Flagellar Regeneration

Many cells move by means of flagella; some of them, like sperm cells, occur in multicellular organisms and others are free-living unicellular organisms like the alga, Chlamydomonas, that we will observe today. Bacteria also may use flagella to move through their environment, but the construction and mechanism of movement of their flagella is very different from the system under study.

The structural basis of flagella in Chlamydomonas is a system of microtubules assembled from the protein tubulin. The cellular tubulin pool contains two kinds of globular molecules: alpha- and beta-tubulin. These two have similar amino acid sequences, and they combine with each other to form heterodimers. When the dimers are attached to each other (polymerized), they form a hollow tube that can be seen in an end-on view to have 13 tubulin molecules around the wall. Each of the 13 tubulin molecules is in a chain along the length of the tubule and that chain is referred to as a protofilament. The cylinder of protofilaments can be 25 μm (or more) in length and it as a diameter of about 24 nm. Within a protofilament, the alpha- and beta-tubulins alternate and the globular proteins in adjacent protofilaments are slightly staggered, forming a helical array. (See text fig 16-9, p.520.)

Tubulin monomers can form dimers and assemble into microtubules in vitro, and that assembly occurs most readily at physiological temperatures and in the presence of Mg++ and guanosine triphosphate (GTP.) Orderly assembly is greatly facilitated if a "nucleation site" is provided by microtubule-associated proteins (MAPs), and it appears that tubulin dimers add onto one end of the microtubule only. If temperatures are lowered and/or a high concentration of Ca++ is present, the tubule tends to disassemble into free dimers. This depolymerization occurs at the opposite end from polymerization. (See text fig. 16-12, p. 522.)

Chlamydomonas that have lost their flagella for any reason can regenerate them by reassembly of tubulin available in the cytoplasm. This process requires an hour or more at room temperature, and the growing flagella can be measured at prescribed time intervals to determine the rate of reassembly. The objective of this lab is to measure that rate in a control condition and in the presence of two inhibitors – colchicine and cycloheximide. Colchicine is an inhibitor of microtubule assembly, acting by binding free tubulin dimers in the cytoplasm. Cycloheximide is a powerful inhibitor of protein synthesis, leading to cessation of tubulin synthesis.

Before flagellar regeneration can be observed, the flagella must be removed from the organisms; this is done by pH-shock in which the pH of the medium is rapidly lowered to 4.5 and then restored. In order to measure regenerating flagella, it is necessary to kill and stain the organisms in Lugol’s iodine so that the flagella will not be moving and the contrast will be enhanced. Once the organisms are fixed, observations can be made at any time. You will also need to have a microscope equipped with an ocular micrometer, since that is the means by which measurements will be taken.

**Procedures:**

1. You should work in teams of three, with each team member being responsible for one
condition – control, colchicine, or cycloheximide. The person assigned the colchicine condition should also measure the flagella in a normal, non-deflagellated sample at the beginning and at the end of the experiment. One group of students will carry out the deflagellation procedure for the entire class.

2. For each culture condition, set up 10 fixation test tubes in a rack; label each tube with the condition and time: 0, 10, 20, 30, 40, 50, 60, 75, 90, 105 minutes. Remember that you will need two tubes for the non-deflagellated culture at 0 and 105 minutes. Add one drop of Lugol’s iodine and two drops of Medium I to each tube.

3. **Deflagellation** is performed on a 40 ml- aliquot from an actively growing *Chlamydomonas* culture; the sample should be placed in a 100-ml beaker with a magnetic stir bar.

   a. Place the beaker on a magnetic stirrer at low – moderate speed and lower a pH electrode into the solution. Lower the pH to 4.5 rapidly (within 30 seconds) by adding 0.5 N acetic acid dropwise.

   b. After 30 seconds, restore the pH to 6.8 with dropwise addition of 0.5 N KOH.

   c. Divide the culture into three equal samples in conical centrifuge tubes; centrifuge labelled **deflagellated 1, 2,** and 3.

   d. Decant and discard the supernatant containing the flagella. Add 10 ml of the following to each labelled tube: tube 1 = Medium I; tube 2 = Medium I with 10 μg/ml cycloheximide. Gently resuspend the cells in each tube with a Pasteur pipet; use separate pipets for each culture. **DO NOT PIPETTE ANYTHING BY MOUTH!**

   e. Pour the contents of each tube into a labeled 50-ml Erlenmeyer flask; add 10 ml of non-deflagellated culture to a 4th flask. Place the four flasks into a fluorescent lamp. **PROCEED IMMEDIATELY WITH THE EXERCISE!**

4. **Record** times from the initial (time 0) sample. For each time, remove two drops of the appropriate culture and place it in its labeled tube; swirl the tubes gently to facilitate fixation.

   Once cells are fixed, they can be set aside for measurement, although you should try to score flagella whenever you have a break. Sample the cultures at the times above (0, 10, 20, 30, 40, 50, 60, 75, 90, 105 minutes); each time, make sure that there is no residual culture in the pipet from the previous sampling. **Make sure that you do not interchange pipets among**
the different cultures.

5. **Measurement** of flagellar length is done by observing a drop of the fixed sample placed on a clean slide with a coverslip. Using low power, find an area with a concentration of cells.

With the ocular micrometer and the oil immersion lens, measure the length in 15 cells that have at least one fairly straight flagellum. Both flagella are the same length, so only one need be measured. Record your measurements on a data sheet and calculate an average length for each time and condition. Record these averages and write them on the board also so that the data from the entire class is available.

**Points to Ponder:**

1. What is the basic process which is occurring in the control culture?

2. How do the results differ in the three conditions?

3. What are the differences in the mechanisms of action of colchicine and cycloheximide and how does that explain the results of this exercise?

4. Would you expect the results of these treatments to differ if the culturing were done in the dark instead of in the light? Why?

5. What effect would you expect if we reincubated the control culture at low temperature (near 0oC)?


Bregman suggests the following references for further information.


Rosenbaum, J.L., et al., 1969 Flagellar elongation and shortening in Chlamydomonas: