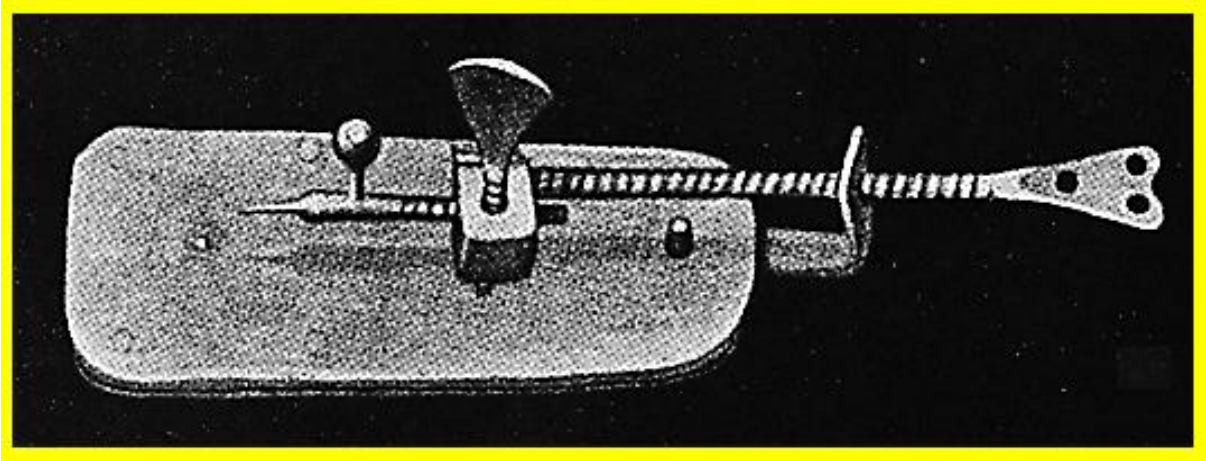
Origins, Cells and Elementary Staining Methods of Microbiology

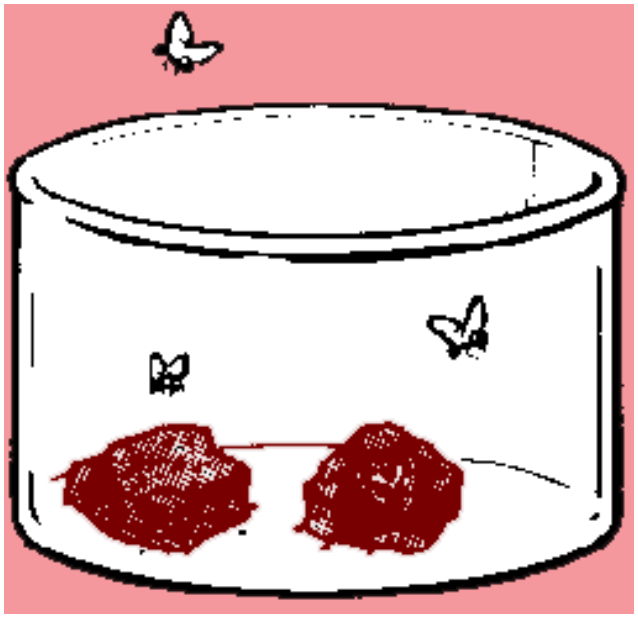
A Rapid Overview

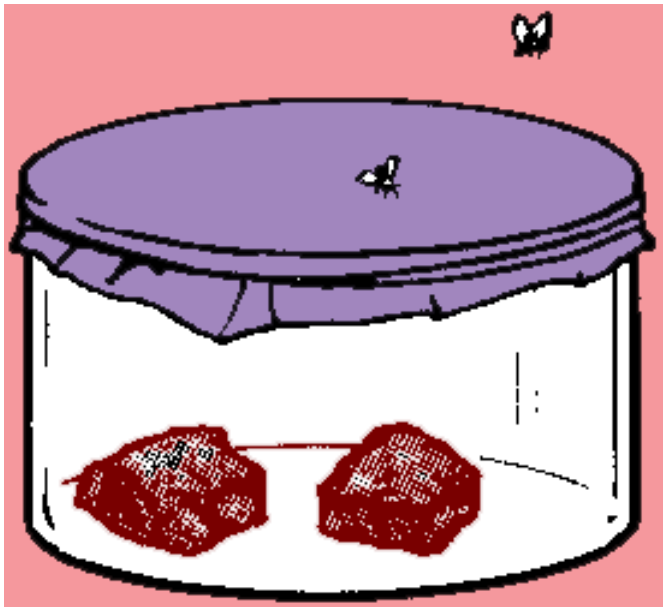
- In order to have an appreciation of today's microbiological database, it is necessary to step back in time and see how Microbiology began.
- From earliest days (Leviticus), the Jews were aware that leprosy and gonorrhea were contagious. At that time, though, the contagiousness was attributed to supernatural causes.

- In 1546, an Italian, Hieronymus Fracastorius published his paper, *De Contagione*.
- He presented the concept that epidemic disease was due to the transmission of an agent from one individual to another.
- His work, like that of Spallanzini's (coming up shortly), was ahead of its time and largely "blown off" by the scientific community.

- In 1665, a Dutchman, Anton van Leeuwenhoek, with a lens of his own making, looked at water and saw "little animals more than a thousand times less than the eye of a full grown louse".
- He called them "animalcules".
- In 1683, he saw various sorts of microorganisms in scrapings from his own teeth.
- In both cases, he had made his own microscopes, Figure below, with which to examine small particles.







- In 1668, another Italian, Francesco Redi, showed that putrefying flesh did not give rise to maggots if flies were excluded from the putrefying flesh.

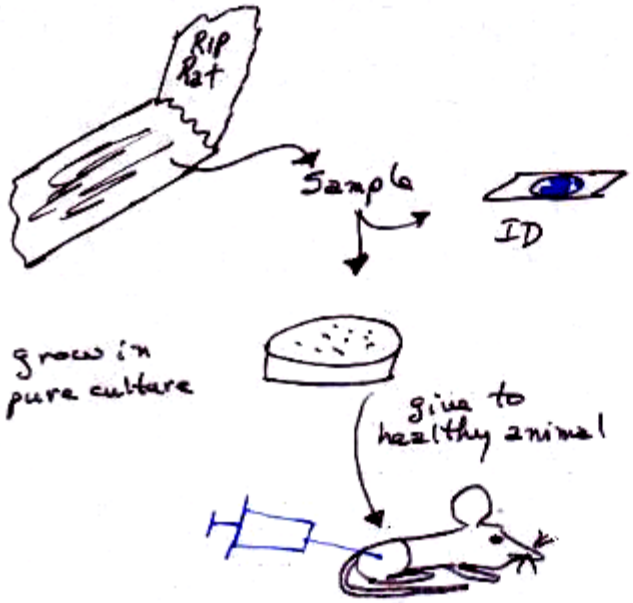
- In 1765 and 1776, Lazzaro Spallanzini, another Italian researcher, showed that "animalcules" failed to appear in infusions if the flasks were boiled long enough and stringent precautions were taken to prevent the entry of air.
- Spallanzini's work (just like Fracastorius') was ahead of its time and regression to the concept of spontaneous microbial generation occurred.

- Between 1835 and 1836, Agostino Bassi, an Italian scientist, demonstrated that a disease in silk worms was contagious and could be transmitted naturally by direct contact or infected food, or experimentally by means of a pin previously sterilized in a flame.
- The causative agent was later shown to be a fungus.
- In later writings, he argued that this theory of "contagion by living organisms" was obviously applicable to human beings.

- A French scientist, Louis Pasteur, proved that the conversion of sugar to alcohol in the production of beer and wine was caused by the activity of living microorganisms. He confirmed Spallanzini's results using a curved-neck flask, Figure right, concluding that microbes are not spontaneously generated from dead organic matter.

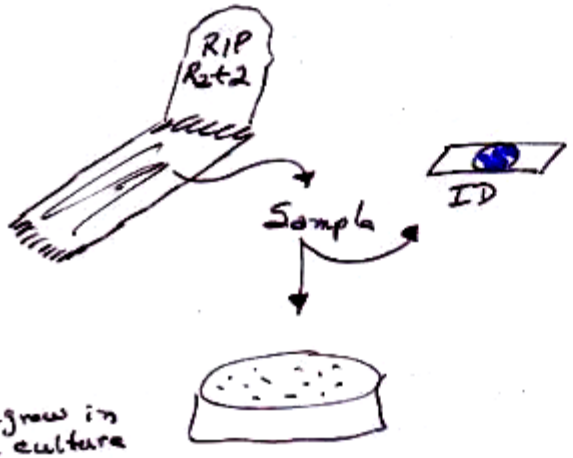
- A German Microbiologist, Robert Koch, is credited with the complete establishment of the germ theory of disease. His theory is now called Koch's Postulates. In short, Koch's postulates may be summarized as follows:
  1. **The same pathogen MUST be present in every case of the disease.**
  2. **The pathogen MUST be isolated from the diseased host and grown in pure culture.**
  3. **The pathogen MUST cause disease when a healthy animal is injected with it.**
  4. **The pathogen MUST be isolated from the second animal and MUST be shown to be the original organism in pure culture.**
- In 1876, Koch proved that *Bacillus anthracis* was the causative agent of anthrax. In 1881, he demonstrated streaking bacteria on gelatin to obtain pure cultures. Agar is now the medium of choice. In 1882, he announced that a microorganism, Mycobacterium tuberculosis, caused *tuberculosis*.

Koch's Postulates



① Same pathogen MUST be present in ⅋ case of the disease.

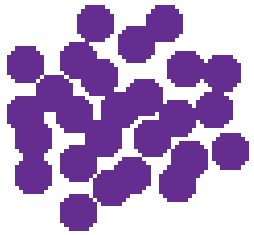
② Pathogen MUST be isolated from diseased host + grown in pure culture.

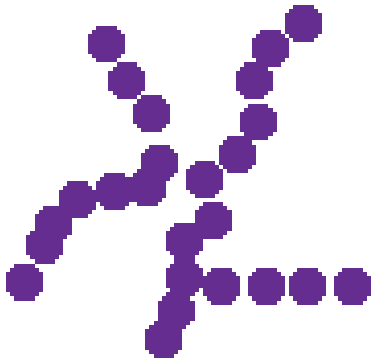


③ Must cause disease when healthy animal injected ≥ it

④ Pathogen must be isolated from 2ⁿᵈ animal + must be shown to be the original organism.

- Between 1880 and 1882, Alexander Ogston, a Scotsman, showed that cocci (spherically shaped bacteria) produced inflammation and suppuration and were the main cause of acute abscesses.
- He discovered and named *Staphylococcus* (staphyle = grape-like clusters) and differentiated them from *Streptococcus* (strepto = chains).





- In 1892 and, then, again, in 1898, Ivanowsky and Beijerinck, respectively, independently showed that mosaic disease of the tobacco plant could be transmitted to healthy plants by means of tissue juices freed from bacteria by filtration.
- Loeffler and Frosch in 1898 reported that foot and mouth disease of animals could be transmitted by bacteria-free filtrates.
- These experiments demonstrated that there were other pathogens capable of causing diseases that were smaller than bacteria.

- Twort and d'Herelle in 1915 and 1917, respectively, showed that bacteria may also become infected by phages (a class of virus).
- In 1940, Chain, Florey and Fleming opened the antibiotic era by showing that penicillin (PCN) was an effective chemotherapy agent.
- In 1949, Enders showed that poliovirus was growable in tissue culture. It soon became clear that viruses were studyable in any well-equipped lab.

- West Nile virus was discovered in the West Nile area of Uganda in 1937, then spread to Mediterranean and temperate parts of Europe.
- In 1960, it was observed in horses in Egypt and France.
- Between the 1950s and 1999, there were sporadic epidemics in Israel, South Africa, Romania and in Russia.
- In September 2002, American researchers reported the first polio–like paralysis stemming from West Nile virus. Infectious disease specialists in Ontario began seeing West Nile patients hooked up to ventilators, unable to move or breathe.

- Mad-Cow Disease, otherwise known as bovine spongiform encephalopathy (BSE), has been in the world news in recent years with an increasing number of cases in England and elsewhere in Europe.
- The disease is believed to have started as mutation of a protein molecule in a cow around 1970 in England, and it is believed to be spread by molecules called prions.
- On March 20, 1996 it was announced in England that the disease had crossed the species barrier and appeared to be infecting people in England.
- In February 2003 there have been 122 BSE human deaths so far in England, and 132 total worldwide.

- Chlamydia was discovered in the late 1970s and is neither a typical bacteria nor a virus.
- It is very small in size, like a virus, and has some characteristics of bacteria but can't manufacture its own energy the way bacteria or viruses can. Instead, it acts like a parasite, entering cells and using their energy.
- It is caused by an organism known as *Chlamydia trachomatis*, but it is not always easy to detect.
- Ten percent of the time, people who have chlamydia will test negative for it.

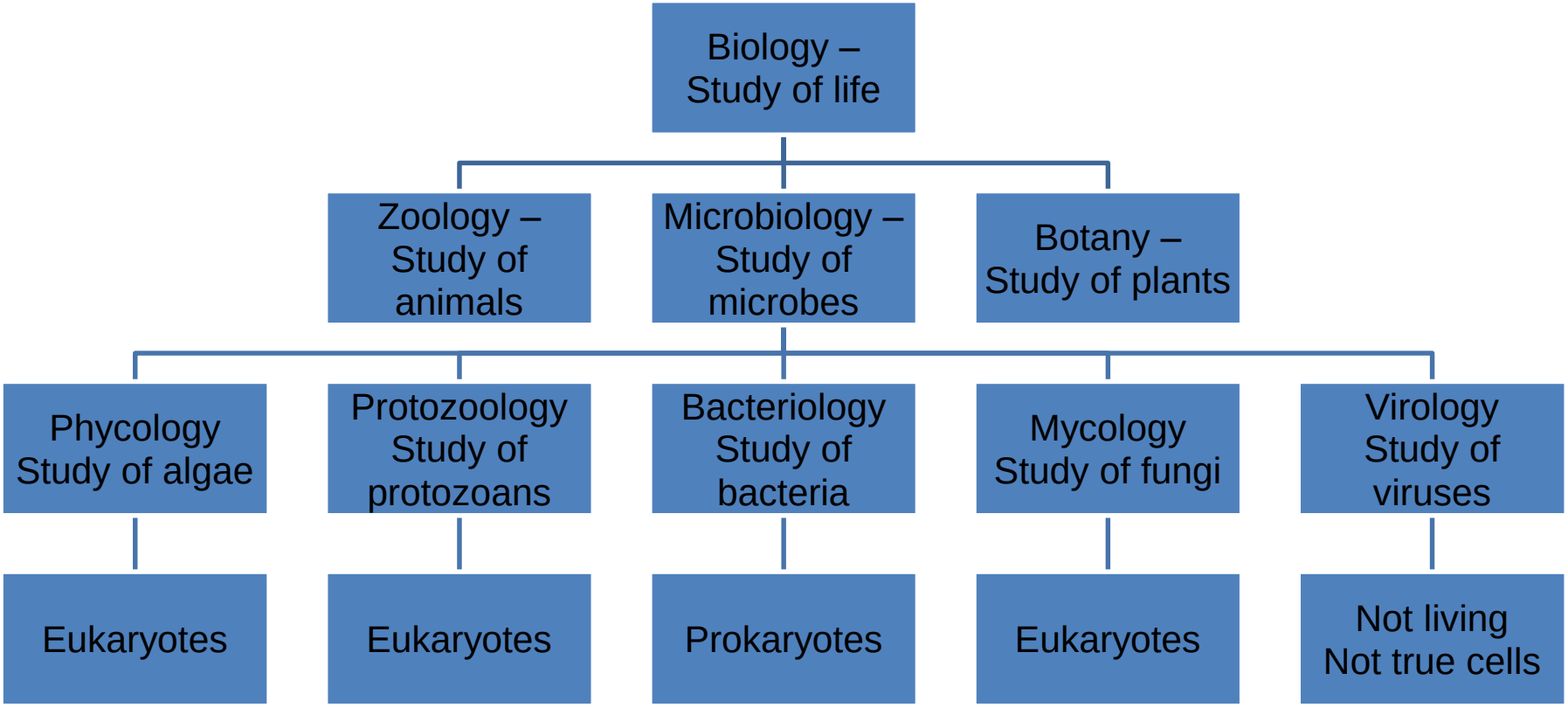
- Ebola virus, a lethal microbe originating in Africa that kills in two weeks of infection, was discovered in 1976.

- In 1977, statistical eradication of smallpox was demonstrated through international cooperation.
- This eradication must be taken with a certain grain of salt, however.
- The statistical eradication of a disease generally occurs as follows: 300 or more of the cases are identified in an area.
- Stringent vaccination programs are instigated.
- After there has been no disease for a pre-determined period of time, the disease is determine to be eradicated.
- Use caution when reading /hearing of this sort of eradication: Mother Nature has ways of bringing these diseases back, indeed, many in the scientific community are concerned about the return of smallpox since HIV has appeared in our society.

- A team of Pliva's researchers, Gabrijela Kobrehel, Gorjana Radobolja-Lazarevski and Zrinka Tamburasev led by Dr Slobodan Dokic, discovered azithromycin in 1980.
- It was patented in 1981, and was later found by Pfizer's scientists while going through patent documents.
- In 1986 Pliva and Pfizer signed a licensing agreement, which gave Pfizer exclusive rights for the sale of azithromycin in the Western Europe and United States.
- Pliva brought their azithromycin on the market in Central and Eastern Europe under the brand name of **Sumamed** in 1988, and Pfizer **Zithromax** in 1991.
- Azithromycin's name is derived from the **az**ane-substituent and ery**thromycin**.
- Its accurate chemical name is (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-13-[(2,6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11- [[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one.

- In mid-March 2003, it was announced to the world that there was a new pneumonia-like disease (with an incubation period of 2 to 7 days) causing a spreading epidemic in the world.
- A new disease epidemic of Severe Acute Respiratory Syndrome (SARS), started in Guangdong Province in China in November 2002, and then spread to other countries in Asia: Hong Kong, Vietnam, Indonesia, Phillipines, Thailand.
- It had also spread to Canada and the U.S..
- Many initial cases had been linked to a Hotel in Hong Kong.

Cells of Microbiology



Classification/Nomenclature of Bacteria

- On the basis of their morphology, staining reactions, nutrition, metabolism, antigenic structure, chemical composition and genetic homology, bacteria have been classified into orders, families, genera and species.
- Within a species, bacteria differing from each other in minor respects are variously designated as groups, types or varieties. Some properties may be characteristic of particular staining methods.
- Each distinct kind of bacterium is assigned a name indicating its genus and species -- in that order.
- The generic -- binomial -- name is often abbreviated in a standard manner. The tables, following, describe some of the binomial classifications:

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Positive	**Stains purple by the Gram Stain**			
Cocci	**Spherically shaped bacteria**			
	Staphylococcus	**aureus**	**S. aureus**	**Impetigo**
	Streptococcus	**pneumoniae**	**S. pneumoniae**	**Pneumonia**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Positive	**Stains purple by the Gram Stain**			
Rods	**Rod-shaped bacteria, a.k.a., bacilli**			
aerobes	**These microorganisms grow in the presence of air [oxygen]**			
	Bacillus	**anthracis**	**B. anthracis**	**Anthrax**
	Mycobacterium	**tuberculosis**	**M. tuberculosis**	**Tuberculosis**
	Nocardia	**asteroides**	**N. asteroides**	**Broncho-pneumonia**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Positive	**Stains purple by the Gram Stain**			
Rods	**Rod-shaped bacteria, a.k.a., bacilli**			
anaerobes	**These microorganisms grow in the absence of air [oxygen]**			
	Clostridium	**tetani**	**C. tetani**	**Tetanus**
	Actinomyces	**israelii**	**A. israelii**	**Lung abscesses**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Positive	**Stains purple by the Gram Stain**			
Rods	**Rod-shaped bacteria, a.k.a., bacilli**			
Facultative anaerobes	**Will grow under any conditions**			
	Corynebacterium	**diphtheriae**	**C. diphtheriae**	**Diphtheria**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Negative	**Stains pinkish/orange by the Gram Stain**			
Cocci	**Neisseria**	**gonorrhea**	**N. gonorrhea**	**Gonorrhea**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Negative	**Stains pinkish/orange by the Gram Stain**			
Aerobic rods	**Pseudomonas**	**aeruginosa**	**P. aeruginosa**	**Burn infections, urinary tract infections (UTI), pneumonia**
	Bordatella	**pertussis**	**B. pertussis**	**Whooping cough**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Gram Negative	**Stains pinkish/orange by the Gram Stain**			
Facultative anaerobes	**Escherichia**	**coli**	**E. coli**	**Enteritis**
	Klebsiella	**pneumoniae**	**K. pneumoniae**	**Pneumonia**
	Yersinia	**pestis**	**Y. pestis**	**Plague**
	Vibrio	**cholerae**	**V. cholerae**	**Cholera**
	Haemophilus	**influenzae**	**H. influenzae**	**Otitis media, meningitis**

Binomial Classification of Bacteria of Major Pathogenic Significance				
Characteristic	**Genus**	**Species**	**Abbreviation**	**Disease caused**
Spiral	**Treponema**	**pallidum**	**T. pallidum**	**Syphilis**
Cell Wall Deficient Organism	**Mycoplasma**	**pneumoniae**	**M. pneumoniae**	**Primary atypical pneumonia; walking pneumonia**

Most microorganisms received their names in ways that makes them fairly simple to remember -- with the exceptions like Erysipelothrix rhusiopathiae.
Some examples are listed in the table below:

How'd They Get Their Names?	
Streptococcus lactis	Chain of spheres; produce lactate in milk
Escherichia coli	Escherich's bacillus; in the colon
Salmonella typhi	Salmon's bacillus; typhoid fever
Staphylococcus aureus	Clusters of spheres; golden pigment
Trichomonas vaginalis	Flagellate; vaginitis
Saccharomyces cerevisiae	Sugar fungus; ferments beer

Bacteria come in different sizes. The table, below, compares the size of various microorganisms with red blood cells:

Relative Sizes of Selected Microorganisms			
RBC	8-10μ	Rickettsiae*	About 200-300 nm
Large protozoan	About 100 μ	Chlamydiae	About 100 nm
Yeast/Fungal Cell	About 30 μ	Poliovirus	About 10 nm
Bacterium	About 1 μ	*Limit of the compound microscope	

- There are three fundamental morphological forms of bacteria recognized:
  - spherical (coccus),
  - straight rod (bacillus -- do NOT confuse this with the genus Bacillus) and
  - curved or spiral rods (Vibrio, Campylobacter, Spirillum, Spirochaetes, Helicobacter).
- Within these three general categories there is great diversity.
- This diversity includes bacteria shaped like tennis rackets, clubs, chains, diplococci (two cocci joined together), kidney bean shaped, clusters, spirals and coccobacilli.
- On average, cocci are about 1m in diameter, rods are roughly 2-5 μ long and about 0.5-1 μ wide and spirochaetes average 5-20 μ long and about 0.1-0.2 μ wide.

Fungi

- Fungi are nature's decomposers
- Occasionally fungi from the non-pathogenic Phyla ARE pathogenic, e.g.,
  - Ascomycota: Candida, Trichophyton
  - Basidiomycota: Cryptococcus
- Generally under some sort of immunocompromisation that is either acquired "naturally" or iatrogenically

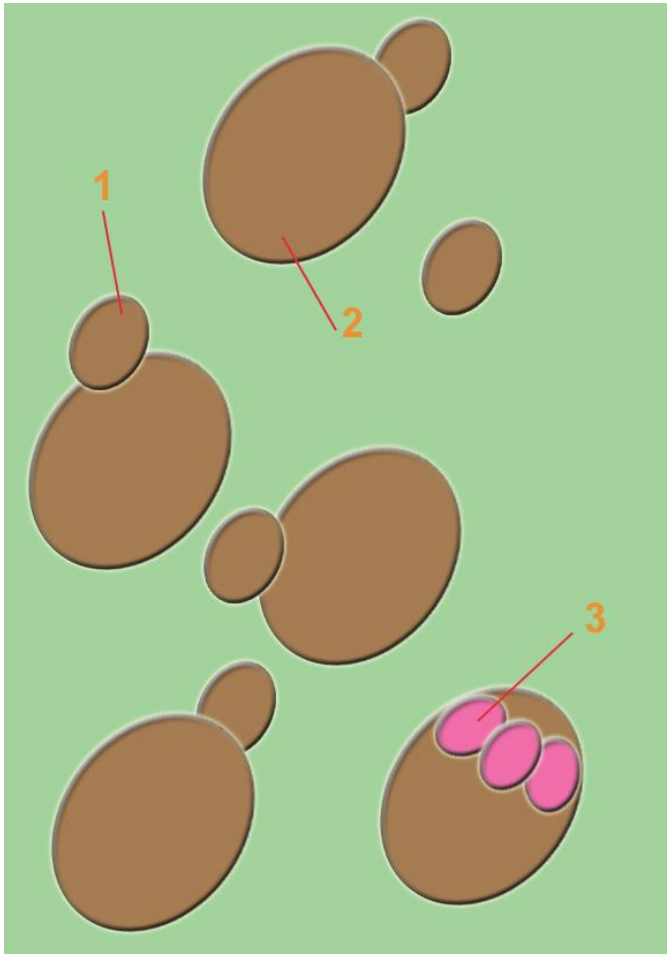
Fungi			
Ascomycotina	Basidiomycotina	Deuteromycotina	Zygomycotina
Ascomyces	Basidiomyces	Imperfect fungi	Phycomyces
Septate hyphae	Septate hyphae	Septate hyphae	Non-septate hyphae
Trichophyton, Blastomyces	Cryptococcus	Epidermophyton, Candida	Rhizopus
Generally not pathogenic	Generally not pathogenic	Are pathogenic	Generally not pathogenic

Subcutaneous, Cutaneous and Superficial mycoses	Skin, hair, nails; generally chronic and resists disease; generally does not affect patient's general health
Deep mycoses	a.k.a. systemic; may be systemic and may be fatal; these organisms live free in nature in soil or decaying organic debris; typical tissue reaction = granuloma with varying degrees of necrosis and varying degrees of formation of abscesses
Pathogenic fungi	As a rule do NOT produce toxins; induce hypersensitivity reactions

Fungal Growth Conditions: A Selected Few	
Grown on Sabouraud's agar	Glucose, beef extract, agar, pH 5.0; does NOT readily support bacterial growth
Sporothrix schenckii	cream colored to black, folded, leathery colonies within 3-5 days
Coccidioides immitis	white to tan colored cottony colonies
Histoplasma capsulatum	white to tan colored cottony colonies
Candida albicans	soft, cream colored colonies with yeasty odor
Cryptococcus neoformans	shiny, mucoid, cream colored colonies
Aspergillus fumigatus	gray-green colonies

Fungal Cells -- 3

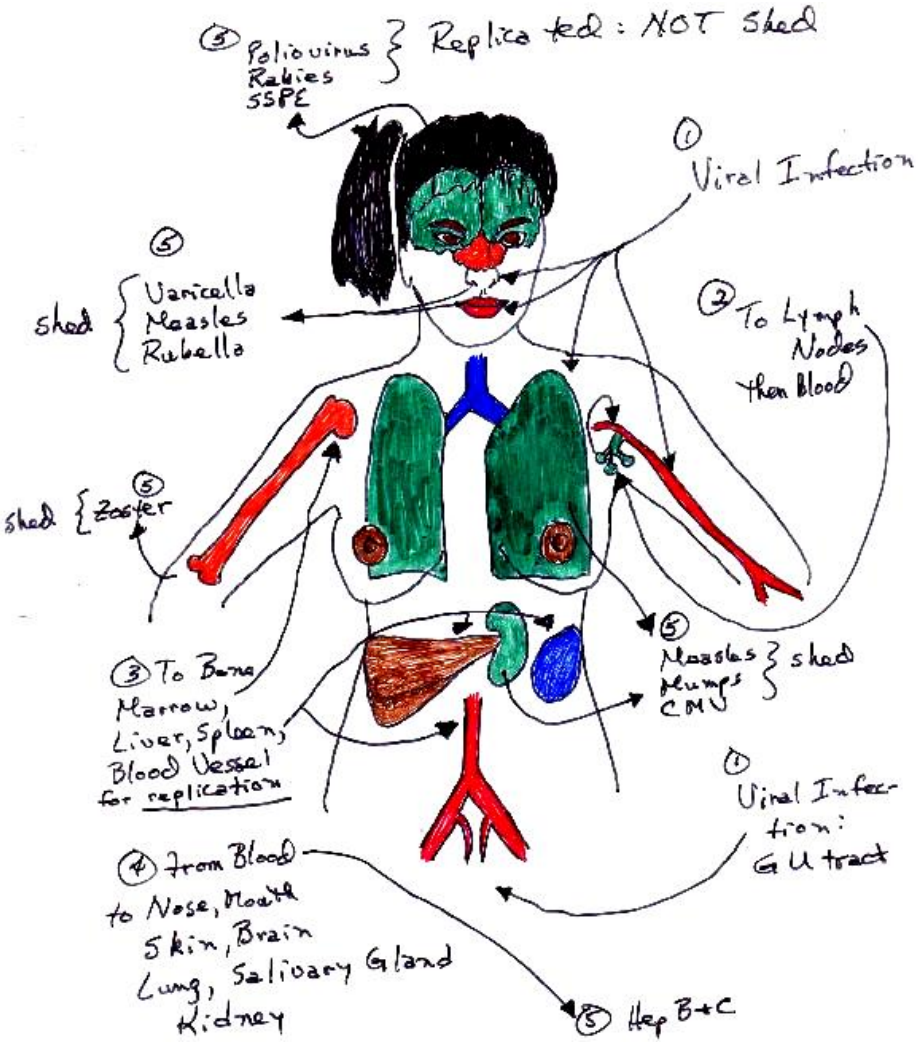
1. Bud
2. Mother cell
3. Budding scars



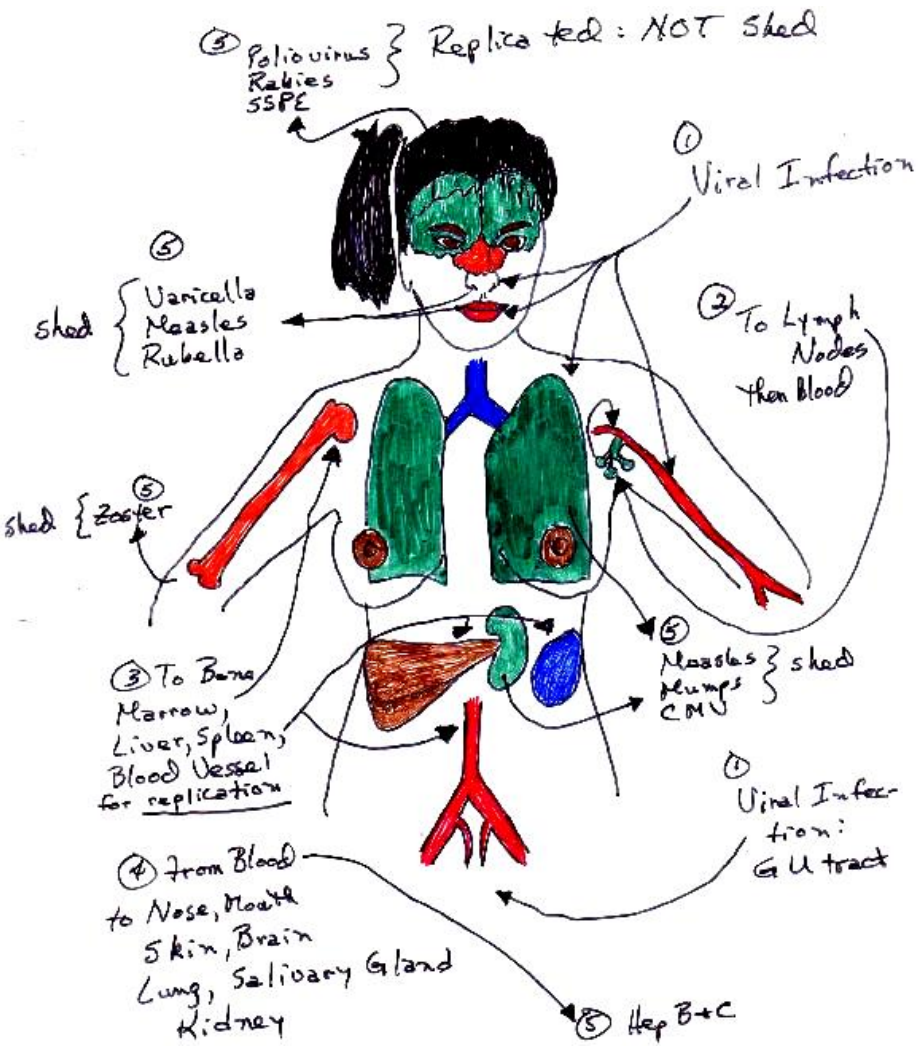
Viruses

- One of the most important things to remember about viruses is that they are not living organisms.
- They are particles that have the capability of being reproduced by our own cellular machinery and contain some of the necessary elements for that replication, e.g., reverse transcriptase in HIV.
- Whenever a virus is referred to as "live", it means that the virus is capable of causing disease.
- An attenuated "live" virus is a virus that is capable of eliciting an immune response in the body, may cause a lighter form of the disease or may cause no noticeable form of disease, at all.

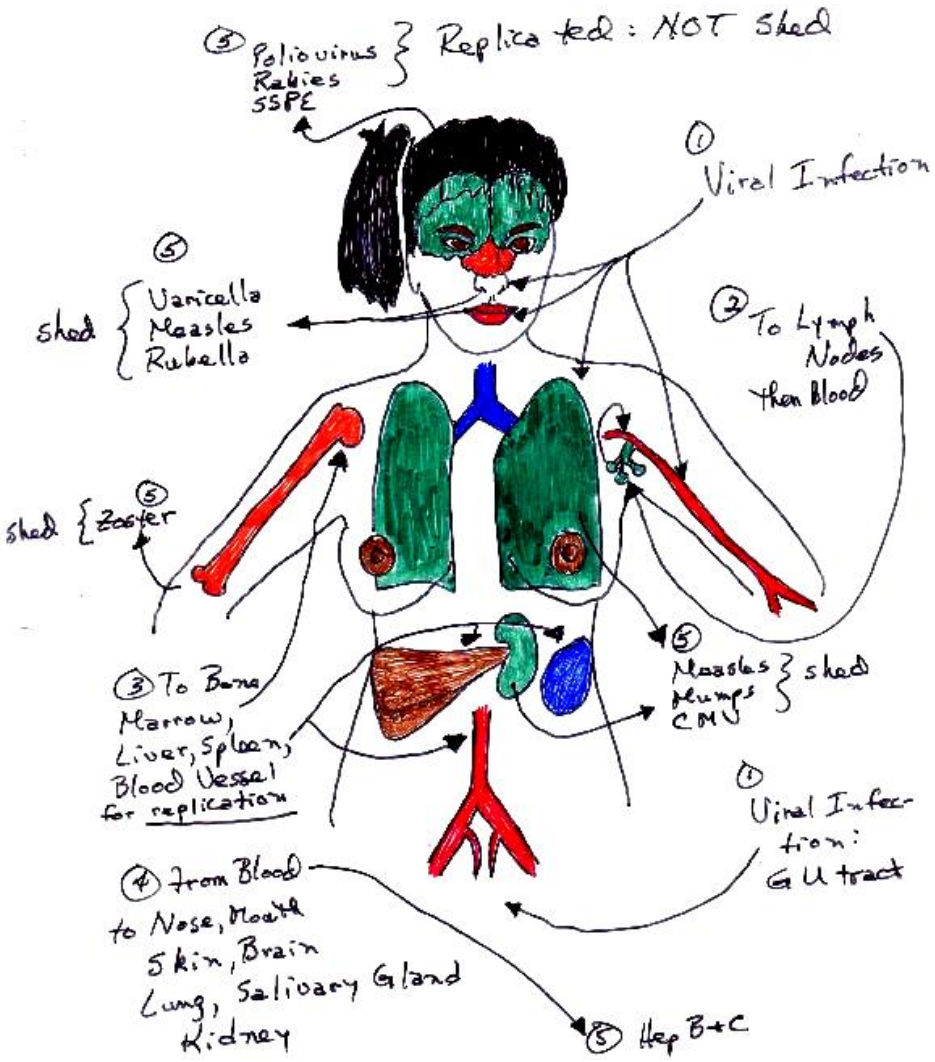
- Figure, right and following, illustrates sites of viral entry and viral replication and viral shedding.
- We become infected by viruses by either breathing them in, ingesting them or injecting them into our bodies.
- They may also be passed to us via the genitourinary tract.
- They then pass into our lymph nodes, thence into our blood.



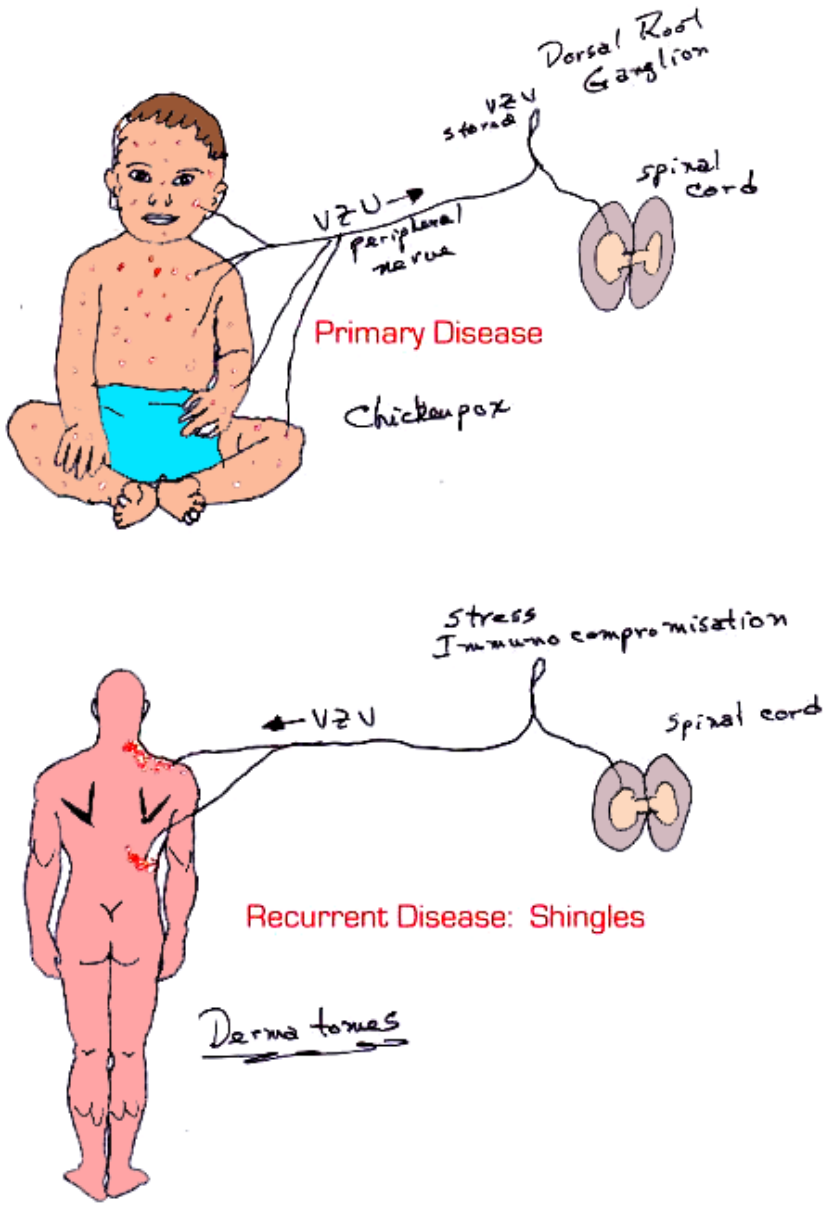
- The viral particles then travel to the bone marrow, liver, spleen and other blood vessels for further replication.
- Once replicated, the viral particles are released from our bodies via the nose, mouth, skin, brain, lung, salivary glands, kidneys, genitourinary system and bowel.



- Varicella, measles and rubella are shed from the nose and mouth; lungs and kidneys shed measles, mumps and cytomegaloviruses; blood sheds Hepatitis B and C viruses and HIV; the bowel sheds Hepatitis A and E viruses; skin sheds varicella zoster virus; the brain replicates, but does not shed, poliovirus, rabies virus and may develop SSPE (subacute sclerosing panencephalitis -- a complication of measles that will be discussed later).



- Figure, right, illustrates how VZV is stored in dorsal root ganglia following chickenpox, then re-released as adults when stress or immunocompromisation provide proper conditions for VZV expression as shingles. Note that shingles follows dermatomes.



The following information is from "Current Pediatric Diagnosis & Treatment, Edition 12", pp. 44, 1037, regarding chicken pox:

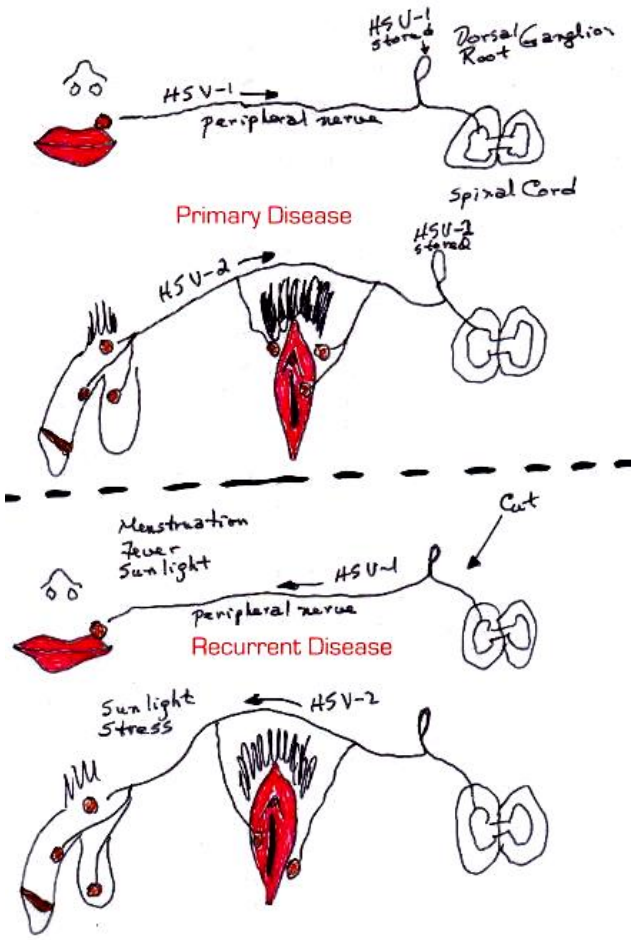
- "Congenital varicella is rare (5% after infection acquired during the first or second trimester) but does cause a recognizable constellation of findings, including limb hypoplasia, cutaneous scars, microcephaly, cortical atrophy, chorioretinitis, and cataracts. Perinatal exposure (5 days before to 2 days after delivery) can cause severe to fatal disseminated varicella. ... Prophylaxis and therapy are available for perinatal varicella. If maternal varicella develops within the perinatal risk period, 1.25 mL of varicella immune globulin (VIG) should be given. If this has not been done, the illness can be treated with intravenous Acyclovir.
- Very premature infants (< 28 weeks' gestation) who are exposed postnatally should receive varicella immune globulin because of poor antibody transfer across the placenta early in pregnancy.", p. 44.

- "Varicella pneumonia usually afflicts immunocompromised, pregnant, or older patients and may be fatal. Cough, dyspnea, tachypnea, rales, and cyanosis occur several days after onset of rash.
- Hemorrhagic varicella lesions may be seen without other complications. Usually due to autoimmune thrombocytopenia, hemorrhagic lesions can occasionally represent idiopathic disseminated intravascular coagulation (DIC; purpura fulminans).
- Varicella may be life threatening in immunosuppressed patients (especially those with leukemia or lymphoma or those receiving high doses of steroids). Their disease is complicated by severe pneumonitis, hepatitis, and encephalitis. Varicella exposure in such patients must be evaluated immediately.
- Neonates born to mothers who develop varicella from 5 days before to 2 days after delivery are at high risk of severe or fatal (5%) disease and must be given varicella-zoster immune globulin (VZIG) and followed closely.
- Unusual complications of varicella include optic neuritis, transverse myelitis, orchitis, and arthritis.", p. 1037.

- NOTE: Although this information doesn't come right out and say it, it seems that most of these cases of chicken pox are in women who have no demonstrable titers of anti-VZV.
- That is not to say that pregnant women who have had chicken pox won't, potentially, have the same sorts of problems when exposed to people with active chicken pox or shingles.
- Hence, I recommend that pregnant women refrain from caring for or coming into contact with those with active VZV infections during their first and third trimesters.

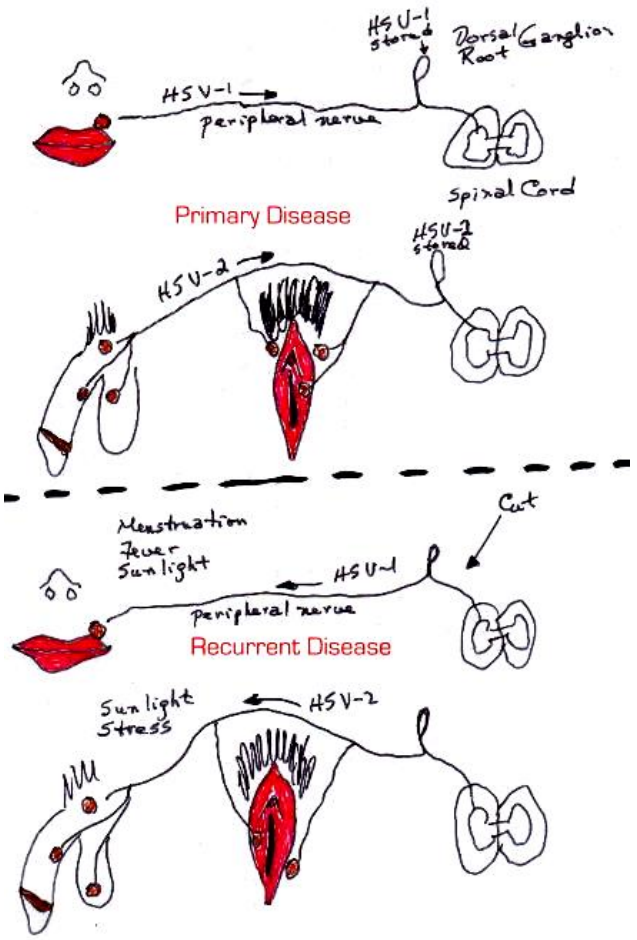
HSV – now called HHV

- The top portion of the figure, right, illustrates how HSV-1 and 2 invade the body after the body is infected with these viruses.
- The common thread to these two viruses is that they are transported to a dorsal root ganglion where they are each stored following the initial infection.

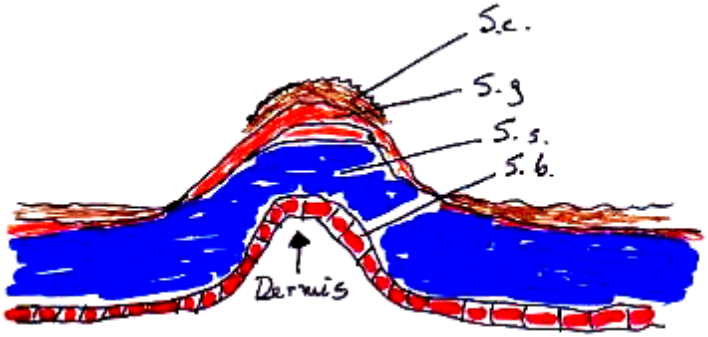


HSV – now called HHV

- Menstruation, stress, sunlight, sectioning the nerve between the cord and dorsal root ganglion all trigger release of HSV-1, causing a recurrence of the disease as evidenced by the appearance of a new lesion.
- The same factors cause the re-release of HSV-2 with the appearance of new genital lesions.



- Figure, right, illustrates the mechanics behind the formation of a wart, caused by **HPV**.
- Also keep in mind that condyloma accuminatum, genital warts, is also caused by HPV -- specifically, HPV6d.
- These lesions tend to be soft, pink, cauliflower-like lesions and occur on the external genitalia, in the vagina, on the cervix and in the rectum.
- The risks of developing cervical cancer and perianal cancer increase with a history of genital warts.
- 79% of common warts are caused by HPV2; 14% by HPV1 and plantar warts (on the plantar surface of the foot) are caused by HPV1a.
- Those with penile carcinoma tend to be co-infected with either HPV16 or HPV18; those with cervical cancer tend to be co-infected with HPV16.



Stratum spinosum contains cells actively replicating HPV DNA for migration into Stratum corneum + Stratum granulosum

Staining Reactions of Micro-Organisms

Includes Samples of Mechanisms of Bacterial Action

Flame Alternative

1. Bacti-Cinerator IV – Operating Instructions (per the User's Manual © 2003 Tyco Healthcare Group LP)
2. Plug cord in to outlet.
3. Turn switch to the "on" position (it will light up).
4. Allow to heat up for at least 15 minutes – at that time, the interior of the chamber is 816° C!!!
5. Obtain an inoculating loop with an insulated handle and carefully insert it inside the ceramic chamber – do not scrape the sides of the ceramic chamber with the loop – it can split.
6. Hold the loop in the back of the chamber for a minimum of 10 seconds.
7. The loop need not glow red as it does in a Bunsen burner to be sterilized.
8. Remove loop and let cool – about 15-30 seconds.
9. Use loop to obtain bacterial sample, streak or smear media or slide, then repeat steps 4-7 and set aside.
10. Repeat steps 4-7 every time you use the loop.
11. Turn off the Bacti-Cinerator IV at the end of the lab period or if everyone is done using it.

Staining Reactions of Microorganisms

- All staining techniques begin with preparing the sample. This involves one of two methods:

Method 1:

1. Mark a target circle on a microscope slide with a China marking pencil about the size of your thumbnail.
2. Flame (or bacticinerate) a bacteriological loop from the loop to where it joins the handle. You want to get each area red-hot.
3. Let it cool to room temperature WITHOUT touching anything.
4. Dip it into a sample of water and place the loop-ful of water in the target circle.
5. Reflame the loop and let cool.
6. Carefully remove a single colony of bacteria (certainly no more than the amount of pus you'd get out of a medium sized zit when it pops) from your solid media sample and place it in the water in your target circle.
7. Mix the water and bacteria together inside the target circle to make a thin smear.
8. Let air dry.

Method two:

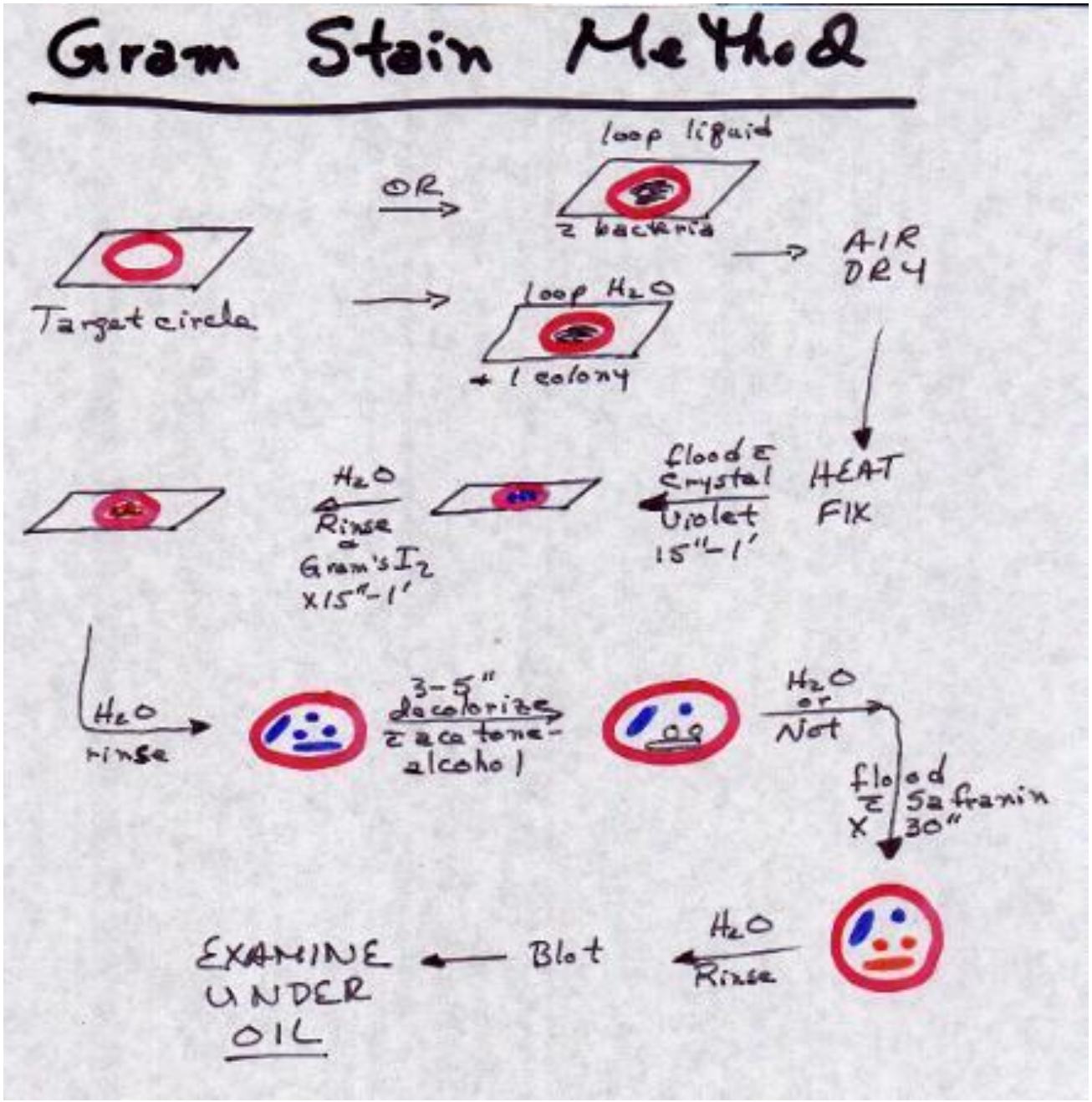
1. Instead of using water and bacterial colonies, use a drop of liquid media that has bacteria growing in it.
2. To remove the bacteria-containing sample, you remove the cap with the pinky finger of your strong hand and pass the open neck of the tube through the flame 2-4 times in your weak hand.
3. Dip your flamed (or bacticinerated) loop into the broth with the other fingers of your strong hand, remove the sample without touching the sides or neck of the tube and reflame the neck of the tube.
4. Replace the cap on the tube and smear the sample inside your target circle.
5. Flame (or bacticinerate) your loop to re-sterilize it.
6. Allow the sample to air dry.

- Regardless of the staining technique, all slides are prepared as above.

- With the exception of the capsule stain, the next step in the majority of microbiological staining is to heat fix the slide.
- This process kills the bacteria and fixes them to the slide so they won't wash off during staining or rinsing.
- To do this, you pass the slide through the flame of a Bunsen burner 2-5 times or until it feels like baby's milk on your wrist -- touch it carefully to your wrist so you don't get burned!
- Once you've heat fixed the slide, you may stain it as directed.
- Below (on the following slides) are the techniques for the Simple stain, Gram stain, Acid-fast stain and Spore stain.
- All of these techniques work best in our little part of the world -- techniques vary by location, altitude and quality of chemicals.

Simple Stain

- The first method is the Simple stain.
- This stain is very ... SIMPLE ... to perform, hence its name.
- To your heat-fixed slide, add methylene blue to the target circle for 1 minute.
- After the one-minute is past, rinse it with water and blot it dry with bibulous paper.
- It's ready for viewing under the microscope on oil-immersion.
- Everything in this slide will appear blue.
- The only information you can get from this method is the morphology of the microorganisms.


Gram Stain Method
loop liquid
OR
2 bacteria
AIR DRY
Target circle
loop H_2O
1 colony
HEAT FIX
flood Crystal Violet 15"-1'
H_2O Rinse
Gram's I_2 x15"-1'
H_2O rinse
3-5" decolorize 2 acetone-alcohol
H_2O or Not
flood 2 x Safranin 30"
H_2O Rinse
Blot
EXAMINE UNDER OIL

For the Student: Gram Stain Summarized

- The primary dye here is crystal violet. The mordant (a chemical that makes the dye stick better) is Gram's iodine. The counterstain (the stain that will stain another color after a decolorization step is used) is safranin. There are many techniques for this method. Indeed it is one of the simpler, faster, but most under-utilized diagnostic stain method around. As long as the Gram's iodine is left on the sample for as long as the crystal violet was, this technique is very amenable to flexibility. Pour crystal violet onto the target circle and leave it there for 15 seconds to 1 minute. Rinse it off with water. Pour Gram's iodine on the target circle and leave it there for 15 seconds to 1 minute. Rinse it off with water. The next two steps are critical, time-wise. Decolorize the stain with acetone-alcohol for 3-5 minutes, then immediately do one of the following: 1) rinse with water and then stain with safranin for 30 seconds or 2) immediately stain with safranin for 30 seconds. Then rinse the safranin off with water, blot dry with bibulous paper and the sample is ready for examination on the microscope under oil-immersion. The information one gets here is not only morphology, but also Gram reaction: purple is Gram positive and pinkish-orange is Gram negative. This information may be utilized in a sort of "choked shotgun" manner to prescribe more specific antibiotics for bacterial infections than if one simply went by statistics and used a more broad-spectrum antibiotic to "shotgun" the infection.

- As mentioned above, there are two classes of bacteria by this method: 1) Gram positive (purple because they retain the crystal violet after decolorization) and 2) Gram negative (pinkish-orange because they lose the crystal violet during decolorization and take the safranin counterstain).
- Gram positive species vary in the tenacity with which they retain the violet stain and some may be largely decolorized. Additionally, older cultures give rise to false negative reactions: it is usually safe to assume that organisms giving a doubtful reaction are Gram positive.
- Furthermore, bacteria are stained evenly and deeply by basic dyes. Basophilia is due to the high amount of RNA that is distributed uniformly through the cytoplasm.

There are differential qualities between the two classes of Gram classified bacteria:

1. The composition of the cell walls is different: gram positive bacteria are high in teichoic acid and peptidoglycan and little to no lipopolysaccharide (LPS); gram negative bacteria have no teichoic acid, are low in peptidoglycan and are high in LPS.
2. Gram positive organisms have less synthetic ability and so more complex nutritional requirements.
3. Gram positive bacteria produce primarily exotoxins (there are exceptions); gram negative bacteria produce only endotoxins.

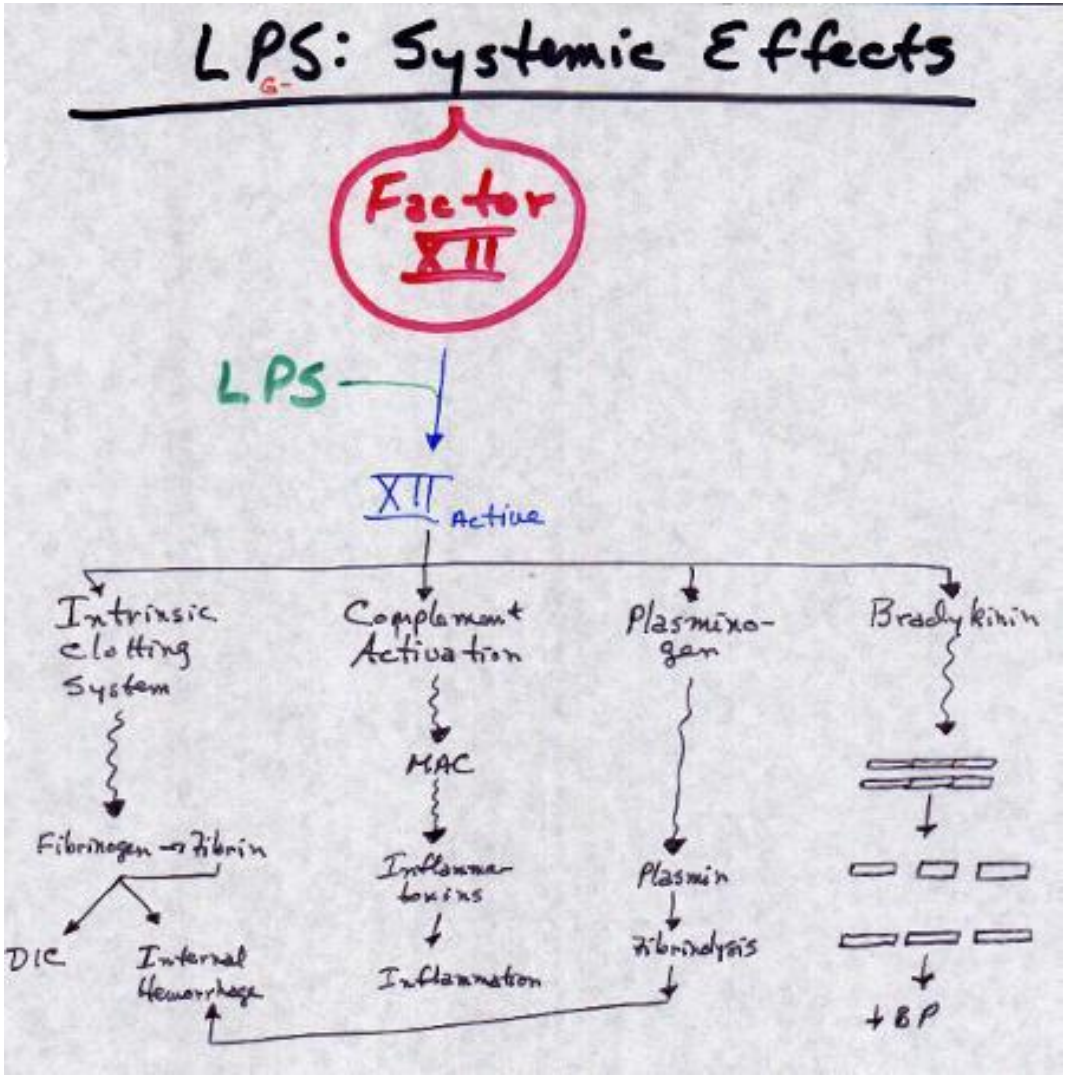
4. Gram positive bacteria are very resistant to mechanical damage -- this explains why they remain on our skin regardless of how well we wash and scrub.
5. The two types of bacteria have different spectra of susceptibility to chemotherapeutic agents, disinfectants, dyes, simple chemicals and enzymes.
6. Gram positive bacteria tend to be more susceptible to PCN; gram negative bacteria tend to be more susceptible to TET (tetracycline).

- The value in this system is that there are only four different "kinds" of bacteria: Gram positive rods or cocci and Gram negative rods or cocci. **In General:**

1. All cocci of medical importance are gram positive EXCEPT for the genera Neisseria, Branhamella (now Moraxella) and Veillonella.
2. All rods of medical importance are Gram negative EXCEPT for the genera Lactobacillus, Mycobacterium, Corynebacterium, Bacillus and Clostridium.
3. If the Gram stain is questionable it is reported as gram positive.

Endotoxins vs Exotoxins

- **Endotoxins** are part of the cell walls (LPS; Figure right) and are released upon bacterial death. They are released only from gram negative bacteria. They are very temperature stable: at temperatures above 60° C, they are still toxic. They have very low antigenicity, so vaccines are uncommon. 10's to 100's of micrograms cause death. They raise body temperature (cause fever) increase hemorrhage, increase swelling in tissues and induce vomiting and diarrhea. Typical bacteria include coliform bacteria. The effects of LPS, systemically, are summarized in Figure, right.



- **Exotoxins** are excreted by the living cell into its environment. These toxins are excreted in high concentrations.
- They are produced by gram-positive bacteria (only rarely by gram negative bacteria). Their composition is polypeptide and they are unstable at temperatures above 60°C.
- They have very high antigenicity and are used in the formation of antitoxins (non-toxic toxoids) by heat treatment, formalin or ether treatments for immunizations.
- Less than microgram quantities cause death.
- It has been approximated that a 6-oz. bottle of Clostridium botulinum exotoxin would be enough to decimate the world's population.
- Exotoxins are located in the cytosol of the living cell. They interfere with synapses, protein synthesis (translation), they increase capillary permeability and increase water elimination.
- Typical bacteria include the spore formers, e.g., aerobic spore formers (Bacillus) and the anaerobic spore formers (Clostridium).

The table, below, summarizes exotoxins released from various microbes and the disease caused by them:

Exotoxin	Example	Gram reaction	Effected structure	Disease
Neurotoxin	C. botulinum	+	Neuromuscular junction	Botulism
Neurotoxin	C. tetani	+	CNS	Tetanus
a toxin	C. perfringens	+	General	Gas gangrene
Diphtheria toxin	C. diphtheriae	+	General	Diphtheria
Enterotoxin	S. aureus	+	Nerve cells	Food poisoning
a toxin	S. pyogenes	+	General	Pyogenic infections
Hemolysins	Strep/Staph	+	Lyse RBC	Septicemia
Guinea pig toxin	Y. pestis	-	General	Bubonic plague

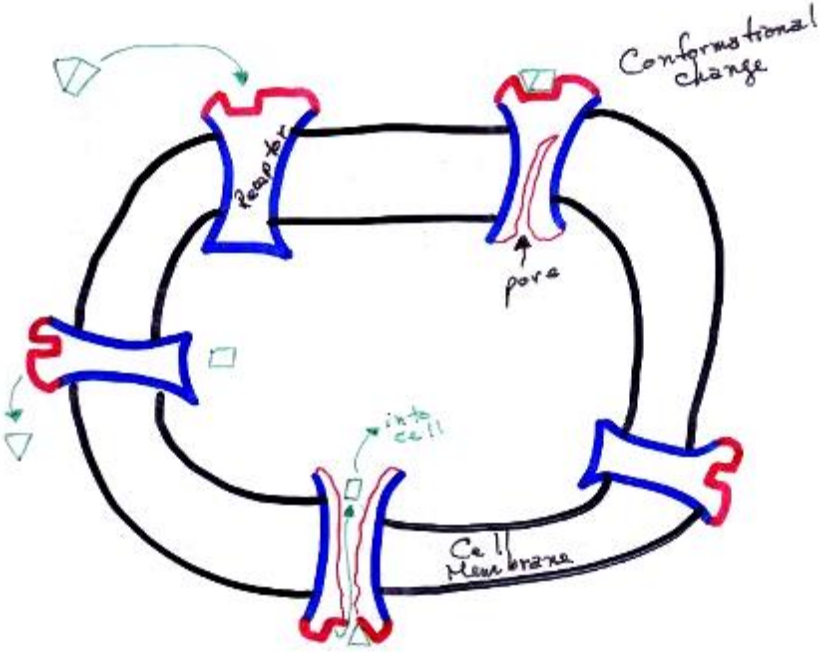
ASIDE

- Endemic disease is when the disease is at a constant in a constant geographical region with low morbidity (diseased state) rate.
- Epidemic disease is when the disease has a morbidity rate increased above "normal" with a high mortality rate or will cause public harm.
- Pandemic disease is an over-grown epidemic, e.g., the geographical are has enlarged significantly, and the number of people with the disease is increasing at an alarming rate with increased mortality (death rate) rate.

End of Aside

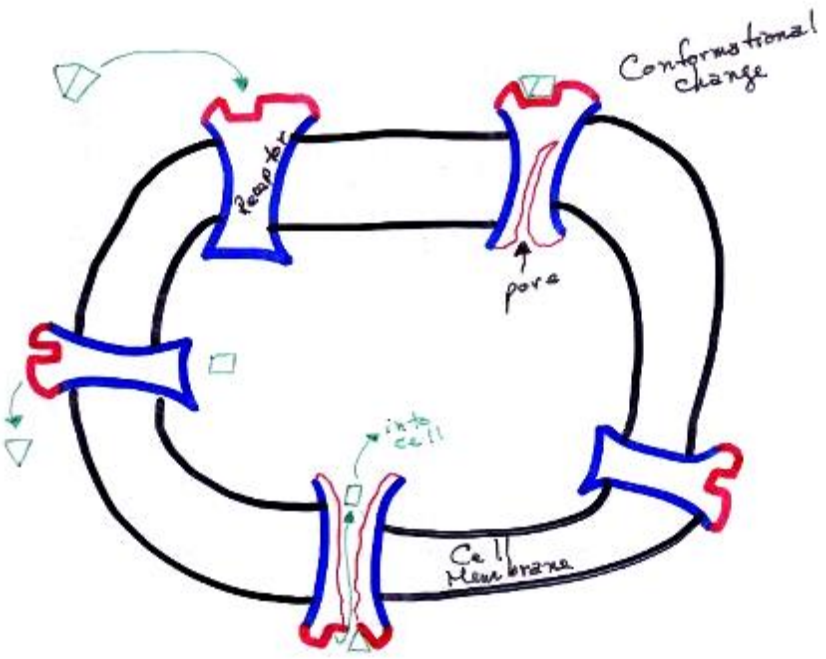
Exotoxin Mechanisms of Action

- Exotoxins invade cells by a mechanism repeated throughout nature: receptor binds the exotoxin, internalizes a part of the exotoxin that exerts its effects inside the cell, right. In a nutshell, the exotoxin binds to an integral protein in the cell membrane of the host.



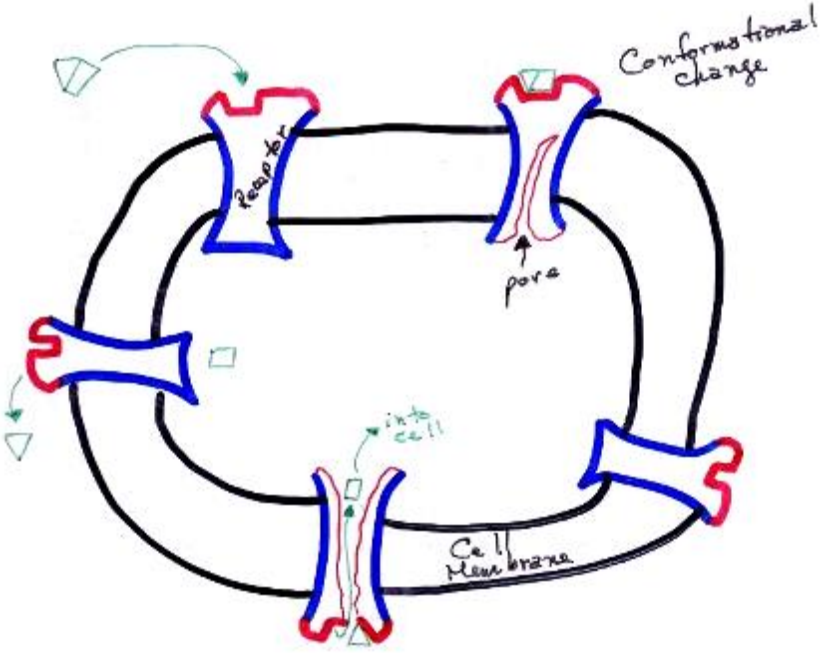
Exotoxin Mechanisms of Action

- This binding causes a conformational change in the integral protein which permits the entry of a part of the exotoxin (the portion that lacks cell-binding capability (**the square in this image**), but that is responsible for the toxic effects inside the cell; this portion is enzymatically active) and then releases another part of the exotoxin (the portion that is non-toxic and biologically inactive, but that is required to cause the conformational change in the integral protein [receptor] to convert to a pore to permit the biologically active portion inside the cell to cause disease (**the triangle in this image**)).

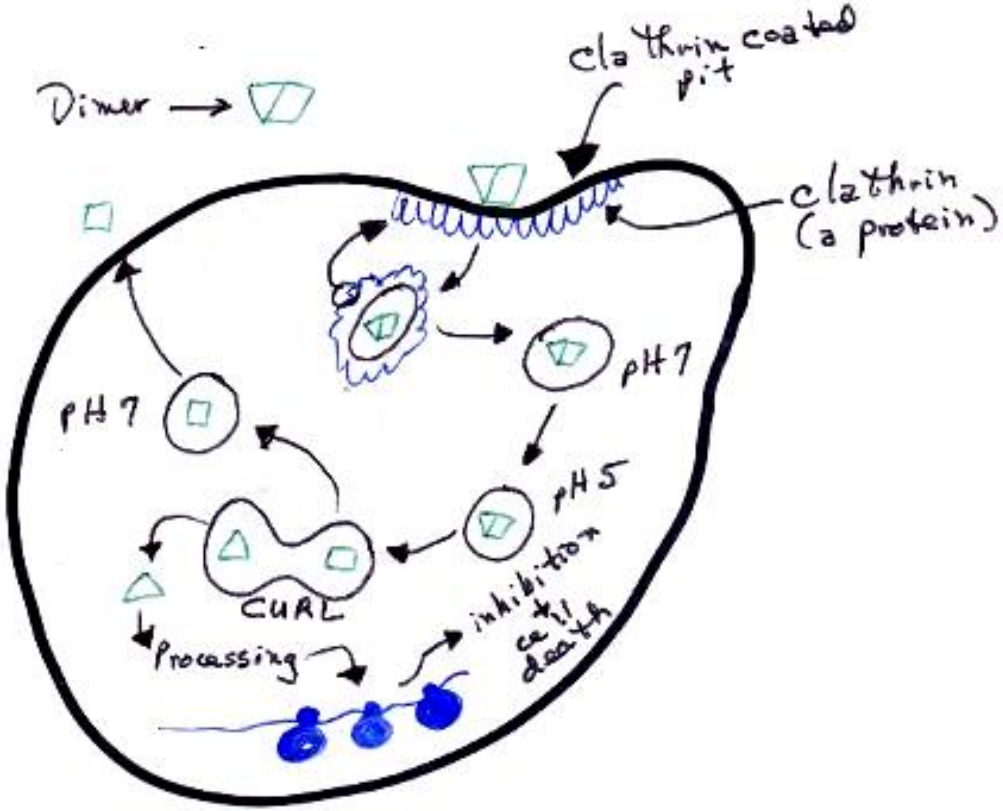


Exotoxin Mechanisms of Action

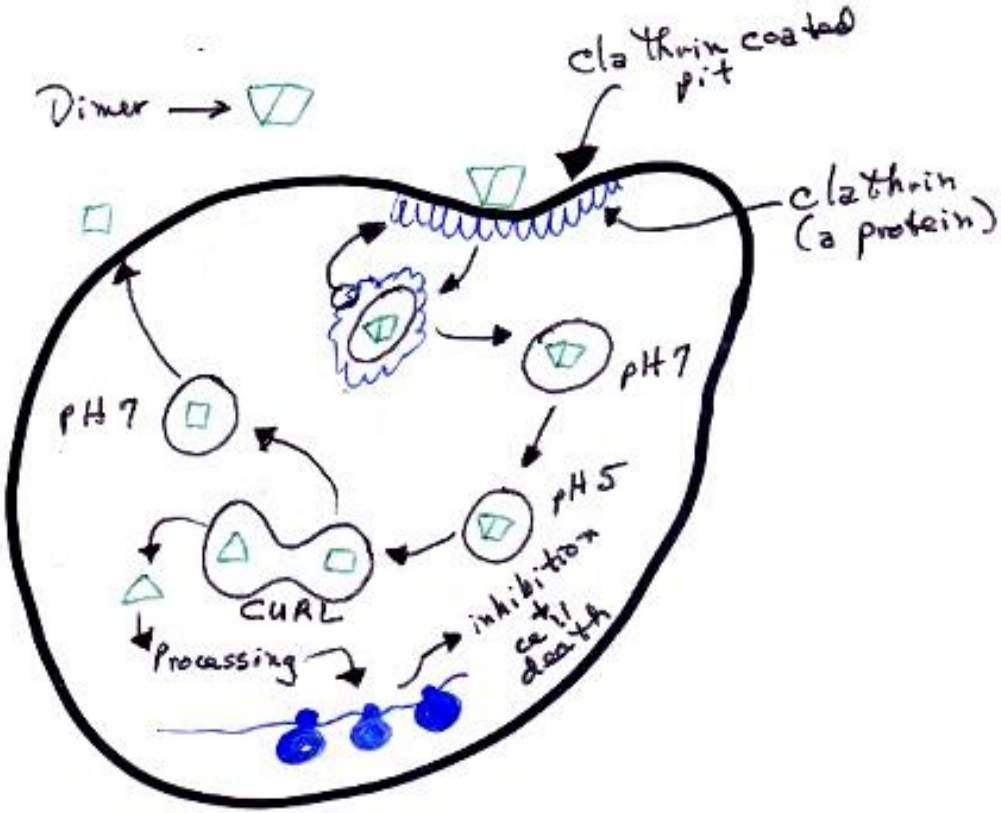
- Examples of exotoxins that work this way, include cholera toxin that increases cAMP levels in the cell that causes diarrhea and botulinum toxin that lowers Ach levels in the NMJ and causes flaccid paralysis.



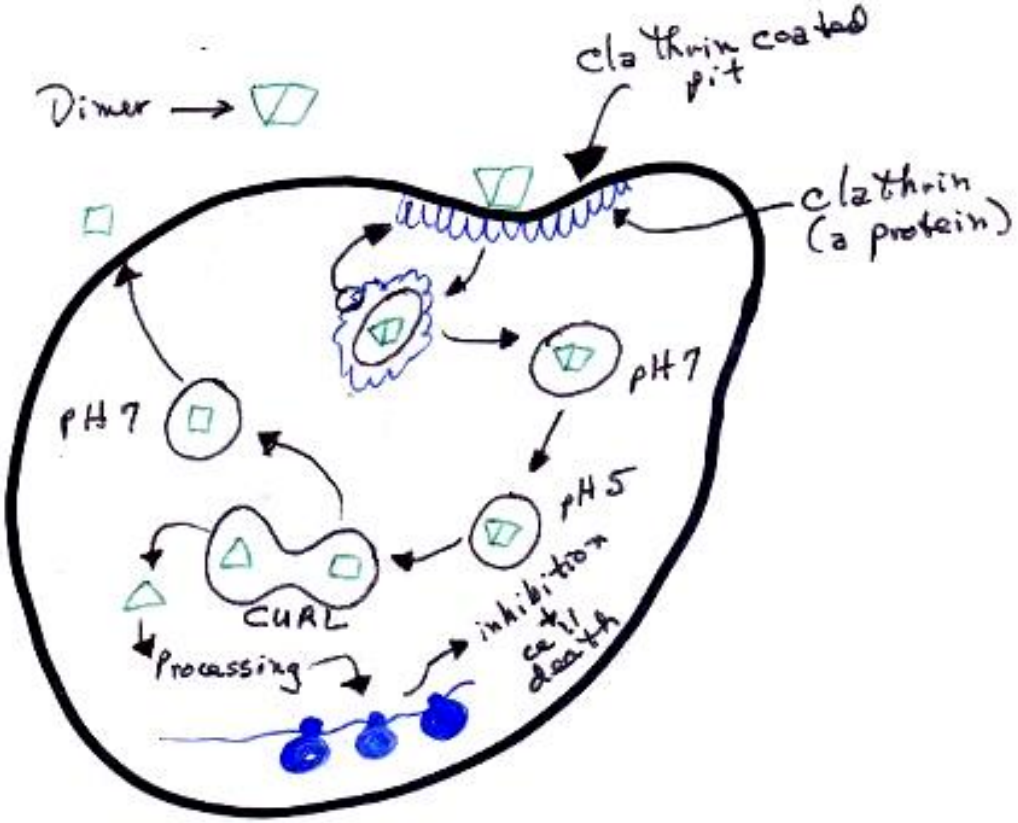
- Intracellularly, the exotoxin, e.g., Diphtheria toxin, exerts its effects following internalization either by Receptor Mediated Endocytosis or via internalization of the exotoxin once it is detected by a clathrin-coated pit (similar to how LDL is taken up in the liver), below.



- Once the pit has internalized, the clathrin is recycled to the cell surface and the endosome differentiates to a pH of about 7. Differentiation to a pH of about 5 continues at which point the now acidic endosome differentiates to a CURL (review A&P I).

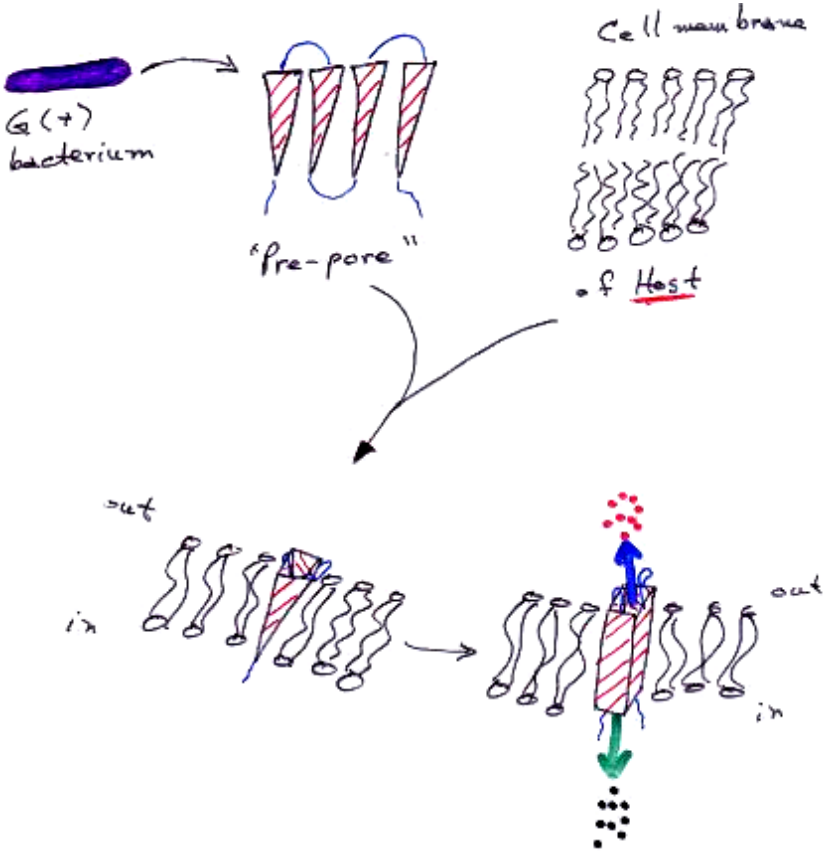


- The catalytically inactive portion of the exotoxin is released from the cell. The catalytically active portion undergoes processing, then interferes with translation. This interference inhibits translation and causes cell death.

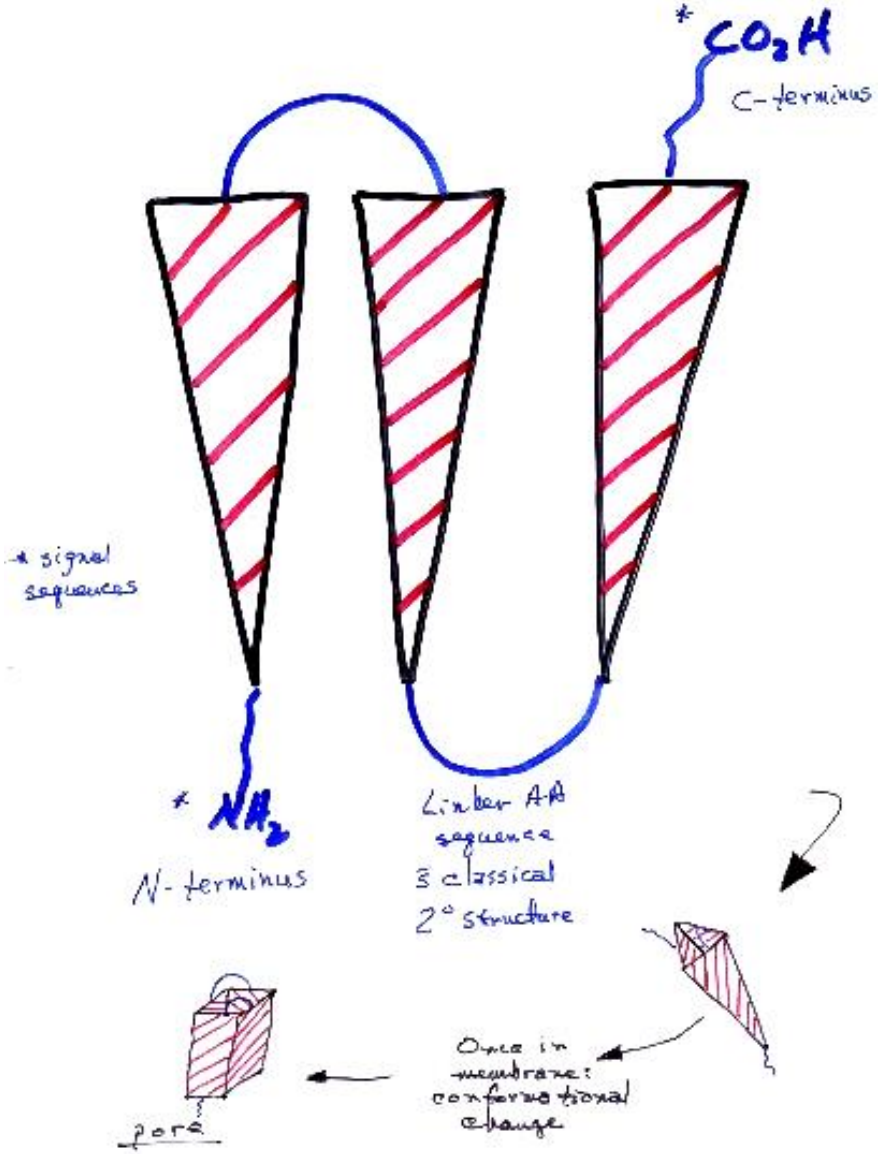


Pore-Forming Toxins

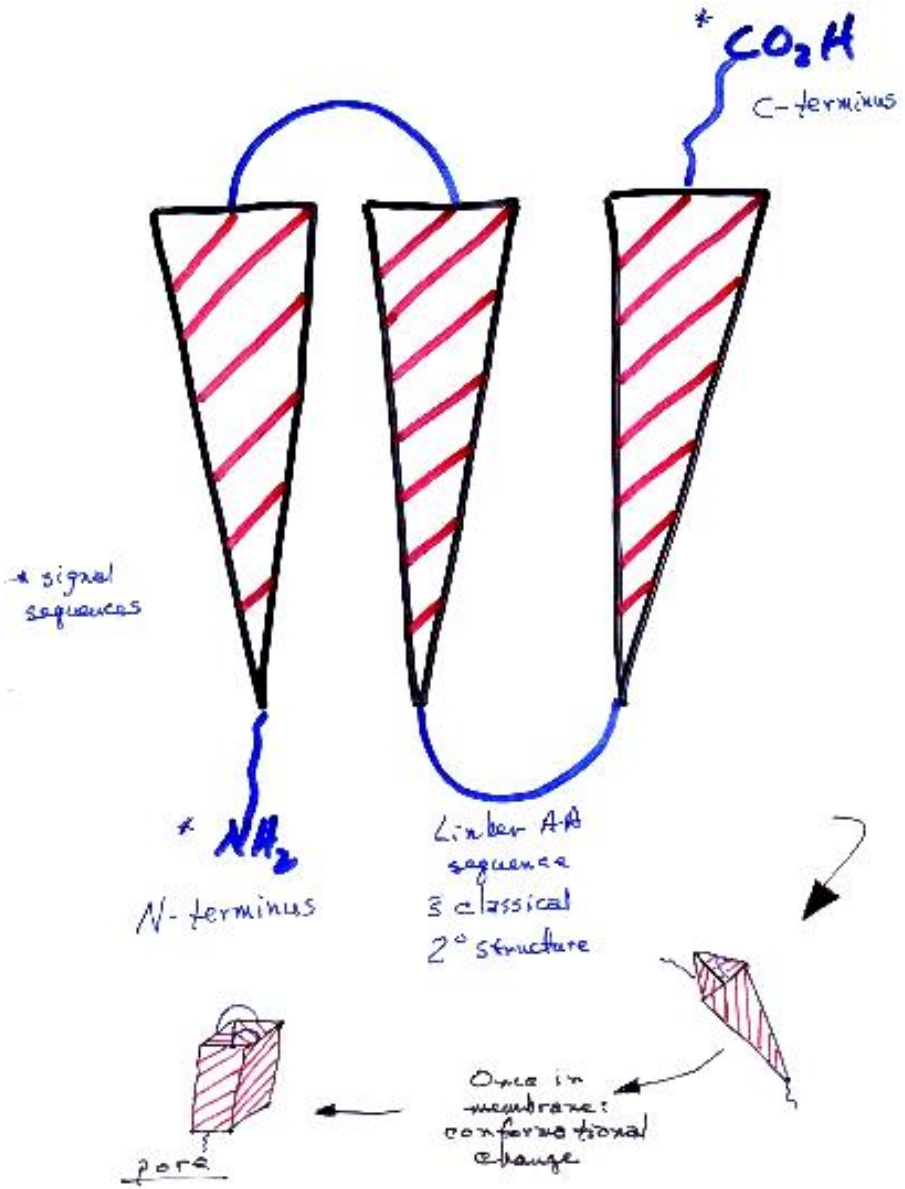
- The first pore-forming exotoxin causes electrolyte imbalance in cells attacked by the bacterium.
- The bacterium first secretes the "pre-pore" unassembled.
- When it "recognizes" the host cell membrane, the pre-pore undergoes a conformational change and inserts itself into the membrane -- much like a spigot into a maple tree to collect maple sap.
- When it is completely inserted inside the membrane, the pre-pore undergoes another conformational change that opens the pore.
- This allows ions and other particles to both exit and enter the cell, causing its death.



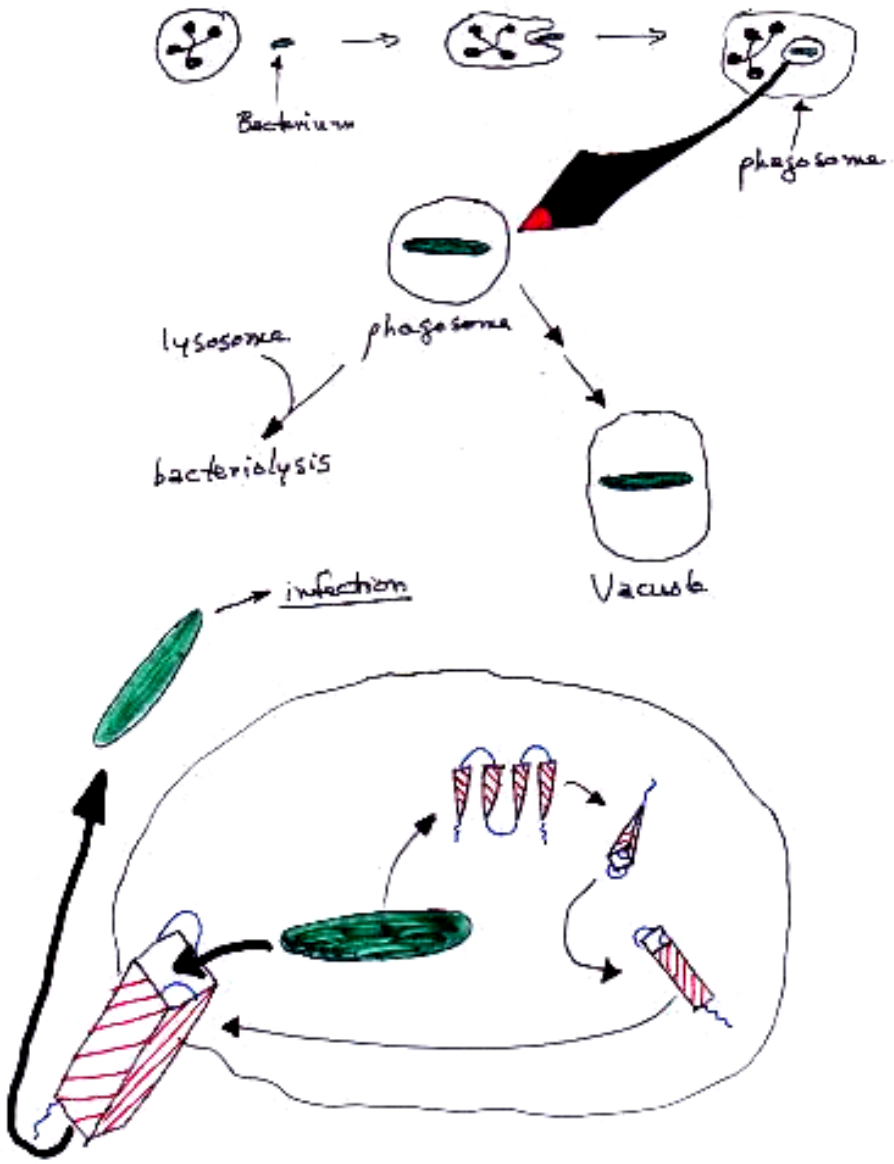
- A blow-up of the pre-pore. Note the presence of linker amino acid (AA) sequences that hold the pre-pore "pieces" together. These sequences have no classical secondary structure. The function of the N-terminal and C-terminal sequences is that of a signal sequence. The signal sequence is the portion of the protein that "identifies" the membrane into which the pore will be inserted.



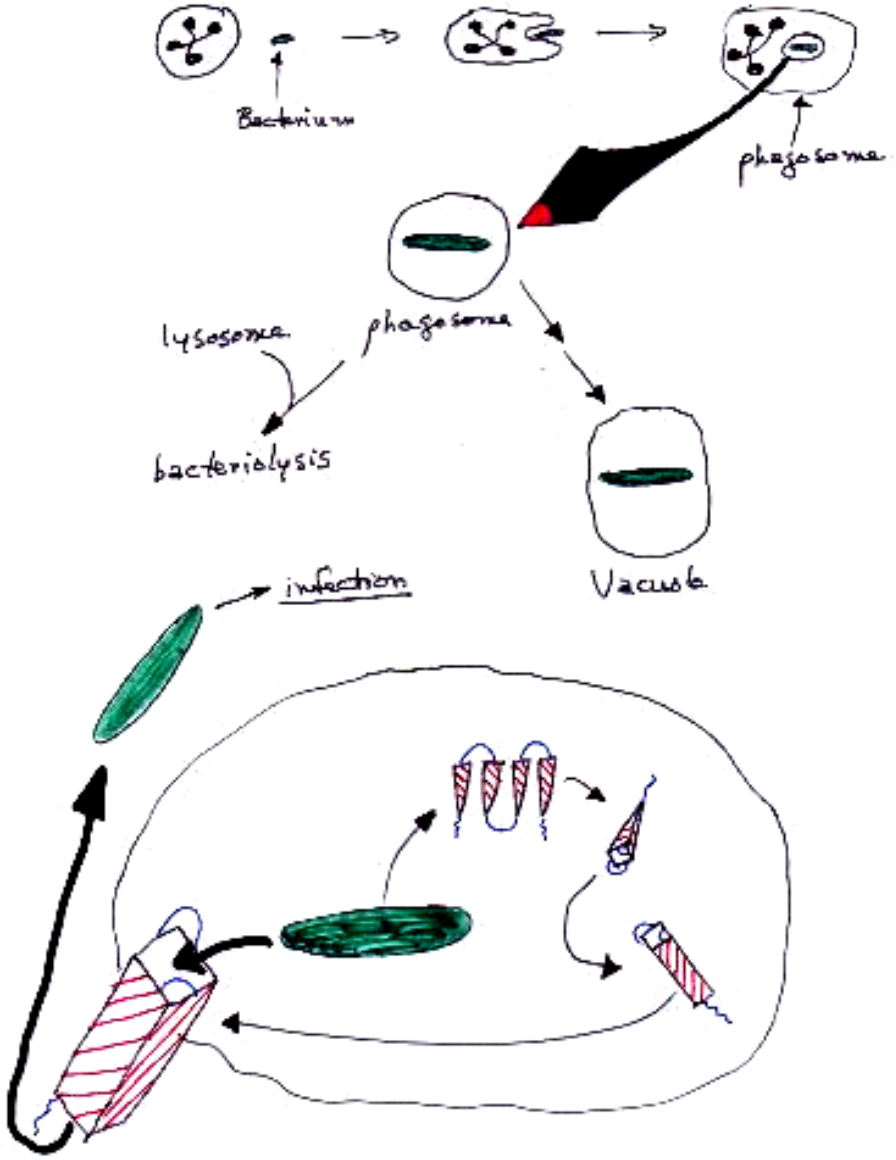
- The pore portions, represented by the wedges, have a functional amino acid sequence that gives the pore its function. All 3 of the proteins are complimentary, so that attractive forces will "fuse" them together in the membrane, making the wedge that will, upon complete insertion, change its shape to make the pore.



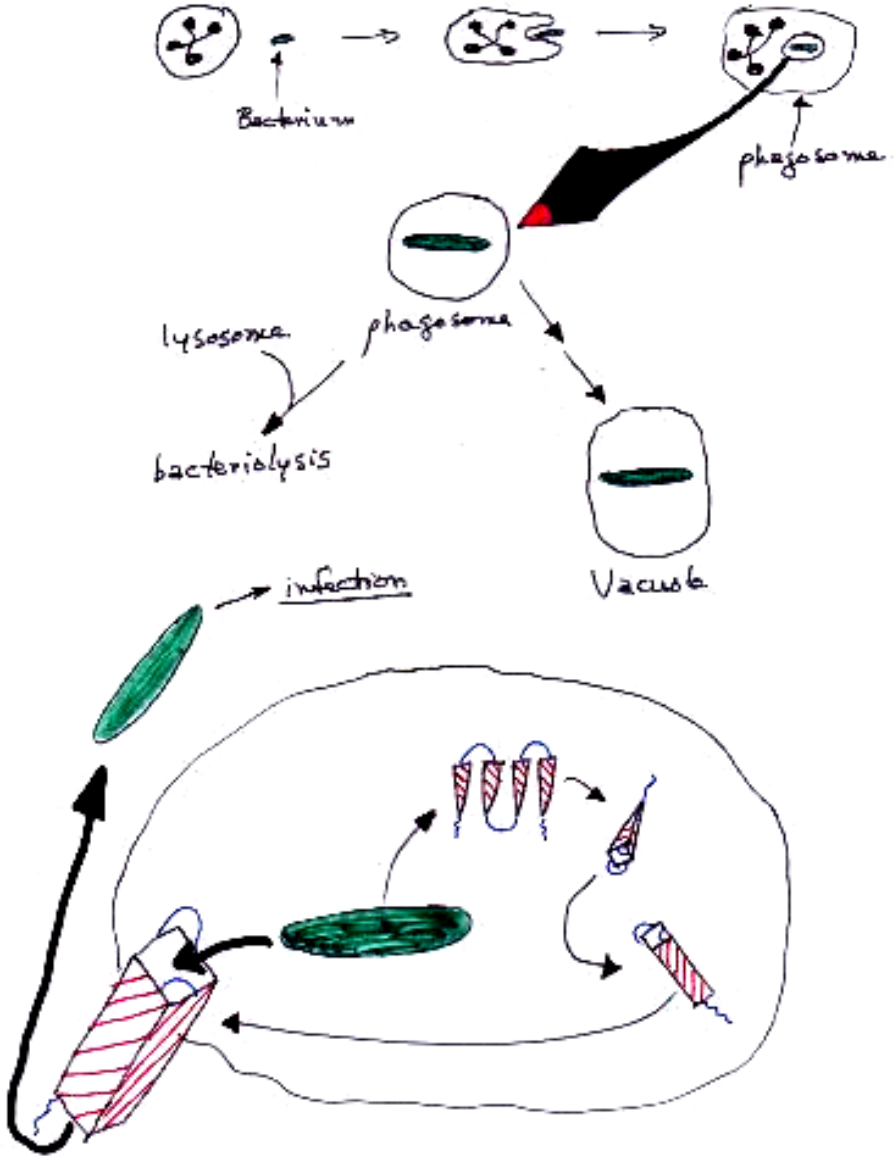
- Figure, right, illustrates perhaps the scariest of the exotoxin-derived pores: the **bacterial escape pore (BEP).**
- Remember that bacteria are not normal inside our tissues or our blood.
- When they are detected in our tissues or blood, phagocytes are mobilized to inactivate these bacteria by phagocytosis.
- Once the bacterium has been internalized, it is temporarily stored in a phagosome (an eating pocket).



- In some instances, the phagosome will differentiate into a vacuole. It is while in the vacuole that the bacterium releases its BEP. Much like the pore that causes cellular electrolyte imbalances, the pore is released in a "pre" form, it arranges itself complimentarily and inserts itself into the vacuolar membrane.

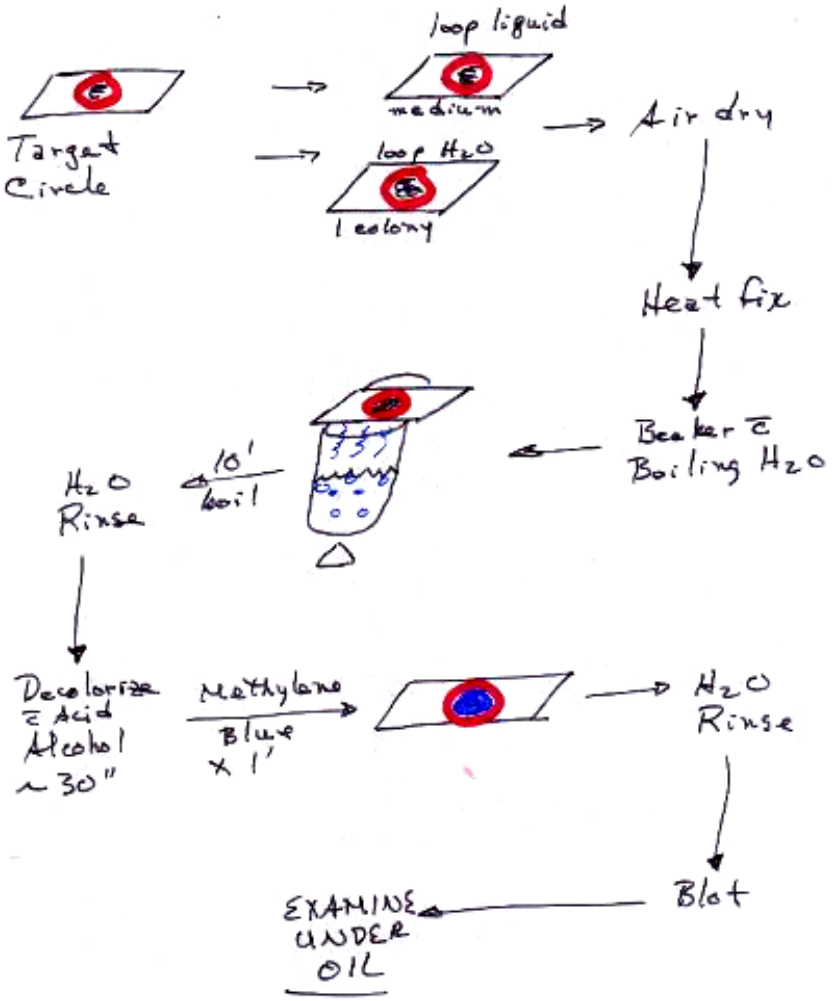


- Once it is inserted, it changes shape, again, and the bacterium escapes the vacuole and phagocytosis, infecting the cell and expediting its death.



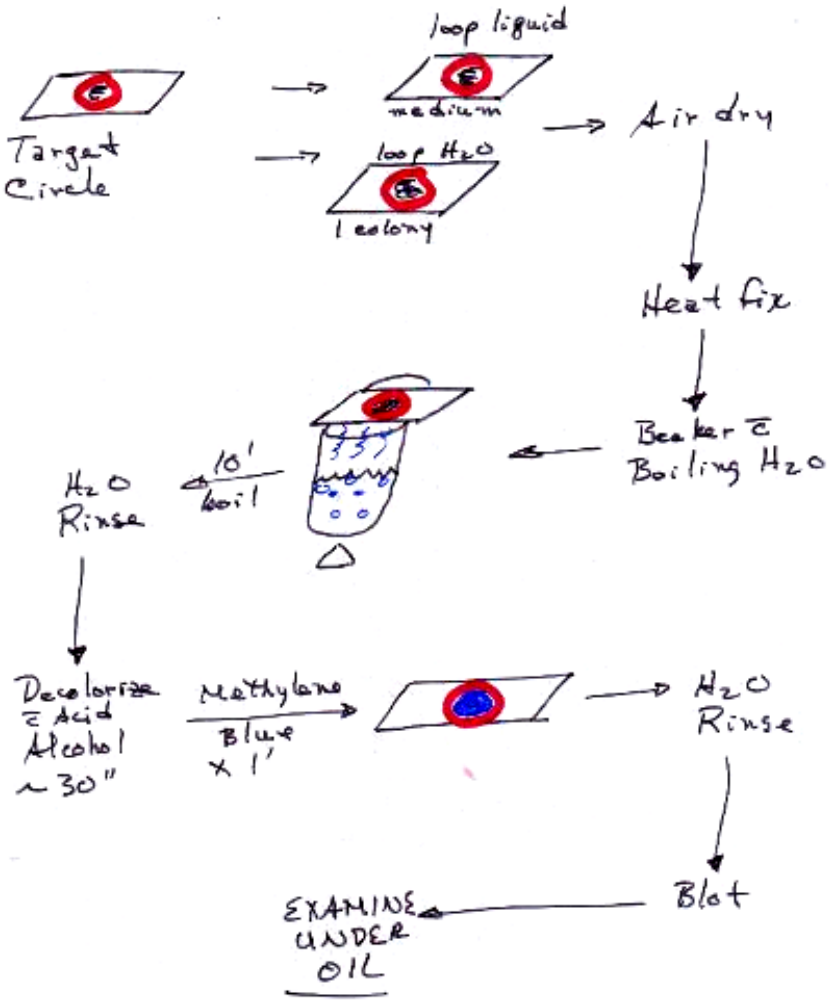
Acid-Fast Stain

- Organisms of the genus Mycobacterium don't stain well by Gram's method, but take up hot carbol fuchsin and hold it so well that they resist decolorization with strong mineral acids (nitric, hydrochloric or sulfuric acid with ethyl alcohol).
- **Point of caution: ethyl alcohol (EtOH) is lipophilic.**
- **Each of the three acids is caustic to skin.**
- Since they are mixed with EtOH, the potential burn that this can cause will be worse than just getting burned by the acids alone.
- That's because the EtOH "drags" the acids deeper into the skin faster than by themselves.



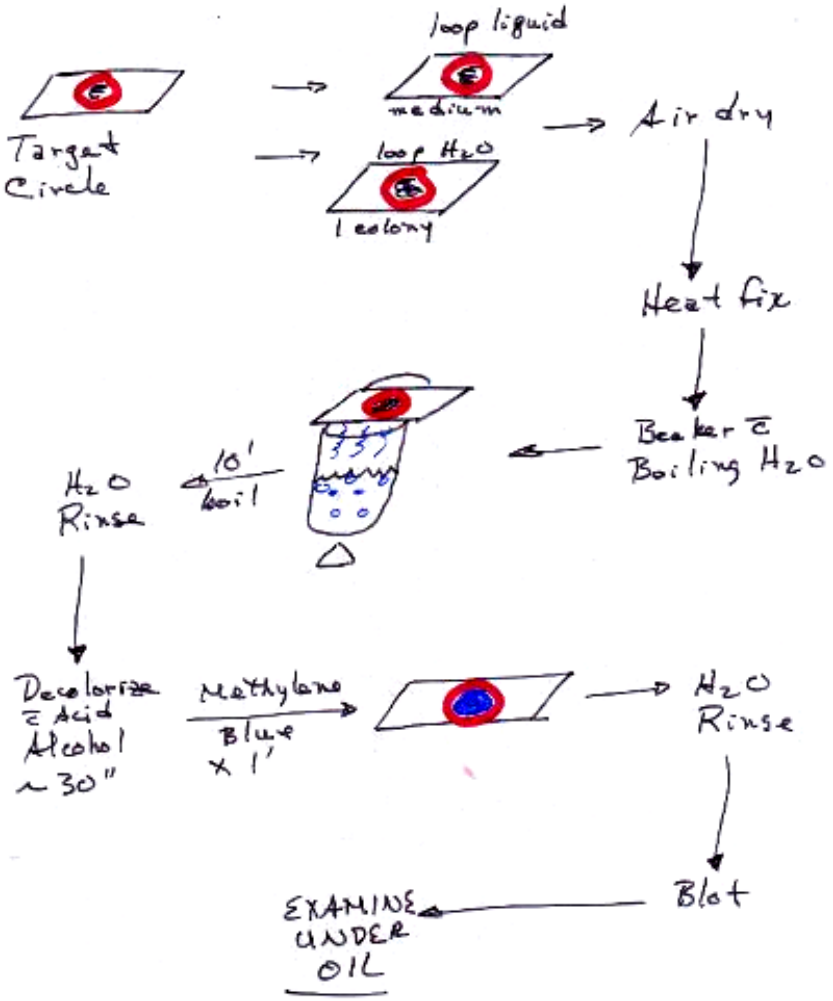
Acid-Fast Stain

- Place your heat-fixed slide on the top of a beaker with boiling water in it. Cover the target circle with carbol fuchsin and keep it moist as it heats. Continue boiling the water beneath the slide for 10 minutes. After the 10 minutes have passed, remove the slide and allow it to cool to room temperature. Rinse it, then, with acid-alcohol for about 30 seconds or until no more fuchsia color comes from the target circle.



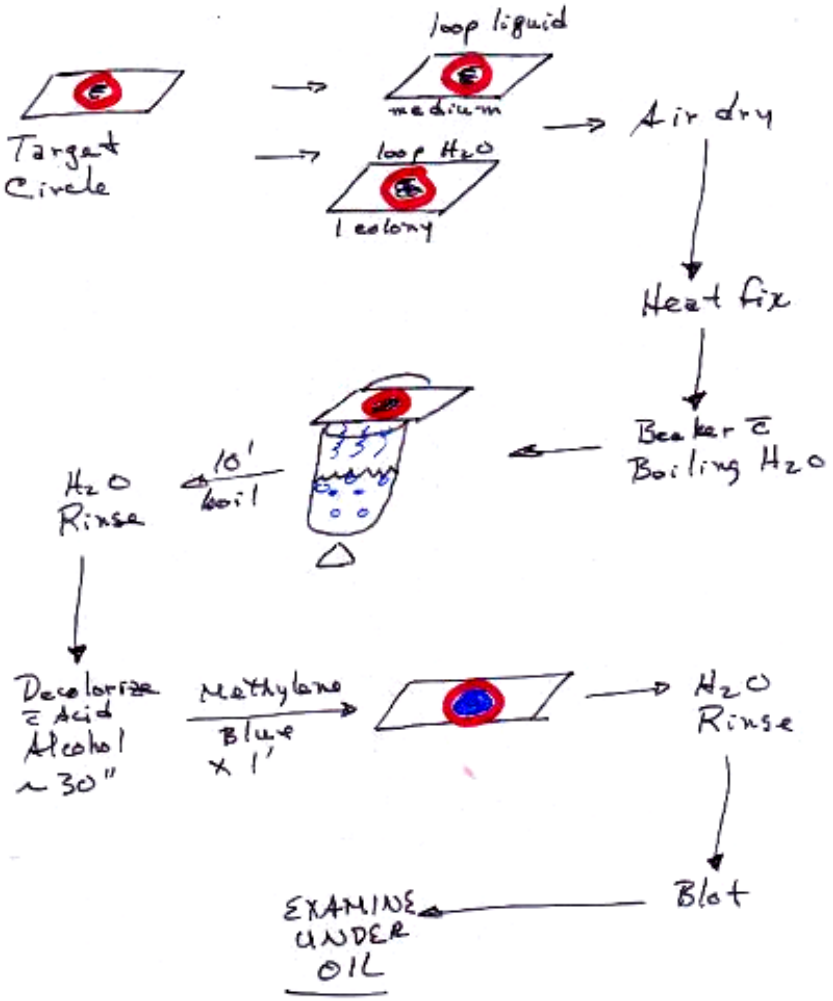
Acid-Fast Stain

- Counter-stain with methylene blue for one minute, then rinse with water and blot dry with bibulous paper and the sample is ready for examination under the microscope on oil immersion.



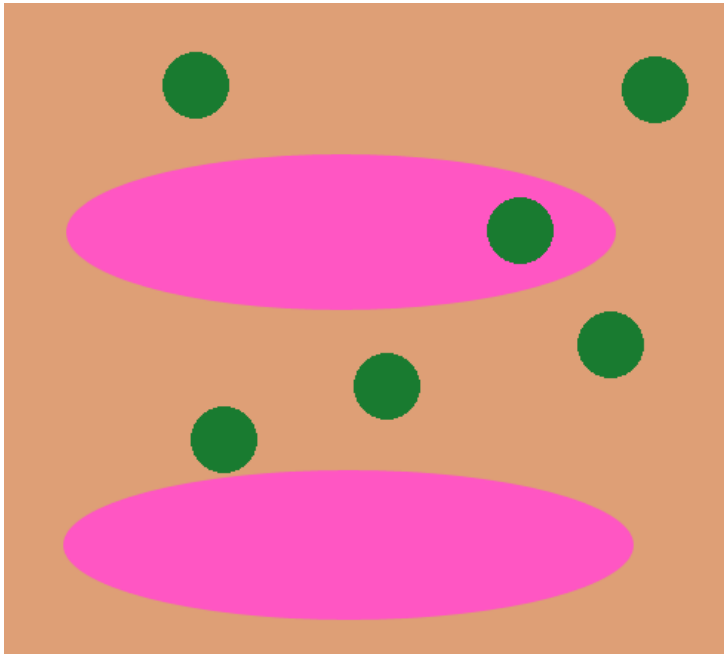
Acid-Fast Stain

- What you'll see, here, are a blue background and Mycobacterium tuberculosis and other acid-fast bacteria (AFB) show up as bright red/fuchsia rods that show up against this blue background, i.e., the acid alcohol does not remove the fuchsia from these microorganisms. This method may be modified by using a fluorescent dye in place of the carbol fuchsin. This may then be illuminated with UV light. This method is much simpler to use for the detection of AFB.



Spore Stain

- The last staining technique to be discussed is the spore stain. This stain is for the observation of free spores in the media or endospores -- spores inside the bacterium -- which is indicative of infections that are of great seriousness. Place your heat-fixed slide on the top of a beaker of boiling water and cover the target circle with malachite green. Keep it moist during the 10 minute boiling period. After 10 minutes, remove the slide from the top of the beaker and allow it to cool to room temperature. Rinse the slide until no more green comes off the target circle, then counterstain with basic fuchsin for 1 minute. Rinse it off with water, blot dry with bibulous paper and it's ready for viewing under the microscope on oil immersion. The resulting spores and endospores are stained green and the sporangia/bacteria are stained fuchsia.



Bacterial Movements

1. True Motility: progression of bacterium relative to other organisms -- due to flagella.
2. Brownian Movement: jerky movement due to molecular bombardment.
3. Streaming Movements: in one direction due to currents in the fluid.

- The **chief motile bacteria** include most varieties of E. coli, nearly all species of Salmonella, Proteus, Pseudomonas, Vibrio and Campylobacter, and some species of Bacillus and Clostridium.

- The **chief non-motile bacteria** include all species of Shigella, Brucella, Haemophilus and Mycobacterium, nearly all species of Corynebacterium, and all cocci of medical importance. Organisms with obvious capsules do not produce flagella and are non-motile, e.g., K. pneumoniae, C. perfringens and B. anthracis.

- Motile bacteria possess chemosensors and show positive and negative chemotaxis, i.e., they move towards some chemicals (mostly sugars and amino acids; positive chemotaxis) and are repelled by others (harmful substances and bacterial excretory substances; negative chemotaxis).
- In terms of their pathogenicity and power of invading the tissues, motile bacteria show no advantage over those that do not possess flagella.