

## COC Biotechnology Program



### Gel Filtration

Version 12-12-03

- **Chromatography is used by scientists to separate one substance from another.**
- **In this experiment you will use gel filtration to separate a mixture of protein and salt.**
- **A plastic column is filled with tiny porous beads. Small molecules running through the column temporarily get trapped in the pores in the beads. Big molecules do not fit in the pores and flow between the beads and therefore exit the column before the small molecules.**
- **Purification of molecules based on size is used in the manufacture of foods and medicines as well as in scientific research and medical labs.**

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## I. OBJECTIVES:

1. To separate a protein from salt using a Sephadex column
2. To understand the principles of gel-filtration

## II. BACKGROUND

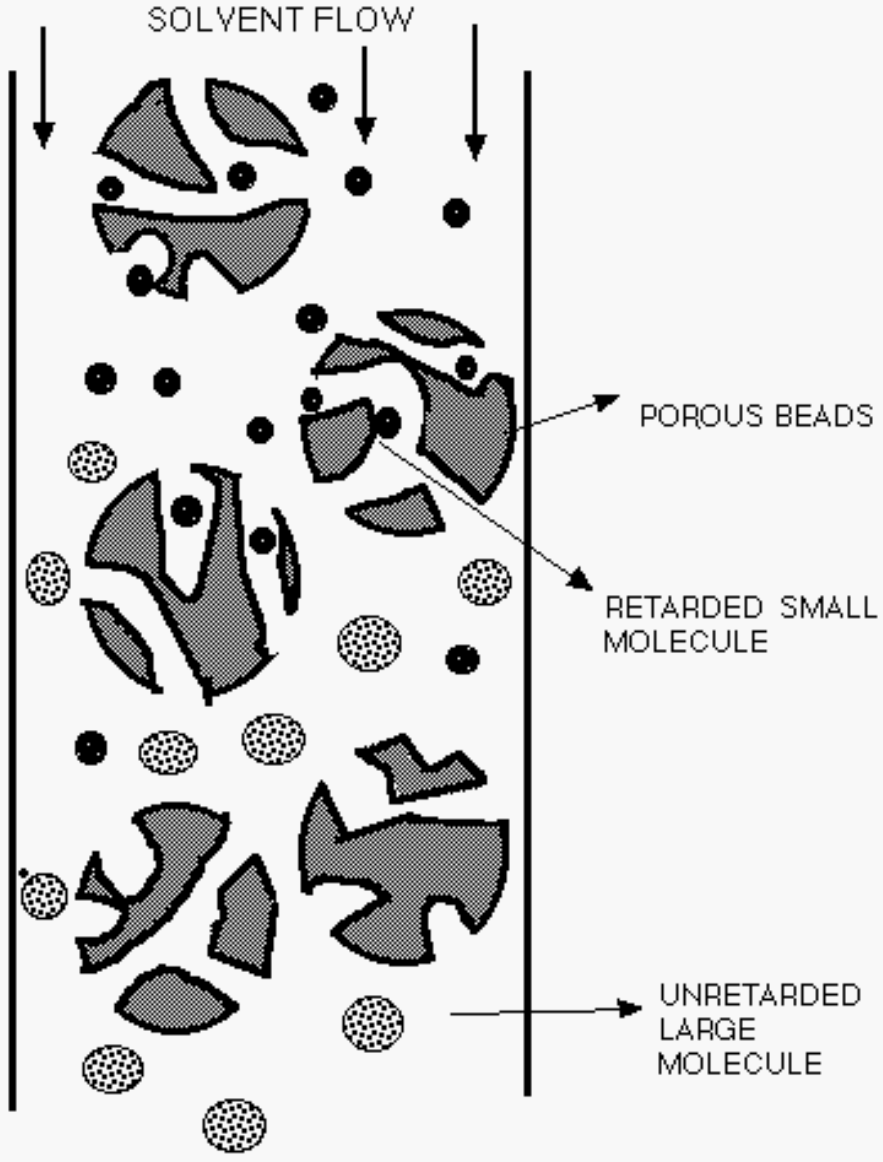
In the first experiment, you separated the colored dyes in Kool-Aid using a C<sub>18</sub> Sep-Pak. You learned that dye molecules could be separated because one dye was more polar than the other. Another method of separating mixtures using gel filtration separates molecules according to their size. The chromatography column in this case is filled with microscopic beads with pores running through them. As a liquid solution containing a mixture of different sized molecules flows through the column, molecules small enough to enter the pores in the beads move slowly within beads and from bead to bead, while molecules too large to enter the pores travel more quickly between the beads. Since larger molecules are excluded from the interior of the beads, the separation is sometimes called **size-exclusion chromatography**. Sephadex (the commercial name for the beads used in this lab) and similar products are available with many different sized pores, so that a scientist can select a pore size just right for a particular job. Gel filtration is important because it can separate molecules gently so that they are undamaged for further study. Gel filtration is used in research, industrial testing, and medical laboratories. It plays a key role in the purification of enzymes and other proteins, polysaccharides, nucleic acids and other molecules. In today's experiment, you will be using a pre-packed Sephadex column to separate a protein from the salt solution in which it is dissolved. The protein is albumin from hen's egg, dissolved in a solution of NaCl. Separating a protein from a salt is called "desalting," a common procedure. For example, a scientist may wish to purify certain proteins from blood. Blood contains salts similar to those in sea water, so one of the first tasks in purifying blood proteins is to free them from the salts. The albumin molecules are thousands of times larger than the sodium and chloride ions present in solution. Both the salt and the protein are colorless, so detection will involve the use of a conductivity meter and colorimetric assay. Since NaCl ions conduct an electrical current, you will detect the salt quantity by measuring the degree of electrical conductivity in each sample. In the Bradford's assay for protein, a small amount of a red indicator solution is added to each sample. If no protein is present, the solution turns brownish or a light blue. If protein is present, the solution turns an intense blue because the indicator color is changed as it binds to a protein.

Predict whether the first samples to come through the Sephadex column will contain salt or protein. Explain your reasoning.

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# GEL FILTRATION CHROMATOGRAPHY



### III. SOP/Lab Activities:

Important: Always note any addenda that are posted by the instructor.

#### 1. Check-List of Materials for a Lab Station

- A. \_\_\_\_\_ 12 2.0 ml microfuge tubes (round bottom)
- B. \_\_\_\_\_ marking pen
- C. \_\_\_\_\_ test tube rack
- D. \_\_\_\_\_ Sephadex G-25 column mounted on support/ring stand.
- E. \_\_\_\_\_ 2 plastic transfer pipets
- F. \_\_\_\_\_ albumin/NaCl solution: 1.8 ml in a plastic tube
- G. \_\_\_\_\_ Bradfords reagent in a plastic tube
- H. \_\_\_\_\_ conductivity/salt meter
- I. \_\_\_\_\_ cup to clean electrodes
- J. \_\_\_\_\_ squeeze bottle of deionized water
- K. \_\_\_\_\_ cup for waste from the column
- L. \_\_\_\_\_ Bradford color chart

#### 2. **PROCEDURE - PRELIMINARY STEPS:**

- A. Fill the squeeze bottle with deionized water.
- B. Remove top and bottom caps from the column, and let the deionized water left in the column during storage drain out of the column into a waste cup. Place the caps where they won't be lost.
- C. Label the 10 test tubes 1 to 10, and line them up in the test tube rack.
- D. Label one tube "positive control", and with a transfer pipette, add 19 drops (0.9 ml) of the albumin/NaCl mixture. Rinse pipet well with deionized water to get rid of any residual protein or salt. Label one tube "negative control" and with a transfer pipette, add 19 drops of deionized (DI) water. Set both tubes in the back of the test tube rack.

#### 3. **THE SEPARATION**

- A. When the column has drained, carefully position the test tube rack with its marked test tubes directly under the column. Test tube #1 should be ready to collect the first drops from the column.
- B. Using your transfer pipette gently transfer the remaining albumin/NaCl mixture onto the top of the column (as was noted in the pre-lab lecture).
- C. Collect 19 drops (0.9 ml) in tube #1 and then move on to tube #2.
- D. Continue to collect 19 drop samples in the correct sequence in the test tubes. When the column stops dripping see step E.
- E. By the second or third tube, the entire original sample will have entered the beads, and the column will stop dripping. At this stage, carefully add deionized water from the squeeze bottle onto the column to a height of about three centimeters. Keep counting 19 total drops per tube. When you have collected more drops and the column has again stopped dripping, carefully add more deionized water onto the column to fill it. Once you have collected all ten 0.9 ml samples, replace the

caps on the column, trapping the remaining water in the column for storage. Be sure there is at least one centimeter of deionized water stored in the column.

**4. TEST FOR ELECTRICAL CONDUCTIVITY TO INDICATE THE PRESENCE OF NaCl.**

A. Close tube # 1, invert it 2 or 3 times to mix, then immerse the conductivity meter electrodes into the liquid. Read and record the salt concentration (mg/oz) on your data table. If the meter jumps between two values, record the average of the two.

B. Rinse the electrodes off by squirting deionized water from the squeeze bottle over them. Let the drops fall into a waste cup. Gently shake off clinging water drops.

C. Measure and record the salt concentration for each of the other tubes by repeating above procedure. Remember to squirt the electrodes with deionized water between samples.

D. Measure and record the salt concentration of the two controls.

**5. TEST FOR PROTEIN: THE BRADFORD TEST**

A. With a transfer pipet add exactly 10 drops of Bradfords reagent to each sample and the controls. Be careful not to contaminate the pipet by allowing it to touch the samples.

B. On the negative control note the light blue color that forms. This indicates that no protein is present and should be useful when determining the protein concentration with the color chart.

C. Close each tube securely. Invert each sample several times to thoroughly mix the indicator with the sample. On your data table record your impression of the color, and record the approximate protein concentration for each tube, using the color chart. Use color descriptions such as dull blue, bright blue, blue gray, gray brown, and so on. If your sample is between two colors on the chart, estimate the protein concentration.

**6. INTERPRETING YOUR DATA**

Make two graphs of your data with the tube number along the X axis. Put salt concentration (mg/oz) along the Y axis of one graph and protein concentration ( $\mu\text{g/ml}$ ) along the Y axis of the other graph. These graphs or the table of data should be in your lab notebook. It is not necessary to reproduce both the graph and table in your lab notebook.



**IV. Post-Lab Questions/Activities:** The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

1. Were you able to separate the protein from salt? Explain how you came to this conclusion.
2. Which of your tubes contains the most concentrated protein that is salt free? Explain how you concluded this.
3. Which of your tubes contains the most concentrated salt solution with the least amount of protein? Explain how you concluded this.
4. Is there one of your samples that you might choose to run through the column a second time? Which one and why?
5. Which tube (only one tube) proves that the original solution contained a high concentration of protein and a high concentration of salt?
6. Why did the salt and protein tube total concentrations (1-10) not equal the concentration of the original amount added to the column? FYI, the concentrations added were 100 mg/oz for salt and 200  $\mu\text{g/ml}$ . Discuss the discrepancies in the salt and protein concentration data separately.
7. See next page before trying the following question. When the experimenter ran the unknown protein through the same column, it was eluted at 50 ml. Use your graph to determine its molecular weight. What is the approximate molecular weight of the unknown protein?

## **V. Notebook Entries:**

Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

### **Your intro should:**

- Define gel filtration and basis of size and pressure source.
- Albumin and salt solution: that each substance was quantified.

### **Results should be:**

- Table of data or graph, (table preferred), NOT BOTH.

### **Discussion should consider the following:**

- Effectiveness of the process.
- Evidence that separation did occur.
- Why did control vials equal total sum of fractions (both salt and albumin).
- Ideas as to how to improve protocol.

The previous lab protocol can be reproduced for educational purposes only. It has been developed by Jim Wolf, and/or those individuals or agencies mentioned in the references.

### **References:**

California Lutheran University Enriched Science Program: [www.clunet.edu](http://www.clunet.edu)

**Exercise:** A scientist used a Sephadex G-100 column to determine the molecular weight of a newly discovered protein. The G-100 Sephadex beads have a larger pore size than the G-25 beads that you have just used. In the G-100 beads the pore size is large enough so that some of the proteins can enter the beads. The scientists ran three standard proteins (A, B, and C) with known molecular weights through the column and measured the elution volume. The elution volume is the total volume of water that drips from the column after adding the sample until the most concentrated protein sample is collected. Using the data below plot the data for the standard proteins. Note: the following example is a practice exercise, and should not appear in your lab notebook. It is still fair game for quizzes, exams, etc...

	<b>Molecular Weight</b>	<b>Elution Volume</b>
Protein A	95,000 A.M.U.	10.0 ml
Protein B	70,000 A.M.U.	25.0 ml
Protein C	13,000 A.M.U.	70.0 ml

