

Column Chromatography

Chromatography is the process use to separate molecules based on SOME physical property of the molecule:

- **Mass (i.e. size)**
- **Charge**
- **Affinity for ligands or substrates**
- **Hydrophobic interactions**

Two phases in EVERY chromatography experiment:

- **Stationary phase: a surface or resin that is inert**
- **Mobile phase: Comprised of the solvent and the sample (eluant). Introduction of the mobile phase can either be done by a gravity feed (siphoning) system or by a pumping device (usually peristaltic). In most of the figures for this chapter (see below), gravity feed systems are used.**

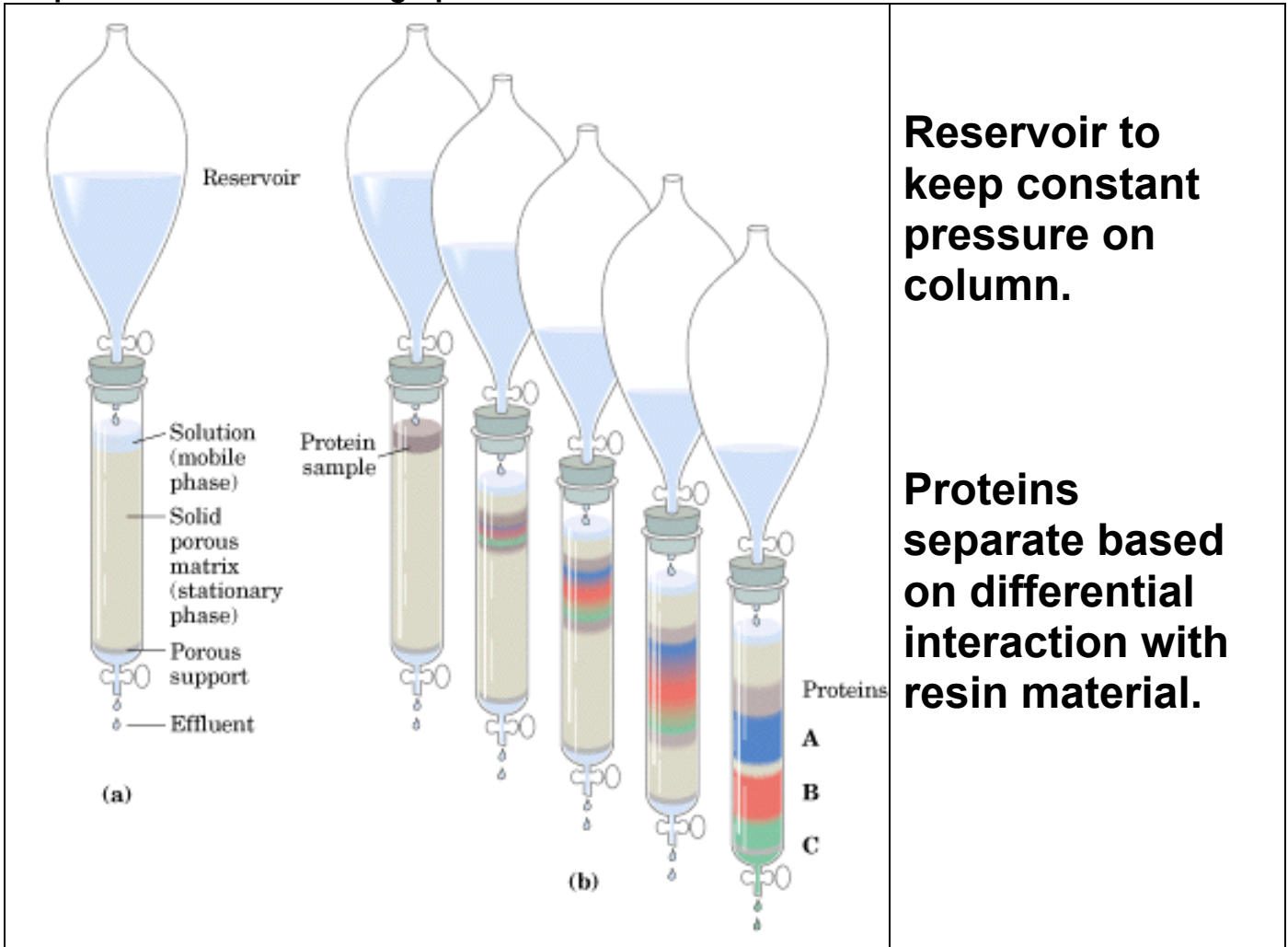
Separation occurs due to VARYING degrees of interaction of sample with the stationary phase.

Interaction of sample with stationary phase can be modulated by changing the solvent conditions (i.e. pH, ionic strength, competitive ligands, etc.).

For column chromatography: stationary phase is referred to as resin or gel or matrix.

Three primary types of RESINS:

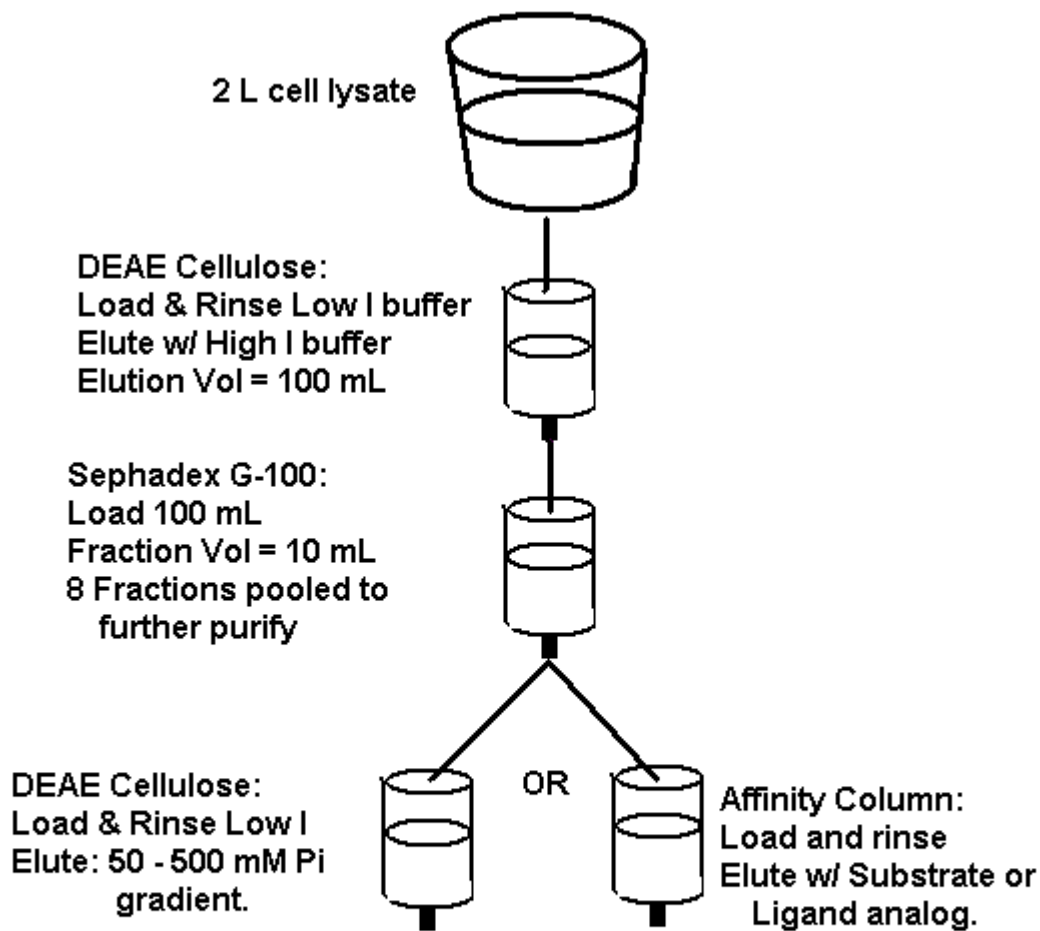
- **Gel Filtration = Size Exclusion (SEC) = Molecular Sieve**
- **Ion Exchange (IEC)**
- **Affinity (ligands, substrates, or “tags”)**



All column chromatographic methods attempt to purify a single protein from many other proteins based on differential interactions. Rarely can this be accomplished using one type of column!

(inserted on Sunday)

**“Never follow a seal act with another seal act”
(old Vaudeville saying)**



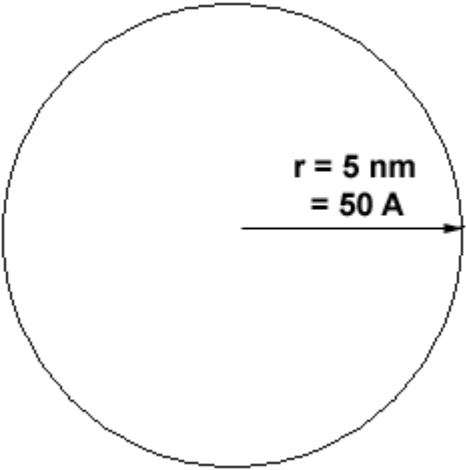
Size Exclusion Chromatography

Uses:

1. Separation and purification of proteins.
2. Determination of Molecular Weight.
3. Desalting (i.e. removing small molecule salts) protein samples.
4. Change the pH and ionic strength of the buffer that the protein is in.

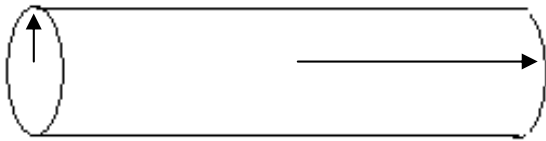
Molecules separated according to their Stokes Radii: the effective radius of a particle moving through a medium. Minimized for a perfect sphere, increases as you deviate from sphere.

Assume for spherical (globular) proteins: radius of protein + 1st hydration sphere is proportional to molecular mass or weight.

 <p>A diagram of a sphere representing a protein. A horizontal arrow points from the center of the sphere to its right edge. Next to the arrow, the text reads: $r = 5 \text{ nm}$ $= 50 \text{ \AA}$</p>	<p>For spherical protein, with a Stokes radius of 5 nm (= 50 Å).</p> <p>Then $V = (4/3)\pi r^3$ $= 524 \text{ nm}^3$</p>
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For non spherical proteins: the radius of the sphere described by the rotation of the molecule about its longest axis.

- **What would be the Stokes radius of a rod-shaped protein that has the same volume (524 nm^3)?**



$$\begin{aligned} \text{Let } r &= 1.5 \text{ nm} \\ V &= 524 \text{ nm}^3 = \pi r^2 h \\ &= \pi (1.5 \text{ nm})^2 h \end{aligned}$$

$$\text{So } h = 74.1 \text{ nm.}$$

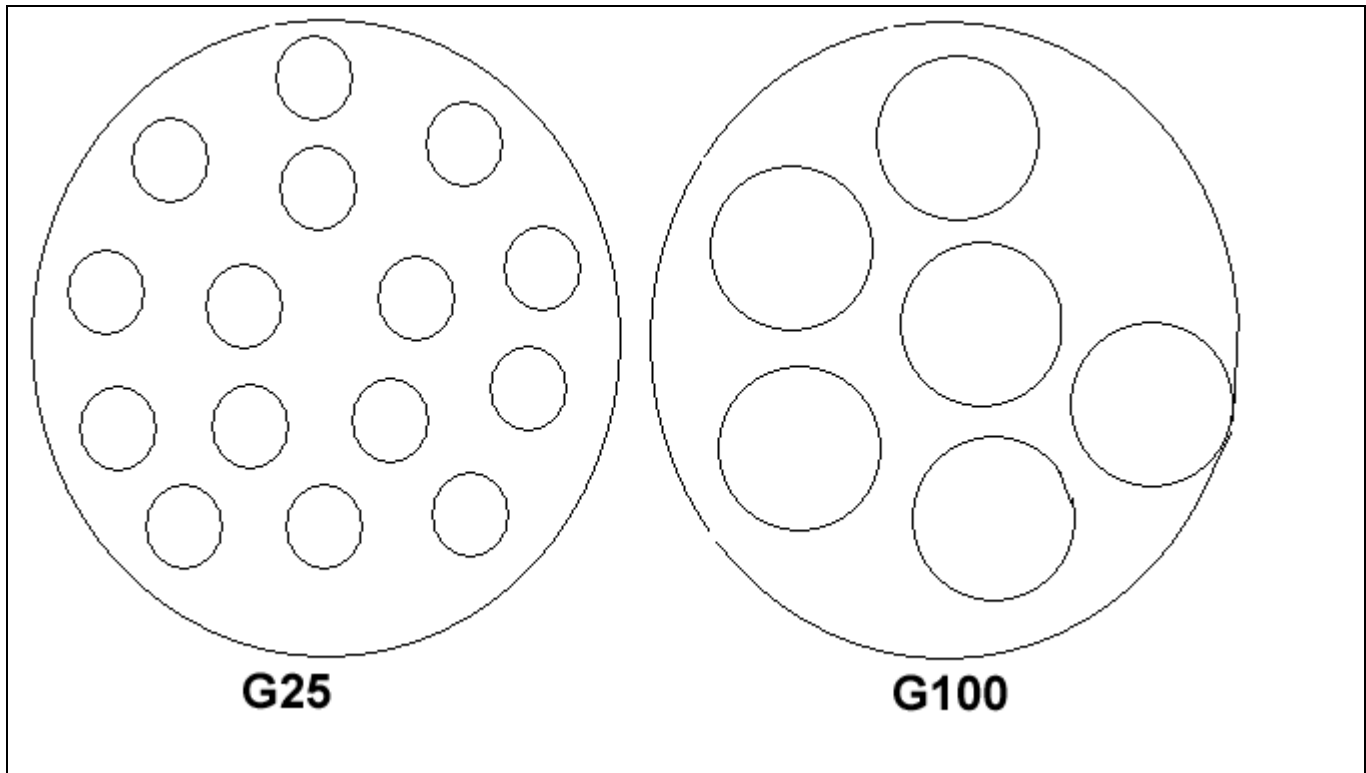
So Stokes radius of cylinder = 37 nm

Or 7X that of a sphere with same volume!

Both proteins have the same Volume or Mass, but the rod-shaped protein will appear to have a much larger size, due to larger Stokes radius, as it tumbles through a gel filtration column.

How does this influence interaction with SEC resins?

SEC resins are hollow beads prepared by *cross linking* a polymer such as Dextran or Acrylamide. The beads have a Wiffle Ball-like structure with more or less discrete hole or pore sizes.



The pore size is determined by the cross-linker/polymer ratio:

- **High ratio : small pore size → low molecular weight cut-offs.**
- **Low ratio : large pore size → high molecular weight cut-offs.**

Dextran cross-linker figure

Cross-linking results in a limited range of pore sizes, rather than a single precise size. This allows for a molecular weight range over which the resin is effective.

Table 4-1.

TABLE 4-1
Low-Pressure Gel-filtration Chromatography Media

GEL PRODUCT	EXCLUSION SIZE	MW RANGE	MATERIAL	SOURCE
A15M	15×10^6	$8 \times 10^4 - 12 \times 10^5$	Agarose	Biorad
A5M	5×10^6	$4 \times 10^4 - 4 \times 10^5$	Agarose	Biorad
A0.5M	5×10^5	$1 \times 10^4 - 5 \times 10^4$	Agarose	Biorad
Sephacrose 4B	2×10^7	$8 \times 10^3 - 12 \times 10^6$	Dextran	LKB
Sephacrose 6B	1×10^7	$4 \times 10^4 - 4 \times 10^6$	Dextran	LKB
Sephadex G200	5×10^5	$4 \times 10^4 - 2 \times 10^5$	Dextran	LKB
Sephadex G150	2×10^5	$2 \times 10^4 - 12 \times 10^4$	Dextran	LKB
Sephadex G100	1×10^5	$8 \times 10^3 - 8 \times 10^4$	Dextran	LKB
Sephadex G75	6×10^4	$4 \times 10^3 - 4 \times 10^4$	Dextran	LKB
Sephadex G50	4×10^4	$2 \times 10^3 - 3 \times 10^4$	Dextran	LKB
Sephadex G25	3×10^4	$0.8 \times 10^3 - 2 \times 10^4$	Dextran	LKB
Sephadex G10	8×10^3	$0.4 \times 10^3 - 6 \times 10^3$	Dextran	LKB
P100	1×10^5	$8 \times 10^3 - 8 \times 10^4$	Acrylamide	BioRad
P60	6×10^4	$3 \times 10^3 - 4 \times 10^4$	Acrylamide	BioRad
P30	4×10^4	$2 \times 10^3 - 2 \times 10^4$	Acrylamide	BioRad
P10	2×10^4	$2 \times 10^3 - 1 \times 10^4$	Acrylamide	BioRad
P6	6×10^3	$0.8 \times 10^3 - 4 \times 10^3$	Acrylamide	BioRad
P2	8×10^3	$1 \times 10^2 - 2 \times 10^3$	Acrylamide	BioRad

Overview of SEC-protein interactions:

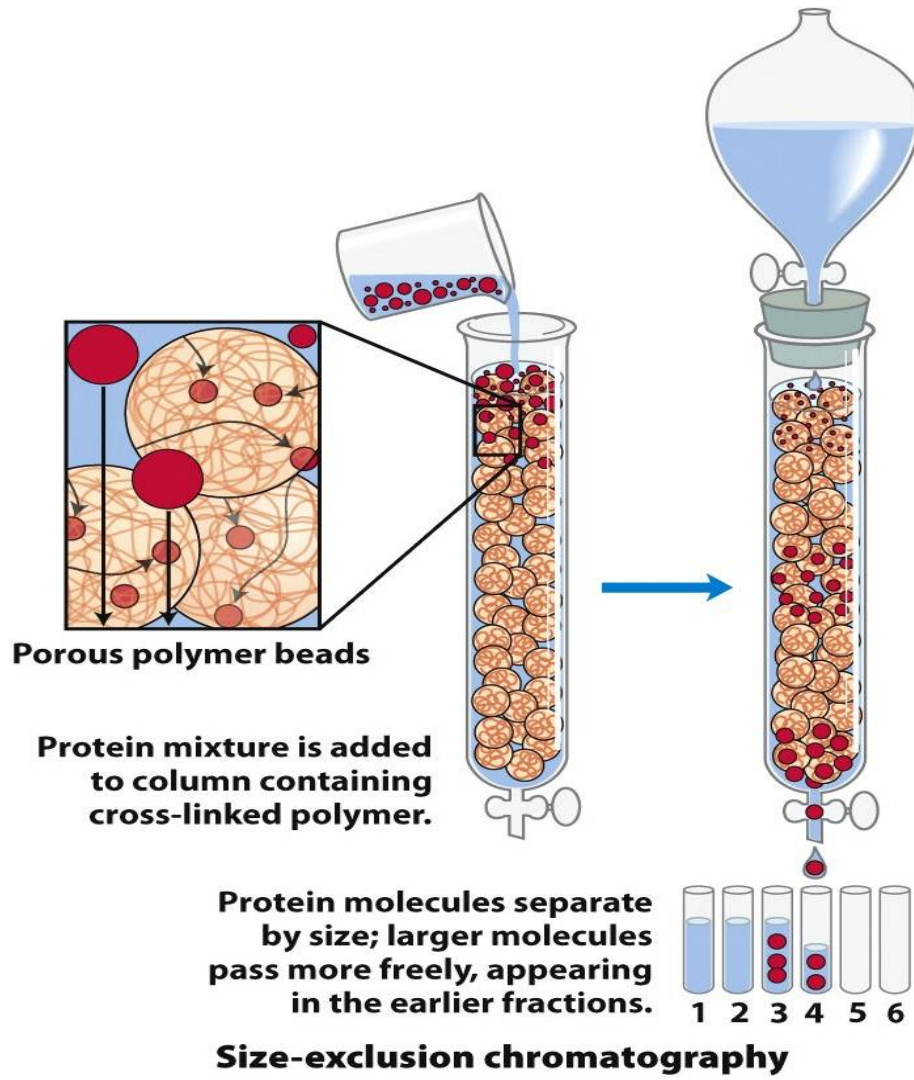


Figure 3-17b
Lehninger Principles of Biochemistry, Fifth Edition
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A word of caution: the larger the pore size (less cross-linking) the more collapsible the resin. Cross-linking adds structural integrity, especially for the dextran based resins (i.e. Sephadex). Under high pressure, the higher MWT cut off resins can collapse because of their LESSER degree of cross-linking, creating an almost impermeable barrier at the bottom of a column. Polyacrylamide (i.e. the Bio-Rad "P" series) resins are significantly less prone to collapse and are often preferred for these applications.

Partition Coefficients and The "Volumes" of SEC

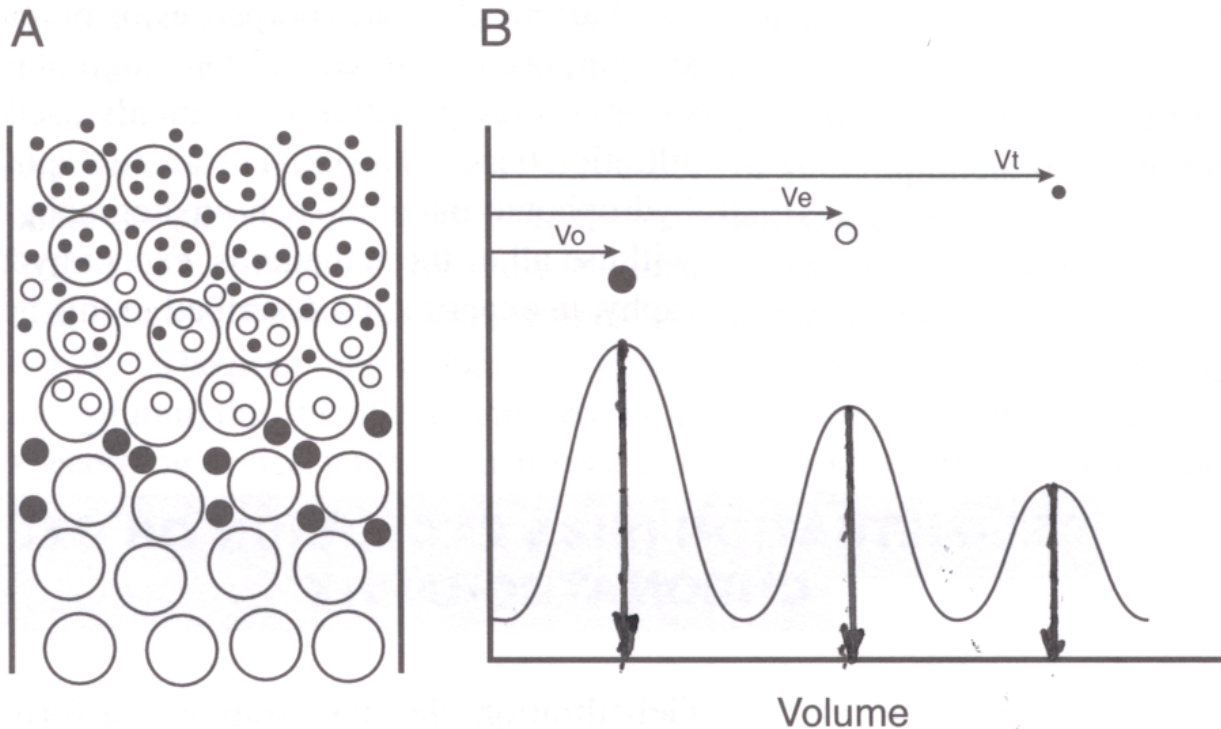
Partition coefficients measure the degree of interaction of a protein with a SEC, i.e. how well the protein partitions on the column.

The degree of interaction of the protein with the SEC resin, e.g. partitioning, is best measured by the **VOLUME** at which the sample elutes from the column.

Volume Definitions:

- V_0 : Void Volume - the volume outside of the beads.
- V_i : Included Volume – the volume within the beads.
- V_g : Gel Volume- the volume of the beads themselves ($\leq 1\%$ of total volume).
- V_{total} : Total Volume of column. $V_t = V_0 + V_i + V_g$.
Since $V_g \sim 0$, then: $V_t = V_0 + V_i$.
- V_e : Elution Volume for a sample.

How are these volumes measured? Referring to following figure, the volumes are calculated from the fraction (of a known volume) at which the material elutes from the column.



Void Volume (V_0) is determined using a material (usually a colored dye such as BLUE DEXTRAN) that is too large to interact with the resin, therefore is not retarded by the resin as it flows through the column.

Total Volume (V_t) is determined by using a material that is very small and interacts maximally with the resin. As in the above case, often a colored small molecular weight species can be used (riboflavin in this experiment).

The volume at which this material elutes includes both the void (outside) and included (inside) volumes.

Partition Coefficient = K_{av} :

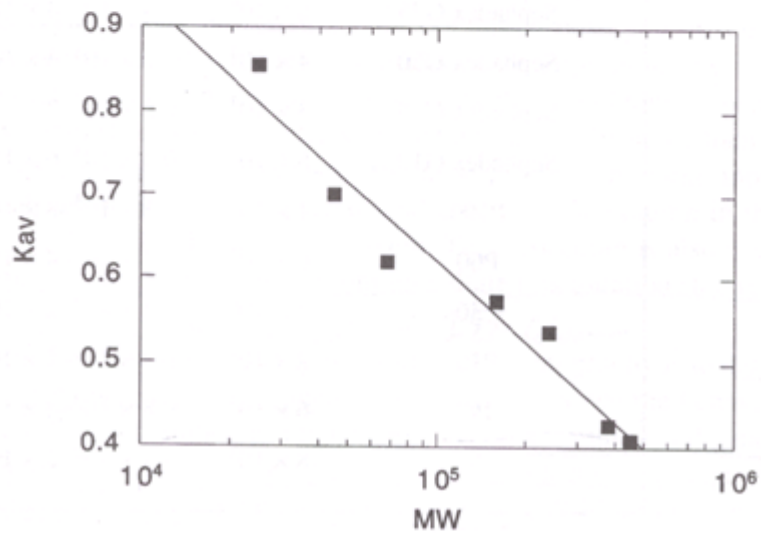
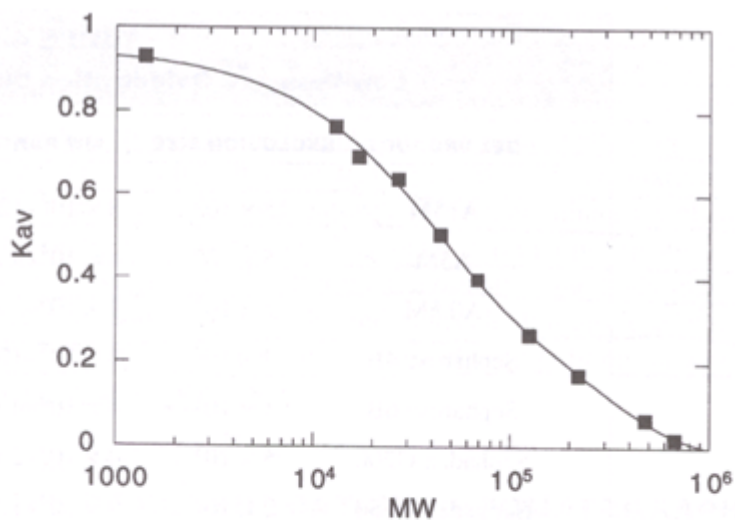
Every protein (or DNA or RNA) elutes from a specific SEC column in a reproducible manner based on its Stokes Radius, which is related to its mass. The measure of this elution behavior is expressed as its Partition Coefficient, K_{av} .

- $K_{av} = (V_e - V_0)/(V_t - V_0) = (V_e - V_0)/V_i$.

Calibration of SEC Columns: The knowledge of the K_{av} for a given protein can be very useful in the determination of the molecular weight (or mass) of a given protein in the following manner:

- 1. A precise volume of a mixture of proteins, Blue Dextran, and a small molecular weight indicator are eluted from the column and their K_{av} values determined.**
- 2. A plot of K_{av} vs. Log Mol. Weight (MW) is constructed.**
- 3. The unknown protein is added to the column using the same volume as used for standards, then eluted from the column. The K_{av} is determined for the unknown and MW is calculated from the plot of K_{av} vs. Log MW.**

Fig. 4-5 Kav vs. Log MW



Resolution of SEC Columns

- Resolution is proportional to $(\text{Length})^{1/2}$.
- But, elution time is also proportional to length
=> YOU MUST BE PATIENT WHEN RUNNING COLUMNS!!!.
- The shallower the slope of a K_{av} vs. Log MW plot (i.e. the broader the range of MWT resolved), the greater the resolution of the column resin.

Plot of K_{av} vs. Log MW for G75, G100, and G200

Problems with SEC

- **Initial equilibration is long and tedious. Involves hydration (swelling), then pulling a vacuum on the resin to remove air from within beads.**
- **CANNOT allow resin to go dry. If so, then repeat equilibration process.**
- **For sugar based resins, algae and bacteria can grow on sugar matrix. Store using a 0.2% NaAzide solution.**
- **Packing of column is critical. Best if done in continuous manner so all of resin settles at same time. Eliminates “banding” in column.**
- **Sephadex resins: Volume decreases with increase in ionic strength.**
- **Flow rate decreases with increase in MW range (i.e. G200 runs slower than G25). Can compensate by using peristaltic pump, BUT...**
- **High pressure can collapse Sephadex beads at bottom of column (use polyacrylamide resins).**
- **Bands are broadened on SEC columns: sample is diluted on SEC columns due to thermal diffusion and frictional effects. Improperly poured columns or columns with**

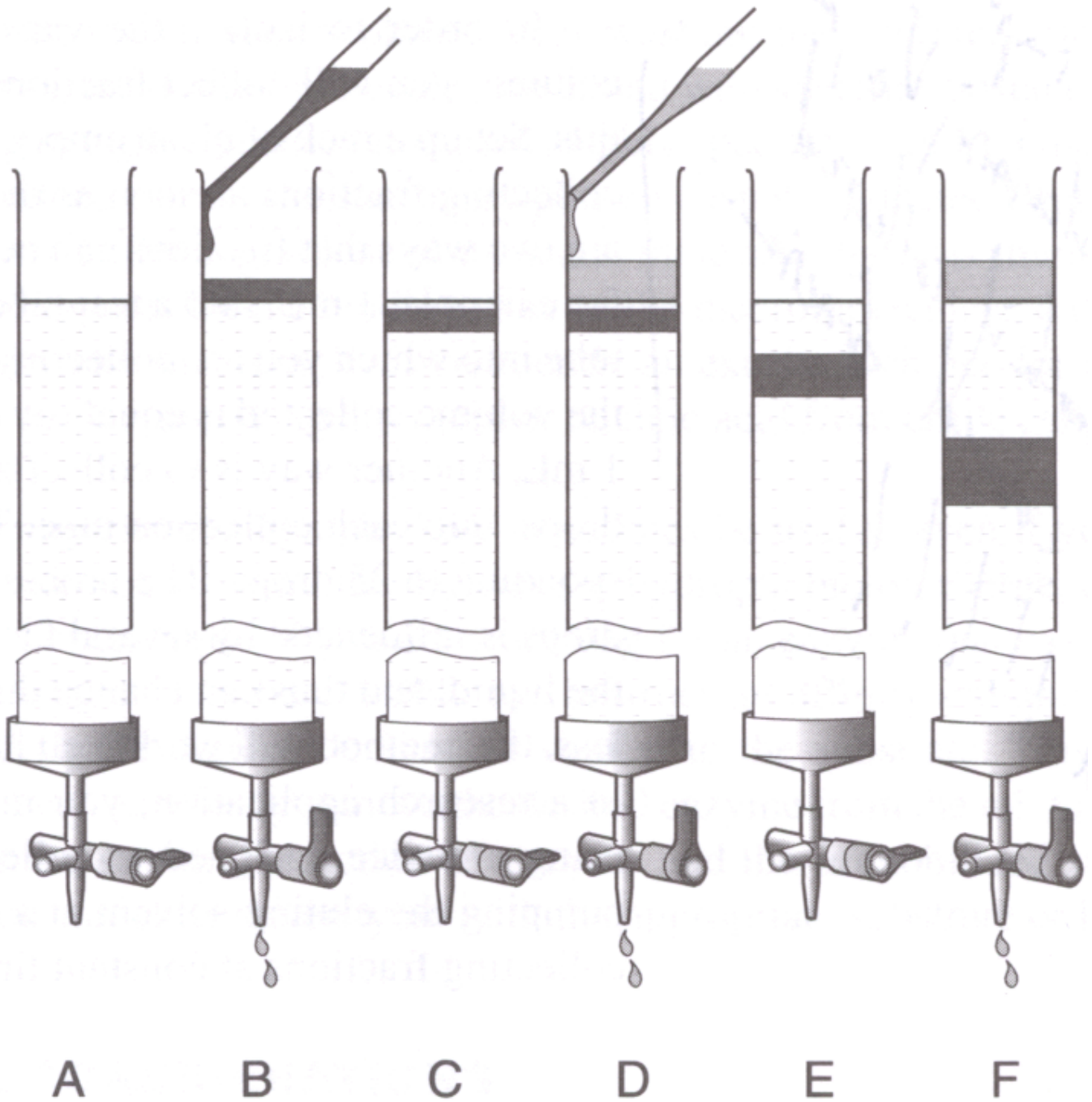
plugged “frits” can result in very erratic band migration.

- **Air bubbles can form in column when taken from cold room to room temp due to expansion of gas volume.**

Room temp → cold: OK

Cold room → room temp: Bad

**Fig. 4-6: Proper Loading of SEC Columns.
Use caution so the surface of the column is not
disturbed!!!**



Ion Exchange Chromatography: DEAE and CMC

Uses:

1. Crude purifications of cell lysates (i.e. batch cuts).
2. Decrease volume of protein sample (i.e. concentrate).
3. Purify proteins according to charge properties of protein and buffer conditions (pH and ionic strength).
4. Either increasing buffer [salt] or changing pH can be used to decrease binding to resin.

Separation based on net charge on protein and electrostatic interactions between protein and charged groups on resin.

<u>Positively Charged AA</u>	<u>Negatively Charged AA</u>
Arg	Asp
Lys	Glu
His	RS ⁻

$$Z_{\text{net}} = (\#)(Z_{\text{pos}}) + (\#)(Z_{\text{neg}})$$

Z_{net} is related to pH and pI:

pH < pI	$Z_{\text{net}} > 0$
pH = pI	$Z_{\text{net}} = 0$
pH > pI	$Z_{\text{net}} < 0$

Often proteins are referred to as Acidic, Basic, or Neutral:

Acidic (lots of Asp, Glu)	pl < 6
Neutral	6 < pl < 8
Basic (lots of Arg, Lys)	pl > 8

Strong vs. Weak Ion Exchangers (see Table 5-2 in text (4-2 below) for names and structures).
Strong: Used to remove small anions or cations. Irreversible binding of ions to resin. Often used for “stripping” purposes.
Weak: Reversibly binds anions or cations (i.e. proteins with negative or positive net charge).

TABLE 4-2
Ion Exchange Groups Used in the Purification of Proteins

FORMULA	NAME	ABBREVIATION
<i>Strong anion</i>		
$-\text{CH}_2\text{N}^+(\text{CH}_3)_3$	trimethylaminoethyl	TAM
$-\text{C}_2\text{H}_4\text{N}^+(\text{C}_2\text{H}_5)_3$	triethylaminoethyl	TEAE
$-\text{C}_2\text{H}_4\text{N}^+(\text{C}_2\text{H}_5)_2\text{CH}_2-\text{CH}(\text{OH})\text{CH}_3$	diethyl-2-hydroxypropylamino-ethyl	QAE
<i>Weak anion</i>		
$-\text{C}_2\text{H}_4\text{N}^+\text{H}_3$	aminoethyl	AE
$-\text{C}_2\text{H}_4\text{N}^+\text{H}(\text{C}_2\text{H}_5)_2$	diethylaminoethyl	DEAE
<i>Strong cation</i>		
$-\text{SO}_3^-$	sulpho	S
$-\text{CH}_2\text{SO}_3^-$	sulphomethyl	SM
$-\text{C}_3\text{H}_6\text{SO}_3^-$	sulphopropyl	SP
<i>Weak cation</i>		
$-\text{COO}^-$	carboxy	C
$-\text{CH}_2\text{COO}^-$	carboxymethyl	CM

In this course:

- **CMC (carboxymethyl) cellulose**
- **DEAE (diethylaminoethyl) cellulose**

Fig. 4-11 How IEC Columns Bind Proteins

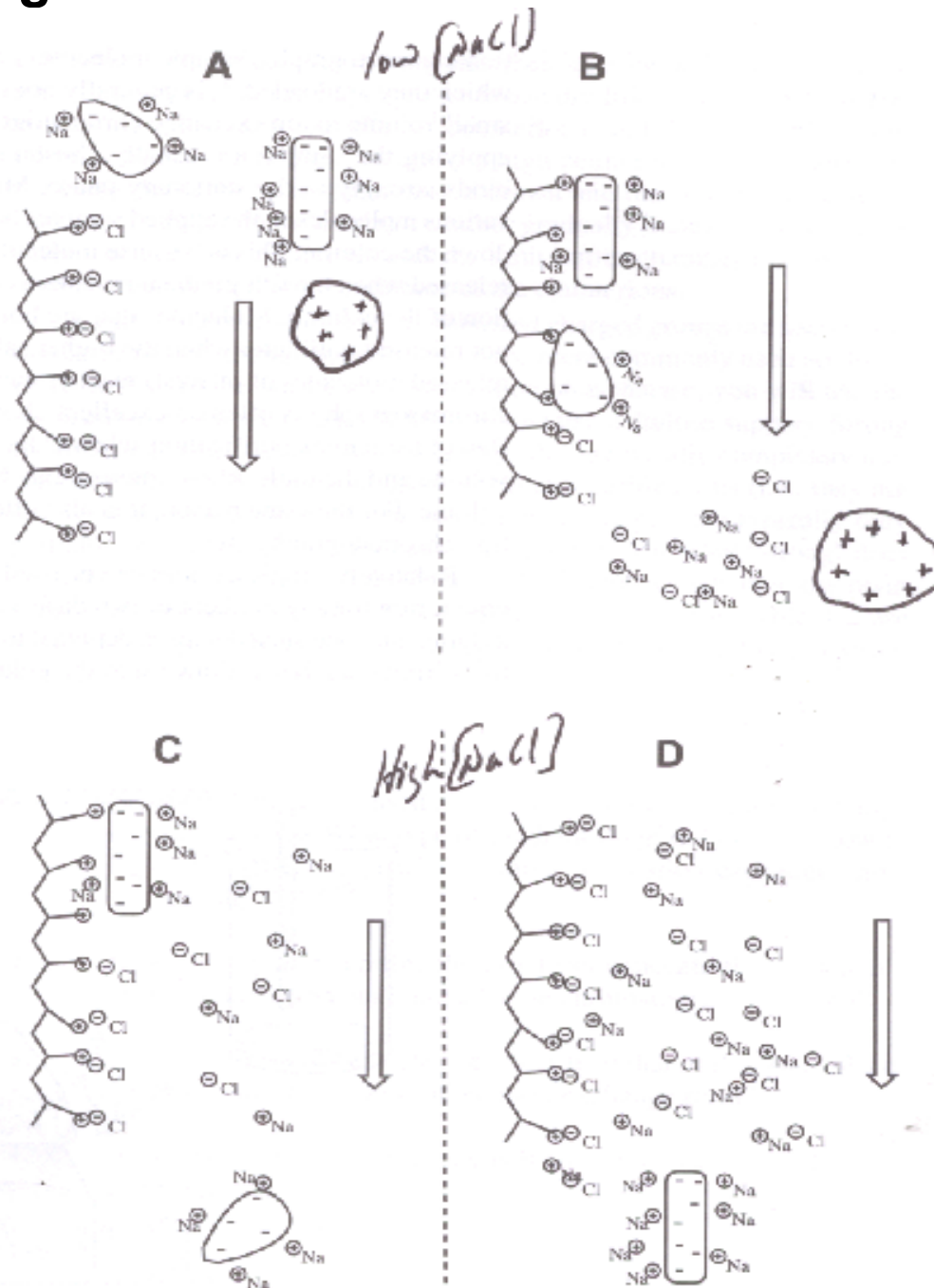
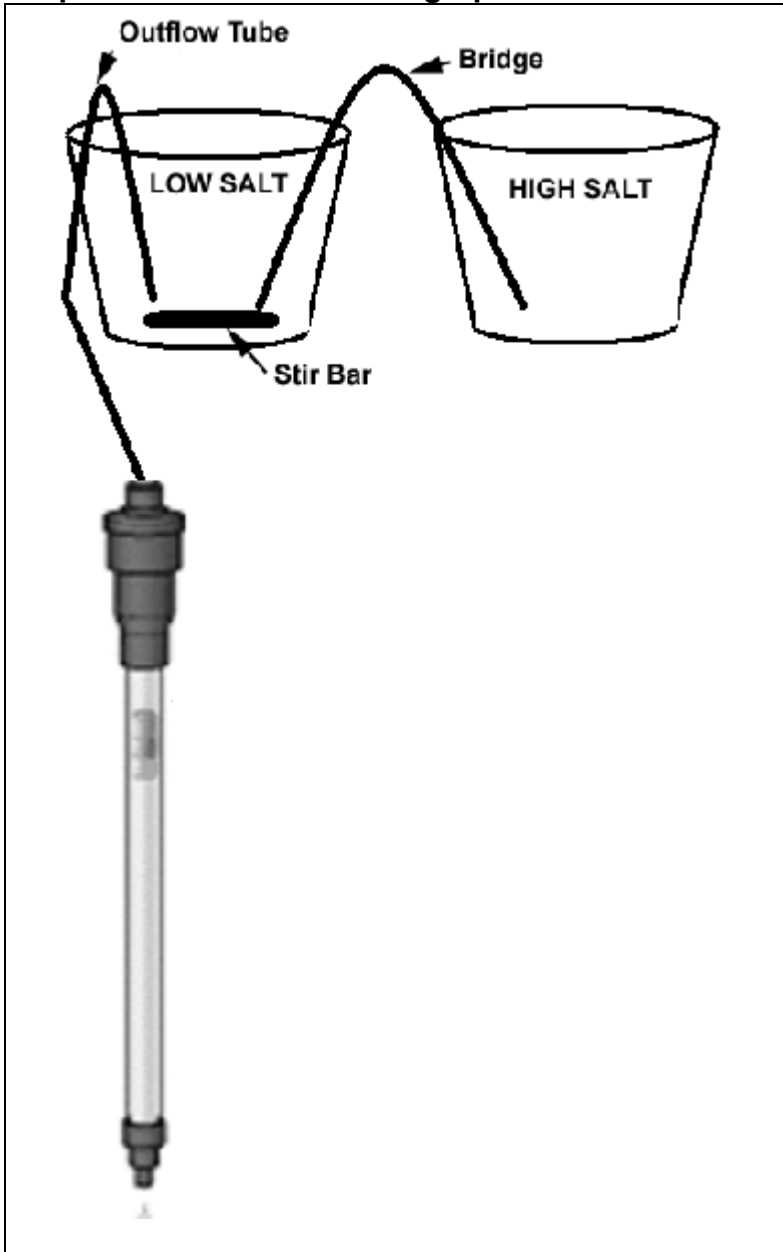


FIGURE 4-11 Illustration of ion-exchange processes occurring when negatively charged proteins are separated on an anion-exchange column. (A) Column is equilibrated with low [NaCl]. (B) Proteins are loaded at low [NaCl] and bind to the matrix, displacing Cl⁻ and Na⁺ ions formerly associated with the matrix and proteins. (C) As [NaCl] increases, the less acidic protein elutes. (D) At higher [NaCl], the more acidic protein elutes.

General Protocols in IEC:

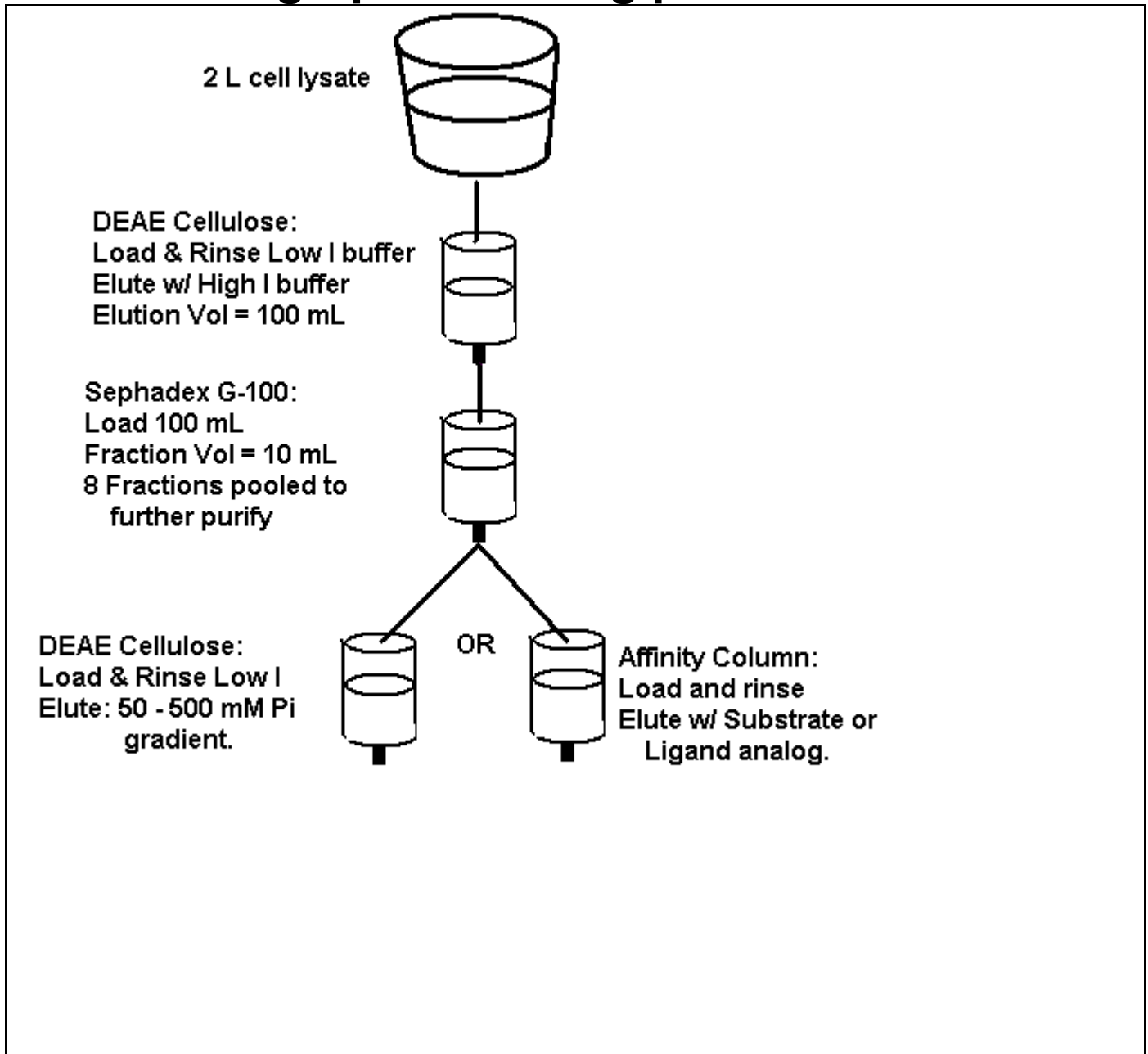
- Resin equilibrated with low ionic strength buffer of proper pH.
- Protein equilibrated in same low I buffer.
- Protein loading volume can be quite large. Protein concentrates on column.
- Elution of protein(s): must disrupt Coulombic interaction of protein with resin by increasing [salt] in buffer OR by altering the charge on the protein by changing buffer pH.
- Buffer changes can be made gradually using a linear gradient or by big STEPS in buffer conditions.
 1. Linear ionic strength or pH gradient often used for high resolution IEC in order achieve maximum purification.
 2. Step gradient: once unwanted proteins are eluted from column, switch to a buffer with dramatically higher ionic strength or different pH. A main goal of this method is to **CONCENTRATE** or **DECREASE** the volume of your sample.



An Ionic Strength Gradient is established as the column siphons buffer from the Outflow Beaker, which in turns siphons from the High Salt Beaker. Mixing the High Salt with the Low Salt solution gradually increases the ionic strength creating a linear gradient flowing onto the column.

Once unwanted proteins have been eluted, switch outflow tube to high salt or high pH buffer.

Combining different types of column chromatographies during purification.



Which steps are used to:

- **concentration protein**
- **separate proteins**
- **concentrate AND separate?**

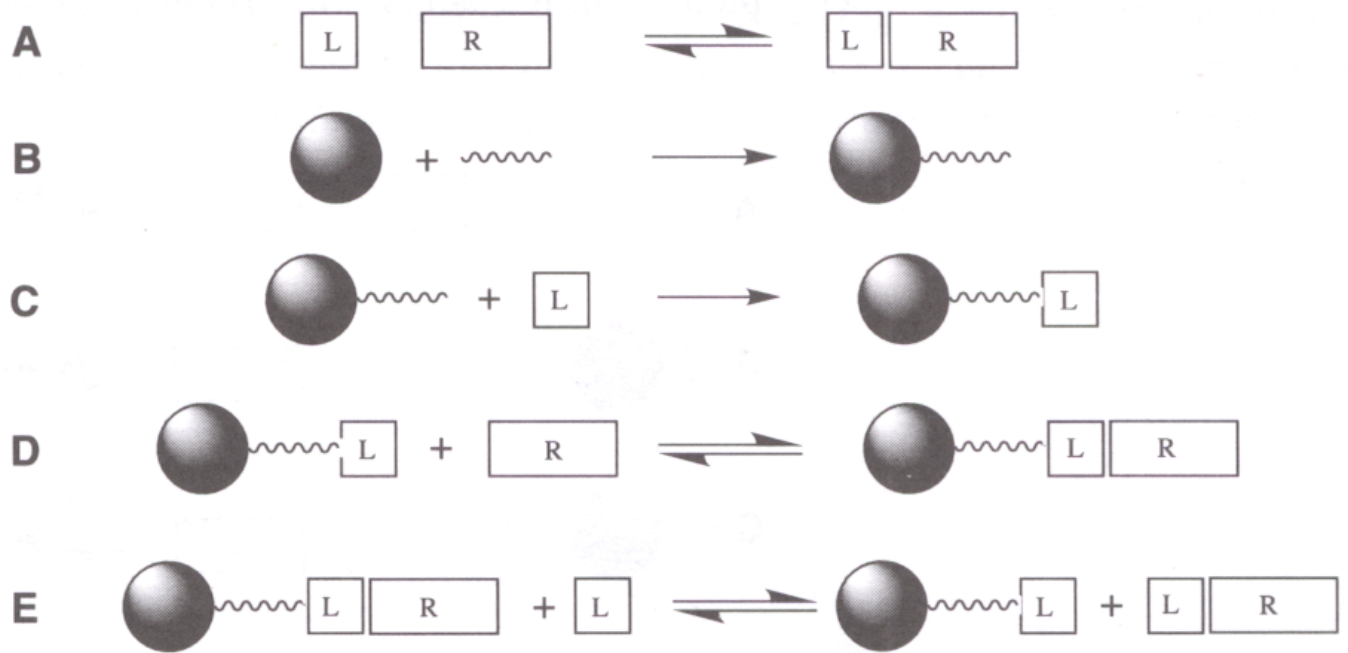
Column Chromatography Part 2: Affinity Chromatography

There are three parts to an affinity resin:

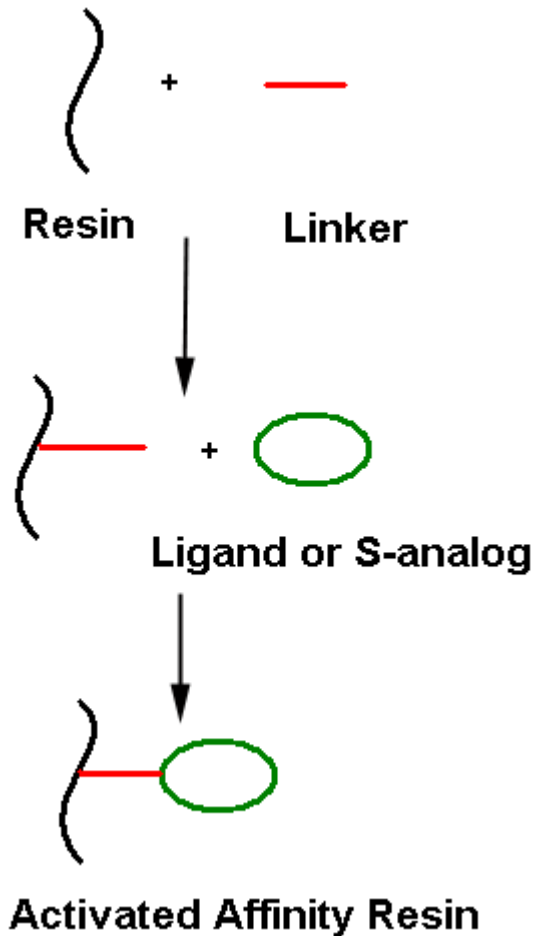
- An activated resin (has reactive sites).
- Activated Linker of defined length.
- Ligand or Substrate analog (inert) that can be bound by linker.

General Procedure:

Figure 4-7 Affinity chromatography



Affinity column resins can be purchased pre-made or you have to carry out the following reaction sequence:



Advantages of Affinity Chromatography:

- **Interactions are highly specific for Receptor:Ligand or Enzyme:Substrate complexes (hydrophobic, van der Waals, hyd. Bonding).**
- **Simplifies purification schemes dramatically.**
- **One-step purification claims by manufacturers are over-stated.**

Disadvantages:

- **If affinity resin not available, must follow procedure to prepare resin.**
- **Resins seem to have finite lifetime, especially Substrate analog resins. They lose effectiveness with time.**

Special Consideration: You do not want to use a Ligand or Substrate that has such a low K_d or K_s that it is difficult to remove from column.

Affinity Column Purification of β -galactosidase:

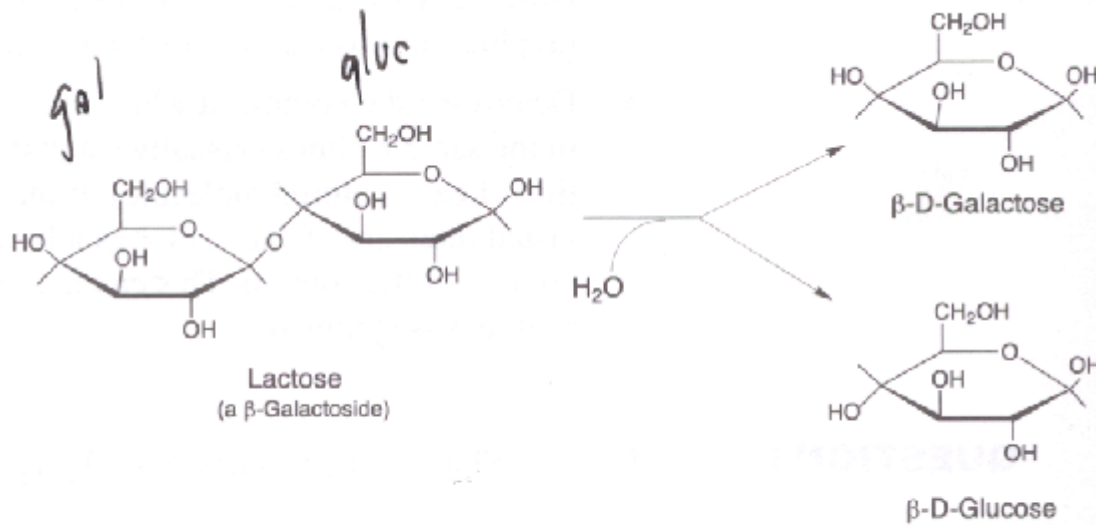
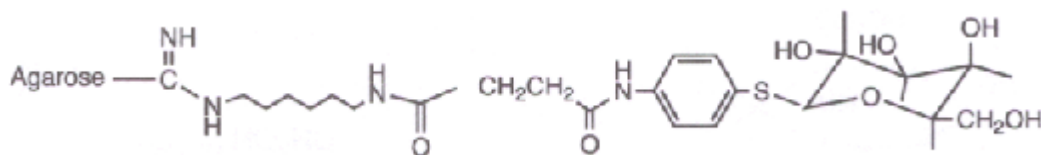


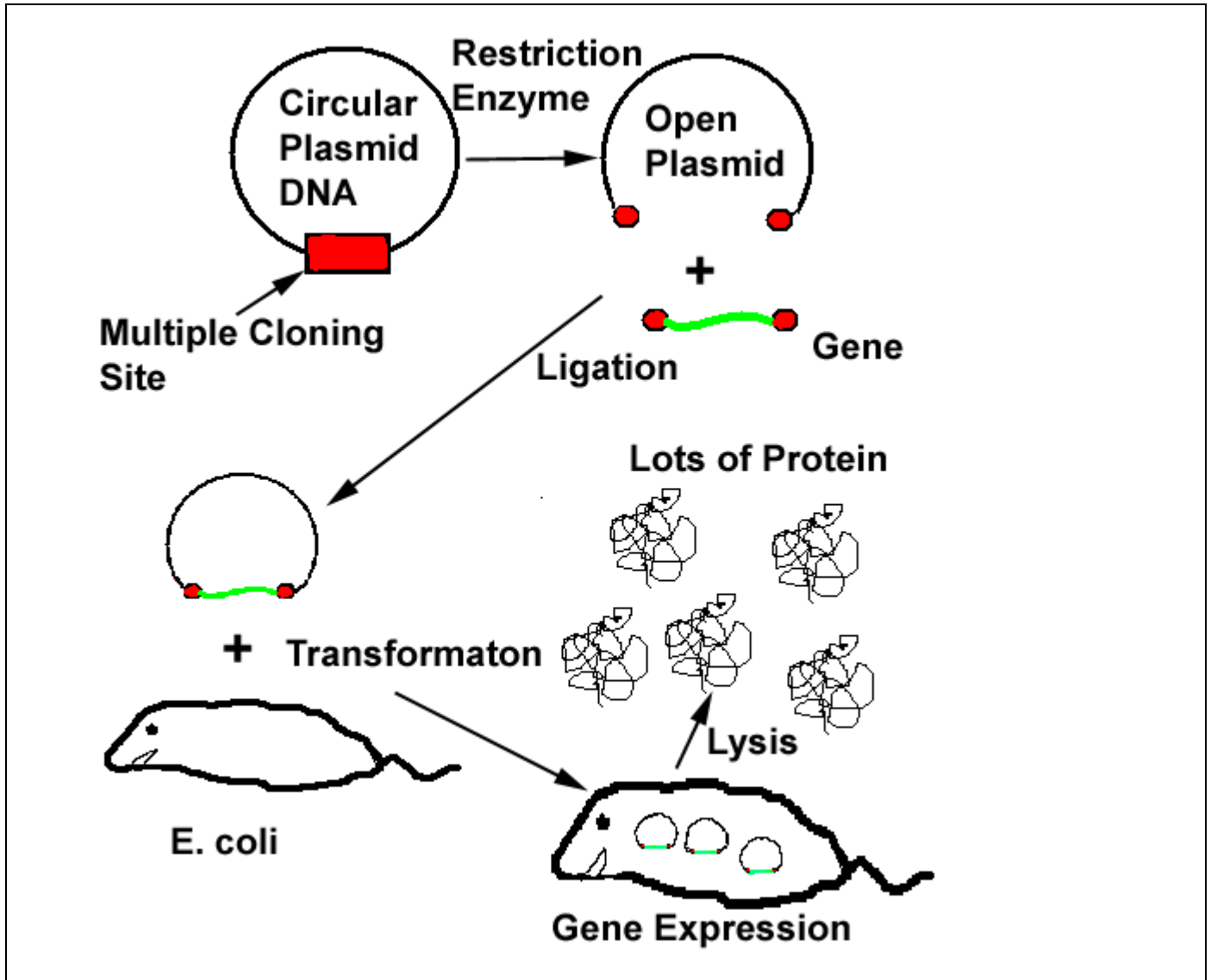
FIGURE 4-14 Reaction catalyzed by β -galactosidase.

We will purify β -galactosidase making use of the fact that it has a high degree of specificity for binding galactose.



p-aminobenzyl-1-thio- β -D-galactopyranoside agarose resin: Why a S-glycosidic linkage instead of an O-glycosidic linkage?

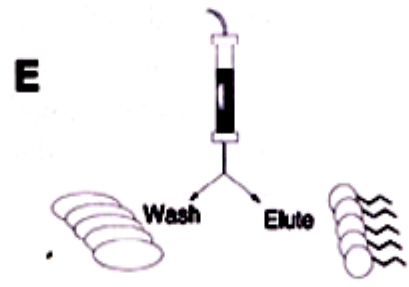
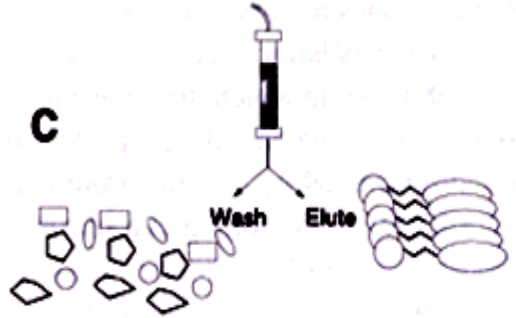
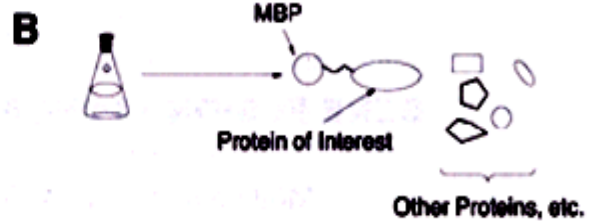
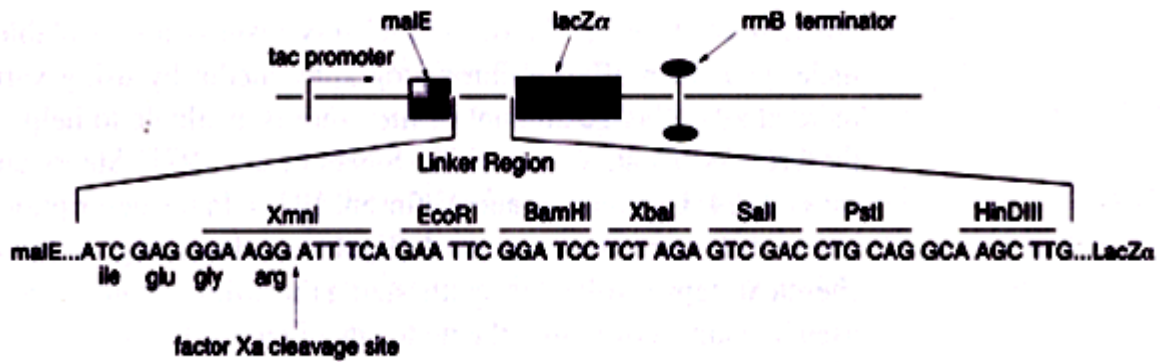
“TAG” or Gene Fusion Columns A Short Primer on the Recombinant Protocol



General Principle:

- **TAG: a short peptide or protein that will interact very strongly with an affinity like column.**
- **The DNA sequence for the TAG is expressed at either the N- or C-terminus of your protein of interest.**
- **Interaction of the TAG with the column, that is specific for the TAG, supposedly allows for a high yield 1-step purification.**
- **Tagging plasmid and Column resin are sold by same manufacturer.**

Maltose Binding Protein Tag:



Common Tagging Systems:

<u>Gene Fusion</u>	<u>Column</u>
Maltose Binding Protein	Maltose analog
Glutathione S Transferase	Glutathione

<u>Peptide Tag</u>	<u>Column</u>
Poly-His (7-8)	Chelated Nickel
Avidin Binding Peptide	Streptavidin

Despite the attractiveness of Gene Fusion systems, there are concerns one should be aware of with these constructs.

The goal of the protein biochemist is to understand how a NATIVE protein functions in the NATIVE organism.

But with Tagged systems we have:

Native (original source)	vs	Wild-type (recombinant)
Wild-type	vs	Tagged (not cleaved)
Wild-type	vs	Cleaved tagged
Cleaved	vs	Native

Although it is often done, it is DANGEROUS to ASSUME (we all know what ASSUME means, don't we?) that any of the recombinant proteins (wild type, tagged not cleaved, or cleaved) will behave EXACTLY like the native protein.

ALWAYS DO A DIRECT COMPARISON!!!