VIII. Sizes of microorganisms

IX. Classification

Nomenclature = binomial system of genus and species
Escherichia coli
variations in members of same species = strains
Escherichia coli K12, E. coli ML, E. coli O157:H7

X. Fields of microbiology
A) Medical and Clinical
B) Veterinary
C) Public Health
D) Immunology
E) Virology
F) Molecular biology, genetics, biotechnology
G) Microbial Physiology and biochemistry/geochemical
H) Aquatic
I) Food

TOPIC 2. SYSTEMATICS AND NOMENCLATURE OF MICROORGANISMS.

Contents

1. Systematics and nomenclature of bacteria.

2. Classification of bacteria.

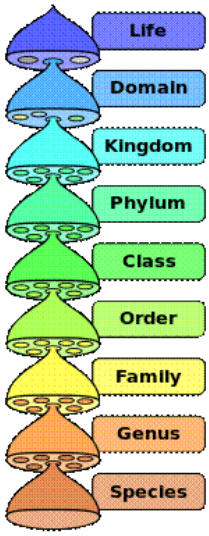

Figure 1. Bacterial taxonomy

The hierarchy of biological classification's eight major taxonomic ranks. Intermediate minor rankings are not shown.

Bacterial taxonomy (fig. 1) is the taxonomy, i.e. the rank-based classification, of bacteria. In the scientific classification established by Carl von Linné, each species has to be assigned to a genus (binary nomenclature), which in turn is a lower level of a hierarchy of ranks (family, suborder, order, subclass, class, division/phyla, kingdom and domain). In the currently accepted classification of Life, there are threedomains (Eukaryotes, Bacteria and Archaea), which, in terms of taxonomy, despite following the same principles have several different conventions between them and between their subdivisions as are studied by different disciplines (Botany, zoology, mycology and microbiology), for example in zoology there are type specimens, whereas in microbiology there are type strains.

Diversity

Main article: Bacteria

Prokaryotes share many common features, such as lack of nuclear membrane, unicellularity, division by binary-fission and generally small size. The various species differ amongst each other based on several characteristics, allowing their identification and classification. Examples include:

- **Phylogeny**: All bacteria stem from a common ancestor and diversified since, consequently possess different levels of evolutionary relatedness (see Bacterial phyla and Timeline of evolution)

- **Metabolism**: Different bacteria may have different metabolic abilities (see Microbial metabolism)
- **Environment**: Different bacteria thrive in different environments, such as high/low temperature and salt (see Extremophiles)
- **Morphology**: There are many structural differences between bacteria, such as cell shape, Gram stain (number of lipid bilayers) or bilayer composition (see Bacterial cellular morphologies, Bacterial cell structure)
- **Pathogenicity**: Some bacteria are pathogenic to plants or animals (see Pathogenic bacteria)

Classification history

Bacteria were first observed by Antonie van Leeuwenhoek in 1676, using a single-lens microscope of his own design. He called them "animalcules" and published his observations in a series of letters to the Royal Society. The name "bacterium" was introduced much later, by Christian Gottfried Ehrenberg in 1838.

Classical classification

Placement

Main article: Monera

Tree of Life in *Generelle Morphologie der Organismen* (1866)

Bacteria were first classified as plants constituting the class *Schizomycetes*, which along with the *Schizophyceae* (blue green algae/*Cyanobacteria*) formed the phylum *Schizophyta*.

Haeckel in 1866 placed the group in the phylum *Moneres* (from μονήρης: simple) in the kingdom *Protista* and defines them as completely structureless and homogeneous organisms, consisting only of a piece of plasma. He subdivided the phylum into two groups:

- die Gymnomoneren (no envelope)
  - *Protogenes* — such as *Protogenes primordialis*, now classed as a eukaryote and not a bacterium
  - *Protamaeba* — now classed as a eukaryote and not a bacterium
  - *Vibrio* — a genus of comma shaped bacteria first described in 1854)
  - *Bacterium* — a genus of rod shaped bacteria first described in 1828, that later gave its name to the members of the Monera, formerly referred to as "a moneron" (plural "monera") in English and "eine Moneren"(fem. plural "Moneres") in German
  - *Bacillus* — a genus of spore-forming rod shaped bacteria first described in 1835
  - *Spirochaeta* — thin spiral shaped bacteria first described in 1835
  - *Spirillum* — spiral shaped bacteria first described in 1832 etc.
- die Lepomoneren (with envelope)
  - *Protomonas* — now classed as a eukaryote and not a bacterium. The name was reused in 1984 for an unrelated genus of Bacteria
  - *Vampyrella* — now classed as a eukaryote and not a bacterium

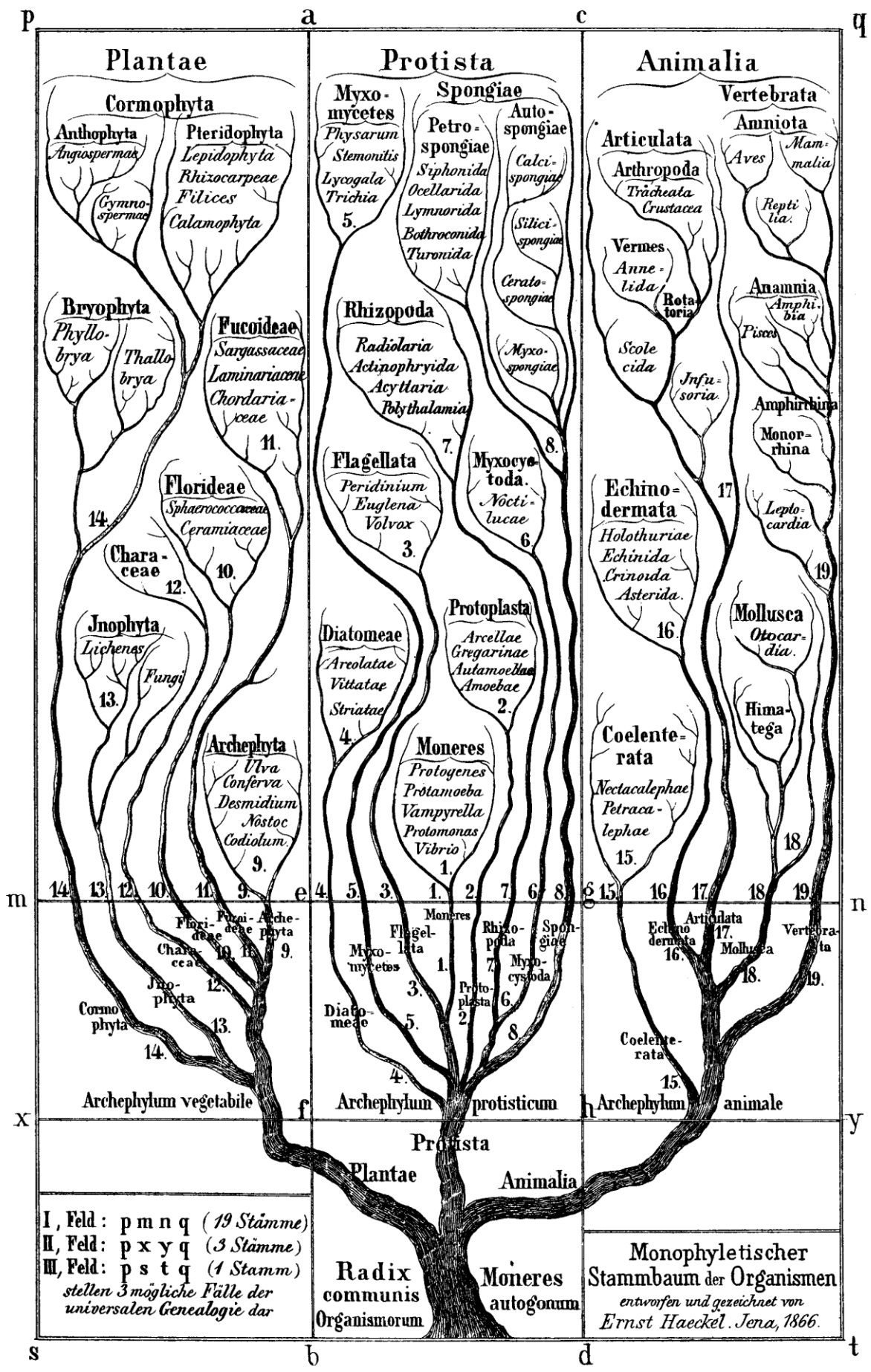

Figure 2. Haeckel's tree of life

The group was later reclassified as the *Prokaryotes* by Chatton.

The classification of *Cyanobacteria* (colloquially "blue green algae") has been fought between being algae or bacteria (for example, Haeckel classified *Nostoc* in the phylum Archephyta of Algae).

in 1905 Erwin F. Smith accepted 33 valid different names of bacterial genera and over 150 invalid names, and in 1913 Vuillemin in a study concluded that all species of the *Bacteria* should fall into the genera *Planococcus*, *Streptococcus*, *Klebsiella*, *Merista*, *Planomerista*, *Neisseria*, *Sarcina*, *Planosarcina*, *Metabacterium*, *Clostridium*, *Serratia*, *Bacterium* and *Spirillum*.

However, different authors often reclassified the genera due to the lack of visible traits to go by, resulting in a poor state which was summarised in 1915 by Robert Earle Buchanan. By then, the whole group received different ranks and names by different authors namely

- *Schizomycetes* (Naegeli 1857)
- *Bacteriaceae* (Cohn 1872,)
- *Bacteria* (Cohn 1872b,)
- *Schizomycetaceae* (DeToni and Trevisan 1889,)

Furthermore the families into which the class was subdivided, changed from author to author and for some such as Zipf the names where in German and not in Latin. The first edition of the Bacteriological Code in 1947 sorted several problems out.

Table 3. Classification history

<table>
<tr><th>Linnaeus
1735</th><th>Haeckel
1866</th><th>Chatton
1925</th><th>Copeland
1938</th><th>Whittaker
1969</th><th>Woese et al.
1990</th><th>Cavalier-Smith
1998</th></tr>
<tr><td>2 kingdoms</td><td>3 kingdoms</td><td>2 empires</td><td>4 kingdoms</td><td>5 kingdoms</td><td>3 domains</td><td>6 kingdoms</td></tr>
<tr><td rowspan="2">(not treated)</td><td rowspan="3">Protista</td><td>Prokaryota</td><td>Monera</td><td>Monera</td><td>Bacteria</td><td rowspan="2">Bacteria</td></tr>
<tr><td rowspan="4">Eukaryota</td><td rowspan="2">Protoctista</td><td rowspan="2">Protista</td><td>Archaea</td></tr>
<tr><td rowspan="4">Eukarya</td><td>Protozoa</td></tr>
<tr><td rowspan="2">Vegetabilia</td><td rowspan="2">Plantae</td><td rowspan="2">Plantae</td><td>Chromista</td></tr>
<tr><td>Plantae</td><td>Plantae</td></tr>
<tr><td>Fungi</td><td>Fungi</td></tr>
<tr><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td><td>Animalia</td></tr>
</table>

Subdivisions based on Gram staining

Despite there being little agreement on the major subgroups of the *Bacteria*, Gram staining results were most commonly used as a classification tool, consequently until the advent of molecular phylogeny, the Kingdom *Prokaryotae* was divided into four divisions, A classification scheme still formally followed by Bergey's manual of systematic bacteriology for tome order.

- ***Gracilicutes*** (gram negative)
  - *Photobacteria* (photosynthetic): class *Oxyphotobacteriae* (water as electron acceptor, includes the order *Cyanobacteriales* = blue green algae, now phylum *Cyanobacteria*) and class *Anoxyphotobacteriae* (anaerobic phototrophs, orders: *Rhodospirillales* and *Chlorobiales*
  - *Scotobacteria* (non-photosynthetic, now the *Proteobacteria* and other gram negative nonphotosynthetic phyla)
- ***Firmacutes*** [sic] (gram positive, subsequently corrected to *Fimicutes*)
  - several orders such as *Bacillales* and *Actinomycetales* (now in the phylum *Actinobacteria*)
- ***Mollicutes*** (gram variable, e.g. *Mycoplasma*)
- ***Mendocutes*** (uneven gram stain, "methanogenic bacteria" now known as the *Archaea*)

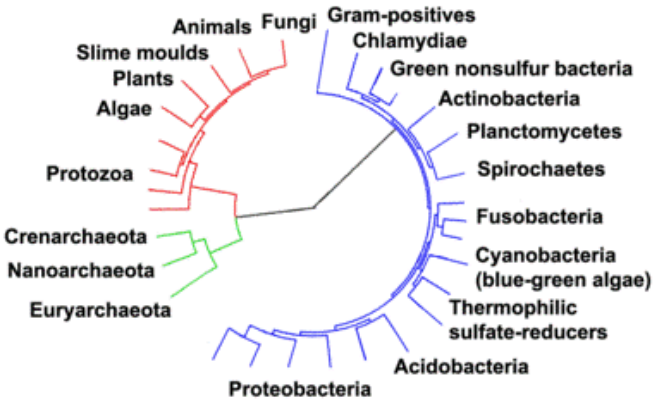

Figure 3. Molecular era.
"Archaic bacteria" and Woese's reclassification

Phylogenetic tree showing the relationship between the archaea and other forms of life. Eukaryotes are colored red, archaea green and bacteria blue. Adapted from Ciccarelli *et al.*

Woese argued that the bacteria, archaea, and eukaryotes represent separate lines of descent that diverged early on from an ancestral colony of organisms. However, a few biologists argue that the Archaea and Eukaryota arose from a group of bacteria. In any case, it is thought that viruses and archaea began relationships approximately two billion years ago, and that co-evolution may have been occurring between members of these groups. It is possible that the last common ancestor of the bacteria and archaea was a thermophile, which raises the possibility that lower temperatures are "extreme environments" in archaeal terms, and organisms that live in cooler environments appeared only later. Since the Archaea and Bacteria are no more related to each other than they are to eukaryotes, the term *prokaryote*'s only surviving meaning is "not a eukaryote", limiting its value.

With improved methodologies it became clear that the methanogenic bacteria were profoundly different and were (erroneous) believed to be relics of ancient

bacteria thus Carl Woese, regarded as the forerunner of the molecular phylogeny revolution, identified three primary lines of descent the *Archaebacteria*, the*Eubacteria* and the *Urkaryotes*, the latter now represented by the nucleocytoplasmic component of the *Eukaryotes*, these lineages were formalised into the rank Domain (*regio* in Latin) which divided Life into 3 domains: the *Eukaryota*, the *Archaea* and the *Bacteria*.This scheme is still followed today.

Subdivisions

Main article: Bacterial phyla

In 1987 Carl Woese divided the *Eubacteria* into 11 divisions based on 16S ribosomal RNA (SSU) sequences, which with several additions are still used today.

Opposition

Some authors have opposed the three domain due to various reasons.

One prominent scientist which opposes the three domain system is Thomas Cavalier-Smith, which proposed that the *Archaea* and the *Eukaryotes* (the *Neomura*) stem from Gram positive bacteria (*Posibacteria*), which in turn derive from gram negative bacteria (*Negibacteria*) based on several logical arguments, which are highly controversial and generally disregarded by molecular biology community (*c.f.* reviewers' comments on, *e.g.* Eric Bapteste is "agnostic" regarding the conclusions) and are often not even mention in reviews (*e.g.*), due to the subjective nature of the assumptions made for logical arguments. However, despite there being a wealth of statistically supported studies towards the rooting of the tree of life between the *Bacteria* and the *Neomura* by means of a variety of methods, including some that are impervious to accelerated evolution, which is claimed by Cavalier-Smith to be the source of the supposed fallacy in molecular methods, there are a few studies which have drawn different conclusions, some of which place the root in the phylum *Firmicutes* with nested archaea for review

Classification of domains and phyla - Hierarchical classific hylum "Korarchaeota"

"*Candidatus* Korarchaeum"

Phylum "Nanoarchaeota"

"*Nanoarchaeum*"

Phylum "Thaumarchaeota"

Order Cenarchaeales

Family "Cenarchaeaceae"

"*Cenarchaeum*"

See also the file Classification of prokaryotes (bacteria): Introduction of prokaryotes (bacteria) w.bacterio.cict.fr/classifphyla.html

See also the file Classification of prokaryotes (bacteria): Introduction

Introduction

The two prokaryotic domains (or empires), "Bacteria" (or "*Eubacteria*") and "Archaea" (or "*Archaeobacteria*"), are subdivided into 35 phyla (or divisions): 30 phyla in the domain "Bacteria", and 5 phyla in the domain "Archaea". This file provides, alphabetically, the list of phyla included in the domains "Bacteria" and "Archaea".

The classes, subclasses, orders, suborders, families and genera included in the phyla are also cited. Taxa above the rank of class are not covered by the Rules of the *Bacteriological Code* (1990 Revision). Such names cannot be validly published and they are cited below in quotes.

Domain "Bacteria"

Phyla: "Acidobacteria"-"Actinobacteria"-"Aquificae"-"Armatimonadetes"-"Bacteroidetes" - "Caldiserica" - "Chlamydiae" - "Chlorobi" - "Chloroflexi"-"Chrysiogenetes" - "Cyanobacteria" -"Deferribacteres" - "Deinococcus-Thermus"-"Dictyoglomi" - "Elusimicrobia" - "Fibrobacteres" - "Firmicutes" - "Fusobacteria" - "Gemmatimonadetes" - "Lentisphaerae" - "Nitrospira" - "Planctomycetes" - "Proteobacteria" - "Spirochaetes" - "Synergistetes" - "Tenericutes" - "Thermodesulfobacteria" - "Thermomicrobia" - "Thermotogae" - "Verrucomicrobia"

Phylum "Acidobacteria"
Class Acidobacteria
Order Acidobacteriales
Family Acidobacteriaceae
Acidicapsa - Acidobacterium - Bryocella - Edaphobacter - Granulicella - Telmatobacter - Terriglobus
Unclassified Acidobacteria Bryobacter
Class Holophagae
Order Acanthopleuribacterales
Family Acanthopleuribacteraceae
Acanthopleuribacter
Order Holophagales
Family Holophagaceae
Geothrix - Holophaga
Phylum "Actinobacteria"
Class Actinobacteria
Subclass Acidimicrobidae
Order Acidimicrobiales
Suborder "Acidimicrobineae"
Family Acidimicrobiaceae
Acidimicrobium - Ferrimicrobium - Ferrithrix - Ilumatobacter
Family Iamiaceae
Iamia
Unclassified "Acidimicrobineae" Aciditerrimonas
Subclass Actinobacteridae
Order Actinomycetales
Suborder Actinomycineae
Family Actinomycetaceae
Actinobaculum - Actinomyces - Arcanobacterium - Falcivibrio - Mobiluncus - Trueperella - Varibaculum
Suborder Actinopolysporineae

Family Actinopolysporaceae
Actinopolyspora
Suborder Catenulisporineae
Family Actinospicaceae
Actinospica
Family Catenulisporaceae
Catenulispora
Suborder Corynebacterineae
Family Corynebacteriaceae
Bacterionema - Caseobacter - Corynebacterium - Turicella
Family Dietziaceae
Dietzia
Family Gordoniaceae
See Nocardiaceae [Zhi *et al*. 2009 propose to combine the families *Nocardiaceae* and *Gordoniaceae* (genera Gordonia, Millisia,and Skermania) in an emended family *Nocardiaceae*]
Family Mycobacteriaceae
Amycolicicoccus - Mycobacterium
Family Nocardiaceae See also Gordoniaceae [Zhi *et al*. 2009 propose to combine the families *Nocardiaceae* and *Gordoniaceae* (genera Gordonia, Millisia and Skermania) in an emended family *Nocardiaceae*] Gordonia - Micropolyspora - Millisia - Nocardia - Rhodococcus - Skermania - Smaragdicoccus - Williamsia
Family Segniliparaceae
Segniliparus
Family Tsukamurellaceae
Tsukamurella
Unclassified suborder Corynebacterineae Hoyosella - Tomitella
Suborder Frankineae
Family Acidothermaceae
Acidothermus
Family Cryptosporangiaceae
Cryptosporangium - Fodinicola
Family Frankiaceae
Frankia
Family Geodermatophilaceae
Blastococcus - Geodermatophilus - Modestobacter
Family Nakamurellaceae
Humicoccus - Nakamurella - Saxeibacter
Family Sporichthyaceae
Sporichthya
Unclassified suborder Frankineae Motilibacter
Suborder Glycomycineae
Family Glycomycetaceae
Glycomyces - Haloglycomyces - Stackebrandtia

Suborder Jiangellineae
Family Jiangellaceae
Jiangella - Haloactinopolyspora
Suborder Kineosporiineae
Family Kineosporiaceae
Angustibacter - Kineococcus - Kineosporia - Pseudokineococcus - Quadrisphaera
Suborder Micrococcineae
Family Beutenbergiaceae
Beutenbergia - Miniimonas - Salana - Serinibacter
Family Bogoriellaceae
Bogoriella - Georgenia - Oceanitalea
Family Brevibacteriaceae
Brevibacterium
Family Cellulomonadaceae
Actinotalea -Cellulomonas - Oerskovia - Paraoerskovia - Sediminihabitans - Tropheryma
Family Demequinaceae
Demequina - Lysinimicrobium
Family Dermabacteraceae
Brachybacterium - Dermabacter - Devriesea - Helcobacillus
Family Dermacoccaceae
Barrientosiimonas - Branchiibius - Calidifontibacter - Demetria - Dermacoccus - Flexivirga - Kytococcus - Luteipulveratus - Yimella
Family Dermatophilaceae
Austwickia - Dermatophilus - Kineosphaera - Mobilicoccus - Piscicoccus
Family Intrasporangiaceae
Aquipuribacter - Arsenicicoccus - Fodinibacter - Humibacillus - Humihabitans - Intrasporangium - Janibacter - Knoellia - Kribbia - Lapillicoccus - Marihabitans - Ornithinibacter -Ornithinicoccus - Ornithinimicrobium - Oryzihumus - Phycicoccus - Serinicoccus - Terrabacter - Terracoccus - Tetrasphaera
Family Jonesiaceae
Jonesia
Family Microbacteriaceae
Agreia - Agrococcus - Agromyces - Alpinimonas - Amnibacterium - Aureobacterium - Chryseoglobus - Clavibacter - Compostimonas - Cryobacterium - Curtobacterium -Diaminobutyricimonas - Frigoribacterium - Frondihabitans - Glaciibacter - Gryllotalpicola - Gulosibacter - Herbiconiux - Homoserinimonas - Humibacter - Klugiella - Labedella - Leifsonia- Leucobacter - Marisediminicola - Microbacterium - Microcella - Microterricola - Mycetocola - Okibacterium - Phycicola - Plantibacter - Pseudoclavibacter - Rathayibacter -Rhodoglobus - Salinibacterium - Schumannella - Subtercola - Yonghaparkia - Zimmermannella
Family Micrococcaceae
Acaricomes - Arthrobacter - Auritidibacter - Citricoccus - Enteractinococcus -

Kocuria - Micrococcus - Nesterenkonia - Pelczaria (rejected name) - Renibacterium - Rothia - Sinomonas- Stomatococcus - Yaniella - Zhihengliuella

Family Promicromonosporaceae

Cellulosimicrobium - Isoptericola - Myceligenerans - Promicromonospora - Xylanibacterium - Xylanimicrobium - Xylanimonas

Family Rarobacteraceae

Rarobacter

Family Ruaniaceae

Haloactinobacterium - Ruania

Family Sanguibacteraceae

Sanguibacter

Family Yaniellaceae

Note: The genus *Yaniella* Li *et al*. 2008 (the type genus of the family *Yaniellaceae* Li *et al*. 2008) is in most 16S rRNA gene sequence-based phylogenetic analyses a member of the family *Micrococcaceae* Pribram 1929 (Approved Lists 1980). According to Schumann *et al*. 2009, it appears debatable whether it deserves the status as the type genus of its own family*Yaniellaceae*. According to Yassin *et al*. 2011, *Yaniellaceae* Li *et al*. 2008 is a later heterotypic synonym of *Micrococcaceae* Pribram 1929 (Approved Lists 1980).

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1. SCHUMANN (P.), KÄMPFER (P.), BUSSE (H.J.) and EVTUSHENKO (L.I.): Proposed minimal standards for describing new genera and species of the suborder *Micrococcineae*.*Int. J. Syst. Evol. Microbiol*., 2009, 59, 1823-1849.

2. YASSIN (A.F.), HUPFER (H.), SIERING (C.), KLENK (H.P.) and SCHUMANN (P.): *Auritidibacter ignavus* gen. nov., sp. nov., of the family *Micrococcaceae*, isolated from an ear swab of a man with otitis externa, transfer of the members of the family *Yaniellaceae* Li *et al*. 2008 to the family *Micrococcaceae* and emended description of the suborder*Micrococcineae*. *Int. J. Syst. Evol. Microbiol*., 2011, **61**, 223-230.

Unclassified suborder Micrococcineae Koreibacter - Luteimicrobium

Suborder Micromonosporineae

Family Micromonosporaceae

Actinaurispora - Actinocatenispora - Actinoplanes - Allocatelliglobosispora - Amorphosporangium - Ampullariella - Asanoa - Catellatospora -Catelliglobosispora - Catenuloplanes -Couchioplanes - Dactylosporangium -Hamadaea - Jishengella - Krasilnikovia - Longispora - Luedemannella -Micromonospora - Phytohabitans - Phytomonospora - Pilimelia -Planopolyspora -Planosporangium - Plantactinospora - Polymorphospora - Pseudosporangium -Rugosimonospora - Salinispora - Spirilliplanes - Verrucosispora - Virgisporangium

Suborder Propionibacterineae

Family Nocardioidaceae

Actinopolymorpha - Aeromicrobium - Hongia - Flindersiella - Kribbella - Marmoricola - Nocardioides - Pimelobacter- Thermasporomyces

Family Propionibacteriaceae

Aestuariimicrobium - Arachnia - Auraticoccus - Brooklawnia - Friedmanniella - Granulicoccus - Luteococcus - Microlunatus - Micropruina - Naumannella - Propionibacterium -Propionicicella - Propioniciclava - Propionicimonas - Propioniferax - Propionimicrobium - Tessaracoccus

Suborder Pseudonocardineae

Family Actinosynnemataceae

Note:

According to Labeda *et al*. 2011, *Actinosynnemataceae* Labeda and Kroppenstedt 2000 emend. Zhi *et al*. 2009 is a later heterotypic synonym of *Pseudonocardiaceae*Embley *et al*. 1989 emend. Labeda *et al*. 2011.

Reference:

1. LABEDA (D.P.), GOODFELLOW (M.), CHUN (J.), ZHI (X.Y.) and LI (W.J.): Reassessment of the systematics of the suborder *Pseudonocardineae*: transfer of the genera within the family *Actinosynnemataceae* Labeda and Kroppenstedt 2000 emend. Zhi *et al*. 2009 into an emended family *Pseudonocardiaceae* Embley *et al*. 1989 emend. Zhi *et al*. 2009.*Int. J. Syst. Evol. Microbiol*., 2011, 61, 1259-1264.

Family Pseudonocardiaceae

Actinoalloteichus - Actinobispora - Actinokineospora - Actinomycetospora - Actinophytocola - Actinosynnema - Alloactinosynnema - Allokutzneria -Amycolata - Amycolatopsis -Crossiella - Faenia - Goodfellowiella -Haloechinothrix - Kibdelosporangium - Kutzneria - Labedaea - Lechevalieria -Lentzea - Prauserella - Pseudoamycolata - Pseudonocardia -Saccharomonospora -Saccharopolyspora - Saccharothrix - Sciscionella - Streptoalloteichus -Thermobispora - Thermocrispum - Umezawaea - Yuhushiella

Suborder Streptomycineae

Family Streptomycetaceae

Actinopycnidium - Actinosporangium - Chainia - Elytrosporangium - Kitasatoa - Kitasatospora - Microellobosporia - Streptacidiphilus - Streptomyces - Streptoverticillium

Suborder Streptosporangineae

Family Nocardiopsaceae

Haloactinospora - Marinactinospora - Murinocardiopsis - Nocardiopsis - Salinactinospora - Spinactinospora - Streptomonospora - Thermobifida

Family Streptosporangiaceae

Acrocarpospora - Herbidospora - Microbispora - Microtetraspora - Nonomuraea - Planobispora - Planomonospora - Planotetraspora - Sphaerisporangium - Streptosporangium -Thermoactinospora - Thermocatellispora - Thermopolyspora

Family Thermomonosporaceae
Actinoallomurus - Actinocorallia - Actinomadura - Excellospora - Spirillospora - Thermomonospora
Unclassified suborder Streptosporangineae Allonocardiopsis - Sinosporangium
Order Bifidobacteriales
Family Bifidobacteriaceae
Aeriscardovia - Alloscardovia - Bifidobacterium - Gardnerella - Metascardovia - Parascardovia - Scardovia
Subclass Coriobacteridae
Order Coriobacteriales
Suborder "Coriobacterineae"
Family Coriobacteriaceae
Adlercreutzia - Asaccharobacter - Atopobium - Collinsella - Coriobacterium - Cryptobacterium - Denitrobacterium - Eggerthella - Enterorhabdus - Gordonibacter - Olsenella -Paraeggerthella - Slackia
Subclass Nitriliruptoridae
Order Euzebyales
Family Euzebyaceae
Euzebya
Order Nitriliruptorales
Family Nitriliruptoraceae
Nitriliruptor
Subclass Rubrobacteridae
Order Gaiellales
Family Gaiellaceae
Gaiella
Order Rubrobacterales
Suborder "Rubrobacterineae"
Family Rubrobacteraceae
Rubrobacter
Order Solirubrobacterales
Family Conexibacteraceae
Conexibacter
Family Patulibacteraceae
Patulibacter
Family Solirubrobacteraceae
Solirubrobacter
Order Thermoleophilales
Family Thermoleophilaceae
Thermoleophilum
Phylum "Aquificae"
Class Aquificae
Order Aquificales

Family Aquificaceae
Aquifex - Calderobacterium - Hydrogenivirga - Hydrogenobacter - Hydrogenobaculum - Thermocrinis
Family Desulfurobacteriaceae
Balnearium - Desulfurobacterium - Phorcysia - Thermovibrio
Family Hydrogenothermaceae
Hydrogenothermus - Persephonella - Sulfurihydrogenibium - Venenivibrio
Unclassified Aquificales Thermosulfidibacter
Phylum "Armatimonadetes"
Class Armatimonadia
Order Armatimonadales
Family Armatimonadaceae
Armatimonas
Class Chthonomonadetes
Order Chthonomonadales
Family Chthonomonadaceae
Chthonomonas
Class Fimbriimonadia
Order Fimbriimonadales
Family Fimbriimonadaceae
Fimbriimonas
Phylum "Bacteroidetes"
Class Bacteroidia
Order Bacteroidales
Family Bacteroidaceae
Acetofilamentum - Acetomicrobium - Acetothermus - Anaerorhabdus - Bacteroides - Capsularis
Family Marinilabiliaceae
Alkaliflexus - Alkalitalea - Anaerophaga - Geofilum - Mangroviflexus - Marinilabilia - Natronoflexus - Thermophagus
Family Porphyromonadaceae
Barnesiella - Butyricimonas - Dysgonomonas - Macellibacteroides - Odoribacter - Oribaculum - Paludibacter - Parabacteroides - Petrimonas - Porphyromonas - Proteiniphilum -Tannerella
Family Prevotellaceae
Hallella - Paraprevotella - Prevotella - Xylanibacter
Family Rikenellaceae
Alistipes - Rikenella
Unclassified Bacteroidales Phocaeicola - Sunxiuqinia
Class Cytophagia
Order Cytophagales
Family Cyclobacteriaceae
Algoriphagus - Aquiflexum - Belliella - Cecembia - Chimaereicella -

Cyclobacterium - Echinicola - Fontibacter - Hongiella - Indibacter -Mongoliicoccus - Mongoliitalea - Nitritalea -Shivajiella

Family Cytophagaceae

Adhaeribacter - Arcicella - Cytophaga - Dyadobacter - Effluviibacter - Ekhidna - Emticicia - Fibrella - Fibrisoma - Flectobacillus - Flexibacter - Huanghella - Hymenobacter - Larkinella -Leadbetterella - Litoribacter - Meniscus - Microscilla - Persicitalea - Pontibacter - Pseudarcicella - Rhodocytophaga - Rhodonellum - Rudanella - Runella - Siphonobacter - Spirosoma -Sporocytophaga

Family Flammeovirgaceae

Aureibacter - Cesiribacter - Fabibacter - Flammeovirga - Flexithrix - Fulvivirga - Limibacter - Marinicola - Marinoscillum - Marivirga - Perexilibacter -Persicobacter - Rapidithrix -Reichenbachiella - Roseivirga - Sediminitomix -Thermonema

Family Rhodothermaceae

Rhodothermus - Rubricoccus - Salinibacter - Salisaeta

Unclassified Cytophagales Chryseolinea - Luteivirga - Ohtaekwangia

Class Flavobacteriia

Order Flavobacteriales

Family Blattabacteriaceae

Blattabacterium

Family Cryomorphaceae

Brumimicrobium - Crocinitomix - Cryomorpha - Fluviicola - Lishizhenia - Owenweeksia - Phaeocystidibacter - Salinirepens - Wandonia

Family Flavobacteriaceae

Actibacter - Aequorivita - Aestuariibaculum - Aestuariicola - Algibacter - Aquimarina - Arenibacter - Aureitalea - Aureivirga - Bergeyella - Bizionia - Capnocytophaga - Cellulophaga -Chryseobacterium - Cloacibacterium - Coenonia - Corallibacter - Costertonia - Croceibacter - Croceitalea - Cruoricaptor - Dokdonia - Donghaeana - Elizabethkingia - Empedobacter -Epilithonimonas - Eudoraea - Euzebyella - Flagellimonas - Flaviramulus - Flavivirga - Flavobacterium - Formosa - Fulvibacter - Gaetbulibacter - Gaetbulimicrobium - Galbibacter -Gangjinia - Gelidibacter - Gillisia - Gilvibacter - Gramella - Hyunsoonleella - Imtechella -Jejuia - Joostella - Kaistella - Kordia - Kriegella - Krokinobacter - Lacinutrix - Leeuwenhoekiella- Leptobacterium - Lutaonella - Lutibacter - Lutimonas - Maribacter - Mariniflexile - Maritimimonas - Marixanthomonas - Meridianimaribacter - Mesoflavibacter - Mesonia - Muricauda -Muriicola - Myroides - Namhaeicola - Nonlabens - Olleya - Ornithobacterium - Persicivirga - Pibocella - Planobacterium - Polaribacter - Pontirhabdus - Postechiella - Pricia - Pseudozobellia - Psychroflexus - Psychroserpens - Riemerella - Robiginitalea - Salinimicrobium - Salegentibacter - Sandarakinotalea - Sediminibacter - Sediminicola - Sejongia -Siansivirga - Sinomicrobium - Snuella - Soonwooa - Spongiibacterium - Stanierella - Stenothermobacter - Subsaxibacter - Subsaximicrobium - Tamlana - Tenacibaculum - Ulvibacter -Vitellibacter - Wautersiella - Weeksella - Winogradskyella - Yeosuana - Zeaxanthinibacter - Zhouia - Zobellia - Zunongwangia

Family Schleiferiaceae
Schleiferia
Class Sphingobacteriia
Order Sphingobacteriales
Family Chitinophagaceae
Balneola - Chitinophaga - Ferruginibacter - Filimonas - Flavihumibacter - Flavisolibacter - Flavitalea - Gracilimonas - Hydrotalea - Lacibacter - Niabella - Niastella - Parasegetibacter -Sediminibacterium - Segetibacter - Terrimonas
Family Saprospiraceae
Aureispira - Haliscomenobacter - Lewinella - Saprospira
Family Sphingobacteriaceae
Mucilaginibacter - Nubsella - Olivibacter - Parapedobacter - Pedobacter - Pseudosphingobacterium - Solitalea - Sphingobacterium
Unclassified Sphingobacteriales Fodinibius
Unclassified "Bacteroidetes" Marinifilum - Prolixibacter - Toxothrix
Phylum "Caldiserica"
Class Caldisericia
Order Caldisericales
Family Caldisericaceae
Caldisericum
Phylum "Chlamydiae"
Class Chlamydiae
Order Chlamydiales
Family Chlamydiaceae
Chlamydia - Chlamydophila
Family Parachlamydiaceae
Neochlamydia - Parachlamydia
Family Simkaniaceae
Simkania
Family Waddliaceae
Waddlia
Phylum "Chlorobi"
Class "Chlorobia" or Chlorobea
Order Chlorobiales
Family Chlorobiaceae
Ancalochloris - Chlorobaculum - Chlorobium - Chloroherpeton - Pelodictyon - Prosthecochloris
Class Ignavibacteria
Order Ignavibacteriales
Family Ignavibacteriaceae
Ignavibacterium
Phylum "Chloroflexi"
Class Anaerolineae
Order Anaerolineales

Family Anaerolineaceae
Anaerolinea - Bellilinea - Leptolinea - Levilinea - Longilinea - Ornatilinea
Class Caldilineae
Order Caldilineales
Family Caldilineaceae
Caldilinea - Litorilinea
Class "Chloroflexi"
Order "Chloroflexales"
Family "Chloroflexaceae"
Chloroflexus - Chloronema - Heliothrix - Roseiflexus
Family Oscillochloridaceae
Oscillochloris
Order "Herpetosiphonales"
Family "Herpetosiphonaceae"
Herpetosiphon
Class Dehalococcoidia
Order Dehalococcoidales
Family Dehalococcoidaceae
Dehalococcoides - Dehalogenimonas
Class Ktedonobacteria
Order Ktedonobacterales
Family Ktedonobacteraceae
Ktedonobacter
Family Thermosporotrichaceae
Thermosporothrix
Order Thermogemmatisporales
Family Thermogemmatisporaceae
Thermogemmatispora
Class Thermomicrobia
Order Thermomicrobiales
Family Thermomicrobiaceae
Thermomicrobium
Subclass Sphaerobacteridae
Order Sphaerobacterales
Suborder "Sphaerobacterineae"
Family Sphaerobacteraceae
Sphaerobacter
Phylum "Chrysiogenetes"
Class Chrysiogenetes
Order Chrysiogenales
Family Chrysiogenaceae
Chrysiogenes - Desulfurispira - Desulfurispirillum
Phylum "Cyanobacteria" (see the file Classification of Cyanobacteria)
Phylum "Deferribacteres"

Class Deferribacteres
Order Deferribacterales
Family Deferribacteraceae
Calditerrivibrio - Deferribacter - Denitrovibrio - Flexistipes - Geovibrio - Mucispirillum
Unclassified Deferribacterales Caldithrix

Phylum "Deinococcus-Thermus"

Class Deinococci
Order Deinococcales
Family Deinococcaceae
Deinobacter - Deinobacterium - Deinococcus
Family Trueperaceae
Truepera
Order Thermales
Family Thermaceae
Marinithermus - Meiothermus - Oceanithermus - Rhabdothermus - Thermus - Vulcanithermus
Phylum "Dictyoglomi"
Class Dictyoglomia
Order Dictyoglomales
Family Dictyoglomaceae
Dictyoglomus
Phylum "Elusimicrobia"
Class Elusimicrobia
Order Elusimicrobiales
Family Elusimicrobiaceae
Elusimicrobium

Phylum "Fibrobacteres"

Class Fibrobacteria
Order Fibrobacterales
Family Fibrobacteraceae
Fibrobacter

Phylum "Firmicutes"

Class Bacilli (or **Firmibacteria**)
Order Bacillales
Family Alicyclobacillaceae
Alicyclobacillus - Kyrpidia - Tumebacillus
Family Bacillaceae
Aeribacillus - Alkalibacillus - Allobacillus - Alteribacillus - Amphibacillus - Anaerobacillus - Anoxybacillus - Aquisalibacillus - Bacillus - Caldalkalibacillus - Caldibacillus - Calditerricola -Cerasibacillus - Falsibacillus - Filobacillus - Geobacillus - Gracilibacillus - Halalkalibacillus - Halobacillus - Halolactibacillus - Lentibacillus - Lysinibacillus - Marinococcus -Microaerobacter - Natribacillus - Natronobacillus - Oceanobacillus - Ornithinibacillus - Paraliobacillus -

Paucisalibacillus - Pelagibacillus - Piscibacillus - Pontibacillus - Psychrobacillus - Saccharococcus - Salibacillus - Salimicrobium - Salinibacillus - Salirhabdus - Salisediminibacterium - Saliterribacillus - Salsuginibacillus - Sediminibacillus - Streptohalobacillus -Tenuibacillus - Terribacillus - Thalassobacillus - Virgibacillus - Viridibacillus - Vulcanibacillus

Family Listeriaceae

Brochothrix - Listeria

Family Paenibacillaceae

Ammoniphilus - Aneurinibacillus - Brevibacillus - Cohnella - Fontibacillus - Oxalophagus - Paenibacillus - Saccharibacillus - Thermobacillus

Family Pasteuriaceae

Pasteuria

Family Planococcaceae

Bhargavaea - Caryophanon - Chryseomicrobium - Filibacter - Jeotgalibacillus - Kurthia - Marinibacillus - Paenisporosarcina - Planococcus - Planomicrobium - Sporosarcina - Ureibacillus

Family Sporolactobacillaceae

Pullulanibacillus - Sinobaca - Sporolactobacillus - Tuberibacillus

Family Staphylococcaceae

Jeotgalicoccus - Macrococcus - Nosocomiicoccus - Salinicoccus - Staphylococcus

Family Thermoactinomycetaceae

Desmospora - Kroppenstedtia - Laceyella - Lihuaxuella - Marininema - Mechercharimyces - Melghirimyces - Planifilum - Seinonella - Shimazuella - Thermoactinomyces -Thermoflavimicrobium

Unclassified Bacillales Chungangia - Exiguobacterium - Gemella - Geomicrobium - Rummeliibacillus - Solibacillus - Thermicanus

Order Lactobacillales

Family Aerococcaceae

Abiotrophia - Aerococcus - Dolosicoccus - Eremococcus - Facklamia - Globicatella - Ignavigranum

Family Carnobacteriaceae

Agitococcus - Alkalibacterium - Allofustis - Alloiococcus - Atopobacter - Atopococcus - Atopostipes - Carnobacterium - Desemzia - Dolosigranulum - Granulicatella - Isobaculum -Lacticigenium - Lactosphaera - Marinilactibacillus - Pisciglobus - Trichococcus

Family Enterococcaceae

Bavariicoccus - Catellicoccus - Enterococcus - Melissococcus - Pilibacter - Tetragenococcus - Vagococcus

Family Lactobacillaceae

Lactobacillus - Paralactobacillus - Pediococcus - Sharpea

Family Leuconostocaceae

Fructobacillus - Leuconostoc - Oenococcus - Weissella

Family Streptococcaceae

Lactococcus - Lactovum - Streptococcus

Class Clostridia
Order Clostridiales
Family Caldicoprobacteraceae
Caldicoprobacter
Family Christensenellaceae
Christensenella
Family Clostridiaceae
Alkaliphilus - Anaerobacter - Anaerosalibacter - Anaerosporobacter - Anoxynatronum - Brassicibacter - Butyricicoccus - Caloramator -Caloranaerobacter - Caminicella - Cellulosibacter- Clostridiisalibacter -Clostridium - Fervidicella - Geosporobacter - Lactonifactor - Lutispora -Natronincola - Oxobacter - Proteiniclasticum - Saccharofermentans - Sarcina -Sporosalibacterium - Tepidimicrobium - Thermobrachium - Thermohalobacter -Thermotalea - Tindallia
Family Defluviitaleaceae
Defluviitalea
Family Eubacteriaceae
Acetobacterium - Alkalibacter - Alkalibaculum - Anaerofustis - Eubacterium - Garciella - Pseudoramibacter
Family Gracilibacteraceae
Gracilibacter
Family Heliobacteriaceae
Heliobacillus - Heliobacterium - Heliophilum - Heliorestis
Family Lachnospiraceae
Acetatifactor - Acetitomaculum - Anaerostipes - Butyrivibrio - Catonella - Cellulosilyticum - Coprococcus - Dorea - Hespellia - Johnsonella - Lachnoanaerobaculum - Lachnobacterium -Lachnospira - Marvinbryantia -Moryella - Oribacterium - Parasporobacterium - Pseudobutyrivibrio - Robinsoniella -Roseburia - Shuttleworthia - Sporobacterium - Syntrophococcus
Family Oscillospiraceae
Oscillibacter - Oscillospira
Family Peptococcaceae
Cryptanaerobacter - Dehalobacter- Desulfitibacter - Desulfitispora - Desulfitobacterium - Desulfonispora - Desulfosporosinus - Desulfotomaculum - Desulfurispora - Pelotomaculum -Peptococcus - Sporotomaculum - Syntrophobotulus - Thermincola - Thermoterrabacterium
Family Peptostreptococcaceae
Anaerosphaera - Filifactor - Peptostreptococcus - Sporacetigenium - Tepidibacter
Family Ruminococcaceae
Acetanaerobacterium - Acetivibrio - Anaerofilum - Anaerotruncus - Ethanoligenens - Faecalibacterium - Fastidiosipila - Hydrogenoanaerobacterium - Papillibacter - Ruminococcus -Sporobacter - Subdoligranulum
Family Syntrophomonadaceae
Dethiobacter - Fervidicola - Pelospora - Syntrophomonas - Syntrophospora - Syntrophothermus - Thermohydrogenium - Thermosyntropha

Unclassified Clostridiales Acetoanaerobium - Acidaminobacter - Anaerobranca - Anaerococcus - Anaerovirgula - Anaerovorax - Blautia - Carboxydocella - Dethiosulfatibacter - Finegoldia -Flavonifractor - Fusibacter - Gallicola - Guggenheimella - Helcococcus - Howardella - Mogibacterium - Murdochiella - Natranaerovirga - Parvimonas - Peptoniphilus - Proteiniborus -Proteocatella - Pseudoflavonifractor - Sedimentibacter - Soehngenia - Sporanaerobacter - Sulfobacillus - Symbiobacterium - Thermaerobacter - Tissierella

Order Halanaerobiales

Family Halanaerobiaceae

Halanaerobium - Halarsenatibacter - Halocella - Haloincola - Halothermothrix

Family Halobacteroidaceae

Acetohalobium - Fuchsiella - Halanaerobacter - Halanaerobaculum - Halobacteroides - Halonatronum - Natroniella - Orenia - Selenihalanaerobacter - Sporohalobacter

Order Natranaerobiales

Family Natranaerobiaceae

Natranaerobius - Natronovirga

Order Thermoanaerobacterales

Family Thermoanaerobacteraceae

Acetogenium - Ammonifex - Brockia - Caldanaerobacter - Caldanaerobius - Caloribacterium - Carboxydibrachium - Carboxydothermus - Desulfovirgula -Gelria - Moorella -Tepidanaerobacter - Thermacetogenium - Thermanaeromonas - Thermoanaerobacter - Thermoanaerobium - Thermobacteroides

Family Thermodesulfobiaceae

Coprothermobacter - Thermodesulfobium

Unclassified Thermoanaerobacterales Caldanaerovirga - Caldicellulosiruptor - Mahella - Syntrophaceticus - Thermoanaerobacterium - Thermosediminibacter - Thermovenabulum -Thermovorax

Class Erysipelotrichia

Order Erysipelotrichales

Family Erysipelotrichaceae

Allobaculum - Bulleidia - Catenibacterium - Coprobacillus - Eggerthia - Erysipelothrix - Holdemania - Kandleria - Solobacterium - Turicibacter

Class Mollicutes: see Phylum "Tenericutes"

Class Negativicutes

Order Selenomonadales

Family Acidaminococcaceae

Acidaminococcus - Phascolarctobacterium - Succiniclasticum - Succinispira

Family Veillonellaceae

Acetonema - Allisonella - Anaeroarcus - Anaeroglobus - Anaeromusa - Anaerosinus - Anaerovibrio - Centipeda - Dendrosporobacter - Dialister - Megamonas - Megasphaera -Mitsuokella - Negativicoccus - Pectinatus - Pelosinus - Propionispira - Propionispora - Quinella - Schwartzia - Selenomonas - Sporolituus - Sporomusa - Sporotalea - Thermosinus -Veillonella - Zymophilus

Class Thermolithobacteria
Order Thermolithobacterales
Family Thermolithobacteraceae
Thermolithobacter
Phylum "Fusobacteria"
Class Fusobacteriia
Order Fusobacteriales
Family Fusobacteriaceae
Cetobacterium - Fusobacterium - Ilyobacter - Propionigenium - Psychrilyobacter
Family Leptotrichiaceae
Leptotrichia - Sebaldella - Sneathia - Streptobacillus
Phylum "Gemmatimonadetes"
Class Gemmatimonadetes
Order Gemmatimonadales
Family Gemmatimonadaceae
Gemmatimonas
Phylum "Lentisphaerae"
Class Lentisphaeria
Order Lentisphaerales
Family Lentisphaeraceae
Lentisphaera
Order Victivallales
Family Victivallaceae
Victivallis
Class Oligosphaeria
Order Oligosphaerales
Family Oligosphaeraceae
Oligosphaera
Phylum "Nitrospira" or "Nitrospirae"
Class "Nitrospira"
Order "Nitrospirales"
Family "Nitrospiraceae"
Leptospirillum - Nitrospira - Thermodesulfovibrio
Phylum "Planctomycetes" or "Planctobacteria"
Class "Planctomycetacia" or Planctomycea
Order Planctomycetales
Family Planctomycetaceae
Aquisphaera - Blastopirellula - Gemmata - Isosphaera - Pirellula - Planctomyces - Rhodopirellula - Schlesneria - Singulisphaera - Telmatocola - Zavarzinella
Class Phycisphaerae
Order Phycisphaerales
Family Phycisphaeraceae
Phycisphaera
Phylum "Proteobacteria"

Class Alphaproteobacteria
Order Caulobacterales
Family Caulobacteraceae
Asticcacaulis - Brevundimonas - Caulobacter - Phenylobacterium
Family Hyphomonadaceae
Algimonas - Hellea - Henriciella - Hirschia - Hyphomonas - Litorimonas - Maribaculum - Maricaulis - Oceanicaulis - Ponticaulis - Robiginitomaculum - Woodsholea
Order Kiloniellales
Family Kiloniellaceae
Kiloniella
Order Kordiimonadales
Family "Kordiimonadaceae"
Kordiimonas
Order Magnetococcales
Family Magnetococcaceae
Magnetococcus
Order "Parvularculales"
Family "Parvularculaceae"
Parvularcula
Order Rhizobiales
Family "Aurantimonadaceae"
Aurantimonas - Aureimonas - Fulvimarina - Martelella
Family Bartonellaceae
Bartonella - Grahamella - Rochalimaea
Family Beijerinckiaceae
Beijerinckia - Camelimonas - Chelatococcus - Methylocapsa - Methylocella - Methyloferula - Methylorosula - Methylovirgula
Family Bradyrhizobiaceae
Afipia - Agromonas - Balneimonas - Blastobacter - Bosea - Bradyrhizobium - Nitrobacter - Oligotropha - Rhodoblastus - Rhodopseudomonas - Salinarimonas - Tardiphaga
Family Brucellaceae
Brucella - Crabtreella - Daeguia - Mycoplana - Ochrobactrum - Paenochrobactrum - Pseudochrobactrum
Family Cohaesibacteraceae
Cohaesibacter
Family Hyphomicrobiaceae
Ancalomicrobium - Angulomicrobium - Aquabacter - Blastochloris - Cucumibacter - Devosia - Dichotomicrobium - Filomicrobium - Gemmiger - Hyphomicrobium - Maritalea -Methylorhabdus - Pedomicrobium - Pelagibacterium - Prosthecomicrobium - Rhodomicrobium - Rhodoplanes - Seliberia - Zhangella
Family Methylobacteriaceae
Meganema - Methylobacterium - Microvirga - Protomonas

Family Methylocystaceae
Albibacter - Hansschlegelia - Methylocystis - Methylopila - Methylosinus - Pleomorphomonas - Terasakiella
Family Phyllobacteriaceae
Aminobacter - Aquamicrobium - Chelativorans - Defluvibacter - Hoeflea - Mesorhizobium - Nitratireductor - Phyllobacterium - Pseudahrensia - Pseudaminobacter - Thermovum
Family Rhizobiaceae
Agrobacterium - Allorhizobium - Carbophilus - Chelatobacter - Ensifer - Kaistia - Rhizobium - Shinella - Sinorhizobium
Family Rhodobiaceae
Afifella - Anderseniella - Lutibaculum - Parvibaculum - Rhodobium - Rhodoligotrophos - Roseospirillum - Tepidamorphus
Family Xanthobacteraceae
Ancylobacter - Azorhizobium - Labrys - Pseudolabrys - Pseudoxanthobacter - Starkeya - Xanthobacter
Unclassified Rhizobiales Amorphus - Bauldia - Vasilyevaea
Order Rhodobacterales
Family Rhodobacteraceae
Actibacterium - Agaricicola - Ahrensia - Albimonas - Albidovulum - Amaricoccus - Antarctobacter - Catellibacterium - Celeribacter - Citreicella - Citreimonas - Defluviimonas -Dinoroseobacter - Donghicola - Gaetbulicola - Gemmobacter - Haematobacter - Hasllibacter - Huaishuia - Hwanghaeicola - Jannaschia -Jhaorihella - Ketogulonicigenium - Labrenzia -Leisingera - Lentibacter -Litoreibacter - Litorimicrobium - Loktanella - Lutimaribacter - Mameliella -Maribius - Marinovum - Maritimibacter - Marivita - Methylarcula - Nautella -Nereida - Nesiotobacter - Oceanibulbus - Oceanicella - Oceanicola - Octadecabacter -Pacificibacter - Palleronia - Pannonibacter - Paracoccus - Pararhodobacter -Pelagibaca -Pelagicola - Pelagimonas - Phaeobacter - Planktotalea - Pontibaca -Ponticoccus - Poseidonocella - Primorskyibacter - Profundibacterium -Pseudorhodobacter - Pseudoruegeria - Pseudovibrio - Rhodobaca - Rhodobacter -Rhodothalassium - Rhodovulum - Roseibaca - Roseibacterium - Roseibium -Roseicitreum - Roseicyclus - Roseinatronobacter - Roseisalinus -Roseivivax -Roseobacter - Roseovarius - Rubellimicrobium - Rubribacterium - Rubrimonas -Ruegeria - Sagittula - Salinihabitans - Salipiger - Sediminimonas - Seohaeicola -Shimia -Silicibacter - Staleya - Stappia - Sulfitobacter - Tateyamaria -Thalassobacter - Thalassobius - Thalassococcus - Thioclava - Thiosphaera -Tranquillimonas - Tropicibacter - Tropicimonas- Vadicella - Wenxinia - Yangia
Order Rhodospirillales
Family Acetobacteraceae
Acetobacter - Acidicaldus - Acidiphilium - Acidisoma - Acidisphaera - Acidocella - Acidomonas - Ameyamaea - Asaia - Belnapia - Craurococcus - Gluconacetobacter - Gluconobacter -Granulibacter - Komagataeibacter - Kozakia - Muricoccus - Neoasaia - Neokomagataea - Paracraurococcus - Rhodopila - Rhodovarius -

Roseococcus - Roseomonas - Rubritepida -Saccharibacter - Stella - Swaminathania - Tanticharoenia - Teichococcus - Zavarzinia

Family Rhodospirillaceae

Azospirillum - Caenispirillum - Conglomeromonas - Constrictibacter - Defluviicoccus - Desertibacter - Dongia - Elstera - Ferrovibrio - Fodinicurvata - Inquilinus - Insolitispirillum -Magnetospira - Magnetospirillum - Marispirillum - Nisaea - Novispirillum - Oceanibaculum - Pelagibius - Phaeospirillum -Phaeovibrio - Rhodocista - Rhodospira - Rhodospirillum -Rhodovibrio -Roseospira - Skermanella - Telmatospirillum - Thalassobaculum - Thalassospira -Tistlia - Tistrella

Unclassified Rhodospirillales Elioraea - Reyranella

Order Rickettsiales

Family Anaplasmataceae

Aegyptianella - Anaplasma - Cowdria - Ehrlichia - Neorickettsia - Wolbachia

Family Holosporaceae

Holospora

Family Rickettsiaceae

Orientia - Rickettsia

Unclassified Rickettsiales Lyticum - Pseudocaedibacter - Symbiotes - Tectibacter

Order Sneathiellales

Family Sneathiellaceae

Oceanibacterium - Sneathiella

Order Sphingomonadales

Family Erythrobacteraceae

Altererythrobacter - Croceicoccus - Erythrobacter - Erythromicrobium - Porphyrobacter

Family Sphingomonadaceae

Blastomonas - Erythromonas - Novosphingobium - Parasphingopyxis - Rhizomonas (rejected name) - Sandaracinobacter - Sandarakinorhabdus - Sphingobium - Sphingomicrobium -Sphingomonas - Sphingopyxis - Sphingosinicella - Stakelama - Zymomonas

Unclassified Alphaproteobacteria Breoghania - Geminicoccus - Rhizomicrobium

Class Betaproteobacteria

Order Burkholderiales

Family Alcaligenaceae

Achromobacter - Advenella - Alcaligenes - Azohydromonas - Bordetella - Brackiella - Candidimonas - Castellaniella - Derxia - Kerstersia - Oligella - Paenalcaligenes - Paralcaligenes -Parapusillimonas - Pelistega - Pigmentiphaga - Pusillimonas - Taylorella - Tetrathiobacter

Family Burkholderiaceae

Burkholderia - Chitinimonas - Cupriavidus - Lautropia - Limnobacter - Pandoraea - Paucimonas - Polynucleobacter - Ralstonia - Thermothrix - Wautersia

Family Comamonadaceae

Acidovorax - Albidiferax - Alicycliphilus - Brachymonas - Caenimonas - Caenibacterium - Caldimonas - Comamonas - Curvibacter - Delftia -Diaphorobacter -

Giesbergeria -Hydrogenophaga - Hylemonella - Kinneretia -Lampropedia - Limnohabitans - Macromonas - Malikia - Ottowia - Pelomonas -Polaromonas - Pseudacidovorax - Pseudorhodoferax -Ramlibacter - Rhodoferax -Roseateles - Schlegelella - Simplicispira - Tepidicella - Variovorax -Verminephrobacter - Xenophilus

Family Oxalobacteraceae

Collimonas - Duganella - Glaciimonas - Herbaspirillum - Herminiimonas - Janthinobacterium - Massilia - Naxibacter - Oxalicibacterium - Oxalobacter - Pseudoduganella - Telluria -Undibacterium

Family Sutterellaceae

Parasutterella - Sutterella

Unclassified Burkholderiales Aquabacterium - Aquincola - Ideonella - Inhella - Leptothrix - Methylibium - Mitsuaria - Paucibacter - Piscinibacter - Rivibacter - Rubrivivax - Sphaerotilus -Tepidimonas - Thiobacter - Thiomonas - Xylophilus

Order Hydrogenophilales

Family Hydrogenophilaceae

Hydrogenophilus - Petrobacter - Sulfuricella - Tepidiphilus - Thiobacillus

Order Methylophilales

Family Methylophilaceae

Methylobacillus - Methylophilus - Methylotenera - Methylovorus

Order Neisseriales

Family Neisseriaceae

Alysiella - Amantichitinum - Andreprevotia - Aquaspirillum - Aquitalea - Bergeriella - Chitinibacter - Chitinilyticum - Chitiniphilus - Chromobacterium - Conchiformibius - Deefgea -Eikenella - Formivibrio - Gulbenkiania - Iodobacter - Jeongeupia - Kingella - Laribacter - Leeia - Microvirgula - Morococcus - Neisseria - Paludibacterium - Prolinoborus -Pseudogulbenkiania - Silvimonas - Simonsiella - Stenoxybacter - Uruburuella - Vitreoscilla - Vogesella

Order Nitrosomonadales

Family Gallionellaceae

Gallionella

Family Nitrosomonadaceae

Nitrosolobus - Nitrosomonas - Nitrosospira

Family Spirillaceae

Spirillum

Order "Procabacteriales"

Family "Procabacteriaceae"

"*Procabacter*"

Order Rhodocyclales

Family Rhodocyclaceae

Azoarcus - Azonexus - Azospira - Azovibrio - Dechloromonas - Dechlorosoma - Denitratisoma - Ferribacterium - Georgfuchsia - Methyloversatilis - Propionibacter - Propionivibrio -Quatrionicoccus - Rhodocyclus - Sterolibacterium - Sulfuritalea - Thauera - Uliginosibacterium - Zoogloea

Unclassified Betaproteobacteria Chitinivorax
Class Deltaproteobacteria or Deltabacteria
Order Bdellovibrionales
Family Bacteriovoracaceae
Bacteriolyticum (illegitimate genus) - Bacteriovorax- Peredibacter
Family Bdellovibrionaceae
Bdellovibrio - Micavibrio - Vampirovibrio
Family Peredibacteraceae (illegitimate)
See Bacteriovoracaceae
Order Desulfarculales
Family Desulfarculaceae
Desulfarculus
Order Desulfobacterales
Family Desulfobacteraceae
Desulfatibacillum - Desulfatiferula - Desulfatirhabdium - Desulfatitalea - Desulfobacter - Desulfobacterium - Desulfobacula - Desulfobotulus - Desulfocella - Desulfococcus -Desulfoconvexum - Desulfofaba - Desulfofrigus - Desulfoluna - Desulfomusa - Desulfonatronobacter - Desulfonema - Desulforegula - Desulfosalsimonas - Desulfosarcina - Desulfospira -Desulfotignum
Family Desulfobulbaceae
Desulfobulbus - Desulfocapsa - Desulfofustis - Desulfopila - Desulforhopalus - Desulfotalea - Desulfurivibrio
Family Nitrospinaceae
Nitrospina
Order Desulfovibrionales
Family Desulfohalobiaceae
Desulfohalobium - Desulfonatronospira - Desulfonatronovibrio - Desulfonauticus - Desulfothermus - Desulfovermiculus
Family Desulfomicrobiaceae
Desulfomicrobium
Family Desulfonatronaceae
Desulfonatronum
Family Desulfovibrionaceae
Bilophila - Desulfobaculum - Desulfocurvus - Desulfomonas - Desulfovibrio - Lawsonia
Order Desulfurellales
Family Desulfurellaceae
Desulfurella - Hippea
Order Desulfuromonadales
Family Desulfuromonadaceae
Desulfuromonas - Desulfuromusa - Malonomonas - Pelobacter
Family Geobacteraceae
Geoalkalibacter - Geobacter - Geopsychrobacter - Geothermobacter - Trichlorobacter
Order Myxococcales

Suborder Cystobacterineae
Family Cystobacteraceae
Anaeromyxobacter - Archangium - Cystobacter - Hyalangium - Melittangium - Stigmatella
Family Myxococcaceae
Angiococcus - Corallococcus - Myxococcus - Pyxidicoccus
Suborder Nannocystineae
Family "Haliangiaceae"
Haliangium
Family Kofleriaceae
Kofleria
Family Nannocystaceae
Enhygromyxa - Nannocystis - Plesiocystis
Suborder Sorangiineae
Family Phaselicystidaceae
Phaselicystis
Family Polyangiaceae
Byssovorax - Chondromyces - Jahnella - Polyangium - Sorangium
Family Sandaracinaceae
Sandaracinus
Order Syntrophobacterales
Family Syntrophaceae
Desulfobacca - Desulfomonile - Smithella - Syntrophus
Family Syntrophobacteraceae
Desulfacinum - Desulfoglaeba - Desulforhabdus - Desulfosoma - Desulfovirga - Syntrophobacter - Thermodesulforhabdus
Unnamed Order
Family Syntrophorhabdaceae
Syntrophorhabdus
Unclassified Deltaproteobacteria Deferrisoma
Class Epsilonproteobacteria
Order Campylobacterales
Family Campylobacteraceae
Arcobacter - Campylobacter - Dehalospirillum - Sulfurospirillum
Family Helicobacteraceae
Helicobacter - Sulfuricurvum - Sulfurimonas - Sulfurovum - Thiovulum - Wolinella
Family "Hydrogenimonaceae"
Hydrogenimonas
Order Nautiliales
Family Nautiliaceae
Caminibacter - Lebetimonas - Nautilia - Nitratifractor - Nitratiruptor - Thioreductor
Class Gammaproteobacteria
Order Acidithiobacillales

Family Acidithiobacillaceae
Acidithiobacillus
Family Thermithiobacillaceae
Thermithiobacillus
Order Aeromonadales
Family Aeromonadaceae
Aeromonas - Oceanimonas - Oceanisphaera - Tolumonas - Zobellella
Family Succinivibrionaceae
Anaerobiospirillum - Ruminobacter - Succinatimonas - Succinimonas - Succinivibrio
Order Alteromonadales
Family Alteromonadaceae
Aestuariibacter - Agarivorans - Aliagarivorans - Alishewanella - Alteromonas - Bowmanella - Catenovulum - Glaciecola - Haliea - Marinimicrobium -Marinobacter - Marinobacterium -Melitea - Microbulbifer - Saccharophagus -Salinimonas
Family Celerinatantimonadaceae
Celerinatantimonas
Family Colwelliaceae
Colwellia - Thalassomonas
Family Ferrimonadaceae
Ferrimonas - Paraferrimonas
Family Idiomarinaceae
Aliidiomarina - Idiomarina - Pseudidiomarina
Family Moritellaceae
Moritella- Paramoritella
Family Pseudoalteromonadaceae
Algicola - Pseudoalteromonas - Psychrosphaera
Family Psychromonadaceae
Psychromonas
Family Shewanellaceae
Shewanella
Unclassified Alteromonadales Eionea - Gilvimarinus - Maricurvus - Pseudoteredinibacter - Teredinibacter
Order Cardiobacteriales
Family Cardiobacteriaceae
Cardiobacterium - Dichelobacter - Suttonella
Order Chromatiales
Family Chromatiaceae
Allochromatium - Amoebobacter - Chromatium - Halochromatium - Isochromatium - Lamprobacter - Lamprocystis - Marichromatium - Nitrosococcus - Pfennigia - Phaeochromatium -Rhabdochromatium - Rheinheimera - Thermochromatium - Thioalkalicoccus - Thiobaca - Thiocapsa - Thiococcus - Thiocystis - Thiodictyon - Thioflavicoccus - Thiohalocapsa -Thiolamprovum - Thiopedia - Thiophaeococcus - Thiorhodococcus - Thiorhodovibrio - Thiospirillum

Family Ectothiorhodospiraceae
Acidiferrobacter - Alkalilimnicola - Alkalispirillum - Aquisalimonas -Arhodomonas - Ectothiorhodosinus - Ectothiorhodospira - Halorhodospira -Natronocella - Nitrococcus - Thioalbus -Thioalkalivibrio - Thiohalospira -Thiorhodospira
Family Granulosicoccaceae
Granulosicoccus
Family Halothiobacillaceae
Halothiobacillus - Thioalkalibacter - Thiofaba - Thiovirga
Family Thioalkalispiraceae
Thioalkalispira - Thiohalophilus - Thioprofundum
Order "Enterobacteriales"
Family Enterobacteriaceae
Arsenophonus - Biostraticola - Brenneria - Buchnera - Budvicia - Buttiauxella - Calymmatobacterium - Cedecea - Citrobacter - Cosenzaea - Cronobacter - Dickeya - Edwardsiella -Enterobacter - Erwinia - Escherichia - Ewingella - Gibbsiella - Hafnia - Klebsiella - Kluyvera - Leclercia - Leminorella - Levinea - Lonsdalea - Mangrovibacter - Moellerella - Morganella -Obesumbacterium - Pantoea - Pectobacterium - Phaseolibacter - Photorhabdus - Plesiomonas - Pragia - Proteus - Providencia - Rahnella - Raoultella - Saccharobacter - Salmonella -Samsonia - Serratia - Shigella - Shimwellia - Sodalis - Tatumella - Thorsellia - Trabulsiella - Wigglesworthia - Xenorhabdus - Yersinia - Yokenella
Order Legionellales
Family Coxiellaceae
Aquicella - Coxiella - Diplorickettsia
Family Legionellaceae
Fluoribacter - Legionella - Sarcobium - Tatlockia
Unclassified Legionellales Rickettsiella
Order Methylococcales
Family Crenotrichaceae
Crenothrix
Family Methylococcaceae
Methylobacter - Methylocaldum - Methylococcus - Methylogaea - Methylohalobius - Methylomarinum - Methylomicrobium - Methylomonas - Methylosarcina - Methylosoma -Methylosphaera - Methylothermus - Methylovulum
Order Oceanospirillales
Family Alcanivoracaceae
Alcanivorax - Fundibacter - Kangiella
Family Hahellaceae
Endozoicomonas - Hahella - Halospina - Kistimonas - Zooshikella
Family Halomonadaceae
Aidingimonas - Carnimonas - Chromohalobacter - Cobetia - Deleya - Halomonas - Halotalea - Halovibrio - Kushneria - Modicisalibacter - Salinicola - Volcaniella - Zymobacter

Family Litoricolaceae
Litoricola
Family Oceanospirillaceae
Amphritea - Balneatrix - Bermanella - Corallomonas - Marinomonas - Marinospirillum - Neptuniibacter - Neptunomonas - Nitrincola - Oceaniserpentilla - Oceanobacter - Oceanospirillum- Oleibacter - Oleispira - Pseudospirillum - Reinekea - Thalassolituus
Family Oleiphilaceae
Oleiphilus
Family "Saccharospirillaceae"
Saccharospirillum
Unclassified Oceanospirillales Salicola - Spongiispira
Order Pasteurellales
Family Pasteurellaceae
Actinobacillus - Aggregatibacter - Avibacterium - Basfia - Bibersteinia - Bisgaardia - Chelonobacter - Gallibacterium - Haemophilus - Histophilus - Lonepinella - Mannheimia -Necropsobacter - Nicoletella - Otariodibacter - Pasteurella - Phocoenobacter - Volucribacter
Order Pseudomonadales
Family Moraxellaceae
Acinetobacter - Alkanindiges - Branhamella - Enhydrobacter - Moraxella - Paraperlucidibaca - Perlucidibaca - Psychrobacter
Family Pseudomonadaceae
Azomonas - Azomonotrichon - Azorhizophilus - Azotobacter - Cellvibrio - Chryseomonas - Flavimonas - Mesophilobacter - Pseudomonas - Rhizobacter - Rugamonas - Serpens
Unclassified Pseudomonadales Dasania
Order "Salinisphaerales"
Family "Salinisphaeraceae"
Salinisphaera
Order Thiotrichales
Family Francisellaceae
Francisella
Family Piscirickettsiaceae
Cycloclasticus - Galenea - Hydrogenovibrio - Methylophaga - Piscirickettsia - Sulfurivirga - Thioalkalimicrobium - Thiomicrospira
Family Thiotrichaceae
Achromatium - Beggiatoa - Cocleimonas - Leucothrix - Thiobacterium - Thiomargarita - Thioploca - Thiospira - Thiothrix
Unclassified Thiotrichales Caedibacter - Fangia
Order "Vibrionales"
Family Vibrionaceae
Aliivibrio - Allomonas - Beneckea - Catenococcus - Echinimonas - Enterovibrio - Grimontia - Listonella - Lucibacterium - Photobacterium - Salinivibrio - Vibrio

Order Xanthomonadales
Family Algiphilaceae
Algiphilus
Family Nevskiaceae
Hydrocarboniphaga - Nevskia
Family Sinobacteraceae
See *Solimonadaceae*.
Sheu *et al*. 2011 propose to transfer the type species of the type genus of the family *Sinobacteraceae* in the genus *Solimonas*. According to Rule 37a, bacteriologists adhering to this proposal must change the name *Sinobacter* to *Solimonas*.
With the now defunct name *Sinobacter* there is now an inconsistency in the nomenclature with the name *Sinobacteraceae*. Therefore, Losey *et al*. 2013 propose the name ¤*Solimonadaceae* fam. nov. to include members of the genera *Solimonas* and *Fontimonas*.
Family Solimonadaceae
Fontimonas - Solimonas
Family Xanthomonadaceae
Aquimonas - Arenimonas - Aspromonas - Dokdonella - Dyella - Frateuria - Fulvimonas - Ignatzschineria - Luteibacter - Luteimonas - Lysobacter - Panacagrimonas - Pseudofulvimonas -Pseudoxanthomonas - Rhodanobacter - Rudaea - Silanimonas - Stenotrophomonas - Thermomonas - Wohlfahrtiimonas - Xanthomonas - Xylella
Unclassified Xanthomonadales Alkanibacter - Singularimonas - Sinobacter - Steroidobacter
Unclassified Gammaproteobacteria Alkalimonas - Arenicella - Chromatocurvus - Congregibacter - Gallaecimonas - Halioglobus - Marinicella - Methylohalomonas - Methylonatrum - Orbus -Plasticicumulans - Porticoccus - Sedimenticola - Simiduia - Solimonas - Spongiibacter - Thiohalobacter - Thiohalomonas - Thiohalorhabdus - Umboniibacter - Zhongshania
Class "Zetaproteobacteria"
Order "Mariprofundales"
Family "Mariprofundaceae"
Mariprofundus
Phylum "Spirochaetes" or "Spirochaetae"
Class Spirochaetes
Order Spirochaetales
Family Brachyspiraceae
Brachyspira - Serpulina
Family Brevinemataceae
Brevinema
Family Leptospiraceae
Leptonema - Leptospira - Turneriella

Family Spirochaetaceae
Borrelia - Clevelandina - Cristispira - Diplocalyx - Hollandina - Pillotina - Sphaerochaeta - Spirochaeta - Treponema
Unclassified Spirochaetales Exilispira
Phylum "Synergistetes"
Class Synergistia
Order Synergistales
Family Synergistaceae
Aminiphilus - Aminobacterium - Aminomonas - Anaerobaculum - Cloacibacillus - Dethiosulfovibrio - Fretibacterium - Jonquetella - Pyramidobacter - Synergistes - Thermanaerovibrio -Thermovirga
Phylum "Tenericutes"
Class Mollicutes (In the next edition of the *Bergey's Manual of Systematic Bacteriology*, the class Mollicutes will be excluded from the phylum "*Firmicutes*" and classified in the phylum "*Tenericutes*" - Reference: K.H. Schleifer and W. Ludwig, personal communication)
Order Acholeplasmatales
Family Acholeplasmataceae
Acholeplasma
Order Anaeroplasmatales
Family Anaeroplasmataceae
Anaeroplasma - Asteroleplasma
Order Entomoplasmatales
Family Entomoplasmataceae
Entomoplasma - Mesoplasma
Family Spiroplasmataceae
Spiroplasma
Order Haloplasmatales
Family Haloplasmataceae
Haloplasma
Order Mycoplasmatales
Family Mycoplasmataceae
Eperythrozoon - Haemobartonella - Mycoplasma - Ureaplasma
Phylum "Thermodesulfobacteria"
Class Thermodesulfobacteria
Order Thermodesulfobacteriales
Family Thermodesulfobacteriaceae
Caldimicrobium - Thermodesulfatator - Thermodesulfobacterium - Thermosulfurimonas
Phylum "Thermomicrobia"
See Phylum "Chloroflexi"
Phylum "Thermotogae"
Class Thermotogae
Order Thermotogales

Family Thermotogaceae
Defluviitoga - Fervidobacterium - Geotoga - Kosmotoga - Marinitoga - Mesotoga - Oceanotoga - Petrotoga - Thermococcoides - Thermosipho - Thermotoga
Phylum "Verrucomicrobia"
Class Verrucomicrobiae
Order Verrucomicrobiales
Family Akkermansiaceae
Akkermansia
Family Rubritaleaceae
Rubritalea
Family Verrucomicrobiaceae
Haloferula - Luteolibacter - Persicirhabdus - Prosthecobacter - Roseibacillus - Verrucomicrobium
Class Opitutae
Order Opitutales
Family Opitutaceae
Alterococcus - Opitutus
Order Puniceicoccales
Family Puniceicoccaceae
Cerasicoccus - Coraliomargarita - Pelagicoccus - Puniceicoccus
Domain "Archaea"
Phyla: "Crenarchaeota" - "Euryarchaeota" - "Korarchaeota" - "Nanoarchaeota" - "Thaumarchaeota"
Phylum "Crenarchaeota"
Class Thermoprotei or Crenarchaeota
Order Acidilobales
Family Acidilobaceae
Acidilobus
Family Caldisphaeraceae
Caldisphaera
Order Desulfurococcales
Family Desulfurococcaceae
Aeropyrum - Desulfurococcus - Ignicoccus - Ignisphaera - Staphylothermus - Stetteria - Sulfophobococcus - Thermodiscus - Thermosphaera
Family Pyrodictiaceae
Hyperthermus - Pyrodictium - Pyrolobus
Order Fervidicoccales
Family Fervidicoccaceae
Fervidicoccus
Order Sulfolobales
Family Sulfolobaceae
Acidianus - Desulfurolobus - Metallosphaera - Stygiolobus - Sulfolobus - Sulfurisphaera - Sulfurococcus
Order Thermoproteales

Family Thermofilaceae
Thermofilum
Family Thermoproteaceae
Caldivirga - Pyrobaculum - Thermocladium - Thermoproteus - Vulcanisaeta
Phylum "Euryarchaeota"
Class Archaeoglobi
Order Archaeoglobales
Family Archaeoglobaceae
Archaeoglobus - Ferroglobus - Geoglobus
Class Halobacteria or Halomebacteria
Order Halobacteriales
Family Halobacteriaceae
Haladaptatus - Halalkalicoccus - Halarchaeum - Haloarchaeobius - Haloarcula - Halobacterium - Halobaculum - Halobellus - Halobiforma - Halococcus - Haloferax - Halogeometricum -Halogranum - Halolamina - Halomarina - Halomicrobium - Halonotius - Halopelagius - Halopenitus - Halopiger - Haloplanus - Haloquadratum - Halorhabdus - Halorientalis - Halorubrum- Halosarcina - Halosimplex - Halostagnicola - Haloterrigena - Halovenus - Halovivax - Natrialba - Natrinema - Natronoarchaeum - Natronobacterium - Natronococcus -Natronolimnobius - Natronomonas - Natronorubrum - Salarchaeum
Class Methanobacteria
Order Methanobacteriales
Family Methanobacteriaceae
Methanobacterium - Methanobrevibacter - Methanosphaera - Methanothermobacter
Family Methanothermaceae
Methanothermus
Class Methanococci or Methanothermea
Order Methanococcales
Family Methanocaldococcaceae
Methanocaldococcus - Methanotorris
Family Methanococcaceae
Methanococcus - Methanothermococcus
Class "Methanomicrobia"
Order Methanocellales
Family Methanocellaceae
Methanocella
Order Methanomicrobiales
Family Methanocorpusculaceae
Methanocorpusculum
Family Methanomicrobiaceae
Methanoculleus - Methanofollis - Methanogenium - Methanolacinia - Methanomicrobium - Methanoplanus
Family Methanoregulaceae
Methanolinea - Methanoregula - Methanosphaerula

Family Methanospirillaceae
Methanospirillum
Unclassified Methanomicrobiales Methanocalculus
Order Methanosarcinales
Family Methanosaetaceae (illegitimate name) Methanosaeta (illegitimate name) - Methanothrix
Family Methanosarcinaceae
Halomethanococcus - Methanimicrococcus - Methanococcoides - Methanohalobium - Methanohalophilus - Methanolobus - Methanomethylovorans - Methanosalsum - Methanosarcina
Family Methermicoccaceae
Methermicoccus
Unclassified "Methanomicrobia" Methanomassiliicoccus
Class Methanopyri
Order Methanopyrales
Family Methanopyraceae
Methanopyrus
Class Thermococci or Protoarchaea
Order Thermococcales
Family Thermococcaceae
Palaeococcus - Pyrococcus - Thermococcus
Class Thermoplasmata
Order Thermoplasmatales
Family Ferroplasmaceae
Acidiplasma - Ferroplasma
Family Picrophilaceae
Picrophilus
Family Thermoplasmataceae
Thermoplasma
Unclassified Thermoplasmatales
Thermogymnomonas

http://www.innvista.com/health/microbes/bacteria/classif.htm
http://www.earthlife.net/prokaryotes/phyla.html
http://web.uct.ac.za/depts/mmi/lectures/bactax/ppframe.html
http://www.gsbs.utmb.edu/microbook/ch003.htm
http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/dental/tutorials/classification/introduction.html
http://www.microbiol.org/WPaper.Gram.htm

TOPIC 3. GENERAL CHARACTERICTICS OF PROKARYOTIC CELLS. GRAM'S METHOD.

Contents

1. **Morphology of bacteria.**
2. **The size and shape of bacteria: spherical, rod-shaped, crimped.**
3. **Morphology and features structure spirochaetes, rickettsia, chlamydia, mycoplasmas, fungi, protozoa.**
4. **Morphology and biology of viruses.**

Morphology of Bacteria

Bacteria (Gk bakterion small staff) are, for the most part, unicellular organisms lacking chlorophyll Their biological properties and predominant reproduction by binary fission relates them to prokaryotes The size of bacteria is measured in micrometres (mcm) and varies from 0.1 mcm (Spiroplasma, Acholeplasma) to 16-18 mcm (Spirillum volutans). Most pathogenic bacteria measure 0.2 to 10 mm. The shape of spherical bacteria represents a certain ratio of surface area ($As = 4\pi r^2$) to volume ($Vs = 4/3\pi r^3$). For those cells having a proper cylindrical shape the formulae will be $At = 2\pi b(b + 2a\}$; $Vi = 2\pi ab^2$, where a is equal to one-half the maximum length, b is equal to one-half the maximum width, and r is equal to the radius of the spherical cell.

The shape as well as the dimensions of microbes is not absolutely constant Morphological differences are found in many bacterial species The organisms are subject to change with the surrounding environmental conditions However, in relatively stable conditions, the microbes are capable of retaining their specific properties (size, shape) inherited during the process of evolution Morphologically, bacteria possess three main forms (Fig. 4). They are either spherical (cocci), rod-shaped (bacteria, bacilli, and clostridia) or spiral-shaped (vibriones and spirilla).

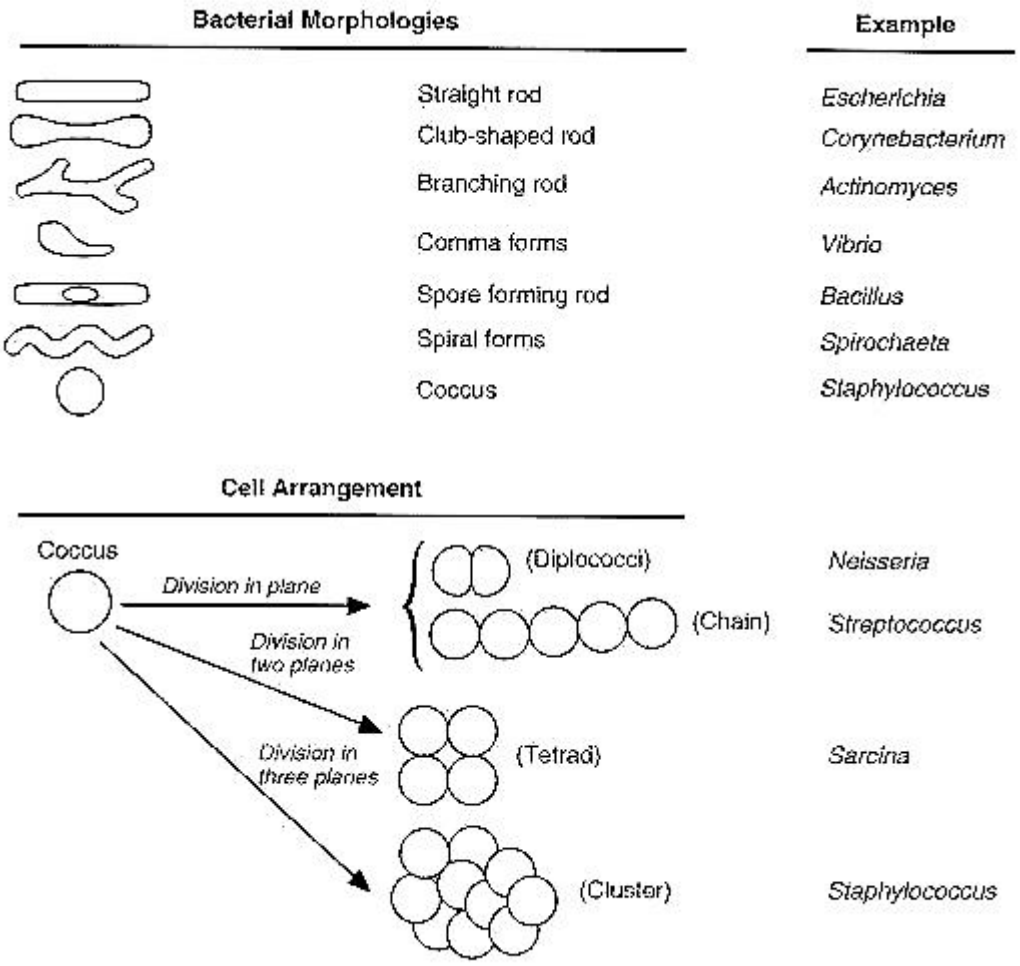

Figure 4. Typical shapes and arrangements of bacterial cells

Cocci (Gk. chokes berry). These forms of bacteria are spherical, ellipsoidal, bean-shaped, and lancelet Cocci are subdivided into six groups according to cell arrangement, cell division and biological properties.

1. **Micrococci** (Monococci, Micrococcus) - Fig. 5. The cells are arranged singly or irregularly They are saprophytes, and live in water and in air (M. agilis, M. roseus, M. luteus, etc.)

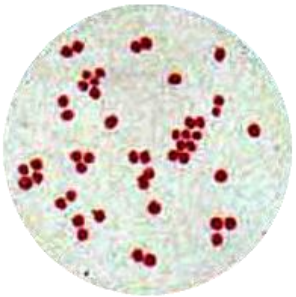

Figure 5. Micrococci

2. **Diplococci** (Gk. diplos double) divide in one plane and remain attached in pairs (Fig. 6). These include, meningococcus, causative agent of epidemic

cerebrospinal meningitis, and gonococcus, causative agent of gonorrhoea and blennorrhoea.

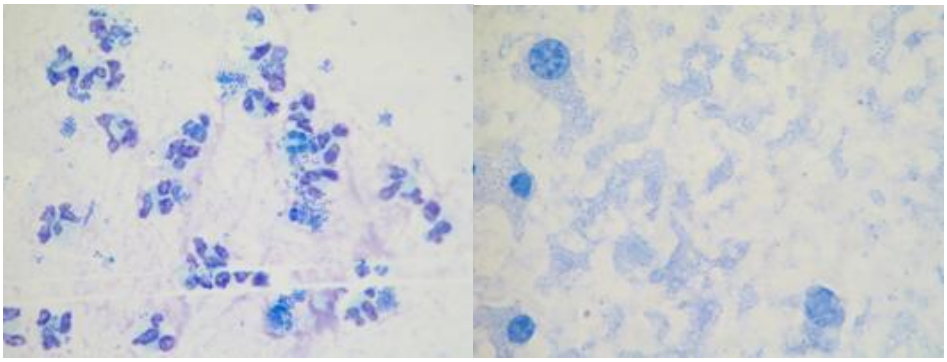

Figure 6. Diplococci

3. **Streptococci** (Gk. streptos curved, kokkos berry) divide in one plane and are arranged in chains of different length (Fig. 7). Some streptococci are pathogenic for humans and are responsible for various diseases.

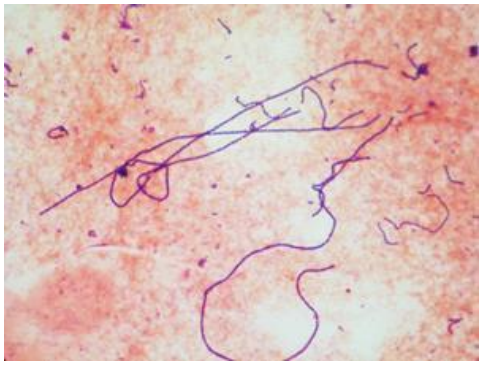

Figure 7. Streptococci

4. **Tetracocci** (Gk. tetra four) divide in two planes at right angles to one another and form groups of ours (Fig. 8). They very rarely produce diseases in humans.

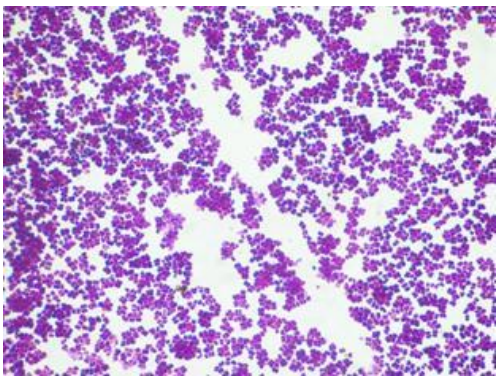

Figure 8. Tetracocci

5. **Sarcinae** (L. sarcio to tie) divide in three planes at right angles to one another and resemble packets of 8, 16 or more cells. They are frequently found in the air. Virulent species have not been encountered (Fig. 9).

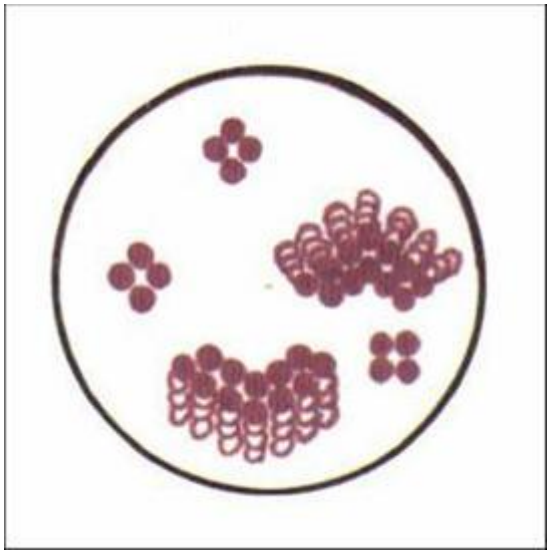

Figure 9. Sarcinae

6. **Staphylococci** (Gk. staphyle cluster of grapes) divide in several planes resulting in irregular bunches of cells, sometimes resembling clusters of grapes Some species of Staphylococci cause diseases in man and animals (Fig. 10).

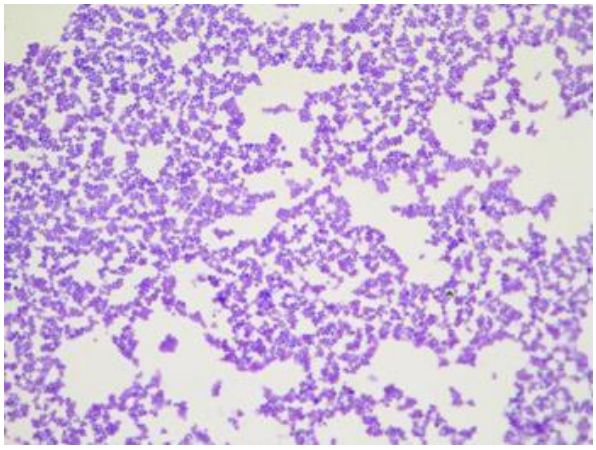

Figure 10. Staphylococci

Rods. Rod-shaped or cylindrical forms (Fig. 11-12) are subdivided into bacteria, bacilli, and clostridia. Bacteria include those microorganisms which, as a rule, do not produce spores (colibacillus, and organisms responsible for enteric fever, paratyphoids, dysentery, diphtheria, tuberculosis, etc.) Bacilli and clostridia include organisms the majority of which produce spores (hay bacillus, bacilli responsible for anthrax, tetanus, anaerobic infections, etc).

Rod-shaped bacteria exhibit differences in form. Some are short (tularaemia bacillus), others are long (anthrax bacillus), the majority have blunted ends, and others have tapered ends (fusobacteria).

According to their arrangement, cylindrical forms can be subdivided into three groups (1) diplobacteria and diplobacilli occurring in pairs(bacteria of pneumonia); (2) streptobacteria or streptobacilli occurringin chains of different length (causative agents of chancroid, anthrax), (3) bacteria and bacilli which are not arranged in a regular pattern (these comprise the majority of the rod-shaped forms).

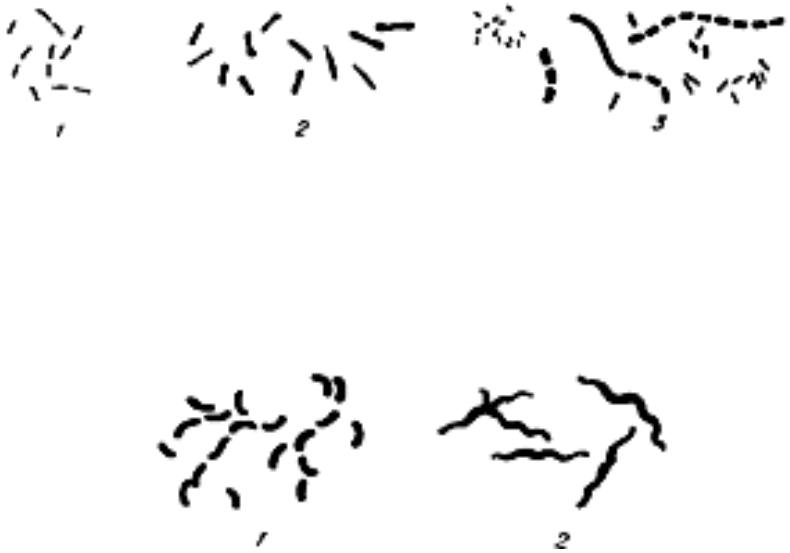

Figure 11. Rod-shaped bacteria and some spiral-shaped bacteria

Fig. 11a: 1 **–** diplobacteria; 2- rods with rounded, sharpened and\or thickened ends; 3- different rod-shaped forms and streptobacteria

Figure 11b: 1-vibriones 2—spirilla

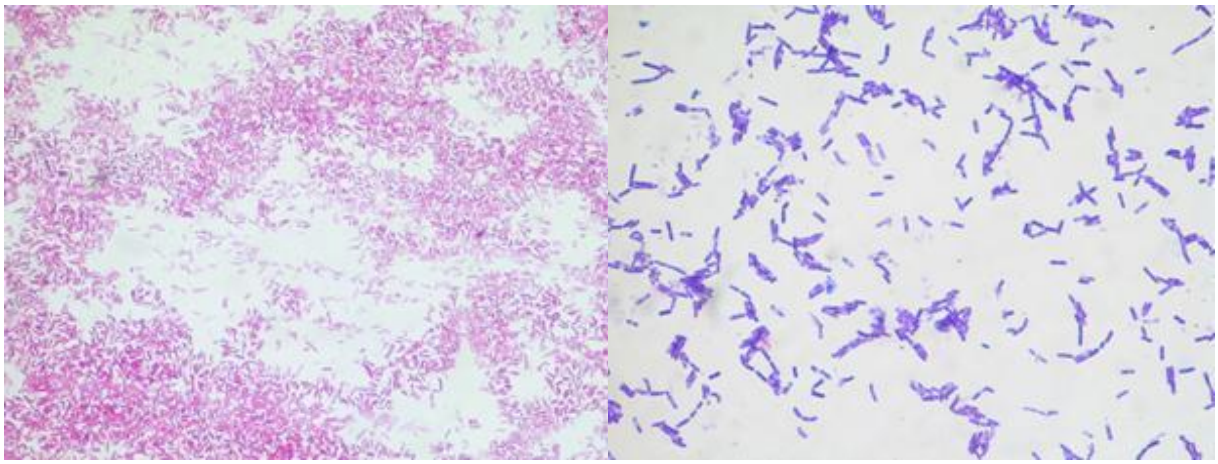

Figure 12. Different rod-shaped forms

Some rod-shaped bacteria have pin-head thickenings at the ends(causative agents of diphtheria); others form lateral branchings (bacilli of tuberculosis and leprosy).

There is a significantly greater number of rod-shaped bacteria than coccal-shaped organisms. This is explained by the fact that in rod-shaped bacteria the ratio of surface area to volume is higher. Thus, a larger surface area is in direct contact with nutrient substances in the surrounding medium.

Spiral-shaped bacteria. Vibriones and spirilla belong to this group of bacteria.

1. Vibriones (L. vibrio to vibrate) are cells which resemble a comma in appearance- Typical representatives of this group are , the causative agent of cholera, and aquatic vibriones which are widely distributed in fresh water reservoirs (Fig.13).

2. Spirilla (L. spira coil) 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 through the bite of rats and other rodents (rat-bite fever, sodoku). Microbes exhibit pleomorphism, they are subject to individual variations, unassociated with age or stage of development, causing the existence of different forms of cells in the same species. They are extremelylabile, and susceptible to changes which are associated with such factors as temperature, nutrients, salt concentration, acidity, metabolites, disinfectants, drugs, and body resistance (Fig.14).

3. Spirochetes: Treponema, Borrelia, Leptospira - (Fig.15).

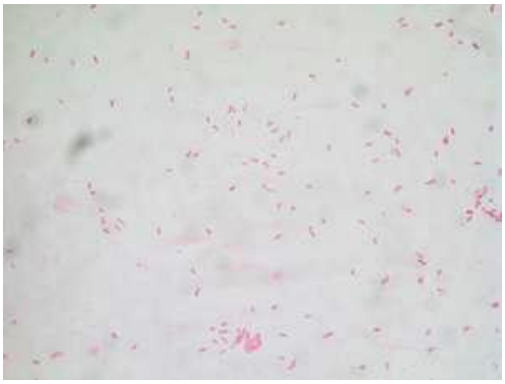

Figure 13. Vibrio comma

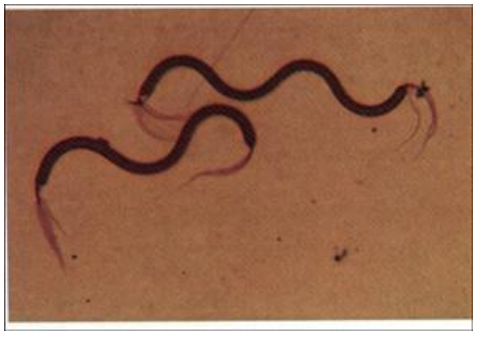

Figure 14. Spirilla

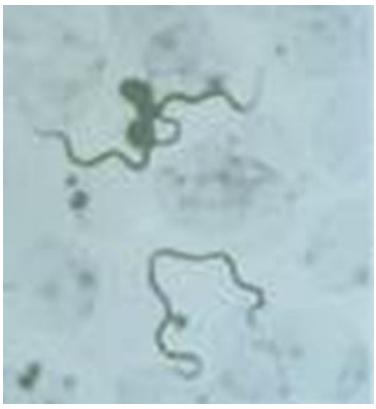

Figure 15. Spirochetes

PROKARYOTIC CELL STRUCTURE

The cytoplasm is enclosed within a lipoprotein cell membrane, similar to the prokaryotic cell membrane (Fig.16). Most animal cells have no other surface layers; many eukaryotic microorganisms, however, have an outer cell wall, which may be composed of a polysaccharide such as cellulose or chitin or may be inorganic, as in the silica wall of diatoms.

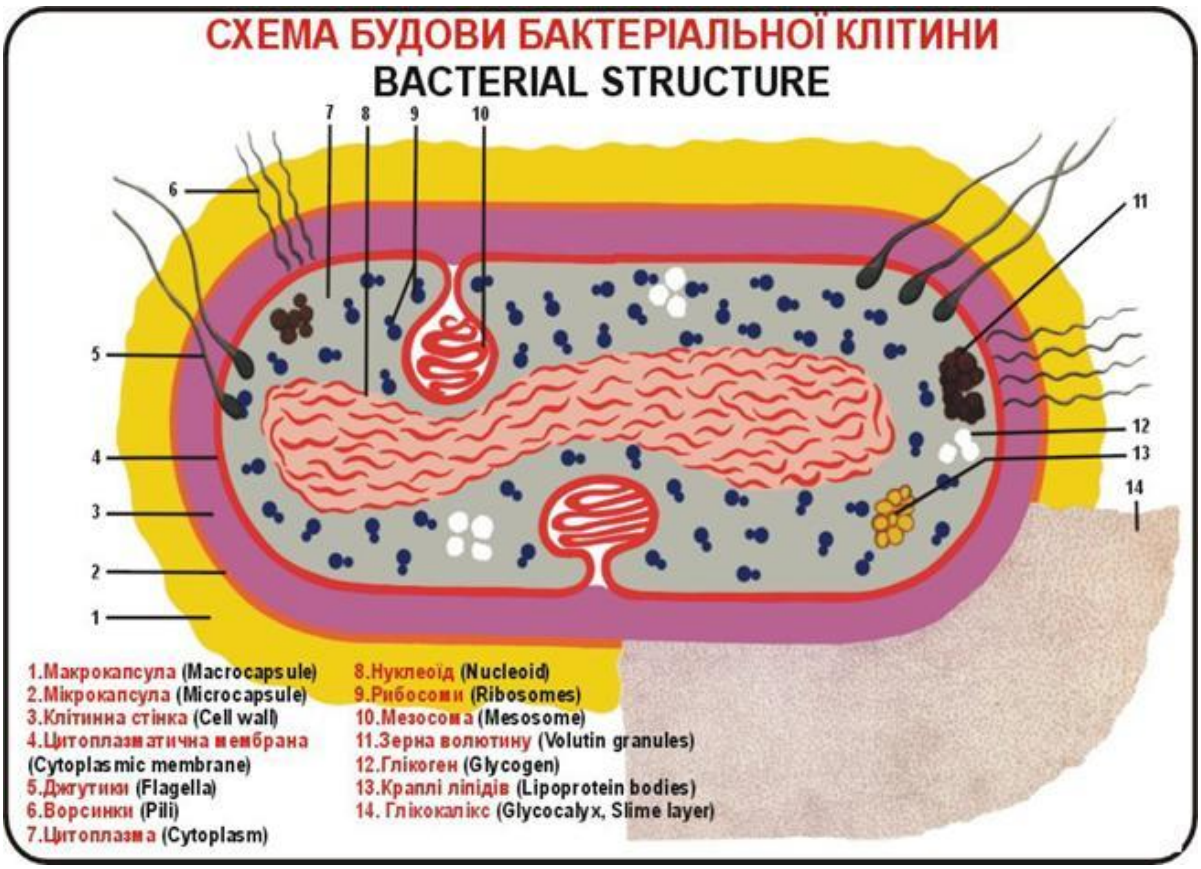

Figure 16. Bacterial structure

The prokaryotic cell is simpler than the eukaryotic cell at every level, with one exception: the cell wall may be more complex (Fig.17).

ОСНОВНІ ВІДМІННОСТІ ПРОКАРІОТІВ І ЕУКАРІОТІВ
DIFFERENCES BETWEEN PROKARYOTIC AND EUKARYOTIC CELLS

Характеристика Characteristic	Прокаріотична клітина Prokaryotic Cells	Еукаріотична клітина Eukaryotic Cells
Генетична інформація Genetic information	Знаходиться в одній хромосомі Found in single chromosome	Знаходиться в парних хромосомах Found in paired chromosomes
Локалізація генетичної інформації Location of genetic information	Нуклеоід Nuclear area (nucleoid)	Обмежене мембраною ядро Membrane-bound nucleus
Ядерце Nucleolus	Відсутнє Absent	Присутнє Present
Гістони Histones	Відсутні Absent	Присутні Present
Екстрахромосомна ДНК Extrachromosomal DNA	У плазмідах In plasmids	В органелах (мітохондрії, хлоропласти) In organelles, such as mitochondria and chloroplasts
Мітотичне веретено Mitotic spindle	Відсутнє	Присутнє Present during cell division
Плазматична мембрана Plasma membrane	Відсутні стероли Fluid-mosaic structure lacking sterols	Присутні стероли Fluid-mosaic structure containing sterols
Внутрішні мембрани Internal membranes	Тільки у фотосинтезуючих бактерій Only in photosynthetic organisms	Обмежують численні органели Numerous membrane-bound organelles
Ендоплазматичний ретикулюм Endoplasmic reticulum	Відсутній Absent	Присутній Present
Дихальні ферменти Respiratory enzymes	Клітинна мембрана Cell membrane	Мітохондрії Mitochondria
Хроматофори Chromatophores	Присутні у фотосинтезуючих бактерій Present in photosynthetic bacteria	Відсутні Absent
Хлоропласти Chloroplasts	Відсутні Absent	Присутні в деяких клітинах Present in some cells
Апарат Гольджі Golgi apparatus	Відсутній Absent	Присутній Present
Лізосоми Lysosomes	Відсутні Absent	Присутні Present
Пероксісоми Peroxisomes	Відсутній Absent	Присутній Present
Рибосоми Ribosomes	70S	80S у цитоплазмі та ендоплазматичному ретикулюмі, 70S в органелах 80S in cytoplasm and on endoplasmic reticulum, 70S in organelles
Цитоскелет Cytoskeleton	Відсутній Absent	Присутній Present
Клітинна стінка Cell wall	Як правило містить пептидоглікан Usually peptidoglycan found on most cells	Целюлоза, хітин Cellulose, chitin, or both found on plant and fungal cells
Джгутики Flagella	Складаються з фібрил флагеліну When present, consist of fibrils of flagellin	Складаються з комплексу мембран зв'язаних структур з характерним розташуванням "9+2" When present, consist of complex membrane-bound structure with "9 + 2" microtubule arrangement
Війки Cilia	Відсутні Absent	Присутні Present
Піли Pili	Присутні Present as attachment or conjugation pili in some prokaryotic cells	Відсутні Absent

Figure 17. The main differences between prokaryotes from eukaryotes

The prokaryotic nucleus can be seen with the light microscope in stained material. It is Feulgen-positive, indicating the presence of DNA. The negatively charged DNA is at least partially neutralized by small polyamines and magnesium ion, but histonelike proteins have recently been discovered in Cytoplasmic Structures

Prokaryotic cells lack autonomous plastids, such as mitochondria and chloroplusts. The electron transport enzymes are localized instead in the cell membrane; in photosynthetic organisms, the photosynthetic pigments are localized in lamellae underlying the cell membrane. In some photosynthetic bacteria, the lamellae may become convoluted and pinch off into discrete particles called chromatophores.

Bacteria often store reserve materials in the form of insoluble cytoplasmic granules, which are deposited as osmotically inert, neutral polymers. In the absence of a nitrogen source, carbon source material is converted by some bacteria to the polymer poly-β-hydroxybutyric acid and by other bacteria to various polymers of glucose such as starch and glycogen. The granules are used as carbon sources when protein and nucleic acid synthesis is resumed. Similarly, certain sulfur-oxidizing bacteria convert excess H_2s from the environment into intracellular granules of elemental sulfur. Finally, many bacteria accumulate reserves of inorganic phosphate as granules of polymerized metaphosphate, called volutin. Volutin granules are also called metachromatic granules because they stain red with a blue dye. They are characteristic features of corynebacteria.

Microtubular structures, characteristic of eukaryotic cells, are generally absent in prokaryotes. In a few instances, however, the electron microscope has revealed bacterial structures that resemble microtubules.

Stains combine chemically with the bacterial protoplasm; if the cell is not already dead, the staining process itself will kill it. The process is thus a drastic one and may produce artifacts.

The commonly used stains are salts. Basic stains consist of a colored cation with a colorless anion (eg, methylene blue chloride"); acidic stains are the reverse (eg, sodium + eosinate"). Bacterial cells are rich in nucleic acid, bearing negative charges as phosphate groups. These combine with the positively charged basic dyes. Acidic dyes do not stain bacterial cells and hence can be used to stain background material a contrasting color (see Negative Staining, below).

The basic dyes stain bacterial cells uniformly unless the cytoplasmic RNA is destroyed first. Special staining techniques can be used, however, to differentiate flagella, capsules, cell walls, cell membranes, granules, nuclei, and spores.

Gram Stain

An important taxonomic characteristic of bacteria is their response to Gram's stain. The gram-staining property appears to be a fundamental one, since the Gram reaction is correlated with many other morphologic properties in phylogenetically related forms. An organism that is potentially gram-positive may appear so only under a particular set of environmental conditions and in a young culture.

The gram-staining procedure begins with the application of a basic dye, crystal violet. A solution of iodine is then applied; all bacteria will be stained blue at this

point in the procedure. The cells are then treated with alcohol. Gram-positive cells retain the crystal violet-iodine complex, remaining blue; gram-negative cells are completely decolorized by alcohol. As a last step, a counter stain such as the red dye safranin) is applied so that the decolorized gram-negative (cells will take on a contrasting color; the gram-positive cells now appear purple.

The Gram-Positive Cell

As previously mentioned, Gram-positive bacteria are characterized by their blue-violet color reaction in the Gram-staining procedure. The blue-violet color reaction is caused by crystal-violet, the primary Gram-stain dye, complexing with the iodine mordant. When the decolorizer is applied, a slow dehydration of the crystal-violet/iodine complex is observed due to the closing of pores running through the cell wall. Because the crystal-violet is still present in the cell, the counter stain is not incorporated, thus maintaining the cell's blue-violet color. If you recall, most cell walls contain peptidoglycan, a molecule made of amino acids and sugar. A distinguishing factor among Gram-positive bacteria is that roughly 90% of their cell wall is comprised of peptidoglycan and a Gram-positve bacteria can have more than 20 layers of peptidoglycan stacked together to form the cell wall. Examples of common Gram-positive cells include Staphylococcus aureus and Streptococcus cremoris, a bacterium used in dairy production.

The Bacterial Cell Wall

The bacterial cell wall is a unique structure which surrounds the cell membrane (Fig. 18). Although not present in every bacterial species, the cell wall is very important as a cellular component. Structuraly, the wall is necessary for:

- Maintaining the cell's characteristic shape - the rigid wall compensates for the flexibility of the phospholipid membrane and keeps the cell from assuming a spherical shape
- Countering the effects of osmotic pressure- the strength of the wall is responsible for keeping the cell from bursting when the intracellular osmolarity is much greater than the extracellular osmolarity
- Providing attachment sites for bacteriophages - teichoic acids attached to the outer surface of the wall are like landing pads for viruses that infect bacteria
- Providing a rigid platform for surface appendages - flagella, fimbriae, and pili all emanate from the wall and extend beyond it.

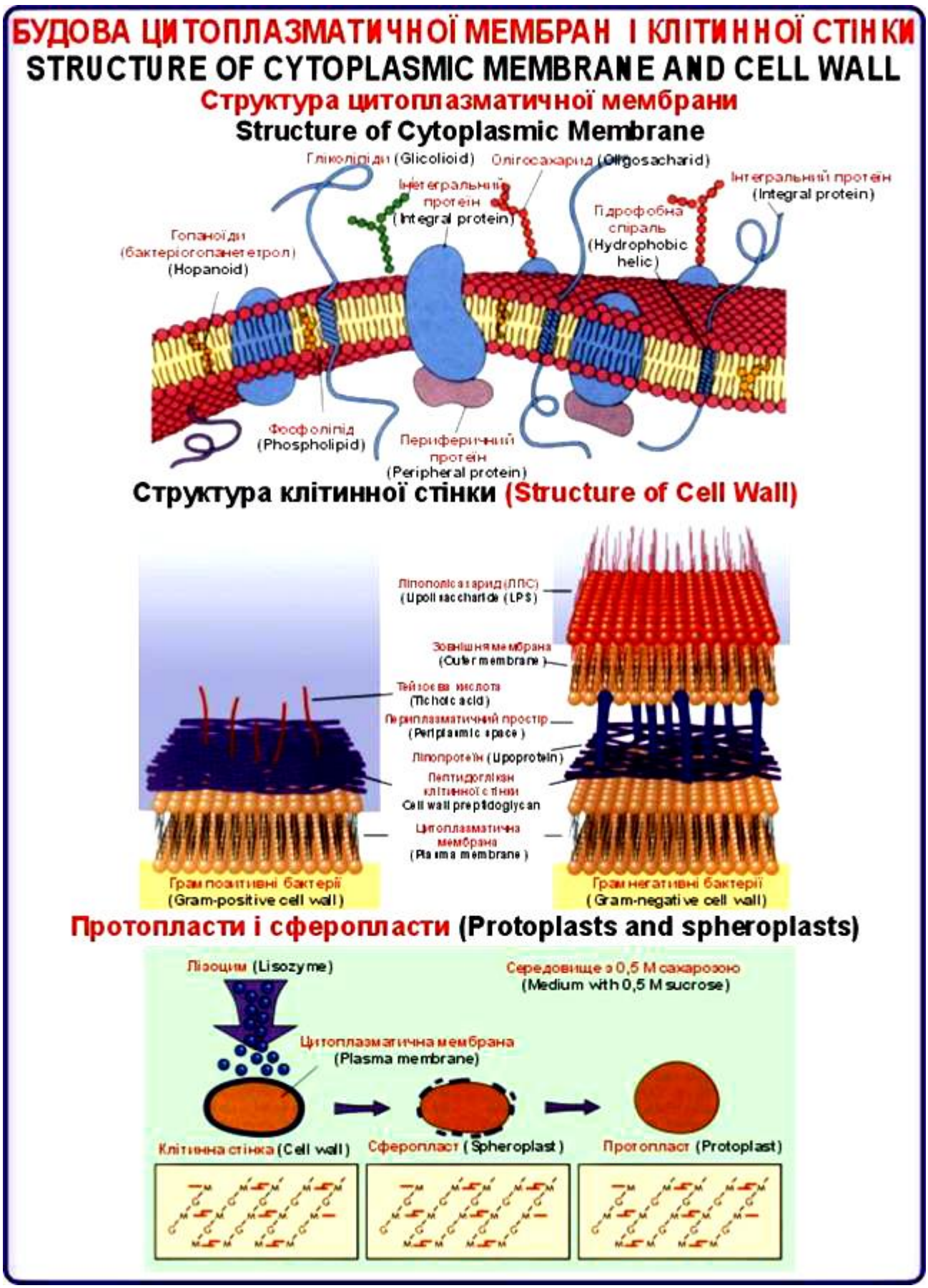

Figure 18. The structure of the cytoplasmic membrane and cell wall

The cell walls of all bacteria are not identical. In fact, cell wall composition is one of the most important factors in bacterial species analysis and differentiation. There are two major types of walls: Gram-positive and Gram-negative. The cell wall of Gram-positive bacteria consists of many polymer layers of peptidoglycan connected by amino acid bridges. A schematic diagram provides the best explanation of the structure. The peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine and N-acetyl-muraminic acid. It's a lot easier to just remember NAG and NAMA. Each peptidoglycan layer is connected, or crosslinked, to the other by a bridge made of amino acids and amino acid derivatives. The particular amino acids vary among different species, however. The crosslinked peptidoglycan molecules form a network which covers the cell like a grid. Also, 90% of the Gram-positive cell wall is comprised of peptidoglycan.

The cell wall of Gram-negative bacteria is much thinner, being comprised of only 20% peptidoglycan. Gram-negative bacteria also have two unique regions which surround the outer plasma membrane: the periplasmic space and the lipopolysaccharide layer. The periplasmic space separates the outer plasma membrane from the peptidoglycan layer. It contains proteins which destroy potentially dangerous foreign matter present in this space. The lipopolysaccharide layer is located adjacent to the exterior peptidoglycan layer. It is a phospholipid bilayer construction similar to that in the cell membrane and is attached to the peptidoglycan by lipoproteins. The lipid portion of the LPS contains a toxic substance, called Lipid A, which is responsible for most of the pathogenic affects associated with harmful Gram-negative bacteria. Polysaccharides which extend out from the bilayer also contribute to the toxicity of the LPS. The LPS, lipoproteins, and the associated polysaccharides together form what is known as the outer membrane. Keep in mind that the cell wall is not a regulatory structure like the cell membrane. Although it is porous, it is not selectively permeable and will let anything pass that can fit through its gaps.

The Gram-Negative Cell

Unlike Gram-positive bacteria, which assume a violet color in Gram staining, Gram negative bacteria incorporate the counter stain rather than the primary stain. Because the cell wall of Gram(-) bacteria is high in lipid content and low in peptidiglycan content, the primary crystal-violet escapes from the cell when the decolorizer is added. This is because primary stains like to bind with peptidoglycan-something the G(-) cell lacks. The pathogenic nature of Gram(-) bacteria is usually associated with certain components of their cell walls, particularly the lipopolysaccharide (endotoxin) layer. The Black Plague, which wiped out a third of the population of Europe, was caused by the tiny G(-) rod, Yersinia pestis. Most enteric (bowel related) illnesses can also be attributed to this group of bacteria.

I. Gram-staining Procedure

Gram-staining is a four part procedure which uses certain dyes to make a bacterial cell stand out against its background. The specimen should be mounted and heat fixed on a slide before you precede to stain it. The reagents you will need to successfully perform this operation are:

- Crystal Violet (the Primary Stain)
- Iodine Solution (the Mordant)
- Decolorizer (ethanol is a good choice)
- Safranin (the Counterstain)
- Water (preferably in a squirt bottle)

Before starting, make sure that all reagents, as well as the squirt-bottle of water, are easily accessible because you won't have time to go get them during the staining procedure. Also, make sure you are doing this near a sink because it can get really messy. Wear the appropriate lab attire.

STEP 1: Place your slide on a slide holder or a rack. Flood (cover completely) the entire slide with crystal violet. Let the crystal violet stand for about 60 seconds. When the time has elapsed, wash your slide for 5 seconds with the water bottle. The specimen should appear blue-violet when observed with the naked eye.

STEP 2: Now, flood your slide with the iodine solution. Let it stand about a minute as well. When time has expired, rinse the slide with water for 5 seconds and immediately precede to step three. At this point, the specimen should still be blue-violet.

STEP 3: This step involves addition of the decolorizer, ethanol. Step 3 is somewhat subjective because using too much decolorizer could result in a false Gram (-) result. Likewise, not using enough decolorizer may yield a false Gram (+) results. To be safe, add the ethanol dropwise until the blue-violet color is no longer emitted from your specimen. As in the previous steps, rinse with the water for 5 seconds.

STEP 4: The final step involves applying the counterstain, safranin. Flood the slide with the dye as you did in steps 1 and 2. Let this stand for about a minute to allow the bacteria to incorporate the safranin. Gram positive cells will incorporate little or no counterstain and will remain blue-violet in appearance. Gram negative bacteria, however, take on a pink color and are easily distinguishable from the Gram positives. Again, rinse with water for 5 seconds to remove any excess of dye.

After you have completed steps 1 through 4, you should dry the slide with bibulous paper or allow it to air dry before viewing it under the microscope (Gram's technique)

Tthe rules of work with immersion system of a microscope.

Positioning the slide

Place the slide specimen-side-up on the stage so that the specimen lies over the opening for the light in the middle of the stage. Secure the slide between (not under) the arms of the mechanical stage. The slide can now be moved from place to place using the 2 control knobs located on the right of the stage.

Adjusting the illumination

Adjust the total light available by turning the flat mirror. Adjust the amount of light coming through the condenser using the iris diaphragm lever located below and to the front of the stage. Light adjustment using the iris diaphragm lever is critical to obtaining proper contrast. For oil immersion microscopy (900X), the iris diaphragm lever should be set almost all the way open (to your left for maximum light).

Obtaining different magnifications

The final magnification is a product of the 2 lenses being used. The eyepiece or ocular lens magnifies 7X, 10X, 15X. The objective lenses are mounted on a turret near the stage. They make magnifications: 10X; 40X, and 90X (black-striped oil immersion lens). Final magnifications are as follows (tabl. 4):

Table 4. Obtaining different magnifications

Ocular lens	X	Objective lens	=	Total magnification
10X	X	10X	=	100X
10X	X	40X	=	400X
10X	X	100X (black)	=	900X

Focusing from lower power to higher power:

a. Rotate the 10X objective until it locks into place (total magnification of 100X).

b. Turn the coarse focus control (larger knob) all the way away from you until it stops.

c. Look through the eyepiece and turn the coarse focus control (larger knob) towards you slowly until the specimen comes into view.

d. Get the specimen into sharp focus using the fine focus control (smaller knob) and adjust the light for optimum contrast using the iris diaphragm lever.

e. If higher magnification is desired, simply rotate the 40X objective into place (total magnification of 400X) and the specimen should still be in focus. (Minor adjustments in fine focus and light contrast may be needed.)

f. For maximum magnification (900X or oil immersion), rotate the 40X objective slightly out of position and place a drop of immersion oil on the slide. Now rotate the black-striped 90X oil immersion objective into place. Again, the specimen should remain in focus, although minor adjustments in fine focus and light contrast may be needed.

Cleaning the microscope

Clean the exterior lenses of the eyepiece and objective before and after each lab using lens paper only. (Paper towel or kim-wipes may scratch the lens.) Remove any immersion oil from the oil immersion lens before putting the microscope away.

Reason for using immersion oil

Normally, when light waves travel from one medium into another, they bend. Therefore, as the light travels from the glass slide to the air, the light waves bend and are scattered (the "bent pencil" effect when a pencil is placed in a glass of water). The microscope magnifies this distortion effect. Also, if high magnification is to be used, more light is needed.

Immersion oil has the same refractive index as glass and, therefore, provides an optically homogeneous path between the slide and the lens of the objective. Light waves thus travel from the glass slide, into glass-like oil, into the glass lens without

being scattered or distorting the image. In other words, the immersion oil "traps" the light and prevents the distortion effect that is seen as a result of the bending of the light waves. Before making a preparation, glass slides are flamed to ensure their additional degreasing.

In preparing (Preparation of a smear) a smear from bacterial culture grown on a solid medium, a drop of isotonic saline or water is transferred onto the precooled glass. A test tube with the culture is taken by the thumb and the index finger of the left hand. The loop is sterilized in the flame. A cotton-wool plug is pinched by a small finger of the right hand, removed from the test tube, and left in this position. The edges of the test tube are flamed and then the loop is introduced into the test tube through the flame. Having cooled the loop against the inner wall of the tube, the loop is touched to the nutrient medium where it meets with the glass wall (if the loop is not sufficiently cooled, it induces cracking and melts the medium). Then the loop is touched to the culture of the microorganisms on the surface of the medium. Then the loop is withdrawn, the edges of the test tube are quickly flamed, the tube is closed with a stopper passed through the flame, and then replaced into the test tube rack. All the above described procedures are made above the flame. The culture sample is placed with the loop into a drop of water on the glass slide and spread uniformly with circular movements on an area of 1– 1.5 cm in diameter, then the loop is flamed.

Then you should do drying an fixation of the smear.

The fixed preparation is placed, the smear upward, on the support. A dye solution is pipetted onto the entire surface of the smear. With Pfeiffer's fuchsine the staining lasts 1-2 min, with alkaline solution of Loeffler's methylene blue or water-alcoholic solution of methylene blue, 3-5 min. (Simple method of staining) Following the staining procedure the dye is dispensed, the preparation is washed with water, dried between sheets of filter paper, and then examined under the oil-immersion objective.

Structure of bacterial cell

The higher resolving power of the electron microscope not only magnifies the typical shape of a bacterial cell but also clearly resolves its prokaryotic organization (Fig. 19).

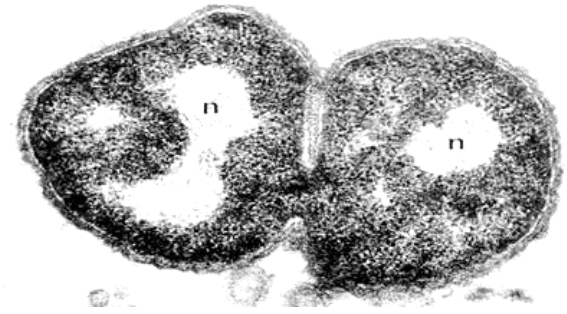

FIGURE 19. Electron micrograph of a thin section of Neisseria gonorrhoeae showing the organizational features of prokaryotic cells. Note the electron-transparent nuclear region (n) packed with DNA fibrils, the dense distribution of ribosomal particles in the cytoplasm, and the absence of intracellular membranous organelles.

The Nucleoid

Prokaryotic and eukaryotic cells were initially distinguished on the basis of structure: the prokaryotic nucleoid the equivalent of the eukaryotic nucleus is structurally simpler than the true eukaryotic nucleus, which has a complex mitotic apparatus and surrounding nuclear membrane. As the electron micrograph in Fig. 2 shows, the bacterial nucleoid, which contains the DNA fibrils, lacks a limiting membrane. Under the light microscope, the nucleoid of the bacterial cell can be visualized with the aid of Feulgen staining, which stains DNA. Gentle lysis can be used to isolate the nucleoid of most bacterial cells. The DNA is then seen to be a single, continuous, "giant" circular molecule with a molecular weight of approximately 3 X 109. The unfolded nuclear DNA would be about 1 mm long (compared with an average length of 1 to 2 μm for bacterial cells). The bacterial nucleoid, then, is a structure containing a single chromosome. The number of copies of this chromosome in a cell depends on the stage of the cell cycle (chromosome replication, cell enlargement, chromosome segregation, etc). Although the mechanism of segregation of the two sister chromosomes following replication is not fully understood, all of the models proposed require that the chromosome be permanently attached to the cell membrane throughout the various stages of the cell cycle.

Bacterial chromatin does not contain basic histone proteins, but low-molecular-weight polyamines and magnesium ions may fulfill a function similar to that of eukaryotic histones. Despite the differences between prokaryotic and eukaryotic DNA, prokaryotic DNA from cells infected with bacteriophage g, when visualized by electron microscopy, has a beaded, condensed appearance not unlike that of eukaryotic chromatin. Plasmids are small circular DNA molecules that can be thought of as carrying extra genes that can be used for special situations. They usually can be dispensed with when not required. There may be several different plasmids in one cell and the numbers of each may vary from only one to 100s in a cell .

Surface Appendages

Surface Layers

The surface layers of the bacterial cell have been identified by various techniques: light microscopy and staining; electron microscopy of thin-sectioned, freeze-fractured, and negatively stained cells; and isolation and biochemical characterization of individual morphologic components of the cell. The principal surface layers are capsules and loose slime, the cell wall of Gram-positive bacteria and the complex cell envelope of Gram-negative bacteria, plasma (cytoplasmic) membranes, and mesosomal membrane vesicles, which arise from invaginations of the plasma membrane. In bacteria, the cell wall forms a rigid structure of uniform thickness around the cell and is responsible for the characteristic shape of the cell (rod, coccus, or spiral). Inside the cell wall (or rigid peptidoglycan layer) is the plasma (cytoplasmic) membrane; this is usually closely apposed to the wall layer.

The topographic relationships of the cell wall and envelope layers to the plasma membrane are indicated in the thin section of a Gram-positive organism (Micrococcus lysodeikticus) in Figure 20-A and in the freeze-fractured cell of a Gram-negative organism (Bacteroides melaninogenicus) in Figure 20-B. The latter shows the typical fracture planes seen in most Gram-negative bacteria, which are weak cleavage planes through the outer membrane of the envelope and extensive fracture planes through the bilayer region of the underlying plasma membrane.

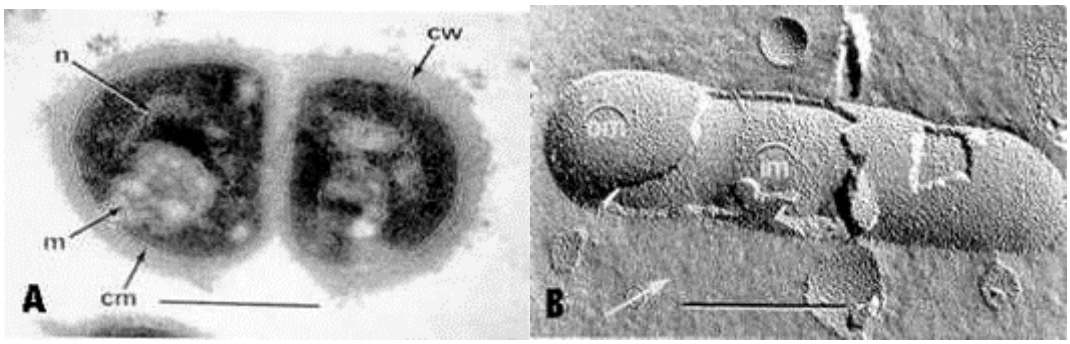

FIGURE 20. (A). Electron micrograph of a thin section of the Gram-positive M lysodeikticus showing the thick peptidoglycan cell wall (cw), underlying cytoplasmic (plasma) membrane (cm), mesome (m), and nucleus (n). (B) Freeze-fractured Bacteriodes cell showing typical major convex fracture faces through the inner (im) and outer (om) membranes. Bars = 1 μm; circled arrow in Fig. B indicates direction of shadowing.

Capsules and Loose Slime

Some bacteria form capsules, which constitute the outermost layer of the bacterial cell and surround it with a relatively thick layer of viscous gel. Capsules may be up to 10 μm thick. Some organisms lack a well-defined capsule but have loose, amorphous slime layers external to the cell wall or cell envelope. The a hemolytic Streptococcus mutans, the primary organism found in dental plaque is able to synthesis a large extracellular mucoid glucans from sucrose. Not all bacterial species produce capsules; however, the capsules of encapsulated pathogens are often important determinants of virulence. Encapsulated species are found among both Gram-positive and Gram-negative bacteria. In both groups, most capsules are composed of highmolecular-weight viscous polysaccharides that are retained as a thick gel outside the cell wall or envelope. The capsule of Bacillus anthracis (the causal agent of anthrax) is unusual in that it is composed of a g-glutamyl polypeptide. Table 2 presents the various capsular substances formed by a selection of Gram-positive and Gram-negative bacteria. A plasma membrane stage is involved in the biosynthesis and assembly of the capsular substances, which are extruded or secreted through the outer wall or envelope structures. Mutational loss of enzymes involved in the biosynthesis of the capsular polysaccharides can result in the smooth-to-rough variation seen in the pneumococci.

The capsule is not essential for viability. Viability is not affected when capsular polysaccharides are removed enzymatically from the cell surface. The exact functions of capsules are not fully understood, but they do confer resistance to phagocytosis and hence provide the bacterial cell with protection against host defenses to invasion.

Capsules are usually demonstrated by the negative staining procedure or a modification of it. One such "capsule stain" (Welch method) involves treatment with hot crystal violet solution followed by a rinsing with copper sulfate solution. The latter is used to remove excess stain because the conventional washing with water would dissolve the capsule. The copper salt also gives color to the background, with the result that the cell and background appear dark blue and the capsule a much paler blue.

Cell Wall and Gram-Negative Cell Envelope

The Gram stain broadly differentiates bacteria into Gram-positive and Gram-negative groups; a few organisms are consistently Gram-variable. Gram-positive and Gram-negative organisms differ drastically in the organization of the structures outside the plasma membrane but below the capsule (Fig. 21): in Gram-negative organisms these structures constitute the cell envelope, whereas in Gram-positive organisms they are called a cell wall.

Most Gram-positive bacteria have a relatively thick (about 20 to 80 nm), continuous cell wall (often called the sacculus), which is composed largely of peptidoglycan (also known as mucopeptide or murein). In thick cell walls, other cell wall polymers (such as the teichoic acids, polysaccharides, and peptidoglycolipids) are covalently attached to the peptidoglycan. In contrast, the peptidoglycan layer in Gram-negative bacteria is thin (about 5 to 10 nm thick); in E coli, the peptidoglycan is probably only a monolayer thick. Outside the peptidoglycan layer in the Gram-negative envelope is an outer membrane structure (about 7.5 to 10 nm thick). In most Gram-negative bacteria, this membrane structure is anchored noncovalently to lipoprotein molecules (Braun's lipoprotein), which, in turn, are covalently linked to the peptidoglycan. The lipopolysaccharides of the Gram-negative cell envelope form part of the outer leaflet of the outer membrane structure.

The organization and overall dimensions of the outer membrane of the Gram-negative cell envelope are similar to those of the plasma membrane (about 7.5 nm thick). Moreover, in Gram-negative bacteria such as E coli, the outer and inner membranes adhere to each other at several hundred sites (Bayer patches); these sites can break up the continuity of the peptidoglycan layer. Table 2 summarizes the major classes of chemical constituents in the walls and envelopes of Gram-positive and Gram-negative bacteria.

The basic differences in surface structures of Gram-positive and Gram-negative bacteria explain the results of Gram staining. Both Gram-positive and Gram-negative bacteria take up the same amounts of crystal violet (CV) and iodine (I). The CV-I complex, however, is trapped inside the Gram-positive cell by the dehydration and reduced porosity of the thick cell wall as a result of the differential washing step with 95 percent ethanol or other solvent mixture. In contrast, the thin peptidoglycan

layer and probable discontinuities at the membrane adhesion sites do not impede solvent extraction of the CV-I complex from the Gram-negative cell.

The above mechanism of the Gram stain based on the structural differences between the two groups has been confirmed by sophisticated methods of electron microscopy. The sequence of steps in the Gram stain differentiation is illustrated diagrammatically in Figure 22. Moreover, mechanical disruption of the cell wall of Gram-positive organisms or its enzymatic removal with lysozyme results in complete extraction of the CV-I complex and conversion to a Gram-negative reaction. Therefore, autolytic wall-degrading enzymes that cause cell wall breakage may account for Gram-negative or variable reactions in cultures of Gram-positive organisms (such as Staphylococcus aureus, Clostridium perfringens, Corynebacterium diphtheriae, and some Bacillus spp).

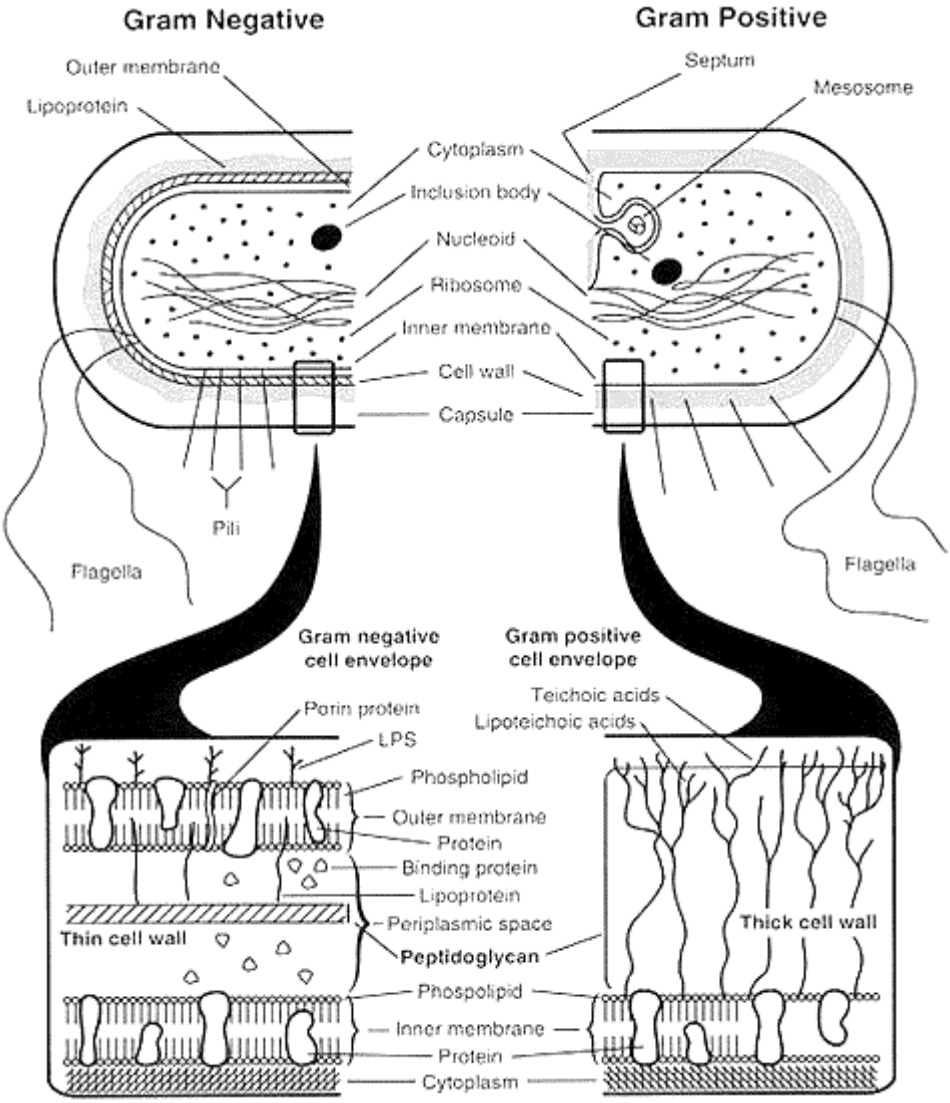

FIGURE 21. Comparison of the thick cell wall of Gram-positive bacteria with the comparatively thin cell wall of Gram-negative bacteria. Note the complexity of the Gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space).

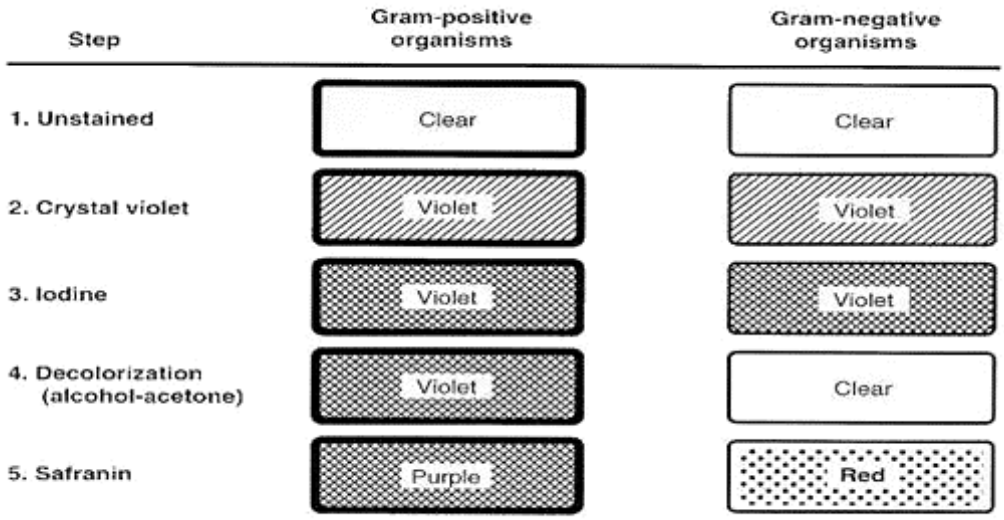

FIGURE 22. General sequence of steps in the Gram stain procedure and the resultant staining of Gram-positive and Gram-negative bacteria

Peptidoglycan

Unique features of almost all prokaryotic cells (except for Halobacterium halobium and mycoplasmas) are cell wall peptidoglycan and the specific enzymes involved in its biosynthesis. These enzymes are target sites for inhibition of peptidoglycan synthesis by specific antibiotics. The primary chemical structures of peptidoglycans of both Gram-positive and Gram-negative bacteria have been established; they consist of a glycan backbone of repeating groups of β1, 4-linked disaccharides of β1,4-N-acetylmuramyl-N-acetylglucosamine. Tetrapeptides of L-alanine-D-isoglutamic acid-L-lysine (or diaminopimelic acid)-n-alanine are linked through the carboxyl group by amide linkage of muramic acid residues of the glycan chains; the D-alanine residues are directly cross-linked to the e-amino group of lysine or diaminopimelic acid on a neighboring tetrapeptide, or they are linked by a peptide bridge. In S aureus peptidoglycan, a glycine pentapeptide bridge links the two adjacent peptide structures. The extent of direct or peptide-bridge cross-linking varies from one peptidoglycan to another. The staphylococcal peptidoglycan is highly cross-linked, whereas that of E coli is much less so, and has a more open peptidoglycan mesh.

The diamino acid providing the e-amino group for cross-linking is lysine or diaminopimelic acid, the latter being uniformly present in Gram-negative peptidoglycans. The structure of the peptidoglycan is illustrated in Figure 23. A peptidoglycan with a chemical structure substantially different from that of all eubacteria has been discovered in certain archaebacteria. Instead of muramic acid, this peptidoglycan contains talosaminuronic acid and lacks the D-amino acids found in the eubacterial peptidoglycans. Interestingly, organisms containing this wall polymer (referred to as pseudomurein) are insensitive to penicillin, an inhibitor of the transpeptidases involved in peptidoglycan biosynthesis in eubacteria.

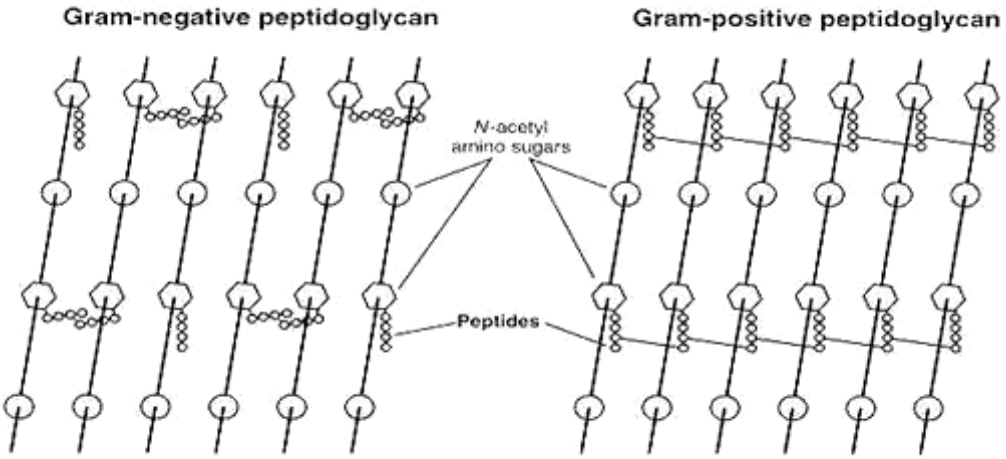

FIGURE 23. Diagrammatic representation of peptidoglycan structures with adjacent glycan strands cross-linked directly from the carboxyterminal D-alanine to the e-amino group of an adjacent tetrapeptide or through a peptide cross bridge ,N-acetylmuramic acid; N-acetylglucosamine.

The ß-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine is specifically cleaved by the bacteriolytic enzyme lysozyme. Widely distributed in nature, this enzyme is present in human tissues and secretions and can cause complete digestion of the peptidoglycan walls of sensitive organisms. When lysozyme is allowed to digest the cell wall of Gram-positive bacteria suspended in an osmotic stabilizer (such as sucrose), protoplasts are formed. These protoplasts are able to survive and continue to grow on suitable media in the wall-less state. Gram-negative bacteria treated similarly produce spheroplasts, which retain much of the outer membrane structure. The dependence of bacterial shape on the peptidoglycan is shown by the transformation of rod-shaped bacteria to spherical protoplasts (spheroplasts) after enzymatic breakdown of the peptidoglycan. The mechanical protection afforded by the wall peptidoglycan layer is evident in the osmotic fragility of both protoplasts and spheroplasts.

There are two groups of bacteria that lack the protective cell wall peptidoglycan structure, the Mycoplasma species, one of which causes atypical pneumonia and some genitourinary tract infections and the L-forms, which originate from Gram-positive or Gram-negative bacteria and are so designated because of their discovery and description at the Lister Institute, London. The mycoplasmas and L-forms are all Gram-negative and insensitive to penicillin and are bounded by a surface membrane structure. L-forms arising "spontaneously" in cultures or isolated from infections are structurally related to protoplasts and spheroplasts; all three forms (protoplasts, spheroplasts, and L-forms) revert infrequently and only under special conditions.

Teichoic Acids

Wall teichoic acids are found only in certain Gram-positive bacteria (such as staphylococci, streptococci, lactobacilli, and Bacillus spp); so far, they have not been

found in gram- negative organisms. Teichoic acids are polyol phosphate polymers, with either ribitol or glycerol linked by phosphodiester bonds; their structures are illustrated in Figure 2. Substituent groups on the polyol chains can include D-alanine (ester linked), N-acetylglucosamine, N-acetylgalactosamine, and glucose; the substituent is characteristic for the teichoic acid from a particular bacterial species and can act as a specific antigenic determinant. Teichoic acids are covalently linked to the peptidoglycan. These highly negatively charged polymers of the bacterial wall can serve as a cation-sequestering mechanism.

Accessory Wall Polymers

In addition to the principal cell wall polymers, the walls of certain Gram-positive bacteria possess polysaccharide molecules linked to the peptidoglycan. For example, the C polysaccharide of streptococci confers group specificity. Acidic polysaccharides attached to the peptidoglycan are called teichuronic acids. Mycobacteria have peptidoglycolipids, glycolipids, and waxes associated with the cell wall.

Lipopolysaccharides

A characteristic feature of Gram-negative bacteria is possession of various types of complex macromolecular lipopolysaccharide (LPS). So far, only one Gram-positive organism, Listeria monocytogenes, has been found to contain an authentic LPS. The LPS of this bacterium and those of all Gram-negative species are also called endotoxins, thereby distinguishing these cell-bound, heat-stable toxins from heat-labile, protein exotoxins secreted into culture media. Endotoxins possess an array of powerful biologic activities and play an important role in the pathogenesis of many Gram-negative bacterial infections. In addition to causing endotoxic shock, LPS is pyrogenic, can activate macrophages and complement, is mitogenic for B lymphocytes, induces interferon production, causes tissue necrosis and tumor regression, and has adjuvant properties. The endotoxic properties of LPS reside largely in the lipid A components. Usually, the LPS molecules have three regions: the lipid A structure required for insertion in the outer leaflet of the outer membrane bilayer; a covalently attached core composed of 2-keto-3deoxyoctonic acid (KDO), heptose, ethanolamine, N-acetylglucosamine, glucose, and galactose; and polysaccharide chains linked to the core. The polysaccharide chains constitute the O-antigens of the Gram-negative bacteria, and the individual monosaccharide constituents confer serologic specificity on these components. Table 5 depicts the structure of LPS. Although it has been known that lipid A is composed of b1,6-linked D-glucosamine disaccharide substituted with phosphomonester groups at positions 4' and 1, uncertainties have existed about the attachment positions of the six fatty acid acyl and KDO groups on the disaccharide. The demonstration of the structure of lipid A of LPS of a heptoseless mutant of Salmonella typhimurium has established that amide-linked hydroxymyristoyl and lauroxymyristoyl groups are attached to the nitrogen of the 2- and 2'-carbons, respectively, and that hydroxymyristoyl and myristoxymyristoyl groups are attached to the oxygen of the 3- and 3'-carbons of the disaccharide, respectively. Therefore, only position 6' is left for attachment of KDO units.

Table 5. The three major, covalently linked regions that form the typical LPS.

Lipid A	Core	O Antigen
Glucosamine β-hydroxymyristate Fatty acids	Ketodeoxyoctonate Phosphoethanolamine Heptose Glucose, galactose, *N*-acetylglucosamine	Polysaccharide chains: repeating units of species-specific monosaccharides, e.g., galactose, rhamnose, mannose and abequose in *S typhimurium* LPS

LPS and phospholipids help confer asymmetry to the outer membrane of the Gram-negative bacteria, with the hydrophilic polysaccharide chains outermost. Each LPS is held in the outer membrane by relatively weak cohesive forces (ionic and hydrophobic interactions) and can be dissociated from the cell surface with surface-active agents.

As in peptidoglycan biosynthesis, LPS molecules are assembled at the plasma or inner membrane. These newly formed molecules are initially inserted into the outer-inner membrane adhesion sites.

Outer Membrane of Gram-Negative Bacteria

In thin sections, the outer membranes of Gram-negative bacteria appear broadly similar to the plasma or inner membranes; however, they differ from the inner membranes and walls of Gram-positive bacteria in numerous respects. The lipid A of LPS is inserted with phospholipids to create the outer leaflet of the bilayer structure; the lipid portion of the lipoprotein and phospholipid form the inner leaflet of the outer membrane bilayer of most Gram-negative bacteria (Fig. 21).

In addition to these components, the outer membrane possesses several major outer membrane proteins; the most abundant is called porin. The assembled subunits of porin form a channel that limits the passage of hydrophilic molecules across the outer membrane barrier to those having molecular weights that are usually less than 600 to 700. Evidence also suggests that hydrophobic pathways exist across the outer membrane and are partly responsible for the differential penetration and effectiveness of certain β-lactam antibiotics (ampicillin, cephalosporins) that are active against various Gram-negative bacteria. Although the outer membranes act as a permeability barrier or molecular sieve, they do not appear to possess energy-transducing systems to drive active transport. Several outer membrane proteins, however, are involved in the specific uptake of metabolites (maltose, vitamin B12, nucleosides) and iron from the medium. Thus, outer membranes of the Gram-negative bacteria provide a selective barrier to external molecules and thereby prevent the loss of metabolite-binding proteins and hydrolytic enzymes (nucleases, alkaline phosphatase) found in the periplasmic space. The

periplasmic space is the region between the outer surface of the inner (plasma) membrane and the inner surface of the outer membrane (Figure 21).

Thus, Gram-negative bacteria have a cellular compartment that has no equivalent in Gram-positive organisms. In addition to the hydrolytic enzymes, the periplasmic space holds binding proteins (proteins that specifically bind sugars, amino acids, and inorganic ions) involved in membrane transport and chemotactic receptor activities. Moreover, plasmid-encoded b-lactamases and aminoglycoside-modifying enzymes (phosphorylation or adenylation) in the periplasmic space produce antibiotic resistance by degrading or modifying an antibiotic in transit to its target sites on the membrane (penicillin-binding proteins) or on the ribosomes (aminoglycosides). These periplasmic proteins can be released by subjecting the cells to osmotic shock and after treatment with the chelating agent ethylenediaminetetraacetic acid.

Intracellular Components

Plasma (Cytoplasmic) Membranes

Bacterial plasma membranes, the functional equivalents of eukaryotic plasma membranes, are referred to variously as cytoplasmic, protoplast, or (in Gram-negative organisms) inner membranes. Similar in overall dimensions and appearance in thin sections to biomembranes from eukaryotic cells, they are composed primarily of proteins and lipids (principally phospholipids). Protein-to-lipid ratios of bacterial plasma membranes are approximately 3:1, close to those for mitochondrial membranes. Unlike eukaryotic cell membranes, the bacterial membrane (except for Mycoplasma species and certain methylotrophic bacteria) has no sterols, and bacteria lack the enzymes required for sterol biosynthesis.

Although their composition is similar to that of inner membranes of Gram-negative species, cytoplasmic membranes from Gram-positive bacteria possess a class of macromolecules not present in the Gram-negative membranes. Many Gram-positive bacterial membranes contain membrane-bound lipoteichoic acid, and species lacking this component (such as Micrococcus and Sarcina spp) contain an analogous membrane-bound succinylated lipomannan. Lipoteichoic acids are structurally similar to the cell wall glycerol teichoic acids in that they have basal polyglycerol phosphodiester 1-3 linked chains. These chains terminate with the phosphomonoester end of the polymer, which is linked covalently to either a glycolipid or a phosphatidyl glycolipid moiety. Thus, a hydrophobic tail is provided for anchoring in the membrane lipid layers (Fig. 21). As in the cell wall glycerol teichoic acid, the lipoteichoic acids can have glycosidic and D-alanyl ester substituents on the C-2 position of the glycerol.

Both membrane-bound lipoteichoic acid and membrane-bound succinylated lipomannan can be detected as antigens on the cell surface, and the glycerol-phosphate and succinylated mannan chains appear to extend through the cell wall structure (Fig. 21). This class of polymer has not yet been found in the cytoplasmic membranes of Gram-negative organisms. In both instances, the lipoteichoic acids and the lipomannans are negatively charged components and can sequester positively charged substances. They have been implicated in adhesion to host cells, but their functions remain to be elucidated.

Multiple functions are performed by the plasma membranes of both Gram-positive and Gram-negative bacteria. Plasma membranes are the site of active transport, respiratory chain components, energy-transducing systems, the H+-ATPase of the proton pump , and membrane stages in the biosynthesis of phospholipids, peptidoglycan, LPS, and capsular polysaccharides. In essence, the bacterial cytoplasmic membrane is a multifunction structure that combines the mitochondrial transport and biosynthetic functions that are usually compartmentalized in discrete membranous organelles in eukaryotic cells. The plasma membrane is also the anchoring site for DNA and provides the cell with a mechanism (as yet unknown) for separation of sister chromosomes.

Mesosomes

Thin sections of Gram-positive bacteria reveal the presence of vesicular or tubular-vesicular membrane structures called mesosomes, which are apparently formed by an invagination of the plasma membrane. These structures are much more prominent in Gram-positive than in Gram-negative organisms. At one time, the mesosomal vesicles were thought to be equivalent to bacterial mitochondria; however, many other membrane functions have also been attributed to the mesosomes. At present, there is no satisfactory evidence to suggest that they have a unique biochemical or physiologic function. Indeed, electron-microscopic studies have suggested that the mesosomes, as usually seen in thin sections, may arise from membrane perturbation and fixation artifacts. No general agreement exists about this theory, however, and some evidence indicates that mesosomes may be related to events in the cell division cycle.

Other Intracellular Components

In addition to the nucleoid and cytoplasm (cytosol), the intracellular compartment of the bacterial cell is densely packed with ribosomes of the 70S type. These ribonucleoprotein particles, which have a diameter of 18 nm, are not arranged on a membranous rough endoplasmic reticulum as they are in eukaryotic cells. Other granular inclusions randomly distributed in the cytoplasm of various species include metabolic reserve particles such as poly-b-hydroxybutyrate (PHB), polysaccharide and glycogen-like granules, and polymetaphosphate or metachromatic granules (volutin granules). They possess high electron density. The volutin granules vary in size from several hundreds of 0.1 to 0.5 mcm.

A characteristic feature of the granules of volutin is their metachromatic stain. They are stained reddish-purple, with methylene blue while the cytoplasm is stained blue.

Volutin was first discovered in the cell of Spirillum volutans (from which it was named), then in Corynebacterium diphtheriae (Fig. 24) and other organisms. The presence of volutin is taken into account in laboratory diagnosis of diphtheria. Lipoprotein bodies are found quite frequently as droplets of fat in certain bacilli and spirilla. They disappear when the cells' are deprived of nutrients, and appear when bacteria are grown on nutrient media of a high carbohydrate content. They are discernible if stained with Sudan or fuchsin.

The presence of volutin granules and lipoprotein bodies is biologically important since they serve as sources of stored food for the bacterium in the case of starvation.

Figure 24. Granules of volutin in Corynebacterium diphtheriae

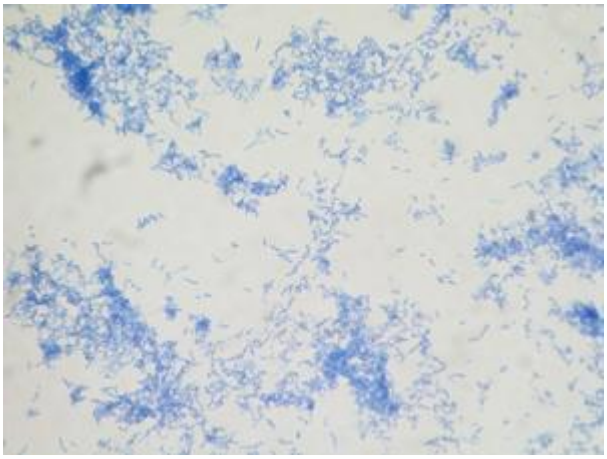

Figure 25. Volutin's granules, Loeffler's technique

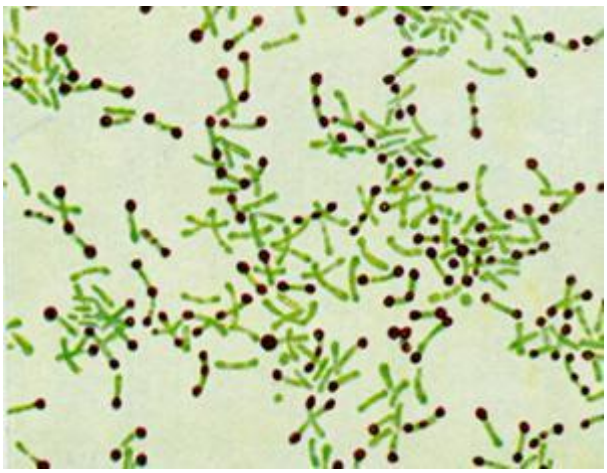

Figure 26. Volutin's granules, Neisser's technique

Glycogen and granulose are intracellular inclusions which can be identified by treating the cell with Lugol's solution. Glycogen stains reddish-brown and granulose

grey-blue. Glycogen granules are prominent in aerobic bacilli. Granulose is frequently found in butyric-acid bacteria, and especially in Clostridium pectinovorum.

Some bacteria contain crystals of a protein nature which have proved to be extremely toxic for certain insect larvae. In the cytoplasm of sulphur bacteria (Beggiatoa) which oxidize hydrogen sulphide, sulphur is deposited in the form of droplets of a colloidal nature. Energy derived from the sulphur is utilized in reducing carbon dioxide.

Granules of amorphic calcium carbonate, the physiological function of which is not yet known, are found in the cytoplasm of some sulphur bacteria [Achromatiun}.

Staining of volutin granules with alkaline methylene blue (by Loeffler's technique). On a fixed smear pour alkaline methylene blue to act for 3-5 min. wash with water, dry with filter paper, and examine under the microscope. The cytoplasm of diphtheria corynebacteria is stained light-blue, while granules of volutin are dark-blue (Fig. 25).

Staining with acetic-acidic methyl violet. A fixed smear is treated for 5-10 min with acetic-acidic methyl violet (methyl violet or crystal violet, 0.25 g, 5 per cent solution of acetic acid, 100 ml). The smear is washed with water and dried. In this case the cytoplasm of diphtheria corynebacteria is stained light lilac, while volutin granules appear dark-lilac.

In complex Neisser’s staining bacterial cells become yellow whereas volutin granules become brown-black (Fig. 26).

Neisser's staining. Staining of volutin granules by this method includes the following stages.

1. A fixed smear is stained with acetic-acidic methylene blue for 1 min, then the dye is poured off, and smear is washed "with water.

2. Pour in Lugol’s solution to act for 20-30 s.

3. Without washing with water, stain the preparation with vesuvin for 1-3 min, then wash it with water and dry.

Ziehl-Neelsen staining is employed for detecting acid-fast tuberculosis and leprosy mycobacteria and some actinomycetes. The acid-fast nature of microorganisms is due to the fact that their cells contain lipids, wax, and oxyacids. Such microorganisms are poorly stained with diluted solutions of dyes. To facilitate the penetration of the stain into the cells of microorganisms, Ziehl’s phenol fuchsine applied onto the preparation is heated over the burner's flame.

Stained microorganisms do not decolorize with weak solutions of mineral acids and alcohol.

Staining of microorganisms by the Ziehl-Neelsen method includes the following stages.

1. Put a slip of filter paper on a fixed smear and pour Ziehl's phenol fuchsine on it (one can use filter paper saturated with a dye and then dried). Heat the smear over the flame until steam rises, then draw it aside for cooling and add a new portion

of the dye. Repeat heating 2-3 times. Allow the smear to cool, take off the filter paper, and wash the preparation with water.

2. The preparation is decolorized by immersing it in or with 5 per cent solution of sulphuric acid and washed several times with water.

3. The preparation is stained with aqueous-alcoholic solution of methylene blue for 3-5 min, washed with water and dried.

Upon staining by Ziehl-Neelsen technique acid-fast bacteria acquire a bright red colour, while the remaining microflora is stained light-blue (Fig. 27).

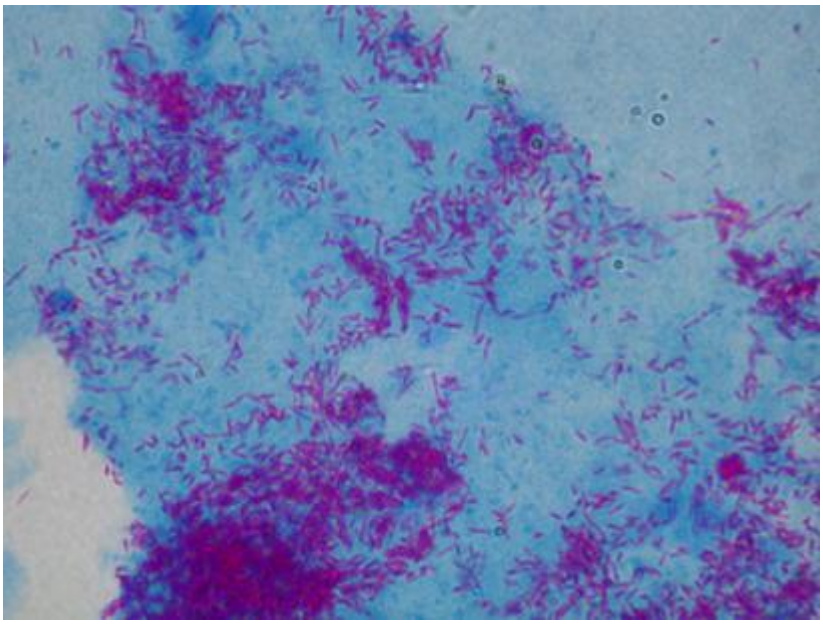

Figure 27. Staining of microorganisms by the Ziehl-Neelsen method

To demonstrate bacterial nucleoid, one can use Feulgen's micro-chemical reaction in which weak acidic hydrolysis is employed. This is accompanied by the release of desoxyribose which subsequently transforms into aldehydes, reacting with colourless fuchsine-sulphurous acid of special Schiff's reagent. The nucleoid is stained red-violet. The nucleoid of microorganisms may also be detected by means of electron microscopic examination of ultrathin sections.

Due to high concentrations of metaphosphates and other phosphorous compounds volutin granules (inclusions in the cytoplasm) are characterized by metachromasia. Upon staining with alkaline methylene blue and acetic-acidic methylene violet, their colour is more intensive as compared to that of the cytoplasm.

Endospores. Endospores are highly heat-resistant, dehydrated resting cells formed intracellularly in members of the genera Bacillus and Clostridium (fig. 28). Endospores are small spherical or oval bodies formed within the cell. A spore is formed at a certain stage in the development of some micro-organisms and this property was inherited in the process of evolution in the struggle for keeping the species intact. Some micro-organisms, principally rod-shaped (bacilli and clostridia), are capable of sporulation. These include the causative agents of anthrax, tetanus,

anaerobic infections, botulism and also saprophytic species living in the soil, water and bodies of animals. Spore formation only rarely occurs in cocci (*Sarcina lutea, Sarcina ureae)* and in spiral forms *(Desulfovibrio desulfuricans).* Sporulation occurs in the environment (in soil and on nutrient media) and is not observed in human or animal tissues.

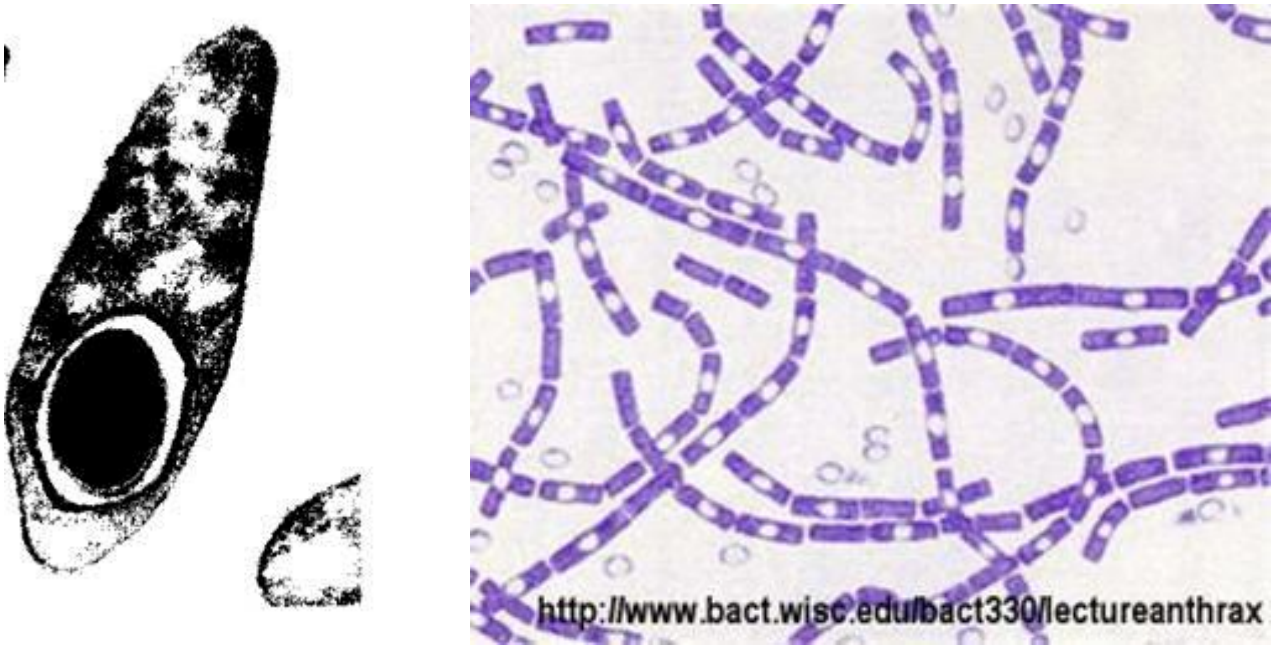

Figure 28. Thin section through a sporulating cell of bacilli

The series of biochemical and morphologic changes that occur during sporulation represent true differentiation within the cycle of the bacterial cell. The process, which usually begins in the stationary phase of the vegetative cell cycle, is initiated by depletion of nutrients (usually readily utilizable sources of carbon or nitrogen, or both).

The cell then undergoes a highly complex, well-defined sequence of morphologic and biochemical events that ultimately lead to the formation of mature endospores. As many as seven distinct stages have been recognized by morphologic and biochemical studies of sporulating Bacillus species: stage 0, vegetative cells with two chromosomes at the end of exponential growth; stage I, formation of axial chromatin filament and excretion of exoenzymes, including proteases; stage II, forespore septum formation and segregation of nuclear material into two compartments; stage III, spore protoplast formation and elevation of tricarboxylic acid and glyoxylate cycle enzyme levels; stage IV, cortex formation and refractile appearance of spore; stage V, spore coat protein formation; stage VI, spore maturation, modification of cortical peptidoglycan, uptake of dipicolinic acid (a unique endospore product) and calcium, and development of resistance to heat and organic solvents; and stage VII, final maturation and liberation of endospores from mother cells (in some species).

When newly formed, endospores appear as round, highly refractile cells within the vegetative cell wall, or sporangium. Some strains produce autolysins that digest the walls and liberate free endospores. The spore protoplast, or core, contains a complete nucleus, ribosomes, and energy generating components that are enclosed within a modified cytoplasmic membrane. The peptidoglycan spore wall surrounds the spore membrane; on germination, this wall becomes the vegetative cell wall. Surrounding the spore wall is a thick cortex that contains an unusual type of peptidoglycan, which is rapidly released on germination. A spore coat of keratinlike protein encases the spore contained within a membrane (the exosporium). During maturation, the spore protoplast dehydrates and the spore becomes refractile and resistant to heat, radiation, pressure, desiccation, and chemicals; these properties correlate with the cortical peptidoglycan and the presence of large amounts of calcium dipicolinate.

Recent evidence indicated that the spores of Bacillus spharicus were revived which had been preserved in amber for more than 25 million years. Their claims need to be reevaluated. The thin section of the spore shows the ruptured, thick spore coat and the cortex surrounding the spore protoplast with the germinal cell wall that becomes the vegetative wall on outgrowth.

The spores of certain bacilli are capable of withstanding boiling and high concentrations of disinfectants. They are killed in an autoclave exposed to saturated steam, at a temperature of 115-125 °C, and also at a temperature of 150-170 °C in a Pasteur hot-air oven.

Sporulation: The sporulation process begins when nutritional conditions become unfavorable, depletion of the nitrogen or carbon source (or both) being the most significant factor Sporulation occurs massively in cultures that have terminated exponential growth as a result of such depletion. Sporulation involves the production of many new structures, enzymes, and metabolites along with the disappearance of many vegetative cell components. These changes represent a true process of differentiation: A series of genes whose products determine the formation and final composition of the spore is activated, while another series of genes involved in vegetative cell function is inactivated. These changes involve an alteration in the specificity of RNA polymerase. The sequence of events in sporulation is highly complex asporogenous mutants reveal at least 12 morphologically or biochemically distinguishable stages, and at least 30 operons (including an estimated 200 structural genes) are involved During the process, some bacteria release peptide antibiotics, which may play a role in regulating sporogenesis.

Morphologically, sporulation begins with the isolation of a terminal nucleus by the inward growth of the cell membrane. The growth process involves an infolding of the membrane so as to produce a double membrane structure whose facing surfaces correspond to the cell wall-synthesizing surface of the cell envelope. The growing points move progressively toward the pole of the cell so as to engulf the developing spore.

The 2 spore membranes now engage in the active synthesis of special layers that will form the cell envelope: the spore wall and cortex, lying between the facing

membranes; and the coat and exosporium, lying outside of the facing membranes. In the newly isolated cytoplasm, or core, many vegetative cell enzymes are degraded and are replaced by a set of unique spore constituents.

In bacilli and clostridia, spores are located (1) *centrally*, in the centre of the cell (causative agent of anthrax); (2) *terminally*, at the ends of the rod (causative agent of tetanus); (3) *subterminally*, towards the ends (causative agents of botulism, anaerobic infections, etc.) (Fig. 29).

In some species of sporulating microorganisms, the spore diameter is greater than the width of the bacterial cell. If the spore is located subterminally, the microbes take on the form of a spindle (closter).

In tetanus clostridia the spore diameter is also greater than the width of the vegetative cell, but the spore is located terminally, and hence the drum-stick appearance.

This property of sporulation is important in characterizing and identifying spore-forming microbes, and also when selecting methods of decontaminating objects, housings, foodstuff's, and other substances. The microbe may lose its ability to sporulate by frequent cultivation on fresh media or by subjecting it to high temperatures.

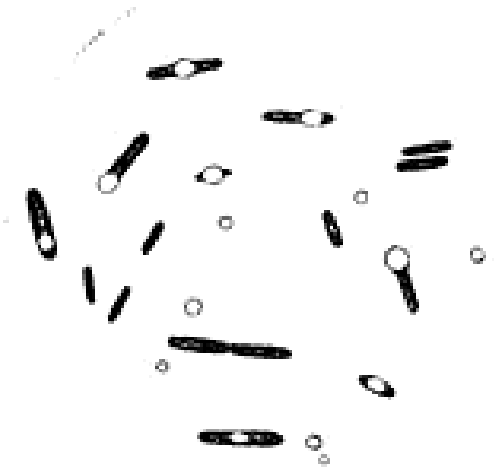

Figure 29. Shapes and arrangement of spores in bacilli and clostridia

Conclusion: Properties of Endospores:

1. Core - the core is the spore protoplast. It contains a complete nucleus (chromosome), all of the components of the protein-synthesizing apparatus, and an energy-generating system based on glycolysis. Cytochromes are lacking even in aerobic species, the spores of which rely on a shortened electron transport pathway involving flavoproteins. A number of vegetative cell enzymes are increased in amount (eg, alanine racemase), and a number of unique enzymes are formed (eg, dipicolinic acid synthetase). The energy for germination is stored as 3-phosphoglycerate rather than as ATP.

The heat resistance of spores is due in pan to their dehydrated state and in part to the presence of large amounts (5-15% of the spore dry weight) of calcium

dipicolinate, which is formed from an intermediate of the lysine biosynthetic pathway. In some way not yet understood, these properties result in the stabilization of the spore enzymes, most of which exhibit normal heat lability when isolated in soluble form.

2. Spore wall - the innermost layer surrounding the inner spore membrane is called the spore wall. It contains normal peptidoglycan and becomes the cell wall of the germinating vegetative cell.

3. Cortex - the cortex is the thickest layer of the spore envelope. It contains an unusual type of peptidoglycan, with many fewer cross-links than are found in cell wall peptidoglycan. Cortex peptidoglycan is extremely sensitive to lysozyme, and its autolysis plays a key role in spore germination.

4. Coat - the coat is composed of a keratinlike protein containing many intramolecular disulfide bonds. The impermeability of this layer confers on spores their relative resistance to antibacterial chemical agents.

5. Exosporium - the exosporium is a lipoprotein membrane containing some carbohydrate.

Germination: the germination process occurs in 3 stages: activation, initiation, and outgrowth.

1. Activation - even when placed in an environment that favors germination (eg, a nutritionally rich medium), bacterial spores will not germinate unless first activated by one or another agent that damages the spore coat. Among the agents that can overcome spore dormancy are heat, abrasion, acidity, and compounds containing free sulfhydryl groups.

2. Initiation - once activated, a spore will initiate germination if the environmental conditions are favorable. Different species have evolved receptors that recognize different effectors as signalling a rich medium: thus, initiation is triggered by L-alanine in one species and by adenosine in another. Binding of the effector activates an autolysin that rapidly degrades the cortex peptidoglycan. Water is taken up, calcium dipicolinate is released, and a variety of spore constituents are degraded by hydrolytic enzymes.

3. Outgrowth - degradation of the cortex and outer layers results in the emergence of a new vegetative cell consisting of the spore protoplast with its surrounding wall. A period of active biosynthesis follows; this period, which terminates in cell division, is called outgrowth. Outgrowth requires a supply of all nutrients essential for cell growth.

Flagella. Motile bacteria are subdivided into creeping and swimming bacteria. Creeping bacteria move slowly (creep) on a supporting surface as a result of wave-like contractions of their bodies, which cause periodic alterations in the shape of the cell. These bacteria include Myxobacterium, Beggiatoa, Thiothrix. Swimming bacteria move freely in a liquid medium. They possess flagella, thin hair-like cytoplasmic appendages measuring 0.02 to 0.05 mcm in thickness and from 6 to 9 mcm in length. In some spirilla they reach a length of 80 to 90 mcm (Fig. 30). Investigations have confirmed that the flagella are made up of proteins the

composition of which differs considerably from that of the bacterial cell proteins (keratin, myosin, fibrinogen).

Figure 30. The flagella of Proteus vulgaris demonstrated by electron microscopy

With the aid of paper chromatography, it has been discovered that the flagellate material contains several ammo acids: lysine, aspartic and glutamic acids, alanine, etc. It has been suggested that the flagella are attached to basal granules which are found in the outlying zones of the cytoplasm The flagella can be observed by dark-field illumination, by special methods involving treatment with mordants, adsorption of various substances and dyes on their surfaces, and by electron microscopy The latter has made it possible to detect the spiral and screw-shaped structure of the flagella. The axial filament of the flagellum consists of two entwined hair-like processes enclosed in a sheath.

According to a pattern in the attachment of flagella motile microbes can be divided into 4 groups: (1) monotrichates, bacteria having a single flagellum at one pole of the cell (cholera vibrio, blue pus bacillus), (2) amphitrichates, bacteria with two polar flagella or with a tuft of flagella at both poles (Spirillum volutans), (3) lophotrichales, bacteria with a tuft of flagella at one pole (blue-green milk bacillus, Alcaligenes faecalis), (4) peritrichales, bacteria having flagella distributed over the whole surface of their bodies (colibacillum, salmonellae of enteric fever and paratyphoids A and B) (Fig. 31).

The above mentioned classification is provisional While studying the flagella under an electron microscope, it was revealed that the flagellum in some monotrichates is not located at the end of the cell, but at the point of transition of the lateral surface to the pole. It has been established that bacteria which once were considered to be monotrichous possess a number of flagella As to amphitrichates, their independent existence is a subject of controversy It has been suggested that the amphitrichate cell is actually comprised of two cells which have been separated incompletely, having flagella at their distal ends (Fig. 32).

Figure 31. Bacterial flagella:
1 – monotrichates, 2 – amphitrichates, 3 – lophotrichates 4 – peritrichates

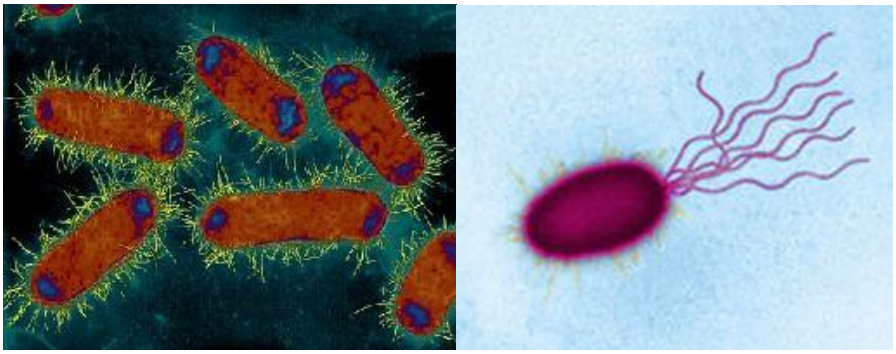

Figure 32. The flagella under an electron microscope

The flagella are main locomotor organoid of bacteria. As the result of their vigorous movements, resembling the twiddling of a corkscrew, the fluid moves along them and the micro-organism moves at a rate of about 50 mcm/sec. The mechanism of the contraction is not quite clear. It has been suggested that the protein of the sheath surrounding the flagellum forms with flagella a bicomponent system which contracts like actomyosin. The contraction of the flagella is due to the existence of two configurations of flagellin molecules differing in ammo acid composition.

The type of motility in bacteria depends on the number of flagella, age and properties of the culture, temperature, amount of chemical substances and on other factors. Monotrichates move with the greatest speed (60 mcm per second). Peritrichates move at rates ranging from 25 to 30 mcm per second. Certain species of motile microbes move at a rate of up to 200 mcm per second.

Motile bacteria also possess the power of directed movements, or taxis. According to the factors under the effect of which motion occurs, chemotaxis, aerotaxis, and phototaxis are distinguished.

Motility in bacteria can be observed by the hanging drop in wet conditions. The determination of motility in microbes is employed in laboratory practice as a means to identify cholera vibrio, dysentery, enteric fever, paratyphoid and other bacteria. However, although the presence of flagella is a species characteristic, they are not always essential to life, since a flagellate forms of motile bacteria exist.

Various types of microbes have pili (cilia, filaments, fimbriae), structures which are much shorter and thinner than the flagella (Fig. 33). They cover the body of the cell and there may be 100 to 400 of them on one cell. Pili are 0.3-1.0 mcm long

and 0.01 mcm wide. It is supposed that cilia are not related to the organs of locomotion and that they serve to attach the microbial cells to the surface of some substrates. Nine different types of pili have been studied. They consist of protein. Just like in the case of flagella, it is not necessary that all bacterial cells have pili. Of most interest are the F-pili within which there is a canal through which the genetic material from the donor to the recipient is transferred during conjugation (see section on conjugation).

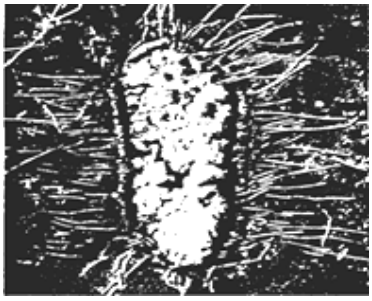

Figure 33. Cilia (pili) of Shigella flexneri demonstrated by electron microscopy

It is possible that the pili contribute to the nutrition of bacteria since they greatly increase the surface area of the bacterial cell. Besides actively moving by means of flagella or by cell contraction, microbes are capable of molecular, passive or brownian movement, due to the thermal molecular motion of the surrounding medium.

Two types of surface appendage can be recognized on certain bacterial species: the flagella, which are organs of locomotion, and pili (Latin hairs), which are also known as fimbriae (Latin fringes). Flagella occur on both Gram-positive and Gram-negative bacteria, and their presence can be useful in identification. For example, they are found on many species of bacilli but rarely on cocci. In contrast, pili occur almost exclusively on Gram-negative bacteria and are found on only a few Gram-positive organisms (e.g., Corynebacterium renale).

Some bacteria have both flagella and pili. The electron micrograph in Fig. 34 shows the characteristic wavy appearance of flagella and two types of pili on the surface of Escherichia coli.

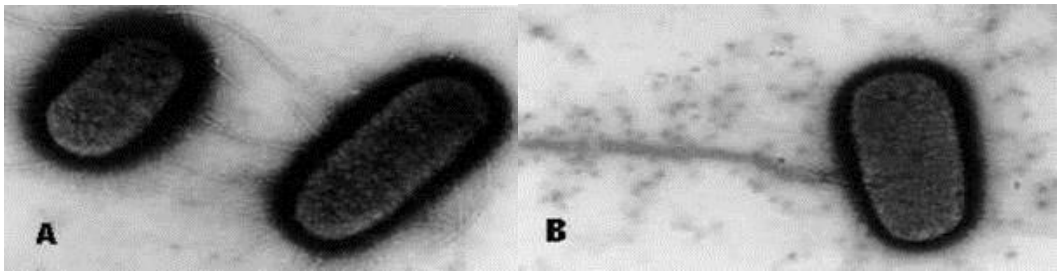

FIGURE 34. (A) Electron micrograph of negatively stained E coli showing wavy flagella and numerous short, thinner, and more rigid hairlike structures, the pili. (B) The long sex pilus can be distinguished from the shorter common pili by mixing E coli cells with a male bacterio phage that binds specifically to sex pili.

MORPHOLOGY AND FEATURES STRUCTURE SPIROCHAETES, RICKETTSIA,CHLAMYDIA, MYCOPLASMAS, FUNGI, PROTOZOA

Morphology and Ultrastructure of Spirochaetes.

Genetically Spirochaetes (L. spira curve, Gk. chaite cock, mane) differ from bacteria and fungi in structure with a corkscrew spiral shape. Their size varies considerably (from 0.3 to 1 5 mcm in width and from 7 to 500 mcm in length). The body of the spirochaete consists of an axial filament and cytoplasm wound spirally around the filament. No special membrane separates the nucleoid from the cytoplasm. Spirochaetes have a three-layer outer membrane. As demonstrated by electron microscopy, they possess a fine cytoplasmic membrane enclosing the cytoplasm The Spirochaetes do not possess the cell wall characteristic of bacteria, but electron microscopy has revealed that they have a thin cell wall (periplast) which encloses the cytoplasm. Spirochaetes do not produce spores, capsules, or flagella. Very delicate terminal filaments resembling flagella have been revealed in some species under the electron microscope.

In spite of the absence of flagella, Spirochaetes are actively motile due to the distinct flexibility of their bodies. Spirochaetes have a rotating motion which is performed axially, a translational motion forwards and backwards, an undulating motion along the whole body of the microorganism, and a bending motion when the body bends at a certain angle. Some species stain blue, others blue-violet, and still others — pink with the Romanowsky-Giemsa stain. A good method of staining Spirochaetes is by impregnation with silver. Staining properties (reaction to stains) are used to differentiate between saprophytic and pathogenic representatives of Spirochaetes.

Classification of Spirochaetes. The order Spirochaetales, family Spirochaetaceae includes the saprophytes (Spirochaeta, Cristispira) representing large cells, 200-500 mcm long, some of which have crypts (undulating crests); the ends are sharp or blunt. They live on dead substrates, in foul waters, and in the guts of cold-blooded animals. They stain blue with the Romanowsky-Giemsa stain. Two pathogenic genera belong to the family Spirochaetaceae (Borrelia, Treponema), and one belong to family Leptospiraceae (Leptospira) [Fig. 35]. The organisms of genus Borrelia differ from Spirochaetes in that their cells have large, obtuse-angled, irregular spirals, the number of which varies from 3 to 10. Pathogenic for man are the causative agents of relapsing fever transmitted by lice (Borrelia hispanica}, and by ticks (Borrelia persica, etc.). These stain blue-violet with the Romanowsky-Giemsa stain.

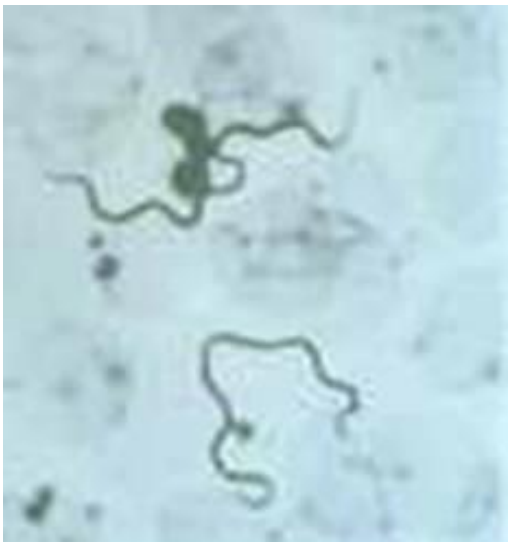

Figure 35. Borrelia

The genus Treponema (Gk. trepein turn, nema thread) exhibits thin, flexible cells with 6-14 twists (Fig. 36). The micro-organisms do not appear to have a visible axial filament or an axial crest when viewed under the microscope. The ends of treponemas are either tapered or rounded, some species have thin elongated threads on the poles. Electron microscopy of ultrathin treponema sections revealed a thin, elastic, and poorly resistant membrane composed of lipids, poliosides, and proteins. The cytoplasmic membrane lends the treponemas a spiral shape. Besides the typical form, there may be treponemas seen as granules, cysts, L-forms, and other structures. The organisms stain pale-pink with the Romanowsky-Giemsa stain. A typical representative is the causative agent of syphilis Treponema pallidum.

Figure 36. Treponema

Organisms of the genus Leptospira (Gk. leptos thin, speira coil) are characterized by very thin cell structure (Fig. 37). The leptospirae form 12 to 18 coils wound close to each other, shaping small primary spirals. The organisms have

two paired axial filaments attached at opposite ends (basal bodies) of the cell and directed toward each other. The middle part of the leptospirae have no axial filament. Due to the presence of the two pairs of axial filaments the leptospirae are capable of quite complexand active movement. During movement the ends of the organisms rotate rapidly at a right angle to the main part of their body. At rest the ends are hooked while during rapid rotary motion they resemble but- tonholes. Secondary spirals give the leptospirae the appearance of brackets or the letter S. The cytoplasm is weakly refractive. They stain pinkish with the Romanowsky-Giemsa stain. Some serotypes which are pathogenic for animals and man cause leptospirosis (Fig. 38).

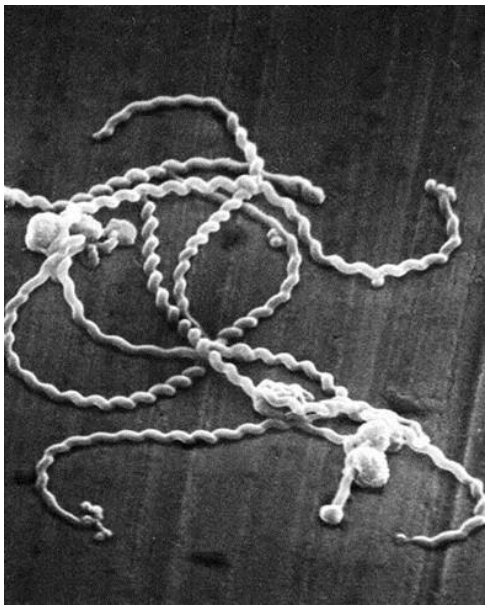

Figure 37. Leptospira

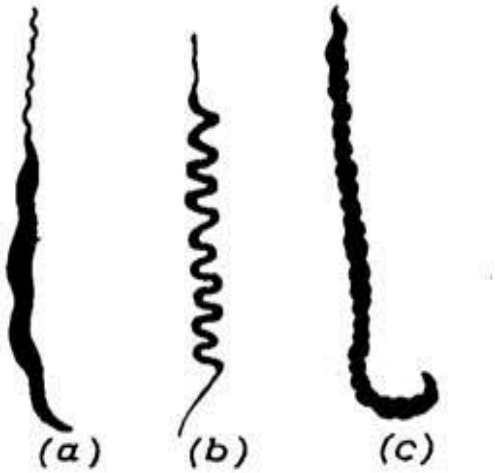

Figure 38. Morphology and structure of Spirochaetales (a – Borrelia, b –Treponema, c – Leptospira)

Morphology and Ultrastructure of Actinomycetes

Actinomycetes (Gk. mykes fungus, aclis ray) are unicellular microorganisms which belong to the class Bacteria, the order Actinomycetales. The body of actinomycetes consists of a mycelium which resembles a mass of branched, thin (0.2-1.2 mcm in thickness), non-septate filaments — hyphae (Fig. 39).

In some species the mycelium breaks up into poorly branching forms. In young cultures the cytoplasm in the cells of actinomycetes is homogeneous, it refracts light to a certain extent, and contains separate chromatin grains. When the culture ages, vacuoles appear in the mycelial cells, and granules, droplets of fat and rod-shaped bodies also occur. The cell wall becomes fragile, breaks easily, and a partial lysis of the cells occurs. In actinomycetes, as in bacteria, differentiated cell nuclei have not been found, but the mycelial filaments contain chromatin.

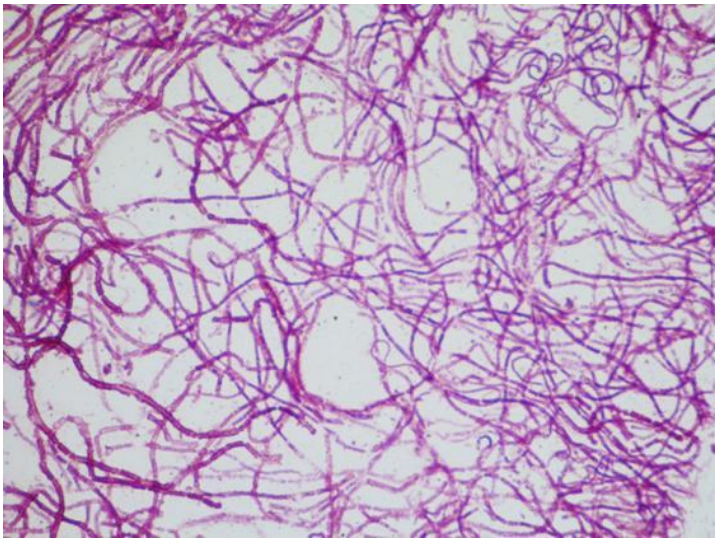

Figure 39. Actinomyces

Classification and morphology of microorganisms granules. The actinomycetes multiply by means of germinating spores attached to sporophores (Fig. 40). and by means of fragmenation where they break up into hyphae.

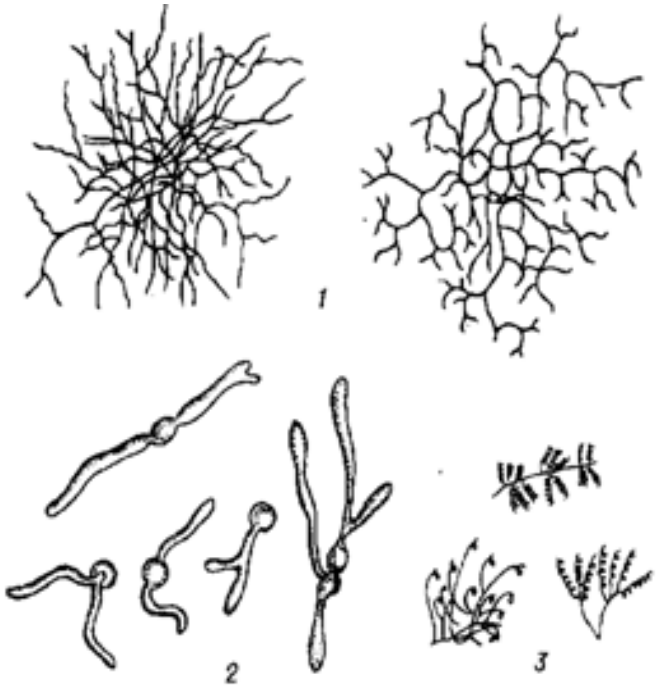

Figure 40. Morphology and structure of actinomycetes

The order Actinomycetales consists of 4 families: Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae, Actinoplanaceae. The family Mycobactenaceae includes the causative agents of tuberculosis, leprosy, and the family Actinomycetaceae, the causative agents of actinomycosis and acid-fast species nonpathogenic for man.

Among the actinomycetes of the family Streptomycetaceae (Fig. 41) are representatives which are capable of synthesizing antibiotic substances. These include producers of streptomycin, chloramphenicol, chlortetracycline oxytetracycline, neomycin, nystatin, etc. No species pathogenic for animals and man are present in the family Actinoplanaceae.

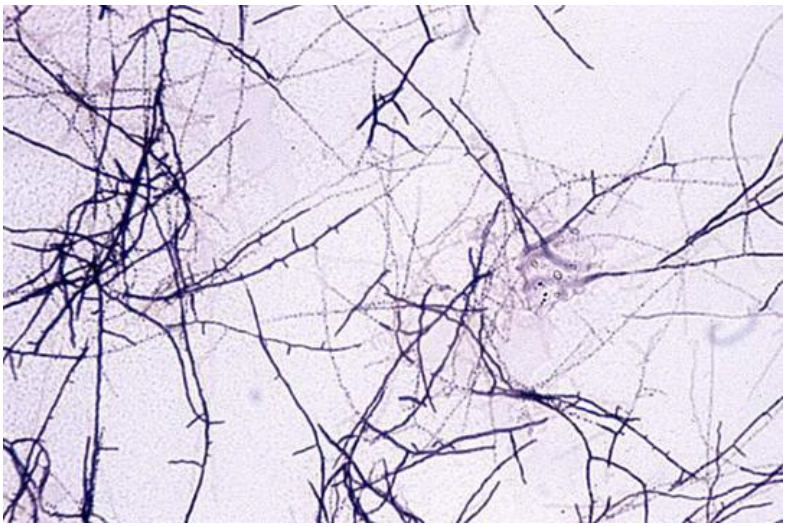

Figure 41. Streptomyces

Morphology and Ultrastructure of Rickettsiae.

Rickettsiae are included in the order Rickettsiales of obligate intra- cellular bacteria containing DNA and RNA, and are pleomorphic organisms (Fig. 42). They live and multiply only within the cells (in the cytoplasm and nucleus) of the tissues of humans, animals, and vectors. Coccoid forms resemble very fine, homogeneous, or single-grain quite often they occur as the diploforms (Fig. 43).

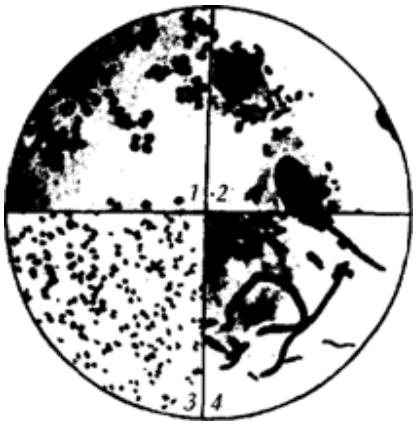

Figure 42. Morphology and Ultrastructure of Rickettsiae.

The Rickettsiae are small (0.3-0.5 x 0.8-2.0 um), Gram-negative, aerobic, coccobacilli that are obligate intracellular parasites of eucaryotic cells. They may reside in the cytoplasm or within the nucleus of the cell that they invade. They divide by binary fission and they metabolize host-derived glutamate via aerobic respiration and the citric acid (TCA) cycle. They have typical Gram-negative cell walls, and they lack flagella. The rickettsiae frequently have a close relationship with arthropod vectors that may transmit the organism to mammalian hosts. The rickettsiae have very small genomes of about 1.0-1.5 millionbases

http://textbookofbacteriology.net/Rickettsia.html

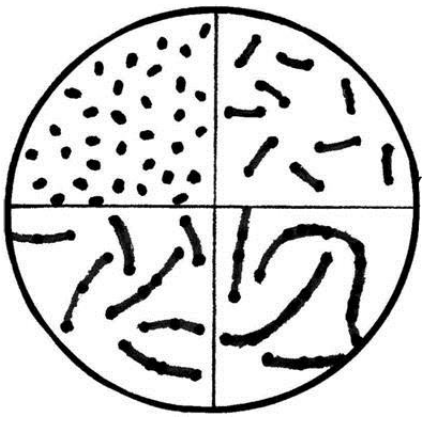

Figure 43. Pleomorphism in rickettsiae: 1 – cocci forms; 2, 3 – small rod-shaped forms; 4 – filamentous forms ovoids about 0.5 mm in diameter

Rickettsia prowazekii, the cause of epidemic typhus, is the prototypical rickettsia (Fig. 44). Typhus has plagued humanity throughout history. The American bacteriologist, Hans Zinsser, to whom this textbook is dedicated, was able to grow the elusive intracellular pathogen and develop a protective vaccine for typhus fever. He wrote a book about the bacterium, published in 1935, *Rats, Lice, and History*: "being a study in biography, which, after 12 preliminary chapters indispensable for the preparation of the lay reader, deals with the life history of typhus fever".

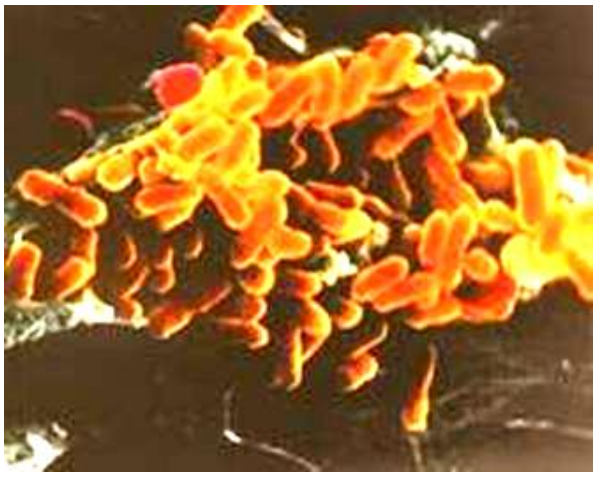

Figure 44.Rickettsia prowazeki

Rickettsia prowazekii has made science news recently since it has been shown to be the probable origin of eucaryotic mitochondria. Its complete genome sequence of 1,111,523 base pairs has been shown to contain 834 protein-coding genes. The functional profiles of these genes show similarities to those of mitochondrial genes. No genes required for glycolysis are found in either *R. prowazekii* or mitochondrial genomes, but a complete set of genes encoding components of the tricarboxylic acid cycle and the respiratory-chain complex is found in both. In effect, ATP production in the rickettsia is the same as that in mitochondria. Many genes involved in the biosynthesis and regulation of biosynthesis of amino acids and nucleosides in free-living bacteria are absent from *R. prowazekii* and mitochondria. Such genes seem to have been replaced by homologues in the nuclear (host) genome. Phylogenetic analyses indicate that *R. prowazekii* is more closely related to mitochondria than it is to any bacterium on the Tree of Life.

Rickettsiae must be grown in the laboratory by co-cultivation with eucaryotic cells, and they have not been grown by in axenic culture. The basis of their obligate relationship with eucaryotic cells has been explained by rickettsial possession of "leaky membranes" that require the osmolarity and nutritional environment supplied by an intracellular habitat.

The rickettsiae, in spite of their small size and obligate intracellular habitat, are a group of alphaproteobacteria, which include many well-known organisms such as *Acetobacter, Rhodobacter, Rhizobium* and *Agrobacterium.* Very few of the

alphaproteobacteria are pathogens of humans. *Brucella, Bartonella, Rickettsia,* and a related intracellular parasite, *Ehrlichia,* are the main exceptions.

The genus *Rickettsia* is included in thebacterial family *Rickettsiaceae* of the order *Rickettsiales*. This genus includes many species associated with human disease, including those in the spotted fever group and the typhus group (figure 45). The rickettsiae that are pathogens of humans are subdivided into three major groups based on clinical characteristics of disease: 1. spotted fever group; 2. typhus group; and 3. scrub typhus group.

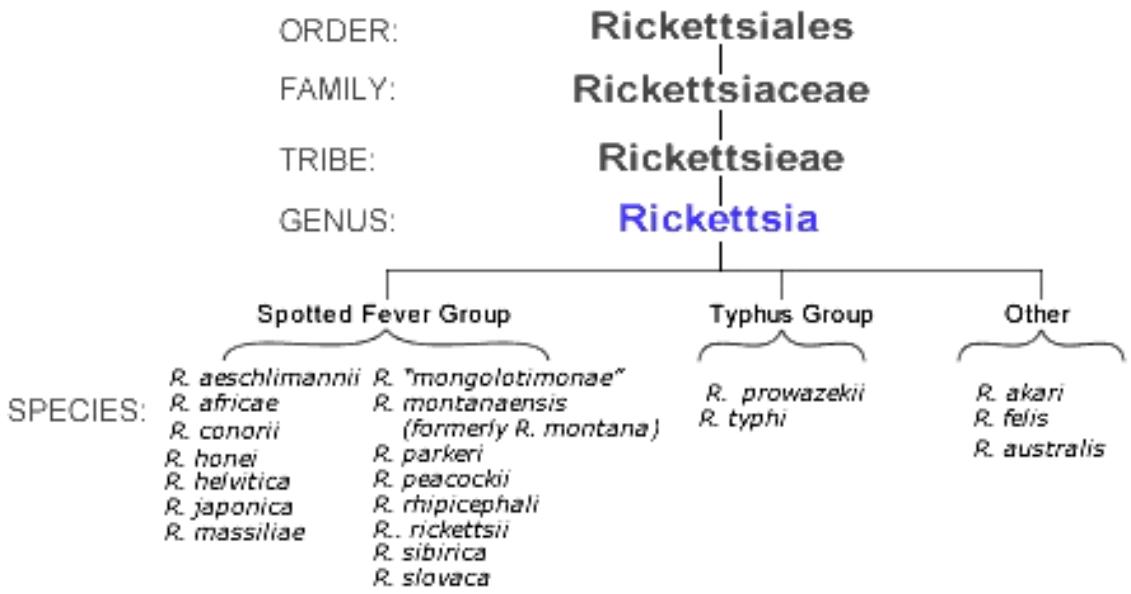

Figure 45. Taxonomic classification of the order *Rickettsiales*

Spotted Fever Group (SFG)

Rickettsia rickettsii is the cause of Rocky Mountain spotted fever (RMSF) and is the prototype bacterium in the spotted fever group of rickettsiae. *Rickettsia rickettsii* is found in the Americas and is transmitted to humans through the bite of infected ticks. The bacterium infects human vascular endothelial cells, producing an inflammatory response. The pathogenesis of RMSF is discussed in some detail below.

Other spotted fever group rickettsiae that produce human rickettsioses include *R. conorii, R. mongolotimonae* and *R. slovaca* (boutonneuse fever and similar illnesses), *R. japonica* (Japanese spotted fever), *R. sibirica* (North Asian tick typhus), *R. africae* (African tick bite fever), *R. helvetica* (perimyocarditis), and *R. honei* (Flinders Island spotted fever). The spotted fever rickettsiae have been found on every continent except Antarctica.

Two "transitional group" (other) rickettsias cause spotted fever-like diseases: *R. akari* (rickettsial pox), and *R. australis* (Queensland tick typhus).

Typhus Group (TG)

Rickettsia prowazekii is the cause of epidemic or louse-borne typhus and is the prototypical bacterium from the typhus group of rickettsiae. *R. prowazekii* infects human vascular endothelial cells, producing widespread vasculitis. In contrast to RMSF, louse-borne typhus tends to occur in the winter. Infection usually is transmitted from person to person by the body louse and, therefore, tends to manifest under conditions of crowding and poor hygiene. The southern flying squirrel is apparently the reservoir in the United States, but the vector involved in transmission from the flying squirrel to humans is unknown. The disease has a worldwide distribution.

Other rickettsiae in the typhus group include *R. typhi* and *R. felis*. Murine typhus is caused by transmission of *R. typhi* from rats, cats and opossums to humans via a flea vector. Murine typhus is found worldwide and is endemic to areas of Texas and southern California in the United States. Although *R. felis* is phylogenetically more closely related to the spotted fever group of rickettsiae than the typhus group, it shares antigens with *R. typhi* and produces a murine typhus-like illness. *Rickettsia felis* has been detected in cat fleas and opossums.

Scrub Typhus Group (STG)

Orientia (Rickettsia) tsutsugamushi is the cause of scrub typhus. Originally called *Rickettsia tsutsugamushi,* this organism was given its own genus designation because it is phylogenetically distinct from the other rickettsiae, though closely related.*Orientia tsutsugamushi* is transmitted to humans by the bite of trombiculid mites (chiggers), which are the vector and host. Scrub typhus occurs throughout much of Asia and Australia.

Rod-shaped rickettsiae are short organisms from 1 to 1.5 mcm in diameter with granules on the ends, or long and usually curved thin rods from 3 to 4 mcm in length. Filamentous forms are from 10 to 40 mcm and more in length: sometimes they are curved and multigranular filaments.

Rickettsiae are non-motile, do not produce spores and capsules and stain well by the Romanowsky-Giemsa stain and the Ziehl-Neelsen stain.

Electron microscopy and cytochemical study have shown that the rickettsiae have an inner (0.06 mcm) and an outer membrane acting as a wall and consisting of three layers. Granules of the ribosome type measuring 2-7 mcm and vacuole-like structures 0.06-0.08 mcm in diameter have been found in the cytoplasm or rickettsiae. Rickettsiae multiply by division of the coccoid and rod-shaped formswhich give rise to homogeneous populations of the corresponding type, and also by the breaking down of the filamentous forms giving rise to coccoid and rod-shaped entities.

Pathogenic rickettsiae invade various species of animals and man. The diseases caused by rickettsiae are known as rickettsioses. A typical representative is Rickettsia prowazekii (the name was given in honour of the scientists, the American Howard Ricketts and the Czech Stanislaus Prowazek), the causative agent of typhus fever.

Rickettsiae pertain to obligate parasites. They live and multiply only in the cells (in the cytoplasm and nucleus) of animals, humans, and vectors.

Genus Chlamydia, family Chlamydiaceae, order Chlamydiales include the causative agents of trachoma, conjunctivitis (inclusion blennorrhoea), inguinal lymphogranulomatosis (Nicolas-Favre disease), and ornithosis. The organisms contain DNA, RNA, nucleoproteins, lipids, and carbohydrates

Chlamydia are obligate intracellular parasites. They are coccal in shape and measure 0.2-1 5 mcm in diameter; reproduction occurs only in the cytoplasm of the cells of the vertebrates. The organisms are characterized by low metabolic activity and are cultivated at 33-41 °C in the yolk sac of a chick embryo They are sensitive to antibiotics of the tetracycline series and are Gram-negative. Chlamydia organisms cause respiratory infections with generalization of the process in birds and affect the respiratory passages, placenta, joints, and the intestinal tract in mammals (Fig. 46).

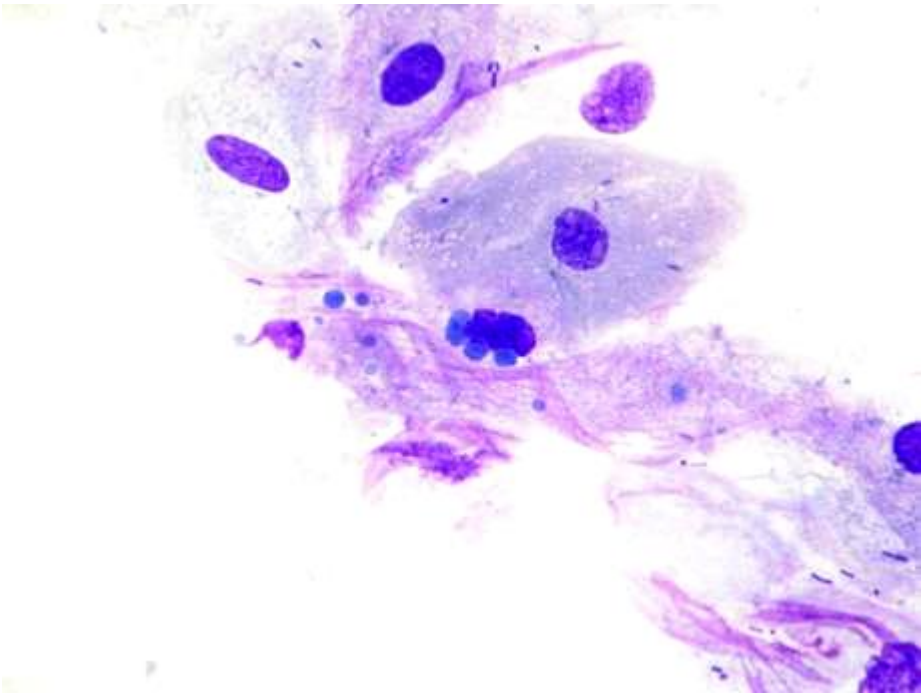

Figure 46. Chlamidia

Mycoplasmas
mashttp://www.ncbi.nlm.nih.gov/books/NBK7637/
w.rain-tree.com/mycoresearch.htm#.UUBkN3lkgUM

General Concepts
Clinical Manifestations

Mycoplasmas are most unusual self-replicating bacteria, possessing very small genomes, lacking cell wall components, requiring cholesterol for membrane function and growth, using UGA codon for tryptophan, passing through "bacterial-retaining" filters, and displaying genetic economy that requires a strict dependence on the host for nutrients and refuge. In addition, many of the mycoplasmas pathogenic for humans and animals possess extraordinary specialized tip organelles that mediate their intimate interaction with eucaryotic cells (Fig. 47). This host-adapted survival is achieved through surface parasitism of target cells, acquisition of essential biosynthetic precursors, and in some cases, subsequent entry and survival intracellularly. Misconceptions concerning the role of mycoplasmas in disease pathogenesis can be directly attributed to their biological subtleties and to fundamental deficits in understanding their virulence capabilities. (Baseman, 1997)

Mycoplasma pneumoniae infection is a disease of the upper and lower respiratory tracts. Cough, fever, and headache may persist for several weeks. Convalescence is slow. *Ureaplasma urealyticum* infection causes nongonococcal urethritis in men, resulting in dysuria, urgency, and urethral discharge (Fig. 48-49).

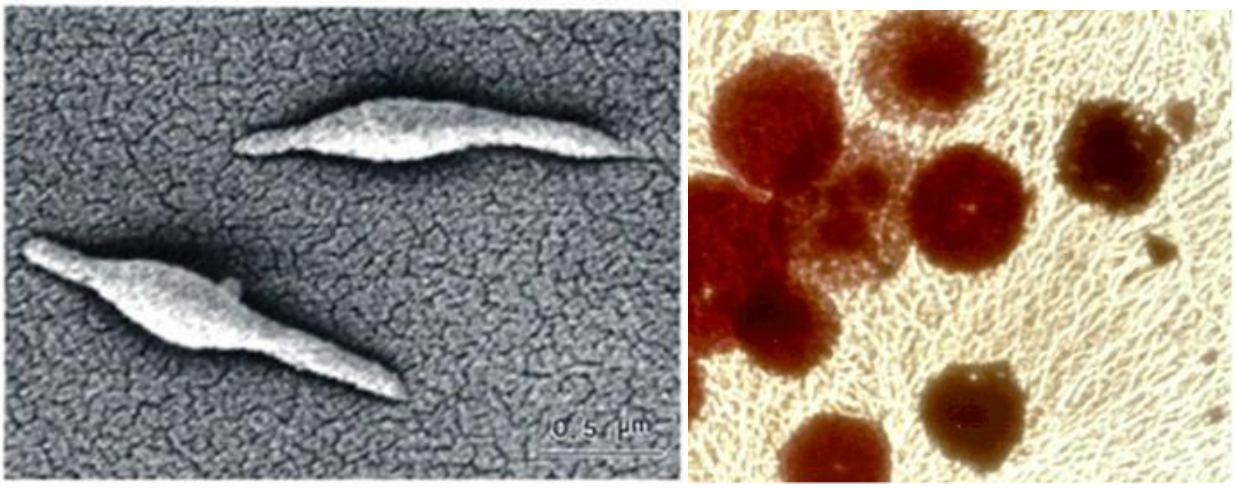

Figure 47. Mycoplasmas

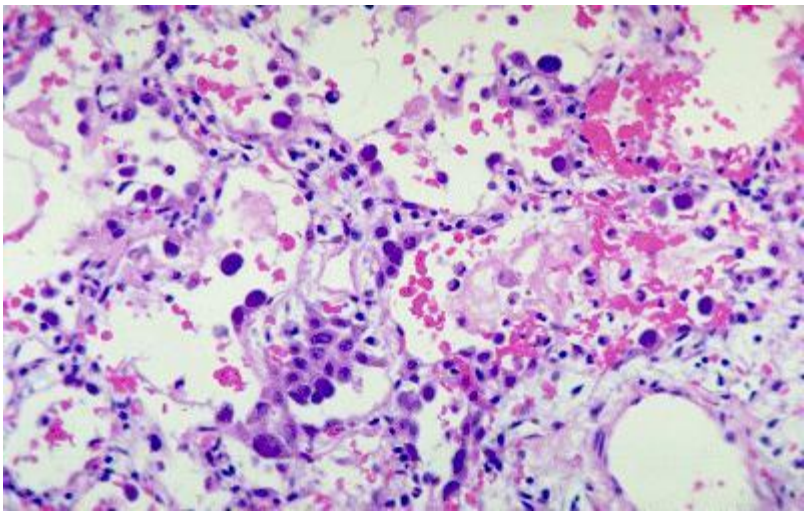

Figure 48. Mycoplasma pneumonia microscopy

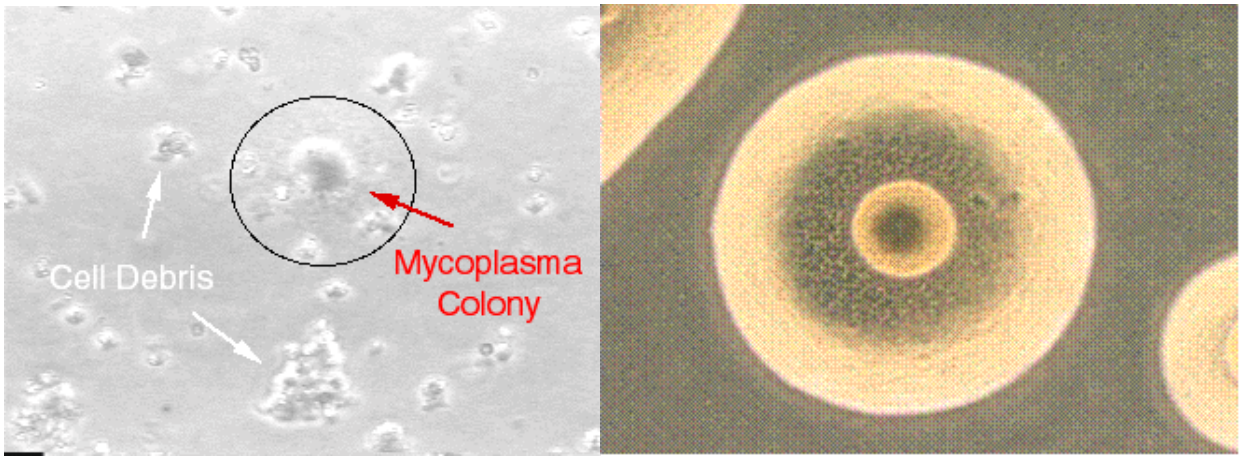

Figure 49. Mycoplasma culture

Structure, Classification, and Antigenic Types

Mycoplasmas are spherical to filamentous cells with no cell walls. There is an attachment organelle at the tip of filamentous *M. pneumoniae, M. genitalium,* and several other pathogenic mycoplasmas. Fried-egg-shaped colonies are seen on agar. The mycoplasmas presumably evolved by degenerative evolution from Gram-positive bacteria and are phylogenetically most closely related to some clostridia. Mycoplasmas are the smallest self-replicating organisms with the smallest genomes (a total of about 500 to 1000 genes); they are low in guanine and cytosine. Mycoplasmas are nutritionally very exacting. Many require cholesterol, a unique property among prokaryotes. Ureaplasmas require urea for growth, another unusual property. Mycoplasmas have surface antigens such as membrane proteins, lipoproteins, glycolipids, and lipoglycans. Some of the membrane proteins undergo spontaneous antigenic variation. Antibodies to surface antigens inhibit growth; various serological tests have been developed and are useful in classification.

The genomes of most Mycoplasma species encode about 600 proteins. For example, The M. genitalium and M. pneumoniae genomes contain 470 and 677

protein-coding gene sequences, respectively, compared with 1,703 protein genes in Haemophilus influenzae and about 4,000 genes in E. Coli. The genomes of M. genitalium and M. pneumoniae have lost the genes involved in certain biosynthetic pathways, such as the genes for amino and fatty acid and vitamin synthesis. Since they are cell wall-deficient bacteria, there is a major reduction in genetic information needed for cell wall biosynthesis. Although Mycoplasma species carry a minimal set of genes involved in energy metabolism and biosynthesis, they still have the essential genes for DNA replication, transcription, translation, and the minimal number of rRNA and tRNA genes. The reduction in mycoplasmal genomes explains their need for host nutritional molecules. A significant number of mycoplasmal genes appear to be devoted to cell adhesion and attachment organelles as well as variable membrane surface antigens to maintain parasitism and evade host immune and nonimmune surveillance systems. Mycoplasma species variably express structurally heterogeneous cell surface antigens. Variations in the genes encoding cell surface adherence molecules reveal distinct patterns of mutations capable of generating changes in mycoplasma cell surface molecular size and antigenic diversity. Variable surface antigenic structures and rapid changes in their expression are thought to play important roles in the pathogenesis of mycoplasmal infections by providing altered structures for escape from immune responses and protein structures that enhance cell and tissue colonization and penetration of the mucosal barrier." (Nicolson, GL 1999)

Pathogenesis

Mycoplasmas are surface parasites of the human respiratory and urogenital tracts. *Mycoplasma pneumoniae* attaches to sialoglycoproteins or sialoglycolipid receptors on the tracheal epithelium via protein adhesins on the attachment organelle. The major adhesin is a 170-kilodalton (kDa) protein, named P1. Hydrogen peroxide and superoxide radicals (O_2^-) excreted by the attached organisms cause oxidative tissue damage. Pneumonia is induced largely by local immunologic and phagocytic responses to the parasites. Sequelae of *M. pneumoniae* infection (mainly hematologic and neurologic) apparently have an autoimmune etiology. Several fastidious mycoplasmas may act as cofactors in activation of the aquired immunodeficiency syndrome (AIDS). Macrophage activation, cytokine induction, and superantigen properties of some mycoplasmal cell components can be considered as pathogenicity factors.

Host Defenses

Ig M antibodies, followed by Ig G and secretory Ig A, are important in host resistance. The importance of cell-mediated immunity is unclear.

Epidemiology

Mycoplasma pneumoniae infection occurs worldwide and is more prevalent in colder months. It affects mainly children ages 5 to 9 years. It is spread by close personal contact and has a long incubation period. *Ureaplasma urealyticum* is spread primarily through sexual contact. Women may be asymptomatic reservoirs.

Diagnosis

Culture of *M. pneumoniae* from sputum or a throat swab is possible, but very slow; therefore diagnosis is usually based on serologic tests. Tests using diagnostic

DNA probes and amplification of specific genomic mycoplasma sequences by the polymerase-chain reaction (PCR) are being developed.

Control

There is no certified vaccine for *M. pneumoniae*. Treatment with erythromycin or tetracyclines is effective in reducing symptoms in both *M. pneumoniae* and *U. urealyticum* infections.

Introduction

Mycoplasmas are the smallest and simplest self-replicating bacteria. The mycoplasma cell contains the minimum set of organelles essential for growth and replication: a plasma membrane, ribosomes, and a genome consisting of a double-stranded circular DNA molecule (Fig. 50-1). Unlike all other prokaryotes, the mycoplasmas have no cell walls, and they are consequently placed in a separate class *Mollicutes* (*mollis*, soft; *cutis*, skin). The trivial term mollicutes is frequently used as a general term to describe any member of the class, replacing in this respect the older term mycoplasmas.

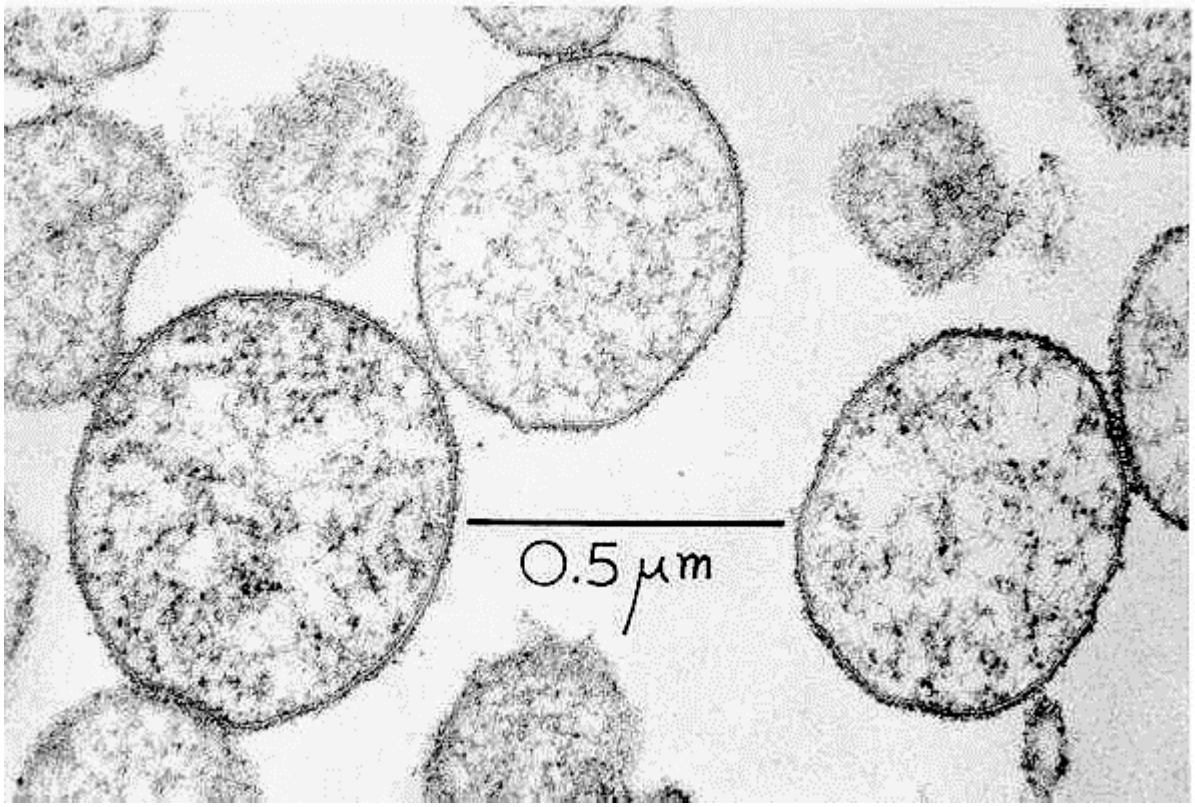

Figure 50-1. Electron micrograph of thin-sectioned mycoplasma cells

Cells are bounded by a single membrane showing in section the characteristic trilaminar shape. The cytoplasm contains thin threads representing sectioned chromosome and dark granules representing ribosomes. (Courtesy of RM Cole, Bethesda, Maryland).

Electron micrograph of thin-sectioned mycoplasma cells. Cells are bounded by a single membrane showing in section the characteristic trilaminar shape. The cytoplasm contains thin threads representing.

Mycoplasmas have been nicknamed the “crabgrass” of cell cultures because their infections are persistent, frequently difficult to detect and diagnose, and difficult to cure. Contamination of cell cultures by mycoplasmas presents serious problems in research laboratories and in biotechnological industries using cell cultures. The origin of contaminating mycoplasmas is in components of the culture medium, particularly serum, or in the flora of the technician's mouth, spread by droplet infection.

Clinical Presentation

Mycoplasmal pneumonia

The term primary atypical pneumonia was coined in the early 1940s to describe pneumonias different from the typical lobar pneumonia caused by pneumococci. Several common respiratory viruses, including influenza virus and adenovirus, were shown to be responsible for a significant number of these pneumonias. From other cases, many of which developed antibodies agglutinating red blood cells in the cold (cold agglutinins), an unidentified filterable agent was isolated by Eaton and associates and was called *Eaton agent*. This agent was identified as a new *Mycoplasma* species after its successful cultivation on cell-free media in 1962. Named *Mycoplasma pneumoniae*, it was the first clearly documented mycoplasma pathogenic for humans.

The effects of *M. pneumoniae* on humans include subclinical infection, upper respiratory disease, and bronchopneumonia. Most human infections do not progress to a clinically evident pneumonia. When pneumonia occurs, the onset generally is gradual and the clinical picture is one of a mild to moderately severe illness, with early complaints referable to the lower respiratory passages. Radiography frequently reveals evidence of pneumonia before physical signs are apparent. Involvement is usually limited to one of the lower lobes of the lungs, and the pneumonia is interstitial or bronchopneumonic. The course of disease varies; remittent fever, cough, and headache persist for several weeks. One of the most consistent clinical features is a long convalescence, which may extend from 4 to 6 weeks. Few fatal cases have been reported. Several unusual complications have been noted, including hemolytic anemia, polyradiculitis, encephalitis, aseptic meningitis, and central nervous system illness such as Guillain-Barré syndrome. In addition, pericarditis and pancreatitis have been observed. These sequelae may be related to the suspected immunopathology of *M. pneumoniae* disease (see below).

Nongonococcal Urethritis and Salpingitis

Growing evidence suggests that *Ureaplasma urealyticum* causes nongonococcal urethritis in men free of *Chlamydia trachomatis,*an established agent of nongonococcal urethritis. The wide occurrence of *U. urealyticum* in sexually active, symptom-free adults hampers research in this field. Evidence is based primarily on the production of nongonococcal urethritis symptoms in ureaplasma-free and chlamydia-free volunteers by intraurethral inoculation of *U. urealyticum* and on a report that this disease could be cured in a chlamydia-free man only when he and his

partner were treated simultanously with tetracycline, which eliminated *U. urealyticum* from both. Ureaplasmas have also been associated with chorioamnionitis, habitual spontaneous abortion, and low-weight infants. *Mycoplasma hominis*, a common inhabitant of the vagina of healthy women, becomes pathogenic once it invades the internal genital organs, where it may cause pelvic inflammatory diseases such as tubo-ovarian abscess or salpingitis.

It has been suggested that *Mycoplasma genitalium*, isolated in 1981 from the urethral discharge of two homosexual men, may account for the tetracycline-responsive, nongonococcal urethritis cases in which chlamydias and ureaplasmas cannot be isolated (about 20 percent of all cases). However, *M. genitalium* is so fastidious that very few clinical isolates have so far been made on the best mycoplasma medium available. Only the recent application of specific PCR amplification of the organism's DNA in clinical specimens has provided experimental proof for the relative prevalence of *M genitalium* in the human urogenital tract and its apparent role in male urethritis.

Mycoplasmas in AIDS and Immunocompromised Patients

The question of whether mycoplasmas act as co-factors in the development of AIDS has attracted much attention recently. Several mycoplasms have so far been incriminated: *M. fermentans*, considered until recently a relatively rare mycoplasma of the human urogenital tract, and *M. penetrans*, a newly-discovered human mycoplasma isolated from several AIDS patients. *M. pirum*, a mycoplasma of an unknown host, has been recently isolated from the blood of a few AIDS patients. While, *in vitro*studies show that these mycoplasmas may markedly enhance pathogenicity of the human immunodeficiency virus, the possibility that the mycoplasmas may simply represent opportunistic agents found in high frequency in patients with AIDS, cannot be ruled out. Yet on the whole, with the increasing incidence of immunocompromised patients (due to AIDS, organ transplantation, etc.) evidence is accumulating for invasion of tissues and the intracellular location of some mycoplasmas, notably *M. fermentans* and *M. penetrans*. Extragenital infections by urogenital mycoplasmas are rather common in neonates, immunosuppressed and/or hypogammaglobulinemic patients; clinical symptoms are expressed frequently as arthritis.

Structure, Classification, and Antigenic Types

Distinguishing Properties

The coccus is the basic form of all mycoplasmas in culture. The diameter of the smallest coccus capable of reproduction is about 300 nm. In most mycoplasma cultures, elongated or filamentous forms (up to 100 μm long and about 0.4 μm thick) also occur. The filaments tend to produce truly branched mycelioid structures, hence the name mycoplasma (myces, a fungus; plasma, a form). Mycoplasmas reproduce by binary fission, but cytoplasmic division frequently may lag behind genome replication, resulting in formation of multinuclear filaments. (Fig. 50-2).

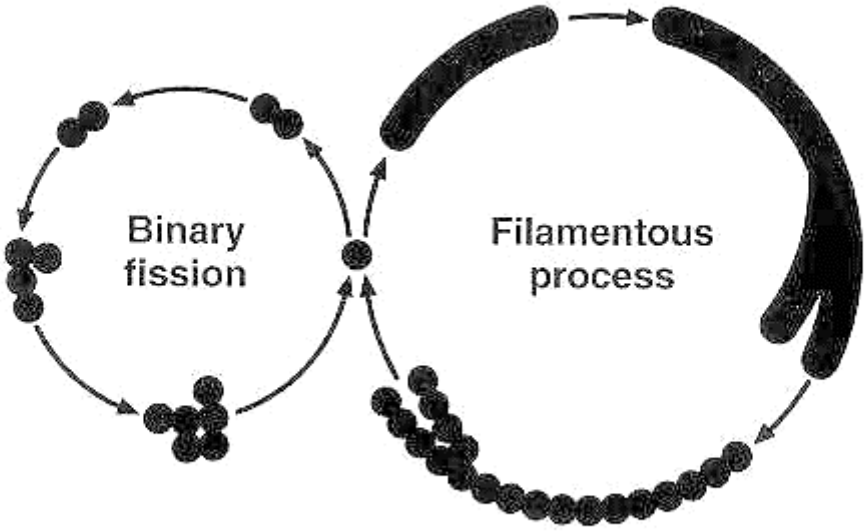

Figure 50-2. Schematic presentation of the mode of mycoplasma reproduction

Cells may either divide by binary fission or first elongate to multinucleate filaments, which subsequently breakup to coccoid bodies. (From Razin S: Mycoplasmas: the smallest pathogenic procaryotes. Isr J Med Sci 17:510, 1981, with permission).

Schematic presentation of the mode of mycoplasma reproduction. Cells may either divide by binary fission or first elongate to multinucleate filaments, which subsequently breakup to coccoid.

Some mycoplasmas possess unique attachment organelles, which are shaped as a tapered tip in *M. pneumoniae* and *M. genitalium. Mycoplasma pneumoniae* is a pathogen of the respiratory tract, adhering to the respiratory epithelium, primarily through the attachment organelle. Interestingly, these two human mycoplasmas exhibit gliding motility on liquid-covered surfaces. The tip structure always leads, again indicating its importance in attachment.One of the most useful distinguishing features of mycoplasmas is their peculiar fried-egg colony shape, consisting of a central zone of growth embedded in the agar and a peripheral one on the agar surface (Fig. 50-3).

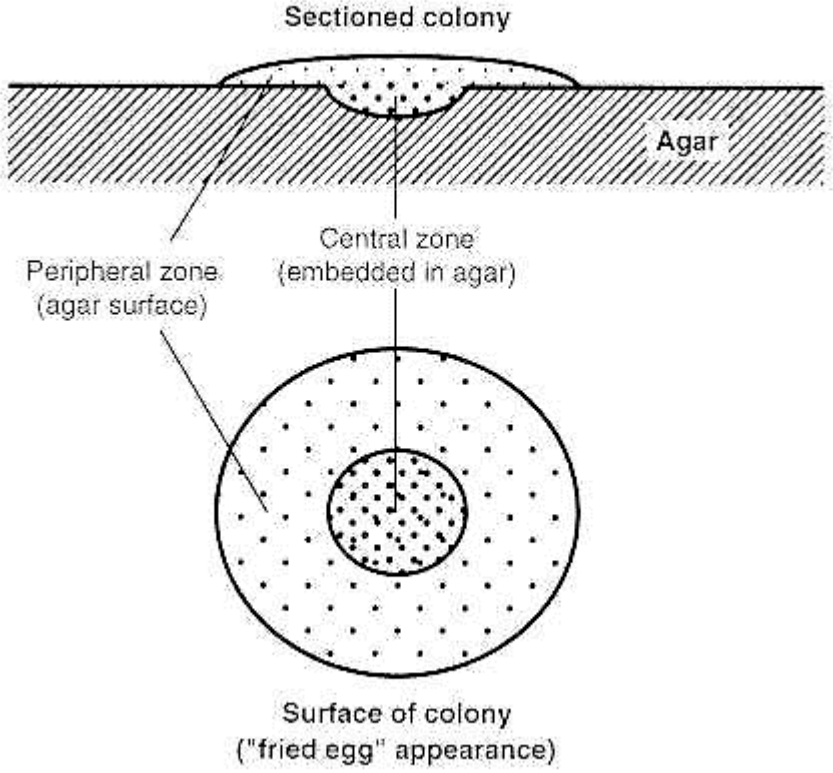

Figure 50-3. Morphology of a typical “fried-egg” mycoplasma colony

Morphology of a typical “fried-egg” mycoplasma colony.

The lack of cell walls and intracytoplasmic membranes facilitates isolation of the mycoplasma membrane in a relatively pure form. The isolated mycoplasma membrane resembles that of other prokaryotes in being composed of approximately two-thirds protein and one-third lipid. The mycoplasma lipids resemble those of other bacteria, apart from the large quantities of cholesterol in the sterol-requiring mycoplasmas.

Membrane proteins, glycolipids, and lipoglycans exposed on the cell surface are the major antigenic determinants in mycoplasmas. Antisera containing antibodies to these components inhibit growth and metabolism of the mycoplasmas and, in the presence of complement, cause lysis of the organisms. These properties are used in various serologic tests that differentiate between mycoplasma species and serotypes and detect antibodies to mycoplasmas in sera of patients (see below).

Molecular Biology

The mycoplasma genome is typically prokaryotic, consisting of a circular, double stranded DNA molecule. The *Mycoplasma* and *Ureaplasma* genomes are the smallest recorded for any self-reproducing). Therefore, there are very few genes; in some mycoplasmas the number is estimated at fewer than 500, about one sixth the number of genes in *Escherichia coli*. Mycoplasmas accordingly express a small number of cell proteins and lack many enzymatic activities and metabolic pathways.

Their nutritional requirements are correspondingly complex, and they are dependent on a parasitic mode of life.

Taxonomy and Properties of Mycoplasmas Capable of Infecting Humans.

The dependence of mycoplasmas on their host for many nutrients explains the great difficulty of cultivation in the laboratory. The complex media for mycoplasma culture contain serum, which provides fatty acids and cholesterol for mycoplasma membrane synthesis. The requirement of most mycoplasmas for cholesterol is unique among prokaryotes. The consensus is that only a small fraction of mycoplasmas existing in nature have been cultivated so far. Some of the cultivable mycoplasmas, including the human pathogen *M. pneumoniae*, grow very slowly, particularly on primary isolation. *Ureaplasma urealyticum,* a pathogen of the human urogenital tract, grows very poorly in vitro, reaching maximal titers of 10^7 organisms/ml of culture.*Mycoplasma genitalium,* another human pathogen, grows so poorly in vitro that only a few successful isolations have been achieved.

Glucose and other metabolizable carbohydrates can be used as energy sources by the fermentative mycoplasmas possessing the Embden-Meyerhof-Parnas glycolytic pathway. All mycoplasmas examined thus far possess a truncated, flavin-terminated respiratory system, which rules out oxidative phosphorylation as an ATP-generating mechanism. Breakdown of arginine by the arginine dihydrolase pathway has been proposed as a major source of ATP in nonfermentative mycoplasmas. Ureaplasmas have a requirement, unique among living organisms, for urea. Because they are non-glycolytic and lack the arginine dihydrolase pathway, it has been suggested, and later proven experimentally, that ATP is generated through an electrochemical gradient produced by ammonia liberated during the intracellular hydrolysis of urea by the organism's urease.

The mycoplasma genome is characterized by a low guanine-plus-cytosine content and by a corresponding preferential utilization of codons containing adenine and uracil, particularly in the third position. Most interesting is the use of the universal stop codon UGA as a tryptophan codon in many mycoplasmas, a rare property found so far only in mycoplasmas and in nonplant mitochondria. Resistance of mycoplasmal RNA polymerase to rifampicin is another property distinguishing mycoplasmas from the conventional eubacteria. However, apart from this resistance to rifampicin, the mycoplasmas are susceptible to antibiotics, such as tetracyclines and chloramphenicol, that inhibit protein synthesis on prokaryotic ribosomes.

Phylogeny

As the smallest and simplest self-replicating prokaryotes, the mycoplasmas pose an intriguing question: do they represent the descendents of exceedingly primitive bacteria that existed before the development of a peptidoglycan-based wall, or do they represent evolutionary degenerate eubacterial forms that have lost their cell walls? The balance of the molecular evidence, based largely on comparison of base sequences of the highly conserved ribosomal RNA (rRNA) molecules, particularly of the 16S rRNA type, favors the hypothesis of degenerative evolution. According to Woese and his colleagues, the mycoplasmas evolved as a branch of the low-guanine-plus-cytosine Gram-positive bacteria and are most closely related to two

clostridia, *Clostridium innocuum* and *C. ramosum.* However, the marked phenotypic and genotypic variability among mycoplasmas has led some workers to conclude that mycoplasmas evolved from a variety of walled bacteria and accordingly have a polyphyletic origin. Woese maintains that the origin of mycoplasmas is monophyletic and explains the great variety of mycoplasmas by a process of rapid evolution characteristic of the group.

Pathogenesis

All mycoplasmas cultivated and identified thus far are parasites of humans, animals, plants, or arthropods. The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts and the joints in some animals. Although some mycoplasmas belong to the normal flora, many species are pathogens, causing various diseases that tend to run a chronic course (Fig. 50-4).

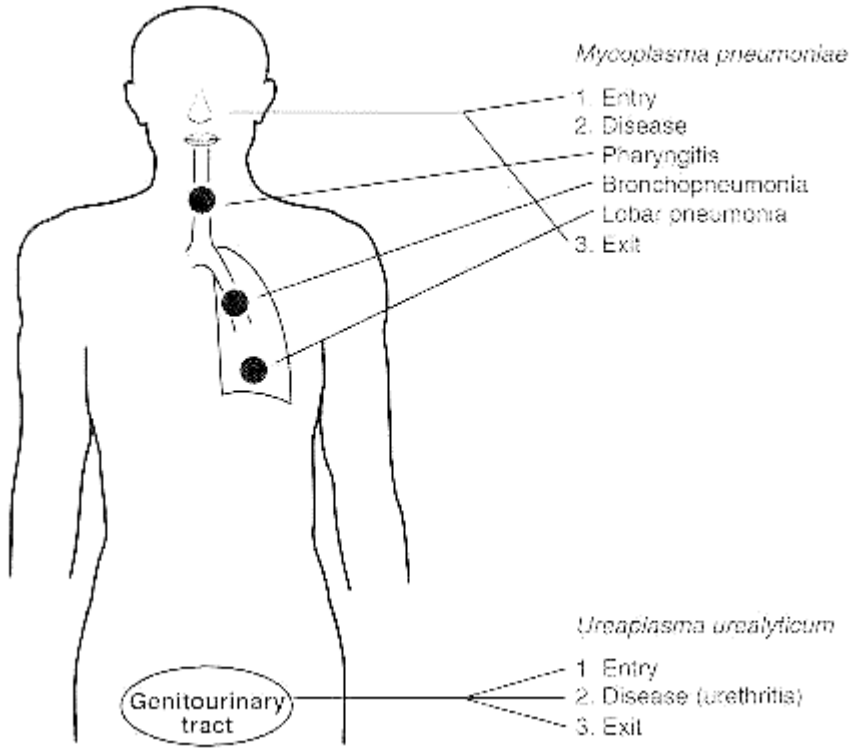

Figure 50-4. Pathogenesis and disease sites of infection by *M. pneumoniae* and *U. urealyticum*

Pathogenesis and disease sites of infection by *M. pneumoniae* and *U. urealyticum*.

Most mycoplasmas that infect humans and other animals are surface parasites, adhering to the epithelial linings of the respiratory and urogenital tracts. Adherence is firm enough to prevent the elimination of the parasites by mucous secretions or urine. The intimate association between the adhering mycoplasmas and their host cells

provides an environment in which local concentrations of toxic metabolites excreted by the parasite build up and cause tissue damage (Fig. 50-5).

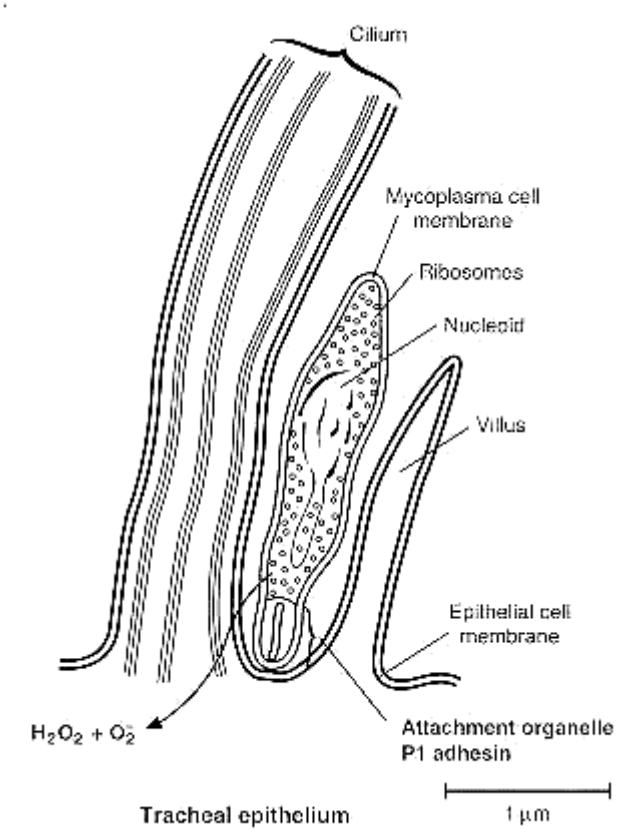

Figure 50-5. Schematic presentation of a *M pneumoniae* organism attaching to the surface of the ciliary tracheal epithelium, as seen by electron microscopy of a thin section

The clustering of the P1 adhesin on the surface of the attachment organelle at the tip of the mycoplasma is depicted. The H_2O_2 and O_2^- excreted by the mycoplasma penetrate into the host cell and cause oxidative damage.

Moreover, because mycoplasmas lack cell walls, fusion between the membranes of the parasite and host has been suggested, and some experimental evidence for it has recently been obtained. Membrane fusion would alter the composition and permeability of the host cell membrane and enable the introduction of the parasite's hydrolytic enzymes into the host cell, events expected to cause serious damage. Recent studies have indicated the presence in mycoplasmas of antigenic variability systems. These systems, some of which are already defined in molecular genetic terms, are responsible for rapid changes in major surface protein antigens. The change in the antigenic coat of the parasite helps it to escape recognition by the immune mechanisms of the host.

Because attachment of *M. pneumoniae* and *M. genitalium* is affected by pretreatment of the host cells with neuraminidase, sialoglycoproteins and/or sialoglycolipids of the host cell membrane appear to be receptor sites for these

mycoplasmas. There is evidence that several *M. pneumoniae* membrane proteins act as adhesins and that they have high affinity for the specific receptors for *M. pneumoniae* on host cells. Monoclonal antibodies to one of these proteins, protein P1 (molecular weight, 170,000 daltons), inhibit attachment of the parasite. Ferritin labeling of the antibodies has shown that P1 concentrates on the tip structure of the mycoplasma, a finding that further supports the notion that the tip serves as an attachment organelle.

The results obtained with *M. pneumoniae* were essentially duplicated recently with *M. genitalium* and showed that in this organism, which closely resembles *M. pneumoniae* morphologically and physiologically, a major adhesin protein, named MgPa, is clustered at the tip organelle. The genes of the major adhesins of *M pneumoniae* (P1) and of *M. genitalium* (MgPa) were cloned and sequenced, allowing the characterization of these proteins. The two adhesins are alike in many respects and in fact contain extensive areas of homology, as expressed also by shared epitopes. These two proteins may be the product of an ancestral gene that underwent a horizontal gene transfer event.

The nature of the toxic factors that damage the mucosal surfaces infected by mycoplasmas is still unclear. Toxins are rarely found in mycoplasmas. Consequently, researchers considered whether the end products of mycoplasma metabolism were responsible for tissue damage. Hydrogen peroxide (H_2O_2), the end product of respiration in mycoplasmas, has been implicated as a major pathogenic factor ever since it was shown to be responsible for the lysis of erythrocytes by mycoplasmas in vitro; however, the production of H_2O_2 alone does not determine pathogenicity, as the loss of virulence in *M. pneumoniae* is not accompanied by a decrease in H_2O_2 production. For the H_2O_2 to exert its toxic effect, the mycoplasmas must adhere closely enough to the host cell surface to maintain a toxic, steady-state concentration of H_2O_2 sufficient to cause direct damage, such as lipid peroxidation, to the cell membrane. The accumulation of malonyldialdehyde, an oxidation product of membrane lipids, in cells exposed to *M. pneumoniae* supports this notion. Moreover, *M. pneumoniae* inhibits host cell catalase by excreting superoxide radicals (O_2^-). This would be expected to further increase the accumulation of H_2O_2 at the site of parasite-host cell contact

Proposed mechanism of oxidative damage to host cells by adhering *M pneumoniae*. by increasing concentrations of H_2O_2 and O_2^-. (Modified from Almagor M.)

There is evidence that both organism-related and host-related factors are involved in the pathogenesis of mycoplasma infections. Mycoplasmas activate macrophages, and induce cytokine production and lymphocyte proliferation; the rat pathogen, *Mycoplasma arthritidis,* produces a potent superantigen. Thus, in the case of *M. pneumoniae*, the host may be largely responsible for the pneumonia by mounting a local immune response to the parasite. Syrian hamsters inoculated intranasally with *M. pneumoniae* show patchy bronchopneumonic lesions consisting of infiltration of mononuclear cells. The ablation of thymic function before the experimental infection prevents development of the characteristic pulmonary

infiltration, but lengthens the period during which the organisms may be isolated from the lungs. When thymic animals are allowed to recover and then reinfected, an exaggerated and accelerated pneumonic process occurs. Epidemiologic data also suggest that repeated infections in humans are required before symptomatic disease occurs: serum antibodies to *M. pneumoniae* can be found in most children 2 to 5 years of age, although the illness occurs with greatest frequency in individuals 5 to 15 years of age.

An immunopathologic mechanism also may explain the complications affecting organs distant from the respiratory tract in some patients infected with *M. pneumonia*. Various autoantibodies have been detected in the sera of many of these patients, including cold agglutinins reacting with the erythrocyte I antigen, and antibodies reacting with lymphocytes, smooth muscle cells, and brain and lung antigens. Serologic cross-reactions between *M. pneumoniae* and brain and lung antigens have been demonstrated, and these antigens are probably related to the glycolipids of *M. pneumoniae* membranes, which are also found in most plants and in many bacteria. Clearly, host reaction varies markedly, as only about half of the patients develop cold agglutinins and complications are rare, even among individuals with anti-tissue globulins.

Host Defenses

Infection with *M pneumoniae* induces the development of serum antibodies that fix complement, inhibit growth of the organism and lyse the organism in the presence of complement. Generally, the first antibodies produced are of the Ig M class, whereas later in convalescence the predominant antibody is Ig.G. Secretory IgA antibodies also develop and appear to be important in host resistance. The first infection in infancy usually is asymptomatic and generates a brief serum antibody response. Recurrent infections generate a more prolonged systemic antibody response and increasing numbers of circulating antigen-responsive lymphocytes. By late childhood, clinically apparent lower respiratory disease, including pneumonia, becomes more common. Therefore, mycoplasma respiratory disease manifestations appear to vary, depending on the state of local and systemic immunity at the time of reinfection. One hypothesis is that local immunity mediates resistance to infection and that systemic immunity contributes substantially to the pulmonary and systemic reaction characteristic of *M. pneumoniae* pneumonia.

The relative importance of humoral and cell-mediated immunity in resistance to respiratory mycoplasma infections is still unclear. For many mycoplasma infections, such as bovine pleuropneumonia, resistance can be transferred with convalescent-phase serum, but this may not be true for all mycoplasma respiratory diseases. For example, resistance of rats to pulmonary disease induced by *M pulmonis* can be transferred only with spleen cells obtained from previously infected animals. Although IgA antibody may be important in resistance to mycoplasmas, other factors seem to be involved in resistance to pulmonary disease, and these factors may not be the same for all mycoplasma infections.

Epidemiology

One of the most puzzling features of *M. pneumoniae* pneumonia is the age distribution of patients. In a survey conducted between 1964 and 1975 of more than 100,000 individuals in the Seattle area, the age-specific attack rate was highest among 5- to 9-year-old children. Rates of *M. pneumoniae* pneumonia in the youngest age group, 0 to 4 years old, were about one-half those in school-age children, but considerably higher than in adults. *Mycoplasma pneumoniae* pneumonia was rarely observed in infants younger than 6 months, suggesting maternally conferred immunity (*Mycoplasma pneumoniae* accounts for 8 to 15 percent of all pneumonias in young school-age children. In older children and in young adults, the organism is responsible for approximately 15 to 50 percent of all pneumonias. Infection with *M pneumoniae* occurs worldwide all year round but shows a predilection for the colder months, apparently because of the greater opportunity for transmission by droplet infection. *Mycoplasma pneumoniae* appears to require close personal contact to spread; successful spreading usually occurs in families, schools, and institutions. The incubation period ranges from 2 to 3 weeks.

Incidence of *M. pneumoniae* pneumonia in Seattle by age, for two epidemics (1966-67 and 1974) and the endemic periods (1967-73). (From Foy HM, Kenny GE, Cooney MK, Allen ID: Long-term epidemiology.

Ureaplasma urealyticum is spread primarily through sexual contact. Colonization has been linked to the frequency of sexual intercourse and the number of sexual partners. Women may be asymptomatic reservoirs of infection.

Diagnosis

Culture is essential for definitive diagnosis (See below).

Culture

A routine mycoplasma medium consists of heart infusion, peptone, yeast extract, salts, glucose or arginine, and horse serum (5 to 20 percent). Fetal or newborn calf serum is preferable to horse serum. To prevent the overgrowth of the fast-growing bacteria that usually accompany mycoplasmas in clinical materials, penicillin, thallium acetate or both are added as selective agents.

For *Ureaplasma* culture, the medium is supplemented with urea and its pH is brought to 6.0. *Ureaplasma* and *M. genitalium* are relatively sensitive to thallium, which is, therefore, omitted from their culture media. For *M. pneumoniae* isolation, nasopharyngeal secretions are inoculated into a selective diphasic medium (pH 7.8) made of mycoplasma broth and agar and supplemented with glucose and phenol red. When *M. pneumoniae* grows in this medium, it produces acid, causing the color of the medium to change from purple to yellow. Broth from the diphasic medium is subcultured to mycoplasma agar when a color change occurs, or at weekly intervals for a minimum of 8 weeks.

Identification

Colonies appearing on the plates can be identified as *M. pneumoniae* by staining directly on agar with homologous fluorescein-conjugated antibody or by demonstrating that a specific antiserum to *M. pneumoniae* inhibits their growth on agar. Colonies of ureaplasmas are usually minute (less than 100 μm in diameter);

because of urea hydrolysis and ammonia liberation, the medium becomes alkaline. When manganous sulfate is added to the medium, the ureaplasma colonies stain dark brown. Isolates can be characterized in more detail by a variety of biochemical and serologic tests. More sophisticated tests, including electrophoretic analysis of cell proteins, DNA-DNA hybridization tests, mycoplasmal DNA cleavage patterns by restriction endonucleases, and PCR tests employing species-specific primers for amplification, may be performed in a research laboratory.

Serodiagnosis and Molecular Probes

Serodiagnosis consists of examining serum samples for antibodies that inhibit the growth and metabolism of the organism or fix complement with mycoplasmal antigens. A fourfold or greater rise in Ig G titer is considered indicative of recent infection, whereas a sustained high antibody titer may not be significant, because a relatively high level of antibody may persist for at least 1 year after infection. A variety of rapid tests based on indirect hemagglutination of erythrocytes or latex particles coated with *M. pneumoniae* antigens have been developed, and some are commercially available.The cold agglutinin test is less useful because only about one-half of patients develop cold agglutinins and because these antibodies also are induced by a great many other conditions. Present techniques for laboratory diagnosis of *M. pneumoniae* infections are of little use to the clinician because recovery by culture and identification of the mycoplasmas take at least 1 to 2 weeks. Methods for rapid laboratory diagnosis, such as direct demonstration of organisms in the respiratory specimens by nucleic acid amplification techniques, have promise but diagnostic kits are not yet commercially available.

Control

Prevention

Chemoprophylaxis of mycoplasma infections is not recommended, and no vaccine is available. Prior natural infection appears to provide the most effective resistance; however, evidence shows that *M. pneumoniae* infections recur at intervals of several years. These observations suggest that immunity to a single natural infection is relatively short-term.

Treatment

The mycoplasmas are sensitive to tetracyclines, macrolides, and the newer quinolones, but are resistant to antibiotics that specifically inhibit bacterial cell wall synthesis. Tetracycline or erythromycin is recommended for treatment of *M. pneumonia* pneumonia, although effective treatment of the symptoms usually is not accompanied by eradication of the organism from the infected host. To prevent recurrence of nongonococcal urethritis caused by *U. urealyticum,* sexual partners should be treated simultaneously with tetracycline. The incidence of tetracycline-resistant strains of *U. urealyticum* and *M. hominis* is on the rise.

- Certain Mycoplasma species can either activate or suppress host immune systems, and they may use these activities to evade host immune responses. For example, some mycoplasmas can inhibit or stimulate the proliferation of normal lymphocyte subsets, induce B-cell differentiation and trigger the secretion of cytokines, including interleukin-1 (IL-1), IL-2, IL-4, IL-6, tumor necrosis factor-a (TNFa), interferons,

and granulocyte macrophage-colony stimulating factor (GM-CSF) from B-cells as well as other cell types. Moreover, it was also found that M. fermentans-derived lipids can interfere with the interferon (IFN)-g-dependent expression of MHC class II molecules on macrophages. This suppression results in impaired antigen presentation to helper T-cells in an experimental animal model. Also, mycoplasmas are able to secret soluble factors that can stimulate proliferation or inhibit the growth and differentiation of immune competent cells.

- Mycoplasmas can target the host white blood cells (lymphocytes/WBC) for intracellular infection, and these cells have the unique ability to cross the blood-brain barrier over into the spinal fluid and d into the host central nervous system (CNS).
- Once inside the host CNS, certain pathogenic mycoplasmas have been reported to activate the CNS hypothalamus/pituitary/adrenal axis and neuroendocrine system. The hypothalamus and pituitary glands form part of the human endocrine system which produces hormones that regulate nearly every bodily function. This involvement is hypothesized to contribute to diseases such as fibromyalgia, chronic fatigue, and some AIDS-related symptoms.[Yirmiya R, 1999]
- Mycoplasma species are known to secrete immune-modulating substances. For example, immune cells are affected by spiralin, a well-characterized mycoplasmal lipoprotein that can stimulate the in vitro proliferation of human peripheral blood mononuclear cells. This stimulation of immune cells results in secretion of proinflammatory cytokines (TNFa, IL-1 or -6). Spiralin can also induce the maturation of murine B-cells.
- Mycoplasmas can escape immune recognition by undergoing surface antigenic variations thus rapidly altering their cell surface structures. Such antigenic variability, the ability to suppress host immune responses, slow growth rates and intracellular locations may explain the chronic nature of mycoplasmal infections and the common inability of a host to suppress mycoplasmal infections with host immune and nonimmune responses.
- Rapid adaptation to host microenvironments by mycoplasmas is usually accompanied by rapid changes in cell surface adhesion receptors for more successful cell binding and entry as well as rapid structural protein changes to mimic host antigenic structures (antigen mimicry). For example, during chronic, active arthritis the size and antigenic diversity of the surface lipoprotein Vaa antigen changes in structure and expression in vivo. Antigenic divergence of Vaa can affect the adherence properties of M. hominis and enhance evasion of host-mediated immunity. Variations in the Vaa genes reveal a distinct pattern of mutations that generate mycoplasma surface variations and thus avoid host immune responses.
- Mycoplasmas can directly suppress host immune responses by initiating or enhancing apoptosis. For example, M. fermentans, a recently discovered mycoplasma found in the urine of HIV and AIDS positive patients, can initiate or enhance concanavalin A-induced apoptosis (programmed cell death) of T-cells. Relatively large amounts of nucleases are also expressed by Mycoplasma species, and these can be released intracellularly to cause degradation of host DNA. Mycoplasmal nucleases

may also be involved in secondary necrosis seen in advanced mycoplasmal infections, as indicated by the occurrence of morphological characteristics of apoptosis (chromatin condensation) and necrosis (loss of membrane integrity and organelle swelling). Although mycoplasmas can release activated oxygen species that may be involved in initiating apoptosis, some Mycoplasma species, such as M. fermentans, express a novel cytolytic activity in a nonlipid protein fraction that has a cytocidal effect not mediated by the known mycoplasmal cytokines like TNFa.

- In addition to apoptosis, mycoplasmas can also release growth inhibitory molecules into their surroundings, such as arginine deaminase. This enzyme can act as a growth-inhibitory substance that suppresses IL-2 production and receptor expression in T cells stimulated by non-specific mitogens, and it can induce the morphologic features of dying cells and DNA fragmentation indicative of apoptosis.
- Hydrogen peroxide and superoxide radicals are generated by adhering mycoplasmas, which induces oxidative stress, including host cell membrane damage.
- Competition for and depletion of nutrients or biosynthetic precursors by mycoplasmas, which disrupts host cell maintenance and function.
- Existence of capsule-like material and electron-dense surface layers or structures, which provides increased integrity to the mycoplasma surface and confers immunoregulatory activities
- High-frequency phase and antigenic variation, which results in surface diversity and possible avoidance of protective host immune defenses
- Secretion or introduction of mycoplasmal enzymes, such as phospholipases, ATPases, hemolysins, proteases, and nucleases into the host cell milieu, which leads to localized tissue disruption and disorganization and chromosomal aberrations and tumor formation.
- Intracellular residence, which sequesters mycoplasmas, establishes latent or chronics

The mycoplasmas belong to the class Mollicutes, order Mycoplasmatales. These bacteria measure 100-150 nm, sometimes 200-700 nm, are non-motile and. do not produce spores. Three stages are observed in the developmental cycle of organisms: (1) small (0.2-0 4 mcm) elementary bodies containing in a compact state the nucleoid genetic material and ribosomes enclosed within a three-layer wall; (2) primary, large (0.8-1.5 mcm), bodies with nucleoid fibrils and ribosomal elements; they are covered with a thin wall and reproduce by fission; the daughter cells reorganize into elementary bodies which may be extracellular and penetrate other cells; (3) intermediate (transitory) stage between the primary and the elementary bodies. Small (elementary) bodies have infectious properties, large (primary) bodies accomplish vegetative function. Growth, reproduction, and maturation of Chlamydia organisms are completed in 40 hours, microcolonies develop within the cytoplasm. Five or six antigens have been detected in the cell wall, which are responsible for the virulent properties of the different strains (Fig. 51).

Figure 51. Mycoplasma

Mycoplasmas are the smallest microorganisms. They were first noticed by Pasteur when he studied the causative agent of pleuropneumonia in cattle. However, at the time he was unable to isolate them in pure culture on standard nutrient media, or to see them under a light microscope. Because of this, these micro-organisms were regarded as viruses. In 1898 Nocard and Roux established that the causative agent of pleuropneumoniacan grow on complex nutrient media which do not contain cells from tissue cultures. Elford using special filters determined the size of the microbe to be within the range of 124-150 nm. Thus, in size mycoplasmas appeared to be even smaller than some viruses.

Since they do not possess a true cell wall, mycoplasmas are characterized by a marked pleomorphism. They give rise to coccoid, granular, filamentous, cluster-like, ring-shaped, filterable forms, etc. Pleomorphism is observed in cultures and in the bodies of animals and man. No two forms are alike. The nuclear apparatus is diffuse. There are both pathogenic and non-pathogenic species. The most typical representative of the pathogenic species is the causative agent of pleuropneumonia in cattle (see section on pathogenic mycoplasmas).

At the present time more than 36 representatives of this order have been isolated, the most minute of all known bacteria. They are found in the soil, sewage waters, different substrates and in the bodies of animals and humans. Since mycoplasmas pass through many filters, and yet grow on media which do not contain live tissue cells, they are considered to be microorganisms intermediate between bacteria and viruses. Chemically, mycoplasmas are closer to bacteria. They contain up to 4 per cent DNA and 8 per cent RNA.

The most typical representatives of the pathogenic species are the causative agents of pleuropneumonia in cattle (Mycoplasma mycoides), acute respiratory infections (Mycoplasma hominis) and atypical pneumonia in humans (Mycoplasma pneumoniae).

FUNGI

http://www.microbiologyonline.org.uk/about-microbiology/introducing-microbes/fungi

Fungi can be single celled or very complex multicellular organisms. They are found in just about any habitat but most live on the land, mainly in soil or on plant material rather than in sea or fresh water. A group called the decomposers grow in the soil or on dead plant matter where they play an important role in the cycling of carbon and other elements. Some are parasites of plants causing diseases such as mildews, rusts, scabs or canker. In crops fungal diseases can lead to significant monetary loss for the farmer. A very small number of fungi cause diseases in animals. In humans these include skin diseases such as athletes' foot, ringworm and thrush (Fig. 52).

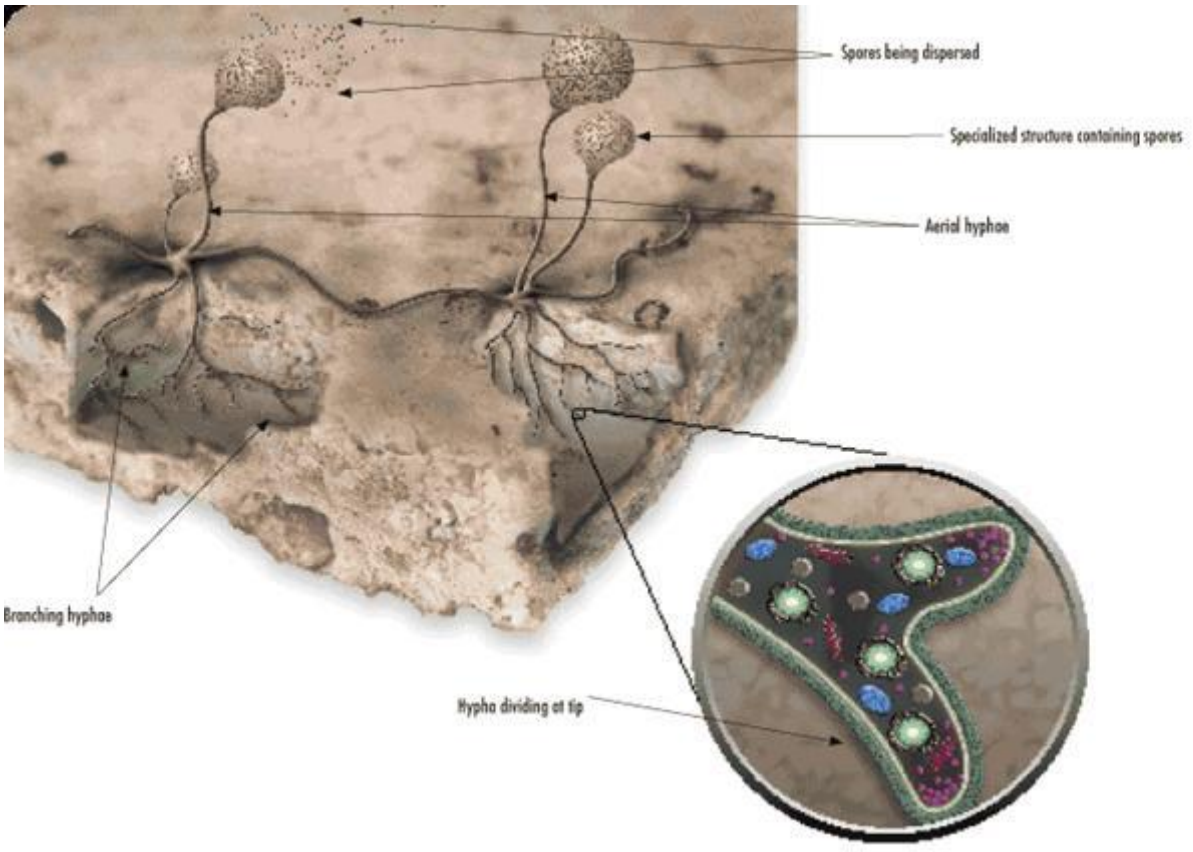

Figure 52. Structure of fungi

Types of fungi

Fungi are subdivided on the basis of their life cycles, the presence or structure of their fruiting body and the arrangement of and type of spores (reproductive or distributional cells) they produce.

The three major groups of fungi are:

- multicellular filamentous moulds

- macroscopic filamentous fungi that form large fruiting bodies. Sometimes the group is referred to as ‘mushrooms’, but the mushroom is just the part of the fungus we see above ground which is also known as the fruiting body.
- single celled microscopic yeasts

Multicellular filamentous moulds

Moulds are made up of very fine threads (hyphae). Hyphae grow at the tip and divide repeatedly along their length creating long and branching chains. The hyphae keep growing and intertwining until they form a network of threads called a mycelium. Digestive enzymes are secreted from the hyphal tip. These enzymes break down the organic matter found in the soil into smaller molecules which are used by the fungus as food.

Some of the hyphal branches grow into the air and spores form on these aerial branches. Spores are specialized structures with a protective coat that shields them from harsh environmental conditions such as drying out and high temperatures. They are so small that between 500 – 1000 could fit on a pin head.

Spores are similar to seeds as they enable the fungus to reproduce. Wind, rain or insects spread spores. They eventually land in new habitats and if conditions are right, they start to grow and produce new hyphae. As fungi can’t move they use spores to find a new environment where there are fewer competing organisms.

Macroscopic filamentous fungi

Macroscopic filamentous fungi also grow by producing a mycelium below ground. They differ from moulds because they produce visible fruiting bodies (commonly known as mushrooms or toadstools) that hold the spores. The fruiting body is made up of tightly packed hyphae which divide to produce the different parts of the fungal structure, for example the cap and the stem. Gills underneath the cap are covered with spores and a 10 cm diameter cap can produce up to 100 million spores per hour.

Yeasts

Yeasts are small, lemon-shaped single cells that are about the same size as red blood cells. They multiply by budding a daughter cell off from the original parent cell. Scars can be seen on the surface of the yeast cell where buds have broken off. Yeasts such as *Saccharomyces*, play an important role in the production of bread and in brewing. Yeasts are also one of the most widely used model organisms for genetic studies, for example in cancer research. Other species of yeast such as *Candida* are opportunistic pathogens and cause infections in individuals who do not have a healthy immune system.

Molds consist of long, branching filaments of cells called hyphae (singular, hypha**)**. A tangled mass of hyphae visible to the unaided eye is a mycelium (plural, mycelia**)**. In some molds, the cytoplasm passes through and among cells of the hypha uninterrupted by cross walls. These fungi are said to be coenocytic fungi. Those fungi that have cross walls are called septate fungi, since the cross walls are called septa.

Yeasts are microscopic, unicellular fungi with a single nucleus and eukaryotic organelles. They reproduce asexually by a process of budding. In this process, a new

cell forms at the surface of the original cell, enlarges, and then breaks free to assume an independent existence.

Some species of fungi have the ability to shift from the yeast form to the mold form and vice versa. These fungi are dimorphic. Many fungal pathogens exist in the body in the yeast form but revert to the mold form in the laboratory when cultivated.
Reproduction in yeasts usually involves spores. Spores are produced by either sexual or asexual means. Asexual spores may be free and unprotected at the tips of hyphae, where they are called conidia (Figure 53). Asexual spores may also be formed within a sac, in which case they are called sporangiospores.

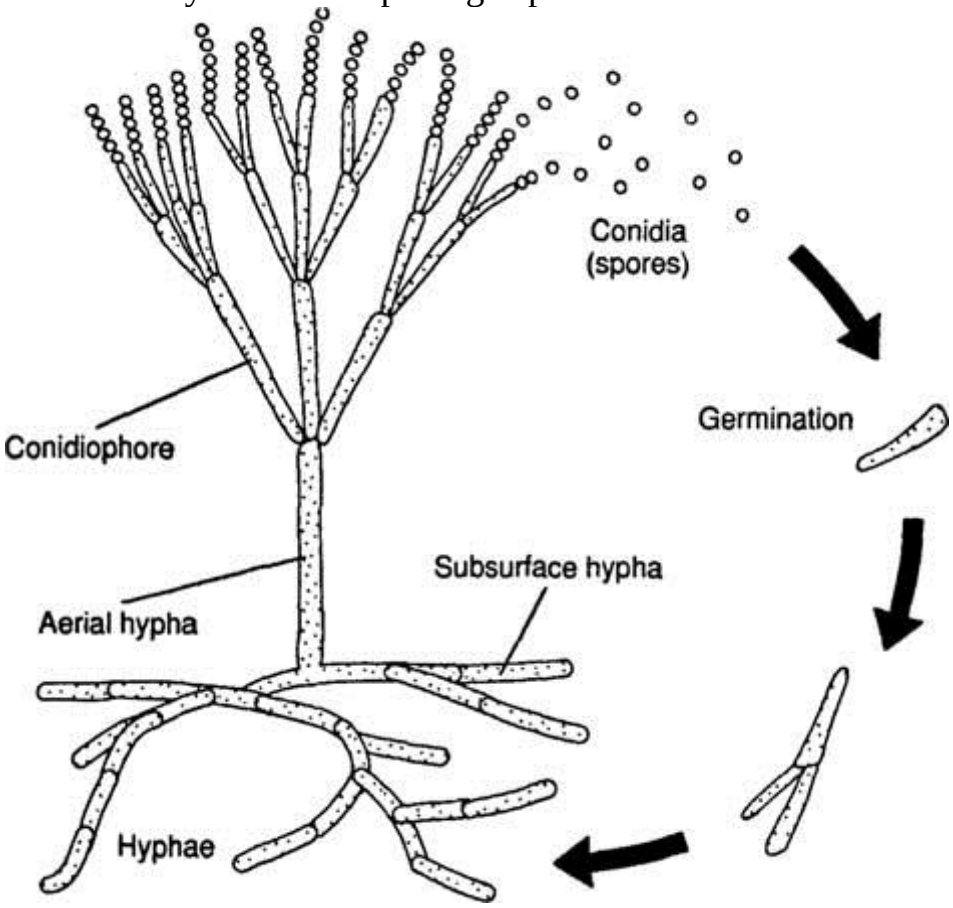

Figure 53. The microscopic structures of a septate fungus showing asexually producedconidia that leave the fungus and germinate to produce a new mycelium

Nutrition. Fungi grow best where there is a rich supply of organic matter. Most fungi are saprobic (obtaining nutrients from dead organic matter). Since they lack photosynthetic pigments, fungi cannot perform photosynthesis and must obtain their nutrients from preformed organic matter. They are the refore chemoheterotrophic organisms**.**

Most fungi grow at an acidic pH of about 5.0, although some species grow at lower and higher pH levels. Most fungi grow at about 25°C (room temperature) except for pathogens, which grow at 37°C (body temperature). Fungi store glycogen for their energy needs and use glucose and maltose for immediate energy metabolism.

Most species are aerobic, except for the fermentation yeasts that grow in both aerobic and anaerobic environments.

(L. fungus a mushroom) belong to plant heterotrophic organisms (eukaryotes) devoid of chlorophyll. The cells of fungi have a differentiated nucleus and many of them multiply by sporulation.They differ greatly from bacteria.

The fungi are marked by various morphology. The main structural component of the vegetative body is the mycelium which is composed of branching colourless filaments (hyphae). In some species the mycelium is non-septate, i. e. formed of a single cell (Mucor mould), in others (higher fungi) it is polycellular (septate). Yeasts are oval or rounded and lack mycelium. The fungus Claviceps purpurea forms a sclerotium which is a firm network of mycelial hyphae (Fig. 54).

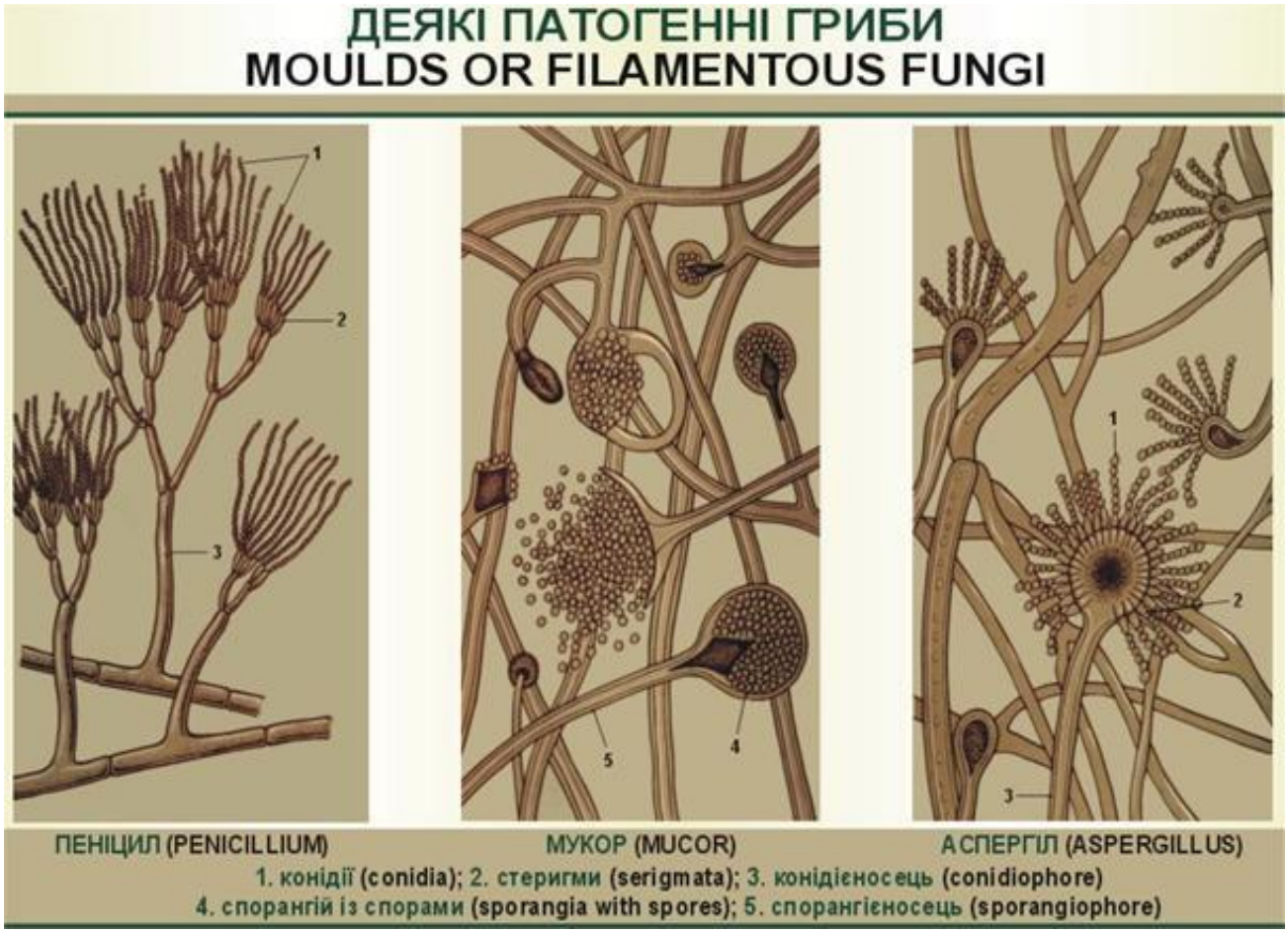

Figure 54. Moulds or filamentous fungi

MODERN CLASSIFICATYIN OF FUNGI

Division Zygomycota. Members of the division Zygomycota are known as zygomycetes. Zygomycetes produce sexual spores known as zygospores (Figure 55), as well as asexual sporangiospores.

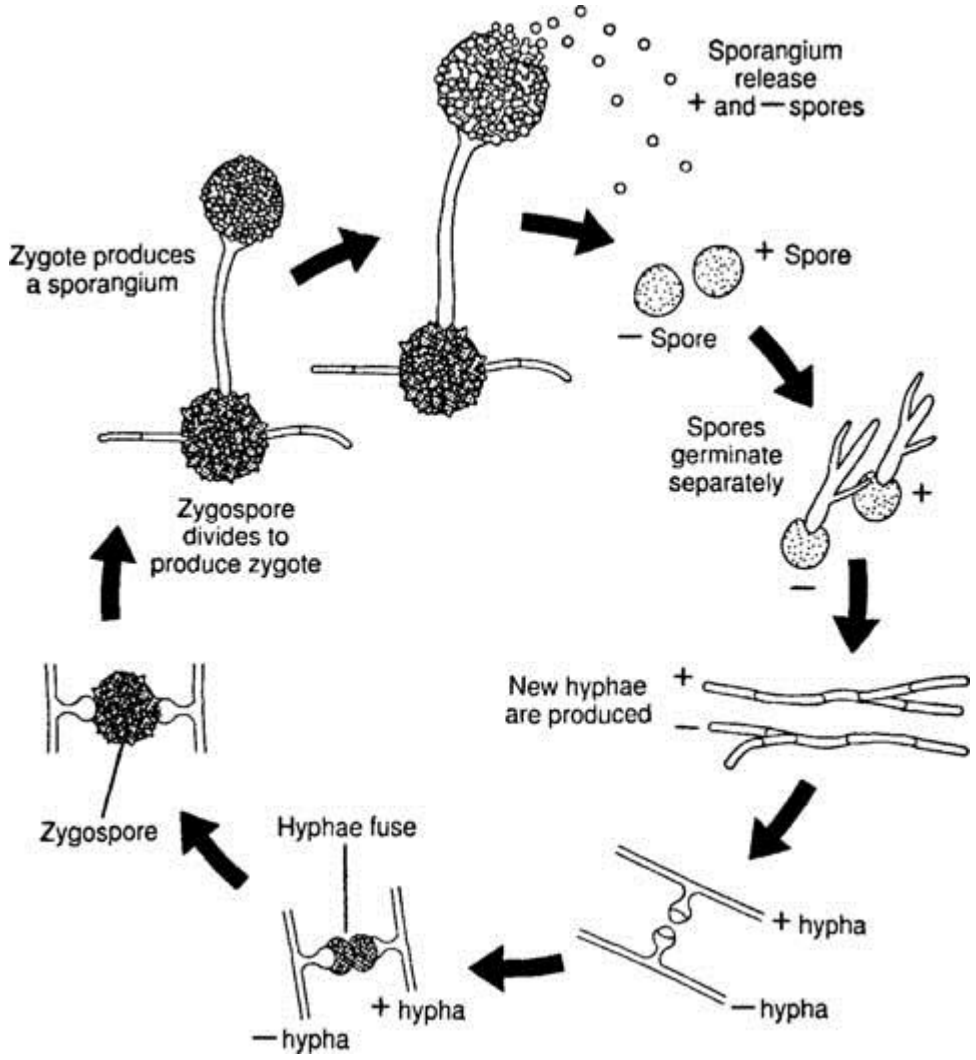

Figure 55. ***Sexual reproduction in the mold*** **Rhizopus stolonifer.** ***Plus and minus mycelia produce sexually opposite hyphae that fuse and give rise to zygospores, which germinate to form new mycelia***

A familiar member of the division is *Rhizopus stolonifer*, a fungus found on fruits, vegetables, and breads. It is the familiar bread mold. It anchors itself to the substratum with special hyphae known as rhizoids. *Rhizopus* is used in the industrial production of steroids, meat tenderizers, industrial chemicals, and certain coloring agents.

Division Ascomycota. Members of the division Ascomycota are referred to as ascomycetes. After sexual fusion of cells has taken place, these organisms form their sexual spores within a sac called an ascus. Therefore, they are called sac fungi**.** Ascomycetes include the powdery mildews and the fungi that cause Dutch elm disease and chestnut blight disease. The research organism *Neurospora crassa* is found within this group. Asexual reproduction in the ascomycetes involves conidia.

Many yeasts are classified in the division Ascomycota. Of particular interest is the fermentation yeast *Saccharomyces*. This yeast is used in the production of

alcoholic drinks, in bread making, and as a source of growth factors in yeast tablets. It is an extremely important research organism as well.

Division Basidiomycota. Members of the division Basidiomycota are referred to as basidiomycetes and are called club fungi. After the sexual cells have united, they undergo division and produce a clubshaped structure called a basidium. Sexually produced basidiospores form at the tips of the basidia. Basidia are often found on huge, visible, fruiting bodies called basidiocarps. The typical mushroom is a basidiocarp.

Basidiomycetes are used as food (for example, mushrooms), but some basidiomycetes are pathogens. One of the organisms of meningitis is the basidiomycete *Cryptococcus neoformans*. The mushroom *Amanita* is poisonous to humans.

Division Deuteromycota. Members of the Deuteromycota division are called deuteromycetes. These fungi lack a known sexual cycle of reproduction and are said to be "imperfect." When its sexual cycle is discovered, a fungus from this division is usually reclassified in one of the other divisions. Among the imperfect fungi are the organisms of athlete's foot and ringworm.

Fungi resemble algae in structure. They have a firm membrane consisting of cellulose, pectin substances, and carbohydrates. Various inclusions are found in the cytoplasm: glycogen, volutin, drops of fat. The cells of fungi may be mononuclear and polynucleate. The nuclei undergo both direct and indirect division. Fungi reproduce by rupture of the mycelium into pieces capable of germinating, by means of chlamydospores and conidia, by sporulation, and by the sexual way. The group of fungi includes saprophytes, parasites, and facultative parasites of plants, animals, and humans.

Chytridiomycetes. Most species inhabit water reservoirs. They lack mycelium or it is present in a rudimentary state. They move by means of pseudopodia. The cells are polynucleate. The Chytridiomycetes undergo a complex developmental cycle. They reproduce by simple division and sporulation. When occurring on a moist substrate, the spores of these fungi absorb water, swell, rupture the membrane, and divide with the production of amoeboid-like cells some of which coalesce and form zygotes which divide and develop into a polynucleate mucous mass. Some species which are pathogenic for plants induce, in particular, cabbage disease ('blackleg') and wart disease of potatoes.

Oomycetes are fungi with non-cellular (non-septate) mycelium. Some species live in water, others in the soil. Water inhabiting oomycetes cause diseases among fish and destroy the roe of fish and frogs. "Other oomycetes parasitize on plants and cause phytophtorosis of potatoes and the fruit of grapes and peronosporosis of sugar beet. The genus Mucor or bread mould belongs to the class Oomycetes (Fig. 56). It consists of a non-septate mycelium in the shape of a much branched cell, from which branch out the fruiting hyphae - sporangiophores with round dilatations at the tips — sporangia. The latter are filled with endospores which provide a means of reproduction. Mucor mould may also reproduce sexually. It is widespread in nature, is often found on vegetables, moist surfaces of objects, and in manure.

A typical representative of Mucor mould is Mucor mucedo.

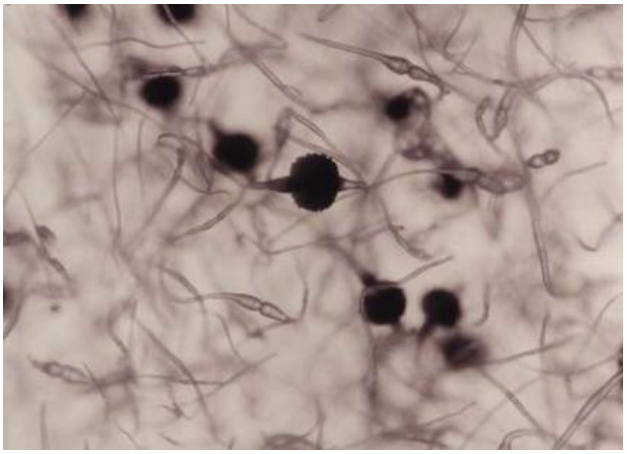

Figure 56. Mucor

Pathogenic species of this mould may cause infections of the lungs and middle ear, and a general severe infectious process in humans, Zygomycetes are soil fungi with a non-cellular mycelium. They reproduce by means of sporangios pores, less frequently by means of conidia. Enzymes secreted by these fungi are used for clarifying juices and pre- paring alcoholic beverages. The class Zygomycetes includes the order Entomophilies, parasites of insects: they cause the death of the larvae of mosquitoes and flies and are used as insecticides. Ascomycetes or sac fungi (35000 species) have a multicellular mycelium. They reproduce sexually by means of ascospores (spores which develop in special spore cases, asci). The organisms reproduce asexually by means of conidia (exospores which bear the function of asexual reproduction in many fungi) – Fig. 57.

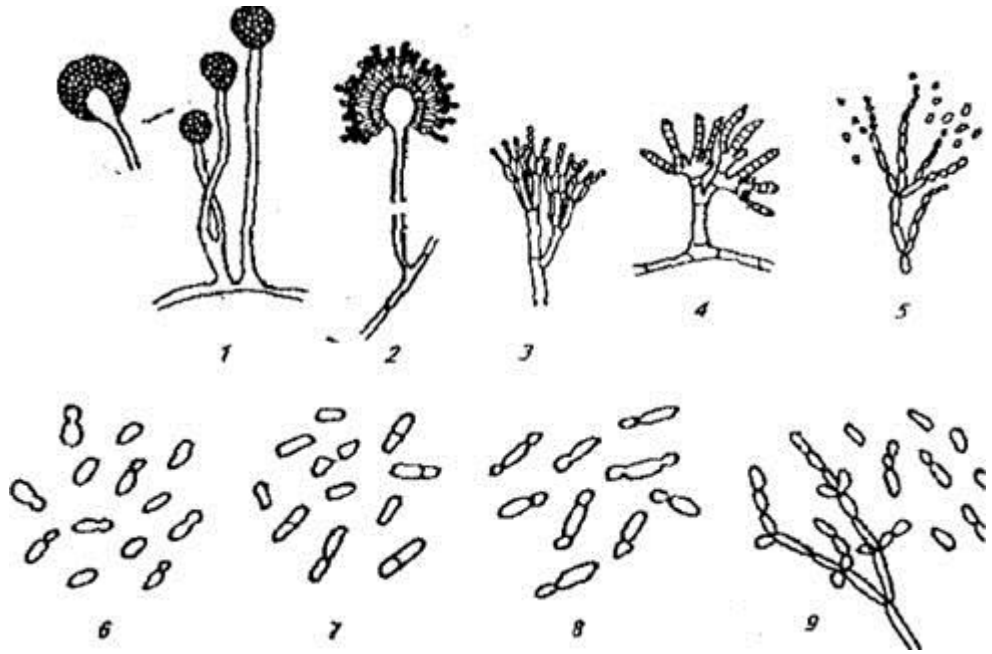

Figure 57. Fungi : 1 — Mucor, 2 — Aspergillus; 3 —- Penicillium; 4 — Fusarium; 5 — Oidium; 6 — Saccharomyces; 7 - Schizosaccharomyces; 8 — Saccharomycoides; 9 — Candida

The genus Aspergillus belongs to the class Ascomycetes (Fig. 58). The fungi have divided septate mycelium, and a unicellular conidiophore which terminates in a fan-like row of short sterigmata from which the spores are pinched off in chains — conidia (Gk. konidion particle of dust).

Figure 58. Aspergillus niger

Microscopic investigations have revealed that the fruiting part of the aspergillus (arrangement of endospores) resembles a jet of water from a watering can, and hence the name 'sprinkler' mould.

A typical representative of aspergilla is Aspergillus niger which is widespread in nature. It is found on moist objects, on bread and jam. Certain species may cause aspergillosis of the lungs, ear, and eye in humans or may infect the whole body.

The genus Penicillium belongs to the class Ascomycetes (Fig. 59). The mycelium and conidiophore are multicellular while the fruiting body is in the shape of a brush. The conidiophore branches towards its upper part and terminates in sterigmata from which even-rowed chains of conidia are pinched off.

Figure 59. Penicillium roqueforti

This genus of fungi is widespread in nature. It is found in fodder, milk products, ink and jam, on moist objects, and old leather. The type species is: Penicillium glaucum. Certain species [Penicillium notation, Penicillium chrysogenium, etc.) are used for producing penicillin which is widely employed in treating many infectious diseases. Some species of this genus of fungi are pathogenic for humans, They cause infections of the skin, nails, ears, upper respiratory tract, lungs. and other organs.

To the class Ascomycetes, the order Saccharomycetales (primary sac fungi) belong the yeasts which are large, oval, round, and rod-shaped cells (Fig. 60).

Figure 60. Yeast

Yeast cells have a double-cell wall and a well defined nucleus. The cytoplasm is homogenous, sometimes of a fine granular structure. It contains inclusions (glycogen, volutin, lipid) and vacuoles, and also filamentous bodies — chondriosomes, which are involved in synthetic processes in the cell. Yeasts multiply by budding, fission, sporulation. Some species of yeasts reproduce sexually. Daughter cells produced by budding from the parent cell transform into independent individuals.

True yeasts are capable of reproducing by sporulation. When there is alack of nutrition. 2. 4, 8 or 16 endospores are formed inside the cells of some species of yeast. The yeast cell forming the ascospores is called the ascus (sac), while sporulating yeasts are known as Ascomyceles.

Many species and varieties of this genus of yeasts are capable of fermenting different carbohydrates. They are widely used in brewing beer, wine making, and baking bread. Typical representatives of these yeasts are Saccharomyces cerevisiae, and Saccharomyces ellipsoides.

A widely used object of genetic research is Neurospora crassa which develops on some bread products as a fluffy, flake-like white or pink mass. The presence of two outwardly indistinguishable forms between which sexual crossbreeding occurs makes it possible to isolate the ascospores and produce pure neurospora lines. Numerous mutants arise under the effect of irradiation which require a definite

metabolite for their development (see section 'Variation in Requirement in Metabolites').

The groups of asporogenic yeasts (family Saccharomycetaceae) includes species pathogenic for humans, which cause severe diseases such as thrush in infants and blastomycosis. They occur due to the suppression of the normal microflora by antibiotics used in the treatment of some infectious diseases and inflammatory processes, as well as in severe diseases in which the protective body forces are weakened.

Claviceps purpurea developing on the grains of rye, wheat, etc. Form a commonly encountered group of Ascomycetes. During flowering the ascospores in the young plants develop into mycelium. The hyphae form a sclerotium (ergot) which takes the place of the grain in the ear and resembles a dark-violet horn. The ergots contain the alkaloid cornutine and sphacelic and ergotic acids which, occurring in rye bread-cause a most severe disease in humans and animals called spasmodic ergotism.

Basidiomycetes, fungi with a multicellular mycelium. These organisms predominantly reproduce sexually by basidiospores (basidia — reproductive organs in which a certain number of spores develop usually 4). The majority of them live on decaying humus and vegetable matter, Certain species are tree parasites. Two hundred species of mushrooms are edible. The fruiting bodies which are commonly known as mushrooms are used as food. Twenty-five species of mushrooms are poisonous. Smut fungi invade grain crops causing a disease known as smut. Rust fungi affect sunflowers, and other plants. They produce orange-coloured sports on infected plants.

Deuteromycetes (Fungi inperfecti) are a rather large group of fungi consisting of a multicellular mycelium without either the asco- or basidio-sporangiophore. but only with conidia. Reproduction is sexual. sexual reproduction is unknown. Among the hyphomycetes which maybe of interest to physicians are: Fusarium graminearum causing intoxication in humans ('drunken bread'), and Fusarium sporotrichiella causing intoxication in man and domestic animals who have eaten the grain crops which had remained in the fields during the winter.

Pathogenic species of imperfect fungi are causative agents of dermatomycoses: favus {Trichophyton schoenleini}, trichophytosis (Trichophyton violaceum), microsporosis {Microsporum canis}, epidermophytosis (Epidermophylon floccosum}.

Protozoa (Gk. *protos* first, *zoon* animal) are unicellular animal organisms more highly organized than bacteria. They have a cytoplasm, a differentiated nucleus, a cell wall which differs in optical properties and primitive organelles. Protozoa reproduce by simple and mullicellular division, sexually, and also by a more complicated process — sexually and asexually (malarial plasmodium). Amoebae, lamblias, and balantidia can produce cysts which are more resistant forms for.survival. Representatives of certain species have two or more nuclei.

A more detailed description and characteristic of protozoa is given in the biology course. The main information on pathogenic species is given in the section on special microbiology.

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Addition materials

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http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/glyco.html
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MORPHOLOGY AND BIOLOGY OF VIRUSES. BASIC METHODS OF VIRUSES CULTIVATION.
METHODS OF INDICATION OF THE VIRUSES
BACTERIAL VIRUSES (BACTERIOPHAGES). STRUCTURE, CLASSIFICATION. SORTS OF INTERACTION OF BACTERIOPHAGES AND BACTERIAL CELLS. VIRULENT AND MODERATE PHAGES.PRACTICAL SIGNIFICANCE OF BACTERIOPHAGE PHENOMENON
SANITARY-BACTERIOLOGIC STUDYING OF WATER, SOIL, AIR. SANITARY-INDICATIVE MICROORGANISMS. MICROBIOLOGIC CONTROL IN STOMATOLOGIC FACILITIES

Viruses are the smallest infectious agents (20-300 nm in diameter), containing one kind of nucleic acid (RNA or DNA) as their genome, usually as single molecule (Fig. 61).

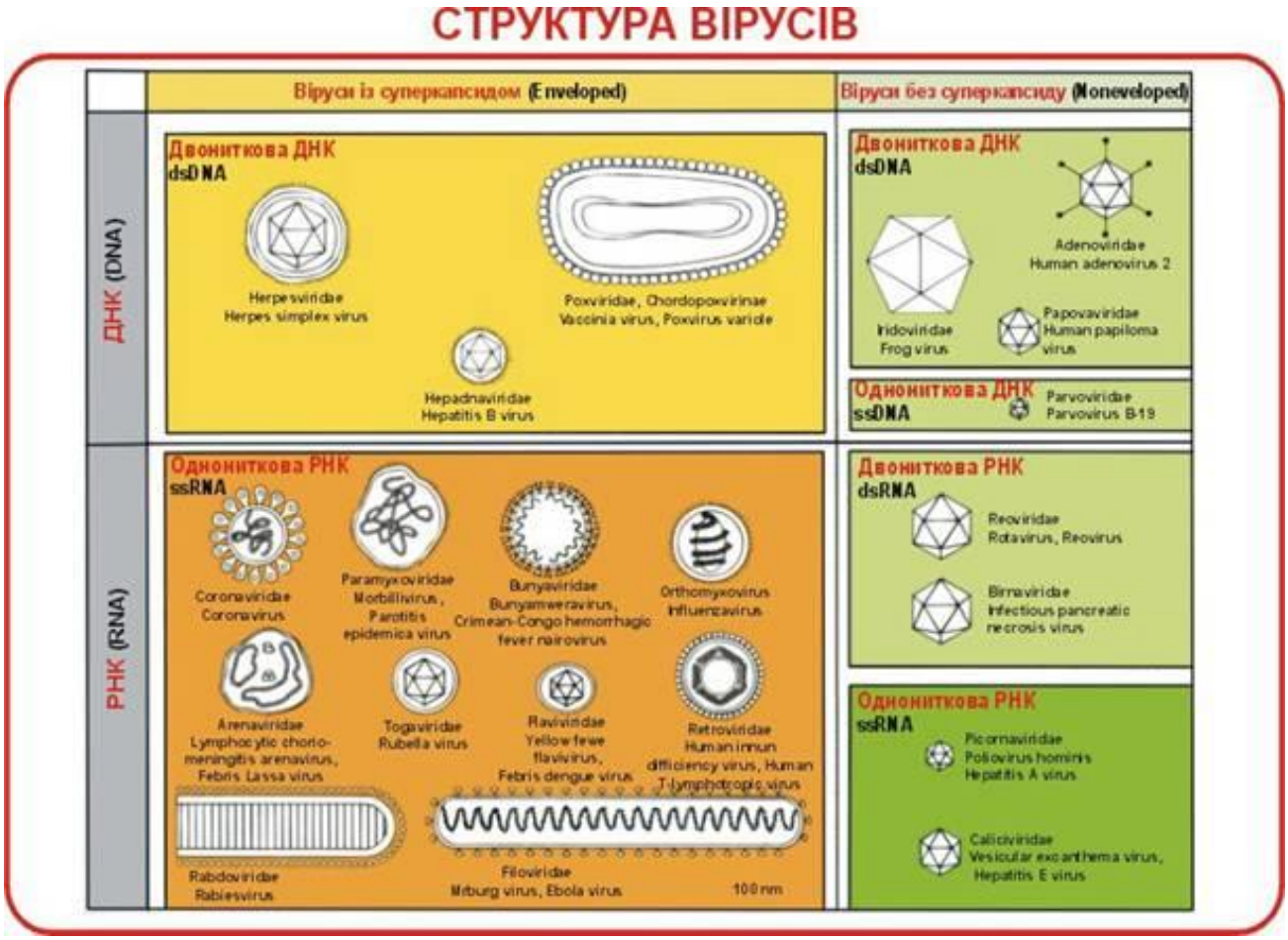


Figure 61. Structure of viruses

The nucleic acid is encased in a protein shell, and the entire infectious unit is termed a virion. Viruses replicate only in living cells. The viral nucleic acid contains information necessary for programming the infected host cell to synthesize a number

of virus-specific macromolecules required for the production of virus progeny. During the replicative cycle, numerous copies of viral nucleic acid and coat proteins are produced. The coat protein assemble together to form the capsid, which encases and stabilizes the viral nucleic acid against the extracellular environment and facilitates the attachment and perhaps penetration of the virus upon contact with new susceptible cells.

The nucleic acid once isolated from the virion, can be hydrolyzed by either ribo- or deoxyribonuclease, whereas the nucleic acid within the intact virus is not affected by such treatment. In contrast, viral antiserum will neutralize the virion because it reacts with the antigens of protein coat. However, the same antiserum has no effect on the free infectious nucleic acid isolated from the virion.

The host range for a given virus may be extremely limited, but viruses are known to infect unicellular organisms such as mycoplasmas, bacteria, and algae and all higher plants and animals.

Much information on virus-host relationships has been obtained from studies on bacteriophages, the viruses that attack bacteria.

Some Useful Definitions in Virology

Capsid: The symmetric protein shell that en closes the nucleic acid genome. Often, empty capsids are by-products of the viral replicative cycle.

Nucleocapsid: The capsid together with the enclosed nucleic acid.

Structural units: The basic protein building blocks of the capsid.

Capsomeres: Morphologic units seen in the electron microscope on the surface of virus particles. Capsomeres represent clusters of polypeptides, which when completely assembled form the capsid.

Virion: The complete infective virus particle, which in some instances (adenoviruses, papovaviruses, picornaviruses) may be identical with the nucleocapsid. In more complex virions (herpesviruses, myxoviruses), this includes the nucleocapsid plus a surrounding envelope.

Detective virus: A virus particle that is functionally deficient in some aspect of replication. Defective virus may interfere with the replication of normal virus.

Pseudovirus: During viral replication the capsid sometimes encloses host nucleic acid rather than viral nucleic acid. Such particles look like ordinary virus, particles when observed by electron microscopy, but they do not replicate. Pseudovirions contain the “wrong” nucleic acid.

Primary, secondary, and tertiary nucleic acid structure: Primary structure refers to the sequence of bases in the nucleic acid chain. Secondary structure refers to the spatial arrangement of the complete nucleic acid chain, i.e., whether it is single- or double-stranded, circular or linear in conformation. Tertiary structure refers to other elements of fine spatial detail in the: helix, eg, presence of supercoiling. break-age points, regions of strand separation.

Transcription: The mechanism by which specific information encoded in a nucleic acid chain is transferred to messenger RNA.

Translation: The mechanism by which a particular base sequence in messenger RNA results in production of a specific amino acid sequence in a protein.

Evolutionary origin of viruses. The origin of viruses is not known. Three hypotheses have been proposed:

(1) Viruses became parasites of primitive cells, and the 2 evolved together. Many viruses today cause no host cell damage and remain latent in the host.

(2) Viruses evolved from parasitic bacteria. While this possibility exists for other obligatory intracellular organisms, eg, chlamydiae, there is no evidence that viruses evolved from bacteria.

(3) Viruses may be components of host cells that become autonomous. They resemble genes that escape the regulatory control of the host cell. There is evidence that some tumor viruses exist in host cells as unexpress genes. The likehood is great that some small viruses evolved in this fashion. On the other hand, large viruses of the pox or herpes groups show very limited resemblance to host cell DNA.

CLASSIFICATION OF VIRUSES. Basis of Classification. The following properties, listed in the order of preference or importance, have been used as a basis for the classification of viruses. The amount of information available in each category is not uniform for all viruses. For some agents, knowledge is at hand about only a few of the properties listed.

(1) Nucleic acid type: RNA or DNA; single-stranded or double-stranded; strategy of replication.

(2) Size and morphology, including type of symmetry, number of capsomeres, and presence of membranes.

(3) Presence of specific enzymes, particularly RNA and DNA polymerases concerned with genome, and neuraminidase necessary for release of certain virus particles (influenza) from the cells in which they were formed.

(4) Susceptibility to physical and chemical agents, especially ether.

(5) Immunologic properties.

(6) Natural methods of transmission.

(7) Host, tissue, and cell tropisms.

(8) Pathology; inclusion body formation.

(9) Symptomatology.

Classification by Symptomatology. The oldest classification of viruses is based on the diseases they produce, and this system offers certain conveniences for the clinician. However, it is not satisfactory for the biologist because the same virus may appear in several groups, since it causes more than one disease depending upon the organ attacked.

A. Generalized Diseases: Diseases in which virus is spread throughout the body via the bloodstream and in which multiple organs are affected. Skin rashes may occur. These include smallpox, vaccinia, measles, rubella, chickenpox, yellow fever, dengue, enteroviruses, and many others.

B. Diseases Primarily Affecting Specific Organs: The virus may spread to the organ through the bloodstream, along the peripheral nerves, or by other routes.

1. Diseases of the nervous system – Poliomyelitis, aseptic meningitis (polio-, coxsackie-, and echoviruses), rabies, arthropod-borne encephalitides, lymphocytic choriomeningitis, herpes simplex, meningoencephalitis of mumps, measles, vaccinia, and "slow" virus infections.

2. Diseases of the respiratory tract – Influenza, parainfluenza, respiratory syncytial virus pneumonia and bronchiolitis, adenovirus pharyngitis, common cold (caused by many viruses).

3. Localized diseases of the skin or mucous membranes – Herpes simplex type 1 (usually oral) and type 2 (usually genital), molluscum contagiosum, warts, herpangina, herpes zoster, and others.

4. Diseases of the eye – Adenovirus conjunctivitis, Newcastle virus conjunctivitis, herpes keratoconjunctivitis, and epidemic hemorrhagic conjunctivitis (enterovirus-70).

5. Diseases of the liver-Hepatitis type A (infectious hepatitis) and type B (serum hepatitis), yellow fever, and, in the neonate, enteroviruses, herpesviruses, and rubella virus.

6. Diseases of the salivary glands – Mumps and cytomegalovirus.

7. Diseases of the gastrointestinal tract – Rotavirus, Norwalk type virus.

8. Sexually transmitted diseases – Until recently, only bacteria *(Neisseria gonorrhoeae, Treponema pallidum,* and *Chlamydia trachomatis)* were included in this category of disease. It is now recognized that herpes simplex virus, hepatitis B virus, papilloma virus, molluscum contagiosum virus, and probably cytomegalovirus are all venereal pathogens.

Classification by Biologic, Chemical, and Physical Properties.

Viruses can be clearly separated into families on the basis of the nucleic acid genome and the size, shape, substructure, and mode of replication of the virus particle. Table 6 shows one scheme used for classification. However, there is not complete agreement among virologists on the relative importance of the criteria used to classify viruses.

Within each family, genera are usually based on antigenicity. Properties of the major families of animal viruses are summarized in Table 6, are discussed briefly below.

DNA-Containing Viruses

A. Parvoviruses: Very small viruses with a particle size of about 20 nm. They contain single-stranded DNA and have cubic symmetry, with 32 capsomeres 2-4 nm in diameter. They have no envelope. Replication and capsid assembly take place in the nucleus of the infected cell. Parvoviruses of rodents and swine replicate autonomously. The adenoassociated satellite viruses are defective, i.e., they require the presence of an adenovirus or a herpesvirus as a "helper". Some satellite viruses occur in humans.

B. Papovaviruses: Small (45-55 nm), ether-resistant viruses containing double-stranded circular DNA and exhibiting cubic symmetry, with 72 capsomeres. Known human papovaviruses are the papilloma (wart) virus and agents isolated from brain tissue of patients with progressive multifocal leukoencephalopathy (JC virus) or

from the urine of immunosuppressed renal transplant recipients (BK virus). In animals, there are papilloma, polyoma, and vacuolating viruses. These agents have a slow growth cycle and replicate within the nucleus. Papovaviruses produce latent and chronic infections in their natural hosts, and all can induce tumors in some animal species.

C. Adenoviruses: Medium-sized (70-90 nm) viruses containing double-stranded DNA and exhibiting cubic symmetry, with 252 capsomeres. They have no envelope. At least 37 types infect humans, especially in mucous membranes, and they can persist in lymphoid tissue. Some adenoviruses cause acute respiratory diseases, pharyngitis, and conjunctivitis. Some human adenoviruses can induce tumors in newborn hamsters. There are many serotypes that infect animals.

D. Herpesviruses: Medium-sized viruses containing double-stranded DNA. The nucleocapsid is 100 nm in diameter, with cubic symmetry and 162 capsomeres. It is surrounded by a lipid-containing envelope (150-200 nm in diameter). Latent infections may last for the life span of the host.

Human herpesviruses include herpes simplex types 1 and 2 (oral and genital lesions); varicella-zoster virus (shingles and chickenpox); cytomegalovirus; and EB virus (infectious mononucleosis and association with human neoplasms). Other herpesviruses occur in many animals.

E. Poxviruses: Large brick-shaped or ovoid (230 x 400 nm) viruses containing double-stranded DNA, with a lipid-containing envelope. All poxviruses share a common nucleoprotein antigen and contain several enzymes in the virion, including a DNA-dependent RNA polymerase. Poxviruses replicate entirely within cell cytoplasm. All poxviruses tend to produce skin lesions. Some are pathogenic for humans (smallpox, vaccinia, molluscum contagiosum), others for animals. (Some of the latter can infect humans, eg, cow-pox, monkeypox).

RNA-Containing Viruses

A. Picornaviruses: Small (20-30 nm), ether-resistant viruses containing single-stranded RNA and exhibiting cubic symmetry. The groups infecting humans are rhinoviruses (more than 100 serotypes causing common colds) and enteroviruses (polio-, coxsackie-, and echoviruses). Rhinoviruses are acid-labile and have a high density; enteroviruses are acid-stable and have a lower density. Picornaviruses infecting animals include foot-and-mouth disease of cattle and encephalomyocarditis of rodents.

B. Reoviruses: Medium-sized (60-80 nm), ether-resistant viruses containing a segmented double-stranded RNA and having cubic symmetry. Reoviruses of humans include rotaviruses, which cause infantile gastroenteritis and have a distinctive wheel-shaped appearance. Antigenically similar reoviruses infect many animals. Orbiviruses constitute a distinct subgroup that includes Colorado tick fever virus of humans and other agents that infect plants, insects, and animals (blue tongue of cattle and sheep).

Table 6. Classification of viruses into families based on chemical and physical properties

Nucleic Acid Core	Capsid Symmetry	Virion: Enveloped or Naked	Ether Sensitivity	No. of Capsomeres	Virus Particle Size (nm)*	Physical Type of Nucleic Acid	No. of Genes (Approx.)	Virus Family
DNA	Icosahedral	Naked	Resistant	32 72 252	18-26 45-55 70-90	SS DS circular DS	3-4 5-8 30	Parvoviridae Papovaviridae Adenoviridae
		Enveloped	Sensitive	162	100**	DS	160	Herpesviridae
	Complex	Complex coats	Resistant***		230 X 400	DS	300	Poxviridae
RNA	Icosahedral	Naked	Resistant	32****	20-30 60-80	SS DS segmented	4-6 10-12	Picornaviridae Reoviridae
		Enveloped	Sensitive	32?	30-90	SS	10	Togaviridae
	Unknown or complex	Enveloped	Sensitive		50-300 80-130 ~100	SS segmented SS SS segmented	10 30 >4	Arenaviridae Coronaviridae Retroviridae
	Helical	Enveloped	Sensitive		90-100 80-120 150-300 70 X 175	SS segmented SS segmented SS SS	>3 >8 >10 >5	Arenaviridae Coronaviridae Retroviridae

"Diameter, or diameter X length.
****The naked virus, i.e., the nucleocapsid, is 100 nm in diameter; however, the enveloped virion varies up to 200 nm.**
*****The genus *Orthopoxvirus,* which includes the better studied poxviruses, eg, vaccinia, variola, cowpox, ectromelia, rabbitpox,**
monkeypox, is ether-resistant. Some of the poxviruses belonging to other genera are ether-sensitive.
******Reoviruses contain an outer and an inner capsid. The inner capsid appears to contain 32 capsomeres, but the number on the outer capsid has not been definitely established. A total of 92 capsomeres has been suggested.**

C. Arboviruses: An ecologic grouping of viruses with diverse physical and chemical properties. All of these viruses (more than 350) have a complex cycle involving vertebrate hosts and arthropods as vectors transmitting the viruses by their bite. Arboviruses infect humans, mammals, birds, and snakes, and mosquitoes and ticks as vectors. Human pathogens include dengue, yellow fever, encephalitis viruses, and others. Arboviruses belong to several groups, including toga-, bunya-, rhabdo-, arena-, and reoviruses, described here.

D. Togaviruses: Most arboviruses of antigenic groups A and B and rubella virus belong here. They have a lipid-containing envelope, are ether-sensitive, and their genome is single-stranded RNA. The enveloped virion measures 40—70 nm. The virus particles mature by budding from the host cell membrane. Some togaviruses, eg, Sindbis virus, possess a 35-nm nucleocapsid and within it a spherical

core 12-16 nm in diameter. Sindbis virus may have 32 capsomeres in an icosahedral surface lattice.

E. Arenaviruses: RNA-containing, enveloped viruses ranging in size from 50 to 300 nm. They share morphologic, biologic, and antigenic properties of arboviruses of the Tacaribe complex, Lassa fever, and lymphocyticchoriomeningitis. Some produce "slow" virus infections.

F. Coronaviruses: Enveloped, 80- to 130-nm particles containing an unsegmented genome of single-stranded RNA; the nucleocapsid is probably helical, 7-9 nm in diameter. They resemble orthomyxoviruses, but coronaviruses have petal-shaped surface projections arranged in a fringe like a solar corona. Coronavirus nucleocapsids develop in the cytoplasm and mature by budding into cytoplasmic vesicles. Human coronaviruses have been isolated from acute upper respiratory tract illnesses— "colds". Coronaviruses of animals include avian infectious bronchitis virus among many others.

G. Retroviruses: Enveloped viruses whose genome contains duplicate copies of high-molecular-weight single-stranded RNA of the same polarity as viral messenger RNA. The virion contains various enzymes including reverse transcriptase (RNA – DNA). Leukemia and sarcoma viruses of animals, foamy viruses of primates, and some "slow" viruses (visna, maedi of sheep) are included.

H. Bunyaviruses: Spherical, 90- to 100-nm particles that replicate in the cytoplasm and acquire an envelope by budding through the cell membrane. The genome is made up of a triple-segmented, single-stranded RNA. About 70 are antigenically related to Bunyamwera virus; 50 others are not but are mor-phologically similar.

Orthomyxoviruses: Medium-sized, 80- to 120-nm enveloped viruses containing a segmented single-stranded RNA genome and exhibiting helical symmetry. Particles are either round or filamentous. Most Orthomyxoviruses have surface projections as part of their outer wall (hemagglutinin, neuraminidase). The internal nucleoprotein helix measures 6-9 nm, and the RNA is made up of 8 segments. During replication, the nucleocapsid is formed in the nucleus, whereas the hemagglutinin and neuraminidase are formed in the cytoplasm. The virus matures by budding at the cell membrane. Orthomyxoviruses are sensitive to dactinomycin. All Orthomyxoviruses are influenza viruses that infect humans or animals.

J. Paramyxoviruses: Similar to but larger (150-300 nm) than Orthomyxoviruses. The internal nucleocapsid measures 18 nm, and the molecular weight of the single-stranded nonsegmented RNA is 4 times greater than that of Orthomyxoviruses. Both the nucleocapsid and the hemagglutinin are formed in the cytoplasm. Paramyxoviruses are resistant to dactinomycin. Those infecting humans include mumps, measles, parainfluenza virus, and respiratory syncytial virus. Others infect animals.

K. Rhabdoviruses: Enveloped virions resembling a bullet, flat at one end and round at the other (Fig 27-35), measuring about 70 x 175 nm. The envelope has 10-nm spikes. The genome is single-stranded RNA. Particles are formed by budding

from the cell membrane. Rabies virus is a member of this group along with many other viruses of animals and plants.

L. Other Viruses: Insufficient information to permit classification. This applies to hepatitis viruses, to agents responsible for some immune complex diseases and for sortie "slow" virus diseases, and to some viruses of gastroenteritis

M. Viroids: Small infectious agents causing diseases of plants and possibly animals and humans. They are nucleic acid molecules (MW 70,000-120,000) without a protein coat. Plant viroids are single-stranded, covalently closed circular RNA molecules consisting of about 360 nucleotides and comprising a highly base-paired rodlike structure with unique properties. They are arranged in 26 double-stranded segments separated by 25 regions of unpaired bases embodied in single-stranded internal loops; there is a loop at each end of the rodlike molecule. These features provide the viroid RNA molecule with structural, thermodynamic, and kinetic properties very similar to those of a double-stranded DNA molecule of the same molecular weight and G + C content. Viroids replicate by an entirely novel mechanism in which infecting viroid RNA molecules are copied by the host enzyme normally responsible for synthesis of nuclear precursors to messenger RNA. Thus, DNA-dependent RNA polymerase purified from healthy plant tissue is capable of synthesizing linear (—) viroid RNA copies of full length from (+) viroid RNA templates in vitro.

The infectious agents of degenerative neurologic disorders such as kuru or Creutzfeldt-Jakob disease, or scrapie of sheep, may fit into this category. (The agent of the latter may be a DNA molecule similar in size to plant viroid RNA).

STRUCTURE AND SIZE OF VIRUSES

Virus Particles

Advances in x-ray diffraction techniques and electron microscopy have made it possible to resolve fine differences in the basic morphology of viruses. The study of virus symmetry in the electron microscope requires the use of heavy metal stains (eg, potassium phosphotungstate) to emphasize surface structure The heavy metal permeates the virus panicle as a cloud and brings out the surface structure of viruses by virtue of "negative staining "

Virus architecture can be grouped into 3 types based on the arrangement of morphologic subunits. (1) those with helical symmetry, eg, paramyxo- and orthomyxovimses, (2) those with cubic symmetry, eg, adenoviruses, and (3) those with complex structures, eg, poxviruses All cubic symmetry observed with animal viruses to date is of the icosahedral pattern. The icosahedron has 20 faces (each an equilateral triangle), 12 vertices, and 5-fold, 3-fold, and 2-fold axes of rotational symmetry. Capsomeres can be arranged to comply with icosahedral symmetry in a limited number of ways, expressed by the formula $N = 10(n\text{-}I)^2 + 2$, where N is the total number of capsomeres and *n* the number of capsomeres on one side of each equilateral triangle shows the number of capsomeres where *n* varies from 2 to 6, in several virus groups.

Icosahedral structures can be built from one simple, asymmetric building unit, arranged as 12 pentamer units and *x* number of hexamer units. The smallest and most basic capsid is that of the phage □X-174, which simply consists of 12 pentamer units Viruses exhibiting icosahedral symmetry can also be grouped according to their tnangulation number, T, which is the number of small triangles formed on the single face of the icosahedron when all its adjacent morphologic subunits are connected by lines. One class has T values of 1,4,9,16, and 25; a second class, values of 3 and 12, and a third class, values of 7, 13, 19, and 21 The number of morphologic units (capsomeres) is expressed by the formula M = 10T + 2. Table 2 shows the tnangulation number for several virus groups This formula for tnangulation number originated in the idea that those viruses would be formed from small subunits so as to give a surface lattice representing the minimum-energy design for closed shells arranged from identical units.

An example of icosahedral symmetry is seen in Fig 62. The adenovirus (n = 6) model illustrated shows the 6 capsomeres along one edge (Fig 62[a]). Degradation of this virus with sodium lauryl sulfate releases the capsomeres in groups of 9 (Fig 62 [b], [c]) and possibly groups of 6 The groups of 9 lie on the faces and include one capsomere from each of the 3 edges of the face, and the groups of 6 would be from the vertices The groups of 9 form the faces of the 20 triangular facets, making the adenovirus icosahedron account for 180 subunits, and the groups of 6 which form the 12 vertices account for 72 capsomeres, thus totaling 252.

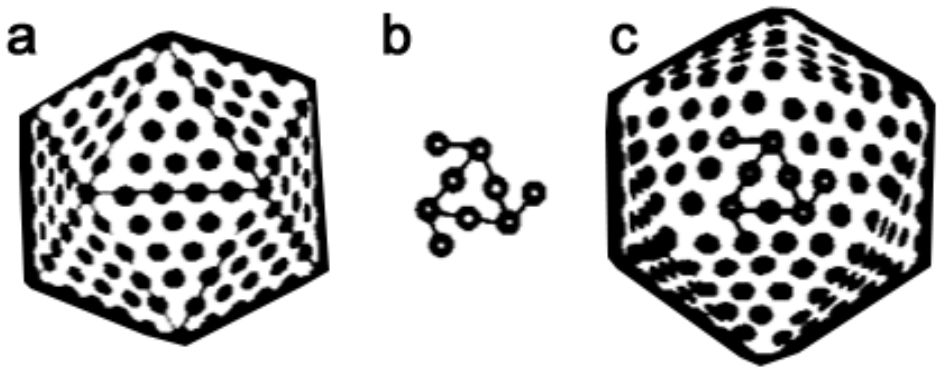


Figure 62. *(a)* Representation of the capsomere arrangement of an adenovirus particle, as viewed through the 2 fold axis of symmetry. (b) Arrangement of capsomere group of 9, obtained by treatment of an adenovirus with sodium lauryl sulfate. (*c)* Orientation of the capsomere group of 9 on the adenovirus particle If the model were marked to show the maximum number of small triangles formed on one face of the icosahedron by drawing a line between each adjacent morphologic subunit, it would yield the tnangulation number for the adenovirus particle, which in this case turns out to be 25.

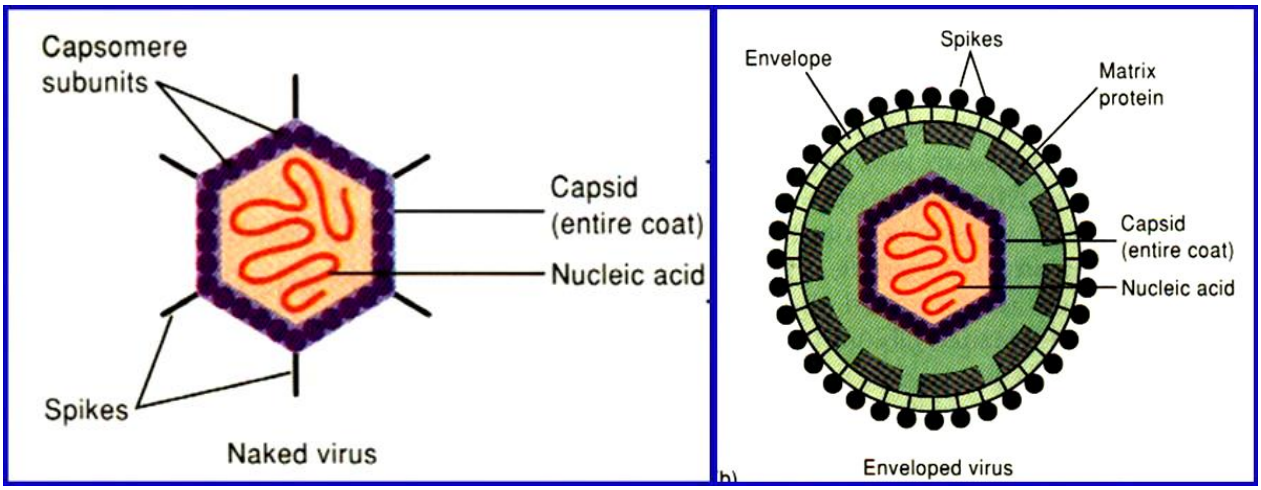


Figure 63. Structure of viruses

Measuring the Size of Viruses. Small size and ability to pass through filters that hold back bacteria are classic attributes of viruses. However, because some bacteria may be smaller than the largest viruses, filtrability is no longer regarded as a unique feature of viruses.

The following methods are used for determining the sizes of viruses and their components.

A. Filtration Through Collodion Membranes of Graded Porosity: These membranes are available with pores of different sizes. If the virus preparation is passed through a series of membranes of known pore size, the approximate size of any virus can be measured by determining which membranes allow the infective unit to pass and which hold it back. The size of the limiting APD (average pore diameter) multiplied by 0.64 yields the diameter of the virus particle. The passage of a virus through a filter will also depend on the physical structure of the virus; thus, only a very approximate estimate of size is obtained.

B. Sedimentation in the Ultracentrifuge: If particles are suspended in a liquid, they will settle to the bottom at a rate that is proportionate to their size. In an ultracentrifuge, forces of more than 100,000 times gravity may be used to drive the particles to the bottom of the tube. The relationship between the size and shape of a particle and its rate of sedimentation permits determination of particle size. Once again, the physical structure of the virus will affect the size estimate obtained.

C. Direct Observation in the Electron Microscope: As compared with the light microscope, the electron microscope uses electrons rather than light waves and electromagnetic lenses rather than glass lenses. The electron beam obtained has a much shorter wavelength than that of light, so that objects much smaller than the wavelength of visible or ultraviolet light can be visualized. Viruses can be visualized in preparations from tissue extracts and in ultrathin sections of infected cells. Electron microscopy is the most widely used method for estimating particle size.

D. Ionizing Radiation: When a beam of charged particles such as high-energy electrons, alpha particles, or deuterons passes through a virus, it causes an energy loss in the form of primary ionization. The release of ionization within the virus particle

proportionately inactivates certain biologic properties of the virus particle such as infectivity, antigenicity, and hemagglutination. Thus, the size of the biologic unit responsible for a given function in a virus particle can be estimated.

E. Comparative Measurements: (See Table 1.) For purposes of reference, it should be recalled that: (1) *Staphylococcus* has a diameter of about 1000 nm. (2) Bacterial viruses (bacteriophages) vary in size (10-100 nm). Some are spherical or hexagonal and have short or long tails. (3) Representative protein molecules range in diameter from serum albumin (5 nm) and globulin (7 nm) to certain hemocyanins (23 nm).

The relative size and morphology of various virus families see Lecture 4. Particles with a 2-fold difference in diameter have an 8-fold difference in volume. Thus, the mass of a pox virus is about 1000 times greater than that of the poliovirus particle, and the mass of a small bacterium is 50,000 times greater.

CHEMICAL COMPOSITION OF VIRUSES

Viral Protein. The structural proteins of viruses have several important functions. They serve to protect the viral genome against inactivation by nucleases, participate in the attachment of the virus particle to a susceptible cell, and are responsible for the structural symmetry of the virus particle. Also, the proteins determine the antigenic characteristics of the virus.

Virus structural proteins may be very specialized molecules designed to perform a specific task: (1) vaccinia virus carries many enzymes within its particle to perform certain functions early in the infectious cycle; (2) some viruses have specific proteins for attachment to cells, eg, influenza virus hemagglutinin;
and (3) RNA tumor viruses contain an enzyme, reverse transcriptase, that makes a DNA copy of the virus RNA, which is an important step in transformation by these viruses.

Viral Nucleic Acid. Viruses contain a single kind of nucleic acid, either DNA or RNA, that encodes the genetic information necessary for the replication of the virus. The RNA or DNA genome may be single-stranded or double-stranded, and the strandedness, the type of nucleic acid, and the molecular weight are major characteristics used for classifying viruses into families (Table 6).

The molecular weight of the viral DNA genome ranges from 1.5 X 10^6 (parvoviruses) to 160 X 10^6 (poxviruses). The molecular weight of the viral RNA genome ranges from 1 x 10^6 (for bromegrass mosaic virus) to 15 x 10^6 (for reoviruses).

The sequence and composition of nucleotides of each viral nucleic acid are distinctive. One of the properties useful for characterising a viral nucleic acid is its guanine + cytosine (G + C) content.

Most viral genomes are quite fragile once they are removed from their protective protein capsid, but some nucleic acid molecules have been examined in the electron microscope without disruption, and their lengths have been measured. If linear densities of approximately 2 X 10^6 per mcm for double-stranded nucleic acid and 1 x 10^6 per mcm for single-stranded forms are used, molecular weights of viral genomes can be calculated from direct measurements (Table 6).

All major DNA virus groups in Table 1 have genomes that are single molecules of DNA and have a linear or a circular configuration. This circle is often supercoiled in the virion.

Viral RNAs exist in several forms. The RNA may be a single linear molecule (eg, picomavirus). For other viruses (eg, orthomyxovirus), the genome consists of several segments of RNA that may be loosely linked together within the virion. The isolated RNA of picomaviruses and toga viruses is infectious, and the entire molecule functions as a messenger RNA within the infected cell. The isolated RNA of other RNA viruses is not infectious. For these virus families, the virions carry an RNA polymerase which in the cell transcribes the genome RNA molecules into several complementary RNA molecules, each of which may serve as a messenger RNA.

Molecular hybridization techniques (DNA to DNA, DNA to RNA, or RNA to RNA) permit the study of transcription of the viral genome within the infected cell as well as the relatedness of different viruses.

The number of genes in a virus can be approximated if one makes certain assumptions about (1) triplet code, (2) the molecular weight of the genome, and (3) the average size of a protein (Table 3).

Viral Lipids. A number of different viruses contain lipids as part of their structure (eg, Sindbis virus [Fig 64]). Such lipid-containing viruses are sensitive to treatment with ether and other organic solvents (Table 3), indicating that disruption or loss of lipid results in loss of infectivity. Non-lipid-containing viruses are generally resistant to ether.

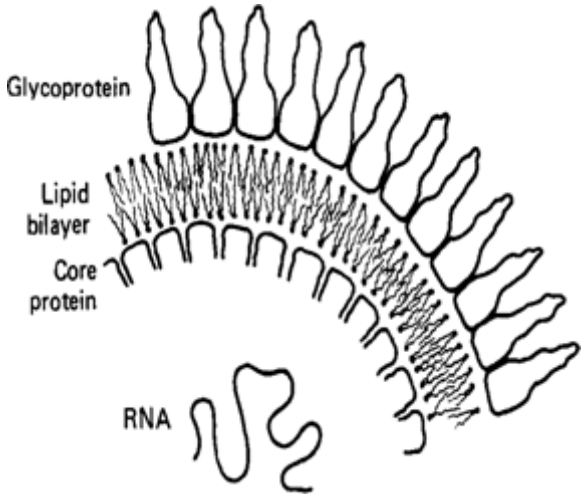


Figure 64. Proposed structure of Sindbis virus

The specific phospholipid composition of a virion envelope may be determined by the ' 'budding " of the virus through specific types of cell membranes in the course of maturation. For example, herpes viruses bud through the nuclear membrane of the host cell, and the phospholipid composition of the purified virus reflects the lipids of the nuclear membrane. The different ways in which various animal viruses acquire an envelope are suggested in Fig 65. Budding of virions occurs only at sites where virus-specific proteins have been inserted into the host cell membrane.

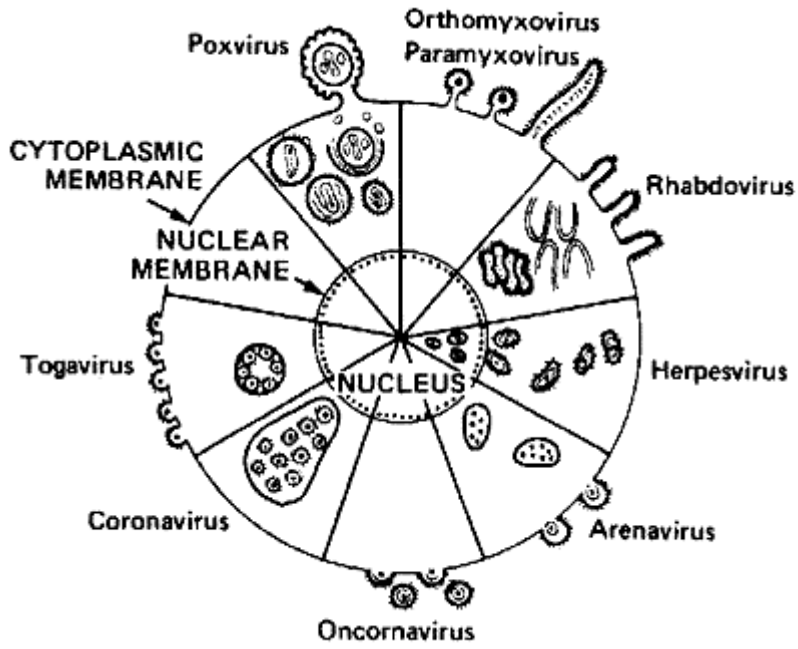


Figure 65. Diagrammatic relationship between lipid-containing viruses and host cell membranes

Glycosphingolipids occur in the surface membrane of animal cells. When cultured cells are transformed by some oncogenic viruses, there are alterations in the various sphingolipids. These may be related to the loss of contact inhibition and to changes in surface antigens that result from viral transformation.

Viral Carbohydrates. Virus envelopes contain glycoproteins. The sugars added to vims glycoproteins often reflect the host cell in which the vims is grown. The glycoproteins are important vims antigens. As a result of their position at the outer surface of the virion, they are frequently involved in the interaction of the vims with neutralizing antibody.

PURIFICATION AND IDENTIFICATION OF VIRUSES

Purification of Virus Particles. For purification studies, the starting material is usually large volumes of tissue culture medium, body fluids, or infected cells. The first step involves concentration of the virus particles by precipitation with ammonium sulfate, ethanol, or polyethylene glycol or by ultrafiltration. Hemagglutination and elution can be used to concentrate myxoviruses. Once concentrated, virus can be separated from host materials by differential centrifugation, density gradient centrifugation, column chromatography, and Rhabdovirus electrophoresis.

The minimal criteria for purity are a homogeneous appearance in electron micrographs and the failure of additional purification procedures to remove “contaminants” without reducing infectivity.

Rate-Zonal Centrifugation. A sample of concentrated virus is layered onto a preformed linear density gradient of sucrose or glycerol, and during centrifugation the virus sediments as a band at a rate determined primarily by the size and weight of the virus particle. Samples are collected by piercing a hole in the bottom of the

centrifuge tube. The band of purified virus may be detected by optical methods, by radiolabeling the virus, or by assaying for infectivity.

Equilibrium Density Gradient Centrifugation. Viruses can also be purified by high-speed centrifugation in density gradients of cesium chloride (CsCI), potassium tartrate, potassium citrate, or sucrose. The gradient material of choice is the one that is least toxic to the virus. Virus particles migrate to an equilibrium position where the density of the solution is equal to their buoyant density and form a visible band. Virus bands are harvested by puncture through the bottom of the plastic centrifuge tube and assayed for infectivity.

Additional methods for purification are based on the chemical properties of the virus surface.

As shown by column chromatography, virus is bound to a substance such as DEAE or phosphocellulose, then eluted by changes in pH or salt concentration. Zone electrophoresis permits the separation of virus particles from contaminants on the basis of charge.

Identification of a Particle as a Virus. When a characteristic physical particle has been obtained, it should fulfill the following criteria before it is identified as a virus particle.

(1) The particle can be obtained only from infected cells or tissues.

(2) Particles obtained from various sources are identical, regardless of the cellular species in which the virus is grown.

(3) The degree of infective activity of the virus varies directly with the number of particles present.

(4) The degree of destruction of the physical particle by chemical or physical means is associated with a corresponding loss of virus activity.

(5) Certain properties of the particles and infectivity must be shown to be identical, such as their sedimentation behaviour in the ultracentrifuge and their pH stability curves.

(6) The absorption spectrum of the purified physical particle in the ultraviolet range should coincide with the ultraviolet inactivation spectrum of the virus.

(7) Antisera prepared against the infective virus should react with the characteristic particle, and vice versa. Direct observation of an unknown virus can be accomplished by electron microscopic examination of aggregate formation in a mixture of antisera and crude virus suspension.

(8) The particles should be able to induce the characteristic disease in vivo (if such experiments are feasible).

(9) Passage of the particles in tissue culture should result in the production of progeny with biologic and serologic properties of the virus.

REACTION TO PHYSICAL & CHEMICAL AGENTS

Heat and Cold. Virus infectivity is generally destroyed by heating at 50-60 °C for 30 minutes, although there are some notable exceptions (eg, hepatitis virus, adenoassociated satellite virus, scrapie virus).

Viruses can be preserved by storage at subfreezing temperatures, and some may withstand lyophilization and can thus be preserved in the dry state at 4 °C or

even at room temperature. Viruses that withstand lyophilization are more heat-resistant when heated in the dry state. Enveloped viruses tend to lose infectivity after prolonged storage even at —90 °C and are particularly sensitive to repeated freezing and thawing; however, in the presence of dimethyl sulfoxide (DMSO) at concentrations of more than 5%, these viruses are stabilized.

Stabilization of Viruses by Salts. Many viruses can be stabilized by molar concentrations of salts, i.e., they are not inactivated even by heating at 50 °C for 1 hour. The mechanism by which the salts stabilize virus preparations is not known. Viruses are preferentially stabilized by certain salts. Molar $MgCl_2$ stabilizes picorna- and reoviruses, molar $MgSO_4$ stabilizes orthomyxo- and paramyxo-viruses, and molar $NazSO_4$ stabilizes herpes-viruses.

The stability of viruses is important in the preparation of vaccines. The ordinary nonstabilized polio-vaccine must be stored at freezing temperatures to preserve its potency. However, with the addition of salts for stabilization of the virus, potency can be maintained for weeks at ambient temperatures, even in the high temperatures of the tropics.

Heating of some virus preparations in the presence of high salt concentrations can be used to remove adventitious agents. For example, heating poliovirus suspensions in molar $MgCI_2$ will inactivate such simian contaminants as SV40, foamy virus, and herpes B virus but has no deleterious effect on the infectivity and potency of poliovirus.

PH. Viruses are usually stable between pH values of 5.0 and 9.0. In hemagglutination reactions, variations of less than one pH unit may influence the result.

Radiation. Ultraviolet, x-ray, and high-energy particles inactivate viruses. The dose varies for different viruses.

Vital Dyes. Viruses are penetrable to a varying degree by vital dyes such as toluidine blue, neutral red, and proflavine. These dyes bind to the viral nucleic acid, and the virus then becomes susceptible to inactivation by visible light. Impenetrable viruses like poliovirus, when grown in the dark in the presence of vital dyes, incorporate the dye into their nucleic acid and are then susceptible to photodynamic inactivation. The coat antigen is unaffected by the process.

Ether Susceptibility. Ether susceptibility can distinguish viruses that possess a lipid-rich envelope from those that do not. The following viruses are inactivated by ether: herpes-, orthomyxo-, paramyxo-, rhabdo-, corona-, retro-, arena-, toga-, and bunyaviruses. The following viruses are resistant to ether: parvo-, papova-, adeno-, picorna-, and reoviruses. Poxviruses vary in sensitivity to ether.

Antibiotics. Antibacterial antibiotics and sulfonamides have no effect on viruses. However, rifampin can inhibit pox virus replication.

Antibacterial Agents. Quaternary ammonium compounds are not effective except for a few viruses. Organic iodine compounds are also ineffective. Larger concentrations of chlorine are required to destroy viruses than to kill bacteria, especially in the presence of extraneous proteins. For example, the chlorine treatment of stools adequate for typhoid bacilli is inadequate to destroy poliomyelitis virus

present in feces. Formalin destroys resistant poliomyelitis and coxsackieviruses. Alcohols such as isopropanol and ethanol are relatively ineffective against certain viruses, especially picornaviruses.

REPLICATION OF VIRUSES

Viruses multiply only in living cells. The host cell must provide the energy and synthetic machinery and also the low-molecular-weight precursors for the synthesis of viral proteins and nucleic acids. The viral nucleic acid carries the genetic specificity to code for all the virus-specific macromolecules in a highly organised fashion. In some cases, as soon as the viral nucleic acid enters the host cell, the cellular metabolism is redirected exclusively toward the synthesis of new virus particles. In other cases the metabolic processes of the host cell are not altered significantly, although the cell synthesises viral proteins and nucleic acids.

During the replicative cycle, viruses transfer genetic information in several ways from one generation to another. The essential theme, however, is that specific mRNAs must be transcribed from the viral nucleic acid for successful expression and duplication of genetic information. Once this is accomplished, viruses use cell components to translate the mRNA. Various classes of viruses use different pathways to synthesize the mRNAs depending upon the structure of the viral nucleic acid. Some viruses (eg, rhabdo-viruses, myxoviruses) carry RNA polymerases to synthesise mRNAs. RNA viruses of this type are called negative-strand viruses, since their single-strand RNA genome is complementary to messenger RNA, which is conventionally designated positive-strand. Table 3 summarises the various pathways of transcription (but not necessarily those of replication) of the nucleic acids of different classes of viruses. Most of the viral mRNAs possess a sequence of polyadenylic acid [Poly (A)] at their 3'-end and an unusual blocked, methylated structure at the 5'-end called a cap. The precise function of these features is yet to be elucidated, but the capped structure appears to enhance initiation of translation. Viral mRNA is not always an exact copy of the genome template, since some mRNAs are processed or spliced to delete certain sequences.

Table 7. Pathways of nucleic acid transcription for various virus classes.

Type of Viral Nucleic Acid	**Intermediates**	**Type of mRNA**	*Example*	**Comments**
± DS DNA	None	+mRNA	Most DNA viruses (eg, herpesvirus, T4 bacteriophage)	
+ SS DNA	± DS DNA	+mRNA	□X bacteriophage	See Bacteriophage
± DS RNA	None	+mRNA	Reovirus	Virion contains RNA

Type of Viral Nucleic Acid	Intermediates	Type of mRNA	*Example*	Comments
				polymerase that transcribes each segment to mRNA.
+ SS RNA	± DS RNA	+mRNA	Picornaviruses, togaviruses	Viral nucleic acid is infectious and serves as mRNA. For togaviruses, smaller + mRNA is also formed for certain proteins.
-SS RNA	None	+mRNA	Rhabdoviruses, paramyxoviruses, orthomyxoviruses	Viral nucleic acid is not infectious; virion contains RNA polymerase which forms + mRNAs smaller than the genome. For orthomyxoviruses, + mRNAs are transcribed from each segment.
+ SS RNA	- DNA,±DNA	+mRNA	Retroviruses	Virion contains reverse transcriptase; viral RNA is not infectious but complementary DNA from transformed cell is.

DS = double-stranded; SS = single-stranded; "–" indicates negative strand; "+" indicates positive strand;
"±" indicates a helix containing a positive and a negative strand

Virus multiplication was first studied successfully in bacteriophages. For animal viruses, some of the steps of the interaction between the infecting virus and susceptible cells have now been elucidated.

The following sections describe the replication of an RNA and a DNA virus.

RNA Virus Replication (Fig 66). Poliovirus contains a single-stranded RNA as its genome. All of the steps are independent of host DNA and occur in the cell cytoplasm. Polioviruses adsorb to cells at specific cell receptor sites (step 1), losing in the process one virus polypeptide (VP4), which may, therefore, be important in adsorption. The sites are specific for virus coat-cell interactions. Whereas intact poliovirus infects only primate cells in culture, the isolated RNA also infects nonprimate cells (rabbit, guinea pig, chick) and completes one cycle of multiplication. Multiple cycles of infection are not observed in nonprimate cells because the resulting progeny possess protein coats and will again infect only primate cells. After attachment, the virus particles are taken into the cell by viropexis (similar to

pinocytosis) (step 2), and the viral RNA is uncoated (step 3). The single-stranded RNA then serves as its own messenger RNA. This messenger RNA is translated (step 4), resulting in the formation of an RNA polymerase that catalyzes the production of a replicative intermediate (RI), a partially double-stranded molecule consisting of a complete RNA strand and numerous partially completed strands (step 5). At the same time, inhibitors of cellular RNA and protein synthesis are produced. Synthesis (+) and (—) strands of RNA probably occurs by similar mechanisms; this is completely elucidated only (+) strands. Here the RI consists of one complete (strand and many small pieces of newly synthesized (strand RNA (step 6). The replicative form (RF) consists of 2 complete RNA strands, one (+) and one (-).

The single (+) strand RNA is made in large amounts and may perform any one of 3 functions: serve as messenger RNA for synthesis of structural proteins, (b) serve as template for continued RI replication, or (c) become encapsidated, resulting mature progeny virions. The synthesis of viral cap proteins (step 7) is initiated at about the same time RNA synthesis.

The entire poliovirus genome acts as its own mRNA, forming a polysome of ~350S, and is translated to form a single large polypeptide that is processed during and after translation to form the various viral polypeptides. Thus, the poliovirus genome serves as a polycistronic messenger molecule. The giant polypeptide is cleaved to form a capsid precursor protein and 2 noncoat proteins one of which undergoes further processing. The capsid precursor protein cleaved into coat proteins VPO, VP 1, and VP3. During encapsidation, VPO is cleaved into coat proteins VP2 and VP4. Completion of encapsidation (step 8) produces mature virus particles that are then released when cell undergoes lysis (step 9).

DNA Virus Replication (Fig 5). In pox virus replication, synthesis of virus components and assembly of virus particles occur wit the cytoplasm of the infected cell. The replication of other DNA viruses (including the adeno-, herpes-, and papovavirus families) differs in that viral DNA is replicated in the nucleus, where viral proteins are synthesized in the cytoplasm, 1 lowed by their migration to and assembly within nucleus. Fig.5 shows the steps in the replication adeno virus, a double-stranded DNA virus. Adsorption (step 1) and penetration (step 2) of the virus into cell are similar to steps described for poliovirus. addition to viropexis, enveloped viruses penetrate fusion of the virus envelope with the plasma membrane, releasing the nucleocapsid into the cytoplasm.

After the virus enters the cell, the protein coat removed (step 3), presumably by cellular enzymes and the viral DNA is released into the nucleus. One both DNA strands are transcribed (step 4) into spec mRNA, which in turn is translated (step 5) to synthesize virus-specific proteins, such as a tumor anti, and enzymes necessary for synthesis of virus DNA. This period encompasses the early virus functions. Host cell DNA synthesis is temporarily elevated an then suppressed as the cell shifts over to the manufacture of viral DNA (step 6). As the viral DNA continues to be transcribed, Iate virus functions become apparent. Messenger RNA transcribed during the later phase of infection <step 6) migrates to the cytoplasm and is translated (step 7). Proteins for virus capsids are synthesized and are transported to the nucleus to be:

incorporated into the complete virion (step 8*)*. The migration of some structural proteins of certain viruses from the cytoplasm to the nucleus can be inhibited when arginine is absent from. the growth medium. Assembly of the: protein subunits around the viral DNA results in the formation of complete virions (step *9)*, which are released after cell lysis.

Summary of Viral RepIication. The molecular events that have been discussed above are summarized in Fig. 67. Virus genomes containing double-stranded (ds) nucleic acid proceed along most of the: steps shown in this figure.

Viruses with single-stranded (ss) nucleic acid only some of the steps. For the orthomyxovirus, the RNA template is utilized for the synthesis of complementary RNA strand that produces the replicative form of the nucleic acid. This in turn serves template for the synthesis of the progeny viral RNA. For the retroviruses, the ssRNA acts as a template for the RNA-dependent DNA polymerase (revertase transcriptase) to synthesize dsDNA. The dsDNA molecules are then used as templates for the transcription and synthesis of ssRNA molecules that serve either as viral mRNA molecules or as viral genomes for encapsidation by the viral structural proteins.

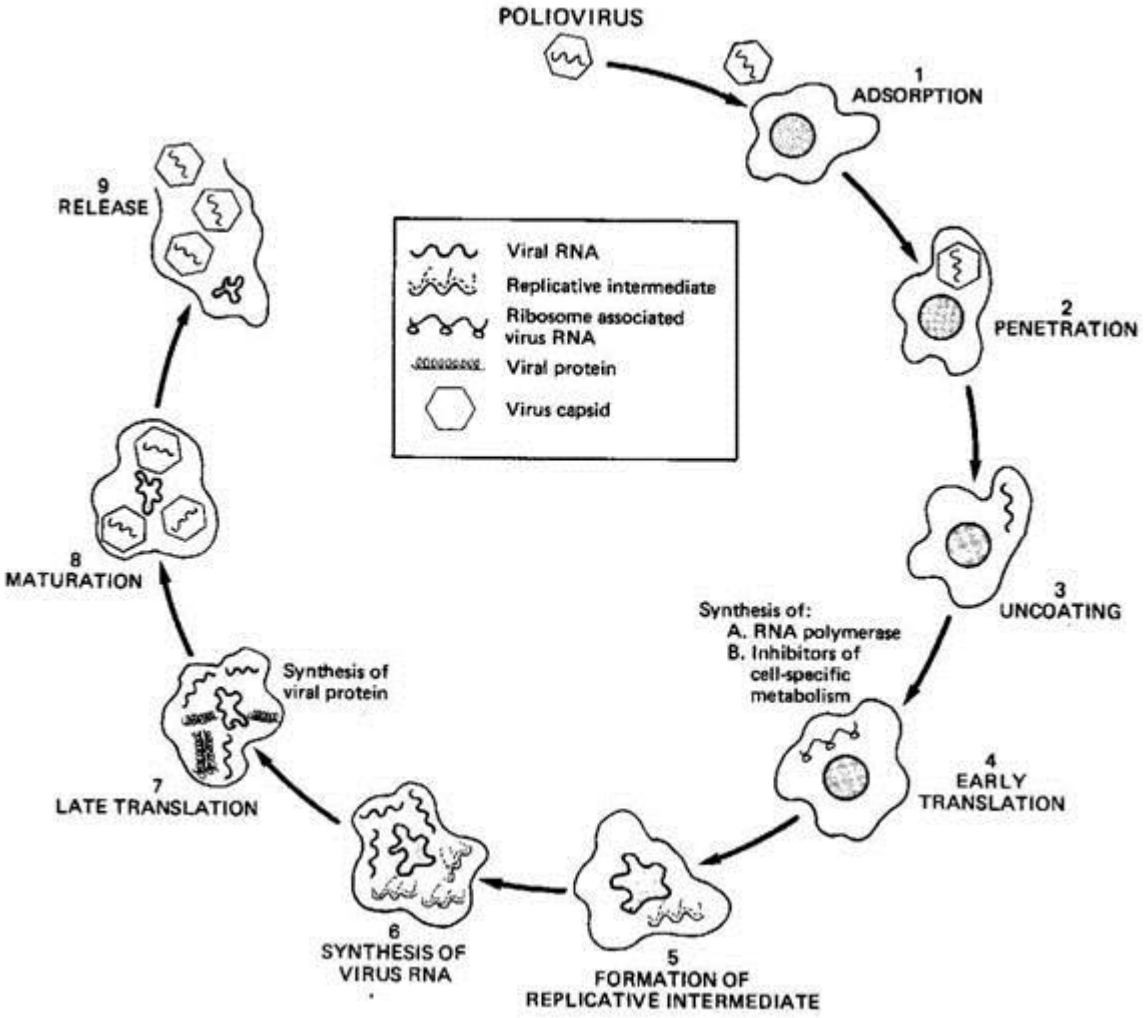


Figure 66. Replication of poliovirus, which containing an RNA genome

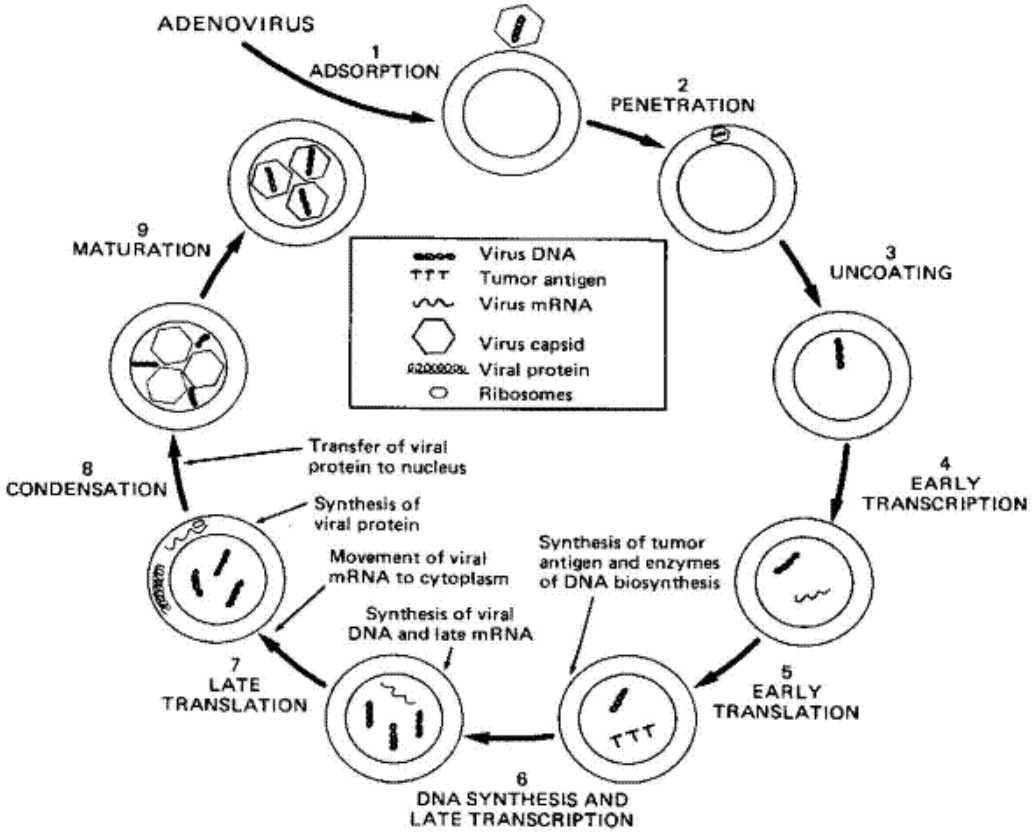


Figure 67. Steps in the replication of adenovirus, which contains DNA in its genome

Control Mechanisms of Virus Replication. In the course of virus replication, all the virus-specified macromolecules are synthesized in a highly organized sequence, although virus components are usually made in excess. In some virus infections, early viral proteins are synthesized soon after infection and late proteins are made only late in infection, after viral DNA synthesis. Early genes may or may not be shut off when late products are made. In addition to these temporal controls, quantitative controls also exist, since not all virus proteins are made in the same amounts. Virus-specific proteins may regulate the extent of transcription of genome or the translation of viral messenger RNA. .Although the exact mechanism of these controls is unknown, we do know something about the mechanism of mRNA synthesis. Small animal viruses and bacteriophages are good models for study of gene expression. Their small size has enabled the total nucleotide sequence of a few small DNA phages and SV40 to be elucidated. This led to the discovery that some pieces of DNA are expressed twice by mRNA, being read off either in 2 reading frames or by 2 mRNA molecules with different starting points being read in the same frame.

One surprising discovery has been the observation that animal "virus mRNA molecules (at least for adeno virus and SV40) are not direct copies of their DNA genomes. In these viruses, the mRNA sequences coding for a given protein are

preceded in the mRNA molecule by short sequences from farther "upstream" on the DNA template, with intervening sequences spliced out. This suggests the possibility that modification and control of virus gene expression could occur at the level of mRNA construction or "splicing".

Laboratory diagnosis of viral infections is based on either detecting a causative agent or demonstrating specific antibodies in the blood.

Virus isolation from a patient is performed, using cell cultures, chicken embryos, or experimental animals. Isolated viruses are identified with such serological tests as virus neutralization, haemagglutination inhibition, and others.

Specific antiviral antibodies in patients' blood are studied over time, using paired sera. An increase in the serum antibody titre becomes diagnostically significant only when it is of at least a fourfold magnitude. Rapid diagnostic techniques (immunoelectron microscopy, immunofluorescence test, radioimmunoassay, enzyme-linked immunosorbent assay, etc.) are increasingly gaining in importance.

VIRUS DETECTION AND IDENTIFICATION IN CELL CULTURES

There are primary, diploid, and continuous cell cultures. *Primary cultures* are obtained directly from animal or human tissue by breaking the intercellular substance with proteolytic enzymes (trypsin, collagenase, pronase). Dissociated (dispersed) cells placed in a culture medium are capable of adhering to the surface of a culture vessel and of proliferating there. Since cells of most primary cultures remain viable for several generations, they may be repeatedly subcultured (passaged). Several passages may produce a *diploid culture*, i.e., a population of fibroblast-like cells which can be rapidly reproduced and endure 30 to 60 passages still retaining their initial sets of chromosomes. Human diploid cells are highly sensitive to numerous viruses and are extensively used in virology. Both human (WI-38, MRC-5, MRC-9, IMR-90, etc.) and animal (cow, swine, sheep, and lamb) diploid cell cultures have been obtained.

Continuous cell cultures can be subcultured endlessly. They are derived from the primary cultures of cells due to their genetic variability during the growing process, rapidly become dominant in the cell population, and have chromosomal sets typical of all continuous cell lines. Continuous (stable) cell cultures have been obtained from various normal and neoplastic human tissues: amnion (A-0, A-1**,** FL), kidneys (Rh), cervical carcinoma (HeLa), laryngeal carcinoma (Hep-2), bone marrow from patients with lung cancer (Detroit-6), human embryo rhabdomyosarcoma (RD), etc.

Continuous cell lines are stored in liquid nitrogen and thawed before use.

Cells are cultivated in glass or plastic vessels of various size and shape, preferably disposable, with sterility strictly observed at all stages of cultivation. Nutrient media for cell cultures contain the whole range of amino acids, vitamins, and growth factors. Commercially available are fluid (medium 199, Eagle's medium, lactic albumin hydrolysate) and dry media or concentrates which are dissolved before use.

There are growth and maintenance culture media. Cell cultures are grown, using growth media enriched with human or animal sera, e.g., bovine or foetal (embryonic) cow serum. The serum makes up 2 to 30 per cent of the medium, depending on the properties of the cell culture and composition of the medium.

The maintenance media are used to preserve the established cell monolayers during virus inoculation. These media contain a smaller amount of serum, or they are added to the culture without it. Before the medium is used, antibiotics are added to it in order to prevent the growth of possible extraneous microorganisms. Culture media are sterilized; if they contain unstable constituents, filtration is carried out. Using buffer systems (commonly, bicarbonate buffer), the pH of the medium is maintained at 7.2-7.6. An indicator is added to the media, e.g., phenol red which becomes orange-yellow in acid medium or crimson in alkaline medium.

Obtaining Cell Culture

Primary cell cultures are obtained from any animal or human embryonic tissue, since embryonic cells have a high ability for growth and proliferation. Cultures are commonly prepared of a mixture of several tissues, such as skin, bone, and muscle. Commonly used are human embryonic fibroblasts, chicken embryonic fibroblast, and human kidney cells. Human embryonic tissues from aborted pregnancies and 8-12-day-old chicken embryos are used for cell cultures.

Preparing cell suspension. The tissue is washed in Hanks' solution or antibiotic-containing phosphate buffer to remove blood, fat, cell detritus or other admixtures, chopped with scissors, and washed once again until the solution **is** clear. Then, it is immersed in trypsin (200-300 ml per 100 g of tissue) and dispersed with a magnetic mixer or pipette. The supernatant containing trypsin-treated cells is decanted and stored in a refrigerator at 4 °C.

Trypsinization is repeated several times. The cell suspension is centrifuged at 600 X g for 5-10 min, resuspended in the medium, and stained with fuchsine, methylene blue, or other dyes. Cell concentration is determined in a Goryaev counting chamber. The suspension is diluted with nutrient medium to a concentration of 400 000-800 000 cells per ml, dispensed into culture flasks, tightly stoppered with rubber plugs, and incubated at 35-37 °C for 48-96 hours (flasks are tilted at a 5 degree angle); thereafter, cultures with well-formed monolayers **are** harvested.

Passage of continuous (stable] cell cultures. After pipetting off the nutrient medium, pour solution of 0.25 per cent trypsin or 0.02 per cent versene warmed to 37 °C into cell-containing flasks and place them in an incubator for 3-5 min. Then, remove trypsin or versene, add a small amount of the nutrient medium into the vessel, and make the cells suspend in the medium by vigorous shaking. Count the cells, adjust the cell concentration to the required level, and dispense the continuous culture into new flasks.

Suspension cultures. Most continuous cell cultures can proliferate in the medium in a suspended state, which is achieved by automatic rotation in a special drum or cultivation in fermenters.

For virus isolation, the medium is decanted from test tubes with established monolayers, and cells are washed several times with Hanks' solution to remove serum

antibodies and inhibitors. A 0.1-0.2-ml portion of the tested material, examined for sterility and appropriately pretreated for virological examination, is placed in every test tube. At least two tubes are used for one test. Some 30-60 min after inoculation, 1 ml of maintenance medium is added to each tube which is then placed in a 37 °C incubator.

If the examined material (e.g., faeces) has a toxic effect on the cell monolayer, it is initially diluted with 1 ml of nutrient medium and, after a 30-60-min exposure to the cell culture, is removed and replaced with the maintenance medium (fig. 68).

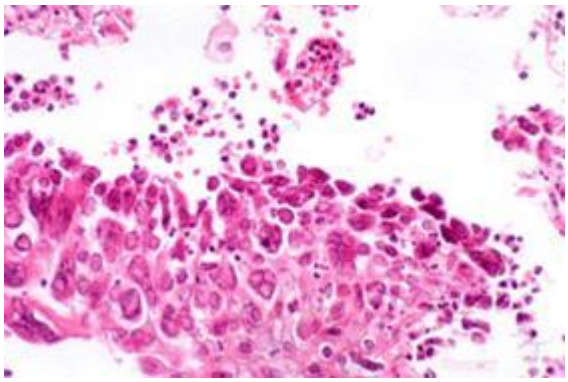


Figure 68. Obtaining Cell Culture

Virus Detection

Detection of viruses in the cell culture is based on their cytopathic effect, electron microscopic identification of intracellular inclusions, immunofluorescence, and haemadsorption and also on interference and the "plaque" formation phenomena. A positive haemagglutination test indicates the presence of a virus in the culture fluid.

A cytopathic effect (CPE) represents degenerative cell alterations resulting from intracellular virus reproduction. It is manifested within the first days after cell culture inoculation with some viruses '(variola, polio, etc.) and much later (in 1-2 weeks) when others (adenoviruses, parainfluenza viruses, etc.) are used. The nature of CPE primarily depends on a virus species.

Monolayer cell degeneration is subdivided into total and partial. ***Total degeneration*** due to such viruses as polio, Coxsackie and ECHO significantly affects monolayer cells, with great numbers of them sloughing off the slide. The remaining separate cells are shrunken (nuclear and cytoplasmic pyknosis) and characterized by double refraction, i.e., strong fluorescence on microscopy.

Partial degeneration of cultured cells falls into several types:

(1) *Racemose formation:* rounding, enlargement, and partial confluence of cells producing characteristic racemose aggregates. Degeneration of this type is caused by adenoviruses.

(2) *Focal degeneration:* local cell injuries (microplaques) appearing against the background of a largely intact monolayer. This type of degeneration is induced by certain strains of variola, variola vaccines, and influenza viruses.

(3) *Symplast formation:* virus-induced cell aggregation resulting in the formation of giant multinuclear cells (symplasts or syncytia). Symplast formation is caused by measles, mumps, parainfluenza, respiratory-syncytial, and herpes viruses.

Certain oncogenic viruses cause malignant transformation of cells provoking their intense proliferation, in other words *changes of a proliferative type.*

If the CPE in infected cell cultures is absent or mild, "blind passages" are performed, i.e., new cell cultures are inoculated with the culture fluid.

Intracellular inclusions occur when certain viruses are reproduced in cell nuclei and cytoplasm (variola, rabies, influenza, herpes viruses, etc.). They are detected by light microscopy after staining a monolayer-carrying slide with the Romanowsky-Giemsa solution or with other dyes, or by the luminescent microscopy, using acridine orange (1:20000).

Depending on a virus type, solitary virions or their crystalloid clusters can. be visualized in cell nuclei and cytoplasm with the electron microscope.

A specific virus antigen can be detected in virus-infected cell cultures. using the direct immunofluorescence test.

Plaque formation. Plaques, or negative virus colonies, are sites of virus-destroyed cells in the agar-coated monolayer. Infective virus activity is quantified by counting these colonies (fig. 69).

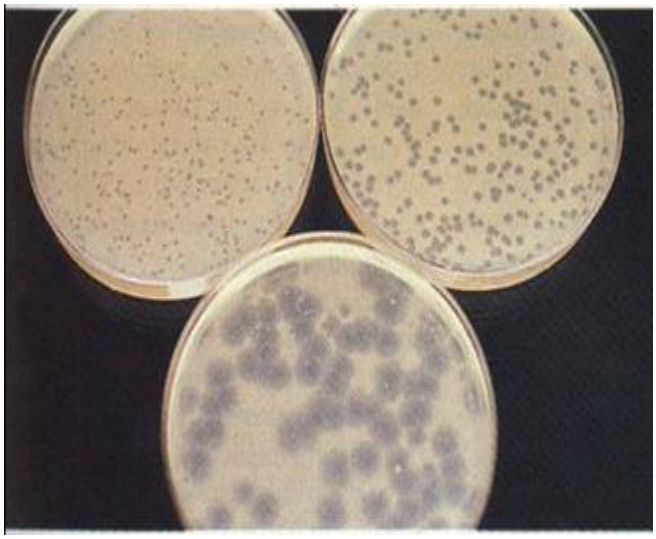


Figure 69. Plaque formation

To obtain the plaques, different dilutions of virus suspension are streaked onto one-layer tissue cultures in Hat vials or Petri dishes and overlaid with a layer of nutrient agar; virus reproduction and CPE are thus confined to initially infected and adjacent cells. Sites of cell degeneration, i.e., plaques, are identified by staining the culture with neutral red which is either included in the composition of the agar layer or added immediately before reading the results. Consisting of dead cells, the plaques are not stained with neutral red and, therefore, are recognized as light spots on a pink-red cell monolayer.

Other techniques of detecting virus plaques in cell cultures are also available, e.g., demonstration of plaque formation under a *bentonite layer*. Finely dispersed purified bentonite is added to a fluid nutrient medium, and the infected cell monolayer is immersed with this mixture. Because of adsorption of bentonite particles on cell surfaces, the monolayer becomes milk-coloured. At sites of virus reproduction (plaques), cells are not covered with bentonite, and are partially or completely stripped off the slide.

Virus plaques are identified under the bentonite nutrient layer, using monolayer cultures of continuous human or animal cells sensitive to the tested virus; 1-2-day-old thin cell monolayers are also suitable for this purpose.

Ten-fold dilutions of the material to be tested are prepared, and at least two culture matrasses (Erienmeyer flasks or penicillin vials) are inoculated with every dilution. After virus adsorption (30-40 min) the monolayers arc washed 3-4 times with sterile sodium chloride solution and coated with a bentonite nutrient layer: bidistilled water (415ml), 5-fiper cent bentonite gel (5 ml), Earle's solution, ten-fold concentrate (50ml), native bovine serum (15 ml), 7.5 per cent sodium hydrocarbonate solution (15 ml), penicillin (200 U/ml), streptomycin or lincomycin (100 U/ml).

An infected cell monolayer in 50-ml Erienmeyer flasks is covered with 20-30 ml and that on bottoms of penicillin vials with 5-6 ml o[bentonite.

Bentonite gel is obtained from dry mineral. Sorbent properties of bentonite are improved by impregnating it with sodium cations. Finally, it is sterilized for 40 min at 111 °C. Bentonite gel may be stored at room temperature for years without any changes in its sorbent properties.

Time of plaque formation under a bentonite layer varies with different viruses. Enterovirus plaque formation is evaluated, for example, in 36-48 hours. Culture flasks are inverted with the monolayer upward, washing degenerated cells away with the medium. Plaques formed by different enteroviruses vary in size, development, and margin patterns.

Since one infective viral particle (virion) produces one plaque, the plaque formation test accurately measures both the number of infective units in the specimen and the neutralizing activity of virus antibodies.

Haemagglutination test (fig. 70) is based on the ability of certain viruses to clump (agglutinate) red blood cells obtained from animals of definite species. Influenza and some other viruses with supercapsid membrane contain the surface antigen haemagglutinin responsible for the erythrocyte agglutination.

The HA test is performed in test tubes, on special plexiglass plates, and in a Takata apparatus. A virus-containing specimen is double-diluted in 0.5 ml of isotonic saline. Half a millilitre of 1°n erythrocyte suspension thrice washed in isotonic saline is added into all test tubes, and 0.5 ml of erythrocyte suspension is mixed with an equal volume of virus-free isotonic sodium chloride solution, to be used as control. The mixture may be incubated at 37°, 20° or 4 °C, depending on the properties of the tested virus.

Test results are assessed at 30-60 rain after complete erythrocyte sedimentation in the control, with the findings reading as follows:

(++++), intense and rapid erythrocyte agglutination with a star-like**,** marginally festooned sediment ("umbrella"}; (++4-), residue of erythrocytes has clearings; (++), a less marked residue; (+), a floccular sediment surrounded with lumps of agglutinated erythrocytes, and (–), a markedly localized erythrocyte sediment ("rouleaus"), as in the control.

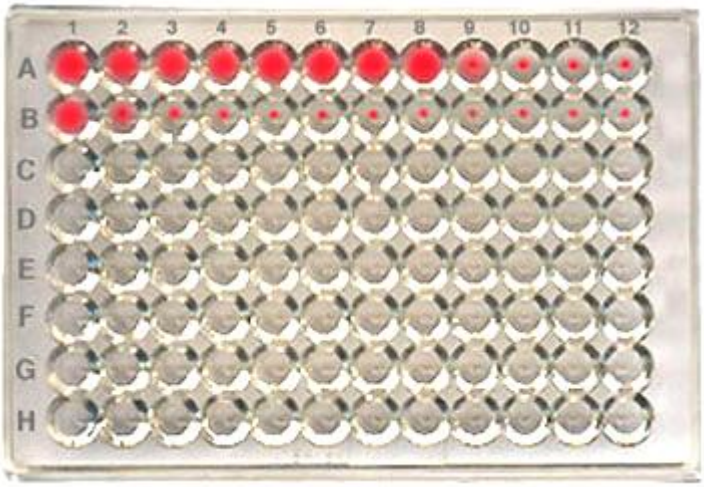


Figure 70. Haemagglutination test

Using HA**,** one can detect the presence of an agglutinating virus in the specimen and determine its titre. The virus titre is defined as the maximum virus dilution at which erythrocyte agglutination still occurs. This dilution is accepted as containing one haemagglutinating unit of the virus.

Results of the haemagglutination test are influenced by several factors: species and individual sensitivity of erythrocytes, temperature and pH of the medium, etc. Furthermore, erythrocyte haemagglutination may be induced by certain microorganisms, such as staphylococci, Escherichia, salmonella, shigella, cholera vibrio El Tor. Therefore, in cases where viruses are detected in bacteria-contaminated material, caution should be exercised while interpreting the results obtained.

Haemadsorption test (fig. 71) makes it possible to reveal the virus before the onset of CPE due to the appearance of the virus-specific antigen (haemagglutinin) on the surface of an infected cell. After a period of incubation appropriate for a virus, 0.2 ml of 0.5 per cent erythrocyte suspension is added to the cell culture (both control and virus-infected) so that the monolayer is covered, and the culture is stored for 15-20 min at 4°, 20° or 37 □C (depending on virus properties). Then, the test tubes are shaken in order to remove unadsorbed erythrocytes, and erythrocyte clusters are counted on single cells or throughout the monolayer by low-magnification microscopy. Uninfected cells should carry no erythrocytes.

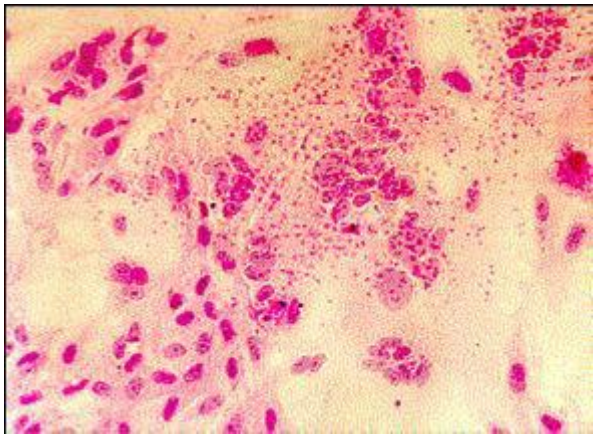


Figure 71. Haemadsorption test

VIRUS DETECTION AND IDENTIFICATION IN CHICKEN EMBRYOS

Virus demonstration in chicken embryos. Viruses are cultivated in 6-15-day-old chicken embryos. The material to be assayed is introduced with a syringe onto the chorio-allantoic membrane (CAM), into the yolk sac, and into the amniotic and allantoic cavity.

To infect the CAM (Fig. 72*)*, the eggshell is treated with iodine and alcohol, punctured above the air sac and a 7x2 mm opening is made laterally at the place of the greatest vascular ramification. Without destroying the shell-underlying membrane, 0.1-0.2 ml of virus-containing material is placed onto the CAM with a short thin needle. The damaged sites of the shell are coated with sterile paraffin or collodion. Then, the embryos are put into an incubator, putting the eggs horizontally.

For inoculation into the allantoic cavity the tested material is introduced through the lateral opening of the shell ID-15 mm deep.

When the amniotic cavity is to be inoculated, the virus-containing specimen is injected through the opening at the obtuse end of the egg; the needle should be directed toward the embryonic body so as to ensure penetration of the virus into different organs and tissues of the embryo. Puncture sites are sealed with paraffin or collodion.

The infected embryos are stored in an incubator at 35-37 °'C for 48-72 hrs, depending on the species of the assayed virus. Then, the eggs are cooled at 4 °C for 18 hrs for maximum constriction of the embryonic blood vessels and opened under sterile conditions. The amniotic and allantoic fluid is aspirated with a syringe, and the membranes and embryo are transferred into sterile Petri dishes.

Virus reproduction in chicken embryos is evidenced by characteristic changes on the CAM. Viruses in the amniotic and allantoic fluid can be recovered by means of HA.

Variola, variola vaccine, and herpes simplex viruses produce plaques on the CAM, which look like whitish convex spots 1 to 2 mm in diameter, with their number corresponding to the number of infectious particles.

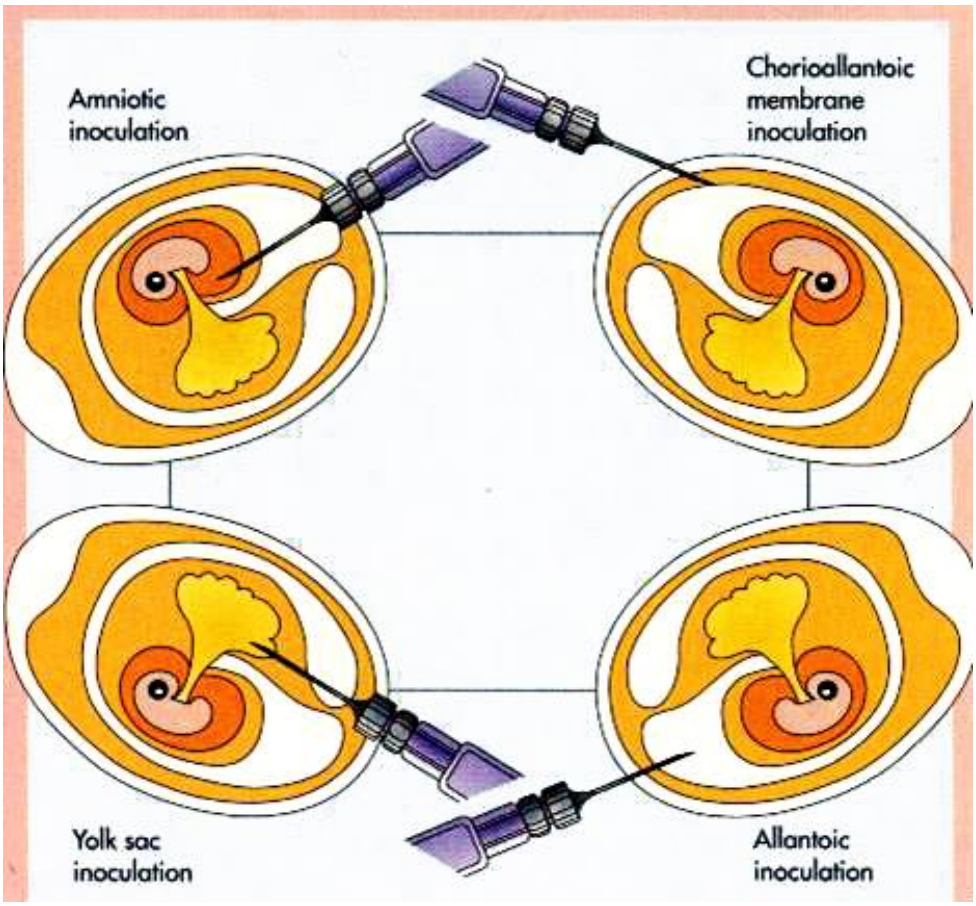


Figure 72. Different methods of chicken embryo inoculation

Erythrocyte agglutination induced by the allantoic and amniotic fluid of infected embryos is a marker of orthomyxovirus and paramyxovirus accumulation. Quantitatively, the virus is determined by the haemagglutination litre (maximum dilution of virus-containing fluid causing erythrocyte agglutination).

Viruses can be titrated on pieces of the chorio-allantoic membrane. Pieces of the shell from an 11-12-day-old chicken embryo with the intact CAM are placed in wells on sterile plates, 0.5-1 ml of virus-containing fluid diluted ten-fold with a buffer (NaCl (8.0 g), KC1 (0.6 g), glucose (0.3 g), $MgCl_2x6H_2O$ (0.05 g), $CaCl_2$-$6H_2O$ (0.8 ml), phenol red 1:100 (10.0 g), NaOH 1M (0.2 ml), gelatine (2.0 g), antibiotics) is added, and the plates are covered with foil and incubated at 35-37 °C. The shell is removed from the wells in 24-72 hrs and 0.5 per cent chicken erythrocyte suspension is added to the remaining medium. A positive HA test indicates virus reproduction (fig. 73).

Different methods of chicken embryo inoculation

Viruses cultivated in chicken embryos are identified with the aid of the *neutralization test* which is interpreted as positive if plaque formation is inhibited on the CAM and haemagglutination is absent; other identification techniques include the *HAI,PG, CF,* and *IF tests.*

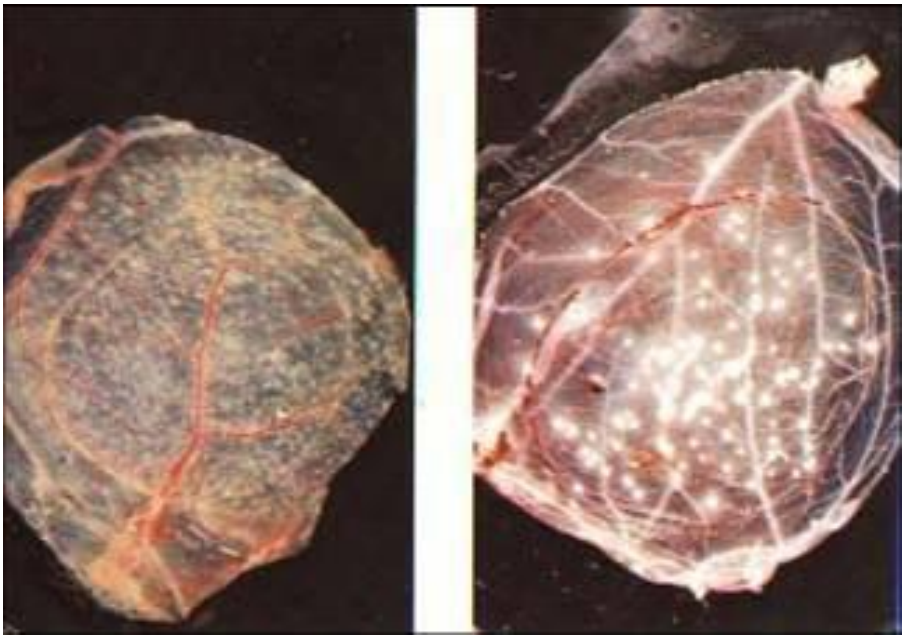


Figure 73. Virus demonstration in chicken embryos

According to the nature of the material to be tested and the procedures utilized, the methods for diagnosing viral infections may be categorized into rapid, viroscopic, virological, and serological (Table 8).

Table 8. Methods of the Diagnosis of Viral Infections

Method	Purpose of examination
Rapid diagnosis	Detection and identification of the virus-specific antigen arid diagnosis viral particles in the patient's material within 2-3 hrs, which is done with the aid of such methods as EM, IEM, IF, RIHA, ELISA, RIA, PG, and HadsSM. Detection and identification of the virus-specific antigen and viral particles in the patient's material or in biological systems following the preliminary cultivation of the virus with the help of EM, IEM, IF, RIHA, ELISA, RIA, PG. HA, HAI, and CF
Virological	Isolation of the virus through its cultivating in sensitive systems, enrichment for the virus, serological identification, and investigation of the biological properties of the virus by means of such reactions as N, CF, PG, HAI, IF, RIA, ELISA, Hads, and Hadsl
Serological	Determination of the growth in the anti-virus antibodies and identification of immunoglobulins by the CF, HAI, N, RH, IF, IHA, RIA, and ELISA tests

VIRUSES OF BACTERIA (BACTERIOPHAGES). MORPHOLOGY, CHEMICAL COMPOSITION, PHASE OF INTERACTION OF BACTERIOPHAGE AND BACTERIAL CELL. PHAGE CONVERSION. PRACTICAL IMPORTANCE OF THE PHENOMENON OF BACTERIOPHAGIA

Bacteria are host to a special group of viruses called bacteriophage, or «phage», which pass through fine-porous filters and develop at the expense of reproducing bacteria (N. Gamaleya, 1892; F. Twort, 1915; F. d'Herelle, 1917). Although any given phage is highly host-specific, it is probable that every known type of bacterium serves as host to one or more phages. Phages have not been successfully used in therapy. They are important, however, because they furnish ideal materials for studying host-parasite relationships, virus multiplication, and molecular genetics.

LIFE CYCLES OF PHAGE AND HOST

Figure 1 summarizes the potential life cycles of bacterial cells infected with double-stranded DNA phages. Single-stranded DNA phages and RNA phages are discussed in later sections. Fig 74 shows the following:

(1) **Life cycle of uninfected bacterium.** An uninfected bacterium may reproduce by binary fission, showing no involvement with phage.

(2) **Adsorption of free phage.** When an uninfected bacterium is exposed to free phage, infection will take place if the cell is sensitive. Bacteria may also be genetically resistant to phage infection; such cells lack the necessary receptors on their surfaces.

When infection takes place, the phage is adsorbed onto the cell surface and the nucleic acid of the phage penetrates the cell. In this state phage nucleic acid is called “vegetative phage”.

(3) **Lytic infection.** The injected vegetative phage material may be reproduced, forming many replicas. These mature be acquisition of protein coats, following which the host cell lyses and free phage is liberated.

(4) **Reduction of vegetative phage to prophage.** Many phages, termed “temperate”, are capable of reduction to prophage as alternative to producing a lytic infection. The bacterium is now lysogenic; after an indeterminate number of cell division, one of its progeny may lyse and liberate invective phage.

(5) **Loss of prophage.** Occasionally a lysogenic bacterium may lose its prophage, remaining viable as an uninfected cell.

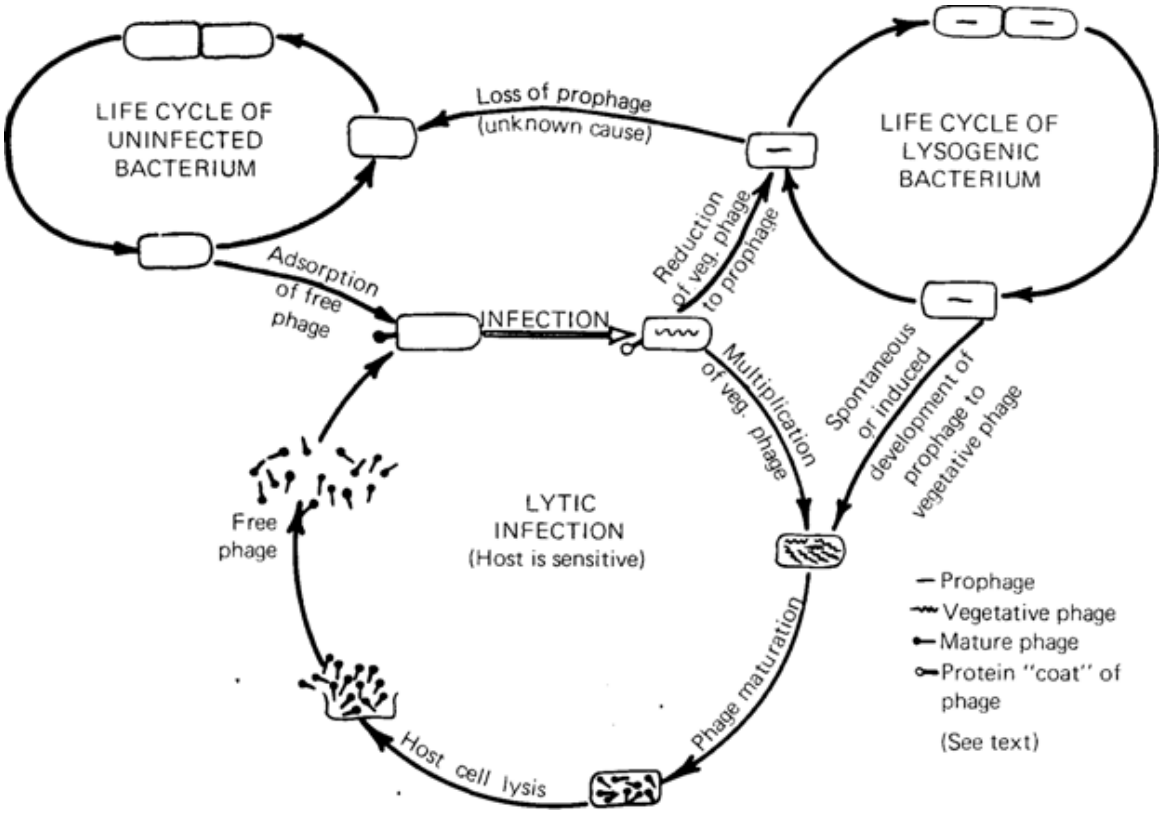


Figure 74. Phage-host life cycles

METHODS OF STUDY

Assay. Since phages (like all viruses) multiply only within living cells, and since their size precludes direct observation except with the electron microscope, it is necessary to follow their activities by indirect means. For this purpose, advantage is taken of the fact that one phage particle introduced into a crowded layer of dividing bacteria on a nutrient agar plate will produce a more or less clear zone of lysis in the opaque film of bacterial growth (Figure 75). This zone of lysis is called a «plaque»; it results from the fact that the initially infected host cell bursts (lysis) and liberates dozens of new phage particles, which then infect neighbouring cells. This process is repeated cyclically until bacterial growth on the plate ceases as a result of exhaustion of nutrients and accumulation of toxic products. When handled properly, each phage particle produces one plaque; any material containing phage can thus be titrated by making suitable dilutions and plating measured samples with an excess of sensitive bacteria. The plaque count is analogous to the colony count for bacterial titration.

Isolation and Purification. In order to study the physical and chemical properties of phage, it is necessary to prepare a large batch of purified virus as free as possible of host cell material. For this purpose, a liquid culture of the host bacterium is inoculated with phage and incubated until the culture is completely lysed. The now clear culture fluid, or lysate, contains in suspension only viral particles and bacterial debris. These materials are easily separated from each other by differential centrifugation. The centrifuged pellet of phage material can be resuspended and

washed in the centrifuge as often as needed and may then be used for chemical and physical analysis in the laboratory or for electron microscopy.

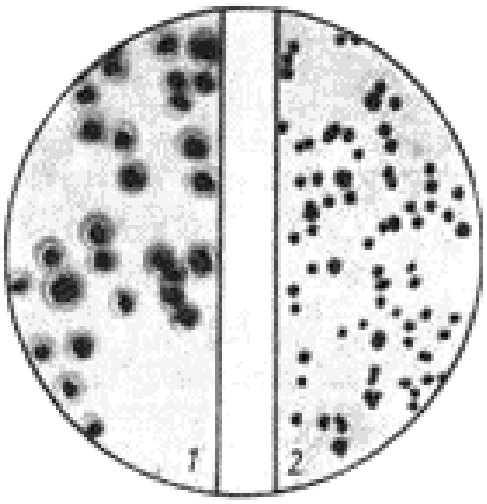


Figure 75. **Different phage plaque types (1 – large colonies; 2 – small colonies)**

PROPERTIES OF PHAGE

One group of phages has been studied more extensively than any other: certain phages that attack *Escherichia coli* strain B (coliphages). Of the numerous coliphages, 7 have been selected for intensive study. Unless otherwise noted, the information given below applies to this group, which has been numbered Tl through T7.

Morphology. A typical phage particle consists of a "head" and a "tail". The head represents a tightly packed core of nucleic acid surrounded by a protein coat, or capsid. The protein capsid of the head is made up of identical subunits, packed to form a prismatic structure, usually hexagonal in cross section. The smallest known phage has a head diameter of 25 nm; others range from 55 x 40 nm up to 100 X 70 nm.

The phage tail varies tremendously in its complexity from one phage to another. The most complex tail is found in phage T2 and in a number of other coli and typhoid phages. In these phages, the tail consists of at least 3 parts: a hollow core, ranging from 6 to 10 nm in width; a contractile sheath, ranging from 15 to 25 nm in width; and a terminal base-plate, hexagonal in shape, to which may be attached prongs, tail fibers, or both. Electron micrographs of phage preparations embedded in electron-dense material such as phosphotungstate show the phages to exist in 2 states: in one, the head contrasts highly with the medium, the sheath is expanded, and the base-plate appears to have a series of prongs. In the second state, the head is of low contrast, the sheath is contracted, and the base-plate is now revealed to have 6 fibers attached to it. The former state presumably represents active phage, containing nucleic acid; the latter state presumably represents phage that has ejected its nucleic acid (eg, into a host cell). These 2 states are diagrammed in Fig. 76.

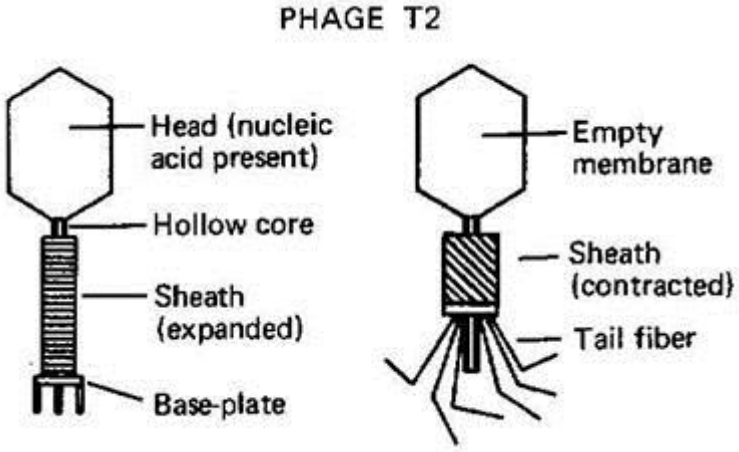


Figure 76. Diagrams of phage T2 on electron micrographic observation

A number of other tail morphologies have been reported. In some of these, sheaths are visible but the contracted state has not been observed; and in one case no sheath can be seen. The phages also vary with respect to the terminal structure of the tail: some have base-plates, some have «knobs,» and some appear to lack specific terminal structures.

The phage tail is the adsorption organ for those phages that possess them. Some phages lack tails altogether; in the RNA phages, for example, the capsid is a simple icosahedron.

Although most phages have the head-and-tail structure described above, some filamentous phages have been discovered that possess a very different morphology. One of these, called «fd,» has been characterized in some detail. It is a rod-shaped structure measuring 6 nm in diameter and 800 nm in length. It contains DNA and protein, which are complexed in a manner that is not completely understood. The DNA may be intertwined with the protein, rather than forming a core.

Chemistry. Phage particles contain only protein and one kind of nucleic acid. Most phages contain only DNA; however, phages that contain only RNA are also known. In the T-even phages, the nucleic acid makes up about 50% of the dry weight and consists of a single molecule (called the phage chromosome) with a molecular weight of 1.3×10^8, sufficient to code for about 200 different proteins of molecular weight 30,000. In phages T2, T4, and T6, a unique base (hy-droxymethylcytosine) is present to which are attached short chains of glucose units. This pyrimidine has never been found in the nucleic acid of the uninfected bacterial host.

The proteins that make up the head, the core, the sheath, and the tail fibers are distinct from each other; in each case, the structure appears to be made of repeating subunits.

An unusual phage called PM2 has been isolated from a culture of a marine pseudomonad. PM2 is a double-stranded DNA phage in which the virion is surrounded by a lipoprotein membrane and contains 2 enzymes: an endonuclease

that converts the phage DNA to the linear form within the host, and a DNA-dependent RNA polymerase.

The resistance of phages to physical and chemical factors is greater than that of the corresponding microbes. Phages withstand high pressures (up to 6000 atmospheres), are resistant to the action of radiant energy, and maintain their activity in a pH range from 2.5 to 8.5. In sealed ampoules phages do not lose their lytic properties for 5-6 and even 12-13 years and can be preserved for relatively long periods in glycerin. Phages perish quickly under the effect of boiling, acids, ultraviolet rays and chemical disinfectants. In relation to resistance phages are intermediate between the vegetative forms of bacteria and spores. Some substances (thymol, chloroform) and enzyme poisons (cyanide, fluoride, dinitrophenol) have no effect on phages, but cause bacteria to perish or inhibit their growth. These preparations are used for maintaining phages in cultures and for destroying bacteria, actinomycetes and fungi.

The specific action of phages. Phages possess both species and type specificity. On solid nutrient medium types Tl, T3, and T7 *E. coli* phages form large colonies, types T2, T4, and T6 produce small colonies. These types also differ morphologically.

The *classification of* phages is based on morphology, chemical structure, type of nucleic acid, and their interaction with the bacterial cell. All phages are divided into DNA- and RNA-containing. The relation of phages to sex differentiation of bacteria is taken into account in determination of additional taxonomic signs. It has been established that one group of phages affects only male bacteria (F^+), another group only female bacteria (F^-), while a third group of phages is indifferent in respect to sex differentiation of cells. Phages are marked by a specific effect on the corresponding bacterial species. Each phage has its own host in which it lives as a parasite and reproduces. Staphylococci have 40 phage types, *E coli* 50, *S. typhi* 56, S.*paratyphi A* 11, *S. schottmueleri B* 7, *Corynebacterium diphtheriae* 19, *Vibrio cholerae* 9, etc.

PHAGE REPRODUCTION

The bacteriophage phenomenon depends on the age of the culture, the concentration of bacteria, phage activity, bacterial phage resistance, composition of the nutrient medium, temperature and other factors. It is manifested in four main phases occurring in succession: adsorption, penetration into the cell, intracellular development, and liberation of phages.

Adsorption. The kinetics of phage adsorption have been thoroughly analyzed, and the process has been shown to be a first-order reaction; the rate of adsorption is proportional to the concentration of both the phage and the bacterium. Under optimal conditions, the observed rates are compatible with the assumption that almost every collision between phage and host cell results in adsorption. If the bacteria are mixed with an excess of phage, adsorption will continue until as many as 300 particles are adsorbed per cell.

Before the phage can be adsorbed onto the host cell, the phage surface must be modified by attachment of positively charged cations (the nature and number of cations varying from one phage to another, for example, bivalent cations Ca^{++}, Mg^{++}) and, in some cases, the amino acid tryptophan. Each phage is quite specific with regard to the cofactors required for adsorption.

The bacterial surface, i.e., the cell wall, is complex and heterogeneous. In gram-negative bacteria, there appear to be 3 distinct layers: an inner layer composed of peptidoglycan, the outer membrane, and lipopolysaccharide. Different bacterial strains are highly specific with regard to the phages that they will adsorb. For example, a strain able to adsorb phages T2, T4, and T6 can give rise to mutants unable to adsorb one or another of these viruses. This specificity has been found to reside in the cell wall; when cell walls are isolated and purified, they exhibit the same adsorption patterns as the cells from which they are prepared. The factors in the cell wall responsible for adsorption appear to be discrete, localized «receptors»; the receptors for phages T3, T4, and T7 reside in the lipopolysaccharide layer, whereas the receptors for phages T2 and T6 reside in the outer membrane. Ability to adsorb phage is obviously a factor in the determination of bacterial sensitivity to infection.

In certain phages (eg, phages T2, T4, T6), the attachment of phage particles (or of empty phage capsids) causes a profound change in the cell membrane: at low phage multiplicities, the membrane becomes permeable to small molecules; and at high multiplicities the cell lyses («lysis from without»). Even a single phage or ghost particle will affect the membrane, causing not only a permeability change but also the inhibition of host DNA and protein synthesis.

Penetration. Phages with contractile tails, such as the T-even phages (Fig 9-3), behave as hypodermic syringes, injecting the phage DNA into the cell. In phage T4, it has been found that the triggering of DNA injection requires the maintenance of a membrane potential by the host cell.

The filamentous DNA phages penetrate the host cell by a different mechanism. The entire phage structure penetrates the cell wall; the major protein of the phage coat is then deposited on the cell membrane, which is penetrated by the phage DNA. A minor coat protein enters the cytoplasm along with the DNA.

Intracellular Development of DNA Phages. Some phages always lyse their host cells shortly after infection, generally in a matter of minutes and usually before the host cell can divide again. (See «lytic infection» cycle in Fig 1.) The process of intracellular development is as follows:

(1) For several minutes following infection (eclipse period), active phage is not detectable by artificially induced premature lysis (eg, by sonic oscillation). During this period, a number of new proteins («early proteins») are synthesized. These include certain enzymes necessary for the synthesis of phage DNA: a new DNA polymerase, new kinases for the formation of nucleoside triphosphates, and a new thymidylate synthetase. The T-even phages (T2, T4, T6), which incorporate hydroxymethylcytosine instead of cytosine into their DNA, also cause the appearance of a series of enzymes needed for the synthesis of hydroxymethylcytosine, as well as an enzyme that destroys the deoxycytidine triphosphate of the host. Later on in the

eclipse period, «late proteins» appear, which include the subunits of the phage head and tail as well as lysozyme that degrades the peptidoglycan layer of the host cell wall. All of these enzymes and phage proteins are synthesized by the host cell using the genetic information provided by the phage DNA.

(2) During the eclipse period, up to several hundred new phage chromosomes are produced; as fast as they are formed, they undergo random exchanges of genetic material (see below).

In many phages, the linear DNA molecule that enters the cell has cohesive ends consisting of short complementary base sequences. Base pairing of these cohesive ends converts the DNA from the linear to the circular form; circularization is completed by a ligase-catalyzed sealing of the single-stranded gaps. Replication then occurs in the circular state, by either a simple-circle or a rolling-circle mechanism.

(3) The protein subunits of the phage head and tail aggregate spontaneously (self-assemble) to form the complete capsid. In the case of a complex capsid such as that of phage T4, capsid formation results from the coming together of 3 independent subassembly lines: one each for the head, the tail, and the tail fibers. Each subassembly proceeds in a defined sequence of protein additions.

(4) Maturation consists of irreversible combination of phage nucleic acid with a protein coat. The mature particle is a morphologically typical infectious virus and no longer reproduces in the cell in which it was formed. If the cells are artificially lysed late in the eclipse period, immature phage particles are found in which the DNA and protein are not yet irreversibly attached, so that the DNA is easily removed.

Lysis and Liberation of New Phage. Phage synthesis continues until the cell disintegrates, liberating infectious phage. The cell bursts as a result of osmotic pressure after the cell wall has been weakened by the phage lysozyme. (The exceptions are the filamentous DNA phages, in which the mature virus particles are extruded through the cell wall without killing the host).

REPLICATION OF RNA PHAGES

When a molecule of viral RNA enters the cytoplasm of the host cell, it is immediately recognized as messenger RNA by the ribosomes, which bind to it and initiate its translation into viral proteins. One such viral protein is a complex enzyme, RNA polymerase. This enzyme brings about the replication of the viral RNA: it polymerizes the ribonucleoside triphosphates of adenine, guanine, cytosine, and uracil, using viral RNA as template.

The first step in the process of RNA replication is the formation of double-stranded intermediates, in which the entering viral RNA strand (called the “plus” strand) is hydrogen-bonded to the complementary «minus» strand synthesized by the polymerase. The polymerase now uses the double-stranded molecule as a template for the repeated synthesis of new plus strands, each new plus strand displacing the previous one from the double-stranded intermediate.

As the newly synthesized plus strands are released from the replicative intermediate, they are either used by the polymerase to form a new double-stranded

intermediate or are assembled into mature virions by the attachment of coat protein subunits.

The complete nucleotide sequence of one RNA phage, MS2, has been determined. It is a single molecule, 3566 nucleotides in length, and contains 3 functional genes coding respectively for the RNA polymerase, the coat protein, and a second protein called the «A protein.» The single-stranded RNA molecule is capable of folding back on itself and forming double-stranded regions by base-pairing; the secondary structure that results appears to play a major role in the regulation of viral RNA replication and translation.

PHAGE GENETICS

Phage particles exhibit the same 2 fundamental genetic properties that are characteristic of organized cells: general stability of type and a low rate of heritable variation.

Phage Mutation. All phage properties are controlled by phage genes and are subject to change through gene mutation. Most of our knowledge concerning the chemical basis of mutation comes from studies on phage genetics.

Phage Recombination. If a bacterium simultaneously adsorbs 2 related but slightly different DNA phage particles, both can infect and reproduce; on lysis, the cell releases both types. When this occurs, many of the progeny are observed to be recombinants. Recombination takes place between pairs of phage DNA molecules and is repeated many times between different, random pairs of replicating phage DNA before maturation. Three-way recombinants are therefore possible in a cell si-multaneously infected with 3 parental phage types.

Genetic Maps. The relative positions on the phage chromosome of mutant loci involved in phage structure or phage reproduction can be determined by a combination of genetic and physical mapping procedures. In genetic mapping, 2 different mutants are propagated simultaneously in the same host cell, and the frequency of their recombination is measured: the lower the frequency, the shorter the distance between the 2 loci. In physical mapping, heteroduplexes are made between single strands of DNA from normal phage and deletion mutants; examination in the electron microscope reveals the location of the deletion in the form of a non-base-paired region.

Some procedures have been used to produce detailed maps of phage chromosomes. An example of such a map is given in Fig 9-5 for the phage X. The genes lettered A-W on the map were originally identified as conditional lethal mutations that were suppressed in a host strain carrying a particular suppressor gene; their functions were later identified by electron microscopy and biochemical analyses.

Phage genomes vary widely in size. The smallest known phage genome, that of the RNA phage MS2, has only 3 genes, as described above. In contrast, the largest phages contain sufficient DNA to code for about 200 proteins of average size; genes are present for coat proteins, morphogenesis, enzymes and regulators of phage replication, glycosylation of phage DNA, inhibitors of host restriction enzymes,

enzymes that degrade host DNA, DNA repair enzymes, recombination enzymes, and proteins involved in integration and excision of prophage DNA.

LYSOGENY

Prophage. Earlier in this chapter it was mentioned that some phages ("temperate phages") fail to lyse the cells they infect and then appear to reproduce synchronously with the host for many generations. Their presence can be demonstrated, however, because every so often one of the progeny of the infected bacterium will lyse and liberate infectious phage. To detect this event it is necessary to use a sensitive indicator strain of bacterium, i.e., one that is lysed by the phage. The bacteria that liberate the phage are called "lysogenic"; when a few lysogenic bacteria are plated with an excess of sensitive bacteria, each lysogenic bacterium grows into a colony in which are liberated a few phage particles. These particles immediately infect neighbouring sensitive cells, with the result that plaques appear in the film of bacterial growth; in the centre of each plaque is a colony of the lysogenic bacterium.

A culture of lysogenic bacteria can also be centrifuged, removing the cells and leaving the temperate phage particles in the supernatant. Their number can be measured by plating suitable dilutions of the supernatant on a sensitive bacterial indicator strain and counting typical plaques.

The release of infectious phage in a culture of lysogenic bacteria is restricted to a very few cells of any given generation. For example, in one bacterial type about 1 in 200 lyse and liberate phage during each generation; in another type it may be 1 in50,000. The remainder of the cells, however, retain the potentiality to produce active phage and transmit this potentiality to their off spring for an indefinite number of generations.

With the rare exceptions mentioned, lysogenic bacteria contain no detectable phage, either as morphologic, serologic, or infectious entities. However, the fact that they carry the potentiality to produce, generations later, phage with a predetermined set of characteristics means that each cell must contain one or more specific noninfectious structures endowed with genetic continuity. This structure is termed «prophage.»

The Nature of Prophage. Two entirely different prophage states are found in different phages. In one state, discovered in phage X, the prophage consists of a molecule of DNA integrated with the host chromosome. The chromosomes of *E coli*and of phage X are circular; the length of the phage chromosome is about one-fiftieth that of the bacterial chromosome. Both the phage and bacterial chromosomes carry a specific attachment site. The bacterial attachment site is immediately adjacent to the *gal* locus (see Fig 77); the phage attachment site is similarly located at a specific point on the phage genetic map. When infects a cell of *E coli*, recombination between the 2 attachment sites occurs, with the result that the 2 circles are integrated (Fig 4). This integration process requires the action of a phage gene product: phage mutants defective in this gene (the *int* locus) are unable to lysogenize the cell.

A number of coliphages are of the type: their prophages integrate with the host chromosome at specific attachment sites. One phage, called Mu, is unusual in that it is capable of integrating at totally random sites on the chromosome, including sites within bacterial genes. Such integrations result in the inactivation of the gene in question and produce the appearance of mutations.

In the other state, discovered in phage PI, the phage chromosome circularizes and enters a state of «quiescent» replication that is synchronous with that of the host; no phage proteins are formed. The prophage in the «PI type» of system is not integrated with the chromosome; its replication is analogous to that of plasmids.

Further Properties of the Lysogenic System

A. Immunity. Lysogenic bacteria are immune to infection by phage of the type already carried in the cell as prophage. When nonlysogenic cells are exposed to temperate phage, many permit phage multiplication and are lysed, while other cells are lysogenized. Once a cell carries prophage, however, neither it nor its progeny can be lysed by homologous phage. Adsorption takes place, but the adsorbed phage simply persists without reproducing and is quickly “diluted out” by continued cell division.

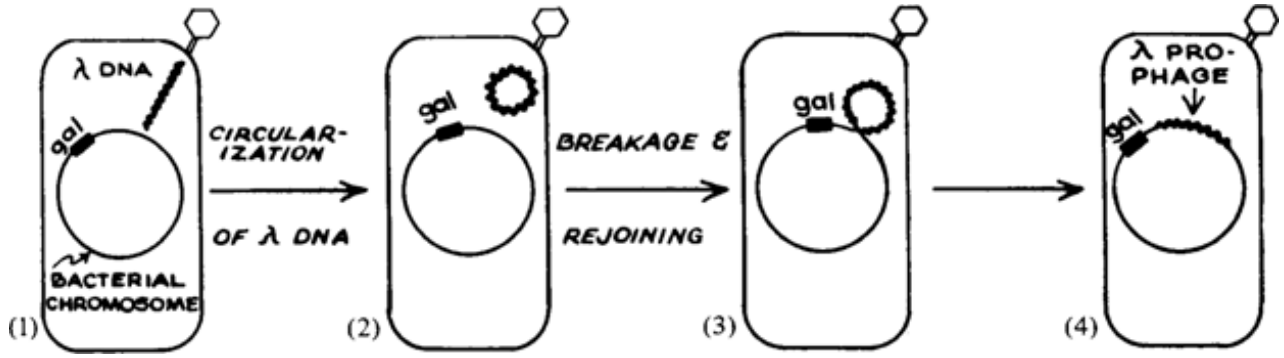


Figure 77. The integration of prophage and host chromosome.

(1) The phage DNA is injected into the host.

(2) The ends of the phage DNA are covalently joined to form a circular element.

(3) Pairing occurs between a sequence of bases adjacent to the *ga*/ locus and a homologous sequence on the phage DNA.

(4) Breakage and reciprocal rejoining («crossing over») within the region of pairing integrates the 2 circular DNA structures. The integrated phage DNA is called prophage. The length of DNA has been exaggerated for diagrammatic purposes. It is actually 1 -2% of the chromosomal length.

It has been shown that temperate phages cause the appearance in the cytoplasm of a repressor substance that inhibits multiplication of vegetative phage. Repressor also blocks the detachment of prophage (which otherwise would occur by the reversal of the integration process described above) as well as the expression of other phage genes (eg, formation of phage proteins). The establishment of the lysogenic state is

thus dependent on the production and action of repressor. The X repressor has been isolated and characterized as a protein that specifically binds to DNA.

The immunity of a lysogenic cell to homologous phage, mediated by a repressor, is clearly different from the phenomenon of «resistance» to virulent phage exhibited by certain bacteria. In the latter case, resistance is caused by failure to adsorb the phage.

B. Induction. «Vegetative phage» is defined as rapidly reproducing phage on its way to mature infective phage, whereas «prophage « reproduces synchronously with the host cell. On rare occasions prophage «spontaneously» develops into vegetative (and later into mature) phage. This accounts for the sporadic cell lysis and liberation of infectious particles in a lysogenic culture. However, the prophage of practically every cell of certain lysogenic cultures can be induced by various treatments to form and liberate infectious phage. For example, ultraviolet light will induce phage formation and liberation by most of the cells in a lysogenic culture at a dose that would kill very few nonlysogenic bacteria.

Induction requires the inactivation or destruction of repressor molecules present in the cell. Phage mutants have been obtained that produce thermolabile repressors: these phages can be induced simply by raising the temperature to 44 °C. Agents such as ultraviolet light that damage host DNA induce prophage development by the following series of reactions the end result of which is the inactivation of phage repressor: (1) The DNA lesions are recognized by specific endonucleases that digest a short segment of one strand. (2) The single-stranded regions thus formed bind a protein called the recA protein (product of the *recA* gene), which acts as a protease. (3) The recA protein cleaves the phage repressor molecules, which have also bound to the single-stranded regions of DNA. Oligonucleotides, produced in the first step, are required to activate repressor cleavage.

C. Mutation to Virulence. When virulent phage is mixed with bacterial cells, all of the infected cells lyse. When temperate phage is mixed with nonlysogenic bacteria, some of the cells reproduce the phage and are lysed, while others are lysogenized.

Temperate phage can mutate to the virulent state. Two types of virulent mutants have been found. In one type, the mutation has made the phage resistant to the repressor, so that it can multiply even in lysogenic cells that are otherwise immune; in the other type, the phage has lost the ability to produce repressor. Virulent mutants of temperate phages are quite different from the naturally virulent phages such as T2. The latter cause the appearance of enzymes that degrade host DNA and stop the synthesis of ribosomal RNA, whereas the former do not interfere with the normal metabolism of the host in this manner.

D. Effect on Genotype of Host. When a lysogenic phage, grown on host «A,» infects and lysogenizes host "B" of a different genotype, some of the cells of host "B" may acquire one or more closely linked genes from host "A". For example, if the phage is grown in a lactose-nonfermenting host, about 1 in every million cells infected becomes lactose-fermenting. The transferred property is heritable. This phenomenon, called «transduction».

In other instances, phage genes may themselves determine new host properties. For example, the toxin of *Corynebacterium diphtheriae* and the toxins of many clostridia are determined by genes carried in prophage DNA. In *Salmonella,* phage infection confers a new antigenic surface structure on the host cell. The acquisition of new cell properties as the result of phage infection is called «phage conversion.» Phage conversion differs from transduction in that the genes controlling the new properties are found only in the phage genome and never in the chromosome of the host bacterium.

Genetic Regulation of Phage Reproduction. The vegetative and prophage modes of temperate phage reproduction are regulated by a complex series of genes that govern the transcription of different segments of the phage DNA.

It should be recalled that □ DNA is circularized immediately after penetration of the cell membrane and that the circular DNA may be replicated and ultimately combined with coat proteins to form mature virions, or alternatively may be integrated into the host chromosome by a recombinational event.

A. Regulation of Vegetative Replication and Maturation. Gene activity involved in productive phage growth occurs in 3 phases: (1) In the «immediate-early» phase, transcription initiates at promoters PL and Pr proceeds left and right respectively, and terminates at the ends of the N and cro genes. These termination sites are designated T[, and TRI; some rightward transcripts extend further, to termination site TRZ. (2) In the «delayed-early « phase, the N gene product (protein) acts as an antitermination factor, allowing the above transcriptions to extend further through the genes for replication, recombination, and regulation. (3) In the «late» phase, the cro protein acts at operators OL and OR to reduce the initiation of early mRNA transcription from promoters PL and PR respectively. Also, Q protein activates rightward transcription from the promoter P'r, which continues through the lysis, head, and tail genes. (Remember that the genome is circular, the m and m' ends being joined during this phase.)

B. Regulation of Lysogenic Development. Different genes are involved in the establishment and maintenance of lysogeny: (1) In the «establishment» phase, the ell and cIII proteins activate leftward transcription from the promoters PE and P_1,thus transcribing the cl and int genes; the ell and cIII proteins also inhibit rightward transcription of the lysis genes. (2) In the «maintenance» phase, the cl protein acts at operators OL and JR to repress nearly all transcription from promoters PL and PR.The cl protein also regulates its own synthesis by controlling leftward transcription from the promoter PM. Transcription from this site is stimulated by low cl protein concentrations and inhibited by high cl protein concentrations.

The choice between the lytic and lysogenic modes of phage development depends on the relative concentrations of the cl protein (the «□ repressor») and the cro protein; the former is required for lysogeny and the latter for lytic growth. Both proteins bind to 3 repressor binding sites within the OR operator; whether transcription from the adjacent PR promotor is inhibited or stimulated depends on their patterns of binding.

C. DNA Replication. Replication starts at the site marked ori and requires the activities of the proteins coded by phage genes 0 and P.

D. Integration and Excision of Prophage. The a-a' attachment site is recognized by the int protein, catalyzing integration by crossing over at a specific attachment site on the host chromosome. Excision, brought about by a second crossover event, requires the activities of both the int and xis proteins.

E. Cleavage of the Circular DNA. Prior to its packaging in virions the circular DNA must be cleaved at a specific site (m-m') to form linear molecules. This requires the activity of the A protein as well as the presence of phage head precursors.

Restriction and Modification. The phenomena of restriction and modificationwere discovered as a result of their effects on phage multiplication. It was observed that if phage □ is grown in *E coli* strain K12, only about 1 in 10^4 particles can multiply in strain B. The few that succeed, however, liberate progeny that infect B with an efficiency of 1.0 but infect strain K12 with an efficiency of 10^{-4}.

It was shown that DNA of particles formed in K 12 is modified by a K12 enzyme so as to be immune to degradation in K12. In strain B, however, the DNA of such particles is rapidly degraded by the restricting enzyme of the host. The few particles that escape restriction are modified by the specific modification enzyme of strain B; the progeny formed are now susceptible to degradation in K12 but not in B. The modifying enzymes have been shown to act by methylating bases at specific sites in the DNA.

Certain temperate phages carry genes that govern the formation of new modification and restriction enzymes in the host. Thus, *E coli* cells carrying PI prophage will degrade all DNA not modified in a Pi-containing cell.

A given restricting enzyme recognizes a particular site on DNA and causes cleavage at that site unless the site has already been protected by the homologous modifying enzyme. Restriction appears to be a mechanism by which a cell protects itself against invasion by foreign DNA. Some phages have been found to have mechanisms for resisting restriction: Phages T3 and T7, for example, produce an early protein that inhibits the host restriction endonuclease; in other cases, the phage codes for enzymes that modify its DNA (eg, by glycosylation) so as to block the action of the restriction enzymes.

Distribution of phages in nature. Phages are wide-spread in nature. Wherever bacteria are found – in the animal body, in body secretions, in water, drainage waters and in museum cultures, conditions may be created for the development of phages. Specific phages have been found in the intestine of animals, birds, humans, and also in galls of plants. and in nodules and legumes. Phage has been isolated from milk, vegetables and fruits.

River water, sea water and drainage waters quite frequently contain an abundance of phages in relation to various microbes including pathogenic (cholera vibrio**,** bacteria of enteric fever, paratyphoid, dysentery) organisms.

Sick people and animals, carriers and convalescents serve as the main source of phages against pathogenic microbes. In sick people the phage can be found not only in the intestinal contents, but in the urine, blood, sputum, saliva, pus, nasal exudate, etc. It is extremely easy to isolate the phage during the period of convalescence. The phage is employed for the determination of species and type specificity of the isolated cultures.This method has been named phage diagnostics.

The discovery of different phages against pathogenic microbes in the environment (water, soil) illustrates the presence in a given area of sick people and animals which excrete the corresponding agents or phages. This can be employed in giving an additional characteristic of the sanitary-epidemic state of water sources and the soil.

The isolation of the phage from the material under investigation has been carried out by a special direct method and an accumulation method. F. Sergienko. G. Katsnelson and M. Sutton. V. Timakov and D. Goldfarb devised a method for the rapid discovery of pathogenic bacteria in the environment with the help of the reaction of successive growth of the titre of the specific phage.

Production of a phage and the determination of its activity. The phage is obtained by adding a special maternal phage into vats with broth cultures, which are kept for one day at 37 C and then filtered. The filtrate is checked for purity, sterility, harmlessness and activity (potency).

Practical importance of the phage in medicine. Arising from the data obtained on the mechanism of phage activity, phages have been used in prophylaxis and medical treatment against dysentery, enteric fever, paratyphoid, cholera, plague, anaerobic. staphylococcal, streptococcal, and other diseases. Bacteriophagia is used in the diagnosis of certain infectious diseases. With the help of special phages the species and types of isolated bacteria of the typhoid-dysentery group, staphylococci. causative agents of plague, cholera, etc.. are determined.

Phages are often very harmful in the manufacture of antibiotics and sour milk products as a result of inhibiting beneficial micro-organisms.

At present due to the introduction of antibiotics into practice phage therapy and prophylaxis of infectious disease are used to a limited extent.

Lysogenic bacteria are most suitable biological models for studying the interaction of the virus and cell. the mechanisms of toxigenicity, the biological efficacy of ionizing radiation, and other problems.

The phage is now used widely as a model in genetic research. The structure and function of the gene may be determined more exactly by means of this model.

http://www.innvista.com/health/microbes/bacteria/classif.htm
http://www.earthlife.net/prokaryotes/phyla.html
http://web.uct.ac.za/depts/mmi/lectures/bactax/ppframe.html
http://www.gsbs.utmb.edu/microbook/ch003.htm
http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/dental/tutorials/classification/introduction.html
http://www.microbiol.org/WPaper.Gram.htm

TOPIC 4. PRINCIPLES OF HEALTH PROTECTION AND SAFETY RULES IN THE MICROBIOLOGICAL LABORATORY. DESIGN, EQUIPMENT, AND WORKING REGIMEN OF A MICROBIOLOGICAL LABORATORY.

Contents:

1. **Equipment of the laboratory**
2. **Rules of work in the laboratory.**
3. **Rules of work at microbiological laboratory with special regime.**
4. **The Department of Microbiology is a place of work dangerous for health with the risk of professional infection.**
5. **Principal microbiological procedures**
6. **Bacterioscopic examination**

Depending on their designation, microbiological laboratories may be bacteriological, parasitological, mycological, virological, immunological, and special (for the diagnosis of particularly virulent infections). A microbiological laboratory usually comprises the following premises: (1) the preparatory room for preparing laboratory glassware, making nutrient media and performing other auxiliary works; (2) washroom; (3) autoclaving room where nutrient media and laboratory glassware are sterilized; (4) room for obtaining material from patients and carriers; (5) rooms for microscopic and microbiological studies comprising one or two boxes. Laboratory animals employed for biological sampling are kept in separate isolated premises (an animal unit).

It is preferable that laboratory rooms should have only one entrance. To facilitate such procedures as washing and treatment with disinfectants, the walls are painted with light-colored oil paint or lined with ceramic tiles, whereas the floors are covered with linoleum.

The infective material is examined in a separate room. The work requiring the observation of a microbiological regimen (inoculation of the material for sterility, contamination of tissue cultures, chicken embryos, etc.) requires special premises (box) whose floor space should be convenient for two workers. Prior to and after work the entire box is treated with disinfectant solutions and irradiated with bactericidal lamps.

Equipment of the laboratory. Laboratory furniture should be simple and convenient. Laboratory tables covered with special enamel, linoleum, or other easily

disinfecting materials are placed near windows. Safe-refrigerators are used for storing microorganism cultures.

The main pieces of equipment in the *bacteriological laboratory* include apparatuses for different types of microscopy, apparatuses for heating (gas and alcohol burners, electrical stoves, etc.), incubators, refrigerators, sterilizing apparatuses (sterilizer, Koch apparatus, Pasteur stove, coagulator, etc.), a centrifuge, distillator, etc.

The used material is rendered safe in the way which is employed in bacteriological laboratories.

Immunological laboratory is furnished with incubators, refrigerators, glassware, and apparatuses necessary to run serological reactions on a wide scale.

The design and furnishing of a virological laboratory somewhat differ from those of a bacteriological one. The premises of a *virological laboratory* should include a box with pre-box inclosure separated by a glass partition where work with cell cultures and chicken embryos is conducted.

Apart from glassware and usual equipment, this type of laboratory should be furnished with chambers of deep and superdeep freezing (-30°-70 °C), refrigerator chambers (-20 □C), centrifuges with a rotation velocity of 1500-3000 X g and over to ensure purification of the virus from ballast substances and its concentration. Other pieces of equipment include a homogenizer to comminute tissues, ovoscope, burners for ampoule soldering, and a vacuum pump.

Before starting the work, the premises are disinfected in a way which is employed for disinfecting the box of microbiological laboratories.

The premises are treated, using disinfectant solutions and bactericidal lamps.

Rules of work in the laboratory.

1. The personnel working at laboratories is supplied with medical coats and kerchiefs or caps. While working in boxes, one should wear a sterile coat, cap, and gauze mash. To make an autopsy of animals, put on an oil cloth apron, oversleeves, and rubber gloves. Special clothes protect the worker and also prevent contamination of the material to be studied with foreign microflora.

2. Eating and smoking in the laboratory are strictly forbidden.

3. Unnecessary walking about the laboratory, sharp movements, and irrelevant conversations should be discouraged.

4. In the process of examination the working place should be kept clean and tidy. Bacteriological loops are rendered harmless by burning them in the burner's flame; used spatulas, glass slides, pipettes, and other instruments are placed into jars with disinfectant solution.

5. Upon the completion of work the nutrient media with inoculated cultures are placed into an incubator; museum cultures, into safe-refrigerators; devices and apparatuses are set up in places specially intended for them. Wipe tables with disinfectant solution and thoroughly wash the hands.

6. If the material to be analyzed or the culture of microorganisms is accidentally spilt onto the hands, table, coat, or shoes, they should be immediately treated with 1 per cent solution of chloramine.

Rules of work at microbiological laboratory with special regime.

Before entering the laboratory, all personnel take off the overcoats at the cloakroom. In the next room with individual closets they take off the remaining clothes and the underwear, put pyjamas, medical coats, kerchief (or cap), socks, and slippers (Set 1 of protective clothes of the fourth type). When working in the autopsy room, put on an anti-plague suit (Set 2), a second autopsy coat, helmet, cotton wool-gauze mask, rubber gloves, oil-cloth apron, and oversleeves. To protect the eyes, one should wear goggles. An anti-plague suit of the first type is put on in the following order: (1) overalls; (2) socks; (3) high boots; (4) helmet; (5) anti-plague coat; (6) cotton wool-gauze mask (place cotton wool tampons over the wings of the nose); (7) goggles; (8) gloves; (9) oil cloth apron and oversleeves (these are put on while working in the autopsy room). A person working with infective material should have a towel soaked in 3 per cent solution of lysol. Upon completion of work immerse gloved hands into 5 per cent solution of lysol for 2 min and repeat this procedure after removing each item of the clothing. The anti-plague suit is taken off in the reverse order, with the exception of gloves which are the last to be taken off. Then. they are folded with the external surface inside and immersed into 5 per cent solution of lysol or I per cent solution of chloramine for 2 hrs. The goggles are put into 70 per cent alcohol.

Following autopsy, instruments and syringes are boiled in lysol for at least 40 min. All used material and corpses of animals are burnt or sterilized.

The Department of Microbiology is a place of work dangerous for health with the risk of professional infection.

Students are allowed to take off their clothes only in a cloakroom that must be locked and the key is placed in a reserved site to prevent any theft. If a student has any valuable things or larger sums of money with him, then he must announce it to his teacher who will secure its safe deposition. However, taking any valuables in is not recommended.

The students come into the hall through the entrance from the waiting room under the teacher's surveillance. They are lending protective coats which must be taken off before entering other departments. It is forbidden to damage these coats, to take away any infective material from the hall, as well as tools and coats. Students' own coats must not be worn.

Students must observe the principles of hygiene. They must disinfect andwash their hands always after contaminating themwithabiologicalmaterial andbefore leaving the hall. For disinfecting hands, 0.5 % chloramine is used for 2 minutes. Then the hands are to be rinsed with warm water and washed with soap. It is forbidden to eat, smoke and drink in the laboratory. It is also necessary to avoid rubbing one's eyes or nose, scratching one'shead,bitingnails,pencils,etc.

The space in front of the building and in the waiting room must be kept clear and quiet.

PRINCIPAL MICROBIOLOGICAL PROCEDURES

A complex of bacterioscopic, bacteriological, serological, allergological, and biological techniques is used in the microbiological diagnosis of bacterial infections. Depending on the nature of the given infectious disease, one of these methods is used as the main one, while the others are supplementary. Such biological substances as blood, faeces, urine, cerebrospinal fluid, bile, etc. serve as the material for microbiological diagnosis.

The main microbiological techniques pertaining to the laboratory diagnosis of bacterial infection are outlined below. Interpretation and specification of each technique with regard to specific infections are presented in the respective sections.

Modern methods of microscopic examination. Contemporary microbiological laboratories employ not only conventional methods of optical **microscopy** in transmitted light (Fig. 78) but also such special ones as dark-field microscopy and phase-contrast, luminescent, and electron microscopy.

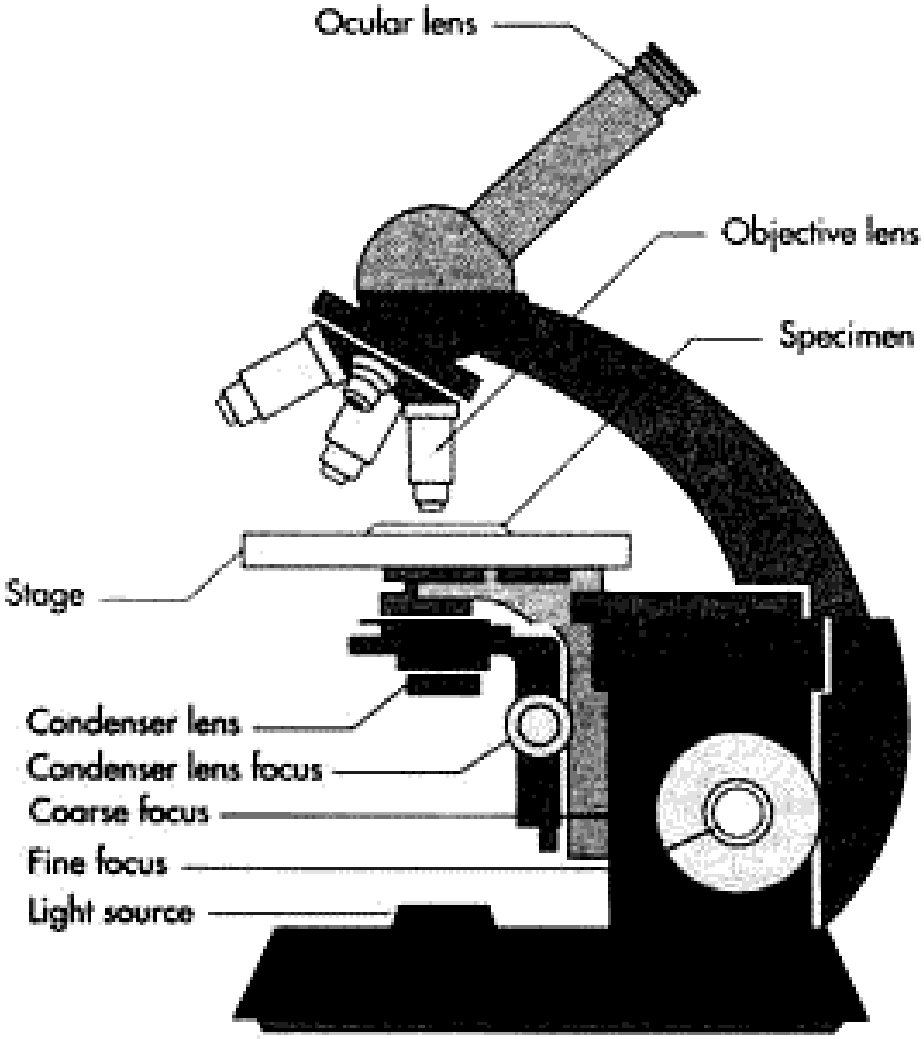


Figure 78. Bacterioscopic examination

Light microscopy. A light microscope is fitted with dry and immersion objectives. A dry objective with a relatively large focal distance and weak

magnification power is ordinarily utilized for studying large biological and histological objects. In examining microorganisms, the immersion objective with a small focal distance and a higher resolving power is predominantly employed.

In microscopic examination with the help of an immersion objective the latter is immersed in oil (cedar, peachy, "immersiol", etc.) whose refractive index is close to that of glass. When such a medium is used, a beam of light emerging from the slide is not diffused and the rays arrive at the objective without changing their direction (Fig. 79).

The resolving power of the immersion objective is about 0.2 mcm. The maximum magnification of modern light microscopes is as high as 2000-3000 (fig. 80).

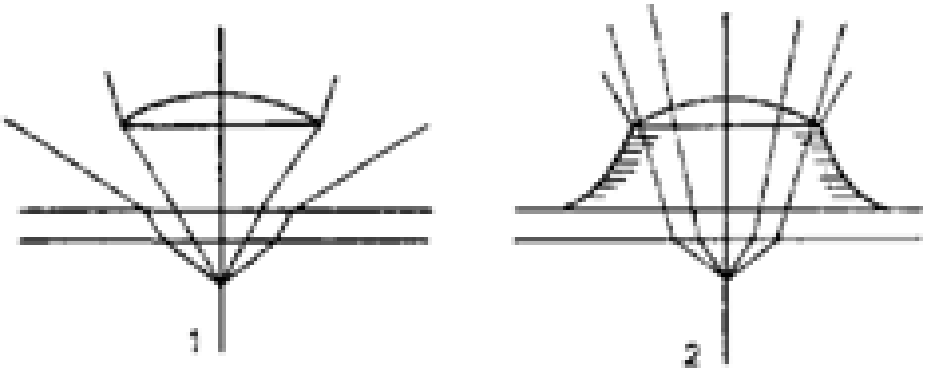


Figure 79. A light microscope.
The course of rays in the dry (1) and oil-immersion (2) systems

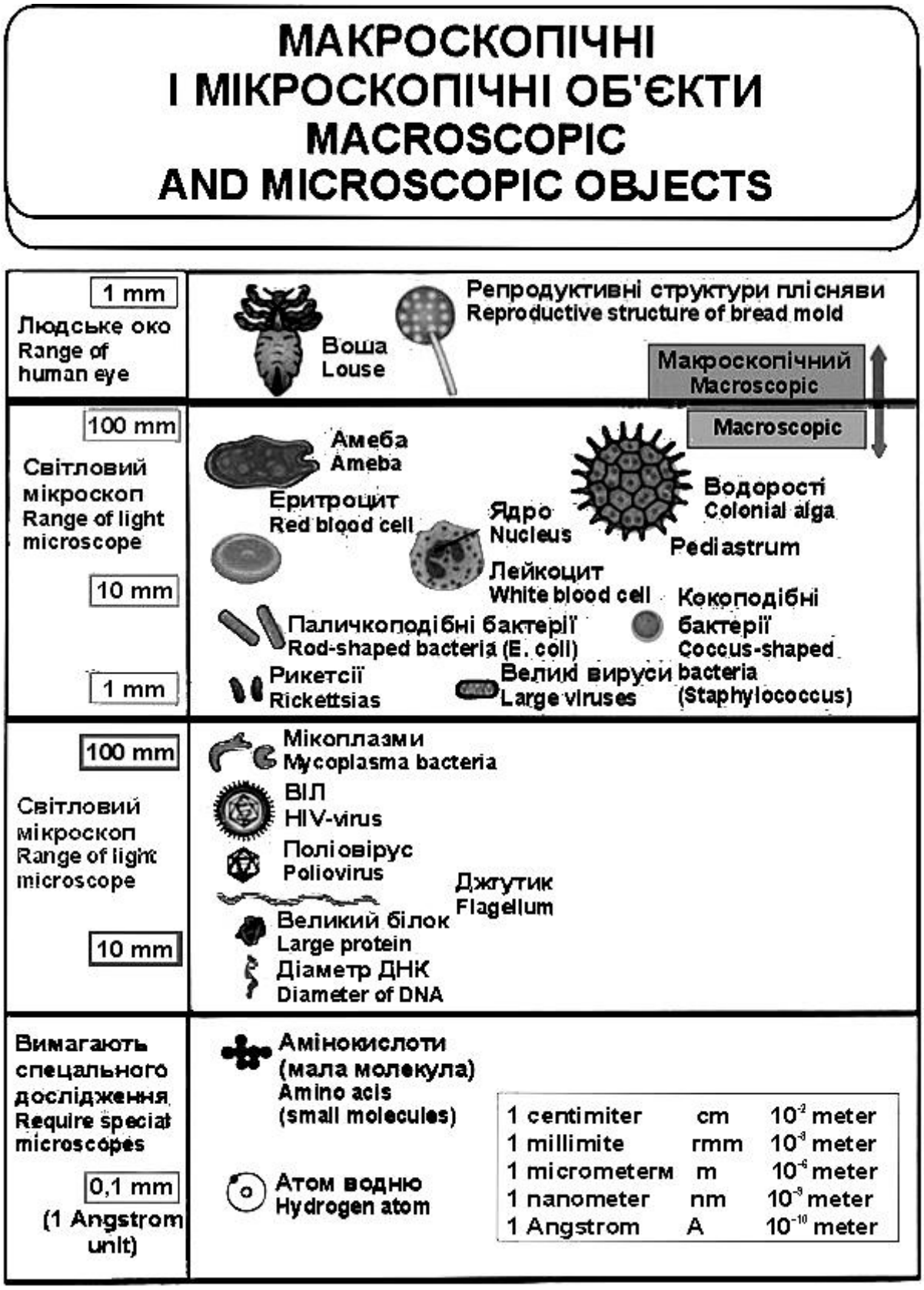


Figure 80. Comparative sizes of different objects

Dark-field microscopy belongs to ultramicroscopic methods. Living objects 0.02-0.06 mcm in size are visualized in lateral illumination in a dark field of vision.

In order to achieve bright lateral illumination, the usual condenser is replaced by a special parabolic condenser in which the central part of the lower lens is darkened, while the lateral surface is mirror (Fig. 81). This condenser intercepts the central portion of the parallel beam of rays forming a dark field of vision. The marginal rays pass through the circular slit, fall on the lateral mirror surface of the condenser, are reflected from it, and concentrate in the focus. On encountering in their path the cells of microorganisms or other optically non-homogeneous structures, the ray of light is reflected from them and gets into the objective. Cells of microorganisms and other objects are brightly illuminated in this case.

An electrical illuminator serves as a source of artificial light. To achieve lateral illumination, one needs a parallel beam of light which is created by means of a flat mirror of the microscope.

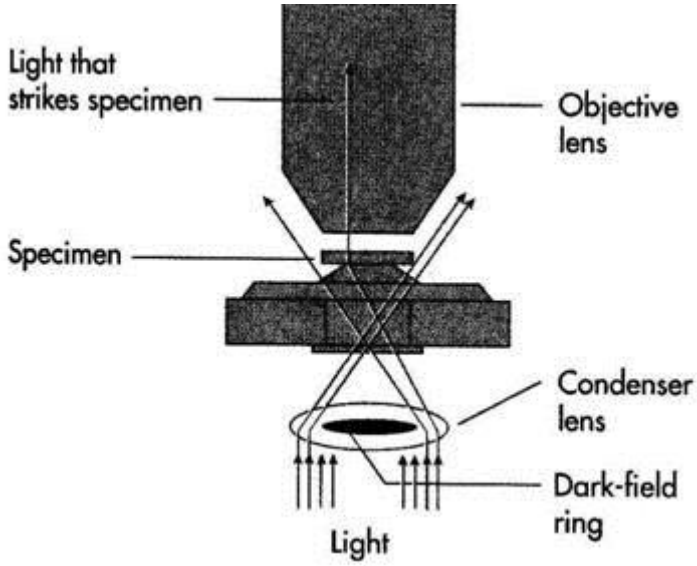


Figure 81. Diagram of a dark-field microscope showing the path of light

The dark-field ring in the condenser blocks the direct passage of light through the specimen and into the objective lens. Only light that is reflected off a specimen will enter the objective lens and be seen.

In dark-field microscopic examination a dry system is typically employed (objective 40). A small drop of the studied material is placed on the slide and covered with a cover-slip, taking care to prevent the formation of air bubbles. A drop of immersion oil is pipetted on the upper lens of the condenser. This oil should fill the space between the condenser and the slide.

Microscopy with an oil-immersion system makes use of a special objective with a diaphragm trapping the rays which pass unobstructed through a homogeneous medium.

Dark-field microscopy is employed for detecting unstained causative agents of syphilis, recurrent typhoid fever, leptospirosis, and other illnesses, as well as for investigating the motility of microorganisms. Yet, dark-field microscopic examination does not allow a good study of their form, to say nothing about their

internal structure. Modified techniques of light microscopy are utilized for this purpose.

Phase-contrast microscopy is based on the fact that the optical length of the light traveling in any substance depends on its refractive index. Light waves transversing through optically denser sites of the object lag in their phase behind the light waves which do. not have to pass through these sites. The intensity of light in this case remains unaltered but the phase of fluctuation, detected by neither eye nor photoplate, is changed. To increase resolution of the-image, the objective is fitted with a special semi-transparent phase plate to create difference in the wave length between the rays of the background and the object. If this difference reaches one-fourth of the wave length, a visually tangible effect occurs when a dark object is clearly seen against a light background (positive contrast) or vice versa (negative contrast) depending on the structure of the phase plate.

Phase-contrast microscopy does not enhance the resolving power of the optical system but helps to elucidate new details of the structure of living microorganisms and to study different stages of their development, the effect on them of chemical agents, antibiotics, and other factors.

Luminescent microscopy. Luminescence (or fluorescence) is the ability of some objects and dyes to fluoresce upon their exposure to. ultraviolet and other short-wave rays of light.

It is commonly accepted to distinguish between inherent (primary) and secondary fluorescence. In primary fluorescence the test object contains substances capable to fluoresce upon their exposure to ultraviolet rays. Most of objects are not inherently fluorescent, so. prior to luminescent microscopy they have to be treated with dyes (fluorochromes) capable to fluoresce. The following substances are usually used as fluorochromes: auramine (for tuberculosis mycobacteria), acridine yellow (for gonococci), coryphosphine (for Corynebacteria of diphtheria), fluoresceinisothiocyanate (FITC) (for making labelled antisera), etc.

A specimen to be examined by luminescent microscopy is prepared in the usual manner, fixed in acetone or ethanol for 5-10 min and exposed to a fluorochrome for 20-30 min. Thereafter, the resultant preparation is washed with tap water for 15-20 min, covered by a cover-slip, and placed under a microscope.

Luminescent microscopes represent ordinary biological microscopes furnished with a bright source of illumination and a set of light filters which isolate a short-wave (ultraviolet or blue-violet) part of the spectrum inducing luminescence. Fluorochromes, binding "with nucleic acids or proteins, form stable complexes that give away yellow-green, orange-red, and brown-red light under the lumimescent microscope.

Fluorescence microscopy has the following advantages as compared with the conventional microscopic methods: colour image; considerable contrasting; possibility to study both live and dead microorganisms, transparent and non-transparent objects; detection of individual bacteria, viruses, and their antigens and possibility of their localization; differentiation of individual components of the cell.

Electron microscopy. In the electron microscope, a beam of electrons passing in vacuum and blocked by an anode is used instead of light. The source of electrons is an electron gun (a tungsten wire heated up to 2500-2900 °C). The optic lenses are replaced by electromagnets, An electrical field of 30 000-50 000 V is generated between the tungsten wire and anode, which imparts high velocity to electrons which arrive at the first electromagnetic lens (a condenser) having passed through the anode opening. At their exit from the condenser the electron rays are accumulated in the plane of the studied object, deviate at different angles due to variable thickness and density of the preparation, and get into the electromagnetic lens of the objective equipped with a diaphragm. Electrons showing only little deviation upon entering the object pass through the diaphragm, while those deviating at a greater angle are retained, which ensures contrasting of the image- The lens of the objective gives an. intermediate enlarged image which is viewed through the viewing window. The projection lens may ensure a multiple magnification of the image; this image is perceived by a fluorescent screen and photographed. The most recent models of electron microscopes permit visualization of particles 1.4 nm in size.

Electron microscopy is extensively used in microbiology for detailed investigation into the structure of microorganisms. It is also employed in virology for diagnostic purposes.

To study preparations under the electron microscope, special films absorbing small numbers of electrons and fixed on supporting meshes are utilized instead of glass slides. Such films are made of collodium, aluminium oxide, and quartz. The material to be studied is thoroughly cleansed of various admixtures and placed on the film. A very thin layer remaining on the film after evaporation of the fluid is subjected to microscopic examination. The electron microscope may also be used for studying sections of tissues, cells, and microorganisms which are obtained with the help of an ultramicrotome. The preparations are contrasted by means of electron-dense (electron-capturing) substances, using such procedures as the spraying with heavy metals and treatment with phosphotungstic acid. uranyl acetate, salts of osmic acid, etc.

Scanning Electron Microscopy. The scanning electron microscope uses a fine beam or spot of electrons that is focused rapidly back and forth over the specimen. As the electrons strike the surface of particles in the sample, secondary electrons are emitted, which are collected by a detector to provide an image of the specimen's surface. This instrument does not require that the sample be sectioned and provides some spectacular three dimensional images. In addition, because the energy of the secondary emitted electron is determined by the identity of the scattering atom, the energy spectrum of these electrons provides information about the location and content of the different elements (tabl.9).

Table 9. Comparison of Various Types of Microscopes

Type of microscope	Maximum useful magnification	Resolution	Description
Bright-field	1,500x	100-200 nm	Extensively used for the visualization of micro organisms; usually necessary to stain specimens for viewing
Dark-field	1.500X	100-200 nm	Used for viewing live microorganisms, particularly those with characteristic morphology; staining not required; specimen appears bright on a dark background
Fluorescence	1,500X	100-200 nm	Uses fluorescent staining; useful in many diagnostic procedures for identifying microorganisms
Phase contrast	1.500X	100-200 nm	Used to examine structures of living microorganisms; does not require staining
TEM (trans-mission electron – microscope)	500,000-1,000,000X	0.1 nm	Used to view ultrastructure of microorganisms, including viruses; much greater resolving power and useful magnification than can be achieved with light microscopy
SEM (scanning electron microscope)	10,000-100,000X	1-10 nm	Used for showing detailed surface structures of microorganisms, produces a three-dimensional image

Preparation and Staining of Smears

Preparation and staining of smears, as well as other microbiological procedures, are performed in a prepared working place. The working table should contain only those materials and objects which are necessary for the given examination, namely; the object to be studied (blood, pus, sputum, faeces, etc.), test tubes or dishes with a culture of microorganisms, sterile distilled water or isotonic sodium chloride solution, a stand for a bacteriological loop, a jar with clean glass slides, and felt tip pens. Other necessary items include a gas or alcohol burner, staining solutions, a basin with a supporting stand (bridge) for slides, a washer with water, forceps, filtering paper, a jar with disinfectant solution used for sterilizing preparations and pipettes.

Methods of the treatment of cover-slips. Now cover-slips are boiled in a 1 per cent solution of sodium hydrocarbonate. rinsed with water, immersed in a weak solution of hydrochloric acid, and then rinsed with water once again. The used glass slips and slides are placed in a concentrated sulphuric acid (technical grade) for 2 hrs or in a mixture of sulphuric acid, potassium bichromate, and water (100:50:1000), thoroughly washed with water, boiled in sodium hydrocarhonate solution or ill sodium hydroxide, then washed with water once more, dried with clean linen cloth, and stored in alcohol or an alcohol-ether mixture in jars with ground stoppers. Detergents are also utilized for washing slides. Prior to making smears take slides with a forceps from the solution whore they have been kept and blot them dry. Hold them by the edges with your fingers. A drop placed on the properly prepared glass spreads uniformly and does not assume a spherical form.

Preparation of a smear. Before making a preparation, glass slides are flamed to ensure their additional degreasing.

In preparing a *smear from bacterial culture grown on a solid medium,* a drop of isotonic saline or water is transferred onto the precooled glass. A test tube with the culture is taken by the thumb and the index finger of the left hand. The loop is sterilized in the flame. A cotton-wool plug is pinched by a small finger of the right hand, removed from the test tube, and left in this position. The edges of the test tube are flamed and then the loop is introduced into the test tube through the flame. Having cooled the loop against the inner wall of the tube, the loop is touched to the nutrient medium where it meets with the glass wall (if the loop is not sufficiently cooled, it induces cracking and melts the medium). Then the loop is touched to the culture of the microorganisms on the surface of the medium. Then the loop is withdrawn, the edges of the test tube are quickly flamed, the tube is closed with a stopper passed through the flame, and then replaced into the test tube rack. All the above described procedures are made above the flame. The culture sample is placed with the loop into a drop of water on the glass slide and spread uniformly with circular movements on an area of 1-1.5 cm in diameter, then the loop is flamed.

In preparing a *smear from bacterial culture grown in a fluid nutrient medium,* a drop of the culture is taken with a loop or a Pasteur pipette (the pipette is immersed in disinfectant solution), transferred onto the middle of the flamed glass slide and spread uniformly. On the other side of the glass slide the preparation is delineated by a wax pencil since very thin smears are almost invisible. The number of the analysis or culture is marked on the left side of the glass.

To prepare a *smear from pus or sputum,* two glass slides are used. A small amount of the material is transferred with a sterile loop or needle onto the middle of the glass slide and covered with a second one so that one-third of the surface of both slides remains free. Then, the glass slides are pulled gently aside (Fig. 82), which results in the formation of two large smears of the same thickness.

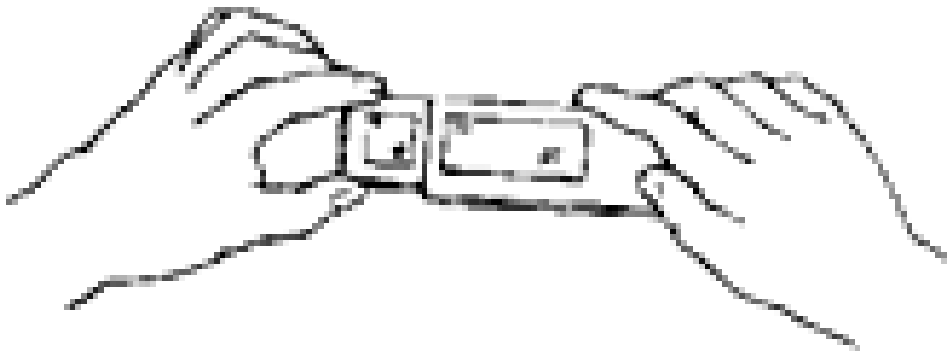


Figure 82. Preparation of a smear from sputum

Blood smear is prepared in the following way. Using a sterile needle, puncture a disinfected fourth finger of the left hand. Wipe away the first drop of blood with a piece of dry cotton wool and touch the thoroughly-cleansed glass slide to the second drop of blood. Quickly put the slide on the table, supporting it with the left hand. Place the end of a second narrower cover slide in touch with the drop of the blood at the 45-degree angle (Fig. 83).

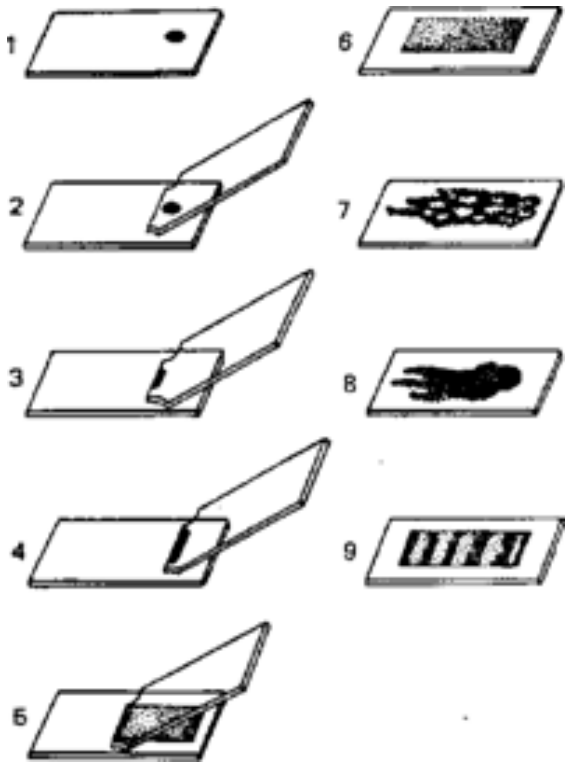


Figure 83. Preparation of a smear from blood:
1-6 — stages of thin smear preparation; *7-9* — inadequately prepared smears

Putting some pressure on the cover slide, smoothly and rapidly move it along the glass slide in the leftward direction stopping it at a distance of 1-1.5 cm from the edge. The correctly prepared smear is yellowish in colour and semitransparent.

Impression preparations are made of the internal organs of cadavers and solid foodstuffs (meat, sausage, ham, etc.). The surface of the organs or of a foodstuff is burnt with a red-hot scalpel, and a piece of the material is cut off from this site. The surface of the section is touched to the glass in two-three places.

Drying and fixation of the smear. Thin smears usually dry rapidly in the air at room temperature; thicker ones are dried in an incubator or by holding them above the flame of a burner. The slide is held by the edges with the thumb and forefinger, the smear upward, while the middle finger is placed under the glass to regulate the degree of its heating and to prevent coagulation of the bacterial protein and destruction of the cell structure. The dried smears are flamed to kill and fix the bacteria on the glass slide, preventing thereby their washing off during staining. The dead microorganisms are more receptive to dyes and present no danger for the personnel working with them. The glass slide is grasped with a forceps or with the thumb and index finger of the right hand, the smear being in the upside position, and passed three times through the hottest part of the burner's flame. Fixation with this technique takes about 5-6 s, with the exposure to the Game being about 2 s.

Blood smears, impression smears, and smears from bacterial culture deforming at high temperature are treated with one of the following fixatives: (1) methyl alcohol (for 5 min); (2) ethyl alcohol (10-15 min); (3) Nikiforov's mixture: equal volumes of ethyl alcohol and ether (10-15 min); (4) acetone (5 min); (5) fumes of osmic acid and formalin (several seconds).

Staining of a smear. Smears are stained with aniline dyes. Chemically, acid, alkaline, and neutral dyes can be distinguished. Alkaline dyes, whose staining portion of the molecule is charged positively, more actively conjugate with a negatively charged bacterial cell.

Staining of bacteria is a complex physicochemical process. Interaction of the dye with the cell substances results in the formation of salts ensuring stability of staining. Relationship between various types of microorganisms and dyes is called a tinctorial property.

The following dyes are employed most extensively: (1) red (basic fuchsine, acid fuchsine, safranine, neutral red, Congo red); (2) blue (methylene blue, toluidine blue, trypan blue, etc.); (3) violet (gentian, methyl or crystal); and (4) yellow-brown (vesuvin, chrysoidine).

All the employed dyes are powder-like or crystalline. Such dyes as basic fuchsine, gentian violet, and methylene blue are usually used to prepare in advance saturated alcoholic solutions (1 g of the dye per 10 ml of 96 per cent alcohol). Saturated alcohol and phenol dye solutions are utilized to prepare water-phenol or water-alcohol solutions to be employed in staining by simple and complex techniques.

Simple techniques of staining make use of only one dye and demonstrate the form of bacteria.

Preparation of dye solutions for simple staining. Basic fuchsine is used for preparing Ziehl's phenol fuchsine which is stable upon storing. Ziehl's fuchsine is employed to stain in red colour the acid-fast microorganisms and spores.

Ziehl's phenol fuchsine

Basic fuchsine	1 g
95 per cent alcohol	10 mg
Crystal phenol	5 g
Glycerol	several drops
Distilled water	100 ml

Fuchsine, together with phenol crystals and some drops of glycerol, is homogenized by grinding in a mortar, adding simultaneously small amounts of alcohol. Then, with the obtained mass being continuously stirred, distilled water is gradually added. The resultant dye is allowed to stand at room temperature for 48 hrs and is then filtered. Shelf life is prolonged.

Pfeiffer's fuchsine

Ziehl's fuchsine	1 ml
Distilled water	9 ml

In using Pfeiffer's fuchsine, the solution should be freshly prepared.

Saturated alcoholic solution of methylene blue

Methylene blue	10 g
95 per cent alcohol	100 ml

Alkaline solution of methylene blue as proposed by Loeffler

Saturated alcoholic solution of methylene blue	30 ml
Sodium hydroxide or potassium hydroxide (1 per cent solution)	1 ml
Distilled water	100 ml

Water-alcoholic solution of methylene blue

Saturated alcoholic solution of methylene blue	10 ml
Distilled water	100 ml

Old solutions of this dye have a better staining ability.

The fixed preparation is placed, the smear upward, on the support. A dye solution is pipetted onto the entire surface of the smear. With Pfeiffer's fuchsine the staining lasts 1-2 min, with alkaline solution of Loeffler's methylene blue or water-alcoholic solution of methylene blue, 3-5 min. Following the staining procedure the dye is dispensed, the preparation is washed with water, dried between sheets of filter paper, and then examined under the oil-immersion objective.

Live staining of microorganisms is made with methylene blue, neutral red, and other weakly poisonous dyes in a 1:10 000 dilution. For this purpose, a drop of the test material is mixed with the dye solution on a glass slide and covered with a cover-slip. Microscopic examination is carried out with a 40X objective.

In a negative method of living bacteria staining by Bum's technique, the bacteria remain unstained against a dark background. In a drop of Indian ink diluted with distilled water 1 to 10 the culture to be tested is introduced and spread uniformly with a loop or the edge of a glass slide. The smear is air dried. Nigrosin, Congo red, and other dyes may occasionally be utilized instead of Indian ink.

Anjesky technique

Spores are most simply observed as intracellular refractile bodies in unstained cell suspensions or as colorless areas in cells stained by conventional methods. The spore wall is relatively impermeable, but dyes can be made to penetrate it by heating the preparation. The same impermeability then serves to prevent decolorization of the spore by a period of alcohol treatment sufficient to decolorize vegetative cells. The latter can finally be counterstained. Spores are commonly stained with malachite green or carbolfuchsin.

To demonstrate bacterial spores, special staining methods proposed by Anjesky, Peshkov, Bitter, Schaeffer-Fulton, and others are used.

Anjesky's staining. A thick smear is dried in the air, treated with 0.5 per cent sulphuric acid, and heated until it steams. Then, the preparation is washed with water, dried, fixed above the flame, and stained by the Ziehl-Neelsen's technique. Spores stain pink-red, the cell appears blue (fig. 84).

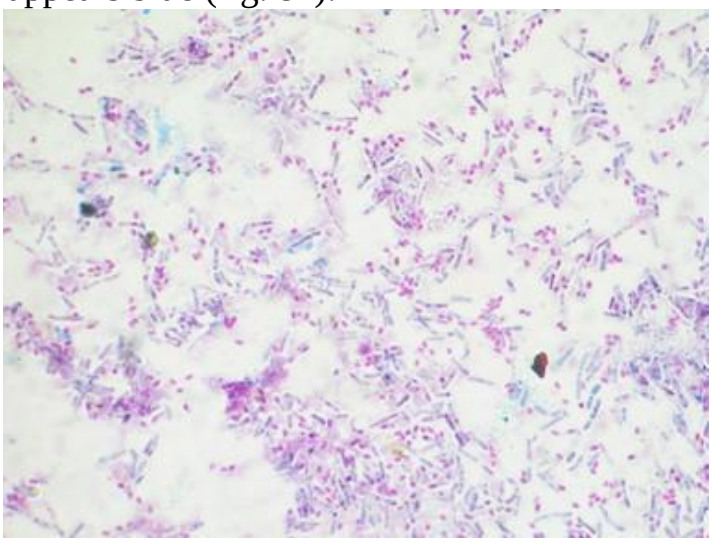


Figure 84. Wet-mount and hanging drop technique

Study of living microorganisms using the wet-mount and hanging-drop techniques. Using living microorganisms, one can study the processes of their propagation and spore formation, as well as the effect on them of various chemical and physical factors. In clinical laboratories living microorganisms are investigated to determine their motility, i.e., indirect confirmation of the presence of flagella. Preparations in this case are made using wet-mount or hanging-drop techniques and then subjected to dry or immersion microscopy. Results are better when dark-field or phase-contrast microscopy is employed.

Wet-mount technique. A drop of the test material, usually 24-hour broth culture of microorganisms, is placed into the centre of a glass slide. The drop is covered with a cover slip in a manner preventing the trapping of air bubbles; the fluid should fill the entire space without overflowing (fig. 85).

An inherent drawback of the wet-mount technique is its rapid drying. In prolonged microscopy it is recommended that the edges of a cover slip be sealed with petrolatum.

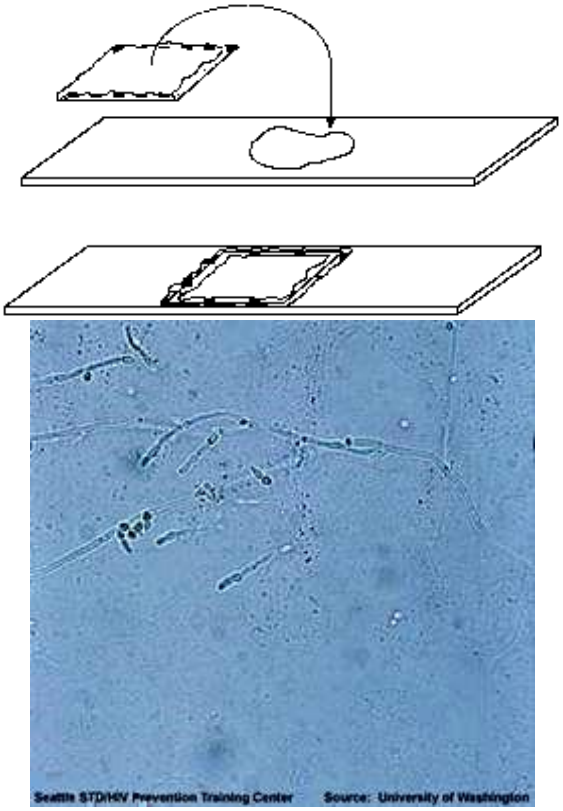


Figure 85. Wet-mount technique

Hanging drop technique. To prepare this kind of preparation, special glass slides with an impression (well) in the centre are utilized. A small drop of the test material is put in the middle of the cover slip. The edges of the well are ringed with petrolatum. The glass slide is placed onto the cover slip so that the drop is in the centre of the well. Then. it is carefully inverted and the drop hangs in the centre of the sealed well, which prevents it from drying (fig. 86).

The prepared specimens are examined microscopically, slightly darkening the microscopic field by lowering the condenser and regulating the entrance of light with a concave mirror. At first low power magnification is used (objective 8 X) to detect the edge of the drop, after which a 40 x or an oil-immersion objective is mounted.

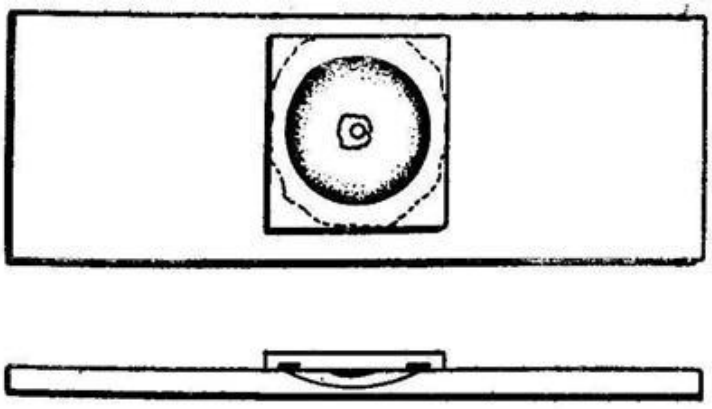


Figure 86. Hanging drop technique

Occasionally, molecular (Brownian) motility is mistaken for the motility of microorganisms. To avoid this error, it should be borne in mind that microorganisms propelled by flagella may traverse the entire microscopic field and make circular and rotatory movements. After the examination the wet-mount and hanging-drop preparations should be immersed in a separate bath with disinfectant solution to kill the microorganisms studied.

CULTIVATION; QUANTIFICATION; INCLUSION BODIES; CHROMOSOME DAMAGE

Cultivation of Viruses

At present, many viruses can be grown in cell cultures or in fertile eggs under strictly controlled conditions. Growth of virus in animals is still used for the primary isolation of certain viruses and for the study of pathogenesis of viruses and of viral on-cogenesis.

A. Chick Embryos: Virus growth in an embryonated chick egg may result in the death of the embryo (eg, encephalitis virus), the production of pocks or plaques on the chorioallantoic membrane (eg, herpes, smallpox, vaccinia), the development of hemagglutinins in the embryonic fluids or tissues (eg, influenza), or the development of infective virus (eg, polio virus type 2) – Fig. 87.

B. Tissue Cultures: The availability of cells grown in vitro has facilitated the identification and cultivation of newly isolated and previously known viruses. There are 3 basic types of cell culture. Primary cultures are made by dispersing cells (usually with trypsin) from host tissues. In general, they are unable to grow for more than a few passages in culture, as secondary cultures. Diploid cell strains are secondary cultures which have undergone a change that allows their limited culture (up to 50 passages) but which retain their normal chromosome pattern. Continuous cell lines are cultures capable of more prolonged (perhaps indefinite)

culture which have been derived from cell strains or from malignant tissues They invariably have altered and irregular numbers of chromosomes.

C. The type of cell culture used for virus cultivation depends on the sensitivity of the cells to that particular virus In the clinical laboratory, multiplication of the virus can be followed by determining the following.

D. 1 The cytopathic effect, or necrosis of cells in the tissue culture (polio-, herpes-, measles-, adenovirus, cytomegalovirus, etc).

E. 2 The inhibition of cellular metabolism, or failure of virus-infected cells to produce acid (eg, enteroviruses).

F. 3 The appearance of a hemagglutinin (eg, mumps, influenza) or complement-fixing antigen (eg, poliomyelitis, varicella, measles).

G. 4 The adsorption of erythrocytes to infected cells, called hemadsorption (parainfluenza, influenza). This reaction becomes positive before cytopathic changes are visible, and in some cases it is the only means of detecting the presence of the virus 5 Interference by a noncytopathogenic virus (eg, rubella) with replication and cytopathic effect of a second, indicator virus (eg, echovirus).

H. 6 Morphologic transformation by an oncogenic virus (eg, SV40, Rous sarcoma virus), usually accompanied by the loss of contact inhibition and the piling up of cells into discrete foci Such alterations are a heritable property of the transformed cells.

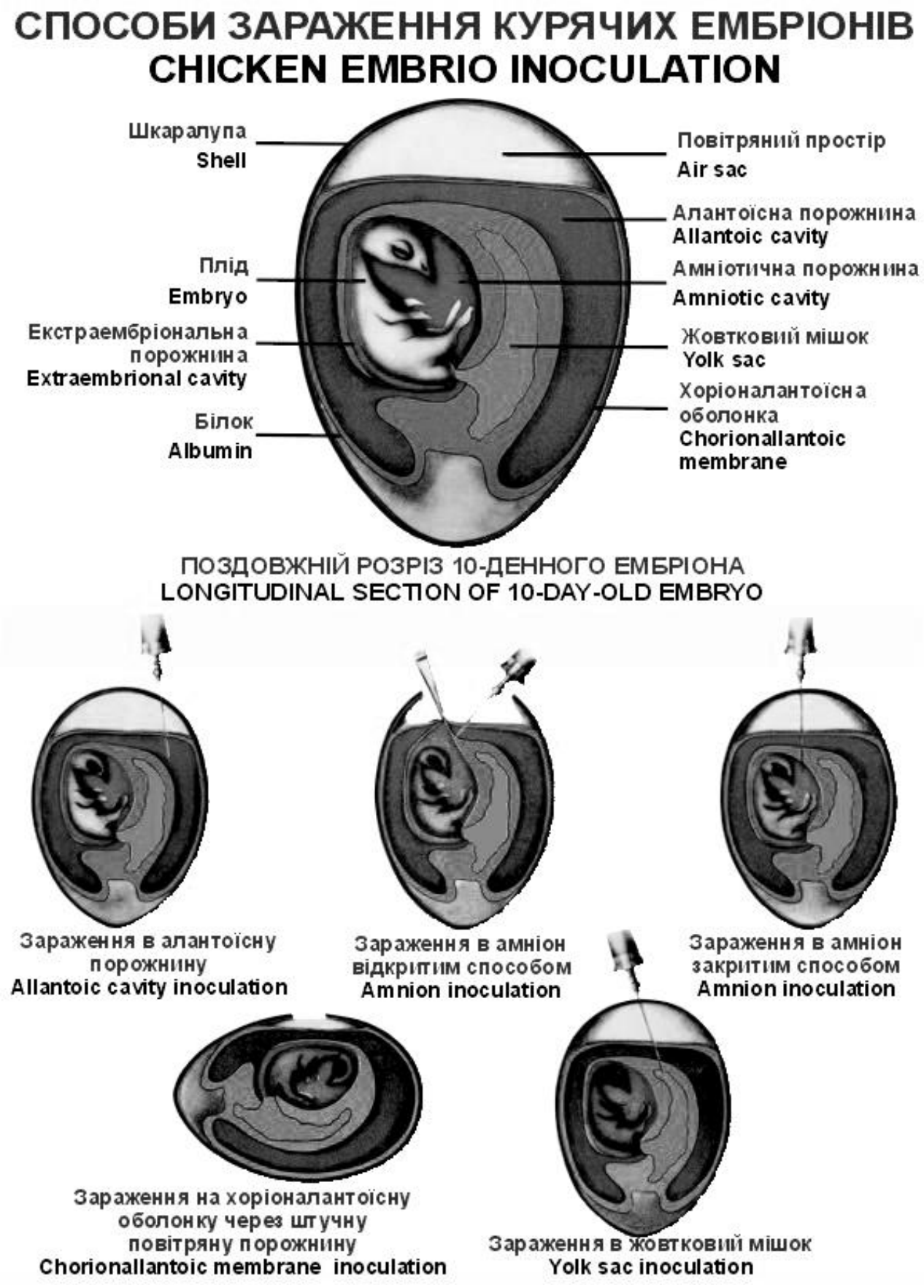


Figure 87. Chicken embrio inoculation

Quantification of Virus

A. Physical Methods: Virus particles can be counted directly m the electron microscope by comparison with a standard suspension of latex particles of similar small size However, a relatively concentrated preparation of virus is necessary for this procedure, and infectious virus particles cannot be distinguished from noninfectious ones.

Hemagglutination. The red blood cells of humans and some animals can be agglutinated by different viruses Both infective and noninfective particles give this reaction, thus, hemagglutination measures the total quantity of virus present The orthomyxoviruses contain a hemagglutinin that is an integral part of the viral envelope Once these viruses have agglutinated with the cells, spontaneous dissociation of the virus from the cells can occur The dissociated cells can no longer be agglutinated by the same virus species, but the recovered virus is able to agglutinate fresh cells. This is due to the destruction of specific mucopolysaccharide receptor sites on the surface of the erythrocyte by the enzyme neuraminidase of the virus particles (fig. 88).

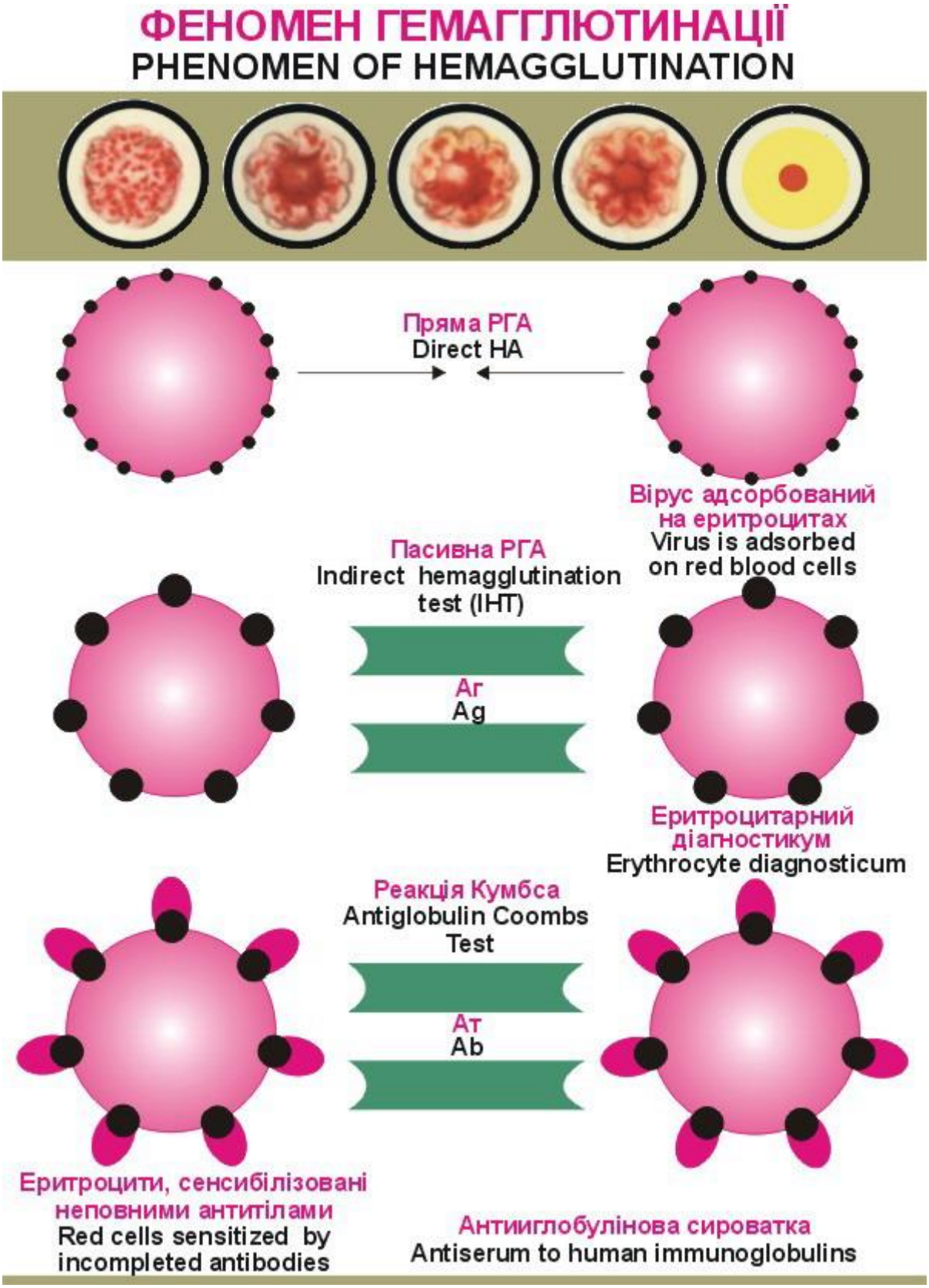


Figure 88. Hemagglutination

Paramyxoviruses growing in cell culture can be detected by hemadsorption Erythrocytes adsorb to each infected cell.

Poxviruses have an agglutinin for red cells (a phosphohpid-protein complex) that can be separated from the infective virus particle.

Arboviruses and others have hemagglutinins that appear to be identical with the virus particle The union between hernagglutinin and red blood cells is irreversible**.**

B. Biologic Methods: Quintal assays depend on the measurement of animal death, animal infection or cytopathic effects in tissue culture upon end point dilution of the virus being tested The titter is exprcssei.1 as the 50% infectious dose (ID5o), which is the reciprocal of the dilution of virus that produces the effect 111 50% of the cells or animals inoculated Precise assays require the use of a large number of test subjects.

The most widely used assay for infectious virus is the plaque assay. Monolayers of host cells are inoculated with suitable dilutions of virus and after adsorption are overlaid with medium containing agar or carboxymethylcellulose to prevent virus spreading Alter several days, the cells initially infected have produced virus that spreads only to surrounding cells, producing a small area of infection, or plaque Under controlled conditions a single plaque can arise from a single infectious virus particle, termed a plaque-forming unit (PFU) The cytopathic effect of infected cells within the plaque can be distinguished from uninfected cells of the monolayer, with or without suitable staining. and plaques can usually be counted macroscopically. The ratio of infectious to physical particles vanes widely, from near unity to less than 1 per 1000.

Certain viruses such as herpes or vaccinia form pocks when inoculated onto the chorionallantoic membrane of the embryonated egg. Such viruses can be quantitated by relating the number of pocks counted to the virus dilution.

Inclusion Body Formation. In the course of virus multiplication within cells, virus-specific structures called inclusion bodies maybe produced. They become far larger than the individual virus particle and often have an affinity for acid dyes (eg, eosin) They may be situated in the nucleus (herpesvirus), in the cytoplasm (pox virus), or in both (measles virus) In many viral infections, the inclusion bodies are the site of development of the virions (the virus factories) In some infections (molluscum contagiosum), the inclusion body consists of masses of virus particles that can be seen in the electron microscope to ripen to maturity within the inclusion body. In others (as in the intranuclear inclusion body of herpes), the virus appears to have multiplied within the nucleus early in the infection, and the inclusion body appears to be a remnant of virus multiplication. Variations in the appearance of inclusion material depend largely upon the fixative used

The presence of inclusion bodies may be of considerable diagnostic aid The intracytoplasmic inclusion in nerve cells, the Negri body, is pathognomonic for rabies (fig. 89).

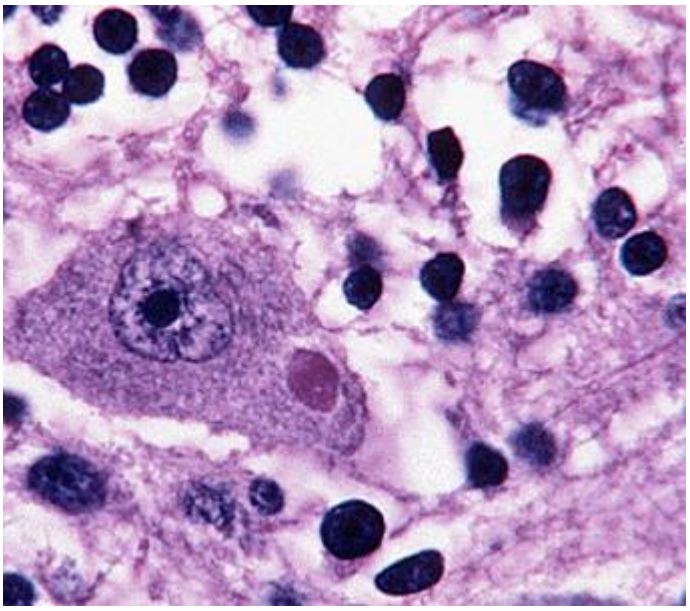


Figure 89. Negri body

Chromosome Damage. One of the consequences of infection of cells by viruses is derangement of the karyotype. Most of the changes observed are random. Frequently, breakage, fragmentation, rearrangement of the chromosomes, abnormal chromosomes, and changes in chromosome number occur Herpes zoster virus interrupts the mitotic cycle of human cells in culture, resulting in formation of micronuclei and fragmentation of some chromosomes. Chromosome breaks have also been observed in leukocytes from cases of chickenpox or measles These viruses, as well as rubella virus, cause similar aberrations when inoculated into cultured cells. Cells infected with or transformed to malignancy by SV40, polyoma, or adenovirus type 12 also exhibit random chromosomal abnormalities.

The Chinese hamster cell has a stable karyotype composed of 22 chromosomes Inoculation of these hamster cells with herpes simplex virus results in chromosome aberrations that arc not random in distribution Most of the breaks occur in region 7 of chromosome No. 1 and in region 3 of the X chromosome The Y chromosome is unaffected Replication of the virus is necessary for induction of the chromosome aberrations. To date, no pathognomonic chromosome alterations have been identified in virusinfected cells in humans.

TOPIC 5. PHYSIOLOGY OF BACTERIA, FUNGI, VIRUSES, MYCOPLASMAS, RICKETTSIA

Contents.

Metabolism refers to all the biochemical reactions that occur in a cell or organism. The study of bacterial metabolism focuses on the chemical diversity of substrate oxidations and dissimilation reactions (reactions by which substrate molecules are broken down), which normally function in bacteria to generate energy. Also within the scope of bacterial metabolism is the study of the uptake and utilization of the inorganic or organic compounds required for growth and maintenance of a cellular steady state (assimilation reactions). These respective exergonic (energy-yielding) and endergonic (energy-requiring) reactions are catalyzed within the living bacterial cell by integrated enzyme systems, the end result being self-replication of the cell. The capability of microbial cells to live, function, and replicate in an appropriate chemical milieu (such as a bacterial culture medium) and the chemical changes that result during this transformation constitute the scope of bacterial metabolism.

The bacterial cell is a highly specialized energy transformer. Chemical energy generated by substrate oxidations is conserved by formation of high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond

$$(R - \overset{\overset{O}{\|}}{C} \sim S - R),$$

such as acetyl ~ S-coenzyme A (acetyl ~ SCoA) or succinyl ~ SCoA.

ADP and ATP represent adenosine monophosphate (AMP) plus one and two high-energy phosphates (AMP ~ P and AMP ~ P~ P, respectively); the energy is stored in these compounds as high-energy phosphate bonds. In the presence of proper enzyme systems, these compounds can be used as energy sources to synthesize the new complex organic compounds needed by the cell. All living cells must maintain steady-state biochemical reactions for the formation and use of such high-energy compounds. From a nutritional, or metabolic, viewpoint, three major physiologic types of bacteria exist: the heterotrophs (or chemoorganotrophs), the autotrophs (or chemolithotrophs), and the photosynthetic bacteria (or phototrophs) (Table 10). These are discussed below.

Heterotrophic Metabolism

Heterotrophic bacteria, which include all pathogens, obtain energy from oxidation of organic compounds. Carbohydrates (particularly glucose), lipids, and protein are the most commonly oxidized compounds. Biologic oxidation of these organic compounds by bacteria results in synthesis of ATP as the chemical energy source. This process also permits generation of simpler organic compounds (precursor molecules) needed by the bacteria cell for biosynthetic or assimilatory reactions.

Table 10. Nutritional Diversity Exhibited Physiologycally Different Bacteria

Required Components for Bacterial Growth				
Physiologic Type	**Carbon Source**	**Nitrogen Source[a] Source[b]**	**Energy Source**	**Hydrogen Source**
Heterotrophic (chemoorganotrophic)	Organic	Organic or inorganic	Oxidation of organic compounds	–
Autotrophic[a]chemolithotrophic)	CO_2	Inorganic	Oxidation of inorganic compounds	–
Photosynthetic Photolithotrophic[b](Bacteria)	CO_2	Inorganic	Sunlight	H_2S or H_2
Cyanobacteria	CO_2	Inorganic	Sunlight	Photolysis of H_2O[c]
Photoorganotrophic (Bacteria)	CO_2	Inorganic	Sunlight	Organic compounds[d]

[a] Common inorganic nitrogen sources are NO_3^- or NH_4^+ ions; nitrogen fixers can use N_2;
[b] Many prototrophs and chemotrophs are nitrogen-fixing organisms;
[c] Results in O_2 evolution (or oxygenic photosynthesis) as commonly occurs in plants;
[d] Organic acids such as formate, acetate, and succinate can serve as hydrigen donors.

The Krebs cycle intermediate compounds serve as precursor molecules (building blocks) for the energy-requiring biosynthesis of complex organic compounds in bacteria. Degradation reactions that simultaneously produce energy and generate precursor molecules for the biosynthesis of new cellular constituents are called amphibolic.

All heterotrophic bacteria require preformed organic compounds. These carbon- and nitrogen-containing compounds are growth substrates, which are used aerobically or anaerobically to generate reducing equivalents (e.g., reduced nicotinamide adenine dinucleotide; NADH + H^+); these reducing equivalents in turn are chemical energy sources for all biologic oxidative and fermentative systems. Heterotrophs are the most commonly studied bacteria; they grow readily in media containing carbohydrates, proteins, or other complex nutrients such as blood. Also, growth media may be enriched by the addition of other naturally occurring compounds such as milk (to study lactic acid bacteria) or hydrocarbons (to study hydrocarbon-oxidizing organisms).

Respiration

Glucose is the most common substrate used for studying heterotrophic metabolism. Most aerobic organisms oxidize glucose completely by the following reaction equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}$$

This equation expresses the cellular oxidation process called respiration. Respiration occurs within the cells of plants and animals, normally generating 38 ATP molecules (as energy) from the oxidation of 1 molecule of glucose. This yields approximately 380,000 calories (cal) per mode of glucose (ATP ~ 10,000 cal/mole). Thermodynamically, the complete oxidation of one mole of glucose should yield approximately 688,000 cal; the energy that is not conserved biologically as chemical energy (or ATP formation) is liberated as heat (308,000 cal). Thus, the cellular respiratory process is at best about 55% efficient.

Glucose oxidation is the most commonly studied dissimilatory reaction leading to energy production or ATP synthesis. The complete oxidation of glucose may involve three fundamental biochemical pathways. The first is the glycolytic or Embden- Meyerhof-Parnas pathway, the second is the Krebs cycle (also called the citric acid cycle or tricarboxylic acid cycle), and the third is the series of membrane-bound electron transport oxidations coupled to oxidative phosphorylation.

Respiration takes place when any organic compound (usually carbohydrate) is oxidized completely to CO_2 and H_2O. In aerobic respiration, molecular O_2 serves as the terminal acceptor of electrons. For anaerobic respiration, NO_3-, SO_4^{2-}, CO_2, or fumarate can serve as terminal electron acceptors (rather than 0_2), depending on the bacterium studied. The end result of the respiratory process is the complete oxidation of the organic substrate molecule, and the end products formed are primarily CO_2 and H_2O. Ammonia is formed also if protein (or amino acid) is the substrate oxidized.

Metabolically, bacteria are unlike cyanobacteria (blue-green algae) and eukaryotes in that glucose oxidation may occur by more than one pathway. In bacteria, glycolysis represents one of several pathways by which bacteria can

catabolically attack glucose. The glycolytic pathway is most commonly associated with anaerobic or fermentative metabolism in bacteria and yeasts. In bacteria, other minor heterofermentative pathways, such as the phosphoketolase pathway, also exist.

Fermentation

Fermentation, another example of heterotrophic metabolism, requires an organic compound as a terminal electron (or hydrogen) acceptor. In fermentations, simple organic end products are formed from the anaerobic dissimilation of glucose (or some other compound). Energy (ATP) is generated through the dehydrogenation reactions that occur as glucose is broken down enzymatically. The simple organic end products formed from this incomplete biologic oxidation process also serve as final electron and hydrogen acceptors. On reduction, these organic end products are secreted into the medium as waste metabolites (usually alcohol or acid). The organic substrate compounds are incompletely oxidized by bacteria, yet yield sufficient energy for microbial growth. Glucose is the most common hexose used to study fermentation reactions.

For most microbial fermentations, glucose dissimilation occurs through the glycolytic pathway. The simple organic compound most commonly generated is pyruvate, or a compound derived enzymatically from pyruvate, such as acetaldehyde, a-acetolactate, acetyl ~ SCoA, or lactyl ~ SCoA. Acetaldehyde can then be reduced by NADH + H+ to ethanol, which is excreted by the cell. The end product of lactic acid fermentation, which occurs in streptococci (e.g., *Streptococcus lactis*) and many lactobacilli (e.g., *Lactobacillus casei, L pentosus*), is a single organic acid, lactic acid. Organisms that ferment glucose to multiple end products, such as acetic acid, ethanol, formic acid, and CO_2, are referred to as heterofermenters. Examples of heterofermentative bacteria include *Lactobacillus, Leuconostoc,* and *Microbacterium* species. Heterofermentative fermentations are more common among bacteria, as in the mixed-acid fermentations carried out by bacteria of the family Enterobacteriaceae (e.g., *Escherichia coli, Salmonella, Shigella,* and *Proteus* species). Many of these glucose fermenters usually produce CO_2 and H_2 with different combinations of acid end products (formate, acetate, lactate, and succinate). Many obligately anaerobic clostridia (e.g., *Clostridium saccharobutyricum, C. thermosaccha-rolyticum*) and *Butyribacterium* species ferment glucose with the production of butyrate, acetate, CO_2, and H_2, whereas other *Clostridum* species (*C. acetobutylicum* and *C. butyricum*) also form these fermentation end products plus others (butanol, acetone, isopropanol, formate, and ethanol).

Electron Transport and Oxidative Phosphorylation

The final stage of respiration occurs through a series of oxidation-reduction electron transfer reactions that yield the energy to drive oxidative phosphorylation; this in turn produces ATP. The enzymes involved in electron transport and oxidative phosphorylation reside on the bacterial inner (cytoplasmic) membrane. This membrane is invaginated to form structures called respiratory vesicles, lamellar vesicles, or mesosomes, which function as the bacterial equivalent of the eukaryotic mitochondrial membrane.

Respiratory electron transport chains vary greatly among bacteria, and in some organisms are absent. The respiratory electron transport chain of eukaryotic mitochondria oxidizes NADH + H+, NADPH + H+, and succinate (as well as the coacylated fatty acids such as acetyl~SCoA). The bacterial electron transport chain also oxidizes these compounds, but it can also directly oxidize, via non-pyridine nucleotide-dependent pathways, a larger variety of reduced substrates such as lactate, malate, formate, a-glycerophosphate, H_2, and glutamate. The respiratory electron carriers in bacterial electron transport systems are more varied than in eukaryotes, and the chain is usually branched at the site(s) reacting with molecular O_2. Some electron carriers, such as nonheme iron centers and ubiquinone (coenzyme Q), are common to both the bacterial and mammalian respiratory electron transport chains. In some bacteria, the naphthoquinones or vitamin K may be found with ubiquinone. In still other bacteria, vitamin K serves in the absence of ubiquinone. In mitochondrial respiration, only one cytochrome oxidase component is found (cytochrome *a +a3* oxidase). In bacteria there are multiple cytochrome oxidases, including cytochromes *a, d, o,* and occasionally *a + a3* .

In bacteria cytochrome oxidases usually occur as combinations of *a1: d: o* and *a + a3*: *o*. Bacteria also possess mixed-function oxidases such as cytochromes P-450 and P-420 and cytochromes *c'* and *c'c'*, which also react with carbon monoxide. These diverse types of oxygen-reactive cytochromes undoubtedly have evolutionary significance. Bacteria were present before O_2 was formed; when O_2 became available as a metabolite, bacteria evolved to use it in different ways; this probably accounts for the diversity in bacterial oxygen-reactive hemoproteins.

Cytochrome oxidases in many pathogenic bacteria are studied by the bacterial oxidase reaction, which subdivides Gram-negative organisms into two major groups, oxidase positive and oxidase negative. This oxidase reaction is assayed for by using N,N,N', N'-tetramethyl-*p*-phenylenediamine oxidation (to Wurster's blue) or by using indophenol blue synthesis (with dimethyl-*p*-phenylenediamine and a-naphthol). Oxidase-positive bacteria contain integrated (cytochrome *c* type:oxidase) complexes, the oxidase component most frequently encountered is cytochrome *o*, and occasionally *a + a3*. The cytochrome oxidase responsible for the indophenol oxidase reaction complex was isolated from membranes of *Azotobacter vinelandii*, a bacterium with the highest respiratory rate of any known cell. The cytochrome oxidase was found to be an integrated cytochrome *c4:o complex*, which was shown to be present in *Bacillus* species. These *Bacillus* strains are also highly oxidase positive, and most are found in morphologic group II.

Autotrophy

Bacteria that grow solely at the expense of inorganic compounds (mineral ions), without using sunlight as an energy source, are called autotrophs, chemotrophs, chemoautotrophs, or chemolithotrophs. Like photosynthetic organisms, all autotrophs use CO_2 as a carbon source for growth; their nitrogen comes from inorganic compounds such as NH_3, NO_3^-, or N_2 (Table 7-1). Interestingly, the energy source for such organisms is the oxidation of specific inorganic compounds. Which inorganic

compound is oxidized depends on the bacteria in question. Many autotrophs will not grow on media that contain organic matter, even agar.

Also found among the autotrophic microorganisms are the sulfur-oxidizing or sulfur-compound-oxidizing bacteria, which seldom exhibit a strictly autotrophic mode of metabolism like the obligate nitrifying bacteria (see discussion of nitrogen cycle below). The representative sulfur compounds oxidized by such bacteria are H_2S, S_2, and S_2O_3. Among the sulfur bacteria are two very interesting organisms; *Thiobacillus ferrooxidans*, which gets its energy for autotrophic growth by oxidizing elemental sulfur or ferrous iron, and *T denitrificans*, which gets its energy by oxidizing S_2O_3 anaerobically, using NO_3^- as the sole terminal electron acceptor. T denitrificans reduces NO_3 to molecular N_2, which is liberated as a gas; this biologic process is called denitrification.

All autotrophic bacteria must assimilate CO_2, which is reduced to glucose from which organic cellular matter is synthesized. The energy for this biosynthetic process is derived from the oxidation of inorganic compounds discussed in the previous paragraph. Note that all autotrophic and phototrophic bacteria possess essentially the same organic cellular constituents found in heterotrophic bacteria; from a nutritional viewpoint, however, the autotrophic mode of metabolism is unique, occurring only in bacteria.

Anerobic Respiration

Some bacteria exhibit a unique mode of respiration called anaerobic respiration. These heterotrophic bacteria that will not grow anaerobically unless a specific chemical component, which serves as a terminal electron acceptor, is added to the medium. Among these electron acceptors are NO_3, SO_4^2, the organic compound fumarate, and CO_2. Bacteria requiring one of these compounds for anaerobic growth are said to be anaerobic respirers.

A large group of anaerobic respirers are the nitrate reducers. The nitrate reducers are predominantly heterotrophic bacteria that possess a complex electron transport system(s) allowing the NO3 ion to serve anaerobically as a terminal acceptor of electrons ($NO_3 \xrightarrow{2e^-} NO_2$; $NO_3 \xrightarrow{5e^-} N_2$; or $NO_3 \xrightarrow{8e^-} NH_3$). The nitrate reductase activity is common in bacteria and is routinely used in the simple nitrate reductase test to identify bacteria (see *Bergey's Manual of Deterininative Bacteriology*, 8th ed.).

The methanogens are among the most anaerobic bacteria known, being very sensitive to small concentrations of molecular O_2. They are also archaebacteria, which typically live in unusual and deleterious environments.

All of the above anaerobic respirers obtain chemical energy for growth by using these anaerobic energy-yielding oxidation reactions.

The Nitrogen Cycle

Nowhere can the total metabolic potential of bacteria and their diverse chemical-transforming capabilities be more fully appreciated than in the geochemical cycling of the element nitrogen. All the basic chemical elements (S, O, P, C, and H) required to sustain living organisms have geochemical cycles similar to the nitrogen cycle.

The nitrogen cycle is an ideal demonstration of the ecologic interdependence of bacteria, plants, and animals. Nitrogen is recycled when organisms use one form of nitrogen for growth and excrete another nitrogenous compound as a waste product. This waste product is in turn utilized by another type of organism as a growth or energy substrate.

The other important biologic processes in the nitrogen cycle include nitrification (the conversion of NH_3 to NO_3 by autotrophes in the soil; denitrification (the anaerobic conversion of NO_3 to N_2 gas) carried out by many heterotrophs); and nitrogen fixation (N_2 to NH_3, and cell protein). The latter is a very specialized prokaryotic process called diazotrophy, carried out by both free-living bacteria (such as *Azotobacter, Derxia, Beijeringeia,* and *Azomona* species) and symbionts (such as *Rhizobium* species) in conjunction with legume plants (such as soybeans, peas, clover, and bluebonnets). All plant life relies heavily on NO3- as a nitrogen source, and most animal life relies on plant life for nutrients.

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. In the laboratory, bacteria are grown in culture mediawhich are designed to provide all the essential nutrients in solution for bacterial growth.

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. The general physiological functions of the elements are outlined in the Table 11.

The above table ignores the occurrence of trace elements in bacterial nutrition. Trace elements are metal ions required by certain cells in such small amounts that it is difficult to detect (measure) them, and it is not necessary to add them to culture media as nutrients. Trace elements are required in such small amounts that they are present as "contaminants" of the water or other media components. As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell. One organism's trace element may be another's required element and vice-versa, but the usual cations that qualify as trace elements in bacterial nutrition are Mn, Co, Zn, Cu, and Mo.

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.

Table 11. 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, SO, 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

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(hetero)trophs. 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 CO_2. Organisms that use organic carbon are heterotrophs and organisms that use CO_2 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 procaryotes may be defined (Table 12).

Table 12. Major nutritional types of prokaryotes

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 procaryotes 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.

This simplified scheme for use of carbon, either organic carbon or CO2, ignores the possibility that an organism, whether it is an autotroph or a heterotroph, may require small amounts of certain organic compounds for growth because they are essential substances that the organism is unable to synthesize from available nutrients. Such compounds are called **growth factors**.

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.

Some bacteria (e.g *E. coli*) do not require any growth factors: they can synthesize all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism. Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids in order to grow. These compounds must be added in advance to culture media that are used to grow these bacteria. The growth factors are not metabolized directly as sources of carbon or energy, rather they are assimilated by cells to fulfill their specific role in metabolism. Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**. Thus, a strain of *E. coli* that requires the amino acid tryptophan in order to grow would be called a tryptophan auxotroph and would be designated *E. coli* trp-.

Some vitamins that are frequently required by certain bacteria as growth factors are listed in Table 13. The function(s) of these vitamins in essential enzymatic reactions gives a clue why, if the cell cannot make the vitamin, it must be provided exogenously in order for growth to occur.

Table 13. Common vitamins required in the nutrition of certain procaryotes

Vitamin	Coenzyme form	Function
p-Aminobenzoic acid (PABA)		Precursor for the biosynthesis of folic acid
Folic acid	Tetrahydrofolate	Transfer of one-carbon units and required for synthesis of thymine, purine bases, serine, methionine and pantothenate
Biotin	Biotin	Biosynthetic reactions that require CO2 fixation
Lipoic acid	Lipoamide	Transfer of acyl groups in oxidation of keto acids
Mercaptoethane-sulfonic acid	Coenzyme M	CH4 production by methanogens
Nicotinic acid	NAD (nicotinamide adenine dinucleotide) and NADP	Electron carrier in dehydrogenation reactions
Pantothenic acid	Coenzyme A and the Acyl Carrier Protein (ACP)	Oxidation of keto acids and acyl group carriers in metabolism
Pyridoxine (B6)	Pyridoxal phosphate	Transamination, deamination, decarboxylation and racemation of amino acids
Riboflavin (B2)	FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide)	Oxidoreduction reactions
Thiamine (B1)	Thiamine pyrophosphate (TPP)	Decarboxylation of keto acids and transaminase reactions
Vitamin B12	Cobalamine coupled to adenine nucleoside	Transfer of methyl groups
Vitamin K	Quinones and napthoquinones	Electron transport processes

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. Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a required salt concentration. In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation-reduction potential.

The manner in which bacteria are cultivated, and the purpose of culture media, vary 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 degrees and remains liquid until cooled to 40 degrees, 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 aquaeous solution (fig. 90).

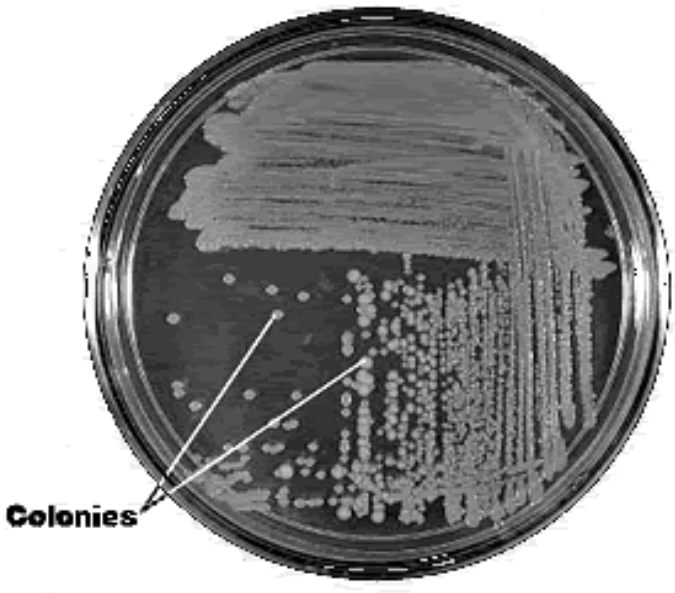


Figure 90. Culture Media for the Growth of Bacteria

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.

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).

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 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 staphylococcus 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 (fig. 91).

Figure 91. Isolation of pure culture

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. However, an enrichment medium may have selective features. An enrichment medium for nonsymbiotic nitrogen-fixing bacteria omits a source of added nitrogen to the medium. The medium is inoculated with a potential source of these bacteria (e.g. a soil sample) and incubated in the atmosphere wherein the only source of nitrogen available is N_2. A selective enrichment medium for growth of the extreme halophile (*Halococcus*) contains nearly 25 percent salt [NaCl], which is required by the extreme halophile and which inhibits the growth of all other procaryotes.

Thus, nutrient media can be subdivided into three main groups:

I. Ordinary (simple) media which include meat-peptone broth, meat-peptone agar, etc.

II. Special media (serum agar, serum broth, coagulated serum, potatoes, blood agar, blood broth, etc.).

Quite often elective media are employed in laboratory practice in which only certain species of bacteria grow well, and other species either grow poorly or do not grow at all. Enriched media are also employed in which the species of interest to the scientist grows more intensively and more rapidly than the accompanying bacteria. Thus, for example, on Endo's medium (elective) the growth of the Gram-positive microbes is inhibited while alkaline peptone water and alkaline meat-peptone agar serve as enriched media for the cholera vibrio. Nutrient media containing certain concentrations of penicillin are elective for penicillin-resistant strains of bacteria, but unfavourable for penicillin-sensitive strains.

III. Differential diagnostic media: (1) media for the determination of the proteolytic action of microbes (meat-peptone gelatine); (2) media for the determination of the fermentation of carbohydrates (Hiss media); media for the differentiation of bacteria which do and do not ferment lactose (Ploskirev, Drigalsky,

Endo. etc.); (3) media for the determination of haemolytic activity (blood agar); (4) media for the determination of the reductive activity of micro-organisms; (5) media containing substances assimilated only by certain microbes.

Besides, in laboratory practice conservation media are used. They are used for primary seeding and transportation of the material under test. They prevent the death of pathogenic microbes and enhance the inhibition of saprophytes. This group of media includes a glycerin mixture composed of two parts 0.85 per cent salt solution, 1 part glycerin, and I part 15-20 per cent acid sodium phosphate, and also a glycerin preservative with lithium salts, a hypertonic salt solution, etc.

At present many nutrient media are prepared commercially as dry powders. They are convenient to work with, are stable, and quite effective.

Non-protein media are widely used for the cultivation of bacteria, on which many heterotrophic microbes including pathogenic species grow well. The composition of these media is complex and includes a large number of components.

When cultivating in synthetic media, the use of the method of radioactive tracers has permitted a more detailed differentiation of microbes according to the character of their biosynthesis.

Selective media are widely used for differentiating prototrophic and ULixotrophic bacteria. Prototrophs grow on a minimum medium which contains only salts and carbohydrates since they themselves are capable til' synthesizing the metaholites necessary for their development. Auxo-Irophs. in distinction, require definite media containing amino acids, vitamins, and other substances.

In consistency nutrient media may be solid (meat-peptone agar, meat-peptone gelatine, coagulated serum, potato, coagulated white of Ihc egg), semisolid (0.5 per cent meat-peptone agar), and liquid (peptone water, meat-peptone broth, sugar broth, etc.).

Physical and Environmental Requirements for Microbial Growth

The procaryotes 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 procaryote, 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 procaryotes 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, procaryotes display a wide range of responses to molecular oxygen O_2 (Table 14).

Table 14. Terms used to describe O_2 Relations of Microorganisms

Group	Environment		O_2 Effect
	Aerobic	**Anaerobic**	
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

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

Obligate anaerobes (occasionally called **aerophobes**) 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 procaryotes 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.

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 O2. 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_20_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or O_2. 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 (Table 15).

Table 15. Distribution of superoxide dismutase, catalase and peroxidase in procaryoteswith 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, Methanogens, Bacteroides)	-	-	-

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 nonphotosynthetic) 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.

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 (Table 16).

Table 16. Minimum, maximum and optimum pH for growth of certain prokaryotes

Organism	Minimum pH	Optimum pH	Maximum pH
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter spp	6.6	7.6-8.6	10.0

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 neutrophilesand 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, and 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.

Enzymes and Their Role in Metabolism

Enzymes, organic catalysts of a highly molecular structure, are produced by the living cell. They are of a protein nature, are strictly specific in action, and play an important part in the metabolism of microorganisms. Their specificity is associated with active centres formed by a group of amino acids.

Enzymes of microbial origin have various effects and are highly active. They have found a wide application in industry, agriculture and medicine, and are gradually replacing preparations produced by higher plants and animals.

With the help of amylase produced by mould fungi starch is saccharified and this is employed in beer making, industrial alcohol production and bread making. Proteinases produced by microbes are used for removing the hair from hides, tanning hides, liquefying the gelatinous layer from films during regeneration, and for dry cleaning. Fibrinolysin produced by streptococci dissolves the thrombi in human blood vessels. Enzymes which hydrolyse cellulose aid in an easier assimilation of rough fodder.

Due to the application of microbial enzymes, the medical industry has been able to obtain alkaloids, polysaccharides, and steroids (hydrocortisone, prednisone, prednisolone. etc.).

Bacteria play an important role in the treatment of caoutchouc, collon. silk. coffee, cocoa, and tobacco: significant processes lake place under their effect which change these substances essentially in the needed direction. In specific weight the synthetic capacity of microorganisms is very high. The total weight of bacterial cytoplasm on earth is much higher than that of animal cytoplasm. The biochemical activity of microbes is of no less general biological importance than that of photosynthesis. The cessation of the existence of microorganisms would lead inevitably to the death of plants and animals.

Enzymes permit some species of micro-organisms to assimilate methane. butane, and other hydrocarbons, and to synthesize complex organic compounds from them. Thus, for example, with the help of the enzymatic ability of yeasts in special-type industrial installations protein-vitamin concentrates (PVC) can be obtained from waste products of petroleum (paraffins), which are employed in animal husbandry as a valuable nutrient substance supplementing rough fodder. Some soil micro-organisms destroy by means of enzymes chemical substances (carcinogens) which are detrimental to the human body because they induce malignant tumours.

Some enzymes are excreted by the cell into the environment (exoenzymes) for breaking down complex colloid nutrient materials while other enzymes are contained inside the cell (endoenzymes).

Depending on the conditions of origin of enzymes there are constitutive enzymes which are constantly found in the cell irrespective of the presence of a catalysing substrate. These include the main enzymes of cellular metabolism (lipase. carbohydrase. proteinase, oxydase, etc.). Adaptive enzymes occur only in the presence of the corresponding substrate (penicillinase, amino acid decarboxylase, alkaline phosphatase, B-galactosidase, etc.). The synthesis of induced enzymes in microbes occurs due to the presence in the cells of free amino acids and with the participation of ready proteins found in the bacteria.

According to chemical properties enzymes can be subdivided into three groups:

1 – enzymes composed only of proteins:

2 – enzymes containing in addition, to protein metallic ions essential for their activity, and assisting m the combination of the enzyme with the substrate, and taking part in the cyclic enzymatic transformations:

3 – enzymes which contain distinct organic molecules (coenzymes. prosthetic groups) essential for their activity. Some enzymes contain vitamins.

Bacterial enzymes are subdivided into some groups:

1. Hydrolases which catalyse the breakdown of the link between the carbon and nitrogen atoms, between the oxygen and sulphur atoms, binding one molecule of water (esterases. glucosidases, proteases. amidases, nucleases, etc.).

2. Transferases perform catalysis by transferring certain radicals from one molecule to another (transglucosidases, transacylases. transaminases).

3. Oxidative enzymes (oxyreductases) which catalyse the oxidation reduction processes (oxidases, dehydrogenases, peroxidases, catalases).

4. Isomerases and racemases play an important part in carbohydrate metabolism. They are found in most species of bacteria. Phosphohexoisomerase,

galactovaldenase, phosphoglucomutase, hosphoglyceromutase pertain to the isomerases.

The absorption of food material by the cell is a rather complex process. Unicellular protozoa are characterized by a holozoic type of nutrition in which hard food particles are swallowed, digested and converted to soluble compounds. Bacteria, algae, fungi, and plants possess a holophytik type of nutrition. They absorb nutrients in a dissolved state. This difference, however, is not essential because the cells of protozoa, just like the cells of plant organisms, utilize nutrient substrates which are soluble in water or in the cell sap, while many bacteria and fungi can assimilate hard nutrients first splitting them by external digestion by means of exoenzymes. During diffusion the dissolved substance is transferred from the region of higher concentration outside the cell into the bacterial cell until the concentration becomes the same. The passage of a solvent through the cytoplasmic membrane of bacteria from a region where it is less concentrated to one where it is more concentrated is performed by osmosis. The concentration gradient and osmotic power on both sides of the cytoplasmic membrane are quite different, and depend on the difference in concentration of many substances contained in the cell and nutrient medium. The transfer of dissolved substances from the nutrient medium to the cell can take place by suction together with the solvent if the membrane is sufficiently porous.

It has been established that the cellular membranes are made up of lipid and protein molecules arranged in a certain sequence. The charged groups of molecules have their ends directed towards the surface of the membrane. On these charged ends the protein layers are adsorbed, composed of protein chains forming a meshwork on the external and internal surfaces of the membrane. The high selectivity which allows the cells to distinguish certain substances from others depends on the presence of enzymatic systems localized on the surface of bacterial cells. Due to the action of these enzymes, the insoluble substances in the membrane become soluble.

The cell membranes play an important role in metabolism. They are capable of changing rapidly their permeability to various substances and regulating in this way the entry of substances into the cell and their distribution in it, and the development of reactions in which these substances participate.

Some bacteria (Salmonella typhimurium} possess rudiments of memory. They recognize whether the medium is favourable or unfavourable to them. They 'run away' from an unfavourable one by means of flagella: when close to a favourable medium (glucose) Salmonella organisms swim to the 'bait'. This ability to recognize the needed direction is probably accomplished by the trial-and-error method.

In the process of bacterial nutrition great importance is attached to exchange adsorption. The active transport of ions takes place due to (he difference in charges on the surface of membranes in the cell wall and the surrounding medium of the micro-organisms. Besides, the role of transporters, as has been suggested, is performed by liposoluble substances X and Y. Compounds are formed with ions of potassium and sodium (KX and NaY) which are capable of diffusing through the cell wall, while the membrane remains unpenetrable for free transporters. Proteins concerned with the transport of amino acids have been isolated from the membranes

of some micro-organisms, and protein systems responsible for the transfer of certain sugars in general and glucose in particular have been revealed.

Practical Use of the Fermentative Properties of Microbes

The widespread and theoretically founded application of microbiological processes in the technology of industries involving fermentation, treatment of flax, hides, farming, and canning of many food products became possible only in the second half of the 19th century. From the vital requirements of a vigorously developing industry, especially of the agricultural produce processing industry, there arose a need for a profound study of biochemical processes. The investigations by Pasteur in this field were prepared to a great extent by the development of industry, organic chemistry, and other sciences.

Microorganisms take part in the cycle of nitrogen (putrefaction), carbon (fermentation), sulphur, phosphorus, iron, and other elements which are important in the vital activity of organisms. Therapeutic muds and brine were produced as the result of the fermentative activity of definite microbial species. Micro-organisms are used as indicators for determining hydrolytic processes in seas and oceans, the soil requirements of fertilizers, and the exact amount of vitamins, amino acids and other substances which cannot be determined by chemical analytical methods. Certain species of microorganisms synthesize antibiotics, enzymes, hormones, vitamins, and amino acids which are industrially prepared and used in medicine, veterinary practice, and agriculture. The synthesis of proteins by means of special species of yeasts has been mastered.

Some soil bacteria are capable of rendering harmless (destroying) certain pesticides used in agriculture as well as chemical carcinogens. Hydrogenous bacteria may be used to produce fodder protein by cultivation on urea or ammonium sulphate. Some bacterial species are used for the control of methane in mines. Methanol, a monocarbon alcohol, is produced from methane by means of microbes.

Of great importance in medical microbiology is the utilization of the specific fermentative capacity of pathogenic bacteria for the determination of their species properties. Many bacteria ferment carbohydrates producing acid or acid and gas, while proteins are fermented with the production of indole, ammonia, hydrogen sulphide, etc.

Fermentative properties of microbes are used in the laboratory diagnosis of infectious diseases, and in studying microbes of the soil, water, and air.

Influence of Environmental Factors on Microbes Effect of Physical Factors

The effect of temperature. Microbes can withstand low temperatures fairly well. The cholera vibrio does not lose its viability at a temperature of -32°C. Some species of bacteria remain viable at a temperature of liquid air (190°C) and of liquid hydrogen (- 253°C). Diphtheria bacilli are able to withstand freezing for three months and enteric fever bacteria are able to live long in ice. Bacillus spores withstand a temperature of –253°C for 3 days. Many microorganisms remain viable at low temperatures, and viruses are especially resistant to low temperatures. Thus, for example, the virus of Japanese encephalitis in a 10 per cent brain suspension does not lose its pathogenicity at -70°C over a period of one year, the causative agents of

influenza and trachoma at -70 C for 6 months and Coxsackie virus at —WC for 1.5 years. Low temperatures halt putrefying and fermentative processes. In sanitary-hygienic practice ice, cellars, and refrigerators for the storage of food products arc used according to this principle.

Only certain species of pathogenic bacteria are very sensitive to low temperatures (e. g. meningococcus, gonococcus, etc.). During short periods of cooling these species perish quite rapidly. This is taken into account in laboratory diagnosis, and materials under test for the presence of meningitis or gonorrhoea are conveyed to the laboratory protected from cold.

At low temperatures the processes of metabolism are inhibited, the bacteria die off as a result of ageing and starvation, and the cells are destroyed under the effect of the formation of ice crystals during freezing. Alternate high and low temperatures are lethal to microbes. It has been established, for instance, that sudden freezing as well as sudden healing causes a decrease in the life activities of pathogenic microbes.

Most asporogenic bacteria perish at a temperature of 58-60 ^{0}C within 30-60 minutes. Bacillus spores are more resistant than vegetative cells. They withstand boiling from a few minutes to 3 hours, but perish under the effect of dry heat at 160-170°C in 1.0-1.5 hours. Heating at 120.6°C at 2 aim steam pressure kills them within 20-30 minutes, Individual and specific variations in the resistance of microbes to high temperatures have different limits and a rather large temperature range.

The inhibition of the activity of catalase. oxydase, dehydrogenase, protein denaturation, and an interruption of the osmotic barrier are the principles of the bacterial action of high temperatures. High temperatures cause a rather rapid destruction of viruses, but some of them (viruses of infectious hepatitis. poliomyelitis, etc.) are resistant to environmental factors. They remain viable long in water, in the faeces of sick people or carriers, and are resistant to heat at 60°C and to small concentrations of chlorine in water.

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 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. Boiling temperature could not inactivate any essential enzyme. Subsequently, procaryotes have been detected growing around black smokers and hydrothermal vents in the deep sea at temperatures at least as high as 115 degrees. Microorganisms have been found growing at very low temperatures as well. In supercooled solutions of H2O 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 procaryotes 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 (Table 17).

Table 17. Terms used to describe microorganisms in relation to temperature requirements for growth

Group	Minimum	Optimum	Maximum	Comments
Psychrophile	Below 0	10-15	Below 20	Grow best at relatively low T
Psychrotroph	0	15-30	Above 25	Able to grow at low T but prefer moderate T
Mesophile	10-15	30-40	Below 45	Most bacteria esp. those living in association with warm-blooded animals
Thermophile	45	50-85	Above 100 (boiling)	Among all thermophiles is wide variation in optimum and maximum T

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. Think how fast milk spoils on the counter top versus in the refrigerator.

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 procaryotes. 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 canola 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.

The effect of desiccation

Micro-organisms have a different resistance to desiccation to which gonococci, meningococci. treponemas, leptospiras. haemoglobinophilic bacteria, and phages are sensitive. On exposure to desiccation the cholera vibrio persists for 2 days. dysentery bacteria — for 7, plague — for 8. diphtheria — for 30, enteric fever — for 70, staphylococci and tubercle bacilli — for 90 days. The dry sputum of tuberculosis patients remains infectious for 10 months, the spores of anthrax bacillus remain viable for 10 years, and those of moulds for 20 years.

Desiccation is accompanied with dehydration of the cytoplasm and denaturation of bacterial proteins. Sublimation is one of the methods used for the preservation of food. It comprises dehydration at low temperature and high vacuum, which is attended with evaporation of water and rapid cooling and freezing. The ice formed in the food is easily sublimated, by-passing the liquid phase. The food may be stored for more than two years. In drying by sublimation all the sugars, vitamins, enzymes, and other components are preserved. Desiccation in a vacuum at a low temperature does not kill bacteria, rickettsiae. or viruses. This method of preserving cultures is employed in the manufacture of stable long-storage, live vaccines against tuberculosis, plague, tularaemia. brucellosis, smallpox, influenza, and other diseases.

Quick freezing of bacterial and viral suspensions at very low temperatures provokes conditions at which crystals do not form, and subsequent disruption of the micro-organisms does not occur.

The effect of light. Some bacteria (purple) withstand the effect of light fairly well. while others are injured. Direct sunlight has the greatest bactericidal action.

Investigations have established that different kinds of light have a bactericidal or sterilizing effect. These include ultraviolet rays (electromagnetic waves with a

wave length of 200-300 nm). X-rays (electromagnetic rays with a wave length of 0.005-2.0 nm), gamma-rays (short wave X-rays), beta- particles or cathode rays (high speed electrons). alpha-particles (high speed helium nuclei) and neutrons.

The experiments in which short waves were used for the disinfection of wards, infectious material, for the conservation of products, the preparation of vaccines, for treating operating rooms and maternity wards. etc., have demonstrated that they have a rather high bactericidal effect. Viruses are very quickly inactivated under the effect of ultraviolet rays with a wave length of 260-300 nm. These waves are absorbed by the nucleic acid of viruses. Longer waves are weaker and do not render vi- ruses harmless.

Viruses in comparison to bacteria are less resistant to X-rays, and gamma-rays. Beta-rays are more markedly viricidal. Alpha-, beta-, and gamma-rays in small doses enhance multiplication but in large doses they are lethal to microbes. Viruses which are pathogenic to animals are inactivated by 44000-280000 roentgens. Thiobacteria which live in uranium ore deposits are highly resistant to radioactive rays. Bacteria were found in the water of atomic reactors at ionizing radiation concentration of 2-3 million rads.

Ionizing radiation can be used for practical purposes in sterilizing food products, and this method of cold sterilization has a number of advantages. The quality of the product is not changed as during heat sterilization which causes denaturation of its component parts (proteins, polysaccharides, vitamins). Radiation sterilization can be applied in the practice of treating biological preparations (vaccines, sera. phages, etc.).

Of interest is the phenomenon of photoreactivation described in 1949 by A. Kelner. If a suspension of bacteria is preliminarily exposed to visible light radiation, it becomes more resistant to ultraviolet radiation. If after exposure to strong ultraviolet light a suspension of colibacilli is irradiated with visible light, marked growth of the bacteria is observed when they are seeded on nutrient media.

The effect of high pressure and mechanical injury on microbes. Bacteria withstand easily atmospheric pressure. They do not noticeably alter at pressure from 100 to 900 aim at marine and oceanic depths of 1000-10000 m. Yeasts retain their viability at a pressure of 500 aim. Some bacteria, yeasts, and moulds withstand a pressure of 3000 aim and phytopathogenic viruses withstand 5000 aim.

The movement of liquid media has a harmful effect on microbes. The movement of water in rivers and streams, undulations in stagnant waters are factors important in self-purification of reservoirs from microbes.

Ultrasonic oscillation (waves with a frequency of about 20000 hertz per second) has bactericidal properties. At present this is used for the sterilization of food products, for the preparation of vaccines, and the disinfection of various objects.

The mechanism of the bactericidal action of ultrasonic oscillation is that in the cytoplasm of bacteria found in an aquatic medium a cavity is formed which is Filled with liquid vapours. A pressure of 10000 atmospheres occurs in the bubble, which leads to disintegration of the cytoplasmic structures. It is possible that highly reactive hydroxyl radicals originate in the cavities formed in the sonified water medium.

Of certain significance in rendering the air harmless is aeroionization. The negatively charged ions have a more lethal effect on the microbes.

Effect of Chemical Factors

Depending on the physicochemical composition of the medium, concentration, the length of contact and temperature chemical substances have a different effect on microbes. In small doses they act as stimulants, in bactericidal concentrations they paralyse the dehydrogenase activity of bacteria.

According to their effect on bacteria, bactericidal chemical substances can be subdivided into surface-active substances, dyes, phenols and their derivatives, salts of heavy metals, oxidizing agents, and the formaldehyde group.

Surface-active substances change the energy ratio. Bacterial cells lose their negative charge and acquire a positive charge which impairs the normal function of the cytoplasmic membrane.

Bactericidal substances with surface-active action include fatty acids and soaps which harm only the cell wall and do not penetrate into the cell.

Phenol, cresol, and related derivatives first of all injure the cell wall and then the cell proteins. Some substances of this group inhibit the function of the coenzyme (diphosphopiridine nucleotide) which participates in the dehydrogenation of glucose and lactic acid. Dyes are able to inhibit the growth of bacteria. The basis of this action is the marked affinity for the phosphoric acid groups of nucleoproteins. Dyes with bactericidal properties include brilliant green. rivanol, tripaflavine, acriflavine, etc.

Salts of heavy metals (lead, copper, fine, silver, mercury) cause coagulation of the cell proteins. When the salts of the heavy metal interact with the protein a metallic albuminate and a free acid are produced.

A whole series of metals (silver, gold, copper, zinc, tin. lead, etc.) have an oligodynamic action (bactericidal capacity). Thus, for example. silverware, silver-plated objects, silver-plated sand in contact with water render the metal bactericidal to many species of bacteria. The mechanism of the oligodynamic action is that the positively charged metallic ions arc adsorbed on the negatively charged bacterial surface. and alter the permeability of the cytoplasmic membrane. It is possible that during this process the nutrition and reproduction of bacteria are disturbed. Viruses also are quite sensitive to the salts of heavy metals under the influence of which they become irreversibly inactivated.

Oxidizing agents act on the sulphohydryl groups of active proteins. More powerful oxidizing agents are harmful also to other groups (phenol, thioelhyl, indole. amine).

Oxidizing agents include chlorine which impairs dehydrogenases, hydrolases. amylases and proteinases of bacteria and which is widely used in decontaminating water, and chloride of lime and chloramine used as disinfectants. In medicine iodine is used successfully as an anti-microbial substance in the form of iodine tincture which not only oxidizes the active groups of the proteins of bacterial cytoplasm, but brings about their denaturation. Potassium permanganate, hydrogen peroxide, and other substances also have oxidizing properties.

Many species of viruses are resistant to the action of ether, chloroform, ethyl and methyl alcohol, and volatile oils. Almost all viruses survive for long periods in the presence of whole or 50 per cent glycerin solution, in Ringer's and Tyrode's solutions. Viruses are destroyed by sodium hydroxide, potassium hydroxide, chloramine, chloride of lime, chlorine, and other oxidizing agents.

Formaldehyde is used as a 40 per cent solution known as formalin. Its antimicrobial action can be explained, as presumed, by its being united to the amino groups of proteins which causes their denaturation. Formaldehyde kills both the vegetative forms as well as the spores. It is applied for decontaminating diphtheria and tetanus toxins as a result of which they are transformed into antitoxins Some viruses (phages, tobacco mosaic virus) inactivated by formalin can sometimes renew their infectivity.

Fabrics possessing an antimicrobial effect have been produced, in which the molecules of the antibacterial substance are bound to the molecules of the material. The fabrics retain the bactericidal properties for a long period of time even after being washed repeatedly. They may be used for making clothes for sick persons, medical personnel, pharmaceutists, for the personnel of establishments of the food industry and for making filters for sterilizing water and air.

Effect of Biological Factors

In nature microorganisms constitute a component of the biocoenosis (a community of plants and animals living in a part of the habitat with more or less homogenous conditions of life).

Microbes are found in nature in associations among which there is a constant struggle for existence. Certain species which adapted themselves to a given medium have more marked antagonistic properties in relation to other species which have fallen into a new habitat. Thus, for example, lactic acid bacteria are antagonistic in relation to the causative agents of dysentery, plague, etc. Blue-pus bacteria inhibit the growth of dysentery, enteric fever microbes, anthrax bacilli, cholera vibrio, causative agents of plague, glanders, and staphylococci. meningococci. etc. The normal inhabitants of the human body (e. g. Colibacilli. enterococci, lactobacilli, microflora of the skin and nasopharynx, etc.) have especially potent antagonistic properties.

For many years a controversy raged on the possibility of intra-species antagonism among microbes. At the present time many scientists have established the antagonistic relationships not only between virulent and non-virulent strains of the same species. These properties are found in certain strains of colibacilli. Streptococcus pneumoniae, enteric fever, and dysentery bacteria, staphylococci. etc.

In certain conditions antagonistic properties appear in microbes due to a lack of nutrients, as a result of which some microbes are forced to feed at the expense of others. This phenomenon was named forced antagonism by I. Schiller. Antagonistic relations have been established by viruses when one virus protects the organism from penetration by another virus. In virology this has been called viral interference.

Among various groups of microbes there are several types of relationships: symbiosis, metabiosis, satellism, synergism, and antagonism. **Symbiosis** represents an intimate mutually beneficial relation of organisms of different species. They

develop together better than separately. Sometimes the adaptation of two organisms becomes so profound that they lose their ability to exist separately (symbiosis of the fungus and blue-green algae, nitrogen-fixing bacteria and cellulose-decomposing bacteria, symbiosis of nodule bacteria with legume plants, various fungi with the roots of plants, yeast-like fungi and lamblias).

Metabiosis is that type of relationship in which one organism continues the process caused by another organism, liberating it from the products of life activities, and thus creating conditions for its further development (nitrifying and ammonizing bacteria).

During **satellism** one of the symbionts known as the favourable microbe incites the growth of the other (some yeasts and sarcinae producing amino acids, vitamins, etc., enhance the growth of microbes more strict in relation to nutrient media).

Synergism is characterized by the increase in the physiological functions of the microbial association (yeasts, lactobacilli, fusobacteria, and Borrelia organisms).

One of the forms of symbiosis is a virus-carrying form ~ a communal existence of some bacteria and protozoa with viruses (lysogenic bacteria retain the corresponding phages for long periods in their cells, during chronic tonsillitis besides the α-haemolytic streptococci, the adenoviruses take part in the infectious process, etc.).

During **antagonistic** relationships there is a struggle for oxygen, nutrients, and a habitat. Modern understanding of the problems of microbiology unfolds complex relationships among organisms and the essence of biological laws.

Biological factors have received widespread application in treating many infectious diseases with the products of the life activities of bacteria, fungi, higher plants, and animal tissues known as antibiotics. These effective drug preparations include penicillin, streptomycin. chloramphenicol. tetracycline, and many others.

In decontaminating the environment from pathogenic microorganisms by antagonism an important role is played by phages widespread in the soil and water and by phytoncides, volatile substances of many plants.

The influence of the environment is taken into account by the physician in combating harmful micro-organisms (sterilization, disinfection), vectors of causative agents of infectious disease (disinsection) and rodents — reservoirs of pathogenic microorganisms (deratization).

Forms of Symbiosis. According to the character of interrelationship with the plant and animal world, microbes can be subdivided into two groups: saprophytes and parasites. Saprophytes include micro-organisms unable to cause disease.

Parasites are microbes which live at the expense of plant and animal bodies. All kinds of associations of the macro-organisms and microorganisms constitute symbiosis in its broadest sense. Symbiosis has different forms: commensalism, mutualism and parasitism.

Commensalisin is a kind of symbiosis (association) of organisms in which one of them lives at the expense of the other without causing it any harm. The overwhelming majority of representatives of the normal microflora of the human body belong to microbe-commensals.

Mutualism is that kind of symbiosis in which both organisms concerned receive mutual benefit from their association. For example, the symbiosis of nodule bacteria with legume plants is characterized by typical mutualism. Nodule bacteria live in plant roots, while the legumes for their nutrition utilize nitrogenous compounds produced by the bacteria from atmospheric nitrogen A commonly encountered mutualism are the numerous lichens (the Arctic reindeer lichen and many others) which are formed of green or blue-green algae and Ascomycetes or Basidiomycetes fungus By means of photosynthesis, the algae provide themselves and the fungus with nutrition, while the fungus protects the algae, supplies them with water and mineral salts Some species of bacteria from the group of intestinal microflora live in symbiosis with animal organisms which they inhabit These microbe- mutualists feed on food remains which enter the lower part of the intestine, while the vitamins which they produce are used by the animals for biocatalytic reactions.

Parasitism is that state of symbiosis in which one organism (parasite) lives at the expense of another (host) and is harmful to it Many microbe-parasites are capable of causing infectious diseases in plants and animals.

Disease-producing species of micro-organisms are known as pathogenic organisms They have adapted themselves in the process of evolutionary development to a parasitic type of nutrition in tissues and fluids of the animal body The susceptible infected organism responds to the entry of the pathogenic microbe by non-specific and specific biological reactions. These are expressed in atypical or typical manifestations of the disease, and also in a variety of defense adaptations.

At one time J. Henle and then R. Koch (1878. 1882) formulated three conditions in the presence of which the given microbe can be recognized as a causative agent of a disease. Henle-Koch's triad consists in the following: (1) the microbe-causative agent should be discovered in all cases during a given disease, and is found neither in healthy persons nor in patients with other diseases; (2) the microbe-causative agent should be isolated from the patient's body in a pure culture: (3) the pure culture of the isolated microbe should cause the same disease in susceptible animals. At present this triad has lost its significance to a considerable degree.

For the origination and development of the infectious process three conditions are necessary. (I) the presence of d pathogenic microbe, (2) its penetration into a susceptible macro-organism, and (3) certain environrnental conditions in which the interaction between the micro-organism and macro-organism takes place.

The interrelationship of the pathogenic micro-organism and the susceptible macro-organism takes place under the complex conditions of the parasite coenosis, that is. in various relationships with other microbes and protozoa

The results of the penetration of pathogenic microbes into the human body depend not only on the reactivity of the macro-organism, but on the normal microflora of the human body), which can express itself antagonistically as well as synergistically

Besides pathogenic organisms, there is a comparatively large group of micro-organisms known as conditionally pathogenic micro-organisms living on the skin, in the intestine, in the respiratory tract and urogenital organs.

Pasteur discovered many of the basic principles of microbiology and, along with R. Koch, laid the foundation for the science of microbiology. In 1857 Napoleon III was having trouble with his sailors mutinying because their wine was spoiling after only a few weeks at sea. Naturally Napoleon was distraught because his hopes for world conquest were being scuttled (pardon the pun) over a little spoiled wine, so he begged Pasteur for help. Pasteur, armed with his trusty microscope, accepted the challenge and soon recognized that by looking at the spoiled wines he could distinguish between the contaminants that caused the spoilage and even predict the taste of the wine solely from his microscopic observations. He then reasoned that if one were to heat the wine to a point where its flavor was unaffected, but the harmful microbes were killed it wouldn't spoil. As we are aware this process, today known as **pasteurization**, worked exactly the way he predicted and is the foundation of the modern treatment of bottled liquids to prevent their spoilage. It is important to realize that pasteurization is NOT the same as sterilization. Pasteurization only kills organisms that may spoil the product, but it allows many microbes to survive, whereas **sterilization** kills all the living organisms in the treated material.

Pasteur also realized that the yeast that was present in all the wine produced the alcohol in wine. When he announced this, a number of famous scientists were enraged, because the current theory of wine production was that wine formation was the result of spontaneous chemical changes that occurred in the grape juice. Pasteur was attacked furiously at scientific meetings, to the point where certain scientists did humorous skits about Pasteur and his tiny little yeast "stills" turning out alcohol. Pasteur had the last laugh however as people all over the world soon realized that if he was right they could control the quality of wine by controlling the yeast that made it. In a short period many others verified his observations and the opposition sank without a sound.

Sterilization of instruments, needles, syringes, etc., is carried out by boiling. Dressings, glassware, and salt solutions are treated in autoclaves.

Pasteurization is widely employed to render milk harmless by heating it at 63°C for 30 minutes or at 71.6-80°C for 15-30 seconds and then cooling it. Pasteurization is also used to prevent the development of harmful microbes which turn wine, beer, and fruit juices sour; it does not destroy vitamins and does not deprive the beverages of their flavour.

DRY HEAT

Incineration. This is an excellent procedure for disposing of materials such as soiled dressings, used paper mouth wipes, sputum cups, and garbage. One must remember that if such articles are infectious, they shoul d be thoroughly wrapped in newspaper with additional paper or sawdust to absorb the excess moisture. Disposable plastic liners for waste containers are inexpensive and may be easily closed on top to prevent scattering of refuse. The wrapping protects persons who must empty the trash cans, and it assures that the

objects do not escape the fire, but it may also protect the microorganisms if incineration is not complete.

Adequate instructions should be given to workers responsable for burning disposable materials to insure complete burning. For example, a sputum cup containing secretions from a patient who has active tuberculosis is filled with paper or sawdust to absorb excess moisture. The cup is then placed in a plastic bag with shredded absorbent paper to prevent spilling If it is burned only on the outside, a soggy mass of dangerous infective material is left on the inside. Other possibilities will occur to the imaginative student

Ovens. Ovens are often used for sterilizing dry materials such as glassware, syringes and needles, powders, and gauze dressings. Petrolatum and other oily substances must also be sterilized with dry heat in an oven because moist heat (steam) will not penetrate materials insoluble in water (fig. 92).

Figure 92. Oven

In order to insure sterility the materials in the oven must reach a temperature of 165 to 170 C (329 to 338 t), and this temperature must be maintained for 120 or 90 minutes, respectively. This destroys all microorganisms, including spores. However, the oven must be maintained at that temperature for the entire time. This means that the oven door must remain closed during the sterilizing time opening the door will cool the articles below effective temperatures so that sterilization cannot be assured. Also, *hot* glassware will shatter immediately) in contact with cool air It is usual practice in a microbiology laboratory to let an oven cool completely before it is opened

It is practical to load the oven with glassware, pipettes, and so forth in the afternoon or evening and turn it on. In the morning, the oven is turned off and by lunchtime it is unloaded. This routine assures sterile glassware, once the setting of the temperature is regulated. A home oven, set at 330 t (model ate temperature), can be used as well as an oven built for laboratory or hospital equipment. It is wise to check the temperature in the oven with an oven thermometer (available at household supply stores)

Items may be secured in brown wrapping paper with a sting, but never with a lubber band. Some types of plastics, like the one used in connecting hoses, are heat-stable in an oven, but most plastics cannot be sterilized in this way.

MOIST HEAT

Boiling Water. Boiling water can never be trusted for absolute sterilization procedures because its maximum temperature is 100 C (at sea level). As indicatedpreviously, spores can resist this temperature Boiling water can generally be used for contaminated dishes, bedding, and bedpans: for these articles neither sterility nor the destruction of spores is necessary) except under very unusual circumstances. All that is desired is disinfection or sanitization. Exposure to boiling water kills all pathogenic microorganisms in 10 minutes 01 less, but not bacterial spores or hepatitis viruses. At altitudes over 5,000 feet the boiling time should be increased by 50 per cent or moiré because water there boils at temperatures of only about 95 C or below (fig. 93).

Figure 93. Sterilizer

Live Steam. Live steam (free flowing) is used in the laboratory in the preparation of culture media or in the home for processing canned foods. It must be remembered that steam does not exceed the temperature of 100 C unless it is under pressure.

To use free flowing steam effectively for sterilization, the fractional method must be used fractional sterilization, or tyndalization, is a process of exposure of substances (usually liquids) to live steam for 30 minutes on each of three successive days, with incubation during the intervals. During the incubations, spores

germinate into vulnerable vegetative forms that are killed during the heating periods. This is a time-consuming process and is not used in modern laboratories. The use of membrane (Millipore) filters or similar rapid methods makes the preparation of heat-sensitive sterile solutions much easier.

Compressed Steam. In older to sterilize with steam certainly and quickly, steam under pressure in the autoclave is used (fig. 94). An autoclave is essentially a metal chamber with a door that can be closed very tightly. The inner chamber allows all air to be *replaced* by steam until the contents reach a temperature far above that of boiling water or live steam. The temperature depends on the pressure, commonly expressed in pounds per square inch, often written as psi. Steam under pressure hydrates rapidly and therefore coagulates very efficiently. Also, it brings about chemical changes somewhat like digestion, called *hydrolysis.* These characteristics give it special advantages in sterilization.

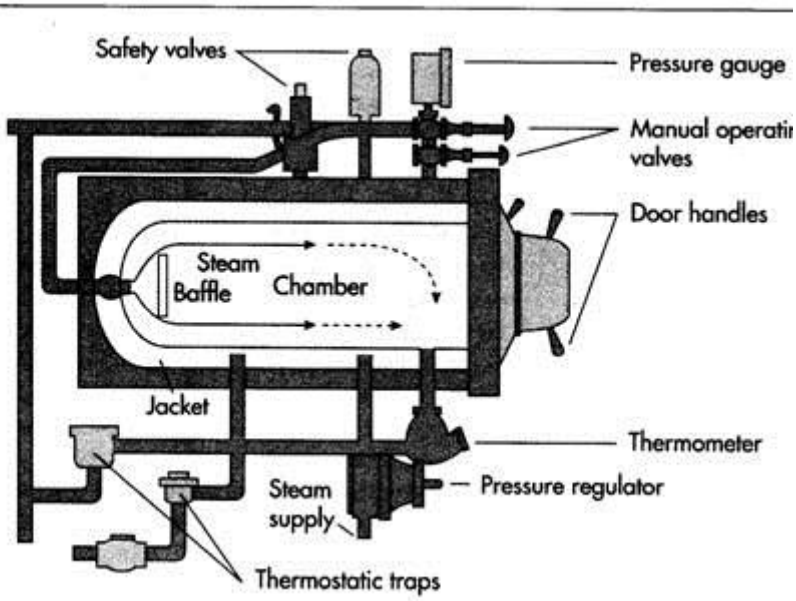


Figure 94. An autoclave

By first allowing all the an in the chamber of the autoclave to escape and be replaced by the incoming steam, the spaces in the interior of masses of material may be brought quickly into contact with the steam. The escape of air is absolute!) essential since sterilization depends on the water vapor. Whenever an is trapped in the autoclave, sterilization is inefficient. One must be sure that:

1. All the air is allowed to escape and is replaced by steam
2. The pressure of the steam reaches at least 1 5 pounds to the square inch (psi) and remains there (In most automatic autoclaves it is now 18 pounds, permitting sterilization to be accomplished in less time)
3. The thermometer reaches at least 121 C without downward fluctuation for 15 minutes (Less time is required when 18 pounds of pressure is used).

If these conditions are met and if the masses 01 bundles are well separated and not too large, the autoclaved material will be sterile.

The actual amount of water present as steam in the pressure chamber is usually small, consequently, the articles sterilize dare not wet with much condenseds team when they are removed from the autoclave.

Allmodern autoclaves are arranged so that all the steam is removed by vacuum after the sterilization period, to prevent dampening the articles inside.

The automatic autoclave is used in many laboratories, has the following settings:

1. Manual—used when the electrical power is off I he operator must then set and time all cycles
2. Slow exhaust—used for a *wet load*, for media or water (for dilutions)
3. Fast exhaust—used for killing microorganisms quickly on and in glass-ware that is to be washed
4. Fast exhaust and dry—used for pipettes, Petri plates, or dressings, a so-called dry loud.

After closing the door tightly, the operator sets the autoclave control to the desired setting, to the time interval that is necessary for the maximum presettemperature and pressure, and to ON. Lights go on as the autoclaving moves from pretimed cycle to cycle finally a bell rings, and the operator turns the settingto OFF and opens the door carefully Asbestos gloves protect the hands, when hot sterile materials are unloaded, but watch the right elbow—the inside of the open door is very hot.

The exhaust trap inside the autoclave must always be cleaned before starting a load, since dirt in the trap may delay the time needed for the various cycles. It is best to do this when the autoclave is still cold.

Since the effectiveness of an autoclave is dependent upon the penetration of steam into all articles and substances, the preparation of packs of dressings isvery important, and the correct placement of articles in the autoclave is essential to adequate sterilization

Substitution of an autoclave for an oven by admitting steam only to the jacket and keeping the chamber dry is not advisablewhensterilizationisnecessarybecausethetemperaturethus achieved(100 C)doe snotkillspores.

The dryness of such an atmosphere may actually preserve some pathogens thatwould be quickly killed in a moist atmosphere

Cleaning Instruments. When sterilizing solutions, the pressure must be allowed to fall gradually so that the solutions will not boil. If the pressure fallsrapidly, violent boiling occurs. Advantage is taken of this fact in autoclaving used surgical instruments. They are immersed in water in a perforated tray. After autoclaving the pressure is reduced suddenly. The water boils violently and washes the instruments clean.

Cleaning by Ultrasonic Energy. Machines are now available for cleaning surgical instruments, syringes, and so on by extremely rapid (ultrasonic) vibrations. These can clean and dry hundreds of instruments (perfectly) every five to ten minutes. They do not sterilize.

Indicators. Many institutions always include some sort of indicator inside bundles being sterilized, such as dyes that change color when the necessary temperature has been maintained for the required time. On glassware and bundles, labels are placed that read Not STERILE before autoclaving or after insufficient autoclaving but read STERILE if sterilization has been fully effective. Another device, similar in principle, is cellulose tape having on it a chemical indicator that changes color when properly heated in the autoclave. One car use wax pellets that melt only at the necessary temperature but may not indicate lapse of time. Strips of paper containing bacterial spores can be dropped into broth in culture tubes after the sterilizing procedure. If the sterilizer has been properly operated, these broth cultures should remain sterile, even after seven days of incubation, since all spores have been lulled 1 hrs method does not give immediate indication of faulty operation, but it does constitute an absolute and permanent record.

Most modern autoclaves have a self-recording thermometer that plots the temperature the instrument has reached and the time of sterilization required for each "load". A permanent record provided in this way often proves to be verve valuable.

STERILIZATION WITHOUT HEAT

For many years heat was the only dependable and practicable means of destroying bacterial endospores. Now, at least three other means of killing microorganisms are available. These are use of the gas *ethylene oxide,* the vapors of *beta propiolactone* (BFL), and certain *electromagnetic radiations* (especially electron beams or cathode rays). The method of ultrasonic vibrations, although quite effective in destroying certain microorganisms, is not a practical means of large scale sterilization. Besides, it produces a heating effect. At present, we can only dream of an ultrasonic "dishwasher" that sanitizes duty dishes, preferably without any water.

Ultraviolet Light. This is satisfactory for the sterilization of smooth sin faces and of air in operating looms, unfortunately, UV radiation has virtually no power of penetration. Mercury-vapor lamps emitting 90 per cent UV radiation at 254 nm are used to decrease airborne infection. Ultraviolet lamps are also used to suppress surface-growing molds and other organisms in meat packing houses, bakeries, storage warehouses, and laboratories Sunlight is a good, inexpensive source of ultraviolet rays, which can induce genetic mutations in microorganisms. In excess, it can cause burns and even cancer

X-Rays. X-rays penetrate well but require very high energy and are costly and inefficient for sterilizing. Their use is therefore mostly for medical and experimental work and the production of mutants of microorganisms for genetic studies

Neutrons. Neutrons are very effective in killing microorganisms but are expensive and hard to control, and they involve dangerous radioactivity

Alpha Rays (Particles). Alpha rays are effective bactericides but have almost no power of penetration

Beta Rays (Particles). Beta rays have a slightly greater power of penetration than alpha lays but are still not practical for use in sterilization

Gamma Rays. These rays are high-energy radiations now mostly emitted from radioactive isotopes such as cobalt-60 or cesium-137, which are readily available by-products of atomic fission Gamma rays resemble x-rays in many respects. The U S Army Quartermaster Corps has used gamma rays and other radiations to sterilize food for military use X rays or gamma rays must be applied in 2 mrad (one mrad is 1/1 000 of a rad; a rad is 100 ergs of absorbed energy per gram of absorbing material) to 4 mrad doses to become a reliable sterilizing treatment of food. Foods exposed to effective radiation sterilization, however, undergo changes in color, chemical composition, taste, and sometimes even odor These problems are only gradually being overcome by temperature control and oxygen removal

Cathode Rays (Electrons). These are used mainly to kill microorganisms on sin faces of foods, fomites, and industrial articles. Since electrons have limitedpowers of penetration, they are at present not very useful for surgical sterilization. However, as a result of research on proper dosage and packaging, cathode laysare being developed for genial purposes such as food processing. This may completely revolutionize the food canning and frozen food industries as well as surgical sterilization techniques.

Pharmaceutical and medical products are adequately sterilized by treatment with a radiation dose of 2,5 mrad . The Association of the British PharmaceuticalIndustry has reported that benzylpenicillin, streptomycin sulfate, and other antibiotics are satisfactorily sterilized by this method In addition, package radiation at dose levels of 2,5 mrad has become common procedure for the sterilization of disposable Petri plates, pipettes, syringes, needles, rubber gloves, tubing, and so on

Sterilization with Chemicals

Ethylene Oxide. This is a gas with the formula CH_2CH_2O. It is applied in special autoclaves under carefully controlled conditions of temperature and humidity Since pure ethylene oxide is explosive and irritating, it is generally mixed with carbon dioxide or another diluent in various proportions 10 per cent ethylene oxide to 90 per cent carbon dioxide (sold as Carboxide), 20 per cent ethylene oxide to 80 per cent carbon dioxide (sold as Oxyfume), or 11 per cent ethylene oxide to 89 per cent halogenated hydrocarbons (sold as Cryoxcide and Benvicide). Each preparation is effective when properly used. Oxyfume is very rapid in action but is more inflammable and moiré toxic than Carboxide, however, Carboxide requires high pressure Cryoxcide is more toxic and more expensive, but it is more convenient and requires less pressure. Other mixtures of ethylene oxide (e.g. , with Freon) are also commercially available All are mote costly and time-consuming than autoclaving with steam

Ethylene oxide is generally measured in terms of milligrams of the pure gas per liter of space. For sterilization, concentrations of 450 to 1,000 mg of gas/liter are necessary. Concentrations of 500 mg of gas/liter are generally effective in about four hours at approximately 1% F (58 C) and a relative humidity of about 40 per cent. Variations in any one of these factors require adjustments of the others For example, if the concentration of gas is increased to 1,000 mg/liter, the time may be reduced to

two hours. Increases in temperature, up to a limit, also decrease the time required. At a relative humidity of 30 per cent, the action of ethylene oxide is about 10 times as rapid as at 95 per cent. The use of ethylene oxide, although as simple as autoclaving, generally requires special instructions (provided by the manufacturers) for each particular situation. At present ethylene oxide is used largely by commercial companies that dispense sterile packages of a variety of products

In general, seven steps are invoked after loading and closing the sterilizing chamber:

1. Draw out nearly all air with a vacuum pump
2. Admit a measured amount of water vapor
3. Admit the requires amount of ethylene oxide gas mixture
4. Raise the temperature to the required degree
5. Hold for the required time, turn off the heat
6. Draw out the gas with the vacuum pump
7. Admit filtered and sterilized air to the chamber

A fully automatic ethylene oxide autoclave requiring only proper supervision is available.

Beta-propiolactone (BPL). At about 20 C this substance is a colorless liquid It has a sweet but very irritating odor It is unstable at room temperatures but may be refrigerated at 4 CG for months without deterioration. Aqueous solutions effectively inactivate some viruses, including those of poliomyelitis and rabies, and alsokill bacteria and bacterial spores. The vapors, in concentrations of about 1,5 mg of lactone per liter of air with a high relative humidity (75 to 80 per cent), at about 25 C, kill spores in a few minutes. A decrease in temperature, humidity, or concentration of the lactone vapors increases the time required to kill spores

Beta-propiolactone is not inflammable under ordinary conditions of use. It is, however, very irritating and may cause blisters if allowed in contact with skin formore than a few minutes. It is not injurious to most materials. BPL appeal s to act by forming chemical compounds with cell proteins. The necessity for high humidities during its use and also its cost are disadvantages Its activity at loom temperatures is a distinct advantage BPL does not penetrate as well as ethylene oxide and is therefore more suitable for disinfecting surfaces (e g , looms, buildings, and furniture) by fumigation

Aqueous solutions of BPL can be used to sterilize biological materials such as virus vaccines, tissues for grafting, and plasma.

Sterilization by Filtration

Many fluids may be sterilized without the use of heat, chemicals, or radiations. This is accomplished mechanically by passing the fluids to be sterilized throughvery fine filters Only fluids of low viscosity that do not contain numerous fine particles in suspension (e.g., silt, erythrocytes), which would clog the filter pores, can be satisfactorily sterilized in this way The method is applicable to fluids that are destroyed by heat and cannot be sterilized in any other way, such as fluids and medications for hypodermic or intravenous use, as well as culture media, especially tissue culture media and their liquid components, e. g , serum (fig. 95).

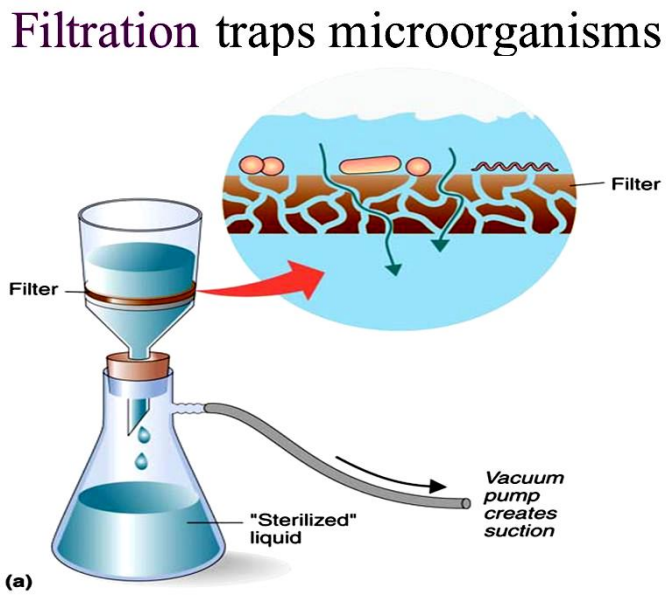


Figure 95. Sterilization by Filtration

Several types of filters are in common use. The Seitz filter, consisting of a mounted asbestos pad, is one of the older filters used. Others consist of diatomaceous earth (the Berkefeld filter), unglazed porcelain (the Chamber-land-Pasteur filters), or sintered glass of several varieties. The Sterifil aseptic filtration system consists of a tubelike arrangement that sucks up fluid around all sides of the tube into a Teflon hose-connected receiving flask. The advantage of this is that the filter is veryinexpensive and can be thrown away when it clogs up

A widely used and practical filter is the membrane, molecular, or Millipore filter. It is available in a great variety of pore sizes, ranging from 0,45 mcm for virus studies to 0,01 mcm. These filters consist of paper-thin, porous membranes of material resembling cellulose acetate (plastic) One common form of these special filters is shown in Figure 9—11 In general, the porcelain, clay, paper, or plastic filtering element is held in some supporting structure, and the fluid to be filtered is forced through the filter into a receptacle by a vacuum or by pressure The filter, support, and receptacle are assembled and autoclaved before use Further details concerning these procedures need not be given here, since sterilization by filtration is rarely used without adequate information pertaining to the specific filtration problem, which would describe the advantages of one type of filter over another.

Summary. The most complete way to dispose of infectious materials is incineration, although precautions must be taken to prevent spilling and to assure that everything is fully burned. Things that cannot be incinerated are sterilized to free them not only of pathogens but of all living organisms. For dry materials, glassware, syringes, dressings, filters, and pipettes, this may be done in a sterilizer oven at 165 °C (329 F) for 2 hours This treatment destroys fungi, bacteria, spores, and viruses

Boiling water cannot be expected to kill bacterial spores, unless applied according to the tyndalization method (fractional sterilization) In order to sterilize with steam certainly and quickly, steam under pressure in an autoclave may be used at 15 psi for 15 minutes The temperature reached should be at least 121 °C, sufficient to destroy all bacteria, their spores, and all other microorganisms

The modern automatic autoclave is really only a glorified pressure cooker. It can be used manually, for a "wet load" of liquid materials, for a "dry load" containing glassware, or with fast exhaust—to kill microorganisms quickly so that contaminated dishes, plates, flasks, and pipettes can be safely washed

Ethylene oxide (in a mixture with carbon dioxide called Carboxide) and beta-propiolactone (BPL) are used routinely in hospitals for gas sterilization of all types of surgical and other materials

Ultraviolet light, employed routinely for sterilization of the air in operating rooms, cannot penetrate like x-rays into materials, but it is readily available in sunlight, which therefore has great bactericidal powers. Commercially, the use of UV in restaurants and so forth is impressive, but, like x-rays, they are inefficient for effective sterilization

Although they are highly bactericidal, neutrons are expensive to produce and difficult to control. Alpha and beta rays are not practical for use, but gamma rays are used widely to sterilize foods and pharmaceuticals Cathode rays (electrons) are applied to food canning, frozen food, and surgical sterilization, and are also used to sterilize disposable Petri plates, pipettes, tubing, and most packaged materials

Many fluids may be sterilized without the use of heat, chemicals, or radiation by means of filters. Several types of filters are in common use, such as the Seitz, the Berkefeld, the Chamberland-Pasteur, sintered glass of many varieties, and membrane filters such as the Millipore The great advantage of modern bacterial filters is that they are disposable.

Antiseptics is of great significance in medical practice. The people of Africa in ancient times knew the methods of treating wounds with the aid of ant bites which healed the edges of the wound no worse than if it had been stitched by modem medical techniques. Sunlight took the place of antiseptic substances. Yet in 1865. N. Pirogov pointed out the necessity of destroying the source of intrahospital infection and tried chlorine water, silver nitrate, iodine and other antiseptic substances in combating wound suppurations. In 1867-J. Lister used phenol extensively as an antiseptic.

The science of antiseptics played a large role in the development of surgery. The practical application of microbiology in surgery brought a decrease in the number of postoperative complications, including gangrene, and considerably diminished the death rate in surgical wards. J. Lister highly assessed the importance of antiseptics and the merits of L. Pasteur in this field.

This trend received further development after E. Bergman and others who introduced aseptics into surgical practice representing a whole system of measures directed at preventing the access of microbes into wounds. Aseptics is attained by disinfection of the air and equipment of the operating room, by sterilization of

surgical instruments and material, and by disinfecting the hands of the surgeon and the skin on the operative field. Film and plastic isolators are used in the clinic for protection against the penetration of micro-organisms. Soft surgical Him isolators attached to the operative field fully prevent bacteria from entering the surgical wound from the environment, particularly from the upper respiratory passages of the personnel of the operating room. A widespread use of aseptics has permitted the maintenance of the health and lives of many millions of people.

Modem methods of aseptics have been perfected to a considerable extent. Consequently almost all operations are accompanied with primary healing of wounds without suppuration, while the incidence of postoperative septicaemia has been completely eliminated.

Controls of sterilization. We use chemical and biological controls of sterilzation products with the purpose of checking of effectivity its. The matter of these procedures consist of some steps (actions). There are three knids of media which we use for control of sterilization, whereas sugar broth of Hotinger, thiglicol media, Saburo broth. We put into these media (sugar broth of Hotinger, thiglicol media, Saburo broth) some products have been sterilized before and put into thermostat till 14 days. But there are one exception, all innoculated media we keep in thermostat 14 days at 37 ^{0}C (centigrate) and just innoculated with checking products Saburo media at 22 ^{0}C (centigrate). After this period of 14 days we examine growth on these media. For example, when growth on media are absent we might make conclusion about effective sterilization process.

Now, some words about chemical controls of sterilization. There are some chemical substances, which have certain point of smelt. Fe., powder of serum (point of smelt is 119 ^{0}C (centigrate)), benzoic acid (point of smelt is 120-122 ^{0}C (centigrate)), beta naphthol (point of smelt is 123 ^{0}C (centigrate)), mannose (point of smelt is 132 ^{0}C (centigrate)). We sterilized products with steam and pressure into autoclave we put into its the closed test-tubes with these chemical substances and some quantity of aniline dyes. The even colored contents of these test-tubes show that the temperature get according level and process of sterilization was effective.

The treating (processing) of arms before operation. The treating of arms of medical personnel which take part in the operation are necessary, The different chemical substances are used for surgical treating of arms, f.e., mixture of 1.71 ml per litre of hydrogen peroxide (H_2O_2) 30-33% and 0.81 ml per litre of formic acid 85%, which call “C-4”, chlorhexidini solution.

Before treating arms we are washing them with soap (without brush) during 1minutes and drying out. After that we are treating arms with “C-4”, and drying with napkin, and put gloves on arms. With regards to method of arms treating with chlorhexidini: we are performing the same washing procedure underlighted upon and processing arms for first by sterile cotton napkin for second by wads with 0.5% spirituosae solution of chlorhexidini during 2-3 minutes.

Respiration in Bacteria

Respiration in bacteria is a complex process which is accompanied with the liberation of energy required by the micro-organism for the synthesis of different

organic compounds. Many microbes similar to vertebrates and plants utilize the molecular oxygen in the air for respiration.

The concept of respiration as a process of oxidation of organic substances with the production of energy has undergone considerable changes due to the discovery of anaerobic microbes unable to exist in the presence of oxygen. Pasteur established that the energy necessary for the life activity of some species of microbes is obtained in the process of fermentation (liberation of energy without the participation of oxygen).

All microbes according to type of respiration can be subdivided into obligate aerobes, facultative anaerobes and obligate anaerobes.

1. Obligate aerobes which develop well in an atmosphere containing 21 per cent of oxygen. They grow on the surfaces of liquid and solid nutrient media (brucellae, micrococci, tubercle bacilli, etc.).

2. Facultative anaerobes which can reproduce even in the absence of molecular oxygen (the majority of pathogenic and saprophytic microbes).

3. Obligate anaerobes for which the presence of molecular oxygen is a harmful growth-inhibiting factor (causative agents of tetanus, botulism, anaerobic infections, etc.).

Aerobic bacteria in the process of respiration oxidize different organic substances (carbohydrates, proteins, lipids, alcohols, organic acids, and other compounds). During complete oxidation of one gram-molecule of glucose a definite number of calories is liberated which corresponds to the potential energy store accumulated in the carbohydrate molecule during its photosynthesis in green plants from carbon dioxide and water

During incomplete (partial) aerobic oxidation, less energy is released corresponding to the degree of oxidation

A typical representative of the facultative aerobes is the colibacillus which in a carbohydrate medium begins to develop first as an anaerobe breaking down the carbohydrates by fermentation. Then it begins to utilize oxygen and grows like an aerobe, oxidizing the products of fermentation (lactic acid) farther to carbon dioxide and water. Facultative aerobes have a considerable advantage, as they can live in aerobic and anaerobic conditions.

Respiration in anaerobes takes place by fermentation of the substrate with the production of a small amount of energy. In the fermentation of one gram-molecule of glucose considerably less energy is produced than during aerobic respiration.

The mechanism of anaerobic respiration takes place in the following way. If carbohydrates make up the oxidizing substrate, then preliminarily they are broken down with the help of auxiliary enzymes. Thus, for example, glucose is phosphorylated employing ATP and ADP. As a result, hexose diphosphale is produced which under the influence of the enzyme aldolase breaks down into two components: phosphogly-ceraldehyde and dioxyacetone phosphate. The latter under the effect of oxyisomerase is transformed into phosphoglyceraldehyde and later on after a sequence of reactions produces pyruvic acid. This stage is the last in the anaerobic phase of transformation of carbon. The later stages are specific and are completed with the production of end products.

Anaerobic processes include alcohol fermentation by yeasts, lactic acid fermentation by lactobacilli, and butyric acid fermentation by butyric acid clostridia.

Anaerobes ferment mostly nitrogen-free compounds causing fermentation. However, there is no sharp boundary between the erobic and anaerobic types of respiration. Thus. for example, yeasts can change the anaerobic type of respiration to aerobic respiration. First of all, they break down sugar into alcohol and carbon dioxide, and during increased aeration glucose is broken down into water and carbon dioxide.

The presence of obligate anaerobes explains the rather great adaptability of living things and the completeness of the cycle ofsubstances in nature.

It has been established by investigations that the respiration in bac- teria takes place under the influence of enzymes of the oxidase and dehydrogenase types, which have a marked specificity and a multilateral activity. The oxidase and dehydrogenase processes of respiration are closely interconnected, supplementing each other, but at the same time differing in biological role and in enzymes.

The intensity of the processes of aerobic respiration depends on the age of the culture, temperature, and nutrient substrates. Actively growing cultures use 2500-5000 cu mm of oxygen per 1 mg of dry matter of bacleria per hour while starved cultures or cultures completely deprived of nitrogen nutrients require only 10-150 cu mm. A young culture produces considerably more heat energy than it uses for its synthetic and other life processes. A certain part of this energy is released into the environment. For instance, the colibacillus in the process of assimilation uses 31 per cent of the energy released, blue us bacteria – 28 per cent, Proteus vulgaris — 20 per cent, and salmonellae of enteric fever – 12 percent. The production by some microbes of an excess of heat energy in manure, turf and garbage can ause spon- taneous heating and spontaneous combustion.

In manure and garbage dumps due to the effect of the high temperature produced by thermophilic microbes, the eggs laid by flies and also the eggs of worms are unable to develop.

Increased respiration and an increased metabolism depend on the rate of cell reproduction, on the increase of the protein synthesis in the cell, which causes an increase in the reduction properties of the medium in which the microbes develop.

Biological oxidation comprises the removal of a negatively-charged electron, reduction - the addition of a negatively-charged electron.

Between the hydrogen acceptor (yellow enzyme) and oxygen there are intermediate hydrogen carriers w hich are participants of the long chain of the catalyst of biological oxidation.

The electrons are carried by cytochromes and which are protein molecules bound with a chemical group of the haem. The haem contains an iron atom capable of undergoing oxidation and reduction alternately. Besides cytochromes, a new substance has been discovered, a carrier of electrons, called ubiquinon or cocnzyme.

Thus the processes of respiration in bacteria are very complex and represent a long chain of a sequence of oxidation-reduction reactions with the participation of many enzyme systems transporting the electrons from the system of the most

negative potential to the system of the most positive potential. During gradual and fractional liberation of energy in respiration and during intermediate transport of hydrogen, the activity of cellular reactions increases. The biochemical mechanisms of respiration are described in detail in biochemistry textbooks.

The habitat of micro-organisms greatly influences the character of respiration. Thus, for example, upon cultivating the cholera-like vibrio in a medium containing glucose, its aerobic respiration can be decreased as a result of which it acquires the properties of a facultative anaerobe. Yeasts are also capable of changing their type of respiration depending on the presence or absence of oxygen.

G. McLeod explained that the toxic effect of oxygen on anaerobes is due to the production of hydrogen peroxide in the presence of oxygen. Anaerobes are unable to produce catalase. Only H,0, but not oxygen itself is toxic. However, this cannot be a complete explanation. Anaerobes can grow if there is oxygen in the medium, which does nt kill microbes, but only inhibits their life activities. Upon the addition of reducing agents to the medium, the microbes begin to grow. as reducing agents lower the oxidation-reduction potential. Glucose and other reducing substances act in the same way.

V. Engelhardt considers that in the presence of a high oxidation-reduction potential, the inactivation of vitally important enzymes takes place. Anaerobes then lose their ability to feed normally, and to carry out constructive processes. Hence they perish from starvation, and not from intoxication by oxygen or H_20_2. The oxidation-reduction potential (rH,) is one of the factors on which the oxidation-reduction reactions in the nutrient medium depend. The oxidation-reduction potential expresses the quantitative character of the degree of aerobiosis. It becomes minimal upon saturating the medium with hydrogen, and maximal upon saturating the medium with oxygen. M. dark proposed to designate the unit of the oxidation-reduction potential as rH,-the negative logarithm of the partial pressure of gaseous hydrogen." The range of rHs from 0 to 42,6 characterizes all degrees of saturation of an aqueous solution with hydrogen and oxygen- Aerobes exist within the limits of rH, from 14 to 20 and more, facultative aerobes from 0 to 20 and more, and anaerobes from 0 to 12.

Aerobes are adapted to existence at a higher oxidation-reduction potential, anaerobes — at a lower rH,. Anaerobes are not passive micro-organisms, and they themselves cause the low rH, in the medium.

Seeded cultures of anaerobes prior to reproduction lower the rH, from 20-22 to 1-5. Thus anaerobes are characterized by a rather marked capability to adapt the medium to their requirements. Aerobes also have these properties, and they guard themselves from an excess of oxygen by a reduction barrier.

Upon controlling the oxidation-reduction potential of the nutrient medium, conditions can be obtained for the growth of anaerobes in the presence of oxygen by lowering the rH,, and also by cultivating the aerobes in anaerobic conditions by increasing the rH, of the medium.

The oxidation-reduction potential drops sharply when the bacterial culture dies, when it is lysed by a phage and when it is affected by lysozyme.

When preparing nutrient media the composition of the nutrient energy-yielding material, the reaction of the medium (pH), and its oxidation-reduction potential (rH;) are all taken into consideration.

Bacterial growth

Colony is bacterial cells of the same species which have grown from one bacterial cell on solid medium as isolated accumulation. Bacteriological investigation is based on isolating a pure culture of the causal organism and its identification. The term pure culture refers to a population of microorganism of the same species isolated on a nutrient medium.

The Medium. The technique used and the type of medium selected depend upon the nature of the investigation. In general, 3 situations may be encountered; (1) one may need to raise a crop of cells of a particular species that is on hand; (2) one may need to determine the numbers and types of organisms present in a given material; or (3) one may wish to isolate a particular type of microorganism from a natural source.

A. Growing Cells of a Given Species: Microorganisms observed microscopically to be growing in a natural environment may prove exceedingly difficult to grow in pure culture in an artificial medium. Certain parasitic forms, for example, have never been cultivated outside the host. In general, however, a suitable medium can be devised by carefully reproducing the conditions found in the organism's natural environment. The pH, temperature, and aeration are simple to duplicate; the nutrients present the major problem. The contribution made by the living environment is important and difficult to analyze; a parasite may require an extract of the host tissue, and a free-living form may require a substance excreted by a microorganism with which it is associated in nature.

B. Microbiologic Examination of Natural Materials: A given natural material may contain many different microenvironments, each providing a niche for a different species. Plating a sample of the materials under one set of conditions will allow a selected group of forms to produce colonies but will cause many other types to be overlooked. For this reason it is customary to plate out samples of the material using as many different media and conditions of incubation as is practicable, Six to 8 different culture conditions are not an unreasonable number if most of the forms present are to be discovered.

Since every type of organism present must have a chance to grow, solid media are used and crowding of colonies is avoided. Otherwise, competition will prevent some types from forming colonies.

C. Isolation of a Particular Type of Microorganism: A small sample of soil, if handled properly, will yield a different type of organism for every microenvironment present. For fertile soil (moist, aerated, rich in minerals and organic matter) this means that hundreds or even thousands of types can be isolated, This is done by selecting for the desired type. One gram of soil, for example, is inoculated into a flask of liquid medium that has been made up for the purpose of favoring one type of organism, eg, aerobic nitrogen fixers (Azotobacter). In this case the medium contains no combined nitrogen and is incubated aerobically. If cells of Azotobacter are present

in the soil, they will grow well in this medium forms unable to fix nitrogen will grow only to the extent that the soil has introduced contaminating fixed nitrogen into the medium. When the culture is fully grown, therefore, the percentage of Azotobacter in the total population will have increased greatly; the method is thus called 'enrichment culture. Transfer of a sample of this culture to fresh medium will result in further enrichment of Azotobacter, after several serial transfers, the culture can be plated out on a solidified enrichment medium and colonies of Azotobacter isolated.

Liquid medium is used to permit competition and hence optimal selection, even when the desired type is represented in the soil as only a few cells in a population of millions. Advantage can be taken of "natural enrichment." For example, in looking for kerosene oxidizers, oil-laden soil is chosen, since such soil is already an enrichment environment for such forms.

Enrichment culture, then, is a procedure whereby the medium is prepared so as to duplicate the natural environment (“niche”) of the desired microorganism, thereby selecting for it. An important principle involved in such selection is the following: The organism selected for will be the type whose nutritional requirements are barely satisfied. Azotobacter, for example, grows best in a medium containing organic nitrogen, but its minimum requirement is the presence of Nhence it is selected for in a medium containing N; as the sole nitrogen source. If organic nitrogen is added to the medium, the conditions no longer select for Azotobacter but rather for a form for which organic nitrogen is the minimum requirement.

When searching for a particular type of organism in a natural material, it is advantageous to plate the organisms obtained on a differential medium if available. A differential medium is one that will cause the colonies of a particular type of organism to have a distinctive appearance. For example, colonies of Escherichia coli have a characteristic iridescent sheen on agar containing the dyes eosin and methylene blue (EMB agar). EMB agar containing a high concentration of one sugar will also cause organisms which ferment that sugar to form reddish colonies. Differential media are used for such purposes as recognizing the presence of enteric bacteria in water or milk and the presence of certain pathogens in clinical specimens from patients.

For selection the pure culture of microorganisms, it follows to separate numerous bacteria which are in tested material, one from other. It is possible to attain by methods which are based on two principles – ***mechanical*** and ***biological separation*** of bacteria (table 18).

Table 18. Bacterial growth

Mechanical principle	Biological principle
METHODS 1. Factional dilutions (L. Pasteur's technique) 2. Pour plate technique (Dilution in solid nutrient media by R. Koch's technique) 3. Spread plate technique (Superficial dispersions by Drigalsky's technique) 4. Streak plate technique	METHODS Take into account: – Respiration type (Fortner's method); – bacterial motility (Shukevich's method – resistance to acids (acid fast bacteria); – sporulation; – temperature optimum; – selective sensitiveness of laboratory animals to the bacteria and so on.

Methods based on mechanical principle

Method of factional dilutions (L. Pasteur's technique) is based on mechanical disconnection of microorganisms by serial dilution in liquid nutrient media. The main lack of this technique: we can not make control the amount of microbal is tested tubes (fig. 96).

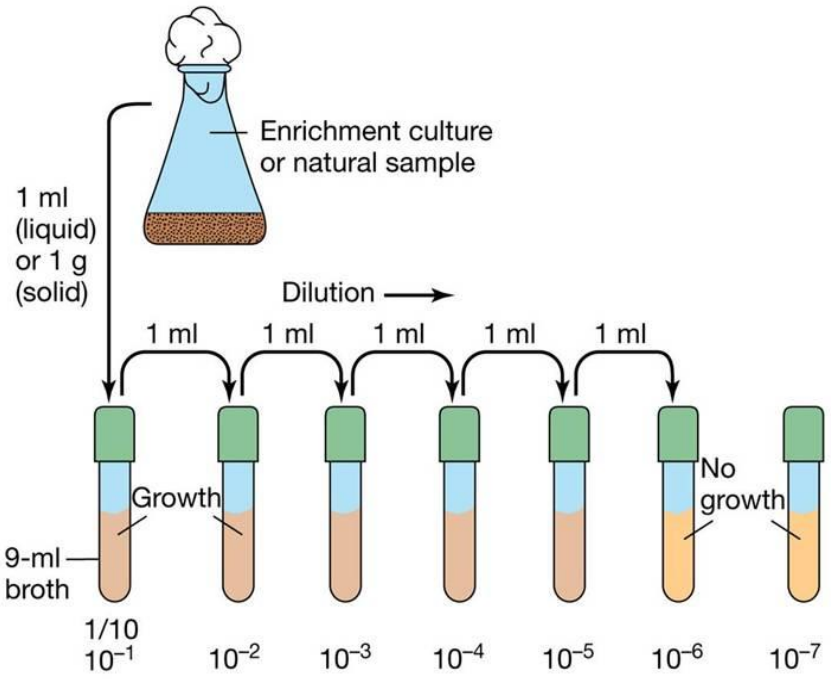


Figure 96. Method of factional dilutions (L. Pasteur's technique)

Pour plate technique (Dilution in solid nutrient media by R. Koch's technique) is based on dilution of microbes and pouring the tested material with gelatin. After cooling the gelatin isolated colonies of microorganisms are formed and

they easily can be transferred on a fresh nutrient medium by a platinum loop for obtaining a microbial pure culture (fig. 97).

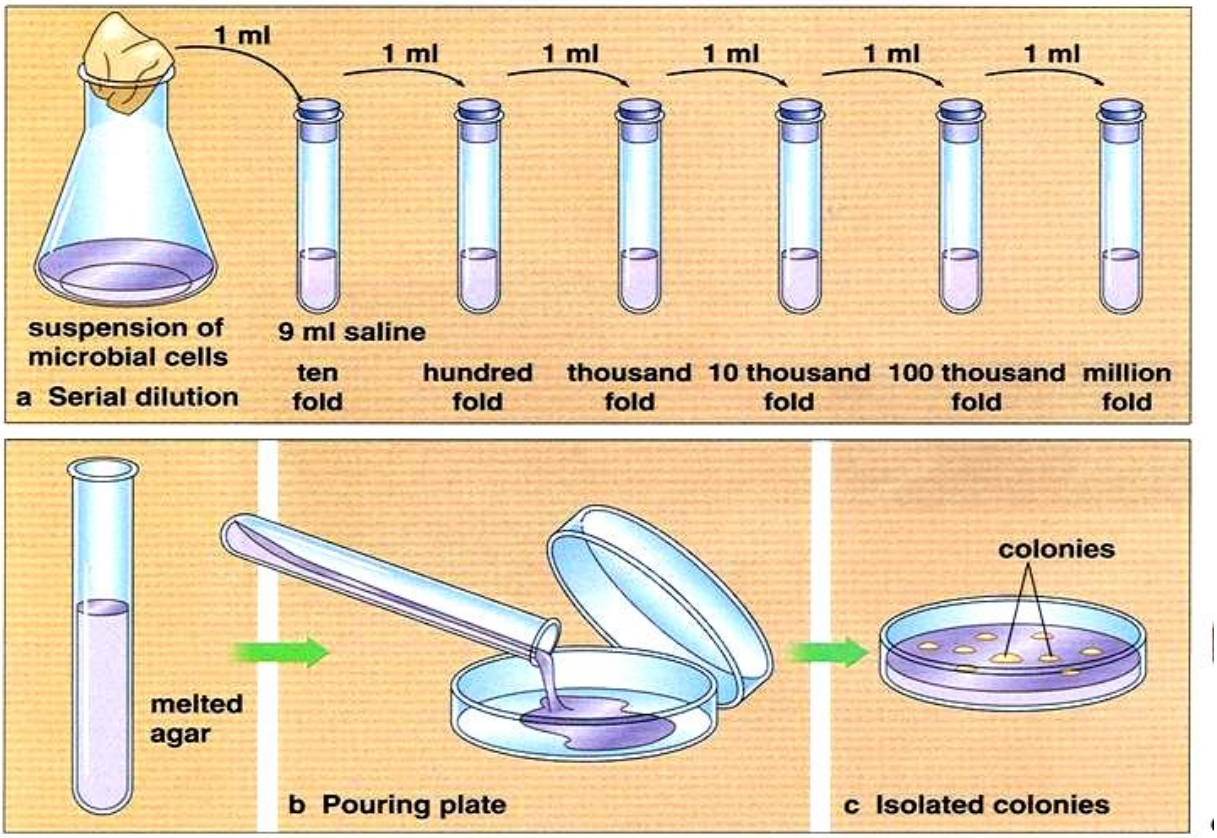


Figure 97. Dilution in solid nutrient media by R. Koch's technique

Spread plate technique (Superficial dispersions by Drigalsky's technique) is more perfect method which is widely widespread in everyday microbiological practice. There is quantitative technique that allows the determination of the number of bacteria in a sample (fig. 98-101).

Stages:

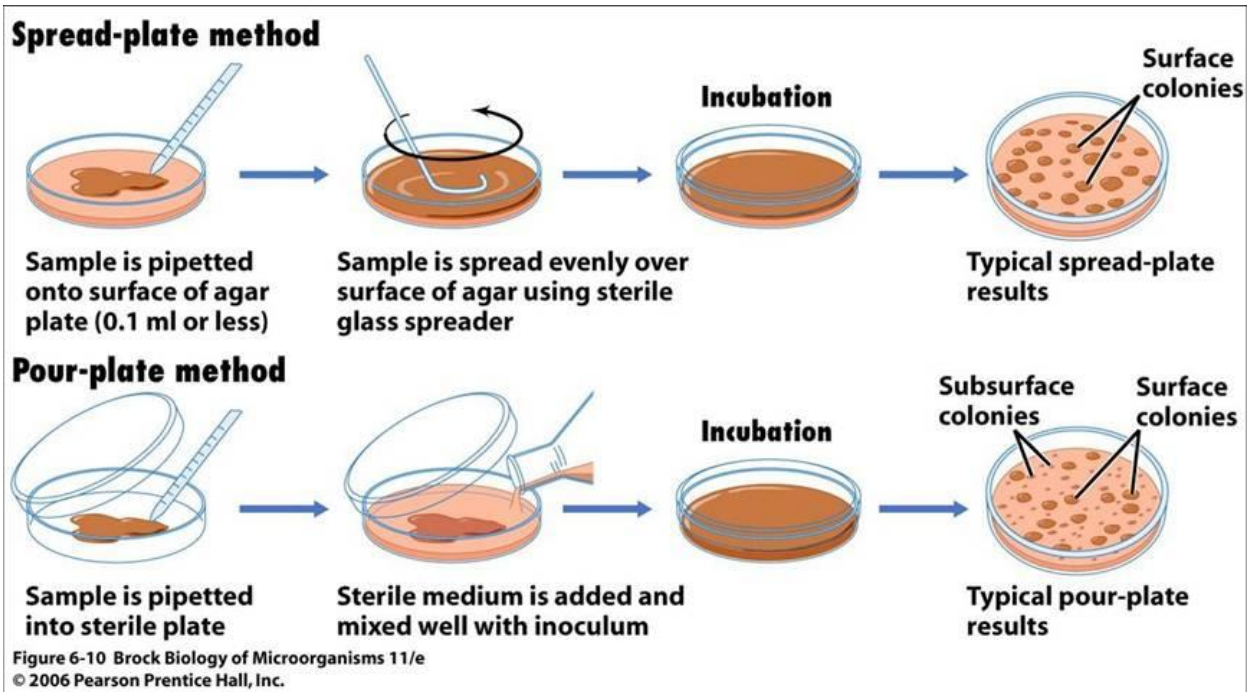
 Pipette the required amount of bacteria (from your dilution) on the surface of the Petri plate.

 Spread the inoculum over the surface of the agar medium using a hockey stick (spatula).

 Repeat this action on 3-4 Petri plates without sterilization of the hockey stick.

 Incubate the plate inverted at 37 ^{o}C.

There must be different number of microbial colony on the Petri plates.



Fugure 98. Superficial dispersions by Drigalsky's technique

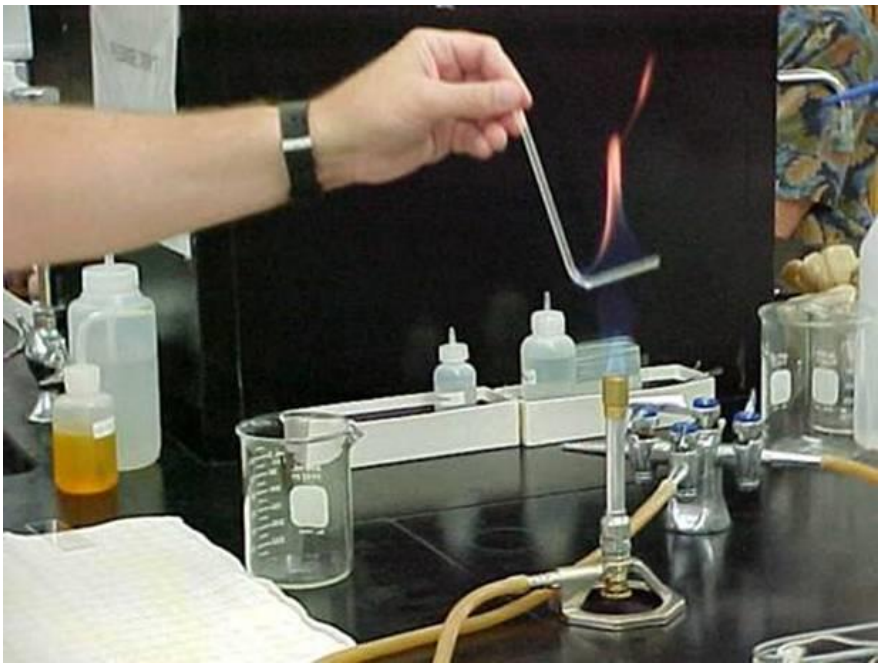


Figure 99. Hockey stick (spatula).

Figure 100. Spread plate technique

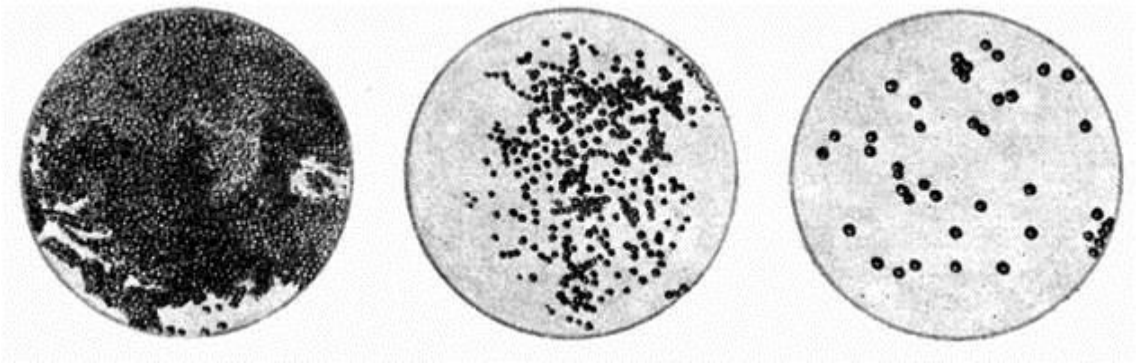


Figure 101. There must be different number of microbial colony on the Petri plates.

Streak plate technique.

ADVANTAGES:

- Spread millions of cells over the surface;
- Individual cells deposited at a distance from all others;
- Divide forming distinct colonies;
- Distinct colonies do not touch any other colonies;
- Clone of a single bacteria pure culture

You streak the plate on 3 different portion of the Petri plate, so you can draw the section that you will streak on the bottom of your plate.

Stages:

- Using a sterile loop take a loopful of your bacteria from the broth
- Streak a vertical line
- Then streak gently across section 1
- Zig-zag pattern until a 1/3 of the plate is covered
- Do not dig into the agar
- Sterilize the loop let it cool
- Rotate the plate about 90 degrees and spread the bacteria from the first streak into a second area
- Do only one streak (or very few) in the first area and once you are in the second area do not go back to the first
- Do a zig-zag pattern until the 2nd area is covered
- Sterilize again do the same for 3rd area
- Make sure that your red hot loop is cool enough prior to touch the bacteria
- After you waited a few seconds
- Stab it into the agar in a position away from bacteria will cool it
- If you stab where bacteria are production of aerosol
- Incubate the plate inverted at 37 ^{o}C.

In a day it is necessary to examine the colonies for future investigation (fig. 102-103).

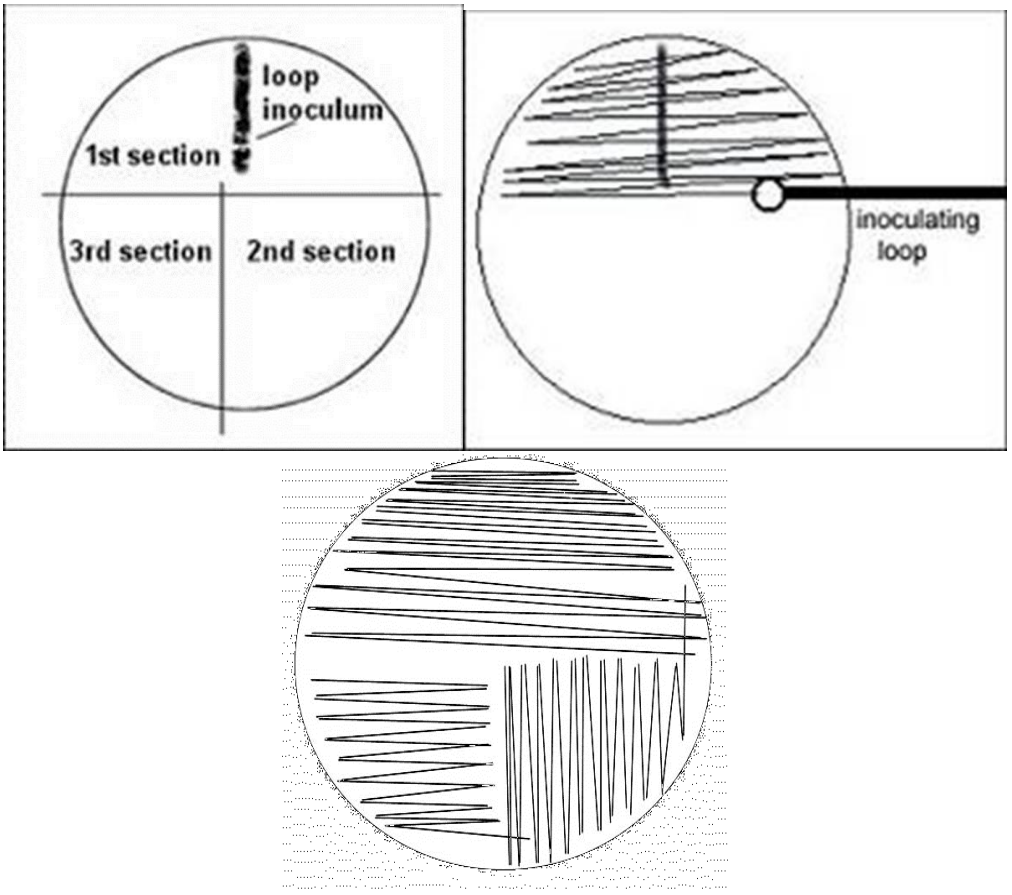


Figure 102. Streak plate technique

Or:

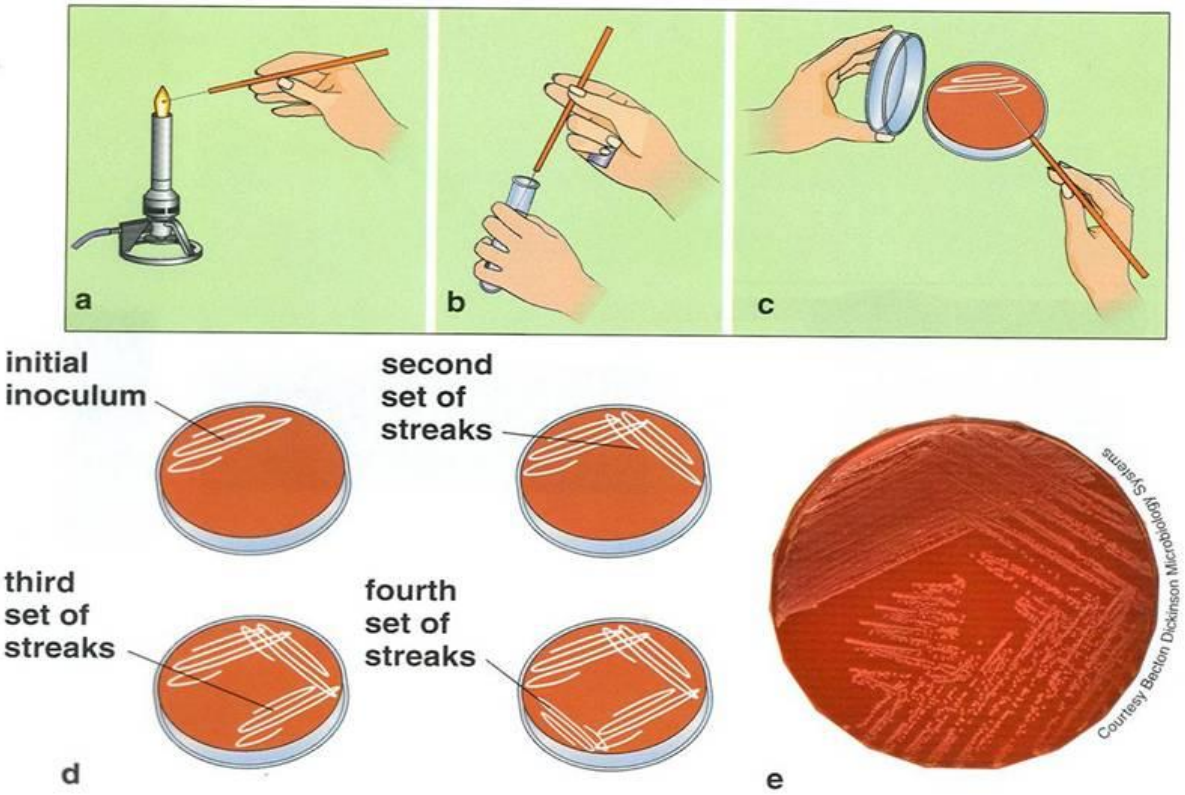


Figure 103. Streak plate technique

Thus, substantial advantage of Pour plate technique (dilution in solid nutrient media by R. Koch's technique), spread plate technique (superficial dispersions by Drigalsky's technique), and streak plate technique consists in that they create the ability to obtain isolated (distinct) colonies of microorganisms which can be transferred on slant agar for pure culture obtaining.

Methods based on biological principle

Biological principle of disconnection of bacteria foresees the purposeful search of methods which take into account the numerous features of microbal species. Among the most widespread methods it is possible to select the followings:

1. Respiration type. All of microorganisms according to the type of respiration are divided into two basic groups:*aerobic (Corynebacterium diphtheriae, Vibrio cholerae and others)* and *anaerobic (Clostridium tetani, Clostridium botulinum, Clostridium perfringens and so on)*. If tested material from which it follows to select anaerobic bacteria to warm up preliminary, and then cultivate in anaerobic terms, these bacteria will grow exactly.

2. *Sporulation*. It is known that some microbes (bacilli and clostridia) form endospores. There are *Clostridium tetani, Clostridium botulinum, Clostridium perfringens, Bacillus subtilis, Bacillus cereus* among them. Spores are resistant against different external environment factors. That's why, if tested material would

be heated previously and then inoculated in nutrient medium spore-forming bacteria would be grown.

3. Resistance of microbes against acids and alkali. Some microbes *(Mycobacterium tuberculosis, Mycobacterium bovis)* as a result of their chemical structure features are resistant agains acids. That's why tested material with this bacteria previously is treated with 10 % sulfuric acid and later inoculated on proper nutrient medium An extraneous flora perishes, and mycobacteria as a result of their resistance to acids grow.Vibrio cholerae is a halophylic bacterium, and for its growth it is inoculated in 1 % alkaline peptone water. Already in 4-6 hrs it growth like a tender bluish pellicle on the surface of medium.

4. Bacteria motility. Some microbes *(Proteus vulgaris)* have a tendency to creeping growth and is able to spread quickly on the surface of moist nutrient medium because they have flagella. So such bacteria are inoculated in the drop of condensation liquid which appears after the cooling the slant agar. In 16-18 hrs they spread on all surface of nutrient medium. If material from the upper part of agar would be taken we will have a pure culture of microbe.

***5.* A susceptibility of microbes to different chemicals, antibiotics etc.** As a result of features of metabolism some bacteria have a different susceptibility to some chemical factors. For example, staphylococci, aerobic bacilli can grow in nutrient media which have 7,5–10 % to the sodium chloride. That is why for the selection of these bacteria this substance is added into yalk-salt afar and mannitol-salt agar for their selection. Other bacteria under the influence of such concentration of sodium chloride do not grow practically.

Some antibiotics (nistatin) is used for inhibition for pathogenic fungi growth if it is necessary to obtain only bacteria. Adding the Penicillin in nutrient medium inhibit the growth only gram-positive bacteria. Presence of Furazolidon makes favorite condition for Corynebacteria and Micriococci.

6. Ability of microorganisms to penetrate through unharmed skin. Some pathogenic bacteria *(Yersinia pestis)* as a result of presence a lot of aggression enzymes are able to penetrate through an intact skin. For this purpose body wool of laboratory animal is shaven and tested material with different bacteria a rubbed in this skin area. Later some microbes may be obtain from the blood or internal organs.

7. A sensitiveness of laboratory animals is to the exciters of infectious diseases. Some laboratory animals show a high susceptibility to the different microorganisms.For example, after any method of *Streptococcus pneumoniae* introduction into a mouse generalized pneumococcal infection are developed. An analogical picture is observed after injection of *Mycobacterium tuberculosis* into Guinean pig or Mycobacterium bovis into the rabbit.

8. Temperature optimum. The cardinal temperatures:

- Minimum
- Optimum
- Maximum

Microorganisms can be grouped by the temperature ranges they require

- Psychrophiles, low temperature optima (4°C) – Polaromonas vacuolata
- Mesophils midrange (39°C) – Escherichia coli
- Thermophiles high (60°C) – Bacillus stearothermophilus
- Hyperthermophiles very high (>80°C) – Thermococcus celer

In everyday practice bacteriologists use such concepts as a species, a strain and pure culture of microorganisms.

Species – a collection of bacterial cells which share an overall similar pattern of traits in contrast to other bacteria whose pattern differs significantly

A ***strain*** is a subset of a bacterial species differing from other bacteria of the same species by some minor but identifiable difference. A *strain* is "a population of organisms that descends from a single organism or pure culture isolate. Strains within a species may differ slightly from one another in many ways."

Culture: population of microorganisms grown under well defined conditions.

Pure culture – one that contains one type of microorganism.

Isolation and identification of a pure culture

First day

1. Microscopic examination of the tested material.

2. **Streaking of the material tested onto nutrient media (solid, liquid).**

Second day

1. Investigation of the cultural properties.
2. Sub-inoculation of colonies onto solid media to enrich for a pure culture.

Third day

1. Checking of the purity of the isolated culture.
2. Investigation of biochemical properties: (a) sugarlytic, (b) proteolytic.
3. Determination of antigenic properties.
4. Study of phagosensitivity, phagotyping, colicinogensitivity, colicinogenotyping, sensitivity to antibiotics, and other properties.

Main Principles of the Cultivation of Microorganisms

Bacterial cultivation. In laboratory conditions microorganisms can be grown in nutrient media in incubation chambers maintained at a constant temperature. According to the type of heating, incubation chambers can be subdivided into electric, gas and kerosene. Each incubation chamber has a thermoregulator which maintains a constant temperature. Temperature conditions are of great importance for the growth and reproduction of bacteria. In relation to conditions of temperature all micro-organisms can be subdivided into three groups: psychrophilic (Gk. psychros cold, philein love), mesophilic (Gk. mesos intermediate), thermophilic (Gk. thermos warm). Microorganisms may reproduce within a wide temperature regimen range of –10 to +80 °C.

Of great importance in the life activities of bacteria is the concentration of hydrogen ions in the nutrient medium, i. e. pH, which is expressed by the negative logarithm of the concentration of hydrogeons. The pH characterizes the degree of acidity or alkalinity, from extremely acid (pH 0) to extremely alkaline (pH 14) conditions.

During evolution each microbial species adapted itself to existence within certain limits of hydrogen ion concentration beyond the range of which its life processes are unable to take place; It has been suggested that pH influences the activity of enzymes. Depending on the pH, weak acids in an acid medium occur as molecules, and in an alkaline medium as ions. Saprophytes can live in conditions within a wide range of a pH from 0.6 to 11.0, while pathogenic species of microbes grow within certain limits of hydrogen ion concentration Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a required salt concentration. In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation reduction potential.During the whole history of microbiology nutrient media have gradually been perfected. Before Pasteur only infusions and decoctions were used as media for growing microbes. Pasteur and Nageli introduced non-protein media for the cultivation of microbes. Koch and Loeffler employed meat broth, peptone, and sodium chloride for growing microbes. This medium is a meat-peptone broth from which meat-peptone agar is prepared by adding 1-2 per cent industrial agar.

Agar (in Malayan - jelly) is compact fibrous material produced from some seaweed, forms in water solutions a solid gel. Agar contains 70-75% polysaccharides, 2-3% proteins and other nitrogen-containing substances, 2-4% ashes. Main components of agar high molecular weight substances — agarose and agaropectin. Agar dissolves in water while heating and solidifies at room temperature. It is manufactured as colourless plates or powder.

Because of the ability of agar upon cooling to give the nutrient medium a solid gel consistency, and due to its high resistance towards the microbial enzymes, it has received wide application in bacteriological techniques for preparing semisolid, solid, and dry nutrient media.For the preparation of nutrient media M. Hottinger suggested the use of products of the tryptic breakdown of proteins which do not contain peptones, but contain the low molecular polypeptides and free amino acids. L. Martin employed papain as an enzyme for the break-down of proteins. In recent years all the essential amino acids and vitamins used for the cultivation of bacteria have been obtained in a pure state.

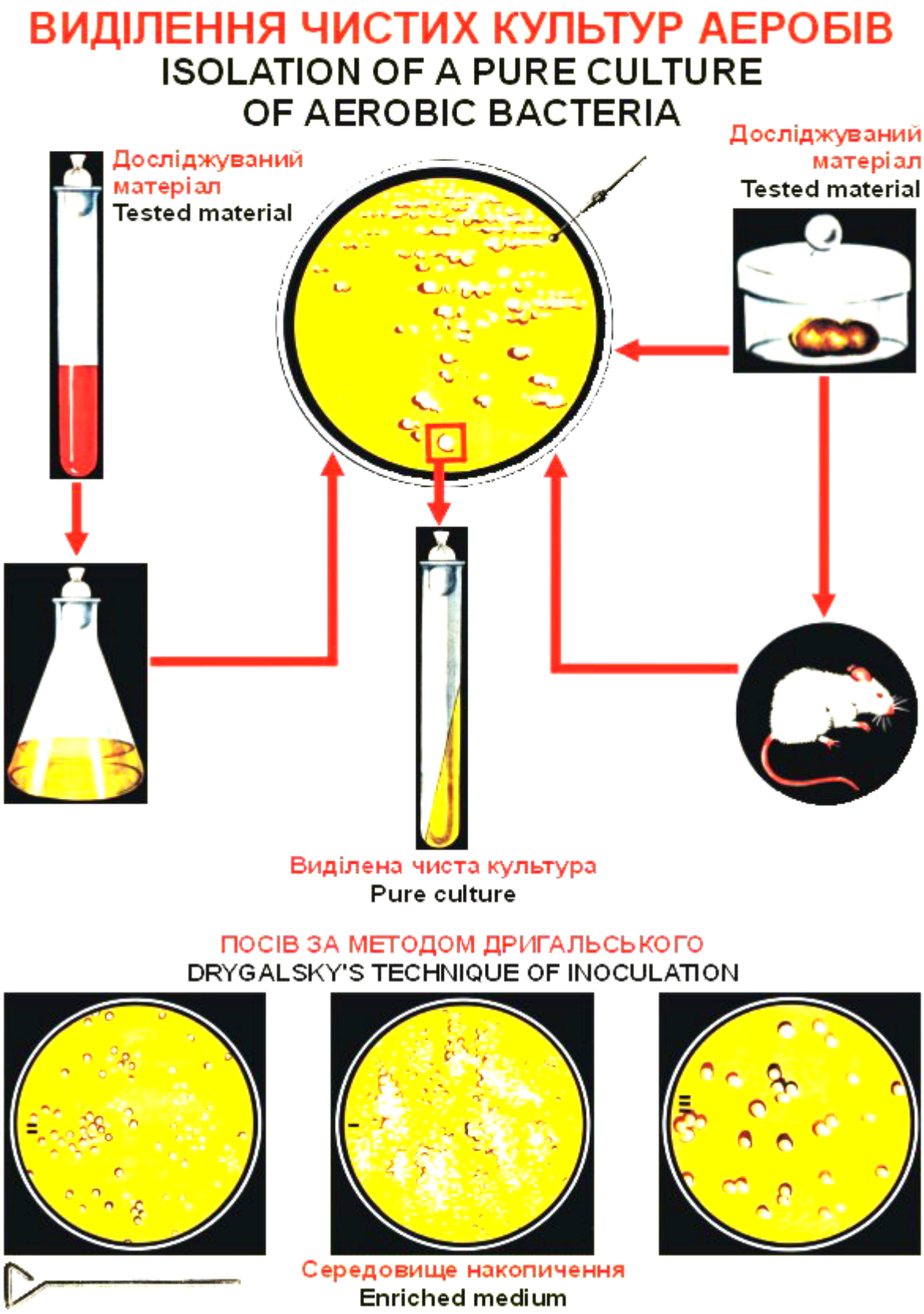


Figure 104. Isolation and Identification of Pure Culture of Aerobic Bacteria

First day. Prepare smears of the tested material and study them under the microscope. Then, using a spatula or a bacteriological loop, streak the material onto a solid medium in a Petri dish. This ensures mechanical separation of microorganisms on the surface of the nutrient medium, which allows for their growth in isolated colonies. In individual cases the material to be studied is streaked onto the liquid enrichment medium and then transferred to Petri dishes with a solid nutrient medium. Place these dishes in a 37 ^{0}C incubator for 18-24 hrs. (fig. 105-106)

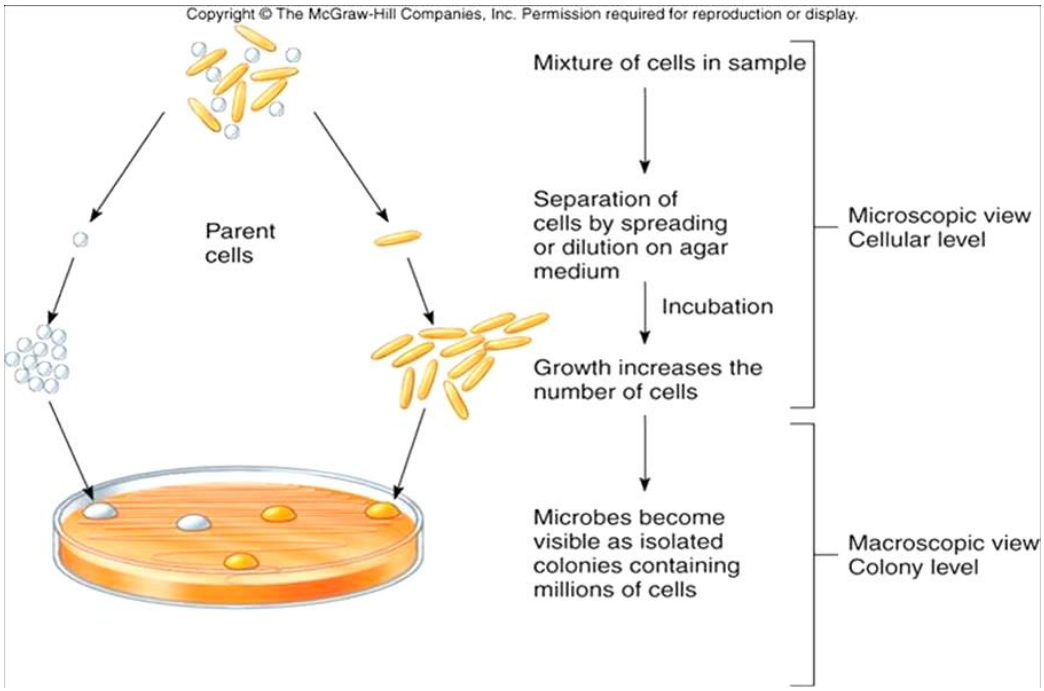


Figure 105. . Isolation of Pure Culture of Aerobic Bacteria

Figure 106. Incubator

Second day. Following a 24-hour incubation, the cultural properties of bacteria (nature of their growth on solid and liquid nutrient media) are studied (fig. 107).

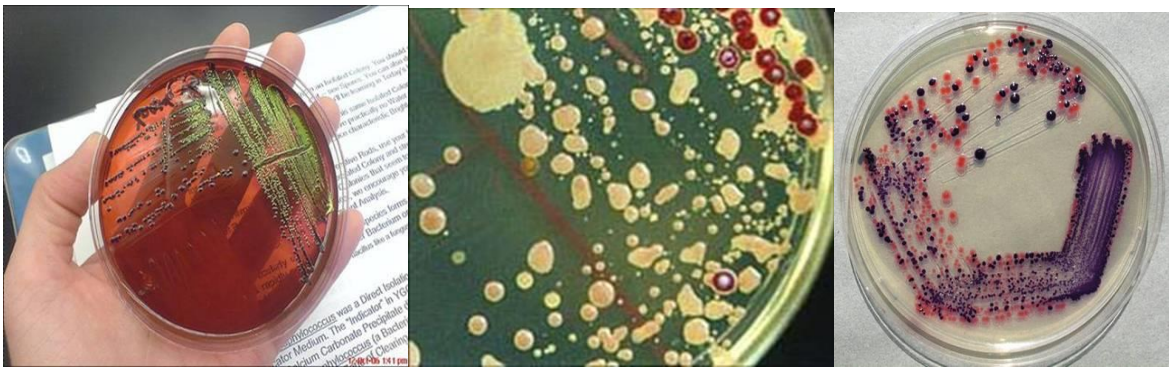


Figure 107. The cultural properties of bacteria are studied

Macroscopic examination of colonies in transmitted and reflected light. Turn the dish with its bottom to the eyes and examine the colonies in transmitted light. In the presence of various types of colonies count them and describe each of them. The following properties are paid attention to; **(a)** size of colonies (largo, 4-5 mm in diameter or more; medium, 2-4 mm; small, 1-2 mm; minute, less than 1 mm); **(b)** configuration of colonies {regularly or irregularly rounded, rosette-shaped, rhizoid, etc.); **(c)** degree of transparency (non-transparent, semitransparent, transparent).

In a reflected light, examine the colonies from the top without opening the lid. The following data are registered in the protocol: **(a)** colour of the colonies (colourless, pigmented, the colour of the pigment); **(b)** nature of the surface (smooth, glassy, moist, wrinkled, lustreless, dry, etc.); **(c)** position of the colonies on the nutrient medium (protruding above the medium, submerged into the medium; flat, at the level of the medium; flattened, slightly above the medium).

Microscopic examination of colonies. Mount the dish, bottom upward, on the stage of the microscope, lower the condenser, and, using an 8 x objective, study the colonies, registering in the protocol their structure (homogeneous or amorphous, granular, fibriliar, etc.) and the nature of their edges (smooth, wavy, jagged, fringy, etc.).

Use some portion of the colonies to prepare Gram-stained smears for microscopic examination. In the presence of uniform bacteria, transfer the remainder of colonies to an agar slant for obtaining a sufficient amount of pure culture. Place the test tubes with the inoculated medium into a 37 °C incubator for 18-24 hrs.

Third day. Using the culture which has grown on the agar slant prepare smears and stain them by the Gram method. Such characteristics as homogeneity of the growth, form, size, and staining of microorganisms permit definite judgement as to purity of the culture. To identify the isolated pure culture, supplement the study of morphological, tinctorial, and cultural features with determination of their enzymatic

and antigenic attributes, phago- and bacterio-cinosensitivity, toxigenicity, and other properties characterizing their species specificity.

To demonstrate carbohydrate-splitting enzymes, Hiss' media are utilized. When bacteria ferment carbohydrates with acid formation, the colour of the medium changes due to the indicator present in it. Depending on the kind and species of bacteria studied, select media with respective mono- and disaccharides (glucose, lactose, maltose, sucrose), polysaccharides (starch, glycogen, inulin), higher alcohols (glycerol, mannitol). In the process of fermentation of the above substances aldehydes, acids, and gaseous products (CO_2, H_2, etc.) are formed.

To demonstrate proteolytic enzymes in bacteria, transfer the latter to a gelatin column. Allow the inoculated culture to stand at room temperature (20-22 °C) for several days, recording not only the development of liquefaction per se but its character as well (laminar, in the form of a nail or a fir-tree, etc.)

Proteolytic action of enzymes of microorganisms can also be observed following their streaking onto coagulated serum, with depressions forming around colonies (liquefaction). A casein clot is split in milk to form peptone, which is manifested by the fact that milk turns yellowish (milk peptonization).

More profound splitting of protein is evidenced by the formation of indol, ammonia, hydrogen sulphide, and other compounds. To detect the gaseous substances, inoculate microorganisms into a meat-peptone broth or in a 1 per cent peptone water. Leave the inoculated cultures in an incubator for 24-72 hrs.

To demonstrate indol by Morel's method, soak narrow strips of filter paper with hot saturated solution of oxalic acid (indicator paper) and let them dry. Place the indicator paper between the test tube wall and stopper so that it does not touch the streaked medium. When indol is released by the 2nd-3rd day, the lower part of the paper strip turns pink as a result of its interaction with oxalic acid.

The telltale sign of the presence of ammonia is a change in the colour of a pink litmus paper fastened between the tube wall and the stopper (it turns blue). Hydrogen sulphide is detected by means of a filter paper strip saturated with lead acetate solution, which is fastened between the tube wall and the stopper. Upon interaction between hydrogen sulphide and lead acetate the paper darkens as a result of lead sulphide formation.

To determine catalase, pour 1-2 ml of a 1 per cent hydrogen peroxide solution over the surface of a 24-hour culture of an agar slant. The appearance of gas bubbles is considered as a positive reaction. Use a culture known to contain catalase as a control.

The reduction ability of microorganisms is studied using methylene blue, thinning, litmus, indigo carmine, neutral red, etc. Add one of the above dyes to nutrient broth or agar. The medium decolorizes if the microorganism has a reduction ability. The most widely employed is Rothberger's medium (meat-peptone agar containing 1 per cent of glucose and several drops of a saturated solution of neutral red). If the reaction is positive, a red colour of the agar changes into yellow, yellow-green, and fluorescent, while glucose fermentation is characterized by cracks in the medium.

Antigen properties of the isolated culture are investigated by the agglutination test and other serological tests.

Species identification of aerobic bacteria is performed by comparing their morphological, cultural, biochemical, antigenic, and other properties.

On solid nutrient media microbes form colonies of different shapes and sizes which are ggregations of individuals connected by bands of cytoplasm providing for a certain structure of bacterial groupings. The colonies may be flat. convex, dome-shaped, or pitted; their surface – smooth (S-forms). rough (R-forms). ridged, or bumpy; their edges may be straight, serralcd. fibrous, or lasseled. The shape of the colonies also differs: e.g. round, rosette-shaped, star-shaped, tree-like. According lo their size the colonies may be divided into large (4-5 mm in diameter), intermediate (2-4 mm), small (1-2 mm), and dwarf (less than 1 mm).

The colonies differ in their consistency, density, and colour. They may be transparent and opaque, coloured and colourless, moist, dry, and slimy.

In liquid nutrient media microbes grow producing a duffuse suspen- sion. film. or precipitate visible to the naked eye.

The growth of bacteria in the laboratory is carried out in test tubes, Petri dishes, and flasks.

In institutes for production of vaccines the cultivation of aerobes is carried out by deep stab methods. This method permits a more rational use of the nutrient substrate, and a large microbial mass can be obtained. The cultures are grown in reactors. Aeration is produced by passing a stream of air through the medium. The method of aeration is used in laboratory investigations to promote rapid growth of bacteria and to study some processes of metabolism.

Reproduction in microbes takes place more intensively in a flowing nutrient medium which is constantly being renewed. For this purpose a spare tank with nutrient medium is installed, from which the mediumenters the cultivator and is carefully mixed with the culture (fig. 108).

Figure 108. Colonies of a different structure

After this the excess of cultural fluid together with the suspended bacterial cells flows out. When the rate of flow of cells from the cultivator is equal to the rate of reproduction, the number of the microbial population remains constant.

Modem plant equipment is supplied with devices for automatic control over reproduction and other microbiological processes.

In usual laboratory conditions anaerobes develop in stationary or portable anaerostats containing rarefied air up to 1-8 mm or in vacuum desiccators (fig. 109-110).

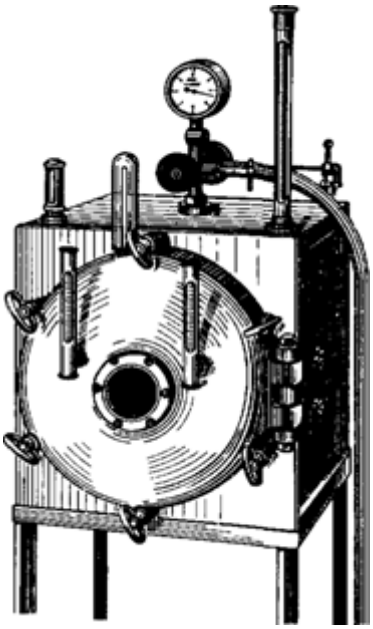


Figure 109. Stationary anaerostat (jar)

Figure 110. Portable anaerostat

For successfully cultivating anaerobes it is necessary to seed a large amount of material into the nutrient medium. The nutrient medium should have a certain viscosity which is attained by adding 0.2 per cent agar. The air is removed by boiling

prior to seeding, and to inhibit thesubsequent entry of air, the medium is covered with a layer of oil 0.5-1 cm thick. Anaerobiosis is obtained by the adsorption of oxygen on porous substances (pumice, cotton wool, coal) and by adding reducing substances (carbohydrates, peptone, cysteine. pieces of liver, spleen, kidneys, brain, etc.). After seeding, the test tubes are filled up with liquid vaseline. Growth of the anaerobes is usually carried out on a Kitt-Tarozzi

With regards to obtaining microorganisms in pure culture, are based on mechanical divorced of bacteria tested material inoculate onto surface media in Petri dish by bacteriological loop or pipette and after that streak plating evenly. After that again that glass spatula (don't burn through the flame) was used for streak plating onto the same second media in Petri dish.The seeding has been done by bacteriological loop too. With that purpose in upper part of Petri dish has been made dense streaking, set free bacteriological loop from superfluous material. After that are made paralel streaks at the last part of the agar. Somever are applied method of laminar dilution, the matter of this method is a stiring diferrent serial dillution tested materials with melting and colling agar in tubes.After that its are flooded into Petri dishes and put down into incubator.The tested materials are boiled of short duration or heat on 80 ^{0}C for destroy bacteria without spores. The spores of microorganisms leave still alive and ater reinoculate this materials they are grown.

Fortner method. The agar media is divided into two parts. Onto the one part inoculate E.coli or Serratia marcescens (these microorganisms absorb intensively oxygen) and onto second part taested material. Closely stop up this Petri dish by parafin and put down into the thermostat. This method is used for obtainig anerobe culture.

Reproduction and Growth of Microorganisms

Reproduction in microbes constitutes the ability of self-multiplication, i.e. the increase in the number of individuals per unit volume The growth of micro-organisms represents the increase of the mass of bacterial cytoplasm as a result of the synthesis of cellular material.

Bacteria reproduce by simple transverse division, vegetative reproduction, which occurs in different planes and produces many kinds of cells (clusters, chains, pairs, packets, etc.). They also reproduce by budding, by means of the cleavage of segmented filaments, by reproducing cells similar to spores, by producing minute motile conidia. And by conjugation, which brings us closely to the concept of sexual reproduction in bacteria DNA replication is an important condition in the process of amitotic binary fission of bacteria, the hydrogen bonds are ruptured and two DNA strands are formed, each one is contained in the daughter cells The single-stranded DNA are eventually linked by means of hydrogen bonds and again form double-chain DNA responsible for genetic information DNA replication and cell fission occur at a definite rate characteristic of each species. Actinomycetes and many fungi (phycomycetes, ascomycetes, etc) reproduce predominantly by sporulation

The transverse division of bacteria is not only a process of cell division of one mother cell into two equal daughter cells, but represents d constant separation of daughter cells from the mother cell, the former in their turn become mother cells.

After a certain number of generations, the mother cells age and perish. This explanation has annulled the metaphysical concept of 'bacterial immortality'.

The rate of cell division differs among bacteria It depends on the species of microbe, the age of the culture, on the nutrient medium, temperature, concentration of carbon dioxide, and on many other factors.

The length of the generation off coli, Clostridium perfringens, Streptococcus faecalis is 15 minutes, while for the cells of a mammalian tissue culture it is 24 hours Thus, bacteria reproduce almost 100 times faster than cells of tissue culture The increase in the number of cells can be expressed in the following way:

0— 1— 2— 3 — 4— 5— n number of generations

The total amount of bacteria (N) after n generations will be equal to 2^n per cell of seeded material If we take the original amount of bacteria inoculated into the nutrient medium as a single individual, and the time for one division as 30 minutes, then theoretically the total amount of bacteria produced per 24 hours would be equal $N=2^{48}$. Upon division every 20 minutes, in 36 hours the microbial mass will be equal to 400 tons. Thermophilic microbes divide even more rapidly.

However, in natural as well as in artificial conditions, the reproduction of bacteria is of a considerably smaller scale. It is limited by the effect of a number of environmental factors. Reproduction in bacteria conforms to certain laws. Fig. 1 illustrates schematically the rate of reproduction of bacteria in arbitrary units, and the size of the bacterial population expressed as the logarithm of the numbers of live cells per millimeter of the medium.

There are eight principal phases of reproduction which are designated on the diagram by Roman numerals.

1. An initial stationary phase represents the time from the moment of seeding the bacteria on the nutrient medium. Reproduction does not occur in this phase. The length of the initial stationary phase after seeding is 1-2 hours.

2. The lag phase of reproduction during which bacterial reproduction is not intensive, while the growth rate is accelerated. The second phase may last almost two hours.

3. Phase of exponential (logarithmic) growth which is characterized by a maximal division rate and decrease in cell size. The length of this period ranges from 5 to 6 hours.

4. Phase of negative growth acceleration during which the rate of bacterial reproduction ceases to be maximal, and the number of dividing cells diminishes. This phase lasts almost two hours.

5. A maximal stationary phase when the number of newly produced bacteria is almost equal to the number of dead organisms. This phase continues for two hours.

6. Accelerated death phase during which the equilibrium between the stationary phase and the bacterial death rate is interrupted. This continues for 3 hours.

7. Logarithmic death phase when the cells die at a constant rate. This continues almost 5 hours.

8. Decelerated death-rate phase in which those cells which remain alive enter a dormant state (fig. 111).

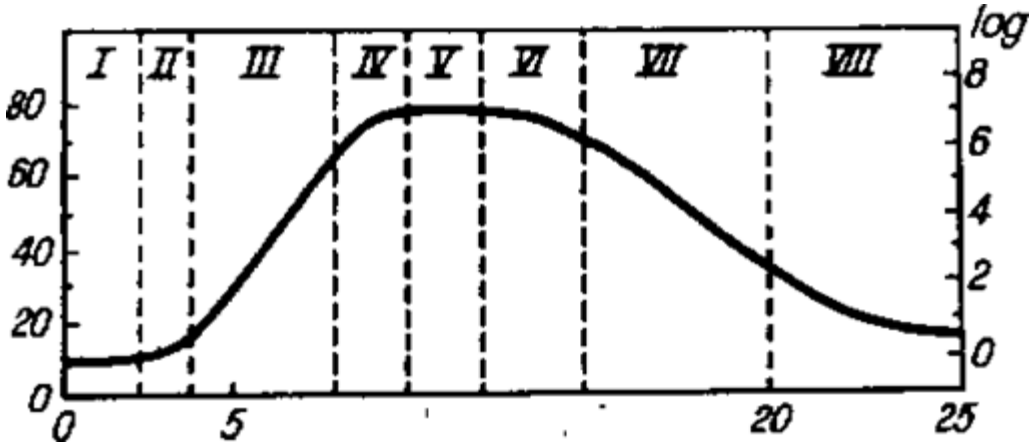


Figure 111. Graph of the reproduction of bacteria

The length of these phases is arbitrary, as it can vary depending on the bacterial species and the conditions of cultivation. Thus, for example, the colibacilli divide every 15-17 minutes, salmonellae of enteric fever — every 23 minutes, pathogenic streptococci — every 30 minutes, diphtheria bacilli — every 34 minutes and tubercle bacilli — every 18 hours.

Main Principles of the Cultivation of Microorganisms

Bacterial cultivation. In laboratory conditions microorganisms can be grown in nutrient media in incubation chambers maintained at a constant temperature. According to the type of heating, incubation chambers can be subdivided into electric, gas and kerosene. Each incubation chamber has a thermoregulator which maintains a constant temperature. Temperature conditions are of great importance for the growth and reproduction of bacteria. In relation to conditions of temperature all micro-organisms can be subdivided into three groups: psychrophilic (Gk. psychros cold, philein love), mesophilic (Gk. mesos intermediate), thermophilic (Gk. thermos warm). Microorganisms may reproduce within a wide temperature regimen range of –10 to +80 °C.

Of great importance in the life activities of bacteria is the concentration of hydrogen ions in the nutrient medium, i. e. pH, which is expressed by the negative logarithm of the concentration of hydrogeons. The pH characterizes the degree of acidity or alkalinity, from extremely acid (pH 0) to extremely alkaline (pH 14) conditions.

During evolution each microbial species adapted itself to existence within certain limits of hydrogen ion concentration beyond the range of which its life processes are unable to take place; It has been suggested that pH influences the activity of enzymes. Depending on the pH, weak acids in an acid medium occur as molecules, and in an alkaline medium as ions. Saprophytes can live in conditions within a wide range of a pH from 0.6 to 11.0, while pathogenic species of microbes grow within certain limits of hydrogen ion concentration Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and

carbohydrate substances, vitamins, a required salt concentration. In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation reduction potential.

During the whole history of microbiology nutrient media have gradually been perfected. Before Pasteur only infusions and decoctions were used as media for growing microbes. Pasteur and Nageli introduced non-protein media for the cultivation of microbes. Koch and Loeffler employed meat broth, peptone, and sodium chloride for growing microbes. This medium is a meat-peptone broth from which meat-peptone agar is prepared by adding 1-2 per cent industrial agar.

Because of the ability of agar upon cooling to give the nutrient medium a solid gel consistency, and due to its high resistance towards the microbial enzymes, it has received wide application in bacteriological techniques for preparing semisolid, solid, and dry nutrient media.

For the preparation of nutrient media M. Hottinger suggested the use of products of the tryptic breakdown of proteins which do not contain peptones, but contain the low molecular polypeptides and free amino acids. L. Martin employed papain as an enzyme for the break-down of proteins. In recent years all the essential amino acids and vitamins used for the cultivation of bacteria have been obtained in a pure state.

Isolation and Identification of Pure Culture of Anaerobic Bacteria

Methods of obtaining anaerobic conditions. Taking into account that free molecular oxygen is toxic for obligate anaerobic bacteria, the main condition of such microorganisms cultivation is limitation of its access. There are some methods (mechanical, physical, biological) which allow providing it.

Toxic forms of oxygen

- Certain oxygen derivatives are toxic to microorganisms.
- Oxygen in its ground state is triplet oxygen ($^3O_2$).
- Toxic forms of oxygen include singlet oxygen $^1O_2$, (superoxide anion) O_2^-, hydrogen peroxide H_2O_2 and hydroxyl radical (OH^-).

As molecules have an unpaired electron, they are very reactive and cause destruction.

Enzymes that destroy toxic oxygen

- Enzymes are present in cells that can neutralise most toxic forms of oxygen.
- Catalase
- Peroxidase
- Superoxide dismutase

Physical methods.

1. Before inoculation of bacteria on/in nutrient media it is necessarily to regenerate them for deletion of surplus oxygen (boiling them for 15-20 min in water bath, quickl cooling to the necessary temperature).

2. For warning oxygen penetration into nutrient medium it must be covered with the layer of sterile vaseline oil or paraffin (for liquid media).

3. A column of nutrient media in test tubes must be quite high (10-12 cm). Oxygen, as a rule, penetrates into the column of medium on a depth up to 2 cm, that

is why favourable conditions for cultivation of anaerobic microbes create below (Fig. 112).

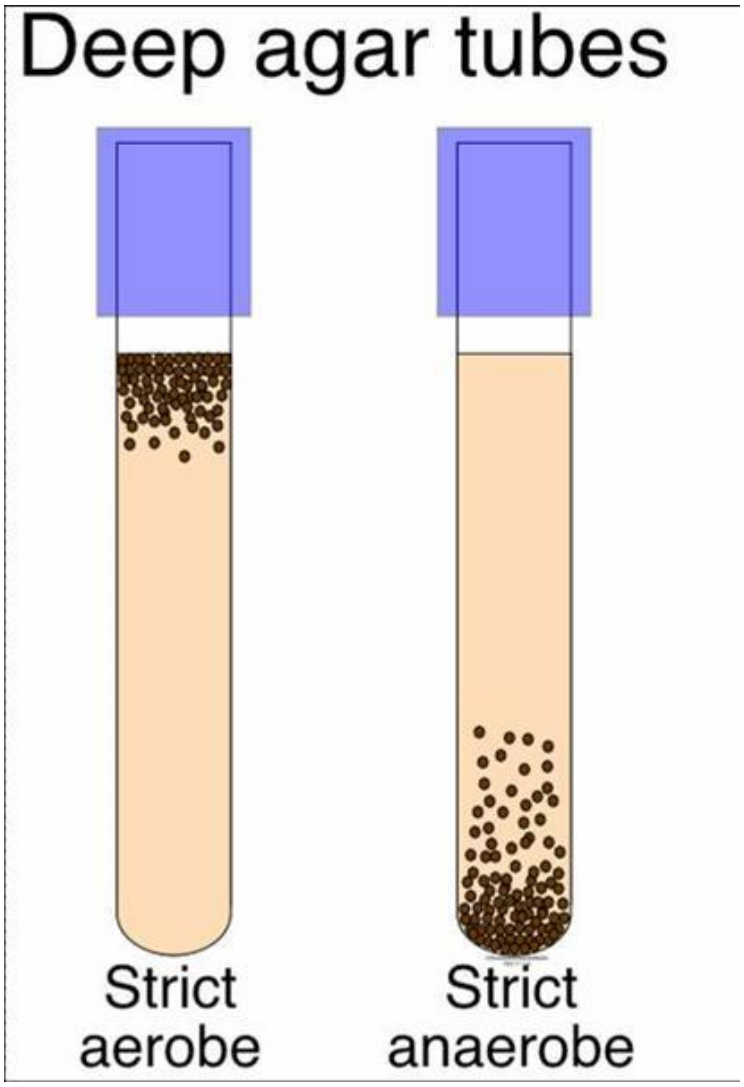


Figure 112. Anaerobic Bacteria

4. An evacuation and replaceable method foresees the use of anaerobic jar. They are hermetically sealed metallic or plastic jars from which it is possible to pump out oxygen and replace it by special gases (helium, nitrogen, argon). Triple gas mixturewhich consists of nitrogen 80 %, carbon dioxide 10 %, and hydrogen 10 % is used. Sometimes natural gas may be used. For a deoxygenation in the jar palladic catalysts are used. For absorption of aquatic steams calcium chloride, silicagel and others substances are used in the jars (fig. 113).

Figure 113. An evacuation and replaceable method foresees the use of anaerobic jar

5. Place the burning candle into the flask or jar with Petri plates (fig. 114).

Figure 114. Isolation of Pure Culture of Anaerobic Bacteria

Chemical methods foresee the use of substances absorb an oxygen (alkaline solution of pyrogallol, sodium hydrosulphite ($Na_2S_2O_4$).

There may be used special reduced substances: cysteine (0,03-0,0,5 %), thioglycolic acid or sodium thioglycolate (0,01-0,02 %), sodium sulphide, ascorbic acid (0,1 %), different sugars.

Such functions have pieces of animals parenchymatous organs (liver, kidneys, heart) or even plants (potato).

The degree of deoxygenation or degree of nutrient medium reduction may be measured by indicators (rezazurine, neutral red, phenosafranine).

3. Use of the special gas generating systems which allow to create oxygen-free conditions in the jars, transport plastic packages and so on. One of most widespread there is the system of **"Gas Generating Box"**.

The GasPak™ EZ Gas Generating Pouch Systems are single-use systems that produce atmospheres suitable to support the primary isolation and cultivation of anaerobic, microaerophilic, or capnophilic bacteria by use of gas generating sachets inside single-use resealable pouches. The GasPak EZ Gas Generating Sachet consists of a reagent sachet containing inorganic carbonate, activated carbon, ascorbic (citric) acid and water. When the sachet is removed from the outer wrapper, the sachet becomes activated by exposure to air. The activated reagent sachet and specimens are placed in the GasPak EZ Incubation Container and the container is sealed. The sachet rapidly reduces the oxygen concentration within the container. At the same time, inorganic carbonate produces carbon dioxide.

Anaerobic environment-action: The gas generator envelope is activated by the addition of water; Hydrogen generated from a sodium borohydride tablet combines with the oxygen in the jar in the presence of the palladium catalyst to form water, removing the oxygen.

Anaerobic conditions are achieved rapidly, generally within 1 hour of incubation; the carbon dioxide concentration is approximately 4-10%. At 35 □C, the Gas Pak methylene blue anaerobic indicator becomes decolorized at 4-6 hours (fig. 115-116).

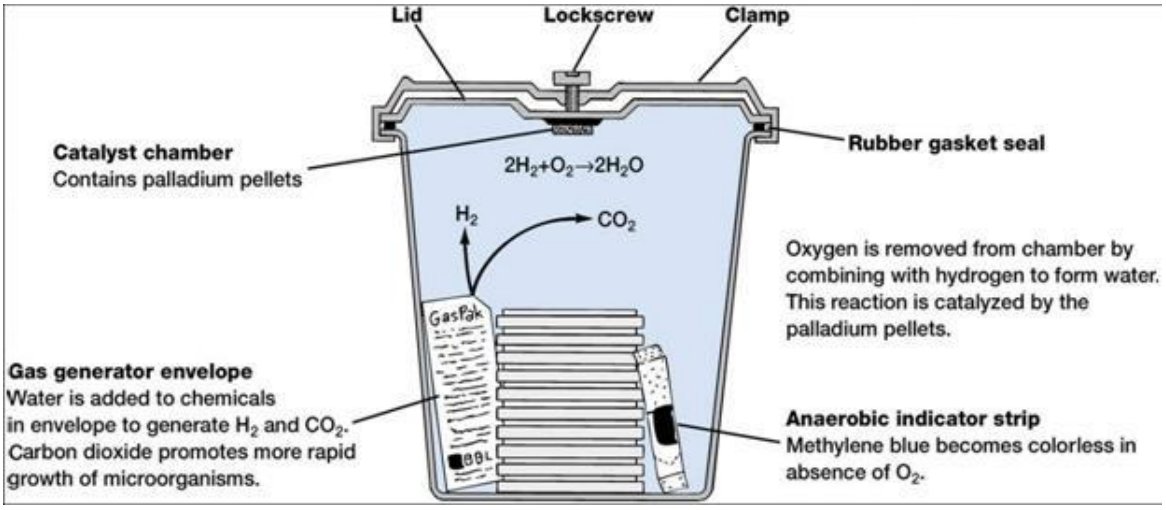


Figure 115. Anaerobic environment-action

Figure 116. Gas Pak with indicator strip and CO_2 generator pack

Biological methods.

1. Fortner's method. A method includes general cultivation on nutrient medium an aerobic and an anaerobic microorganisms. At first part of nutrient medium in Petri plate aerobic bacteria (Serratia marcescens) are inoculated, at second – tested material with anaerobic bacteria. The edges of cup are closed hermetically (e.g. with paraffin). In a few days the colonies both aerobic and anaerobic microbes grow. Serratia marcescens forms pink or colourless colonies, and when there are violations of hermetic conditions – bright red ones. The colonies of anaerobic microbes grow on other half of Petri plate.

2. Hennel's technique ("watch glasses technique"). There is original modification of previous one. Tested material with anaerobic bacteria is inoculated on the square 2-2,5 cm in diameter. Later it is covered by special convex glass where is nutrient medium and Serratia marcescens on it. Aerobic microbes (Serratia spp.) taking an oxygen create favourable conditions for anaerobes growth.

Now the stationary anaerobic boxes for cultivation of anaerobic bacteria are made (fig. 118).

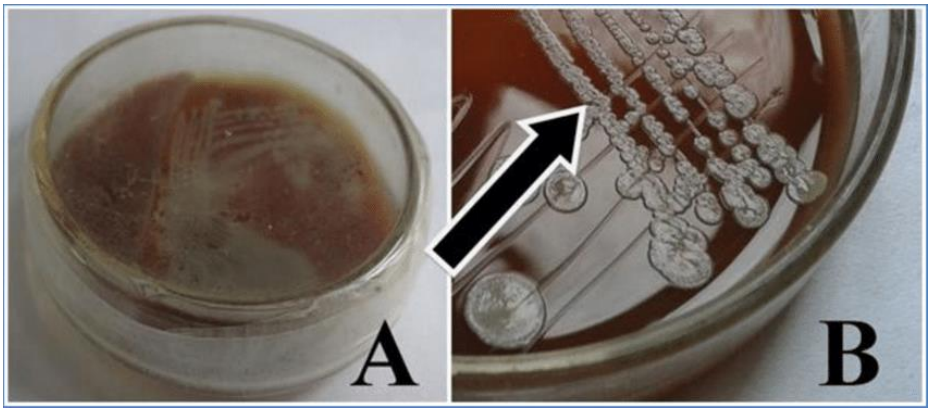


Figure 117. Fortner's method of culture & (B) Close-up view. Arrow points to the small, irregular, rough Actinomyces colonies

Figure 118. The stationary anaerobic boxes

One of the main requirements in cultivating anaerobic bacteria is removal of oxygen from the nutrient medium. The content of oxygen can be reduced by a great variety of methods: immersing of the surface of the nutrient medium with petrolatum, introduction of microorganisms deep into a solid nutrient medium, the use of special anaerobic jars.

First day. Inoculate the studied material into Kitt-Tarozzi medium (nutrient medium): concentrated meat-peptone broth or Hottinger's broth, glucose, 0.15 per cent agar (pHl 7.2-7.4).

To adsorb oxygen, place pieces of boiled liver or minced meat to form a 1-1.5 cm layer and pieces of cotton wool on the bottom of the test tube and pour in 6-7 mi of the medium. Prior to inoculation place the medium into boiling water for 10-20 min in order to remove air oxygen contained in it and then let it cool. Upon isolation of spore forms of anaerobes the inoculated culture is reheated at 80 '"C for 20-30 min to kill non-spore-forming bacteria. The cultures are immersed with petrolatum and placed into an incubator. Apart from Kitt-Tarozzi medium, liquid media containing 0.5-1 per cent glucose and pieces of animal organs, casein-acid and casein-mycotic hydrolysates can also be employed.

Casein-acid medium', casein-acid hydrolysate, 0.5 1; 10 per cent yeast extract, 0.35 1: 20 per cent corn extract, 0.15 1; millet, 240 g; cotton wool, 25 g. The medium is poured into flasks with millet and cotton wool and sterilized for 30 min at 110^0C. Use casein-mycotic hydrolysate to obtain casein-mycotic medium.

Second day. Take note of changes in the enrichment medium, namely, the appearance of opacification or opacification in combination with gas formation. Take broth culture with a' Pasteur pipette and transfer it through a layer of petrolatum onto the bottom of the test tube. Prepare smears on a glass slide in the usual manner, then

flame fix and Gram-stain them.During microscopic examination record the presence of Gram-positive rod forms (with or without spores). Streak the culture from the enrichment medium onto solid nutrient media. Isolated colonies are prepared by two methods.

1. Prepare three plates with blood-sugar agar. To do it, melt and cool to 45 °C 100 ml of 2 per cent agnr on llottinger's broth, then add 10-15 ml of deftbrinated sheep or rabbit blood and 10 ml of 20 per cent sterile glucose. Take a drop of the medium witli microorganisms into the first plate and spread it along the surface, using a glass spatula. Use the same spatula to streak tlic culture onto tlie second and then third plates and place them into an anaerobic jar or other similar devices at 37 "C for 24-48 hrs (Zoisslcr's method).

2, Anaerobic microorganisms are grown deep in a solid nutrient medium (Veinherg's method of sequential dilutions). The culture from the medium is taken with a Pasteur pipette with a soldcd tip and transferred consecutively into the 1st, 2nd, and 3rd test tubes with 10 ml of isotonic sodium chloride solution. Continue to dilute^ transferring the material into the 4th, 5th. and 6th thin-walled test tubes (0.8 cm in diameter and 18 cm in height) with melted and cooled to 50 °C meat-peptone agar or Wilson-Blair medium (to 100 ml of melted meat-peptone agar with 1 per cent glucose add 10 ml of 20 per cent sodium sulphite solution and 1 ml of 8 per cent ferric chloride). Alter agar has solidified, place the inoculated culture into an incubator.

On the third day, study the isolated colonies formed in tlie plates and make smears from the most typical ones. The remainder is inoculated into Kitt-Tarozzi medium. The colonies in the test tubes are removed by means of a sterile Pasteur pipette or the agar column may be pushed out of the tube by steam generated upon warming the bottom of the test tube. Some portion of the colony is used to prepare smears, while its remainder is inoculated into Kitt-Tarozzi medium to enrich pure culture to be later identified by its morphological, cultural, biochemical, toxicogenic, antigenic, and other properties.

The Vinyale-Veyone’s method is used for mechanical protection from oxygen. The seeding are made into tube with melting and cooling (at 42 ^{0}C) agar media.

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. Nutrient media should be easily assimilable, and they should contain a known amount of nitrogen and carbohydrate substances, vitamins, a required salt concentration. In addition they should be isotonic, and sterile, and they should have buffer properties, an optimal viscosity, and a certain oxidation-reduction potential (tabl. 19).

Table 19. Three categories of media classification

Three Categories of Media Classification

Physical State (Medium's Normal Consistency)	Chemical Composition (Type of Chemicals Medium Contains)	Functional Type (Purpose of Medium)*
1. Liquid 2. Semisolid 3. Solid (can be converted to liquid) 4. Solid (cannot be liquefied)	1. Synthetic (chemically defined) 2. Nonsynthetic (not chemically defined)	1. General purpose 2. Enriched 3. Selective 4. Differential 5. Anaerobic growth 6. Specimen transport 7. Assay 8. Enumeration

**Some media can serve more than one function. For example, a medium such as brain-heart infusion is general purpose and enriched; mannitol salt agar is both selective and differential; and blood agar is both enriched and differential.*

The manner in which bacteria are cultivated, and the purpose of culture media, vary 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 degrees and remains liquid until cooled to 40 degrees, 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 aquaeous solution.

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.

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).

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 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 staphylococcus 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 (tabl. 20).

Table 20. Differential media

Differential Media

Medium	Substances That Facilitate Differentiation	Differentiates Between
Blood agar	Intact red blood cells	Types of hemolysis
Mannitol salt agar	Mannitol, phenol red, and 7.5% NaCl	Species of *Staphylococcus* NaCl also inhibits the salt-sensitive species
Hektoen enteric (HE) agar	Brom thymol blue, acid fuchsin, sucrose, salicin, thiosulfate, ferric ammonium citrate, and bile	*Salmonella, Shigella,* other lactose fermenters from nonfermenters Dyes and bile also inhibit gram-positive bacteria
Spirit blue agar	Spirit blue dye and oil	Bacteria that use fats from those that do not
Urea broth	Urea, phenol red	Bacteria that hydrolyze urea to ammonia
Sulfur indole motility (SIM)	Thiosulfate, iron	H_2S gas producers from nonproducers
Triple-sugar iron agar (TSIA)	Triple sugars, iron, and phenol red dye	Fermentation of sugars, H_2S production
XLD agar	Lysine, xylose, iron, thiosulfate, phenol red	*Enterobacter, Escherichia, Proteus, Providencia, Salmonella,* and *Shigella*
Birdseed agar	Seeds from thistle plant	*Cryptococcus neoformans* and other fungi

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. However, an enrichment medium may have selective features. An

enrichment medium for nonsymbiotic nitrogen-fixing bacteria omits a source of added nitrogen to the medium. The medium is inoculated with a potential source of these bacteria (e.g. a soil sample) and incubated in the atmosphere wherein the only source of nitrogen available is N_2. A selective enrichment medium for growth of the extreme halophile (*Halococcus*) contains nearly 25 percent salt [NaCl], which is required by the extreme halophile and which inhibits the growth of all other procaryotes.

Thus, nutrient media can be subdivided into three main groups:

I. Ordinary (simple) media which include meat-peptone broth, meat-peptone agar, etc.

II. Special media (serum agar, serum broth, coagulated serum, potatoes, blood agar, blood broth, etc.).

Quite often **elective media** are employed in laboratory practice in which only certain species of bacteria grow well, and other species either grow poorly or do not grow at all. Enriched media are also employed in which the species of interest to the scientist grows more intensively and more rapidly than the accompanying bacteria. Thus, for example, on Endo's medium (elective) the growth of the Gram-positive microbes is inhibited while alkaline peptone water and alkaline meat-peptone agar serve as enriched media for the cholera vibrio. Nutrient media containing certain concentrations of penicillin are elective for penicillin-resistant strains of bacteria, but unfavourable for penicillin-sensitive strains.

III. Differential diagnostic media: (1) media for the determination of the proteolytic action of microbes (meat-peptone gelatine); (2) media for the determination of the fermentation of carbohydrates (Hiss media); media for the differentiation of bacteria which do and do not ferment lactose (Ploskirev, Drigalsky, Endo. etc.); (3) media for the determination of haemolytic activity (blood agar); (4) media for the determination of the reductive activity of micro-organisms; (5) media containing substances assimilated only by certain microbes.

Besides, in laboratory practice conservation media are used. They are used for primary seeding and transportation of the material under test. They prevent the death of pathogenic microbes and enhance the inhibition of saprophytes. This group of media includes a glycerin mixture composed of two parts 0.85 per cent salt solution, 1 part glycerin, and I part 15-20 per cent acid sodium phosphate, and also a glycerin preservative with lithium salts, a hypertonic salt solution, etc.

At present many nutrient media are prepared commercially as dry powders. They are convenient to work with, are stable, and quite effective.

Non-protein media are widely used for the cultivation of bacteria, on which many heterotrophic microbes including pathogenic species grow well. The composition of these media is complex and includes a large number of components.

When cultivating in synthetic media, the use of the method of radioactive tracers has permitted a more detailed differentiation of microbes according to the character of their biosynthesis.

Selective media are widely used for differentiating prototrophic and ULixotrophic bacteria. Prototrophs grow on a minimum medium which contains only

salts and carbohydrates since they themselves are capable til' synthesizing the metaholites necessary for their development. Auxo-Irophs. in distinction, require definite media containing amino acids, vitamins, and other substances.

In consistency nutrient media may be solid (meat-peptone agar, meat-peptone gelatine, coagulated serum, potato, coagulated white of Ihc egg), semisolid (0.5 per cent meat-peptone agar), and liquid (peptone water, meat-peptone broth, sugar broth, etc.).

Enzymes and Their Role in Metabolism

Enzymes, organic catalysts of a highly molecular structure, are produced by the living cell. They are of a protein nature, are strictly specific in action, and play an important part in the metabolism of microorganisms. Their specificity is associated with active centres formed by a group of amino acids.

Enzymes of microbial origin have various effects and are highly active. They have found a wide application in industry, agriculture and medicine, and are gradually replacing preparations produced by higher plants and animals.

With the help of amylase produced by mould fungi starch is saccharified and this is employed in beer making, industrial alcohol production and bread making. Proteinases produced by microbes are used for removing the hair from hides, tanning hides, liquefying the gelatinous layer from films during regeneration, and for dry cleaning. Fibrinolysin produced by streptococci dissolves the thrombi in human blood vessels. Enzymes which hydrolyse cellulose aid in an easier assimilation of rough fodder.

Due to the application of microbial enzymes, the medical industry has been able to obtain alkaloids, polysaccharides, and steroids (hydrocortisone, prednisone, prednisolone. etc.).

Bacteria play an important role in the treatment of caoutchouc, collon. silk. coffee, cocoa, and tobacco: significant processes lake place under their effect which change these substances essentially in the needed direction. In specific weight the synthetic capacity of microorganisms is very high. The total weight of bacterial cytoplasm on earth is much higher than that of animal cytoplasm. The biochemical activity of microbes is of no less general biological importance than that of photosynthesis. The cessation of the existence of microorganisms would lead inevitably to the death of plants and animals.

Enzymes permit some species of micro-organisms to assimilate methane. butane, and other hydrocarbons, and to synthesize complex organic compounds from them. Thus, for example, with the help of the enzymatic ability of yeasts in special-type industrial installations protein-vitamin concentrates (PVC) can be obtained from waste products of petroleum (paraffins), which are employed in animal husbandry as a valuable nutrient substance supplementing rough fodder. Some soil micro-organisms destroy by means of enzymes chemical substances (carcinogens) which are detrimental to the human body because they induce malignant tumours.

Some enzymes are excreted by the cell into the environment (exoenzymes) for breaking down complex colloid nutrient materials while other enzymes are contained inside the cell (endoenzymes).

Depending on the conditions of origin of enzymes there are constitutive enzymes which are constantly found in the cell irrespective of the presence of a catalysing substrate. These include the main enzymes of cellular metabolism (lipase. carbohydrase. proteinase, oxydase, etc.). Adaptive enzymes occur only in the presence of the corresponding substrate (penicillinase, amino acid decarboxylase, alkaline phosphatase, B-galactosidase, etc.). The synthesis of induced enzymes in microbes occurs due to the presence in the cells of free amino acids and with the participation of ready proteins found in the bacteria.

According to chemical properties enzymes can be subdivided into three groups:

1 – enzymes composed only of proteins:

2 – enzymes containing in addition, to protein metallic ions essential for their activity, and assisting m the combination of the enzyme with the substrate, and taking part in the cyclic enzymatic transformations:

3 – enzymes which contain distinct organic molecules (coenzymes. prosthetic groups) essential for their activity. Some enzymes contain vitamins.

Bacterial enzymes are subdivided into some groups:

1. **Hydrolases** which catalyse the breakdown of the link between the carbon and nitrogen atoms, between the oxygen and sulphur atoms, binding one molecule of water (esterases. glucosidases, proteases. amidases, nucleases, etc.).

2. **Transferases** perform catalysis by transferring certain radicals from one molecule to another (transglucosidases, transacylases. transaminases).

3. **Oxidative enzymes** (oxyreductases) which catalyse the oxidation reduction processes (oxidases, dehydrogenases, peroxidases, catalases).

4. **Isomerases and racemases** play an important part in carbohydrate metabolism. They are found in most species of bacteria. Phosphohexoisomerase, galactovaldenase, phosphoglucomutase, hosphoglyceromutase pertain to the isomerases.

The absorption of food material by the cell is a rather complex process. Unicellular protozoa are characterized by a holozoic type of nutrition in which hard food particles are swallowed, digested and converted to soluble compounds. Bacteria, algae, fungi, and plants possess a holophytik type of nutrition. They absorb nutrients in a dissolved state. This difference, however, is not essential because the cells of protozoa, just like the cells of plant organisms, utilize nutrient substrates which are soluble in water or in the cell sap, while many bacteria and fungi can assimilate hard nutrients first splitting them by external digestion by means of exoenzymes. During diffusion the dissolved substance is transferred from the region of higher concentration outside the cell into the bacterial cell until the concentration becomes the same. The passage of a solvent through the cytoplasmic membrane of bacteria from a region where it is less concentrated to one where it is more concentrated is performed by osmosis. The concentration gradient and osmotic power on both sides of the cytoplasmic membrane are quite different, and depend on the difference in concentration of many substances contained in the cell and nutrient medium. The transfer of dissolved substances from the nutrient medium to the cell can take place by suction together with the solvent if the membrane is sufficiently porous.

It has been established that the cellular membranes are made up of lipid and protein molecules arranged in a certain sequence. The charged groups of molecules have their ends directed towards the surface of the membrane. On these charged ends the protein layers are adsorbed, composed of protein chains forming a meshwork on the external and internal surfaces of the membrane. The high selectivity which allows the cells to distinguish certain substances from others depends on the presence of enzymatic systems localized on the surface of bacterial cells. Due to the action of these enzymes, the insoluble substances in the membrane become soluble.

The cell membranes play an important role in metabolism. They are capable of changing rapidly their permeability to various substances and regulating in this way the entry of substances into the cell and their distribution in it, and the development of reactions in which these substances participate.

Some bacteria (Salmonella typhimurium} possess rudiments of memory. They recognize whether the medium is favourable or unfavourable to them. They 'run away' from an unfavourable one by means of flagella: when close to a favourable medium (glucose) Salmonella organisms swim to the 'bait'. This ability to recognize the needed direction is probably accomplished by the trial-and-error method.

In the process of bacterial nutrition great importance is attached to exchange adsorption. The active transport of ions takes place due to (he difference in charges on the surface of membranes in the cell wall and the surrounding medium of the micro-organisms. Besides, the role of transporters, as has been suggested, is performed by liposoluble substances X and Y. Compounds are formed with ions of potassium and sodium (KX and NaY) which are capable of diffusing through the cell wall, while the membrane remains unpenetrable for free transporters. Proteins concerned with the transport of amino acids have been isolated from the membranes of some micro-organisms, and protein systems responsible for the transfer of certain sugars in general and glucose in particular have been revealed.

Bases of bacterial Identification

Identification is the determination of whether an organism (or isolate in the case of microorganisms) should be placed within a group of organisms known to fit within some classification scheme.

Through the early part of the twentieth century, there appeared to be a general feeling that the same battery of observations and tests could be used to characterize and identify any kind of bacterium. But as different, "exotic" types of bacteria were discovered, it was found that they would tend not to grow in the standard test media nor even in the usual conditions of incubation. Obligate parasites and strict anaerobes were among the emerging groups of bacteria needing special methods for growth and characterization. By the 1930s, a standard descriptive chart was developed for uniformity in recording the characteristics of the "aerobic saprophytes" (which are equivalent to what we call the "commonly-found chemoheterotrophs" in our general courses today)..

As we now know, a huge battery of tests done at once to identify an unknown organism would result in a lot of media and time being wasted dealing with irrelevant tests. (Time and media are money!) Thus we would like to proceed in stages, running

those tests which are applicable to what basic knowledge we have about our unknown. That is, a very different set of tests would be run on a gram-negative rod compared to a gram-positive coccus.

There is no medium (differential or otherwise) that can possibly support the growth of all of the different species of bacteria. As an example, many different formulations exist for media to detect glucose fermentation, based on nutrient requirements of various groups of bacteria. Also, when running the standard test for oxygen relationship with Thioglycollate Medium, consider that (1) many organisms (including a lot of chemoheterotrophs!) cannot grow in this medium and (2) the medium does not allow for anaerobic growth which is due to phototrophy (more specifically, metabolism in the presence of light as performed by the non-oxygen-evolving photosynthetic bacteria) or anaerobic respiration (the use of alternate electron acceptors such as nitrate and sulfate).

In Bergey's Manual and other bacteriological texts, reference is made to the oxygen relationships of various bacteria – that is, how bacteria metabolize and replicate (if at all) in the presence or absence of oxygen.

Molecular oxygen (O_2) is the electron acceptor utilized by organisms which obtain energy from respiration (i.e., aerobic respiration). However, it does not function as such for organisms which obtain energy from fermentation, photosynthesis or anaerobic respiration.

For the following discussion, it may be helpful to review basic catabolic processes such as what is covered on this page. Also, a review of respiration vs. fermentation is given here.

I. In the most elementary sense, living organisms can be classified according to "oxygen relationships" as follows: (1) strict (or obligate) aerobes – those which require O_2, (2) strict (or obligate) anaerobes – those which can only grow in the absence of O_2, and (3) facultative anaerobes – those which can grow in the presence or absence of O_2. Practicing bacteriologists do not settle for this oversimplification as we see in the following two sections.

II. Bergey's Manual applies oxygen relationship categories to the chemotrophic bacteria, and the definitions which follow are taken verbatim from those in Bergey's Manual of Determinative Bacteriology (9th ed., 1994) except for the items in brackets:

[Strict or Obligate] Aerobe: An organism that is capable of using oxygen as a terminal electron acceptor [i.e., aerobic respiration], can tolerate a level of oxygen equivalent to or higher than that present in an air atmosphere (21% oxygen), and has a strictly respiratory type of metabolism. Some aerobes may also be capable of growing anaerobically with electron acceptors other than oxygen [i.e., anaerobic respiration].

Facultative anaerobe: An organism that can grow well both in the absence of oxygen and in the presence of a level of oxygen equivalent to that in an air atmosphere (21% oxygen). Some are capable of growing aerobically by respiring with oxygen and anaerobically by fermentation [anaerobic respiration is also possible]; others have a strictly fermentative type of metabolism and do not respire

with oxygen. [We form the "aerotolerant anaerobe" category with the latter type; see below.]

Microaerophile: An organism that is capable of oxygen-dependent growth but cannot grow in the presence of a level of oxygen equivalent to that present in an air atmosphere (21% oxygen). Oxygen-dependant growth [i.e., aerobic respiration] occurs only at low oxygen levels. In addition to being able to respire with oxygen, some microaerophiles may be capable of respiring anaerobically with electron acceptors other than oxygen.

[Strict or Obligate] Anaerobe: An organism that is incapable of oxygen-dependent growth and cannot grow in the presence of an oxygen concentration equivalent to that present in an air atmosphere (21% oxygen). Some anaerobes may have a fermentative type of metabolism; others may carry out anaerobic respiration in which a terminal electron acceptor other than oxygen is used. [The primary consideration for defining an organism as a strict anaerobe is its total intolerance of oxygen.]

With these Bergey's Manual definitions, phototrophs would be categorized with difficulty if at all. As one example, the purple non-sulfur photosynthetic bacteria can respire and can also grow anaerobically, but anaerobic growth is associated with the organisms' use of energy derived from light, not (except for certain exceptional strains and species) from fermentation or anaerobic respiration.

III. *Thioglycollate Medium* – which we utilize in our Bacteriology laboratory courses – is a "standard" medium for the determination of oxygen relationships, and it will support the growth of common, easily-grown chemoheterotrophic bacteria. The observed growth patterns of organisms in this medium determine their oxygen relationship designations (strict aerobe, facultative anaerobe, etc.) which correlate with such physiological abilities as respiration, fermentation and the catalase reaction and also whether there is an inhibitory effect on the organism in the presence of air. See the table under the photo below. Thus, a description of a chemoheterotrophic organism as a "strict aerobe" can imply a number of associated characteristics that may be unnecessary to specify separately (able to respire, unable to ferment, catalase-positive, azide-sensitive, etc.).

The amino acids and glucose in the medium can be respired, and glucose is the only fermentable energy source in the medium except for those exceptional organisms such as certain species of Clostridium which can ferment amino acids.

With Thioglycollate Medium, we are able to differentiate two distinct patterns of growth for those classified in the Bergey's Manual definitions (above) as "facultative anaerobes":

Those which are indifferent to oxygen and have a strictly fermentative type of metabolism grow evenly throughout the medium. We term such an organism an aerotolerant anaerobe and set that off as an additional category of oxygen relationship (added to the list of four above).

Those left in the facultative anaerobe category show greater concentration of growth at the top of the medium where oxygen is present and aerobic respiration is then possible. Comparing the degree of growth under aerobic vs. anaerobic

conditions can be a good demonstration of the relative efficiencies of aerobic respiration and fermentation when it comes to generation of cell mass.

The terms "facultative" and "aerotolerant" are always meant to modify another term such as "anaerobe" and they should not be used by themselves. Describing an organism as simply "facultative" may mean "facultative anaerobe," "facultative phototroph," or a variety of other things.

One must consider the following limitations of Thioglycollate Medium:

Many organisms (including a lot of chemoheterotrophs) cannot grow in this medium for one reason or another.

No allowance is given in the medium or method for anaerobic growth (1) with alternate electron acceptors (such as nitrate) or (2) in light (such as what is seen with the anoxygenic photosynthetic bacteria). Thus, an organism which may be termed a "strict anaerobe" in the more general sense – i.e., one which cannot tolerate oxygen and can only obtain energy by reactions which do not involve O_2 – would only show anaerobic growth in this test if it were capable of fermentation of the glucose in this medium.

The results in Thioglycollate Medium can be difficult to read. As shown in the table below, an organism's oxygen relationship designation can be determined by a combination of other methods which can be used as a check to see if the medium is showing the correct results – i.e., (1) testing for fermentation in Glucose Fermentation Broth, (2) performing the catalase test, and (3) testing if the organism can grow in the presence of oxygen. These methods tend to be quite reliable and can be utilized if Thioglycollate Medium is not available or even specified for use in the identification process. With that in mind, Thioglycollate Medium could be considered redundant.

The results we see in Thioglycollate Medium are shown below. (Note that microaerophiles are not included.) The accompanying table gives related information (fig. 119 and table 21).

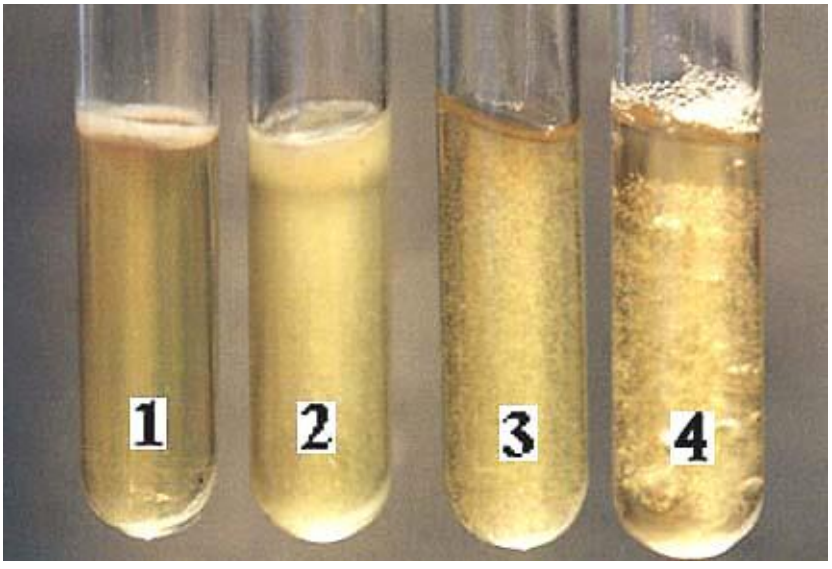


Figure 119. Bases of bacterial Identification

Table 21. Bacterial Identification

Corresponding tube no. above	1	2	3	4
Oxygen relationship designation	**STRICT (OBLIGATE) AEROBE**	**FACULTATIVE ANAEROBE**	**AEROTOLERANT ANAEROBE**	**STRICT (OBLIGATE) ANAEROBE**
Aerobic respiration*	+	+	–	–
Fermentation*	–	+	+	+
Ability to grow aerobically (oxygen tolerance)	+	+	+	–
Ability to grow anaerobically	–	+	+	+
Catalase reaction	+	+	–	–
Reaction in Glucose O/F Medium (for those able to grow well in medium)	O or –	F		
Response to sodium azide in a growth medium	**SENSITIVE**	**SENSITIVE** (under aerobic conditions)	**RESISTANT**	**RESISTANT**

* These are the basic things tested for in this medium. Whether or not any organism can obtain energy by anaerobic respiration or phototrophy is not relevant to these designations of oxygen relationships.

So, in becoming a practicing bacteriologist, one will see that there is more to this concept than whether bacteria simply "like" or "don't like" oxygen – which, unfortunately, is the extent to which oxygen relationships are too-often and unconscionably taught.

IV. Rather than (or in addition to) using "oxygen relationships" as descriptive terms – however they may be determined or defined – we can characterize and classify bacteria more consistently and comprehensively by applying the method(s) of energy generation of which an organism is capable:

aerobic respiration
anaerobic respiration
fermentation
anoxygenic phototrophy
oxygenic phototrophy

Remember that Thioglycollate Medium tests for an organism's ability to perform aerobic respiration and/or fermentation – the results of which give us the "oxygen relationship" categories for those organisms which can grow in the medium under the incubation conditions provided. Anaerobic growth in this medium is only associated with fermentation.

V. The following summary may help to explain how media formulations can allow anaerobic growth for organisms capable of doing so for one reason or another. The same organism – a typical strain of E. coli – was inoculated into tubes 1, 2 and 3, and a "facultative phototroph" was inoculated into tube 4.

In Tube #1, we have a medium containing peptone and agar plus other nutrients a "typical organism" (i.e., a commonly-found, easy-to-grow chemo- or photoheterotroph) might require for metabolism and replication – except that nothing is included which would support anaerobic growth such as glucose (or something else that could be fermented) or nitrate (or some other electron acceptor/"oxygen substitute" that could be used in anaerobic respiration). After inoculation of this medium and incubation in the dark, any growth would be due to aerobic respiration with the growth only at the top of the medium. There would be no anaerobic growth except for some rare, exceptional organisms which can ferment amino acids (fig. 120).

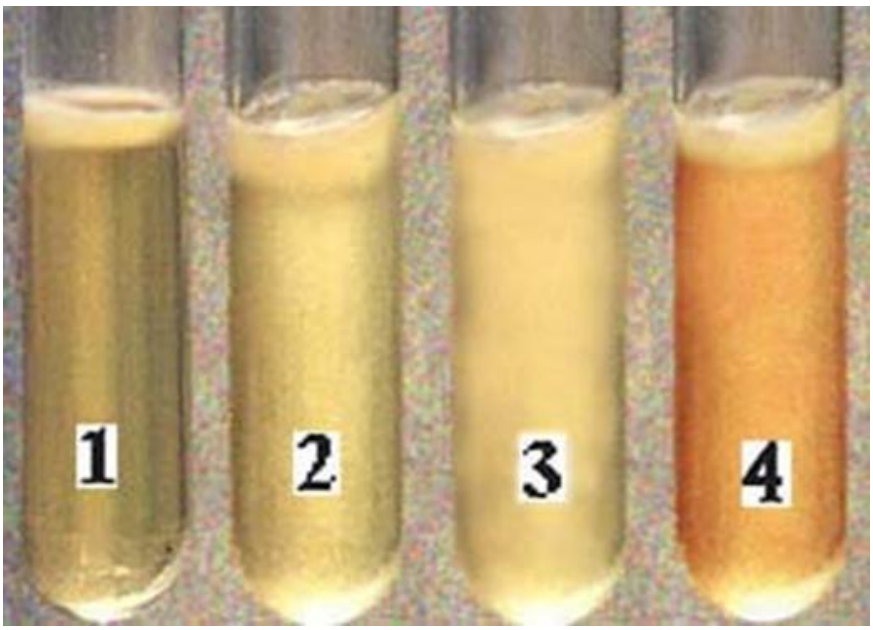


Figure 120. Thioglycollate Medium

Tube #2 is the same medium as in #1, but glucose has been added. After incubation (in the dark), any anaerobic growth would be due to fermentation of the glucose. Thus the medium can be used to detect whether or not an organism can respire (aerobically) or ferment. An example of such a medium is the Thioglycollate Medium we use to test common chemoheterotrophs for "oxygen relationships" (discussed above).

Tube #3 is the same medium as in #1, but potassium nitrate has been added. After incubation (in the dark), any anaerobic growth would be due to anaerobic respiration where the organism is using nitrate as the electron acceptor. In Bact. 102 (Exp. 7), we do a test in a broth medium for nitrate reduction; with reagents we can detect nitrite formation, and with the Durham tube we can detect N2 gas formation. One can probably see why we would not want to include nitrate in the Thioglycollate Medium above.

Tube #4 is the same medium as in #1, but we have incubated the tube in the presence of light. With light as the ultimate energy source, anaerobic growth would be due to anoxygenic phototrophy. This is the basis for the test we do in Bact. 102 (Exp. 11.1) to see if our isolates of purple non-sulfur bacteria are either "strict phototrophs" (just anaerobic growth in the light) or "facultative phototrophs" (anaerobic growth in the light, plus aerobic growth due to aerobic respiration whether in the dark or the light). Click here for a summary of this test.

Glucose Fermentation Broth and O/F Medium

First, a quick review of respiration vs. fermentation: we deal mostly with chemotrophic bacteria – primarily the chemoheterotrophs. Depending on the abilities of any specific chemotrophic organism and the environment in which it is found, the catabolic pathway is involved with either oxidative or substrate-level phosphorylation. If the former, the organism is obtaining energy by respiration; if the latter, the process is fermentation. Relative comparisons are made between respiration and fermentation in the following outline. (The three kinds of "phosphorylation" are diagrammed under respiration, fermentation and phototrophy on the catabolism page.)

Respiration:

There is a greater variety of potential substrates (amino acids, sugars, etc.). The substrate is more completely broken down than by fermentation.

A relatively smaller variety of end products is produced. A small amount of acidic intermediates can accumulate when respiring organisms catabolize sugars (such as for Pseudomonas species which utilize the Entner-Douderoff pathway). ATP is generated by oxidative phosphorylation wherein relatively more ATP is generated than by substrate-level phosphorylation, and oxygen is utilized as the terminal electron acceptor. Certain respiring organisms can use an alternate terminal electron acceptor such as nitrate under anaerobic conditions; this situation is termed anaerobic respiration. Relatively more cell mass is generated.

Fermentation:

A smaller variety of substrates can be fermented. Many organisms which can ferment sugars will not ferment amino acids. The substrate is less completely broken down. A relatively larger variety of end products is produced. Much acid (and possibly gas) is produced when sugars are fermented. ATP is generated by substrate-level phosphorylation wherein relatively less ATP is generated than by oxidative phosphorylation. Oxygen is not involved in the process. Relatively less cell mass is generated.

Both Glucose Fermentation Broth and Glucose O/F Medium include the following major ingredients:

Glucose – a sugar from which most common chemoheterotrophic bacteria can obtain energy – by fermentation and/or respiration. Glucose can also be utilized as a source of carbon, but these media include a large number of potential carbon sources (amino acids as well as glucose), and whether or not glucose is used as a carbon source cannot be directly determined from the reactions seen in these media.

Peptone – a commonly-used medium ingredient which mainly supplies amino acids (sources of nitrogen, carbon, sulfur and energy for many bacteria). It is a crude preparation of a partially-digested protein, and a peptone solution can serve as a complete medium for a number of organisms such as E. coli. If too much peptone (relative to glucose) is incorporated in the medium, detection of acidic products of fermentation or respiration may not be possible, as overabundance of ammonium (which is alkaline) released from the breakdown of amino acids can neutralize the acids.

pH indicator – the pH indicators employed in these media turn yellow under acidic conditions. Brom-cresol purple is in Glucose Fermentation Broth (which also contains the Durham tube), and brom-thymol blue is in Glucose O/F Medium.

Glucose Fermentation Broth

Testing whether an organism can ferment glucose is one of the basic, primary tests in the identification of chemoheterotrophic bacteria. For this test we routinely use a "Glucose Fermentation Broth."

Fermentation of glucose results in the abundant production of acidic end products, the presence of which can be detected by the pH indicator in the medium.

Many organisms produce gas – either CO_2 alone or a mixture of H_2 and CO_2. H2 is insoluble and is detected by bubble formation in a Durham tube placed in the medium.

Note the examples shown below.

Tube 1: No fermentation. The pH indicator remains purple. There can still be growth due to the use of amino acids as sources of energy (usually by respiration).

Tubes 2A and **2B**: Fermentation with the production of acid (yellow color) but no gas. A slight amount of acid is seen in tube 2A, but fermentation is still recorded for this tube.

Tubes 3A and **3B**: Fermentation with the production of acid (yellow color) and insoluble gas (bubble in Durham tube). Tube 3B shows an alkaline reaction on top; this is simply due to deamination of amino acids whose alkaline reaction has not been over-neutralized by the acid diffusing through the tube from fermentation (fig. 121).

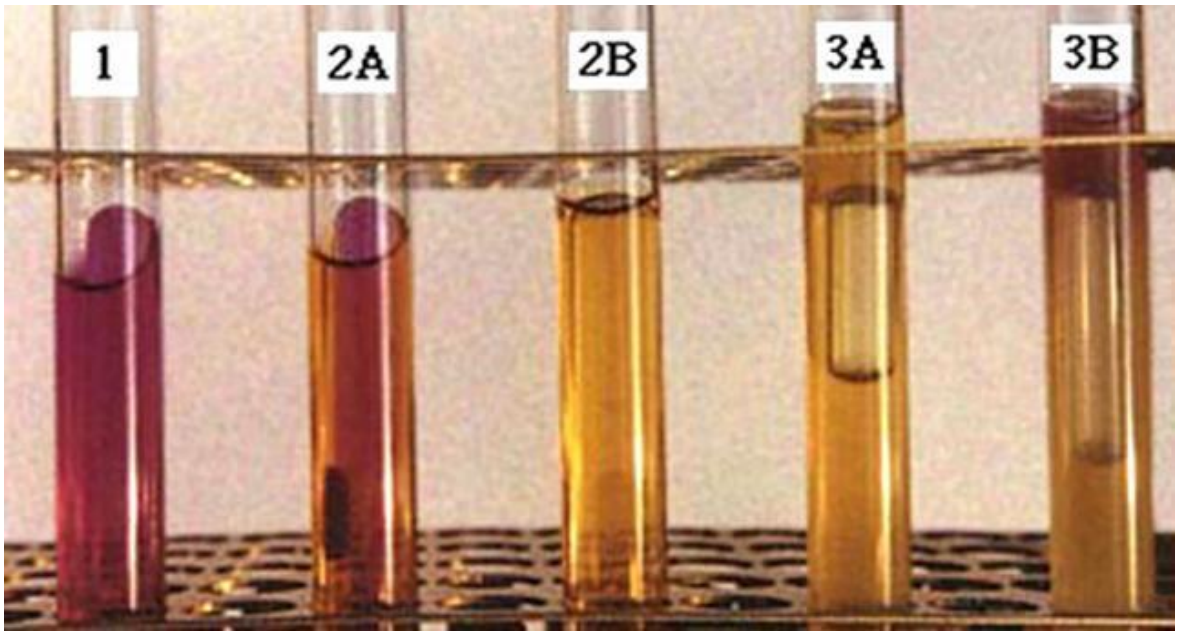


Figure 121. Glucose Fermentation Broth

Furthermore, with any of the reactions shown here, amino acids and/or glucose can be used as sources of carbon, but determination of what is or is not used as a carbon source cannot be made with this medium.

Glucose O/F Medium

The original intention of this medium was to be able to differentiate between gram-negative bacteria (1) that can ferment, (2) that only catabolize glucose by respiration and (3) that do not catabolize glucose at all. This differentiation is not as important in the identification of gram-positive bacteria, and it so happens that gram-positive bacteria do not grow in Glucose O/F Medium well (if at all) anyway – probably because of some sensitivity to the pH indicator. It is a waste of time and money to use this medium to characterize gram-positive cultures.

Whether an organism can respire or ferment glucose can be tested with Glucose O/F Medium. A small amount of acid production can be associated with glucose respiration. The original paper which describes the medium gives the example of the Entner-Douderoff pathway that is utilized by a variety of generally gram-negative bacteria (including Pseudomonas) to convert glucose to pyruvate – an alternative method of pyruvate formation to that of the Embden-Meyeroff pathway. (Pyruvate is further oxidized to CO2 in the aerobic respiration process.) Among the intermediates in the Entner-Douderoff pathway are forms of gluconic acid. So, where a strictly aerobic organism producing this acid is growing – i.e., at the top of the medium where O_2 is available – a net acidic reaction will be seen. However, this acidic reaction would be rendered indistinguishable if the organism were a facultative anaerobe – in which case the large amount of acid (produced by fermentation in the anaerobic environment of the tube) would be diffusing throughout the entire medium.

Duplicate tubes are inoculated for each organism, and the medium in one of the tubes is overlayed with mineral oil. Mineral oil does not in itself cause anaerobic

conditions but rather prevents oxygen from continuing to diffuse into the medium. After incubation, one looks for the presence and location of growth and acid.

It is important to emphasize that this medium contains relatively less peptone and more glucose than Glucose Fermentation Broth, so the acid associated with respiration can be detected in the aerobic part of the non-overlayed tube – if it is not made indistinguishable by acid production from fermentation which turns both tubes yellow throughout.

Note the examples shown below. For each pair of tubes, the tube on the right was overlayed with mineral oil after inoculation.

First pair of tubes: Tubes were inoculated with a strict aerobe which neither respires nor ferments glucose – therefore no acidic reaction. The blue alkaline reaction shows up where there is growth at the top of the "aerobic" tube. This is the negative reaction.

Second pair of tubes: Tubes were inoculated with a strict aerobe which respires but does not ferment glucose. The small amount of acid associated with respiration shows up where there is growth at the top of the "aerobic" tube. This is the "O" reaction, typical for most species of Pseudomonas. (The alkaline reaction from amino acid deamination is overneutralized.)

Third pair of tubes: Tubes were inoculated with a facultative anaerobe – i.e., one which can respire (with O_2) and ferment. Acid from the fermentation of glucose diffuses throughout both the "aerobic" and "anaerobic" tubes. This is the "F" reaction, typical of the enterics. (The alkaline reaction from amino acid deamination is overneutralized. Also, one cannot discern any acid production that might be associated with respiration.) – fig. 122 and table 22.

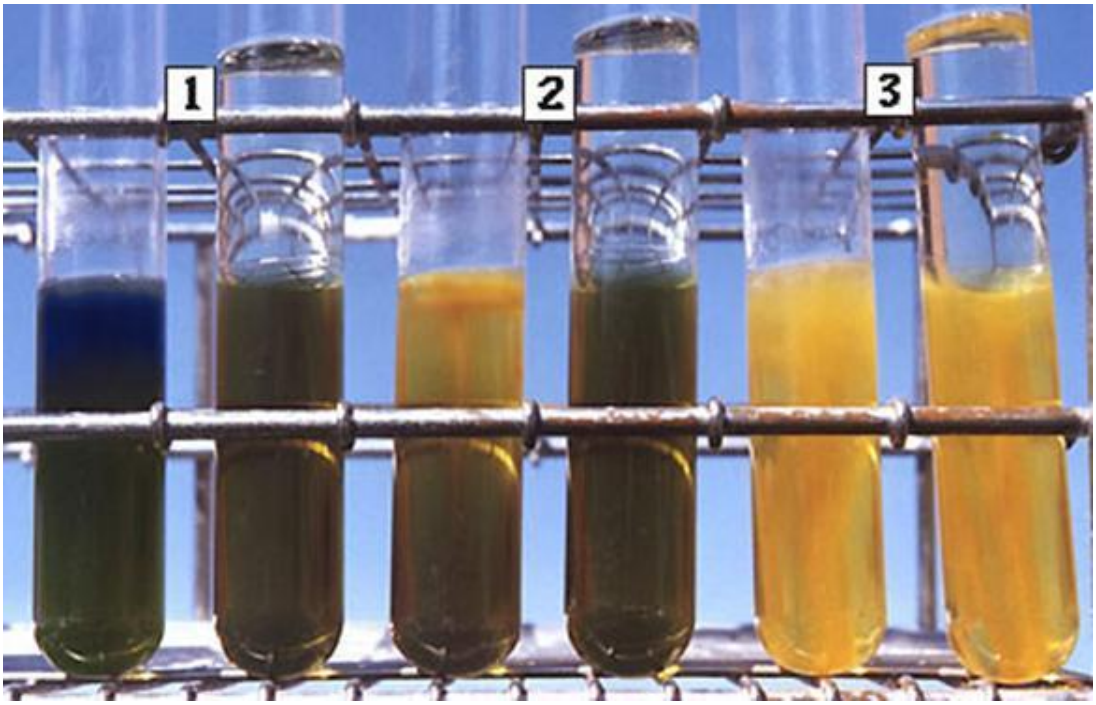


Figure 122. Glucose O/F Medium

Table 22. Glucose O/F Medium

Pair of Tubes	1st	2nd	3rd
Aerobic deamination of amino acids – not relevant to glucose catabolism (This occurs in upper part of open tube)	+ (Blue alkaline reaction seen; not over-neutralized by any acid production.)	+ (Alkaline reaction overneutralized by acid.)	+ (Alkaline reaction overneutralized by acid.)
Respiration of glucose (Acid seen in upper part of open tube)	–	+	This cannot be discerned due to the high amount of acid produced from fermentation.
Fermentation of glucose (Acid diffuses throughout both tubes)	–	–	+
Reaction recorded	Negative	O	F

Additional comparisons between these two media are here

The following examples show the behavior of four organisms in both Glucose Fermentation Broth and Glucose O/F Medium. The organisms are gram-negative except Staphylococcus epidermidis which grows weakly in Glucose O/F Medium.

"EC" = Escherichia coli
"PF" = Pseudomonas fluorescens
"AF" = Alcaligenes faecalis
"SE" = Staphylococcus epidermidis
"1" = Glucose O/F Medium
"2" = same with mineral oil overlay
"3" = Glucose Fermentation Broth

For certain groups of bacteria, different formulations of Glucose Fermentation Broth are employed which satisfy special growth requirements. For example, clinical streptococci and dairy lactobacilli require a much richer basal medium than that provided by peptone. Likewise, a variation of Glucose O/F Medium is used for the characterization of Staphylococcus and Micrococcus. Bergey's Manual and other reference books give more specific information.

As genotypic characterization (determination of the DNA and RNA characteristics of our bacteria) is becoming more widely practiced, we may soon be back to one standard of characterizing and identifying bacteria. This time it will be universally applicable as all bacterial genera and species become uniformly defined according to genotypic uniqueness. We hope that the results of the phenotypic tests we run will correlate with the genotypic characteristics and bring about accurate and useful identification of our organisms.

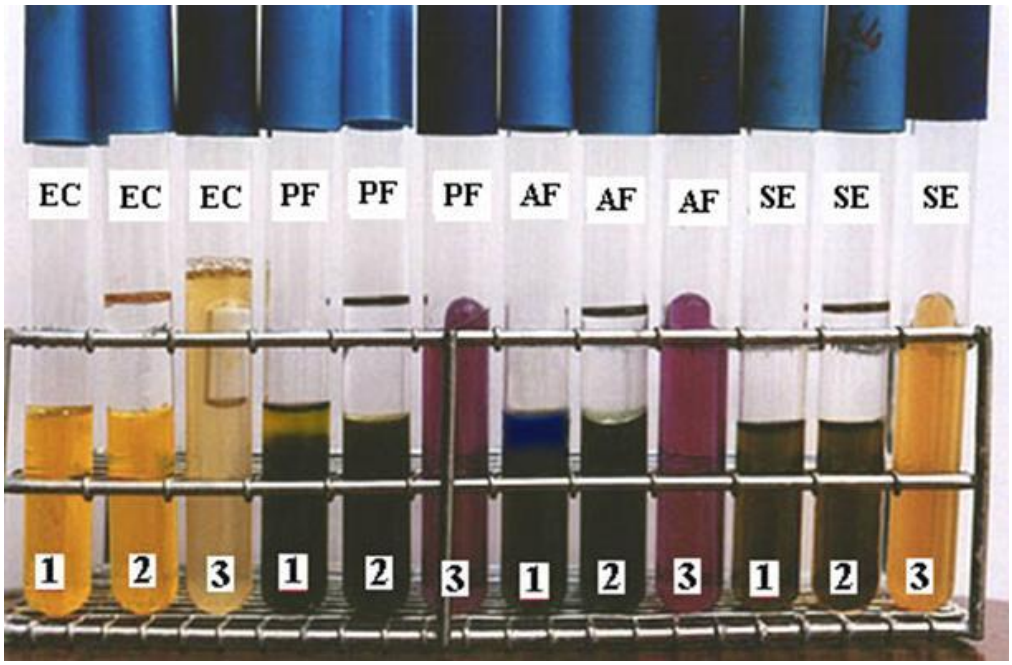


Figure 123. Glucose O/F Medium

Table 23. Glucose O/F Medium

Reaction types	Escherichia coli	Pseudomonas fluorescens	Alcaligenes faecalis	Staphylococcus epidermidis
Organism ferments glucose (and can grow anaerobically as a result)	+ (Also produces H_2 and CO_2)	–	–	+
Acidic reaction from fermentation seen throughout both media	+ (Note gas in Durham tube)	–	–	(Weak growth in Glucose O/F Medium)
Organism respires glucose	+	+	–	+
Acidic reaction from respiration seen in open Glucose O/F Medium tube	Can't tell due to acid from fermentation permeating medium	+	– (Alkaline reaction from peptone becomes apparent)	Can't tell due to acid from fermentation permeating medium
Reaction recorded for Glucose O/F Medium	**F**	**O**	–	(weak F)

In the table below, a few commonly-found and easily-grown chemoheterotrophic genera are sorted out based on various "primary tests" which include the use of Both Glucose Fermentation Broth and Glucose O/F Medium include. Thebenzidine test which has been used effectively for the presence of iron-porphyrin compounds such as cytochromes and the true catalase enzyme. Some organisms possess the enzyme cytochrome a_3 oxidase as part of the electron transport system in respiration; this enzyme is responsible for a positive reaction in the oxidase test where the dye tetramethyl-p-phenylenediamine is reduced to a purple compound.Further tests (not indicated) would then be done to determine positively the genus identification and also the likely species. You can go where the experts are and consult the latest editions of Bergey's Manual of Systematic Bacteriology andBergey's Manual of Determinative Bacteriology for more information. Bergey's Manual of Systematic Bacteriology is a multi-volume set, and the first volume of the new, 2nd edition is out now but may not be specifically helpful for the organisms listed in the table below. Bergey's Manual of Determinative Bacteriology is mainly used for identification, but the present 9th edition has become quite dated in that respect.The idea for the format of the following table comes from the classic Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd edition, revised by S. T. Cowan (1974, Cambridge University Press). This table of often-isolated chemoheterotrophic bacteria was put as a guide in targeting likely names of genera to pin on the "nature isolates." An X marks the place where a certain pattern of characteristics matches up with a possible genus. Considering additional characteristics of the isolate, one can consult Bergey's Manual or The Prokaryotes for this genus and related genera (on nearby pages) for a more definitive identification (table 24).

Table 24. Definitive identification of bacteria

Gram reaction (young culture)	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
shape	coccus (clusters)	coccus (clusters)	coccus (chains)	coccus (tetrads)	rod	rod	irreg. rod	rod	rod	rod	rod	rod	rod	rod	rod	coccus (pairs)
aerobic growth	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
anaerobic growth	–	+	+	+	+	–	–	+	+	–	–	–	+	+	+	–
endospores	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
motility (Motility Medium)	–	–	–	–	–	+	–	+ or –	+ or –	+ or –	+ or –	+ or –	–	+	+	–
catalase reaction	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+	+
benzidine reaction	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+	+
oxidase reaction	+	–	–	–	–	–	–	–	+ or –	+ or –	+	+	–	–	+	+
glucose fermentation to acid **or** to acid+gas	–	+	+	+	+	–	–	+ (or –)	+	–	–	–	+	+	+	–
Glucose O/F Medium											–	O	F	F	F	O
Micrococcus	**X**															
Staphylococcus		**X**														
Streptococcus			**X**													
Lactococcus			**X**													
Enterococcus			**X**													
Leuconostoc			**X**													
Pediococcus			**X**	**X**												
Aerococcus				**X**												
Lactobacillus					**X**											
Kurthia						**X**										
Arthrobacter							**X**									
Clostridium								**X**								
Bacillus									**X**	**X**						
Alcaligenes											**X**					
Pseudomonas												**X**				
Klebsiella													**X**			
Shigella													**X**			
Salmonella														**X**		
Escherichia														**X**		
most other enteric genera														**X**		
Aeromonas															**X**	
Chromobacterium															**X**	
Neisseria																**X**

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Methods of examination of bacterial susceptibility to antibiotics. The main principles of rational antibiotic therapy of diseases.

Various chemical substances comparatively harmless for the macroorganism but with a lethal action on pathogenic micro-organisms are widely used in medical practice for treating patients with infectious diseases and in some cases for prophylaxis.

This method was known long ago to ancient people, and was used for treating certain diseases. The Peruvian Indians discovered the therapeutic action of cinchona bark, and in the 18th century cinchona bark was brought to Europe. The inhabitants of Brazil successfully employed the root of the ipecacuanha for treating amoebiasis. Mercury has been extensively employed in the therapy of syphilis. In the middle of the 16^{th} century this method became known to the people of Europe.

The basis of modem chemotherapy was founded by P. Ehrlich and D. Romanowsky, who formulated the main scientific principles and the essence of chemotherapy. They showed that in the treatment of each infection a substance should be found which, during injection into the diseased body, will bring the least harm to it and cause the most destructive action to the pathogenic (causative) agent. P. Ehrlich devised the principles of synthesis of medicinal substances by chemical variations: methylene blue, derivatives of arsenic–salvarsan ("'606"), neosalvarsan ("914"). By the further development of chemistry new medicinal preparations could be obtained.

Extensive experimental and clinical tests of chemopreparations were carried out by E Metchnikoff.

Chemopreparations should have a specific action, a maximal therapeutic effectiveness, and a minimal toxicity for the body.

As a characteristic of the quality of a medicinal preparation, P. Ehrlich introduced the *chemotherapeutic index* which is the ratio of the maximal tolerated dose to the minimal curative dose:

$$\frac{\textbf{maximal tolerated dose (DT—Dosis tolerata)}}{\textbf{minimal curative dose (DC—Dosis curativa)}} > 3$$

The chemotherapeutic index should not be less than 3 Chemotherapeutic preparations include a number of compounds used in medicine

Arsenic preparations (novarsenol, myarsenol, aminarsone, osarsol, etc.) are administered in syphilis, relapsing fever, trypanosomiasis, amoebiasis, balantidiasis, anthrax, sodoku, and other diseases.

Bismuth preparations (basic bismuth nitrate, xeroform, basic bismuth salicylate, bioquinol, bismoverol, bithiurol, pentabismol, etc.) are used against enterocolitis and syphilis

Antimony compounds (tartaric antimony potassium salt, stibenil, stibozan, surmine, solusurmine, etc.) are used for treating patients with leishmaniasis and venereal lymphogranulomatosis.

Mercury preparations (mercury salicylate, mercuric iodide, mercury cyanide, calomel, unguentum hydrargyri cinereum containing metallic mercury, etc.) are prescribed for treating patients with syphilis and are used as antiseptics in pyogenic diseases.

Acridine preparations (rivanol, tripaflavine, acriflavine, acricide, flavicide, etc.) are recommended for pyogenic diseases and inflammatory processes of the pharynx and nasopharynx

Antimalarial substances include more than 30 preparations, e g , chinine hydrochloride, quinine sulphate, mepacrine (acrichine), rodochin (plasmocide), proguanyl (bigumal), pyrimethamine (chloridine), resochine, quinocide sulphones and sulphonamides, sulphadiamine, etc.

Alkaloid preparations (emetine, etc.) are used for treating patients with amoebiasis.

Sulphonamide preparations. The introduction into practice of compounds of the sulphonamide group (streptocid, ethasole, norsulphazol, sulphazine, methylsulphazine, sulphadimezin, urosulphan, phthalazole, sulgine, sulphacyi, soluble sulphacyl, disulphormin, etc.) marked a revolution in the chemotherapy of bacterial infections.

Sulphonamide preparations are used for treating pyogenic diseases, tonsillitis, scarlet fever, erysipelas, pneumonia, dysentery, anaerobic infections, gonorrhoea, cystitis, venereal lymphogranulomatosis, psittacosis, ornithosis, trachoma, blennorrhoea in the newborn, etc.

There are several points of view concerning the mechanism of action of sulphonamides on microbes.

Antibiotics (Fr. *anti* against, *bios* life) are chemical substances excreted by some micro-organisms which inhibit the growth and development of other microbes (in recent years several antibiotics have been obtained semisynthetically) – fig. 124.

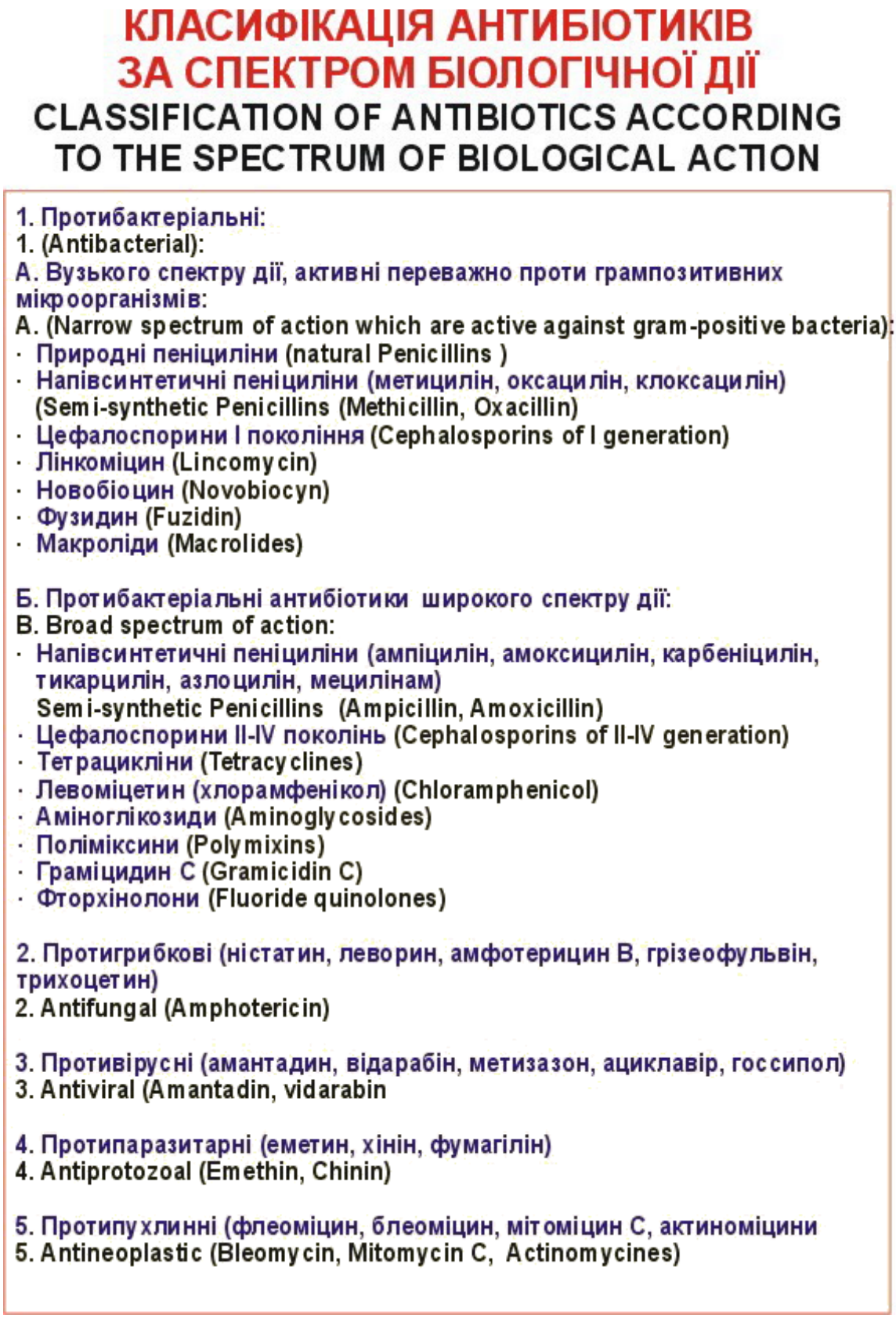

Figure 124. Classification of antibiotics

Antibiotics are obtained by special methods employed m the medical industry For the production of antibiotics strains of fungi, actmomycetes, and bacteria are

used, which are seeded in a nutrient substrate After a definite growth period the antibiotic is extracted, purified and concentrated, checked for inocuousness and potency of action In composition a number of antibiotics (penicillin, streptomycin, gramicidin, etc) have optically distorted molecules The antibacterial properties of some antibiotics are associated with optical inversion of their molecules which have the same physicochemical properties as normal molecules and can easily be bound to the enzyme Since they lack the ability to participate in biochemical reactions, this binding is accompanied by a blockade of enzymes, and consequently, a growth inhibition followed by death of the microorganism.

According to the character of action, antibiotics are subdivided into bacteriostatic (tetracyclines, chloramphenicol, and others) and bactericidal (penicillines, ristomycin, and others). Each antibiotic is characterized by a specific antimicrobial spectrum of action. Some antibiotics are inactivated in the presence of animal and plant proteins. Only a few antibiotics have a powerful antibacterial action, which does not decrease in the presence of protein matter of animal tissues and at the same time is not toxic (in certain concentrations) for the human being.

The mechanism of action of antibiotics varies. Penicillin inhibits the synthesis of polymers of the bacterial cell wall (it hinders the use of muramic acid by bacteria), which leads to an increase of cells incapable of multiplication. Sometimes the action of penicillin leads to the formation of L-forms in the shape of pleomorphic protoplasmic structures. Thus, penicillin has a lethal effect not on the given population, but on its off-spring. The selective action of penicillin on microbes hinders the penetration of glutamic and other amino acids through the cytoplasmic membrane of pathogenic cocci unable to synthesize amino acids which are vitally important for the existence of these bacteria. Penicillin inhibits the ability of the bacterial cell to absorb protein components — aminoacids, and it inhibits the synthesis of the enzyme system and also of adaptive enzymes (fig. 125).

Streptomycin inhibits the incorporation of some amino acids in protein synthesis and attacks the bacterial enzyme with the participation of which the introduction of pyruvic acid into the tricarbonic acid cycle by its union with oxalacetic acid takes place. This antibiotic inhibits the activity of biotin-containing enzymes catalysing the union of carbon dioxide with carbonic acids; it disturbs reading of the genetic code and synthesizes leucine instead of alanine.

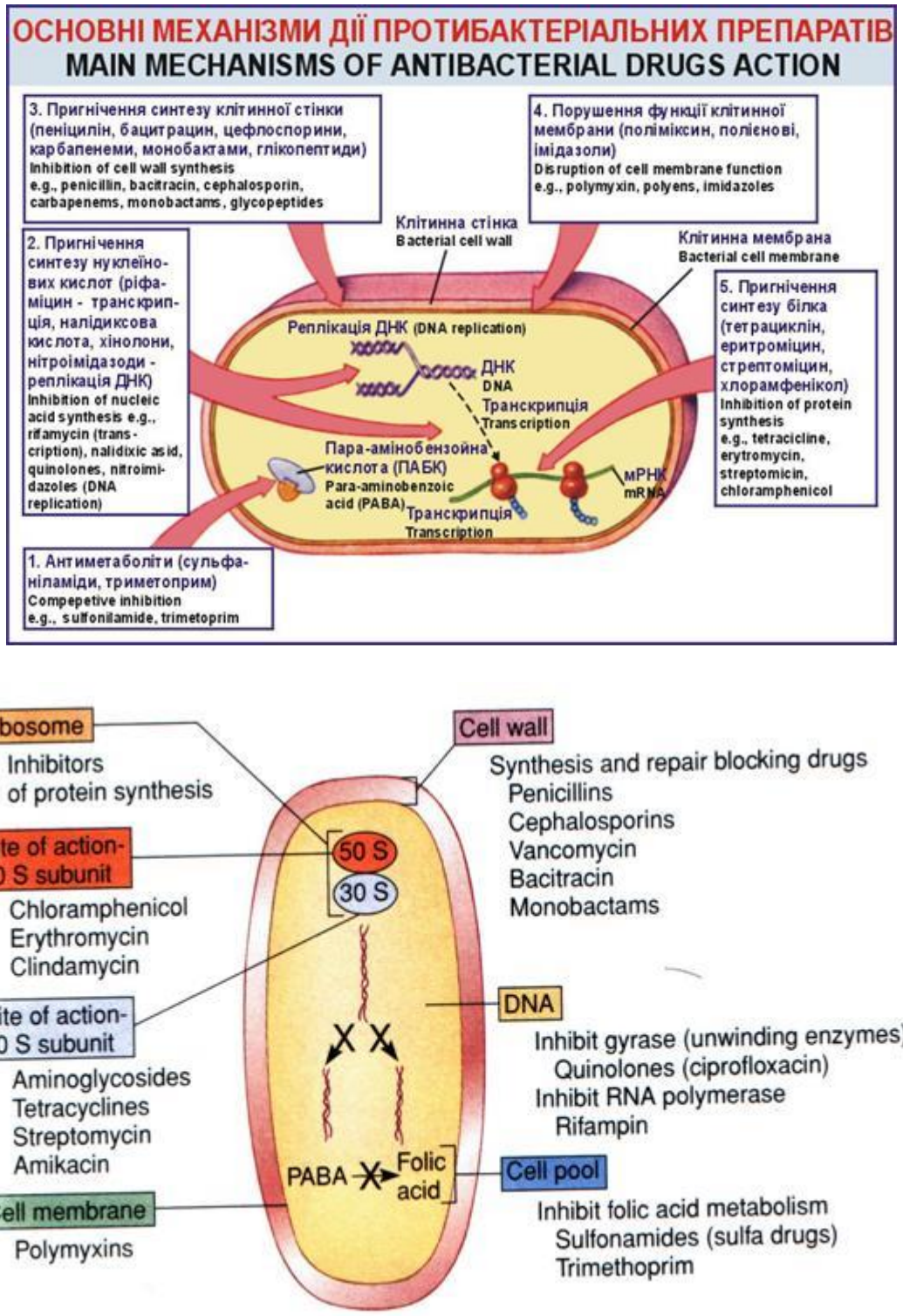

Figure 125. Main mechanisms of antibacterial drugs action

Of special interest is the mechanism of action of streptomycin on tubercle bacilli. This preparation does not have a sterilizing action, but inhibits the respiration of tubercle bacilli, which leads to the inhibition of cell reproduction and toxin formation. At the same time stimulation of tissue respiration occurs in the patient as well as an increase in the ability of the macro-organism to destroy tubercle bacilli and their toxins.

The selective action of streptomycin on the tubercle bacillus is due to the fact that the permeability of cell membranes in the bacilli and in the tissue cells of animals and man differs due to the dissimilar chemical structure of the cytoplasm of these organisms.

There are data showing that streptomycin inhibits the capacity of bacterial cells of the colibacillus to oxidize fumaric and glutamic acids. This leads to an inhibition of adaptive enzyme production.

Chloramphenicol is a specific inhibitor of the biosynthesis of bacterial protein. It comes into action with the peptidyl transferase area of 50S ribosome. Competing with the aminoacyl end of the aminoacyl tRNA, chloramphenicol blocks the formation of the peptide bond.

Tetracyclines, lincomycin, erythromycin, kanamycin, neomycin, spectinomycin, sparsomycin, fucidine and others belong to the group of antibiotics which inhibit protein biosynthesis in bacteria at the ribosome level. The antibiotic rifampicin suppresses protein biosynthesis by inhibiting the activity of RNA polymerase.

Antifungal antibiotics impair the intactness of the cytoplasmic membrane in fungi; antineoplastic antibiotics suppress the synthesis of nucleic acids in bacterial and animal cells and bind with DNA which serves as the matrix for RNA synthesis; bruneomycin leads to sharp inhibition of the synthesis of DNA or to its destruction.

There are various hypotheses and theories which have not entirely revealed the mechanism of action of antibiotics, and this question has not been completely solved.

The activity of antibiotics is expressed in international units (IU). Thus, for example, 1 IU of penicillin (Oxford unit) is the smallest amount of preparation inhibiting the growth of a standard *Staphylococcus aureus* strain. Recently the method of determining the activity of antibiotics according to the weight of the preparation has received wide application.

One unit of activity (AU) corresponds to the activity of 0.6 micrograms (ug) of. the chemically pure crystalline sodium salt of benzylpenicillin. Consequently, in 1 mg of sodium salt of benzylpenicillin there may be 1667 AU, and in 1 mg of potassium salt — 1600 AU. For practical purposes both preparations are manufactured with an activity not less than 1550 AU.

The concentration of dry preparations as well as of solutions is expressed as the number of micrograms of active substance in 1 g of preparation or in 1 mg of solution. Antibiotics are classified according to the chemical structure of the dmg, the molecular mechanism, and the spectrum of activity exerted on the cells.

According to origin, antibiotics are subdivided into the following groups (fig. 126).

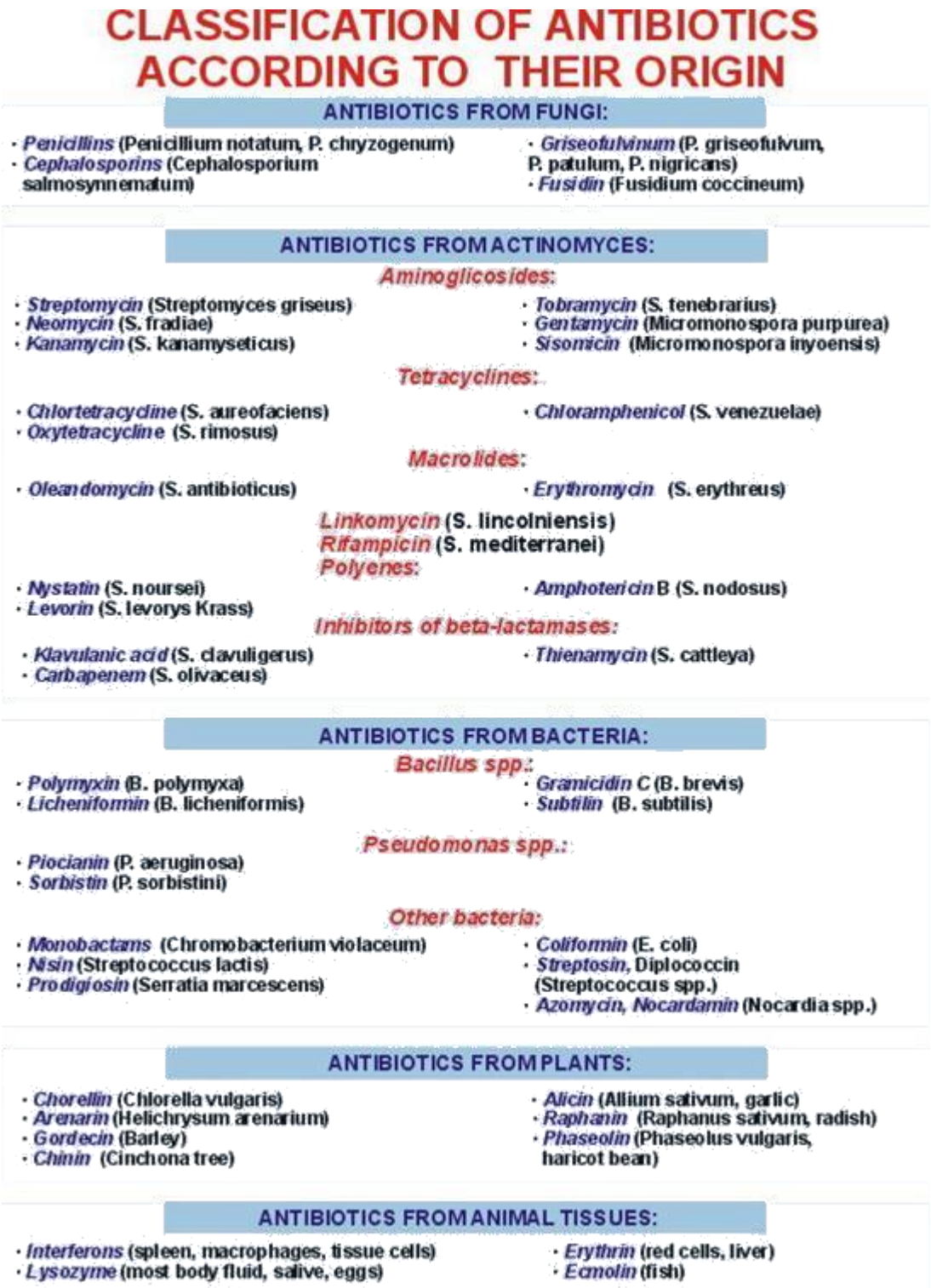

Figure 126. Classification of antibiotics according to their origin

Antibiotics produced by fungi. Penicillin is produced by the fungi *Penicillium notatum*, *Penicillium chrysogenum*, etc. Penicillin is produced as sodium and potassium salts. It dissolves readily in water, but its solutions are not stable. It is a dipeptide consisting of dimethylcysteine and acetylserine.

Penicillin is used in staphylococcal, streptococcal, and meningococcal infections, anaerobic infections, gonorrhoea, syphilis, leptospirosis, anthrax and other diseases.

Penicillin preparations include ecmonovocillin which is a form of long-acting penicillin, maintaining the necessary therapeutic concentration of penicillin in the blood. It is used only for intramuscular injections. The indications are the same as for the application of penicillin.

Semisynthetic penicillins (methicillin, oxacillin) are used in infection with penicillin-resistant staphylococci; ampicillin is prescribed in mixed infections. Resistance, however, develops faster to semisynthetic preparations than to natural ones. Novobiocin and ristomycin cause a favourable therapeutic effect.

Antibiotics produced by actinomycetes. 1. *Streptomycin* is obtained from *Streptomyces griseus*. Chemically it consists of two components: the nitrous base of streptidin and streptobiosamine. Streptomycin is a base and forms salts with acids, which readily dissolve in water and are insoluble in organic solvents. It has a bacteriostatic property in relation to Gram-negative as well as to Gram-positive pathogenic microbes.

Streptomycin has a good therapeutic action on tuberculosis, tuberculous meningitis, plague, brucellosis, tularaemia, whooping cough, etc.

2. *Chloramphenicol* is obtained from the cultural fluid of a strain of *Streptomyces venezuelae,* isolated from the soil in tropical South America. It has a good therapeutic effect during dysentery, enteric fever, typhus fever and other rickettsioses.

3. *Chlortetracycline* (biomycin, aureomycin) is produced by *Streptomyces aureofaciens.* It is employed during staphylococcal infections, pneumonia, subacute septic endocarditis, rickettsioses, amoebiasis, dysentery, whooping cough, gonorrhoea, brucellosis, tularaemia, trachoma, psittacosis, peritonitis, surgical sepsis and other diseases.

4. *Tetracycline* is a derivative of chlortetracycline. It is obtained by reductive dechlorination of chlortetracycline. Tetracycline has a wide spectrum of action, it inhibits many species of Gram-positive, Gram-negative and acid-fast microbes. It also inhibits the development of many rickettsiae and some protozoa. It is used in treating patients with cholera, pneumonia, subacute septic endocarditis, amoebiasis, dysentery, whooping cough, gonorrhoea, in diseases of the urogenital tract, typhus fever and other rickettsioses, and for the prevention of suppurative processes in surgery. Tetracycline hydrochloride is manufactured in the form of pills with pure tetracycline or in combination with nystatin.

5. *Oxytetracycline* (terramycin) is obtained from *Streptomyces rimosus.* In spectrum and mode of action it is close to chlortetracycline. Rondomycin (6-methyl-5-hydroxytetracycline) is a homologue of oxytetracycline. It is absorbed rapidly. Rondomycin possesses a broad spectrum of action (suppresses Gram-positive and Gram-negative bacteria, i. e. cocci, *Salmonella* organisms, *Shigella* organisms, pathogenic *E. coli* serotypes) and is administered per os.

6. *Erythromycin* is obtained from *Streptomyces erythraeus.* It is administered in streptococcal diseases. In experiments on animals it has proved to be effective in diseases caused by Gram-positive and Gram-negative bacteria, rickettsiae, chlamydias, intestinal amoebae and trichomonads. Diphtheria bacilli are quite sensitive to erythromycin

7. *Neomycin* has been isolated from *Streptomyces fradiae.* It has a bacteriostatic action against Gram-negative and Gram-positive bacteria. The preparation is slightly toxic. It is prescribed mainly for the local treatment of suppurative processes, caused by staphylococci which are resistant to penicillin and to other antibiotics, and also during colienteritis, the causative agents of which are the pathogenic serotypes of *E. coli.*

8. *Nystatin* has been extracted from the cultural fluid of *Streptomyces noursei.* It inhibits many pathogenic fungi and some pathogenic protozoa. It is non-toxic when used per os. It has received wide application in treatment of candidiasis.

9. *Kanamycin* is an antibiotic produced by *Streptomyces kanamycetius.* In mode of action it resembles streptomycin and neomycin. It inhibits the growth of Gram-positive and Gram-negative bacteria. Kanamycin is used for treating patients with tuberculosis in whom the causative agent became resistant to antituberculous chemopreparations and antibiotics. It is prescribed for treating anthrax, gonorrhoea and for acute and chronic forms of infections of the urinary tract, and diseases caused by resistant strains of staphylococci.

Cycloserine obtained from *Streptomyces lavendulae* and other actinomycetes belongs to this group of antibiotics. It produces a beneficial therapeutic effect in tuberculosis; it disturbs the synthesis of the cell wall of mycobacteria and other Gram-positive micro-organisms. Oleandomycin obtained from *Streptomyces antibioticus* culture fluid inhibits the vital activity of Gram-positive bacteria, *Mycobactenum tuberculosis*, rickettsia, and *Chlamidobacteriales* organisms. Levorin produced by *Actinomyces levoris* is employed for treating superficial and deep candidiases.

Amphotericin (A and B) are antimycotic antibiotics obtained from *Streptomyces nodosum.* They are effective against yeast-like fungi, pathogens of deep and systemic mycosis, particularly, histoplasmosis, chromomycosis, sporotrichosis (fig. 127).

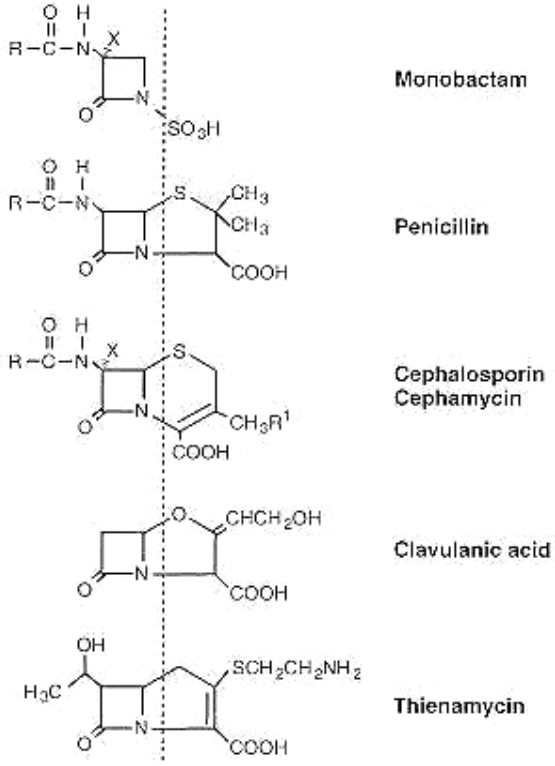

Figure 127. Chemical structure of differens antibiotics

Antibiotics produced by bacteria.

1. *Gramicidin* isolated from a culture of *B. brevis* has a bacteriostatic and bactericidal action on some pyogenic cocci.

2. *Polymyxins* are produced by *Bac. polymyxa*. They are prescribed in diseases caused by Gram-negative bacteria.

Semisynthetic antibiotics. This group includes some penicillins obtained on the basis of6-aminopenicillmic acid, the nucleus of penicillin (methicillin, oxacillin, dioxacillm, ampicillin, etc.). – fig. 128 -130.

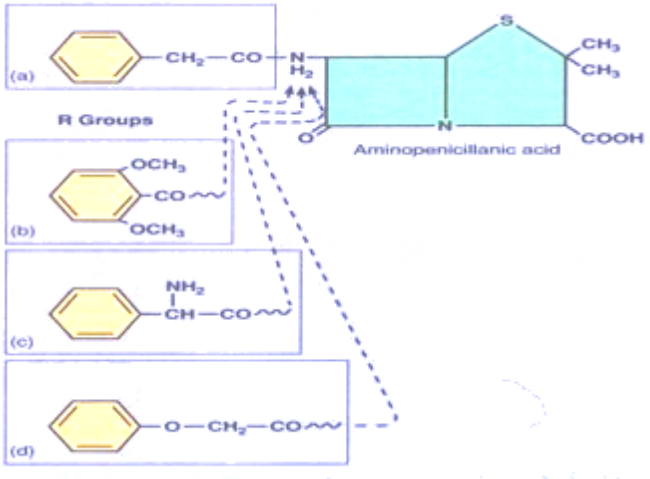

Figure 128. Semisynthetic penicillins

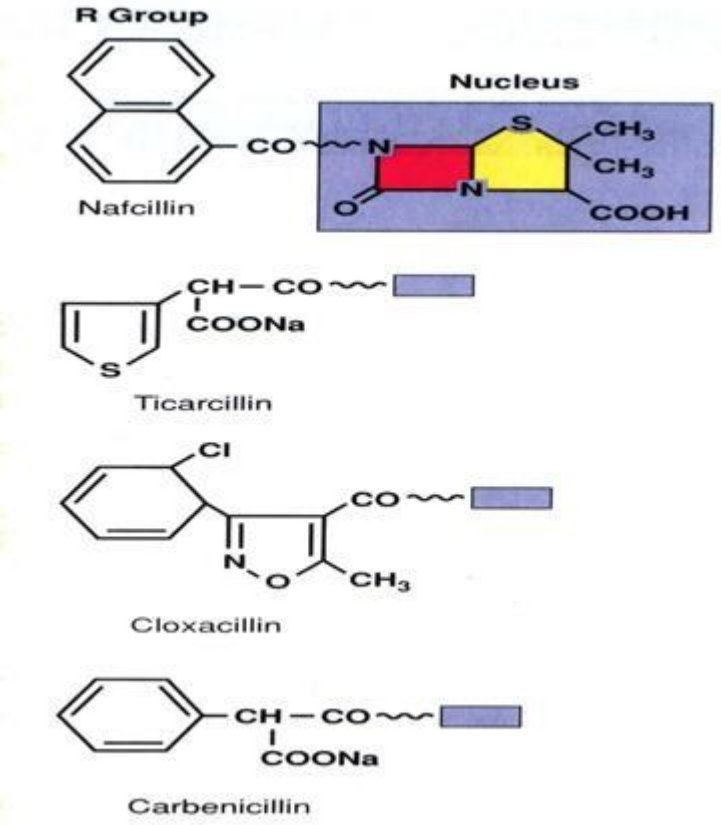

Figure 129. Semisynthetic penicillins

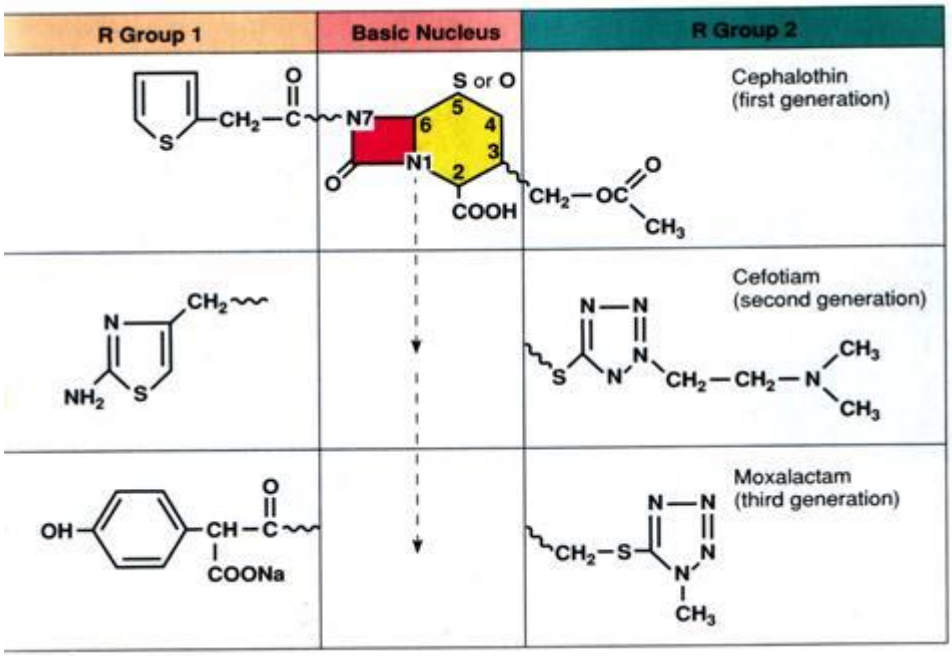

Figure 130. Semisynthetic cephalosporins

The antibiotic levomycetin (an analogue of natural chloramphemcol) is obtained by synthesis. Combined preparations have also been produced on a mass scale, e. g. vitacycline (tetracycline with vitamins C, B(and B, and some others). New medicinal forms of tetracyclines having weaker side effects have been devised.

Methods of examination of antibiotic susceptibility

Method of serial dilutions in a liquid medium. Hottinger's broth (or another medium suitable for the growth of the given micro-organism) is poured by 2-ml portions into test tubes mounted in a tube rack by ten in each row. Prepare antibiotic solution containing 100 U per ml and add 2 ml of this solution into the first test tube. Following thorough mixing, transfer with a new sterile measuring pipette 2 ml of the culture from this tube into the next one, and so on until the ninth tube is reached, from which 2 ml is poured off. The tenth tube containing no antibiotic serves as a control of culture growth.

Wash the 24-hour agar culture of the studied microorganism with isotonic sodium chloride solution, determine the density of the suspension by the turbidity standard, and dilute to a concentration of 10000 microorganisms per ml. A sample of 0,2 ml of the obtained suspension is inoculated into all tubes of the row beginning from the control one. Thus, all tubes contain 1000 microorganisms per 1 ml. The results of the experiment are read following incubation of the tube at 37 °C for 18-20 hrs. The minimal concentration of the antibiotic suppressing the growth of the given microorganism is determined by the last test tube with a transparent broth in the presence of an intensive growth in the control one.

One may also prepare antibiotic solution in molten nutrient agar to subsequently streak the tested culture onto the surface of this medium.

Another approach to antimicrobial susceptibility testing is the determination of the minimum inhibitory concentration (MIC) that will prevent microbial growth (fig. 3). The MIC is the lowest concentration of antimicrobic that prevents the growth of a microorganism in vitro.

The minimum inhibitory concentration indicates the minimal concentration of the antibiotic that must be achieved at the site of infection to inhibit the growth of the microorganism being tested. By knowing the MIC and the theoretical levels of the antibiotic that may be achieved in body fluids, such as blood and urine, the physician can select the appropriate antibiotic, the dosage schedule, and the route of administration. Generally, a margin of safety of 10 times the MIC is desirable to ensure successful treatment of the disease.

MIC is not designed to determine whether the antibiotic is microbicidal. It is, however, also possible to determine the minimal bactericidal concentration (MBC). The MBC is also known as the minimal lethal concentration (MLC) (fig. 3). The minimal bactericidal concentration is the lowest concentration of an antibiotic that will kill a defined proportion of viable organisms in a bacterial suspension during a specified period of exposure. Generally, a 99.9% kill of bacteria at an initial concentration of 10^5-10^6cells/mL during a 17- to 24-hour exposure period is used to define the MBC.

To determine the minimal bactericidal concentration, it is necessary to plate the tube suspensions showing no growth in tube dilution (MIC) tests onto an agar growth medium. This is done to determine whether the bacteria are indeed killed or whether they survive exposure to the antibiotic at the concentration being tested.

Determination of the MIC is adequate for establishing the appropriate concentration of an antibiotic that should be administered for controlling the infection in patients with normal immune response levels. Determination of the MBC is essential for patients with endocarditis (inflammation of the endocardium or lining to the heart) because the patient's immune response cannot be relied on to remove the infecting microorganisms. It is particularly useful in determining the appropriate concentration of an antibiotic for use in treating patients with low erred immune defense responses, such as may occur in patients receiving chemotherapy treatment in cancer (fig. 131).

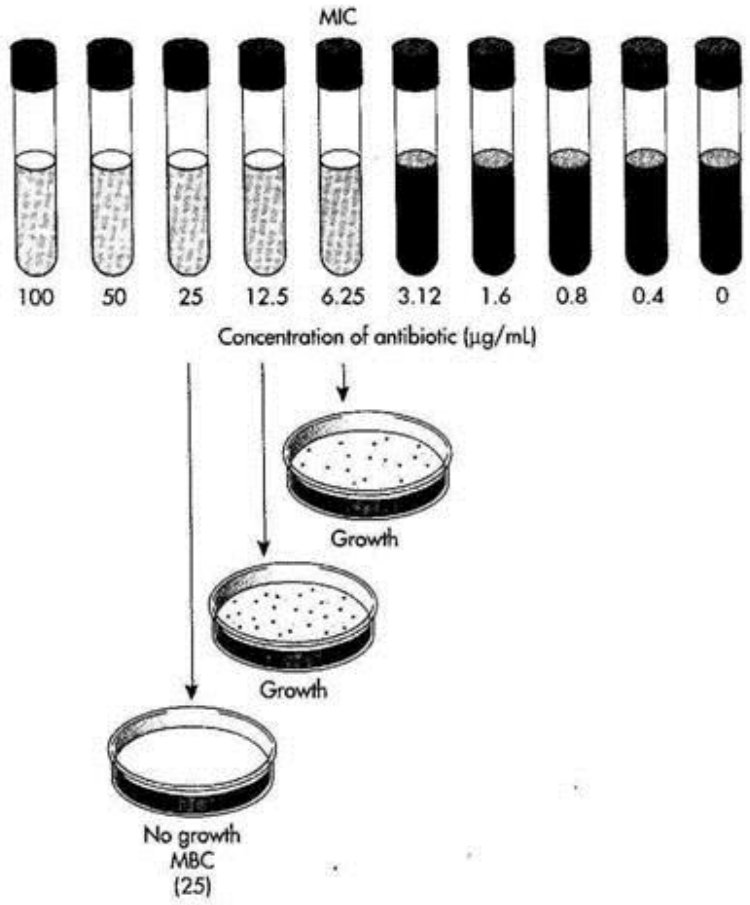

Figure 131. The minimum bactericidal concentration (MBC) of an antibiotic requires the demonstration that microorganisms have lost the ability to reproduce. In this example, although cell growth is inhibited at concentrations of 100 and 6,25 (MIC – 6,25 mg/vL), viable cells remain, which is shown by the formation of colonies (growth) on an agar plate lacking the antimicrobic No vable cells are detected (no growth on agar plates) at 25 mg/mL, which therefore is the MBC.

Disk diffusion technique. Into sterile Petri dishes placed on a horizontal surface, pour 15 ml of solid nutrient medium (most often *2* per cent agar on Hottinger's broth containing 0.11-0.13 per cent of amine nitrogen). On the surface of solidified and slightly dried agar, pour 1 ml of suspension of 24-hour culture of the causative agent or, if no pure culture has been isolated, of the pathological material (pus, exudate) obtained for the study and diluted with isotonic saline. Spread uniformly over the agar surface the bacterial suspension, removing its remainder with a Pasteur pipette. Disks with antibiotics (5-6 disks per plate) are placed onto the surface of the inoculated plate at a distance of 25 mm from its centre. The plates are incubated at 37 °C for 16-18 hrs, after which the results of the test are read by measuring the zones of growth retardation of microorganisms around the disks, including the diameter of the disk itself (fig.132). The size of the zones depends on the degree ofsensitivity of the causative agent to a given antibiotic (tabl. 25). Yet, this method cannot be considered strictly quantitative.

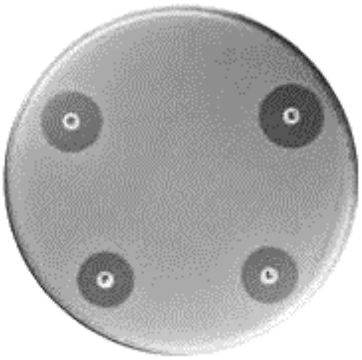

Figure 132. Testing sensitivity of bacteria to antibiotics by the "disk method"

Table 25. Zone Size Interpretive

Antimicrobial agent	Disc code	R = mm or less	I = mm	MS = mm	S = mm or more
Amikacin	AN-30	15	15-16	-	16
Amoxicillin/ clavulanic acid – staphylococci	AmC-30	19	-	-	20
Amoxicillin/ clavulanic acid - other organisms	AmC-30	13	14-17	-	18
Ampicillin – staphylococci	AM-10	28	-	-	29
Ampicillin - G- enterics	AM-10	11	12-13	-	14
Azlocillin	AZ-75	14	15-17	-	13
Aztreonam	ATM-30	15	-	16-21	22

Antimicrobial agent	Disc code	R = mm or less	I = mm	MS = mm	S = mm or more
Carbenicillin – Enterobacteriaceae	CB-100	17	18-22	-	23
Carbenicillin – *Pseudomonas*	CB-100	13	14-16	-	17
Cefamandole	MA-30	14	15-17	-	18
Cefazolin	CZ-30	14	15-17	-	18
Cefonicid	CID-30	14	15-17	-	18
Cefoperazone	CFP-75	15	-	16-20	21
Cefotaxime	CTX-30	14	-	15-22	23
Cefotetan	CTT-30	12	-	13-15	16
Cefoxitin	FOX-30	13	-	15-17	18
Ceftazidime	CAZ-30	14	15-17	-	18
Ceftizoxime – *Pseudomonas*	ZOX-30	10	-	11	-
Ceftizoxime - other organisms	ZOX-30	14	-	15-19	20
Ceftriaxone	CRO-30	13	-	14-20	21
Cefuroxime	CXM-30	14	15-17	-	18
Cephalothin	CF-30	14	15-17	-	18
Chloramphenicol	C-30	12	13-17	-	18
Cinoxacin	CIN-100	14	15-18	-	19
Ciprofloxacin	CIP-5	15	16-20	-	21
Clindamycin	CC-2	14	15-20	-	21
Doxycycline	D-30	12	13-15	-	16
Erythromycin	E-15	13	14-22	-	23
Gentamicin	GM-10	12	13-14	-	15
Imipenem	IPM-10	13	14-5	-	16
Kanamycin	K-30	13	14-17	-	18
Methicillin – staphylococci	DP-5	9	10-13	-	14
Mezlocillin	MZ-75	12	13-15	-	16
Minocycline	MI-30	14	15-18	-	19
Moxalactam	MOX-30	14	-	15-22	23
Nafcillin – staphylococci	NF-1	10	11-12	-	13
nalidixic acid	NA-30	13	14-18	-	19
Netilmicin	NET-30	12	13-14	-	17
Nitrofurantoin	F/M-300	14	15-16	-	17
Norfloxacin	NOR-10	12	13-16	-	17

Antimicrobial agent	Disc code	R = mm or less	I = mm	MS = mm	S = mm or more
Oxacillin – staphylococci	OX-1	10	11-12	-	13
Penicillin	P-10	28	-	-	29
Streptomycin	S-10	11	12-14	-	15
Sulfamethoxazole + trimethoprim	SXT	10	11-15	-	16
Tetracycline	Te-30	14	15-18	-	19
Ticarcillin	TIC-75	11	12-14	-	15
ticarcillin/clavulanic acid	TIM-85	11	12-14	-	15
Tobramycin	NN-10	12	13-14	-	15
Trimethoprim	TMP-5	10	11-15	-	16
Vancomycin	Va-30	9	10-11		12

Resistance of microbes to antibiotics. With the extensive use of antibiotics in medical practice, many species of pathogenic micro-organisms became resistant to them.

Resistance may develop to one or simultaneously to more antibiotics (multiple resistance).

The molecular mechanism of the production of resistance to antibiotics is determined by genes localized in the bacterial nucleoids or in the plasmids, the cytoplasmic transmissible genetic structures.

Resistance to antibiotics occurs as the result of disturbed translation of genetic information and altered synthesis of the polypeptide chain, diminished permeability of the cytoplasmic membrane and cell wall, and the formation, due to the effect of R-plasmids, of enzymes inactivating antibiotics (ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, etc.).

Mutations according to the nucleoid genes, leading to antibiotic resistance, form with a frequency of 10^{-6} to 10^{-12}. Owing to this, the occurrence of simultaneous mutations to two and more antibiotics is excluded; they may develop, however, as the result of independent mutation in a strain primarily resistant to one of the antibiotics.

Resistance to penicillin is linked with penicillinase (B-lactamase) synthesis controlled by one of the genes of the R-factor. Penicillinases are synthesized under the effect of not only the R-factor genes but also the nucleoid genes. Resistance to chloramphenicol is determined by the action of the enzyme — chloramphenicol acetyl-phenicolacetyl transferase coded by the gene of the R-factor. Five enzymes are responsible for the resistance to antibiotics of the aminoglycoside group. Inactivation of antibiotics in the R+ strains with multiple resistance is accomplished by three types of reactions, phosphorylation, acetylation, and adenylation. It has been established that a bacterial cell may be resistant to more than one antibiotic by one gene.(Fig. 133, 134).

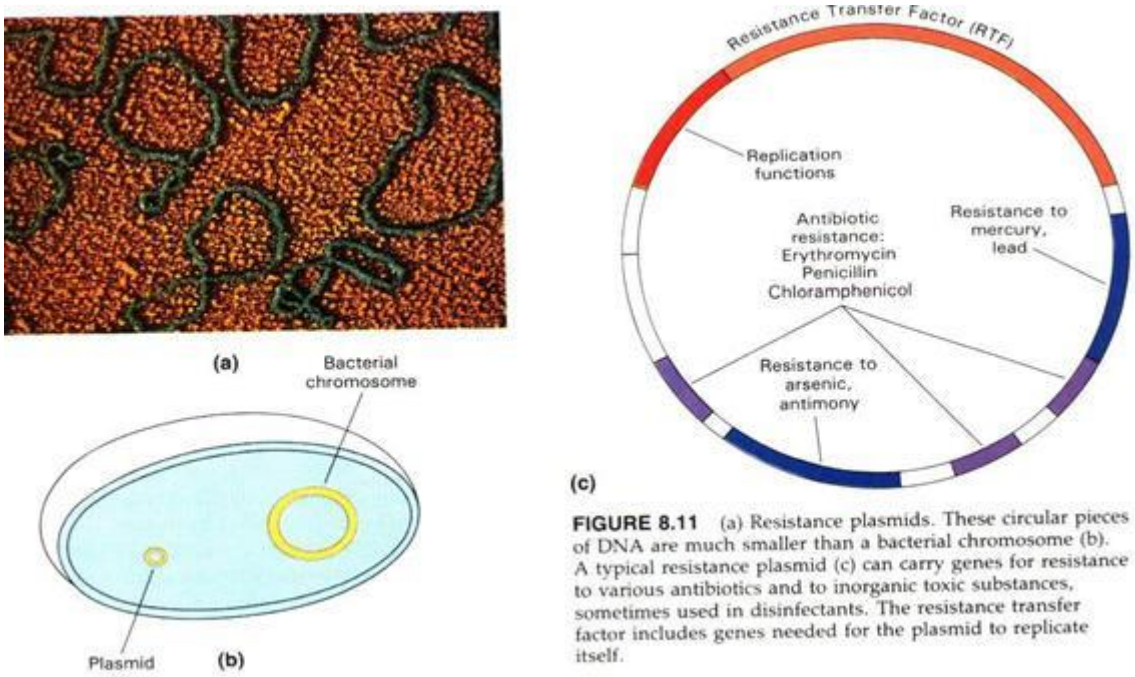

FIGURE 8.11 (a) Resistance plasmids. These circular pieces of DNA are much smaller than a bacterial chromosome (b). A typical resistance plasmid (c) can carry genes for resistance to various antibiotics and to inorganic toxic substances, sometimes used in disinfectants. The resistance transfer factor includes genes needed for the plasmid to replicate itself.

Figure 133. Resistance plasmids (R-factors)

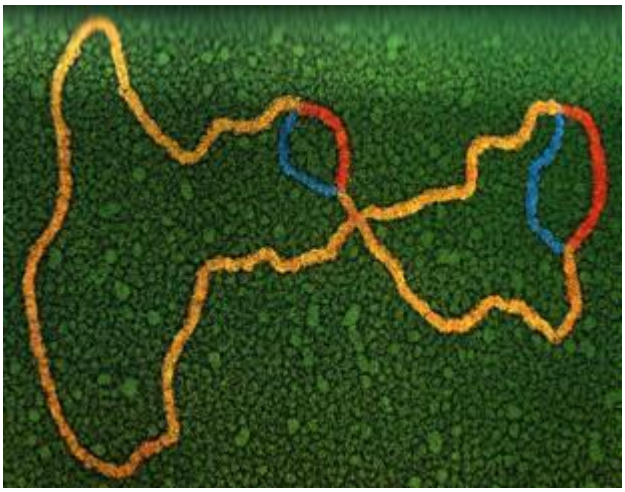

Figure 134. R plasmid and genes of resistance

Due account is given in medical practice to cross-resistance to antibacterial agents which have the same chemical structure. It has been found to exist between preparations of the tetracycline series and new semisynthetic antibiotics (morphocycline, glycocycline, dibiomycin, and ditetracycline), preparations of penicillin (benzylpenicillin, phenoxymethylpenicillin, ephycillin), compounds of the nitrofuran group, and between sulphanilamides.

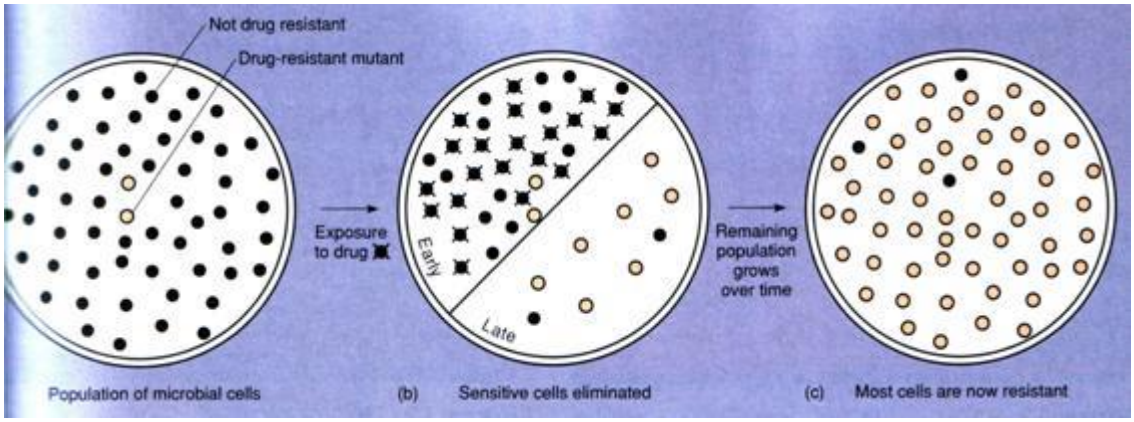

Figure 135. Origin of resistent forms

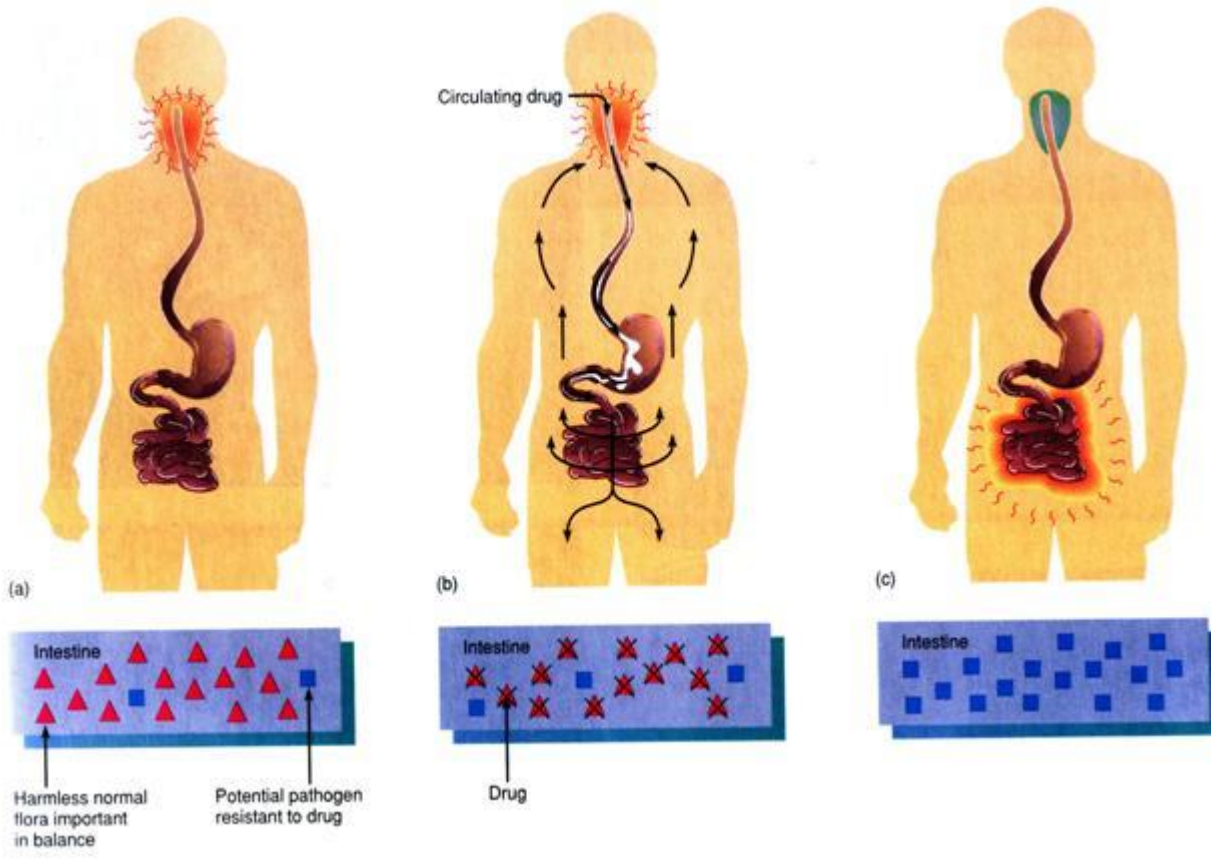

Figure 136. Result of medical treatment of angina by antibiotics

Due to the wide distribution of staphylococci resistant to antibiotics a search for new preparations became necessary. At present a semisynthetic staphylococcal penicillin has been obtained which has a distinct bacteriostatic action on resistant strains of pathogenic staphylococci With the isolation of the penicillin nucleus, 6-aminopenicillinic acid (6APA), it became possible to obtain various derivatives of penicillin.

Dimethylchlorotetracycline from the group of tetracyclines is used for the treatment of many infectious diseases and in doses half as strong as tetracycline. A

good result has been obtained in treatment of inflammatory processes of the urinary tract.

With the discovery of the antibiotic griseofulvin dermatology was enriched with an effective preparation with the help of which diseases of the skin, hair and nails caused by fungi imperfecti could be treated.

Some antibiotics have a poisonous effect on rats, insects and mites. They are used for exterminating rodents and arthropods, the vectors of infectious diseases.

Antibiotics (kormogrisin, chlortetracycline, etc.) stimulate the growth of animals and fowl, and are therefore widely used in agriculture.

Of interest is the very difficult problem of chemotherapy of viral diseases. At present there are no effective drugs against viral infections. This is due to the biological peculiarities of viruses as obligatory intracellular parasites, which must be acted upon by other means than those used in microbial diseases. In recent years many new antibiotics have been obtained which have a good effect in the treatment of murine leucoses. Some of them are employed successfully in agriculture for treating fowl leucoses. Antitumour antibiotics include actinomycins C, D, K, F, etc., carcinophilin, mytomycin, actinoxanthin, chrysomalin, aurantin, sarcomycin.

Side effects of antibiotics. It has been established that large doses of penicillin and streptomycin have a neurotoxic action, tetracyclines affect the liver, chloromycetin has a toxic effect on the haematopoietic organs, and chlortetracycline and oxytetracycline upon intravenous injection may lead to collapse with a lethal outcome.

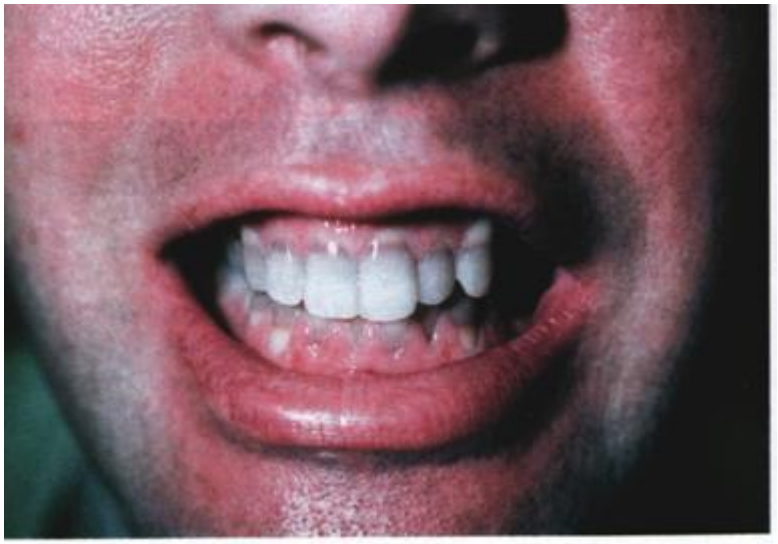

Figure 137. Effect of tetracyclin on decolorization dental enamel

Upon injection of penicillin and streptomycin a rash, contact dermatitis, angioneurotic oedema, anaphylactic reactions or allergic asthma may occur. Quite frequently allergic reactions arise during local application of antibiotics. Of the most practical importance is their indirect action which is mainly due to the development of resistant strains of micro-organisms, sometimes causing furuncles or severe generalized diseases which develop vigorously, in some cases with a lethal outcome. In case of the application of antibiotics with a wide spectrum of action infections may

develop which are caused by resistant strains of *Proteus* and fungi. Staphylococcal colitis proceeds very severely, and is characterized by profuse diarrhoea, dehydration of the body, toxic phenomena, shock and collapse.

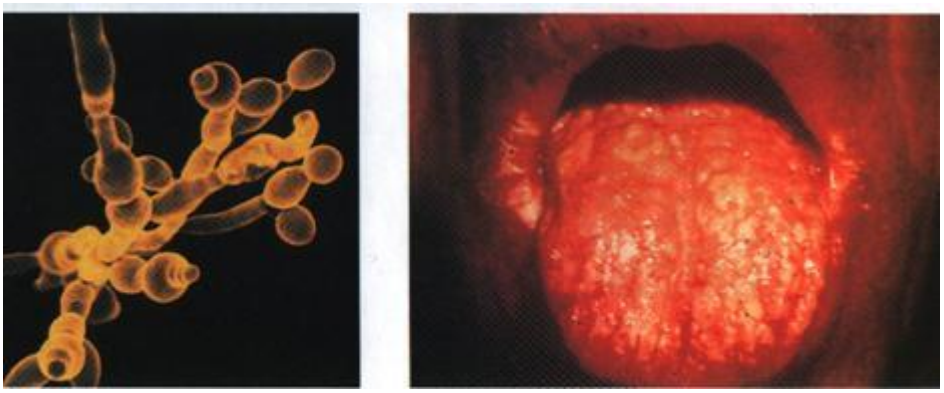

Figure 138. Candidosis

Of great hazard is the formation of resistant staphylococci which cause various postoperative complications — persistent furunculosis and staphylococcal septicaemias.

A severe complication is anaphylactic shock from the use of penicillin in which a rapid drop in blood pressure, cyanosis, superficial breathing, loss of consciousness, and convulsions are observed, and in some cases death occurs. Complications caused by penicillin are characterized by allergic reactions and proceed according to the serum sickness type.

In prolonged use of penicillin or levomycetin (in syphilis and enteric fever) collapse is one of the severe side effects.

Contact dermatitis is an allergic reaction of a medicinal origin. This disease is caused by the action of streptomycin in medical personnel and patients using this preparation over long periods. Quite often allergic manifestations are recorded in the mucous membranes such as hyperaemia and oedema of the pharynx and tongue. In children antibiotics with a wide spectrum of action cause perianal skin hyperaemia, and hyperaemia of the rectal mucosa (fig 139-140).

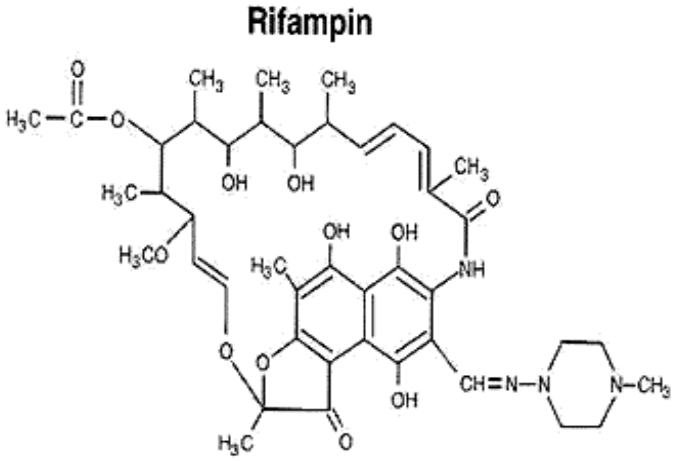

Figure 139. Formula of rifampin

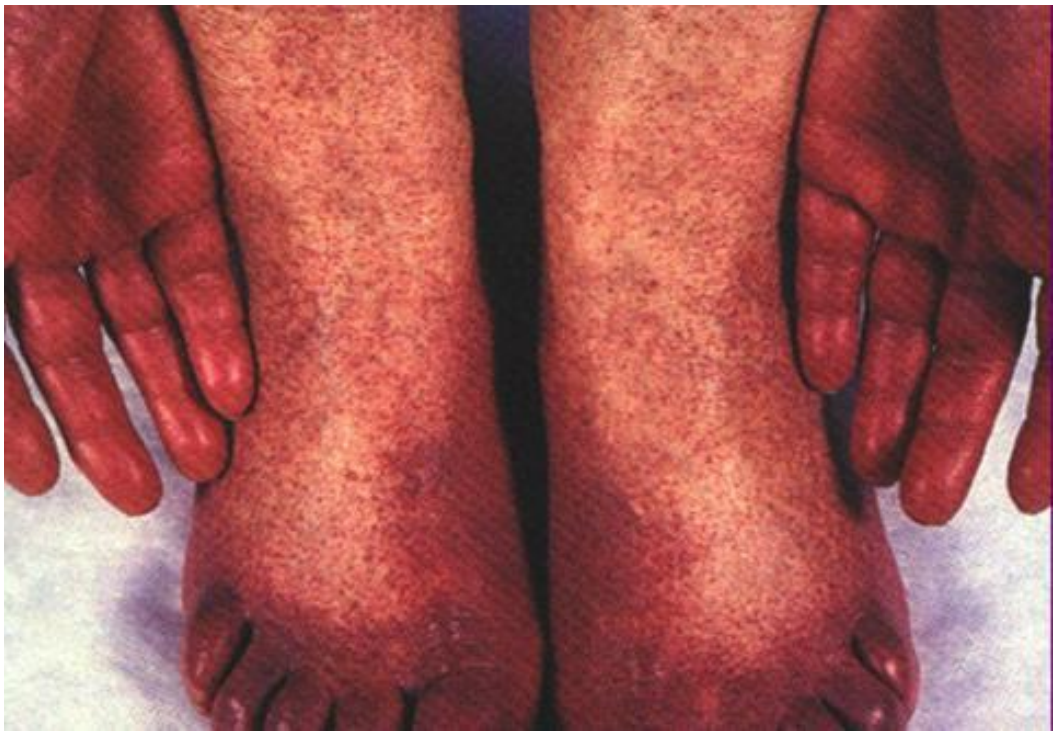

Figure 140. Side effect after the reception of rifampin

In some countries the number of cases of infection with staphylococcal pneumonia in children has increased. It has been suggested that this can be explained partly by the origin of penicillin-resistant strains of staphylococci. The disease has the tendency of becoming complicated with abscesses, empyema, pneumothorax and the formation of cysts.

Antimicrobial agents cause the formation of numerous variants of microbes with weak pathogenicity (atypical strains, filterable forms, L-forms) which lead to the formation of latent forms of infections marked by recurrences and exacerbations.

Antibacterial agents may induce disorders of the genetic apparatus of the macro-organism's cells and cause chromosomal aberrations; some of them possess a teratogenic effect leading to the development of foetal monstrosities if they are taken in the first days of pregnancy.

Due to the wide distribution of antibiotic-resistant pathogenic bacteria, combined treatment is recommended with the use of new antibiotics to which the causative agents of infectious diseases have yet not developed resistance. To prevent the development of resistant forms of microbes, combined preparations are prescribed: penicillin and streptomycin, erythromycin and oxytetracycline, etc.

Combined action of antibiotics

Tetracycline with nystatine are applied for the prevention of candidiasis.

The use of preparations which block selectively R-plasmid replication and those which promote the elimination of antibiotic modifying enzymes is believed to be promising in the control of multiple antibiotic-resistance.

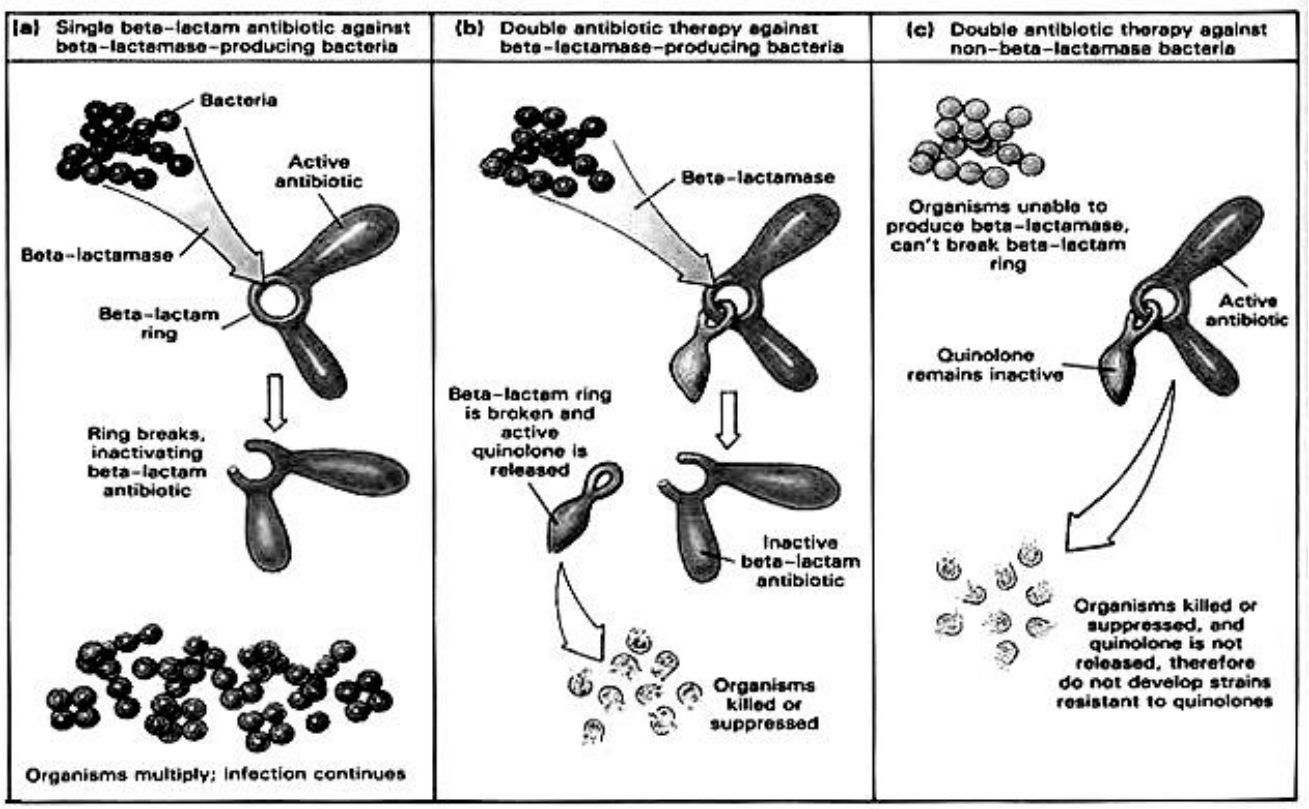

Figure 141. Action of antibiotics

Protein and DNA Sequencing

In the last 25 years, molecular biology has developed rapidly and it is now possible to sequence the proteins from different bacterial species, make large databases of the sequences, and use them as very powerful identification tools. Similar database have been developed for bacterial DNA and bacterial RNA, particularly the RNA that forms the structural components of bacterial ribosomes.

Such techniques are also being used to follow the development of strains of bacterial species that are currently evolving at a very rapid rate. Strains of Chlamydia trachomatis, for example, are known to be exchanging large numbers of genes, forming completely new strains in a very short time. This is worrying – this bacterium is responsible for taking the sight of 8 million people living in developing countries today. Identifying the new strains and studying how they have arisen so quickly is crucial to controlling infection and preventing new cases of blindness.

There are a few basic things regarding 16S ribosomal RNA gene analysis. The actual mechanics of the various parts of this test can be found elsewhere on the web or in an up-to-date textbook, and they may be summarized here in the future. With this comparative test, differences in the DNA base sequences between different organisms can be determined quantitatively, such that a **phylogenetic tree** can be constructed to illustrate probable evolutionary relatedness between the organisms.

The nucleotide base sequence of the gene which codes for 16S ribosomal RNA is becoming an important standard for the definition of bacterial species. Comparisons of the sequence between different species suggest the degree to which

they are related to each other; a relatively greater or lesser difference between two species suggests a relatively earlier or later time in which they shared a common ancestor.

A comparison between eleven species of gram-negative bacteria is illustrated on a separate sequence comparison page, where the sequences are aligned such that similarities and differences can be readily seen when one scrolls to the right or left. Gaps and insertions of nucleic acid bases (the result of "frame-shift" mutations occuring over eons of time as the organisms diverge from common ancestors) which affect long stretches of DNA have to be taken into account for a proper alignment.

In an earlier version of the above-mentioned sequence comparison page, when only four species were compared with each other, a relatively short segment stood out as appearing to be "frame-shifted" when comparing *Pseudomonas fluorescens* with a group of three enterics. This situation is shown as follows with the nucleotide bases of the segment in question shown in red.

Table 26. Protein and DNA Sequencing

Pseudomonas fluorescens	...gctaataccgcatacgtcctacgggagaaagcagggg...
Our new organism, shown below as "AH"	...gctaataccgcataacgtcgcaagaccaaagcggggg...
Budvicia aquatica	...gctaataccgcgtaacgtcgaaagaccaaagcggggg...
Edwardsiella tarda	...gctaataccgcataacgtcgcaagaccaaagtggggg...

One can surmise that a frame-shift mutation – if the bases are not misplaced to the extent that the mutation becomes silent or lethal – could be a "cheap" way to effect a major change in the genotype and subsequent phenotype – perhaps resulting in one of those infamous "leaps" in evolution one hears conjectured about from time to time. Even though the specific sequence within a shifted segment of DNA may not be changed, the shift will result in the nucleotide bases being re-grouped into different triplet codes and read accordingly, and the resulting gene may produce a vastly different protein which can change the appearance or function of a cell to a significant extent. So, when sequences between two species are compared, the organisms may appear to be a bit more closely related if these relatively short frame-shifted segments were taken into consideration. (With long stretches of DNA, one would not expect independant genes farther along the chromosome to be affected.)

When a 1308-base stretch of that part of the chromosome which codes for 16S ribosomal RNA was lined up and analyzed ("manually" when I had a little time to kill) to find the extent to which the above four organisms differed from each other, the percent difference between any two organisms was determined, and the results are summarized as follows:

Table 27. The chromosome which codes for 16S ribosomal RNA

PF	PF			
AH	14.8*	AH		
BA	14.5	3.2	BA	
ET	14.9	4.3	5.0	ET

* An example: The same bases appear in the same sequence, position by position, for each of the two organisms except for 14.8% of the time.

With the percent differences used to denote probable evolutionary distances between the organisms, a **phylogenetic tree** was roughed out to illustrate the relationships. The distances between any two organisms, when read along the horizontal lines, corresponds closely to the percent differences. (The bar at the bottom signifies approximately 1% base difference.)

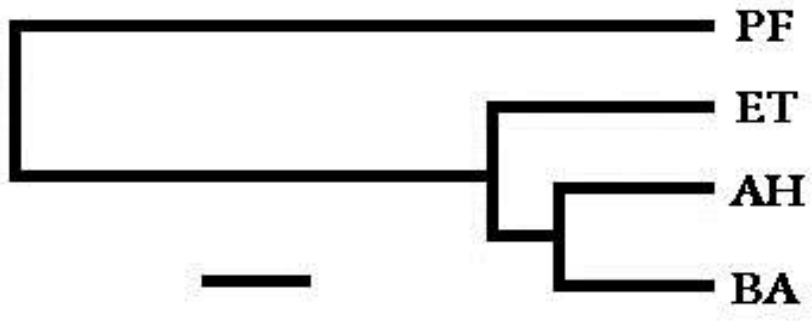

Figure 142. The distances between any two organisms

Databases of various gene sequences are found on the web. Genbank's database was used as the source of the above sequences. And rather than having to line up the sequences and determine the differences manually, a set of programs to analyze sequence data and plot trees are available.

Methods of examination of bacterial susceptibility to antibiotics. The main principles of rational antibiotic therapy of diseases.

Various chemical substances comparatively harmless for the macroorganism but with a lethal action on pathogenic micro-organisms are widely used in medical practice for treating patients with infectious diseases and in some cases for prophylaxis.

This method was known long ago to ancient people, and was used for treating certain diseases. The Peruvian Indians discovered the therapeutic action of cinchona bark, and in the 18th century cinchona bark was brought to Europe. The inhabitants of Brazil successfully employed the root of the ipecacuanha for treating amoebiasis. Mercury has been extensively employed in the therapy of syphilis. In the middle of the 16^{th} century this method became known to the people of Europe.

The basis of modem chemotherapy was founded by P. Ehrlich and D. Romanowsky, who formulated the main scientific principles and the essence of chemotherapy. They showed that in the treatment of each infection a substance

should be found which, during injection into the diseased body, will bring the least harm to it and cause the most destructive action to the pathogenic (causative) agent. P. Ehrlich devised the principles of synthesis of medicinal substances by chemical variations: methylene blue, derivatives of arsenic–salvarsan ("'606"), neosalvarsan ("914"). By the further development of chemistry new medicinal preparations could be obtained.

Extensive experimental and clinical tests of chemopreparations were carried out by E Metchnikoff.

Chemopreparations should have a specific action, a maximal therapeutic effectiveness, and a minimal toxicity for the body.

As a characteristic of the quality of a medicinal preparation, P. Ehrlich introduced the *chemotherapeutic index* which is the ratio of the maximal tolerated dose to the minimal curative dose:

$$\frac{\textbf{maximal tolerated dose (DT—Dosis tolerata)}}{\textbf{minimal curative dose (DC—Dosis curativa)}} > \mathbf{3}$$

The chemotherapeutic index should not be less than 3 Chemotherapeutic preparations include a number of compounds used in medicine

Arsenic preparations (novarsenol, myarsenol, aminarsone, osarsol, etc.) are administered in syphilis, relapsing fever, trypanosomiasis, amoebiasis, balantidiasis, anthrax, sodoku, and other diseases.

Bismuth preparations (basic bismuth nitrate, xeroform, basic bismuth salicylate, bioquinol, bismoverol, bithiurol, pentabismol, etc.) are used against enterocolitis and syphilis

Antimony compounds (tartaric antimony potassium salt, stibenil, stibozan, surmine, solusurmine, etc.) are used for treating patients with leishmaniasis and venereal lymphogranulomatosis.

Mercury preparations (mercury salicylate, mercuric iodide, mercury cyanide, calomel, unguentum hydrargyri cinereum containing metallic mercury, etc.) are prescribed for treating patients with syphilis and are used as antiseptics in pyogenic diseases.

Acridine preparations (rivanol, tripaflavine, acriflavine, acricide, flavicide, etc.) are recommended for pyogenic diseases and inflammatory processes of the pharynx and nasopharynx

Antimalarial substances include more than 30 preparations, e g , chinine hydrochloride, quinine sulphate, mepacrine (acrichine), rodochin (plasmocide), proguanyl (bigumal), pyrimethamine (chloridine), resochine, quinocide sulphones and sulphonamides, sulphadiamine, etc.

Alkaloid preparations (emetine, etc.) are used for treating patients with amoebiasis.

Sulphonamide preparations. The introduction into practice of compounds of the sulphonamide group (streptocid, ethasole, norsulphazol, sulphazine,

methylsulphazine, sulphadimezin, urosulphan, phthalazole, sulgine, sulphacyi, soluble sulphacyl, disulphormin, etc.) marked a revolution in the chemotherapy of bacterial infections.

Sulphonamide preparations are used for treating pyogenic diseases, tonsillitis, scarlet fever, erysipelas, pneumonia, dysentery, anaerobic infections, gonorrhoea, cystitis, venereal lymphogranulomatosis, psittacosis, ornithosis, trachoma, blennorrhoea in the newborn, etc.

There are several points of view concerning the mechanism of action of sulphonamides on microbes.

Antibiotics (Fr. *anti* against, *bios* life) are chemical substances excreted by some micro-organisms which inhibit the growth and development of other microbes (in recent years several antibiotics have been obtained semisynthetically).

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2. Medical Microbiology and Immunology: Examination and Board Rewiew /W. Levinson, E. Jawetz.– 2003.– P.14-16

3. Handbook on Microbiology. Laboratory diagnosis of Infectious Disease/ Ed by Yu.S. Krivoshein, 1989, P. 29-74.

4. Essentials of Medical Microbiology / W.A. Volk at al., – Lippincott-Raven, Philadelphia-New-York

The identification of bacteria is a careful and systematic process that uses many different techniques to narrow down the types of bacteria that are present in an unknown bacterial culture, such as the infected blood of someone dangerously ill with meningitis.

Identification techniques include:

· Morphological (according to the bacterial morphology);

· Cultural (according to the bacterial growth signs in/on different nutrient media);

· Biochemical (according to the bacterial ability to utilize differen substrates);

· Serological (according to the bacterial antigens);

· Bilological (according to the bacterial ability to cause different changes in laboratory animals after their inoculation by microbes);

· Flow cytometry;

· Phage typing;

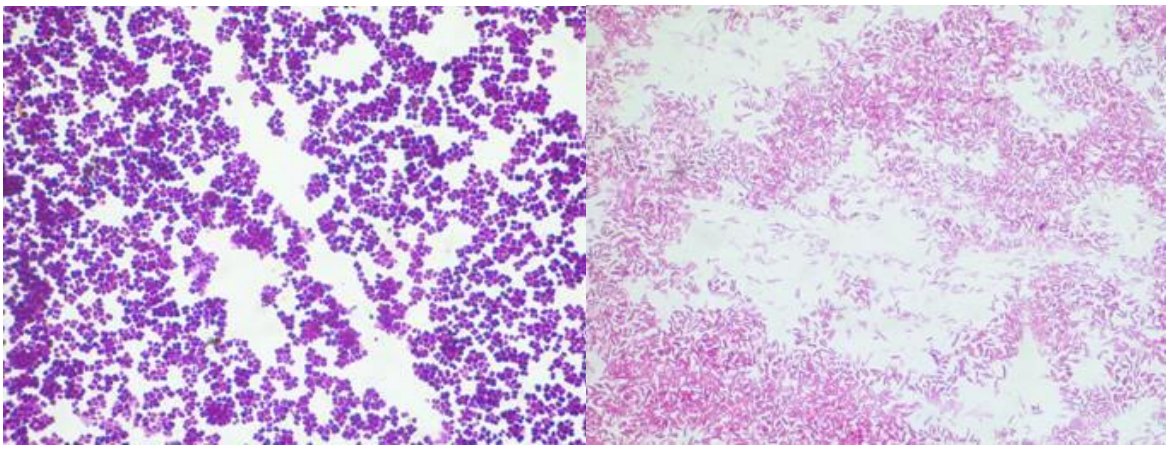
· Protein analysis;

· Comparison of nucleotide sequenses.

Morphological identification (according to the bacterial morphology) – Microscopic morphology

A number of morphological characteristics are useful in bacterial identification. These include the presence or absence of:

cell shape

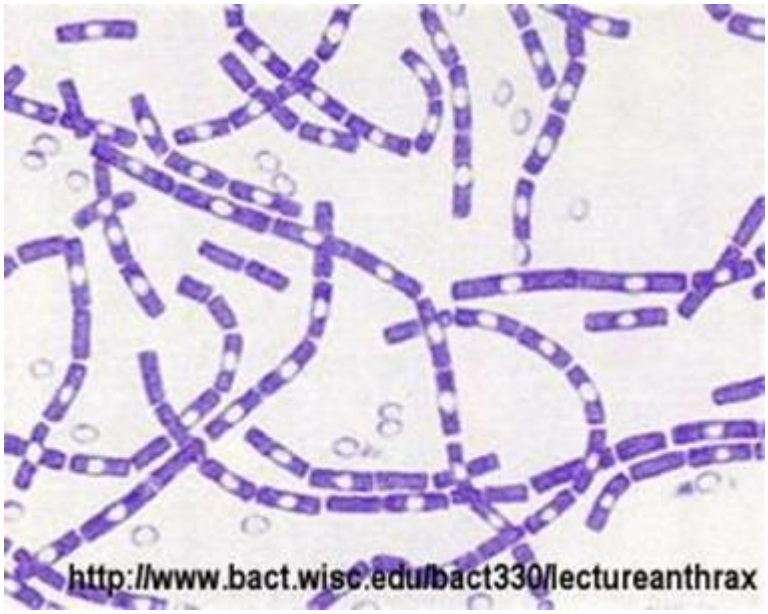


Cocci **Rods**

Figure 143. Cell shape

cell size

endospores



Spores

Figure 144. Cell size, endospores

flagella

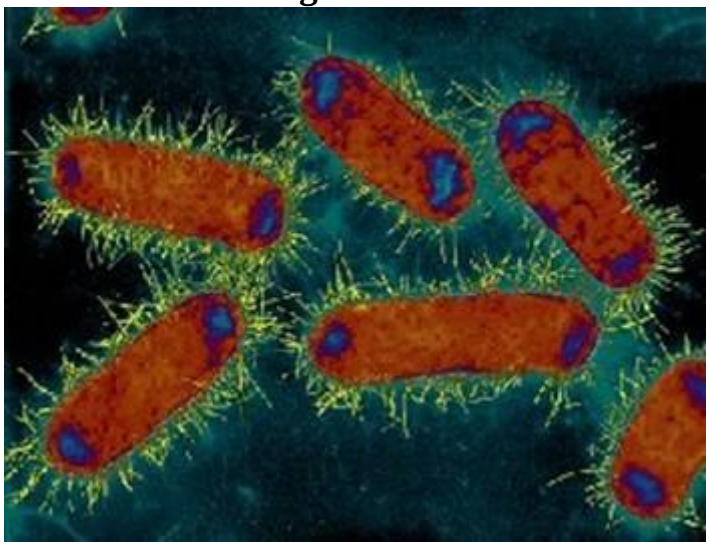


Figure 145. Flagella , glycocalyxetc.

The techniques used at the earliest stages are relatively simple. An unknown sample may contain different bacteria, so a culture is made to grow individual bacterial colonies. Bacteria taken from each type of colony is then used to make a thin smear on a glass slide and this is examined using a light microscope. Viewing the bacteria shows if they are cocci or bacilli or one of the rarer forms, such as the corkscrew shaped spirochaetes.

Gram Staining

Cocci and bacilli can be either gram positive bacteria or gram negative bacteria, depending on the structure of their cell wall. The Gram Stain is named after Hans Christian Gram, a bacteriologist from Denmark who developed the technique in the 1880s. The test is performed on a thin smear of an individual bacterial colony that has been spread onto a glass slide. Gram positive bacteria retain an initial stain, crystal violet, even when the bacterial smear is rinsed with a mixture of acetone and ethanol. The solvent removes the dark blue colour from gram negative bacteria, dissolving away some of the thin cell wall. When a second stain, a pink dye called fuchsin is then added, gram positive bacteria are unaffected by this, as they are already stained dark blue, but the gram negative bacteria turn bright pink. The colour difference can be seen easily using a light microscope.

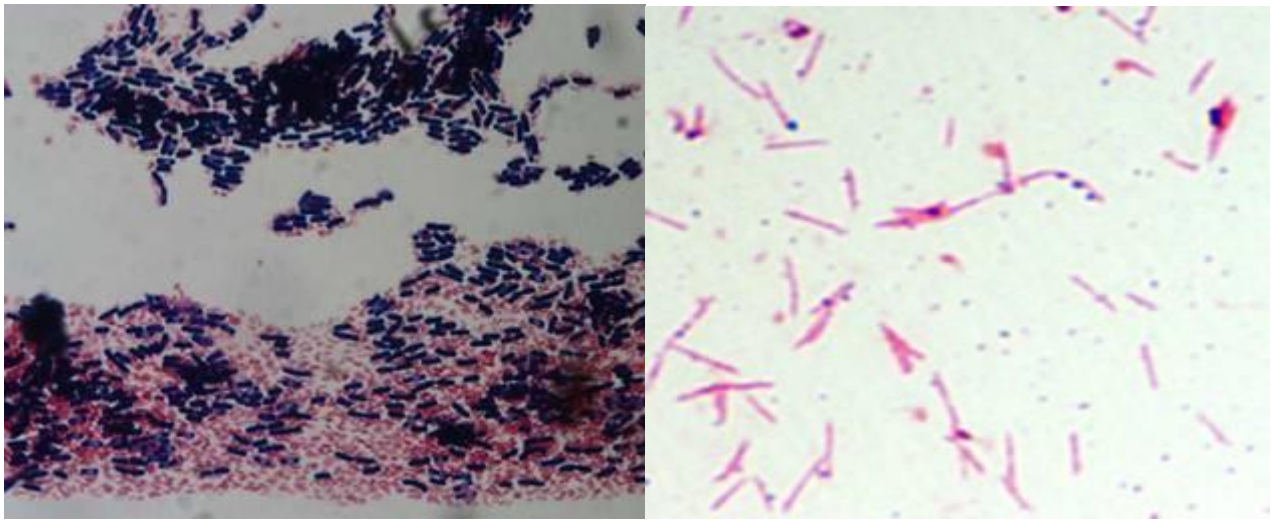


Figure 146. Gram-positive (left) and gram-negative (right) microbes

Acid Fast Bacteria

Spirochaetes such as the Mycobacteria that cause tuberculosis and leprosy do not stain well using the Gram Stain. Other stains that do not wash away with dilute acid are used instead. The bacteria are deeply stained, either bright red against a blue background or red against a green background. Because the stain cannot be removed by washing with acid, organisms stained by these methods are termed acid-fast bacteria.

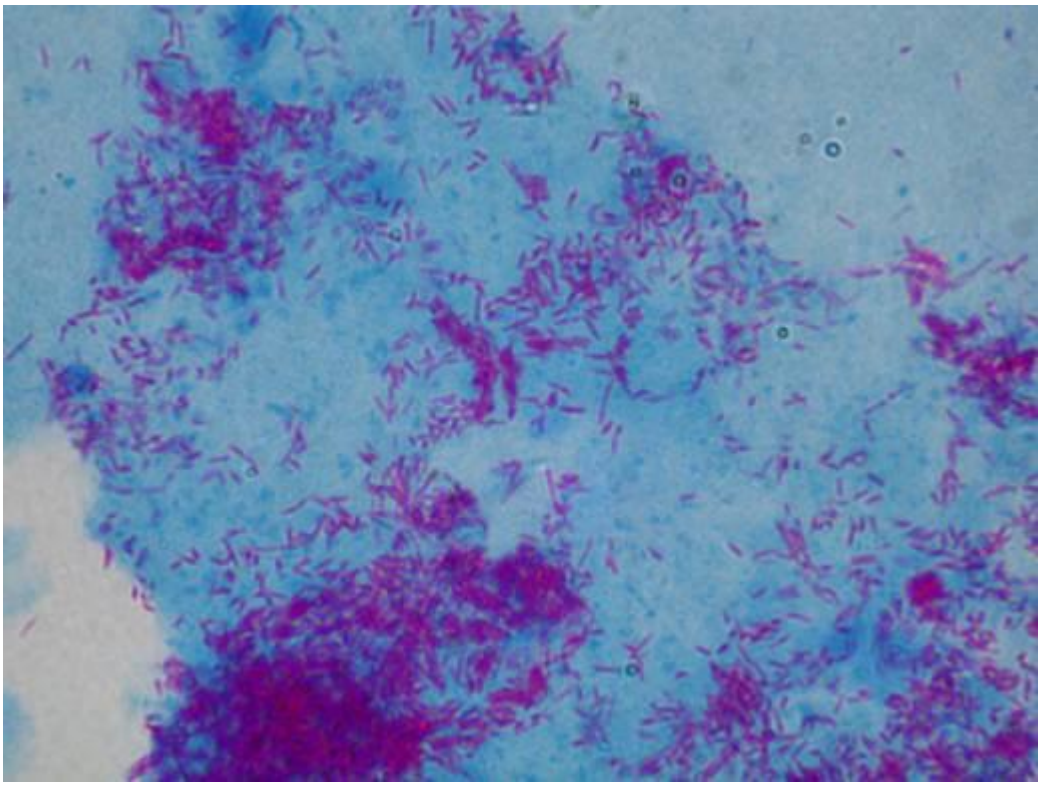


Figure 147. This stains acid-alcohol fast bacteria, e.g. mycobacteria.

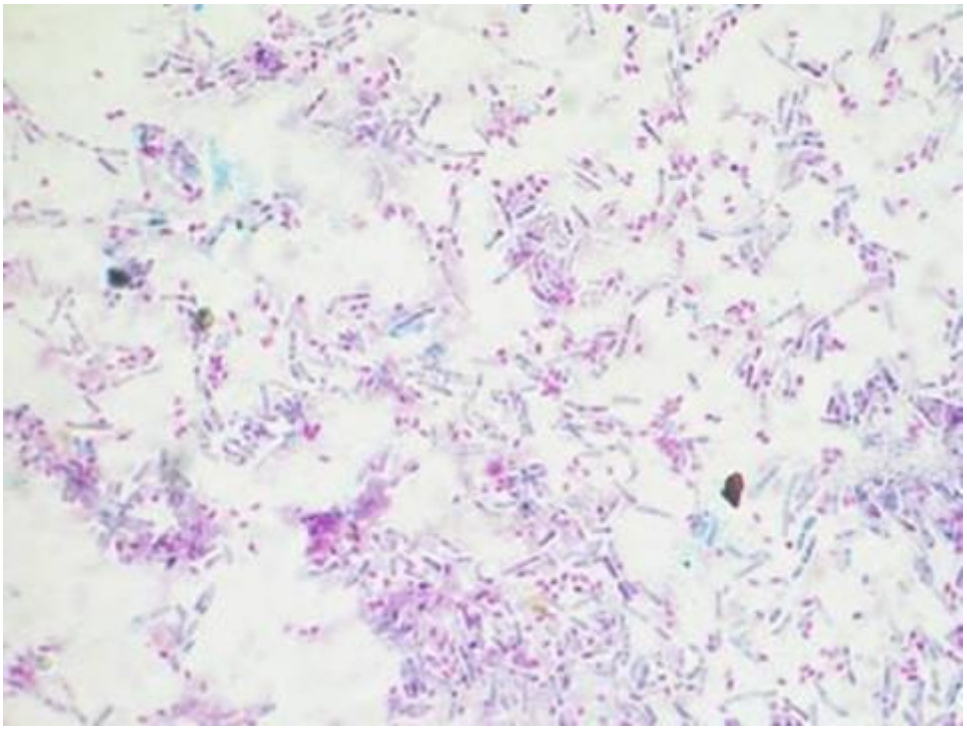


Figure 148. Bacterial spores are seen as red structures: vegetative cells stain blue.

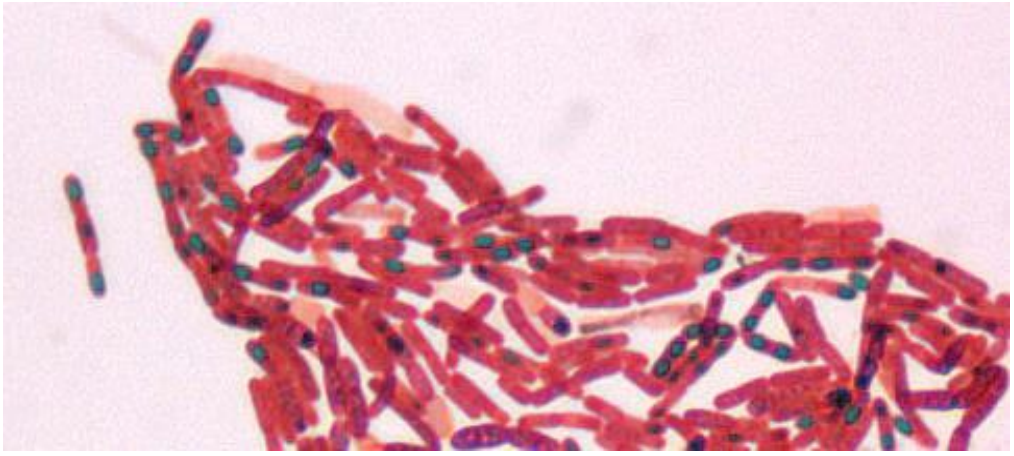


Figure 149. Bacterial spores (Schaeffer-Fulton stain technique)

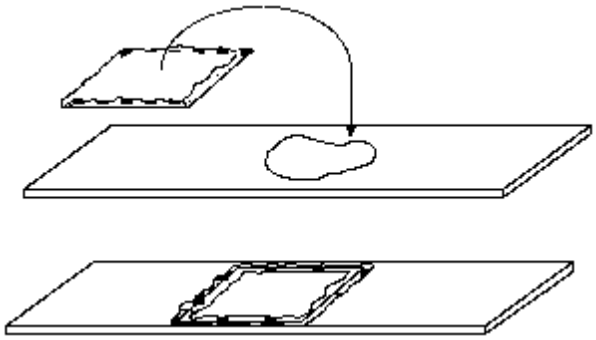
Motility

· Place a drop of liquid culture on a microscope coverslip.

· Invert over a plasticine ring on a microscope slide.

· Examine under x40 objective (high power dry lens).

· Accidental spills may occur during this procedure.



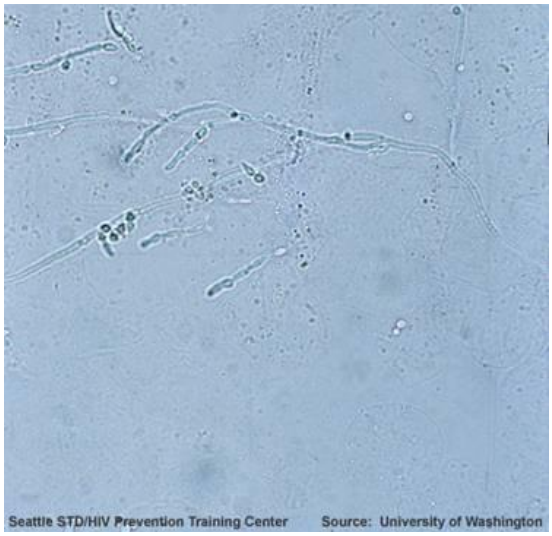


Figure 150. Wet mount technique

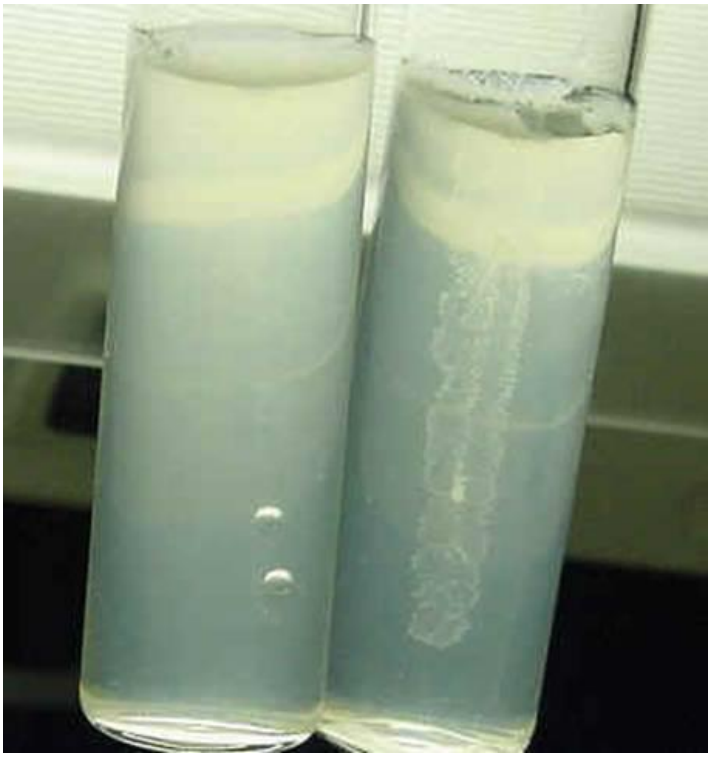


Figure 151. Left – negative test, right – positive

Capsule staining (relief staining with eosin)

· Place a drop of broth culture on one end of a microscope slide.

· Add one drop of eosin solution and leave for one minute.

· Take a second slide and draw its edge back to contact the stained suspension.

· Holding the second slide at 45 degrees, spread a thin layer of fluid along the first slide by a continuous forward movement.

Allow the film to air dry then examine under oil-immersion.

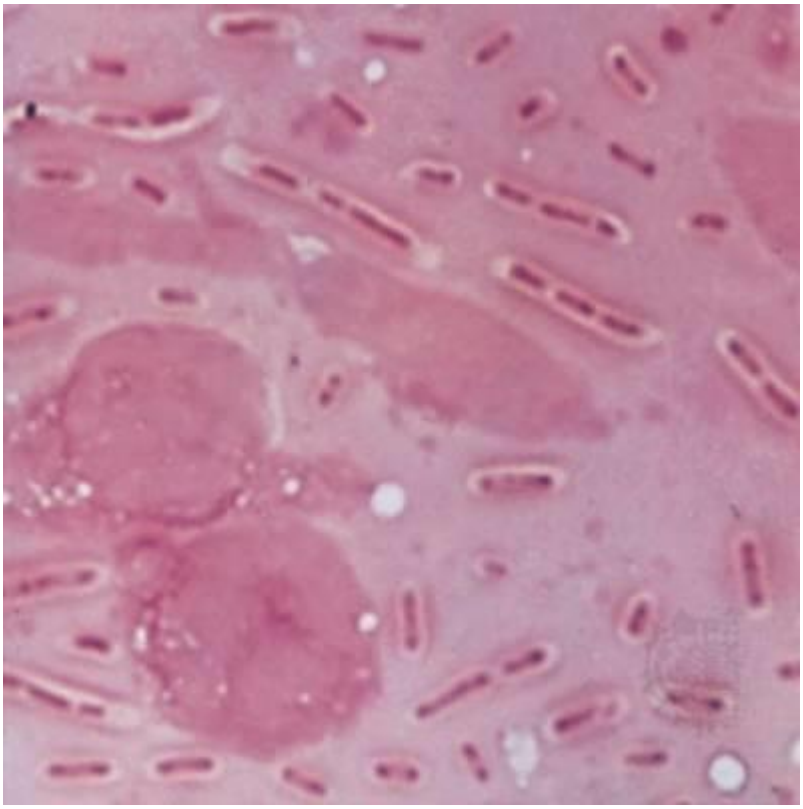


Figure 152. Background material and cells stain red. Capsular material appears as an unstained halo around the cells

Aerobic or Anaerobic?

Finding out whether bacteria are aerobic or anaerobic helps separate them into different categories. It is relatively easy to discover whether a bacterial culture grows in the presence or absence of oxygen. Bacteria that only grow if oxygen is available are called aerobic bacteria. Anaerobic bacteria can grow without oxygen, and some species are killed by oxygen, only surviving in completely oxygen-free environments. These species are described as obligate anaerobes (see above).

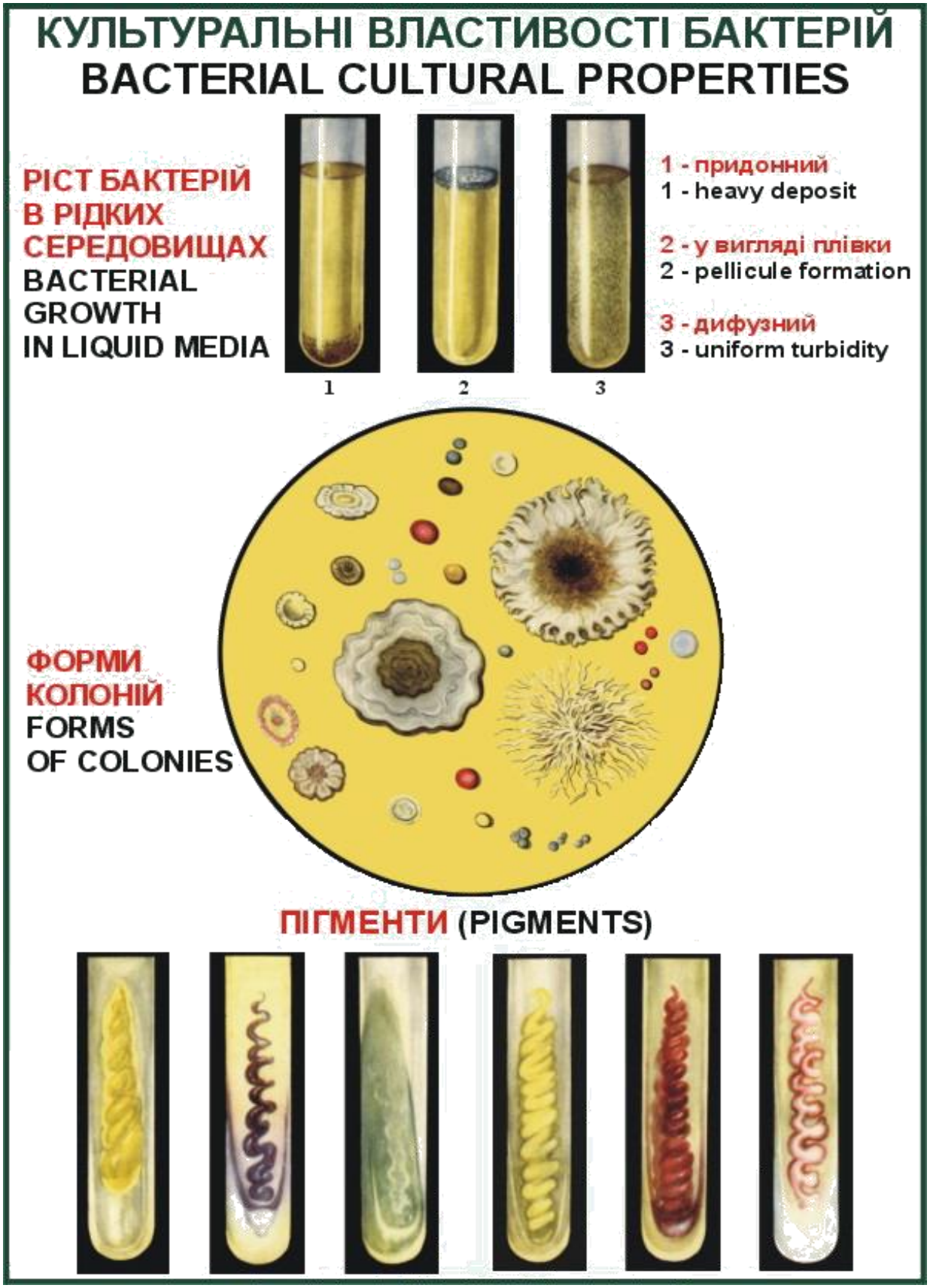


Figure 153. Cultural (according to the bacterial growth signs in/on different nutrient media) – colony morphology

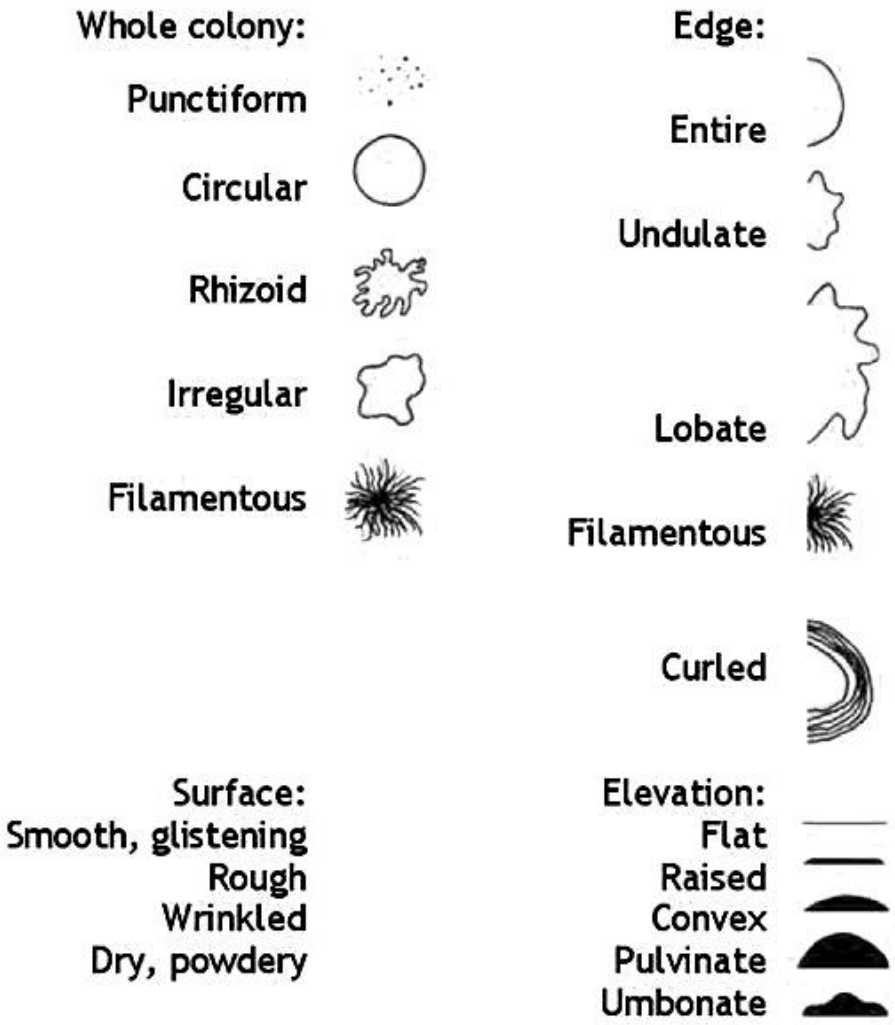


Figure 154. Forms of colonies

Figure 155. Mixed coloniesBiochemical (according to the bacterial ability to utilize differensubstrates) – Enzyme Tests

Within broad types of bacteria, individual species have different metabolic systems and are able to grow using a range of nutrients. Testing bacteria to find out whether they are positive or negative for specific enzymes helps narrow down their identity. For example, Staphylococcus aureus tests positive for the enzyme coagulase, but Staphylococcus epidermidis is negative for this enzyme.

Fermentative properties of microbes are used in the laboratory diagnosis of infectious diseases, and in studying microbes of the soil, water, and air.

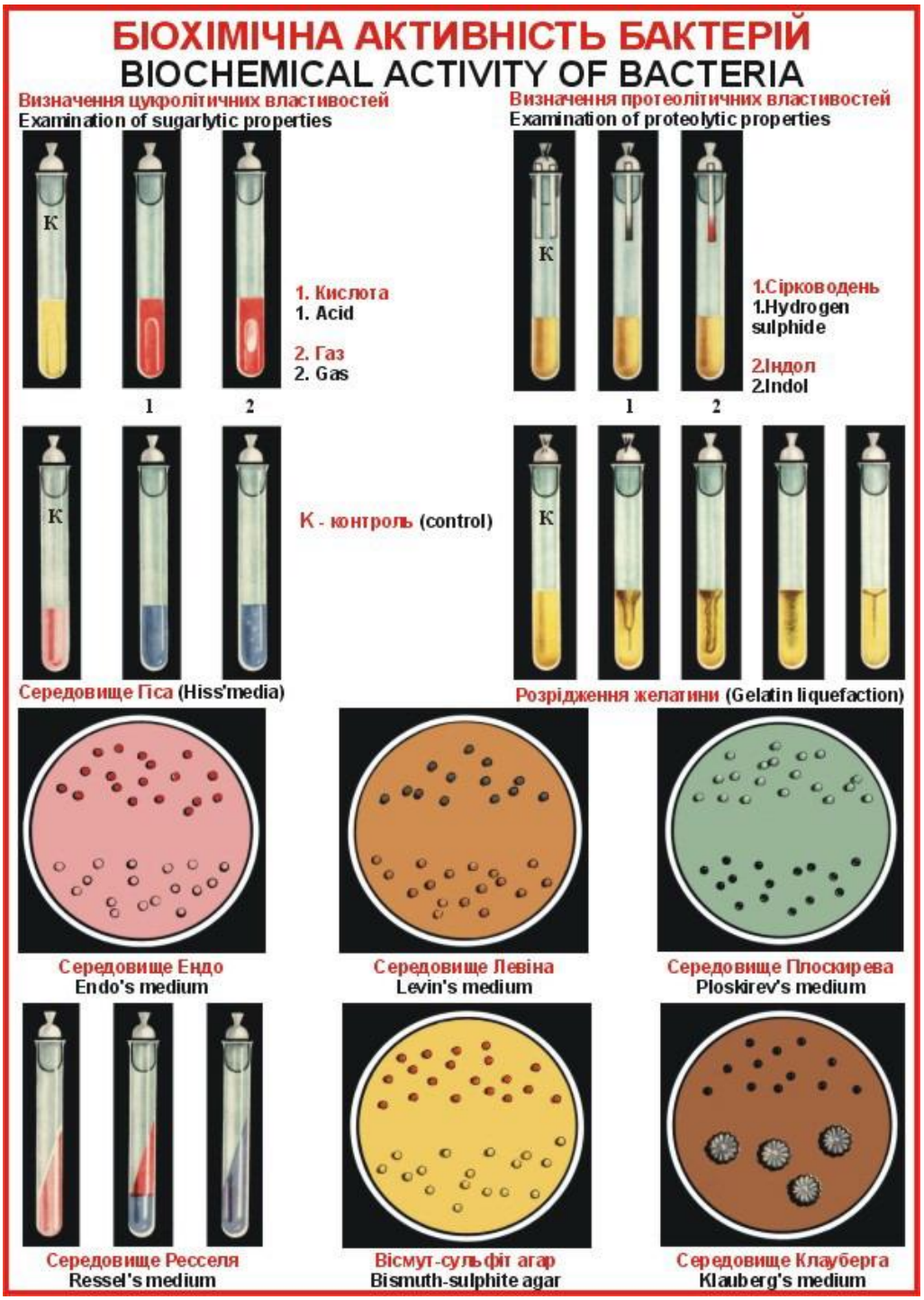


Figure 156. Biochemical activity of bacteria

To identify the isolated pure culture, supplement the study of morphological, tinctorial, and cultural features with determination of their enzymatic and antigenic attributes, phago- and bacterio-cinosensitivity, toxigenicity, and other properties characterizing their species specificity.

To demonstrate carbohydrate-splitting enzymes, Hiss' media are utilized. When bacteria ferment carbohydrates with acid formation, the colour of the medium changes due to the indicator present in it. Depending on the kind and species of bacteria studied, select media with respective mono- and disaccharides (glucose, lactose, maltose, sucrose), polysaccharides (starch, glycogen, inulin), higher alcohols (glycerol, mannitol). In the process of fermentation of the above substances aldehydes, acids, and gaseous products (CO_2, H_2, etc.) are formed.

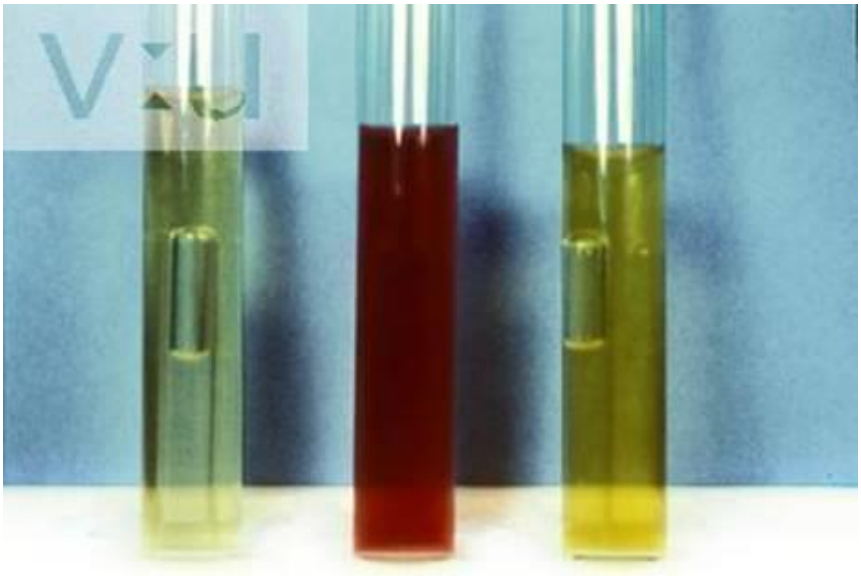


Figure 157. To demonstrate carbohydrate-splitting enzymes, Hiss' media are utilized.

TSI (Triple Sugar Iron) and KIA (Kligler's Iron Agar)

Triple Sugar Iron Agar (TSI) and Kligler's Iron Agar (KIA) are used to determine if bacteria can ferment glucose and/or lactose and if they can produce hydrogen sulfide or other gases. (If an organism can ferment glucose, it is "glucose positive". If it ferments lactose, it is "lactose positive".) In addition, TSI detects the ability to ferment sucrose. These characteristics help distinguish various *Enterobacteriacae*, including *Salmonella* and *Shigella*, which are intestinal pathogens.TSI contains three sugars: glucose, lactose and sucrose. Lactose and sucrose occur in 10 times the concentration of glucose (1.0% versus 0.1%). Ferrous sulfate, phenol red (a pH indicator that is yellow below pH 6.8 and red above it), and nutrient agar are also present. The tube is inoculated by stabbing into the agar butt (bottom of the tube) with an inoculating wire and then streaking the slant in a wavy pattern. Results are read at 18 to 24 hours of incubation.

Reading the Results

A yellow slant on TSI indicates the organism ferments sucrose and/or lactose. On KIA a yellow slant indicates the organism ferments lactose. (Because KIA does not contain sucrose, sucrose fermentation is not detected with KIA tests.) Other results are the same for TSI and KIA. A yellow butt shows that the organism fermented glucose. Black preciptate in the butt indicates hydrogen sulfide production. Production of gases other than hydrogen sufide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube.

Understanding the Results

If an organism ferments glucose only, the entire tube turns yellow due to the effect of the acid produced on phenol red. Because there is a minimal amount of glucose present in the tube, the organism quickly exhausts it and begins oxidizing amino acids for energy. Ammonia is thus produced and the pH rises. Within 24 hours the phenol red indicator reverts to its original red color on the slant. Because TSI/KIA media is poured as a deep slant, the butt has limited oxygen and bacteria are unable to oxidize amino acids there. The butt thus remains yellow.If an organism can ferment lactose and/or sucrose, the butt and slant will turn yellow (as they do from glucose fermentation). However, they remain yellow for at least 48 hours because of the high level of acid products produced from the abundant sugar(s).

KIA resembles TSI in all respects except that KIA contains two sugars (lactose and glucose) while TSI contain three sugars (lactose, glucose and sucrose). Like TSI media, KIA contains 10 times as much lactose as glucose. Thus KIA tests for an organism's ability to ferment glucose or lactose but not sucrose.

If the gas being produced is hydrogen sulfide (H_2S), it reacts with the ferrous sulfate and preciptates out as a black precipitate (ferric sulfide) in the butt. Organisms producing large amounts of hydrogen sulfide (e.g. *Salmonella* and *Proteus*) may produce so much black precipitate that it masks the yellow (acid) color of the butt.

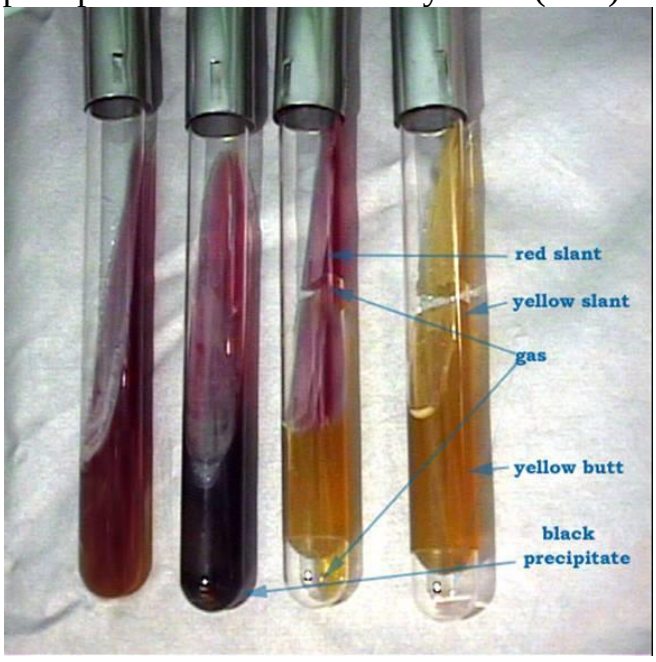


Figure 158. Organisms producing large amounts of hydrogen sulfide

1. Reading results

Figure 159. TSI (Triple Sugar Iron) and KIA (Kligler's Iron Agar)

2. Interpreting results on TSI

Figure 160. Interpreting results on TSI

3. Interpreting results on KIA

To demonstrate proteolytic enzymes in bacteria, transfer the latter to a gelatin column. Allow the inoculated culture to stand at room temperature (20-22 °C) for several days, recording not only the development of liquefaction per se but its character as well (laminar, in the form of a nail or a fir-tree, etc.).

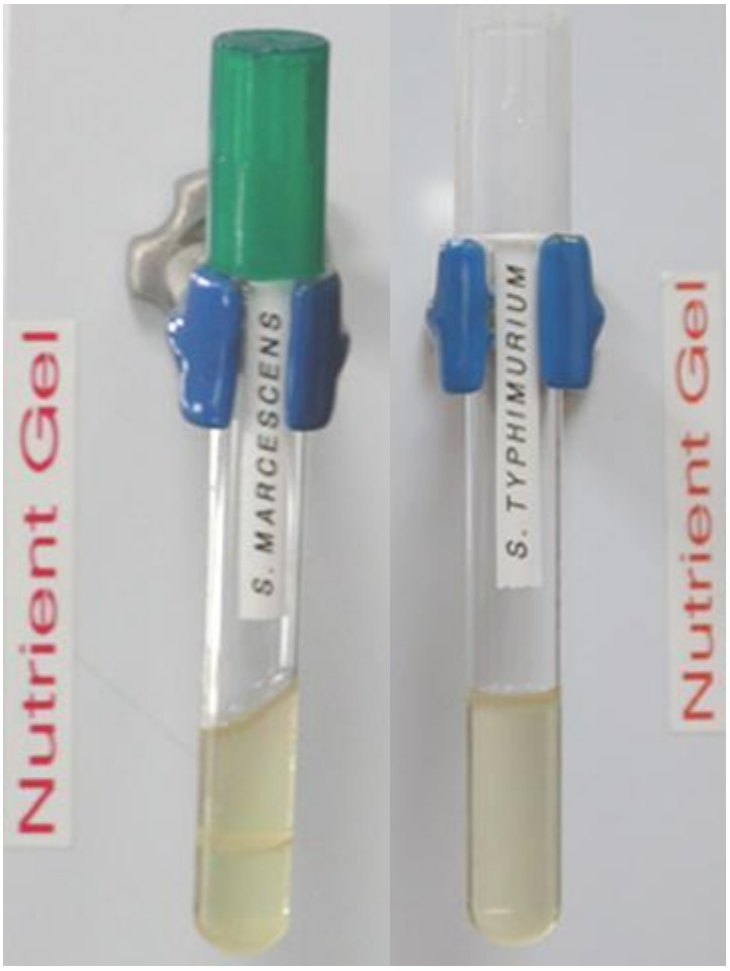


Figure 161. Serratia marcescens on the left is positive for gelatinase production, as evidenced by the liquidation of the media.
Salmonella typhimurium on the right is negative, as evidenced by the solidity of the media.

Proteolytic action of enzymes of microorganisms can also be observed following their streaking onto coagulated serum, with depressions forming around colonies (liquefaction). A casein clot is split in milk to form peptone, which is manifested by the fact that milk turns yellowish (milk peptonization).

More profound splitting of protein is evidenced by the formation of indol, ammonia, hydrogen sulphide, and other compounds. To detect the gaseous substances, inoculate microorganisms into a meat-peptone broth or in a 1 per cent peptone water. Leave the inoculated cultures in an incubator for 24-72 hrs.

To demonstrate indol by Morel's method, soak narrow strips of filter paper with hot saturated solution of oxalic acid (indicator paper) and let them dry. Place the indicator paper between the test tube wall and stopper so that it does not touch the streaked medium. When indol is released by the 2nd-3rd day, the lower part of the paper strip turns pink as a result of its interaction with oxalic acid.

The telltale sign of the presence of ammonia is a change in the colour of a pink litmus paper fastened between the tube wall and the stopper (it turns blue).

Hydrogen sulphide is detected by means of a filter paper strip saturated with lead acetate solution, which is fastened between the tube wall and the stopper. Upon interaction between hydrogen sulphide and lead acetate the paper darkens as a result of lead sulphide formation.

Figure 162. The telltale sign of the presence of ammonia

To determine catalase, pour 1-2 ml of a 1 per cent hydrogen peroxide solution over the surface of a 24-hour culture of an agar slant. The appearance of gas bubbles is considered as a positive reaction. Use a culture known to contain catalase as a control.

The reduction ability of microorganisms is studied using methylene blue, thionine, litmus, indigo carmine, neutral red, etc. Add one of the above dyes to

nutrient broth or agar. The medium decolourizes if the microorganism has a reduction ability. The most widely employed is Rothberger's medium (meat-peptone agar containing 1 per cent of glucose and several drops of a saturated solution of neutral red). If the reaction is positive, a red colour of the agar changes into yellow, yellow-green, and fluorescent, while glucose fermentation is characterized by cracks in the medium.

Bile solubility test (Pure culture)

· Emulsify a few colonies of the test culture in 1ml of saline to form a smooth suspension.

· Add one drop of 10% sodium deoxycholate solution.

· Incubate at 37^{o}C.

· Examine for clearing at 15 minutes, 30 minutes and 60 minutes

Clearing should occur within 30 minutes

In a mixed culture place one drop of 10% sodium deoxycholate solution onto the test colony.

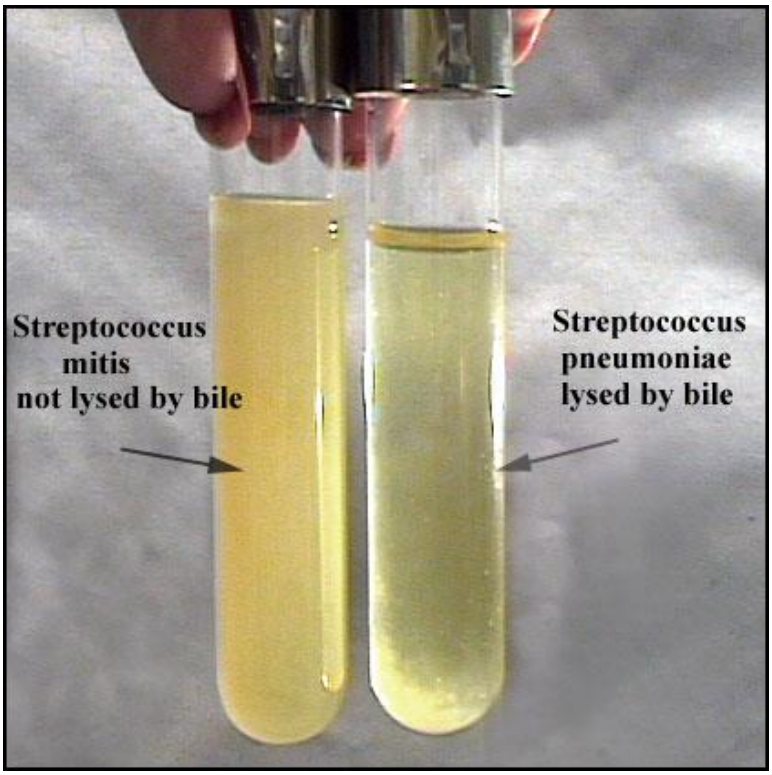


Figure 163. This should lyse within 30 minutes. This method is not entirely reliable, and it is better to purify any suspected colony

Catalase Test

· Using a glass capillary tube, pick a small amount of culture from the plate.

· If possible do not pick from a blood containing medium as the presence of catalase in the medium itself may give a false positive result. This sometimes cannot be avoided.

· Carefully invert the tube and insert it into the hydrogen peroxide solution.

· Tilt the tube so the fluid flows onto the culture material.

· Look for the immediate formation of oxygen bubbles in the tube indicating the activity of catalase.

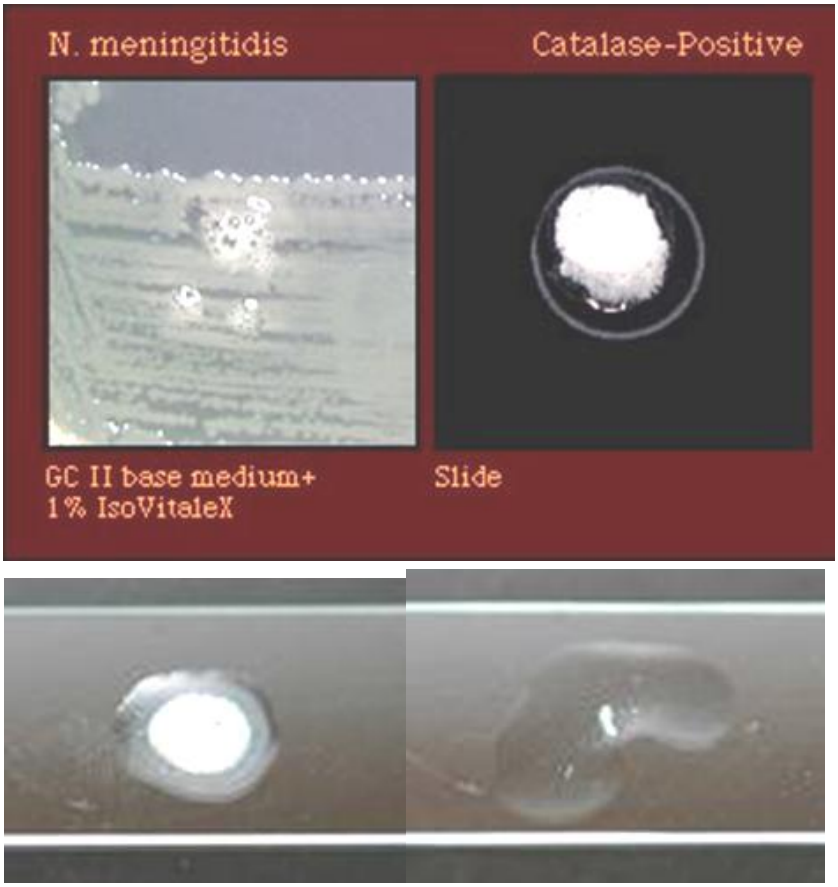


Figure 164. Catalase test positive (left) and negative (right)

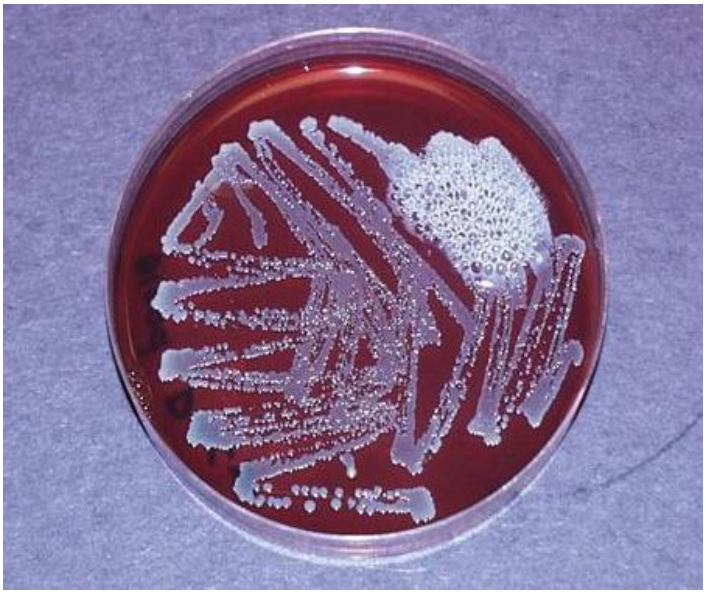


Figure 165. Catalase test

Coagulase Test

A. Slide method:

This test detects the presence of "clumping factor" and is not a true coagulase test.

· Place three separate drops of saline on a clean slide.

· Suspend a loopful of test colony in two of these, and a loopful of control *Staphylococcus aureus* in the third.

· With a sterile loop, add a drop of citrated rabbit plasma to one test and the control suspension.

· Clumping occurring within 10 seconds indicates a positive result.

· The saline control should remain evenly suspended.

B. Tube method:

· Emulsify a few colonies of control *Staphylococcus aureus* and the test isolate into appropriately labelled tubes containing a 1/10 dilution of plasma in 0.85% saline.

· Incubate at 37^{o}C.

· Examine for coagulation at 1, 3 and 6 hours.

Figure 166. Conversion of the plasma into a soft or stiff gel, seen on tilting the tube to a horizontal position indicates a positive result.

DNase Test

· Inoculate sections of tryptose agar medium containing DNA with material from test colonies.

· Controls of known *Staphylococcus aureus* and *Staphylococcus epidermidis* should be inoculated as positive and negative controls.

· Incubate the plate at 37^{o}C for 18-24 hours.

· Flood the plate with lM HCl that precipitates DNA and turns the medium cloudy.

Figure 167. DNase Test

Lecithinase activity results in the production of an opaque zone of precipitation around the area of growth. This precipitation should not be present on that side of the plate previously inoculated with specific α-antitoxin.

Nagler Test

Clostridium perfringens elaborates a variety of exotoxins, one of which is α-toxin (lecithinase or phospholipase C). The following test is used to demonstrate production of this specific toxin.

· Divide an egg yolk plate into two equal sections.

· Spread a loopful of *Clostridium perfringens* antitoxin over half the plate and allow to dry.

· With a single streak, inoculate the plate with a loopful of the test culture, beginning on the untreated side of the plate.

· Incubate at 37^{o}C under anaerobic conditions.

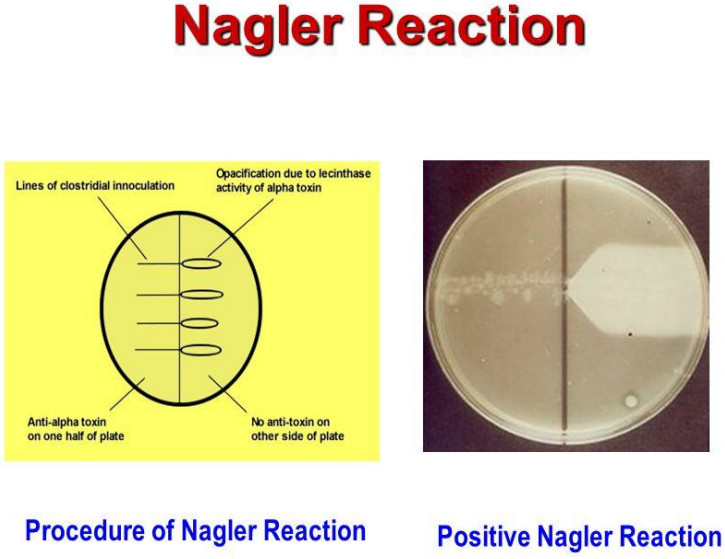


Figure 168. Nagler reaction

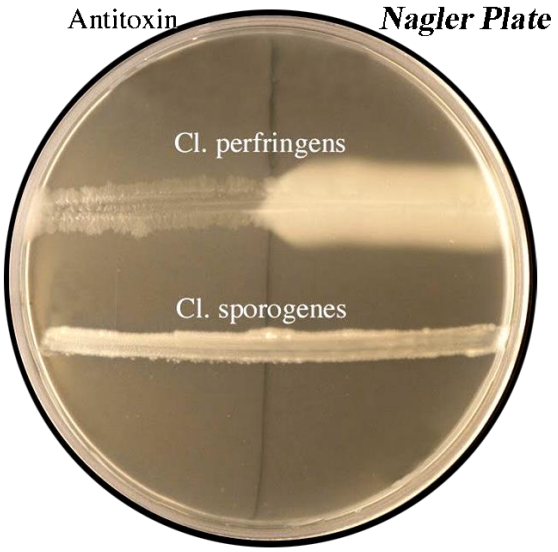


Figure 169. Nagler Test
Lecithinase activity results in the production of an opaque zone of precipitation around the area of growth. This precipitation should not be present on that side of the plate previously inoculated with specific α-antitoxin

Optochin Test

· Divide a blood agar plate into three equal sections.

· Inoculate one with a known *Streptococcus pneumoniae*, another with a viridans streptococcus and the third with the test isolate.

· Care must be taken to keep the cultures separate.

· Place a 5 microgram Optochin disc (ethylene hydrocupreine hydrochloride) in the centre of the plate.

· Incubate at 37^{o}C overnight and observe the zone of inhibition.

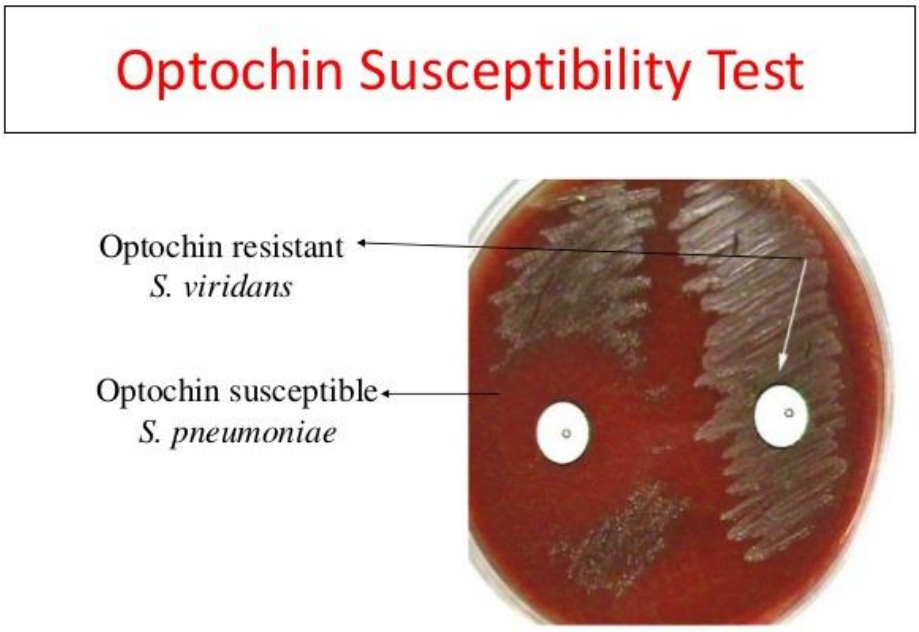


Figure 170. Optochin Test

Oxidase Test

· Dip a sterile swab in freshly prepared oxidase reagent (1% tetra methyl-*para*-phenylene diamine dihydrochloride) then touch the target colony. A positive reaction is indicated by the rapid appearance of a purple colour on the swab where the test bacteria adhere.

Figure 171. Oxidase Test

Alternatively

· Place a drop of freshly prepared oxidase reagent (1% tetra methyl-*para*-phenylene diamine dihydrochloride) on a piece of filter paper in a Petri dish or on a glass slide.

· Leave for 1 minute.

· Using a wooden stick or a glass slide (not a wire loop) rub a small amount of the test colony onto the moistened paper.

· Again, a positive test is indicated by the rapid appearance of a purple colour at this site.

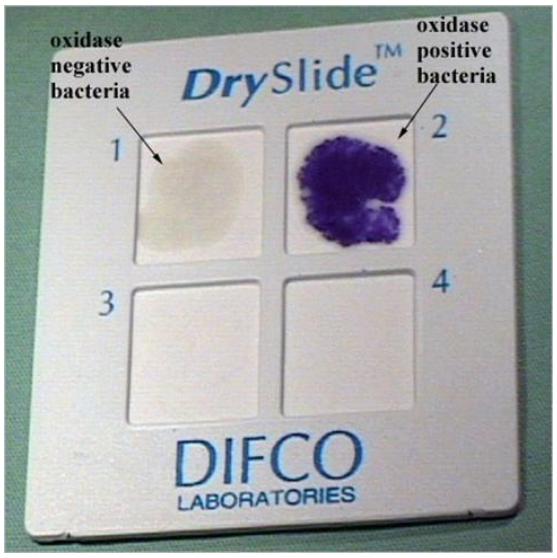


Figure 172. Positive reaction

Indole production - measure the ability to hydrolyse and deaminate tryptophan

- *Klesiella-enterobacter-salmonella-serratia* are mostly negative
- positive-red colour

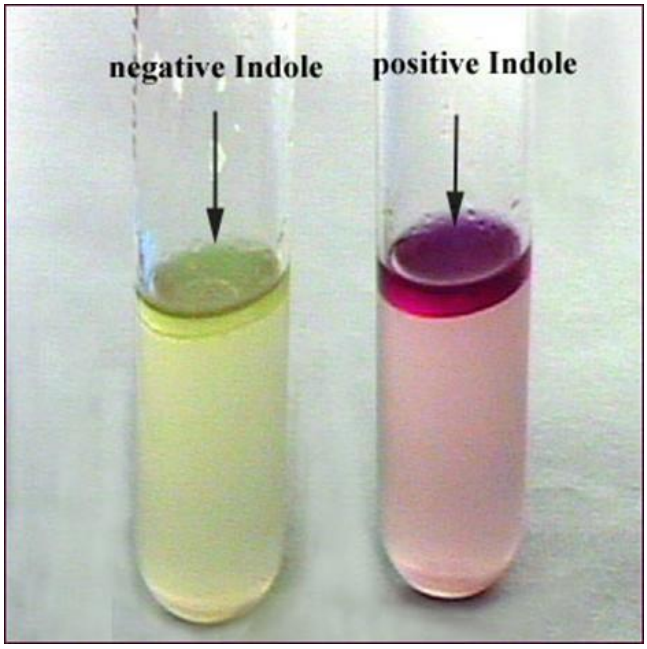


Figure 173. Indole production

Methyl red - methyl red, a pH indicator with a range between 4.4(red) and 6.0(yellow)

- only species that produce suffiicient acids can maintian the pH at below4.4 against the buffer system of the test medium
- most species of Enterobacteriaceae produce strong acids. *Enterobacter-serratia* do not produce enough acids

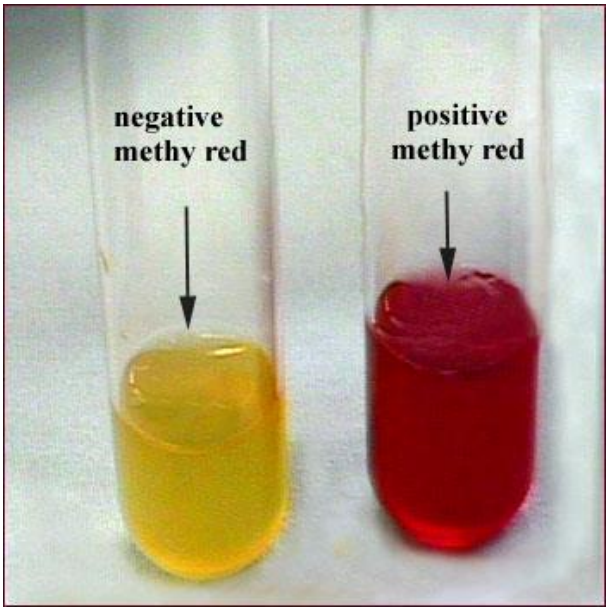


Figure 174. Methyl red

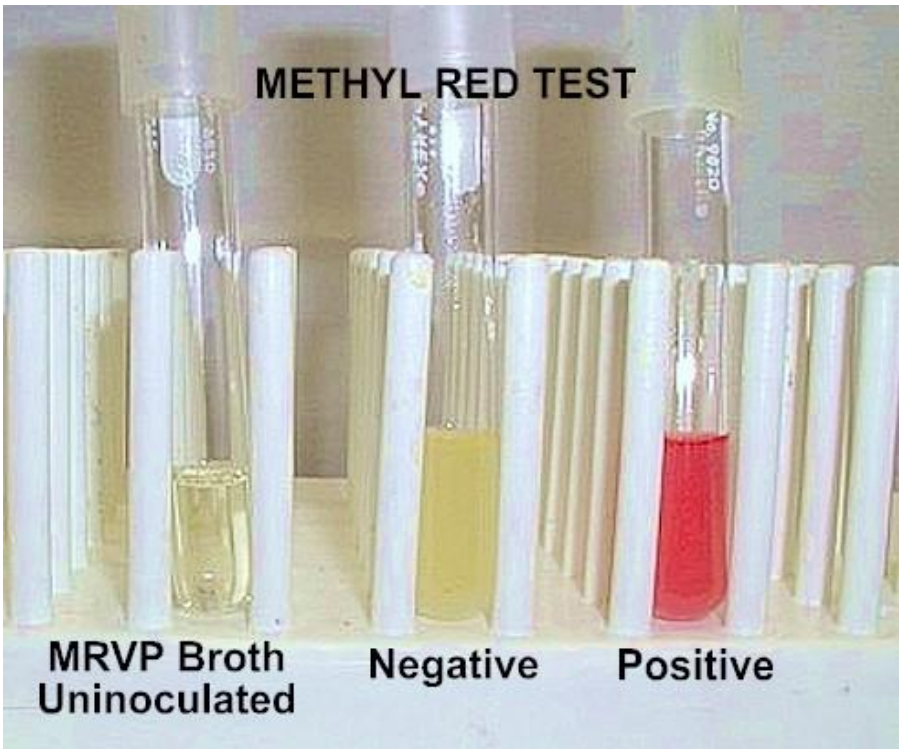


Figure 175. Positive-stable red colour in the surface layer of the medium

Voges-proskauer reaction test

-this test is based on the conversion of acetoin to a red coloured complex through the action of KOH, atmospheric 02 and alpha napthol

-*Klesiella-enterobacter-serratia* is able to perform this pathway

-

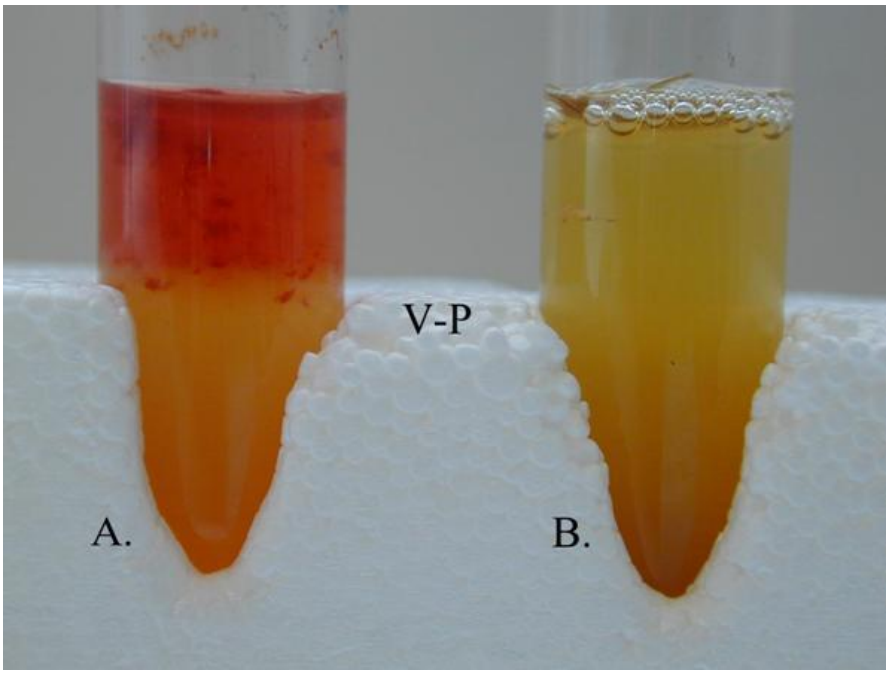


Figure 176. Voges-proskauer reaction test. Red colour at the surface of the medium after 15 mins following the addition of reagents

Citrate utilisation test - some bacteria have the ability to utilize citrate as the sole carbon sourc and turn the medium allkaline due to production of ammonia

-*Escherichia-Edwardisella-shigella-salmonella* cannot utilise citrate as the sole source of carbon

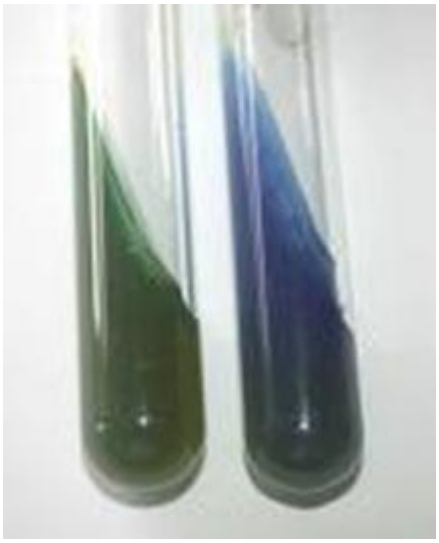


Figure 177. Citrate utilisation test

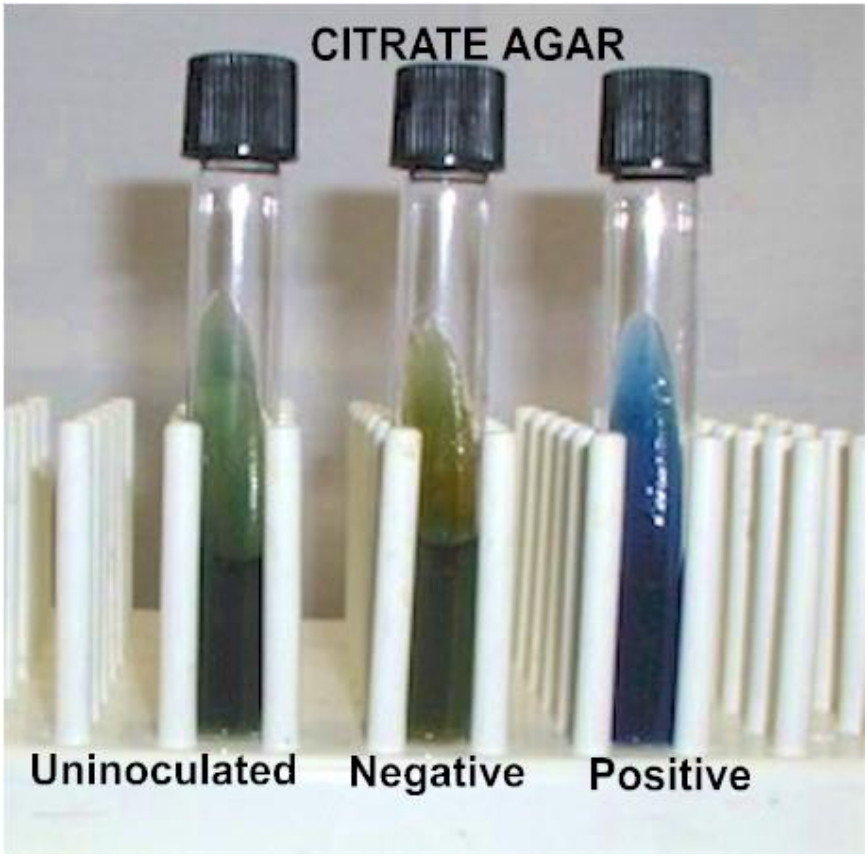


Figure 178. Positive - from colour green to blue

Urease test - some species posses the enzyme urease and able to hydrolyze urea with the release of ammonia and carbon dioxide

- this is used mainly to differentiate urease positive Proteus species from other member of Enterobacteriaceae

- positive-yellowish orange to pink

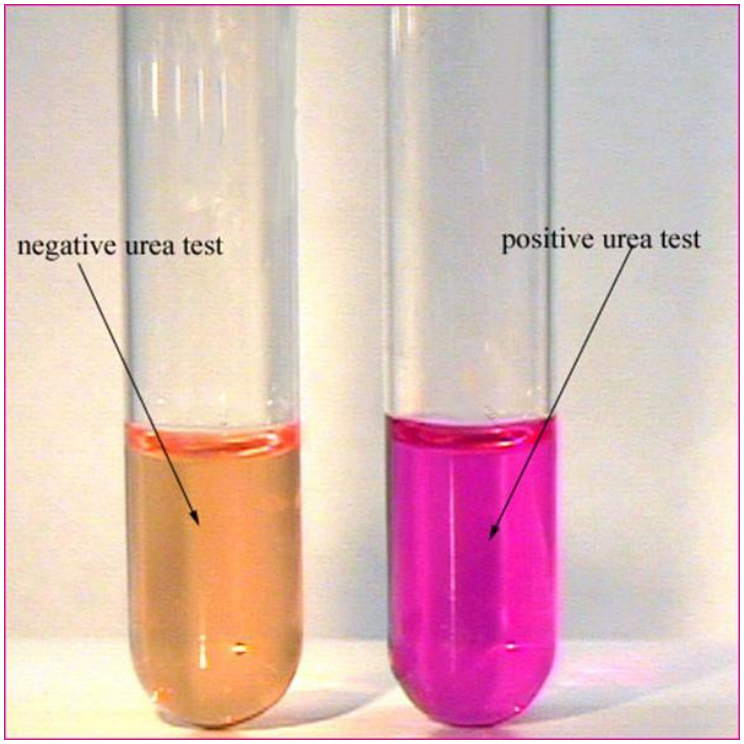


Figure 179. Urease test

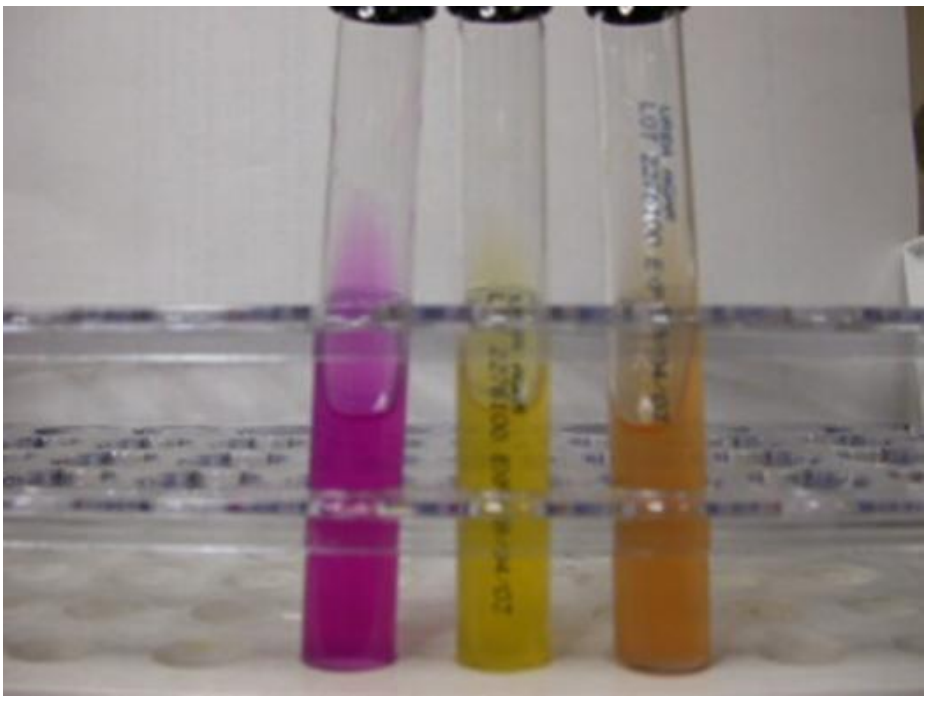


Figure 180. Positive - yellowish orange to pink

The API-20E test kit for the identification of enteric bacteria (bioMerieux, Inc., Hazelwood, MO) provides an easy way to inoculate and read tests relevant to members of the Family *Enterobacteriaceae* and associated organisms. A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrates the dessicated medium in each tube. A few tubes are completely filled (CIT, VP and GEL as seen in the photos below), and some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H_2S, URE).

After incubation in a humidity chamber for 18-24 hours at 37°C, the color reactions are read (some with the aid of added reagents), and the reactions (plus the oxidase reaction done separately) are converted to a seven-digit code. The code is fed into the manufacturer's database via touch-tone telephone, and the computer voice gives back the identification, usually as genus and species. The reliability of this system is very high, and one finds systems like these in heavy use in many food and clinical labs.

Note: Discussion and illustration of the API-20E system here does not necessarily constitute any commercial endorsement of this product. It is shown in our laboratory courses as a prime example of a convenient multi-purpose testing method one may encounter out there in the "real world."

In the following photos:

- Note especially the color reactions for amino acid decarboxylations (ADH through ODC) and carbohydrate fermentations (GLU through ARA).
- The amino acids tested are (in order) arginine, lysine and ornithine. Decarboxylation is shown by an alkaline reaction (red color of the particular pH indicator used).
- The carbohydrates tested are glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. Fermentation is shown by anacid reaction (yellow color of indicator).
- ·Hydrogen sulfide production (H2S) and gelatin hydrolysis (GEL) result in a black color throughout the tube.
- A positive reaction for tryptophan deaminase (TDA) gives a deep brown color with the addition of ferric chloride; positive results for this test correlate with positive phenylalanine and lysine deaminase reactions which are characteristic of *Proteus*, *Morganella* and *Providencia*.

In the first set of reactions:

· Culture "5B" (isolated from an early stage of sauerkraut fermentation) is identified as *Enterobacter agglomerans* which has been a convenient dumping ground for organisms now being reassigned to better-defined genera and species including the new genus *Pantoea*. This particular isolate produces reddish (lactose +), "pimply" colonies on MacConkey Agar which exude an extremely viscous slime as may be seen here; this appearance is certainly atypical of organisms identified as *E. agglomerans* or *Pantoea* in general.

· Culture "8P44" is identified as *Edwardsiella hoshinae*. The CDC had identified this culture (in 1988) as the ultra-rare Biogroup 1 of *Edwardsiella*

tarda which may not be in the API-20E database. This system probably would not be able to differentiate between these two organisms.

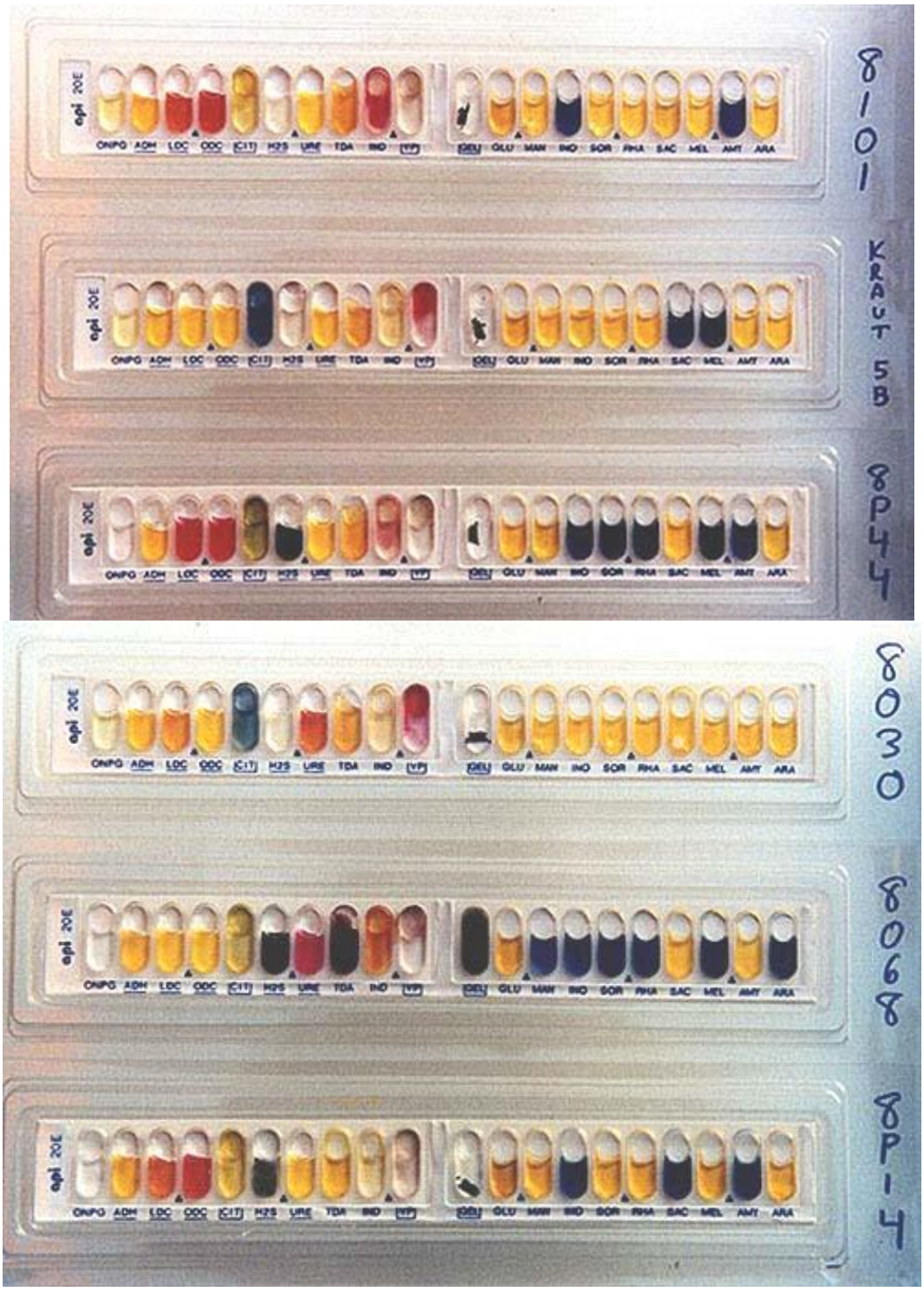


Figure 181. The API-20E test kit for the identification of enteric bacteria

Serological identification (according to the bacterial antigens)

All immunological tests are based on specific antibody-antigen interaction. These tests are called serological since to make them one should use antibody-containing sera.

Serological tests are employed in the following cases: (a) to determine an unknown antigen (bacterium, virus, toxin) with the help of a known antibody; (b) to identify an unknown antibody (in blood serum) with the help of a known antigen. Hence, one component (ingredient) in serological tests should always be a known entity.

The main serological tests include tests of agglutination, precipitation, lysis, neutralization, and their various modifications.

Agglutination Tests

Every individual species of bacterium has a unique collection of 3D shapes on its surface, called antigens. These are formed by the molecules on the outside of the cell wall. When a bacterium infects a human or an animal, the immune system reacts to these antigens, making a specific antibody to each one. Antiserum raised against a known bacterial species can therefore be used to positively identify if that species is present in an unknown culture. A small amount of different antiserum, specific for different bacteria, is used to test a sample. When the result is positive, the bacteria clump together, or agglutinate; when the result is negative, no clumping occurs.

Lancefield Grouping (Streptex Method)

- Emulsify a loopful of the test culture in 0.4 ml of extraction enzyme.
- Incubate at 37^{o}C for 1 hour.
- Add one drop of latex reagent to the appropriate circle of a black tile.
- Next, add one drop of extract to each circle and mix, using a wooden stick.
- Rock gently for one minute.
- Clumping indicates a positive reaction.

Presumptive agglutination test. A presumptive AT is performed on glass slides. Using a Pasteur pipette, transfer several drops of serum of low (1:10-1:20) dilutions and a drop of isotonic saline for control on a grease-free glass slide. Into each drop of the serum as well as in the control drop, inoculate a loopful of 24-hour living culture of the microorganism picked from the surface of a solid nutrient medium or pipette one drop of the suspension of dead microorganisms (diagnosticum). The inoculated culture is thoroughly mixed until the drop of liquid is uniformly turbid.

The reaction takes place at room temperature. Inspect visually the results in 5-10 min; occasionally one may use a 5 X magnifying lens for this purpose. If the glass slides are placed into a humid closed chamber to prevent evaporation, the results of the test may be read in 30-40 min as well.

A positive test is indicated by the appearance in the drop with serum of large or small flakes, readily visible upon rocking of the cover-slip. In a negative test, the fluid remains uniformly turbid.

Figure 182. Presumptive agglutination test

Slide agglutination

In cases where the number of microorganisms is small and the results of the test are difficult to interpret, dry the drop of the inoculated serum, fix the preparation, stain it with Pfeifier's fuchsine, and study under the microscope. In a positive test, a microscopic field is largely free of microorganisms but they are accumulated in some places. In a negative test, microorganisms are uniformly distributed throughout the microscopic field. This test is known as microagglutination.

Bilological identification

Biological examination. Biological study consists of infecting animals for the purpose of isolating the culture of the causative agents and their subsequent examination for pathogenicity and virulence.

Choice of experimental animals depends on the aim of the study. Most frequently used are rabbits, guinea pigs, albino mice, and albino rats. This is explained by the fact that they are susceptible to the causative agents of various infections diseases in man, easy to handle, and propagate readily. Hamsters, polecats, cotton rats, monkeys, birds, etc. may also be occasionally infected.

Specialized, particularly virological, laboratories, make use of genetically standardized, so-called inbred animals (mice, rabbits, guinea pigs, and others).

Working with experimental animals, one should keep it in mind that they may have spontaneous bacterial and viral diseases and latent infections activated as a result of additional artificial inoculation. This hinders the isolation of pure culture of the causative agent and determination of its aetiological role. Gnotobiotes (without microflora) and animals free of pathogenic microorganisms have no such drawback. Currently they include chickens, rats, mice, guinea pigs, pigs, etc.

Laboratory animals are distinguished by their species, age, and individual sensitivity toward microorganisms. Thus, in selecting animals for study it is necessary to take into account their species and age. For instance, sensitivity in mongrel animals may show considerable individual variations. The use of inbred animals with a definite constant susceptibility toward microorganisms excludes individual variations in sensitivity and allows for reproducible results.

Animals are infected for .isolating pure culture of the causative agent in cases where it is impossible to obtain it by any other method (for example, in contamination of the studied objects by extraneous microflora which inhibits growth

of the causative agent and in case of insignificant amounts of microorganisms or their transformation into filtering forms). Thus, in studying decayed corpses of rodents for the presence of plague causative agents, one inoculates (with suspension of the organs or blood) guinea pigs which die 3-7 days later with manifestations of septicaemia. Pure culture of the causative agent is readily isolated from the blood of internal organs.

Contamination of susceptible animals for reproducing the infectious process is used in diseases caused by Rickettsia and viruses.

Injection to mice of material from a patient with tickborne encephalitis brings about paralysis and death in these animals. To determine pathogenicity and virulence of the causative agents of plague, tularaemia, botulism, anthrax, and some viral diseases, cultures obtained from patients arc inoculated into albino mice, guinea pigs. rats, or suckling mice.

Figure 183. Mouse with tetanus signs

Figure 184. Guinea pig with botulism signs

Phage Typing. Bacteriophage (or phage) are viruses that infect bacteria. Phage can be very specific in what bacteria they infect and the pattern of infection by many phage may be employed in *phage typing* to distinguish bacterial species andstrains. The molecules on the surface of the bacterial cell are also targets for bacteriophages (phages for short). These are viruses that infect bacteria and that associate with different bacterial species very specifically. It is therefore possible to identify bacteria by investigating which bacteriophages can bind to their surface.

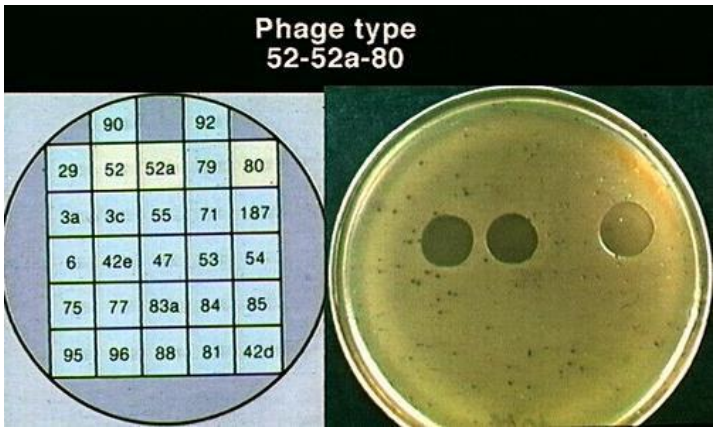


Figure 185. Phage Typing

Protein analysis [gel electrophoresis, SDS-PAGE, establishment of clonality]

I. The size and other differences between proteins among different organisms may be determined very easily employing methods of protein separation using methods collectively known as *gel electrophoresis*.

II. SDS-PAGE:

 One popular technique goes by the name *SDS-PAGE* which stands for *sodium dodecyl sulfate-polyacrylamide gel electrophoresis*

 Note that another name for *SDS* is *sodium lauryl sulfate,* a detergent you will find in many shampoos.

Such methods are very good at detecting small differences between isolates and are especially good at *establishing clonality*.

Protein and DNA Sequencing

In the last 25 years, molecular biology has developed rapidly and it is now possible to sequence the proteins from different bacterial species, make large databases of the sequences, and use them as very powerful identification tools. Similar database have been developed for bacterial DNA and bacterial RNA, particularly the RNA that forms the structural components of bacterial ribosomes.

Such techniques are also being used to follow the development of strains of bacterial species that are currently evolving at a very rapid rate. Strains of Chlamydia trachomatis, for example, are known to be exchanging large numbers of genes, forming completely new strains in a very short time. This is worrying – this bacterium is responsible for taking the sight of 8 million people living in developing

countries today. Identifying the new strains and studying how they have arisen so quickly is crucial to controlling infection and preventing new cases of blindness.

There are a few basic things regarding 16S ribosomal RNA gene analysis. The actual mechanics of the various parts of this test can be found elsewhere on the web or in an up-to-date textbook, and they may be summarized here in the future. With this comparative test, differences in the DNA base sequences between different organisms can be determined quantitatively, such that a phylogenetic tree can be constructed to illustrate probable evolutionary relatedness between the organisms.

The nucleotide base sequence of the gene which codes for 16S ribosomal RNA is becoming an important standard for the definition of bacterial species. Comparisons of the sequence between different species suggest the degree to which they are related to each other; a relatively greater or lesser difference between two species suggests a relatively earlier or later time in which they shared a common ancestor.

A comparison between eleven species of gram-negative bacteria is illustrated on a separate sequence comparison page, where the sequences are aligned such that similarities and differences can be readily seen when one scrolls to the right or left. Gaps and insertions of nucleic acid bases (the result of "frame-shift" mutations occuring over eons of time as the organisms diverge from common ancestors) which affect long stretches of DNA have to be taken into account for a proper alignment.

In an earlier version of the above-mentioned sequence comparison page, when only four species were compared with each other, a relatively short segment stood out as appearing to be "frame-shifted" when comparing *Pseudomonas fluorescens*with a group of three enterics. This situation is shown as follows with the nucleotide bases of the segment in question shown in red.

Table 28. Protein and DNA Sequencing

Pseudomonas fluorescens	...gctaataccgcatacgtcctacgggagaaagcagggg...
Our new organism, shown below as "AH"	...gctaataccgcataacgtcgcaagaccaaagcggggg...
Budvicia aquatica	...gctaataccgcgtaacgtcgaaagaccaaagcggggg...
Edwardsiella tarda	...gctaataccgcataacgtcgcaagaccaaagtggggg...

One can surmise that a frame-shift mutation – if the bases are not misplaced to the extent that the mutation becomes silent or lethal – could be a "cheap" way to effect a major change in the genotype and subsequent phenotype – perhaps resulting in one of those infamous "leaps" in evolution one hears conjectured about from time to time. Even though the specific sequence within a shifted segment of DNA may not be changed, the shift will result in the nucleotide bases being re-grouped into different triplet codes and read accordingly, and the resulting gene may produce a vastly different protein which can change the appearance or function of a cell to a significant extent. So, when sequences between two species are compared, the

organisms may appear to be a bit more closely related if these relatively short frame-shifted segments were taken into consideration. (With long stretches of DNA, one would not expect independant genes farther along the chromosome to be affected.)

When a 1308-base stretch of that part of the chromosome which codes for 16S ribosomal RNA was lined up and analyzed ("manually" when I had a little time to kill) to find the extent to which the above four organisms differed from each other, thepercent difference between any two organisms was determined, and the results are summarized as follows:

Table 29. When a 1308-base stretch of that part of the chromosome which codes for 16S ribosomal RNA

PF	PF			
AH	14.8*	AH		
BA	14.5	3.2	BA	
ET	14.9	4.3	5.0	ET

* An example: The same bases appear in the same sequence, position by position, for each of the two organisms except for 14.8% of the time.

With the percent differences used to denote probable evolutionary distances between the organisms, a **phylogenetic tree** was roughed out to illustrate the relationships. The distances between any two organisms, when read along the horizontal lines, corresponds closely to the percent differences. (The bar at the bottom signifies approximately 1% base difference.)

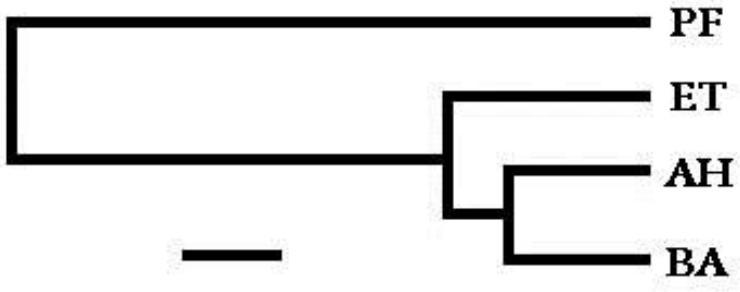


Figure 186. Phylogenetic tree

Databases of various gene sequences are found on the web. Genbank's database was used as the source of the above sequences. And rather than having to line up the sequences and determine the differences manually, a set of programs to analyze sequence data and plot trees are available.