Thus for a system of overall composition $X_B$, referring to Figure 12.5,

$$NX_B = N_T X_A + N_B X_C$$  \hspace{1cm} (12.4.3)

Substituting Equation (12.4.2) into Equation (12.4.3), we get

$$(N_T + N_B)X_B = N_T X_A + N_B X_C$$  \hspace{1cm} (12.4.4)

Rearranging leads to

$$\frac{N_T}{N_B} = \frac{X_C - X_B}{X_B - X_A} = \frac{\text{length of line } BC}{\text{length of line } AB}$$  \hspace{1cm} (12.4.5)

This equation is known as the inverse lever rule. Thus, the ratio of the masses of the top and bottom phases can be calculated simply as the ratio of the length of two segments of the tie line.

### 12.5 Chromatography Scaleup

In this laboratory exercise, elution volume, peak width, resolution, height equivalent of a theoretical plate, and the number of theoretical plates will be determined for a small-scale column and a large-scale column and compared to determine the success of scaleup without significantly changing resolution.

#### 12.5.1 BACKGROUND

Column chromatography is a common procedure for purifying biological compounds such as proteins, nucleic acids, and antibiotics (see Chapter 7). The basis for separating one dissolved species from another in a mixture depends on the interaction of the solute molecules with the solid support (packing) and the liquid mobile phase. The ionic charge, molecular weight, or relative hydrophobicity may be used as the basis for separation. When one wants to separate solutes based on their ionic charge, one uses an ion exchange column.

#### 12.5.2 OBJECTIVES

The goal of this experiment is to obtain hands-on experience in running an ion exchange chromatography column and to learn how to operate the column such that a separation of two different proteins is obtained. Equipment used in this experiment will be chromatography columns and instruments commonly found in purification laboratories. A scaleup calculation will be performed using the Yamamoto (constant resolution) approach.

#### 12.5.3 PROCEDURE

This exercise will require some prelaboratory preparation before the laboratory procedures can be implemented. The prelaboratory preparation will aid in being prepared to make all the appropriate measurements.
Preparatory Preparation

Review the small-scale column chromatogram (Figure 12.6) and calculate the resolution ($R_s$), the gradient slope ($G$), and the Yamamoto number ($Y_m$) for the separation of ribonuclease A and cytochrome c only. Use the relevant equations given shortly. Assume for S Sepharose Fast Flow (the packing that will be used) that $c = 0.3$ and $d_r = 100 \mu m$. Bring these calculations to the laboratory and be prepared to review them at the time of the experiment.

![Small-scale column chromatogram for feed containing carboxyhemoglobin (2.0 mg), ribonuclease A (2.0 mg), and cytochrome C (1.8 mg). (Courtesy of Amersham Biosciences Corp.)](image)

**Figure 12.6** Small-scale column chromatogram for feed containing carboxyhemoglobin (2.0 mg), ribonuclease A (2.0 mg), and cytochrome C (1.8 mg). (Courtesy of Amersham Biosciences Corp.)
Constant Resolution Relationships

The scaleup principle described in Chapter 7 (Section 7.8) is based on the principle of having the same resolution at all levels of scaleup. From Equation (7.4.3) for resolution, using average elution volumes instead of average retention times, we write

$$ R_s = \frac{V_2 - V_1}{\frac{1}{2}(w_2 + w_1)} \quad (12.5.1) $$

where $V_2$ and $V_1$ are the average elution volumes at which components 2 and 1 emerge from the column, respectively, and $w_2$ and $w_1$ are the widths of the respective elution peaks. Peak width $w$ is defined in Figure 7.6, where it is seen that

$$ w = 4\sigma \quad (12.5.2) $$

for peak standard deviation $\sigma$.

From constant resolution theory, we next define a number, $Y_n$, which, when held constant, can be used to calculate scaled operating values. For the purpose of this exercise, the following form is used [see Equation (7.8.11)]:

$$ Y_n = \left( \frac{L}{G(1 - \varepsilon) \mu d_p^2} \right)^{1/2} \quad (12.5.3) $$

where $L$ is bed height, $\mu$ is interstitial flow velocity, $d_p$ is resin particle diameter, $\varepsilon$ is void fraction, and $G$ is the normalized gradient slope, given by Equation (7.8.17). If we assume that void fraction and resin particle size are not changed with scaleup, then the relationship of interest is simply

$$ \left( \frac{Y_{n1}}{Y_{n2}} \right)^2 = 1 = \frac{L_1 G_2 \mu_2}{L_2 G_1 \mu_1} \quad (12.5.4) $$

Finally, for constant resolution, the volume of gradient components to be used in the laboratory column can be determined by writing Equation (7.8.17) as

$$ V_t = \frac{V(c_{aw} - c_{iw})}{G} \quad (12.5.5) $$

where $V$ is the column volume and $V_t$ is the total gradient volume.

Laboratory Procedure

1. With the help of the instructor, determine the proper operating parameters for the particular column with which you will work. Each group may work with a different set of column parameters. Pitfalls, according to experience, could consist of improper chart connections, pumping speed, or calibrations. Assignment of tasks is important.
2. Equilibrate the column with the buffer provided for this purpose, and set up the UV detector and recorder to be sure it is functioning properly.
3. Load the protein mixture onto the column.

4. Separate the proteins from one another by eluting the column with the salt gradient. During this time, record the optical absorbance and conductivity at the outlet, and monitor the elution volume by collecting the eluant in a graduated cylinder.

5. Obtain a copy of the chromatogram (recording of optical density versus time), and record the volumetric data, column parameters, equipment sketch, and any other relevant information needed for completing the report.

6. Re-equilibrate the column(s).

12.5.4 REPORT

Each person will prepare an individual written report using the following outline:

1. One-paragraph summary.

2. Experimental procedure, including equipment sketch.

3. Calculation of \( R_1 \), \( G_1 \), and \( V_m \) for the small-scale column using relationships in Equations (12.5.1), (12.5.2), (12.5.3), and (7.8.17).

4. Graphical summary, including a copy of your experimental chromatogram obtained during the experiment with the salt gradient superimposed.

5. Calculation of values of \( V_f \) and \( w \) in milliliters, height equivalent of a theoretical plate (HETP), number of theoretical plates (\( N \)), and \( R_s \) for the experiment column. Discuss whether the scaleup was or was not successful. \( N \) is determined from the following form of Equation (7.4.2):

\[
N = \frac{V_f^2}{(w/4)^2} \tag{12.5.6}
\]

where \( w \) is the peak width and \( V_f \) is the elution volume to elute a particular separand out of the column. The corresponding "plate height" is

\[
\text{HETP} = \frac{L}{N} \tag{12.5.7}
\]

Each of these values may be calculated for each separand.

6. Concise written discussion of results, errors, and measurement inaccuracies.

7. Scaling calculation performed by describing how you would operate a 10-liter column to give the same resolution as the small-scale column or the large-scale column. Include column diameter, bed height, linear and volumetric flow rates, and total gradient volume calculations for the 10-liter column. The column you choose should be available from Millipore (see Table 12.4), and the flow properties of S Sepharose Fast Flow packing should be considered (Figure 12.7). Discuss why you selected your particular design, and list your assumptions and considerations.

Limit reports to seven pages, including graphs and sketches.
### TABLE 12.4
Characteristics of Moduline³ Acrylic Biochromatography Columns: Sizes and Pressure Ratings

<table>
<thead>
<tr>
<th>Diameter x length (mm)</th>
<th>Adjustable length (mm)</th>
<th>Cross-sectional area (cm²)</th>
<th>Adjustable capacity (liters)</th>
<th>Pressure rating (bar)</th>
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<td>58</td>
<td>0-1.9</td>
<td>4</td>
</tr>
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<td>38</td>
<td>1.9-5.8</td>
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<td>0-3.2</td>
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Source: Millipore, Inc., 2002

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**Figure 12.7** Pressure–flow characteristics for S Sepharose Fast Flow resin columns. BP 113 column: 15 cm bed height and 100 cm² cross-sectional area. BP 252 column: 15 cm bed height and 300 cm² cross-sectional area. (Courtesy of Amersham Biosciences Corp.)