

## Exercise 6

# Uptake and Incorporation of Radioactive Sulfur by *Chlamydomonas*

## Radioisotope Safety Instruction

**Read these instructions before the next laboratory session. You should also review the relevant section your lecture notes and textbook. You will be tested on this material at the start of the laboratory period.**

1) In the following exercise you will be using the radioisotope  $^{35}\text{S}$ . Any time you work with radioactive materials special safety precautions must be taken. Anyone disregarding the safety procedures will be asked to leave the laboratory and will receive a grade of zero for this exercise.

2)  $^{35}\text{S}$  is a beta emitter of moderate energy, with a half-life of approximately 3 months. Since sulfur is an important component of a variety of cell compounds it can become incorporated into the body if ingested. Surface contamination is less dangerous, but should still be avoided. In order to check that no bodily exposure has occurred, each student will be monitored for  $^{35}\text{S}$  contamination before and after the lab period by use of a 'swipe' test.

Take a piece of Kimwipe (about 2.5 cm x 2.5 cm) and wipe your face, palms and fingers with it. Place the Kimwipe in a scintillation vial and put your initials on the lid. **NOTE:** No student will be allowed to leave the laboratory until the instructor has seen the swipe test results. This means that casual entering or leaving the lab is forbidden without expressed permission of the lab instructor.

3) Safety rules

- No eating, drinking, chewing, or smoking during the lab period.
- Wear plastic gloves and safety aprons at all times when working with the radioisotope.
- **Never** mouth pipette any liquids.
- Avoid touching anything that will not be thrown out in the safety bags. This includes your face, clothing, partner, chairs, *etc.*
- Perform all work over the metal tray on the safety cloth, (which will absorb any spills). Place all used materials in the disposal bags provided. The only radioactive material that leaves the bench should be in sealed centrifuge tubes or in vials to be counted.
- **In the event of an accident or spill, do not move - inform the instructor at once!**

## Introduction

Sulfur is one of more than a dozen essential elements required by all organisms. Most autotrophs acquire sulfur, in the form of sulfate ions,  $\text{SO}_4^-$ , directly from their environment. Sulfur uptake by these organisms involves three major steps: 1)  $\text{SO}_4^-$  is moved across the cell membrane. 2) Inside the cell,  $\text{SO}_4^-$  reacts with a variety of organic molecules to form sulfur-containing compounds, including certain amino acids. 3) As protein synthesis takes place, the sulfur-containing amino acids will be incorporated into a variety of cell proteins. Normally, it would not be possible to trace these steps, since one sulfur atom appears the same as all others. However, by providing the cells with radioactive sulfur, in the form of  $^{35}\text{SO}_4^-$ , we can follow the movement of sulfur from the medium into cellular molecules by monitoring the location of the  $\beta^-$  particles produced by  $^{35}\text{S}$  decay. This allows us to study certain aspects of sulfate transport and amino acid and protein synthesis.

To ensure that the cells take up the radioactive sulfur, they will be starved for sulfur for several hours, and the only source of sulfur for the cells will be the  $^{35}\text{SO}_4^-$  that we provide. In order to monitor the uptake and incorporation of  $^{35}\text{S}$ , cells will be removed from the radioactive medium at specified time intervals and cell fractions analyzed for  $^{35}\text{S}$  activity. You can then determine how much radioactive sulfate was extracted from the medium, how much was taken up by the cells, and how much ended up in sulfur-containing amino acids and in proteins.

This kind of use of radioactive materials is called 'pulse' labeling, since the cells get a single, short-term exposure to the isotope. Only those proteins made during the course of today's exercise will be radioactive. If the cells were grown in the presence of the isotope for several generations, then essentially all of the sulfur-containing molecules in the cell would be radioactive. This latter type of use is called 'equilibrium' labeling (Cooper 1977).

## Background Reading

You should review your lecture notes or other references on the use of radioisotopes in cell biology, and you should be prepared for a quiz on safety procedures and the basic objectives of this exercise. In addition, you should review the basic concepts related to ion transport across membranes and protein synthesis.

*Chlamydomonas* Teaching Site: <http://ChlamyTeach.info>

Adams, M., E.Savino, and M. Freeman (1994) Timing and induction of hsp 70 production in *Chlamydomonas reinhardtii*. Biol. Bratislava 49: 623-628.

Cooper, T.G. (1977) The Tools of Biochemistry. Wiley - Interscience, John Wiley and Sons, New York.

Lewin, R.A. (1962) Physiology and Biochemistry of Algae. Academic Press, New York.

Hendee, W.R. (1984) Radioactive Isotopes in Biological Research. Krieger, Malabar, FLA.

Davies, J.P, F. Yildiz, and A.R. Grossman. (1994) Mutants of *Chlamydomonas* with aberrant responses to sulfur deprivation. The Plant Cell 6: 53-63

Yildiz F.H., J.P. Davies, and A.R. Grossman (1994) Characterization of sulfate transport in *Chlamydomonas reinhardtii* during sulfur-limited and sulfur-sufficient growth. Plant Physiol. 104: 981-987

## PROCEDURES

- 1) At the start of the exercise the sulfur-starved cells will still be in non-radioactive, sulfur-free TAP medium<sup>1</sup> (*ca.*  $2-4 \times 10^6$  cells/ml). Approximately 10 ml of suspension will be centrifuged for 5 minutes at  $500 \times g$  to pellet the cells, and the sulfur-free medium discarded.
- 2) Each group should take a **50  $\mu\text{L}$**  sample from the radioactive TAP medium ( $^{35}\text{S}$ , 1.25  $\mu\text{Ci/ml}$ ) and place it into a scintillation vial containing 5.0 ml of scintillation cocktail (Ecolume®; ICN Biomedicals, Inc.). Label this vial **M0** (= Medium; time 0). This will be used to determine the total radioactivity in the medium at the start of the experiment.
- 3) The pellet of cells will then be resuspended in 10 ml of radioactive medium. A member of each group will add 1 ml of the radioactive cell suspension to each of 3 Eppendorf tubes. **The time at which the cells are placed in the radioactive medium is the official time zero ( $T_0$ ) for the experiment.**  
\*\*\* What is the approximate cell density (cells/ml) in your tubes of radioactive cell suspension? \*\*\*
- 4) Your group's tubes should be stored in a rack in your metal pan, for safety.
- 5) After 20 minutes ( $T_{20}$ ) one of the three tubes containing the cell suspension should be centrifuged at **10,000  $\times g$  for 3 minutes** to pellet the cells.
- 6) Carefully remove the tube from the centrifuge and return it to your rack. Remove **50  $\mu\text{L}$**  of the supernatant and add it to a scintillation vial labeled **M20** (Medium; 20 min.). Carefully decant the rest of the supernatant into the liquid waste disposal jar without disturbing the pellet. Be sure to remove as much of the liquid as possible; any supernatant left in the tube will alter the  $^{35}\text{S}$  counts in the next step.

- 7) Add **0.1 ml** of **SDS lysis solution**<sup>2</sup> to the pellet of cells and stir gently with a micropipet. The cells should break open and the resulting solution may be very viscous. (\*\* **Why?** \*\*).
- 8) Add **0.2 ml** 10% TCA<sup>3</sup> to the tube and mix thoroughly with the micropipet. Then, add an additional 0.7 ml of 10% TCA, mix gently, and close the lid on the tube.  
**CAUTION:** TCA is very corrosive- avoid spills.
- 9) Centrifuge the tube for 3 minutes at **10,000 x g** in the micro-centrifuge. The insoluble material (including proteins) should form a pellet at the bottom of the tube.
- 10) Carefully open the tube, remove a **50  $\mu$ L** sample of the supernatant, and add this to a fresh scintillation vial and label it **S20** (= Soluble molecules; 20 min.). Decant the remaining supernatant into the liquid waste container.
- 11) Add **0.2 ml** SDS lysis solution to the pellet. Gently resuspend the pellet with a micropipet (watch out for bubbles!!). When the pellet is evenly distributed, add an additional 0.8 ml of SDS lysis solution, and gently mix. Take a **50  $\mu$ L** sample and place it in a fresh vial labeled **P20** (Precipitate; 20 min.). Cap the Eppendorf tube and discard it into the solid waste bag.
- 12) The three samples (M20, S20 and P20) will be used to determine how much radioactivity is in the medium, soluble compounds and precipitated compounds, respectively. **\*\*\* Will the total amount of radioactivity have changed since time zero? \*\*\***
- 13) Repeat steps 5-12 at 40 and 60 minutes, placing samples into vials labeled M40, S40, P40, *etc.*
- 14) After the 60 minute sample has been processed, pour any radioactive liquids into the liquid waste jar.
- 15) Place all radioactive pipettes, tubes, *etc.* into the radioactive waste bags. You will not be permitted to leave the laboratory until your instructor sees the results of the final swipe test.

- 16) All samples will be counted for one minute in the LKB 1218 Rackbeta liquid scintillation counter. Corrections for background radiation will be made automatically (\*\* How ? \*\*). The results (counts per minute, cpm) will be made available to you tomorrow. From the "raw" counts you can determine how much <sup>35</sup>S activity there was in each fraction at each of the time points.

### Data Analysis

1. Compile your data in a table, as follows:

Time	Medium	Soluble	Precipitate	Total
0				
20				
40				
60				

2. Calculate the class average for each time point. \*\*\* Do you have more confidence in your own group's results, or the class average results? Why? \*\*\*
3. **If there is substantial variation in the total counts among the four time points, convert your counts for each fraction to the % of total counts for that time point.**
4. Plot the CPM for the medium, soluble molecules, and precipitated fractions over the 60 minute experimental period. Join the points with a smooth curve for each sample type. How do you explain the observed curves? Which fraction contained free amino acids? Which fraction contained proteins?
5. While writing up your laboratory report, keep the following questions in mind:

What happens to the amount of radioactivity in each fraction over time?

Do the counts for the three fractions add up to the same total at each time point? Why or why not?

Are the graphs for the different laboratory groups similar? Would you expect them to be?

How fast was each cell taking up radioactive sulfur? Which is a more reliable method of calculating the rate of  $^{35}\text{S}$  uptake, using the disappearance of  $^{35}\text{S}$  activity from the medium, or the appearance of  $^{35}\text{S}$  in the cell fractions?

By what mechanism(s) does  $\text{SO}_4^{2-}$  enter the cells?

What factors might influence the rate of  $^{35}\text{S}$  uptake? Is the mechanism or rate of  $\text{SO}_4^{2-}$  uptake influenced by sulfur-deprivation?

What do you think the graph would look like if the experiment had been continued for another hour?

What proteins were synthesized during your experiment?

What factors might influence the rate of protein synthesis in the cells?

What kinds of questions about cell processes (*e.g.*, amino acid and protein synthesis) can you answer using pulse labeling? Using equilibrium labeling?

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## Notes

1. **TAP Medium** is a culture medium for *Chlamydomonas*. See the ChlamyTeach site for more information. Sulfur-free TAP medium is made by substituting  $\text{MgCl}_2$  for  $\text{MgSO}_4$ .
- 2 **SDS** (sodium dodecyl sulfate; also known as sodium lauryl sulfate) is an ionic detergent, and the most widely used method of solubilizing cell components. Its initial effect is to disrupt the cell membrane, thus liberating the contents. Each protein is then enveloped in a 'cloud' of SDS molecules. Since the SDS molecules are polar, the result is to make the protein soluble. The lysis mixture also contains other components: **aprotinin** is an inhibitor of proteolytic enzymes, and ensures that the proteins are not degraded, while **beta-mercaptoethanol** (the stinky stuff) is a reducing agent that helps to break disulfide bonds within proteins, and thus aids in the solubilization.
- 2 **TCA** (trichloroacetic acid) is a powerful denaturing agent. It causes most macromolecules to aggregate, while leaving low molecular weight compounds in solution. After centrifugation, proteins and nucleic acids will be in the pellet, while most other compounds will be in the supernatant.

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### **Instructor's notes**

Use enough  $^{35}\text{S}$  to provide approximately 25,000 cpm in the initial 50  $\mu\text{l}$  sample.

Transfer the cells to a totally sulfur-free medium about four hours before the lab starts.

Cover tables with safety paper and place a metal pan on each table. All radioactive material should stay inside the pan.

Warn students to avoid tilting the micropipettes back, this can allow radioactive material to enter the barrel.

Provide each group with a different colored marker

Have students look at each fraction both as raw data and also as a % of total counts for each time point