Period 6 (Mar. 5)

Use of stains for light microscopy

Background:

To increase the visibility of cells or their constituents by light microscopy, the cells are often killed and treated with dyes that have an affinity for particular cellular components. As examples we will use two positive stains. The first is the most widely-used stain for bacteria, the gram stain (see below). This stain distinguishes between the envelope structures of so-called gram-negative and gram-positive organisms. According to "Nester," Dr. Hans Christian Gram, a Danish physician working in a morgue in Berlin, developed the gram-staining method in 1884 to distinguish between bacteria that cause pneumonia and eukaryotic cell nuclei in infected mammalian tissues. Oddly, the identity of the person who first had the idea to use this stain to distinguish between the envelope structures of different groups of bacteria is apparently not known. See the attached diagram and explanation of the gram stain from Nester (p. 53).

Outside their cytoplasmic membranes, gram-negative bacteria have a relatively thin murein or osmotically-resistant layer and an outer membrane. By contrast, gram-positive bacteria have a relatively thick murein layer and lack an outer membrane. Crystal violet enters both gram-negative and gram-positive bacteria. Iodine also enters both and forms a complex with crystal violet that is less soluble. Subsequent treatment with decolorizer (a mixture of ethanol and acetone) dissolves the cytoplasmic and outer membranes of gram-negative bacteria and allows leaching of the Crystal violet dye through the relatively thin and porous murein layer. The decolorizer apparently dehydrates the thick murein layer of gram-positive bacteria, closing its pores and restricting loss of the Crystal violet dye. Decolorization is not well understood and its basis remains controversial. The length of treatment with decolorizer is critical to distinguishing between gram-negative and gram-positive organisms because eventually both will lose the Crystal violet dye. In addition some gram-positive cells lose the dye easily and appear gram-negative. The counterstain, Safranin, is not necessary but it imparts color to the gram-negative cells, which makes them more readily visible.

The second stain we will use stains endospores, the resting state of gram-positive bacteria.

Materials:
1. Light microscopes
2. Microscope slides and cover slips (20/pair)
3. Bunsen burners and loops
4. Clothes pins or metal clamps to hold slides
5. Water bottles
6. Culture of *E. coli* K12 grown in Luria Broth (1 ml/pair)

7. Freshly grown culture of *Bacillus subtilis* (1 ml/pair) (From an exponential culture inoculate 0.1 ml → 100 ml LB and 10 µl → 100 ml. Inoculate with shaking overnight)
8. Samples from Winogradsky columns
9. Reagents for gram-staining (enumerated in an attachment)
10. Reagents for staining endospores (enumerated in an attachment)
11. Test tubes and test tube racks for taking samples

Methods:

Gram staining

1. *Preparation of smear*:
   A. Carefully clean a slide with tissue paper so that you can spread a cell suspension evenly into a thin film.
   B. With a sterilized loop place a small amount of the culture to be examined on the slide. (*E. coli* is gram-negative and *B. subtilis* is gram-positive. Examine them separately and together.) REMEMBER, to compare them you must use exactly the same protocol for both.
   C. Alternatively, place a small drop of distilled water on the slide. With a sterilized loop, lightly touch a colony or other material to be examined and transfer a very small quantity of it to the drop (just enough to make the drop very slightly cloudy). You can examine material from the Winogradsky columns this way.
   D. Using the loop spread the drop over a small area of the slide (about the size of a dime.
   E. Hold the slide with a clothes pin or metal clamp. Air dry and then heat-fix the slide over a Bunsen burner. (Do not pass the dried slide through the flame of the Bunsen burner.) Do not overheat. You should be able to put the slide on your wrist.
2. *Staining*:
   A. Cover the fixed film with Crystal violet/ammonium oxalate solution (about 1 drop) for 1 min. Use the clothes pin to hold the slide so you won't stain your hands.
   B. Pour off the stain and wash with distilled water (use a water bottle) for not more than several sec.
   C. Cover with Gram's iodine solution (about 1 drop) for 30 sec.
   D. Pour off the iodine solution, wash with distilled water, and blot dry with bibulous paper.
   E. Add a few drops of Gram's decolorizer (ethanol/acetone) so it trickles down the slide. Rinse off with water after about 5 seconds or when the decolorizer is no longer colored as it flows over the slide. Decolorizing for too long will allow decolorization of gram-positive as well as gram-negative organisms and will defeat the purpose of the stain.
   F. Counterstain with Gram's Safranin solution (about 1 drop) for 20 sec.
   G. Wash with distilled water and blot dry.
3. *Observation*: Examine the slide with immersion oil. It is sometimes helpful to place oil on the sample, put a cover slip over it, and place another drop of oil on the cover slip.

4. *Cleanup*: If stain spills wash well with water immediately to avoid leaving permanent marks on glassware, the lab bench or the sink.

Staining for endospores

1. Prepare smear or slides as for the gram stain. (DO NOT GRAM STAIN!) Use both *B. subtilis*, an endospore former, and *E. coli*, which does not form endospores. You may also examine material from the Winogradsky columns.
2. Stain for 10 min with Malachite Green and rinse with distilled water for about 10 sec.
3. Stain with Safranin solution for 15 sec and rinse with water, blot, and dry.
4. Examine microscopically for bacteria in which most of the cell is stained red and endospores appear as green inclusions. You may also see green spheres free of cells.

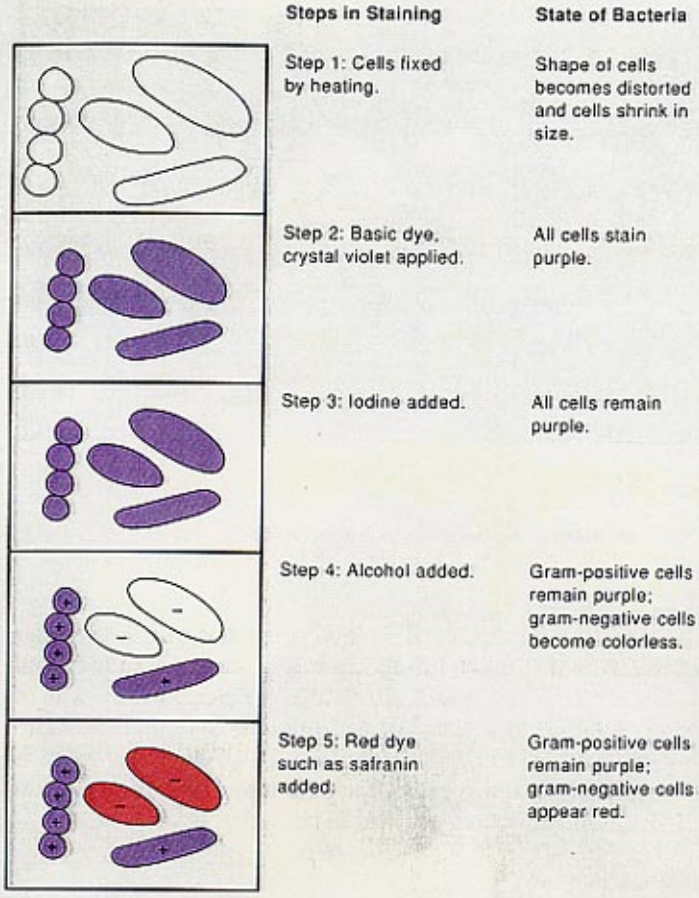
Analysis:

Did staining help you to see *E. coli*, *B. subtilis*, *B. subtilis* spores? How did stains affect your ability to see organisms from the Winogradsky columns or other organisms you examined?

Nester

Gram stain

Nester, E. W., Roberts, C. E., and Nester, M. T. (1995). Microbiology: A Human Perspective, p. 53. Wm. C. Brown Publishers, Dubuque, IA.



A basic purple dye, usually crystal violet, is added to bacteria fixed on a microscope slide. All bacteria that are able to absorb this dye are stained. Next, a dilute solution of iodine is added, which decreases the solubility of the purple dye within the cell by combining with the dye to form a dye-iodine complex. Then, an organic solvent such as ethanol is added. This solvent readily removes the purple dye-iodine complex from some but not all genera of bacteria. A red dye, such as safranin, is then applied, which stains all bacteria. Bacteria that are decolorized by the ethanol appear red and are called **Gram-negative bacteria.** Those that retain the purple dye appear purple, since the purple dye masks the red, and are called **Gram-positive bacteria.**

Figure 3.7

The Gram stain. (*a*) Steps in the Gram staining procedure. (*b*) Photomicrograph of bacteria that are Gram-positive (purple) and Gram-negative (red). The species are *Staphylococcus aureus* and *Escherichia coli*, respectively.

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Stains for light microscopy (period 6). Please replenish after this period for use during later labs.

Reagents for gram stain:

1. Crystal violet/ammonium oxalate solution:
   A. 20 g Crystal violet in 100 ml 95% ethanol
   B. 1 g ammonium oxalate in 100 ml distilled water
   C. distilled water

   Mix: 2 ml A + 80 ml B + 20 ml C. Store in a dropper bottle as Crystal Violet/Ammonium oxalate. WE WILL NEED 20 ml/PAIR

2. Gram's Iodine solution: 1 g I_2 crystals, 2 g KI, and 3g sodium bicarbonate in 300 ml distilled water. WE WILL NEED 20 ml/PAIR

3. Gram's Decolorizing solution: equal volumes of 95% ethanol and acetone. WE WILL NEED 20 ml/PAIR

4. Gram's Safranin counter-stain:
   A. 2.5 g Safranin O in 100 ml 95% ethanol
   B. distilled water

   Mix: 20 ml A with 100 ml B. WE WILL NEED 20 ml/PAIR

Reagents for endospore stain (Bartholomew and Mittler)

1. Malachite green solution: Saturated aqueous solution = approximately 8 g plus 100 ml distilled water. WE WILL NEED 10 ml/PAIR.

2. Safranin solution: 0.25 g Safranin O plus 100 ml distilled water. We will need 10 ml/pair.