Basic Methods in Cellular and Molecular Biology

(Purifying Proteins)

PURIFYING PROTEINS

-The challenge of isolating a single type of protein from the thousands of other proteins present in a cell is a formidable one, but must be overcome in order to study protein function in vitro.

-Recombinant DNA technology can enormously simplify this task by "tricking" cells into producing large quantities of a given protein, thereby making its purification much easier.

-Whether the source of the protein is an engineered cell or a natural tissue, a purification procedure usually starts with **subcellular fractionation to reduce the complexity of the material,** and is then followed **by purification steps of increasing specificity**.

Cells Can Be Separated into Their Component Fractions

To purify a protein, it must first be extracted from inside the cell.
Cells can be broken up in various ways: they can be subjected to osmotic shock or ultrasonic vibration, forced through a small orifice, or ground up in a blender.

These procedures break many of the membranes of the cell (including the plasma membrane and endoplasmic reticulum) into **fragments** that immediately reseal to form small **closed vesicles**.

If carefully carried out, the disruption procedures leave organelles such as nuclei, mitochondria, the Golgi apparatus, lysosomes, and peroxisomes largely intact.

The suspension of cells is thereby reduced to a thick slurry (called a **homogenate** or **extract**) that contains a variety of membrane-enclosed organelles, each with a distinctive size, charge, and density.

Provided that the homogenization medium has been carefully chosen (by trial and error for each organelle), the various components—including the vesicles derived from the endoplasmic reticulum, called **microsomes**—retain most of their original biochemical properties.

Cells Can Be Separated into Their Component Fractions

The different components of the homogenate must then be separated. Such cell fractionations became possible only after the commercial development in the early 1940s of an instrument known as the **preparative ultracentrifuge**, which rotates extracts of broken cells at high speeds.

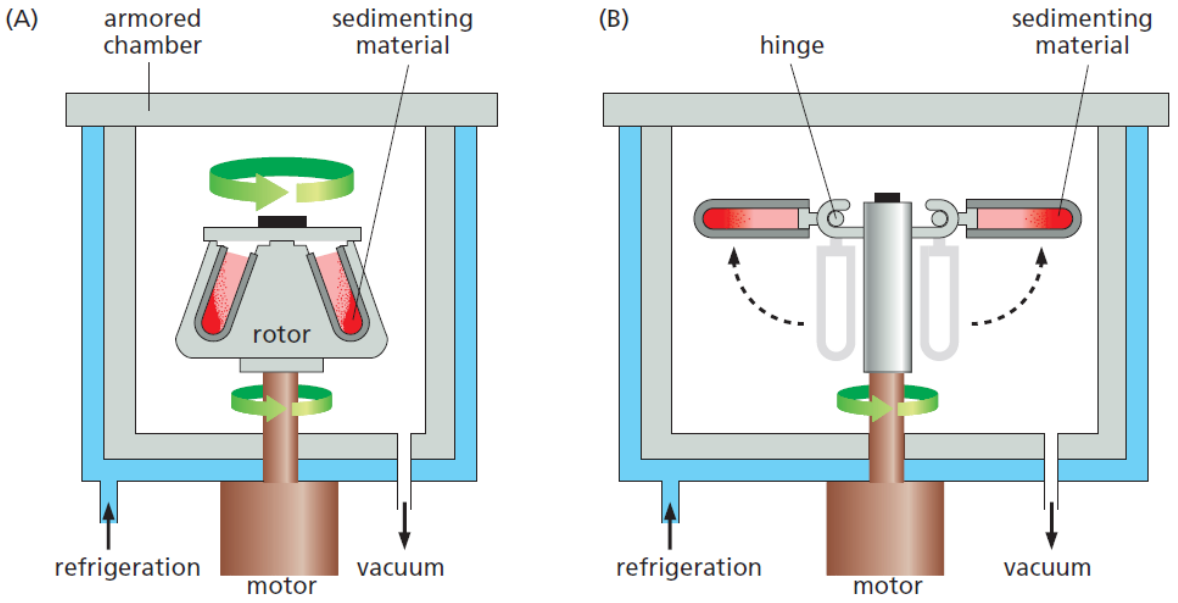


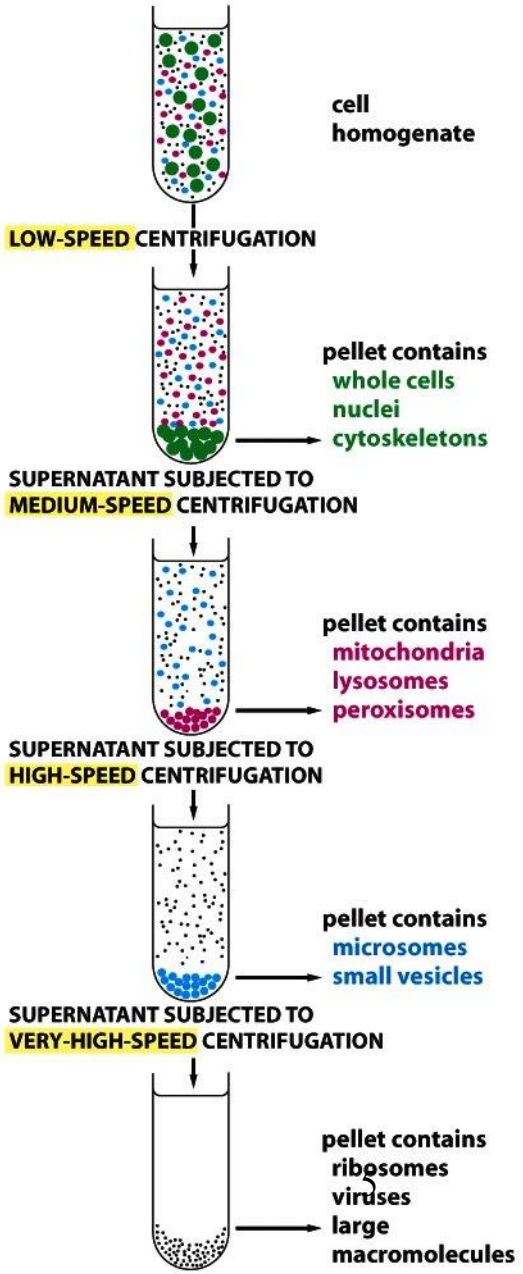
Figure 8–5 **The preparative ultracentrifuge.** (A) The sample is contained in tubes that are inserted into a ring of angled cylindrical holes in a metal *rotor*. Rapid rotation of the rotor generates enormous centrifugal forces, which cause particles in the sample to sediment against the bottom sides of the sample tubes, as shown here. The vacuum reduces friction, preventing heating of the rotor and allowing the refrigeration system to maintain the sample at 4°C. (B) Some fractionation methods require a different type of rotor called a *swinging-bucket rotor*. In this case, the sample tubes are placed in metal tubes on hinges that allow the tubes to swing outward when the rotor spins. Sample tubes are therefore horizontal during spinning, and samples are sedimented toward the bottom, not the sides, of the tube, providing better separation of differently sized components

Cells Can Be Separated into Their Component Fractions

This treatment separates cell components by **size and density**: in general, the largest objects experience the largest centrifugal force and move the most rapidly.

- At relatively low speed, large components such as nuclei sediment to form a pellet at the bottom of the centrifuge tube;
- at slightly higher speed, a pellet of mitochondria is deposited;
- at even higher speeds and with longer periods of centrifugation, first the small closed vesicles and then the ribosomes can be collected.

All of these fractions are impure, but many of the contaminants can be removed by resuspending the pellet and repeating the centrifugation procedure several times.



Repeated centrifugation at progressively higher speeds will fractionate homogenates of cells into their components.
Typical values for the various centrifugation steps referred to in the figure are: low speed:
1000 times gravity for 10 minutes medium speed:
20,000 times gravity for 20 minutes high speed:
80,000 times gravity for 1 hour very high speed:
150,000 times gravity for 3 hour

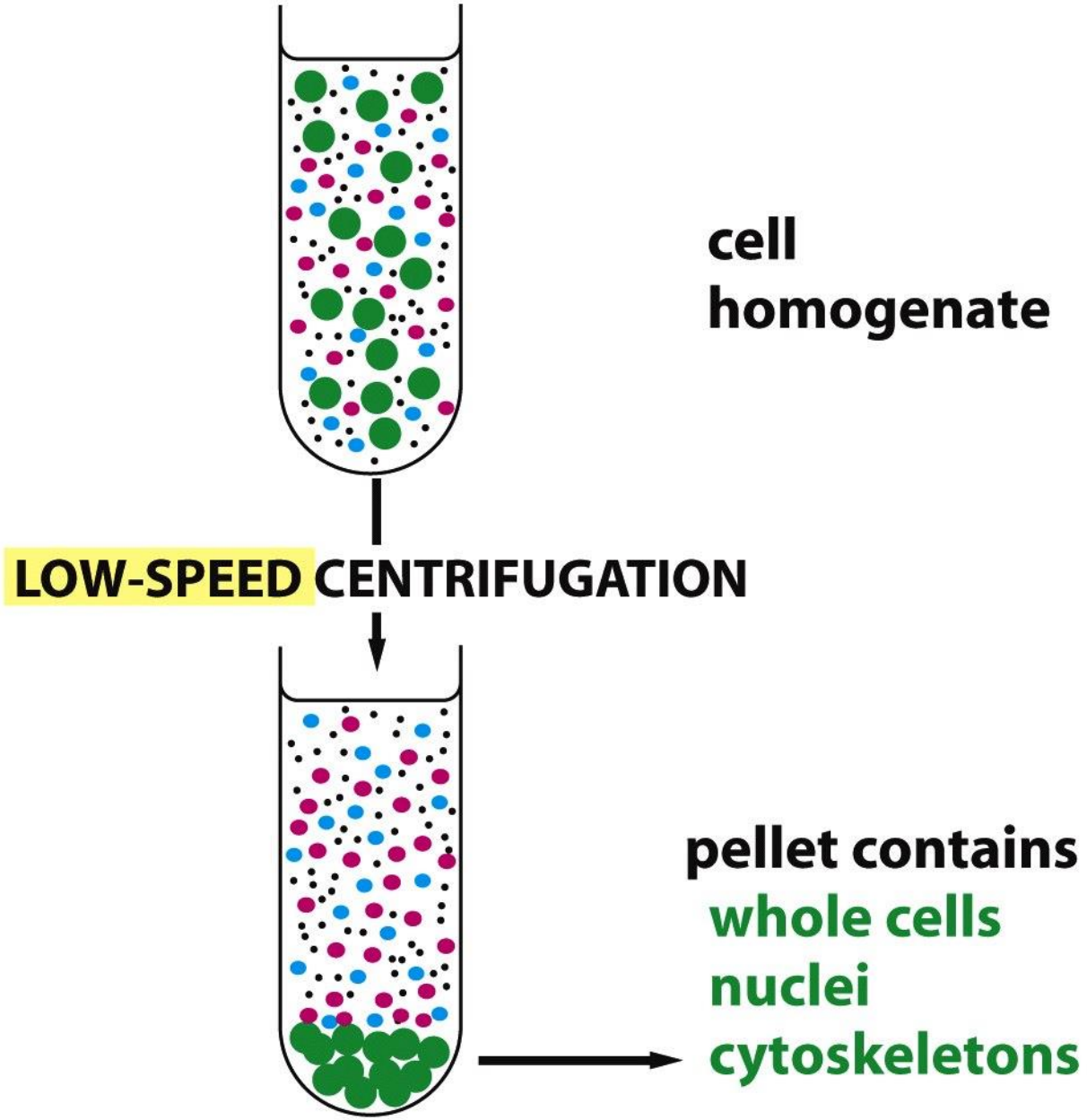


Figure 8-10 (part 1 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

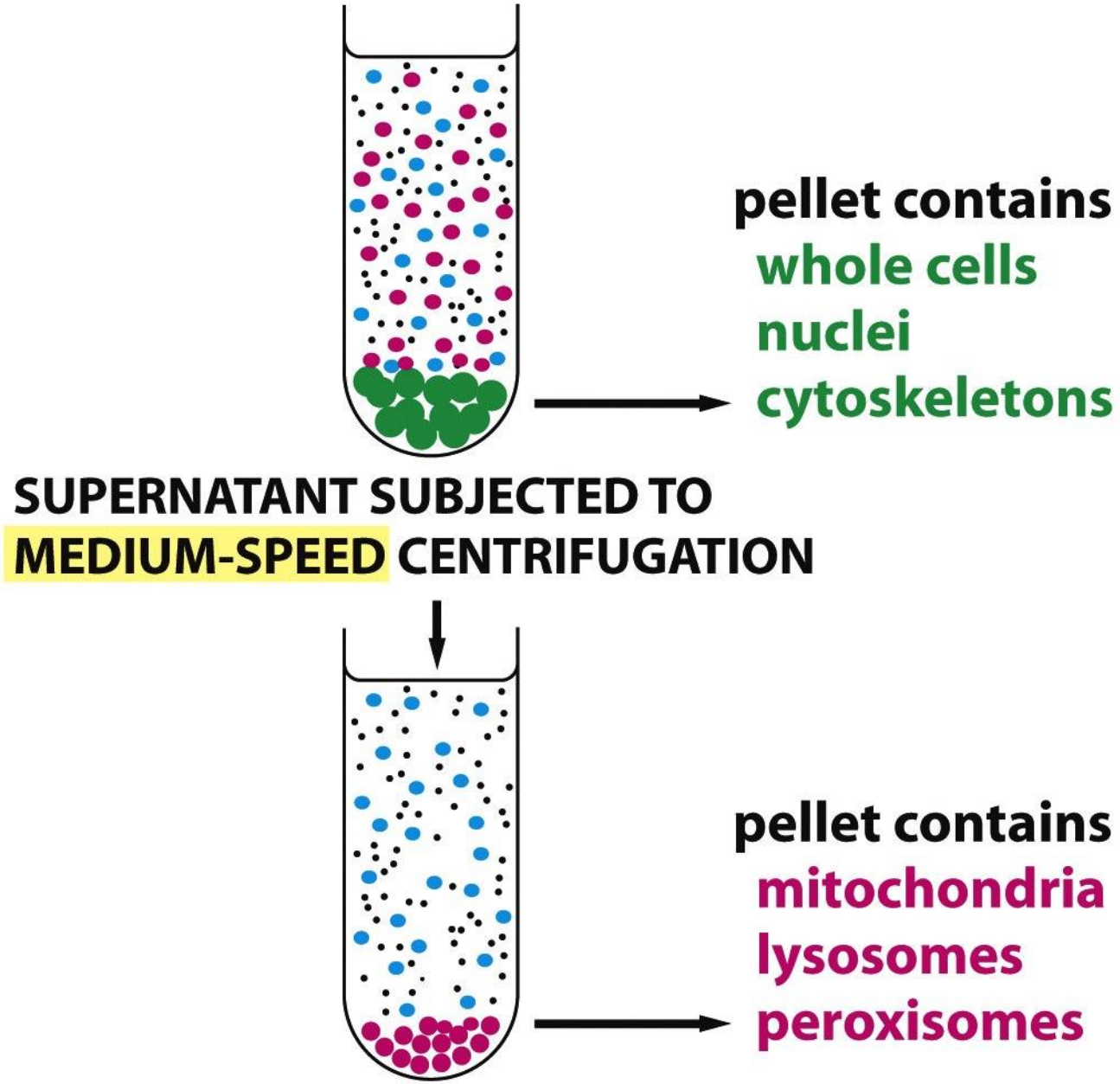


Figure 8-10 (part 2 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

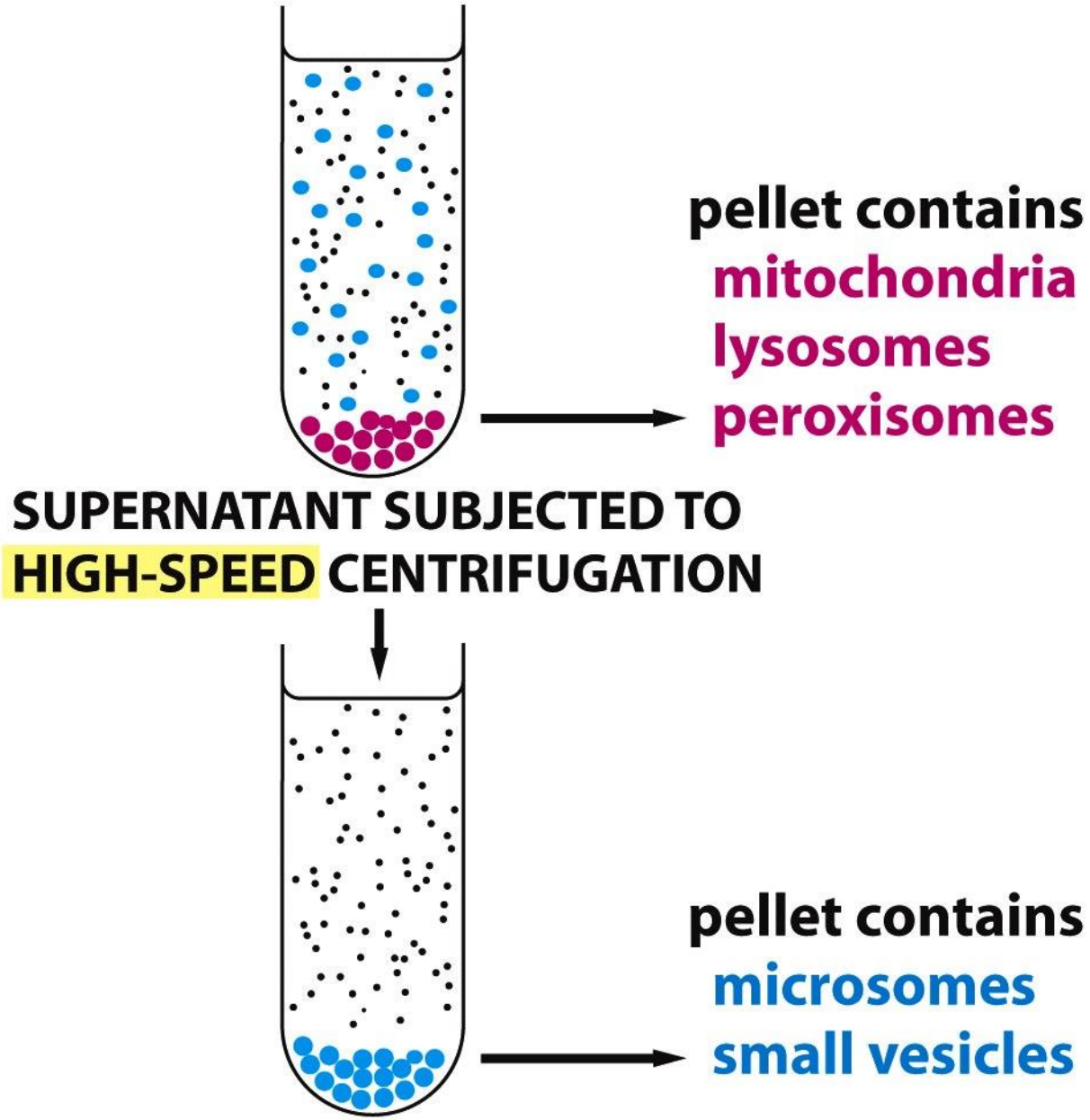


Figure 8-10 (part 3 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

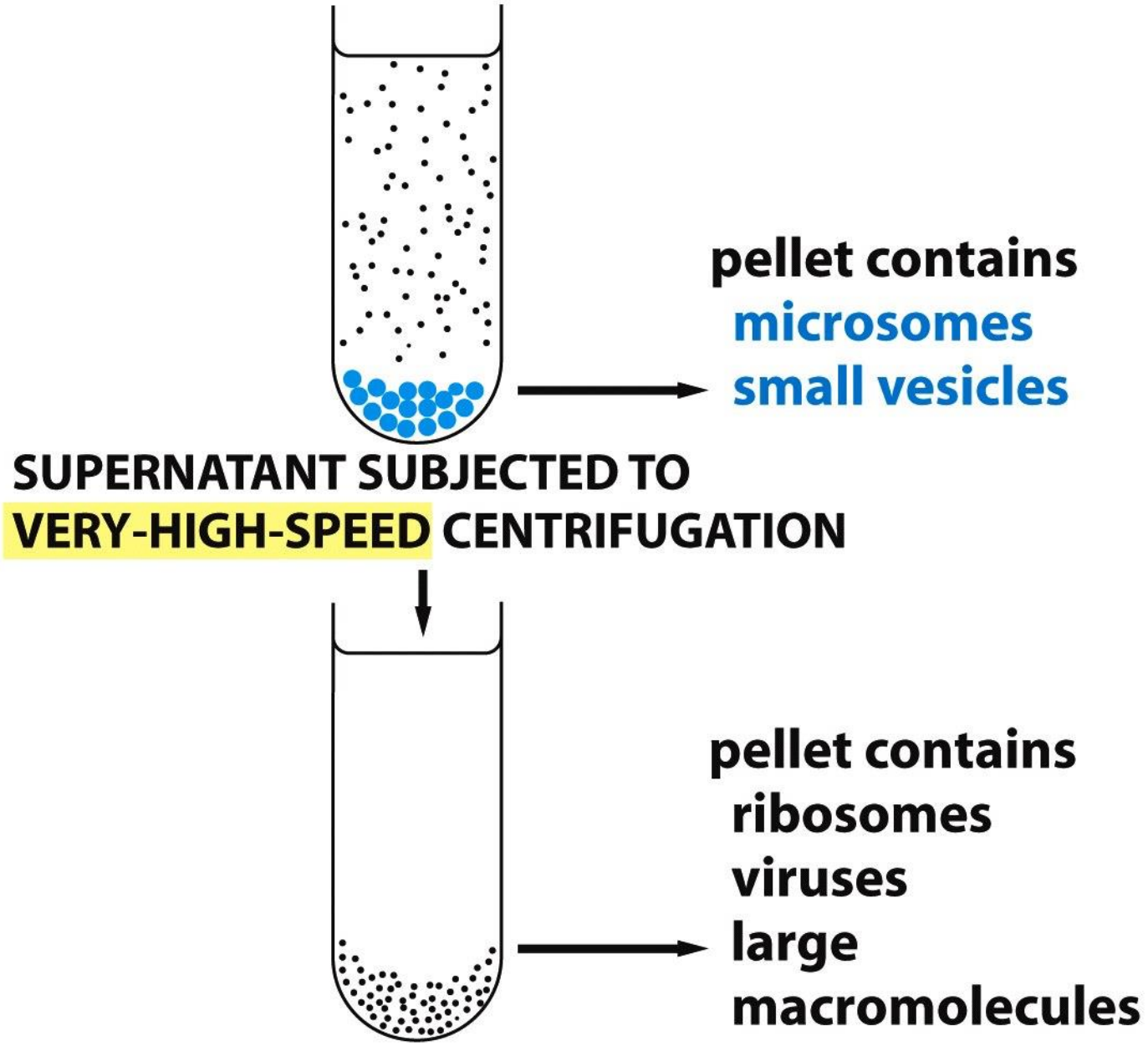


Figure 8-10 (part 4 of 4) *Molecular Biology of the Cell* (© Garland Science 2008)

Cells Can Be Separated into Their Component Fractions

- Centrifugation is the first step in most fractionations, but it separates only components that differ greatly in size.

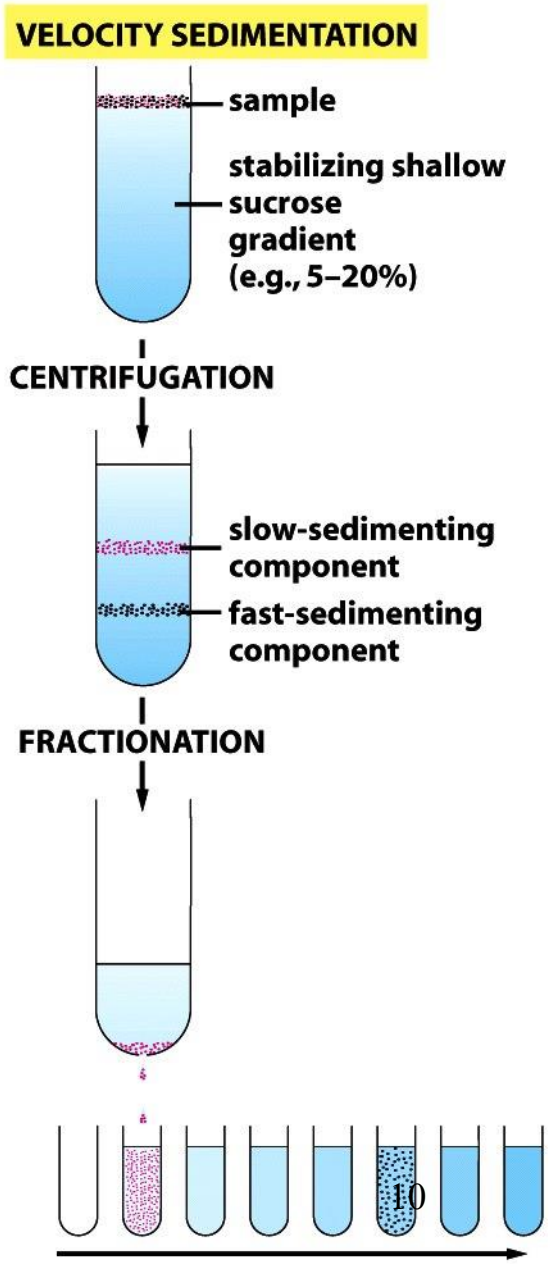
- A finer degree of separation can be achieved by layering the homogenate in a thin band on top of a salt solution that fills a centrifuge tube.

- When centrifuged, the various components in the mixture move as a series of distinct bands through the solution, each at a different rate, in a process called **velocity sedimentation**.

- For the procedure to work effectively, the bands must be protected from convective mixing, which would normally occur whenever a denser solution (for example, one containing organelles) finds itself on top of a lighter one (the salt solution).

- This is achieved by augmenting the solution in the tube with a **shallow gradient of sucrose** prepared by a special mixing device.

- The resulting density gradient—with the dense end at the bottom of the tube—keeps each region of the solution denser than any solution above it, and it thereby prevents convective mixing from distorting the separation.



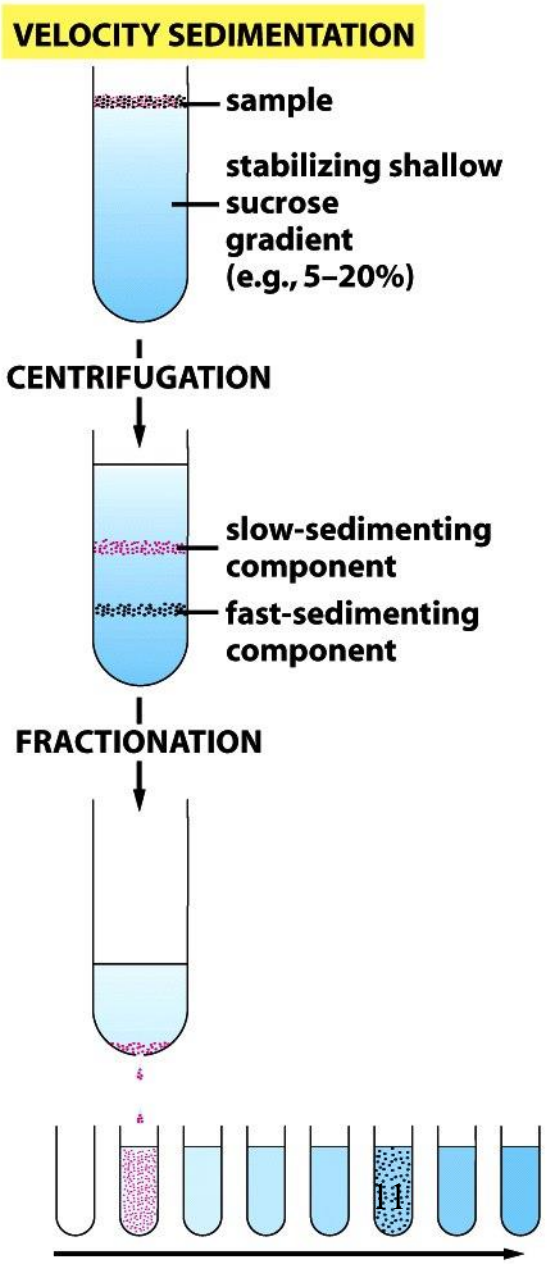
Cells Can Be Separated into Their Component Fractions

- When sedimented through sucrose gradients, different cell components separate into **distinct bands** that can be collected individually.

- The relative rate at which each component sediments depends primarily on its size and shape—normally being described in terms of its **sedimentation coefficient**, or **S value**.

- Present-day ultracentrifuges rotate at speeds of up to 80,000 rpm and produce forces as high as 500,000 times gravity.

- These enormous forces drive even **small macromolecules**, such as tRNA molecules and simple enzymes, to sediment at an appreciable rate and allow them to be separated from one another by size.



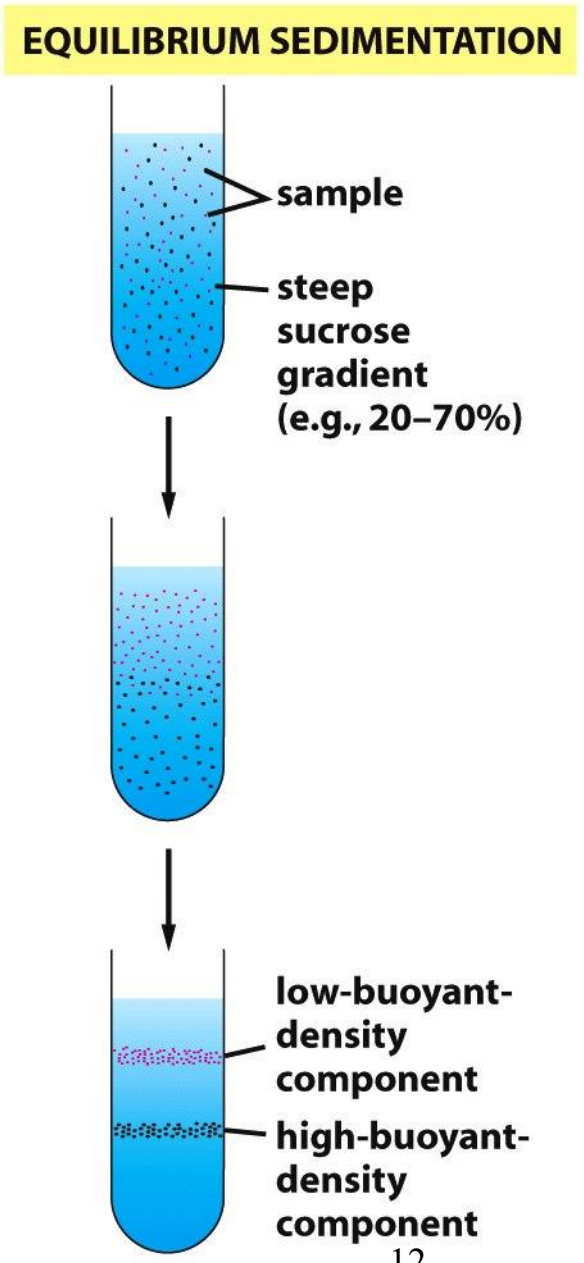
Cells Can Be Separated into Their Component Fractions

- The ultracentrifuge is also used to separate cell components on the basis of **their buoyant density**, independently of their size and shape.

- In this case, the sample is sedimented through a **steep density gradient** that contains a very high concentration of sucrose or cesium chloride.
- Each cell component begins to move down the gradient but it eventually reaches **a position where the density of the solution is equal to its own density**.

- At this point, the component floats and can move no farther.

- A series of distinct bands is thereby produced in the centrifuge tube, with the bands closest to the bottom of the tube containing the components of highest buoyant density.
- This method, called **equilibrium sedimentation**, is so sensitive that it can separate macromolecules that have incorporated heavy isotopes, such as 13C or 15N, from the same macromolecules that contain the lighter, common isotopes (12C or 14N).
- In fact, the cesium-chloride method was developed in 1957 to separate the labeled from the unlabeled DNA produced after exposure of a growing population of bacteria to nucleotide precursors containing 15N; this classic experiment provided direct evidence for the semiconservative replication of DNA.



Cell Extracts Provide Accessible Systems to Study Cell Functions

- Studies of organelles and other large subcellular components isolated in the ultracentrifuge have contributed enormously to our **understanding of the functions of different cell components**.

- Experiments on mitochondria and chloroplasts purified by centrifugation, for example, demonstrated the central function of these organelles in converting energy into forms that the cell can use.

- Similarly, resealed vesicles formed from fragments of rough and smooth endoplasmic reticulum (microsomes) have been separated from each other and analyzed as functional models of these compartments of the intact cell.

- Similarly, highly concentrated cell extracts, especially extracts of **Xenopus laevis (African clawed frog) oocytes**, have played a critical role in the study of such complex and highly organized processes as the cell-division cycle, the separation of chromosomes on the mitotic spindle, and the vesicular-transport steps involved in the movement of proteins from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane.

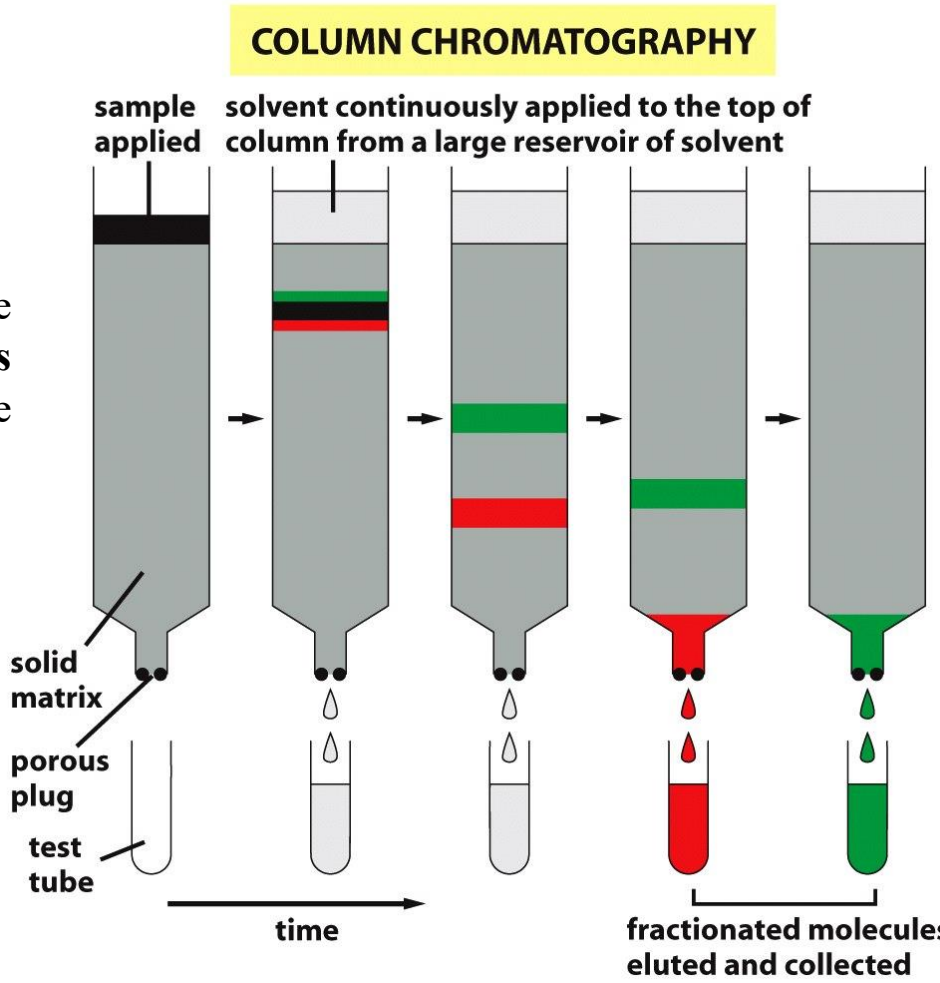
- Cell extracts also provide, in principle, the **starting material** for the complete separation of all of the individual macromolecular components of the cell.

Proteins Can Be Separated by Chromatography

Proteins are most often fractionated by **column chromatography**, in which a mixture of proteins in solution is passed through a column containing a **porous solid matrix**.

Different proteins are retarded to different extents by their interaction with the matrix, and they can be collected separately as they flow out of the bottom of the column.

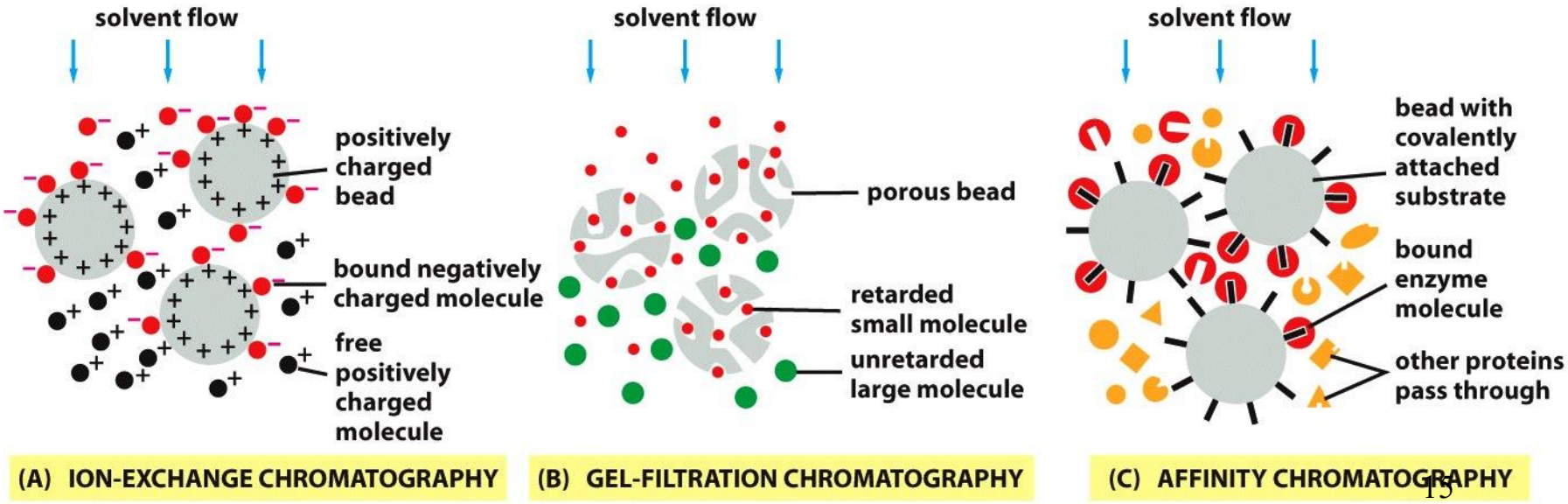
- Because various components of the sample travel **at different rates** through the column, they are fractionated into different tubes.



Proteins Can Be Separated by Chromatography

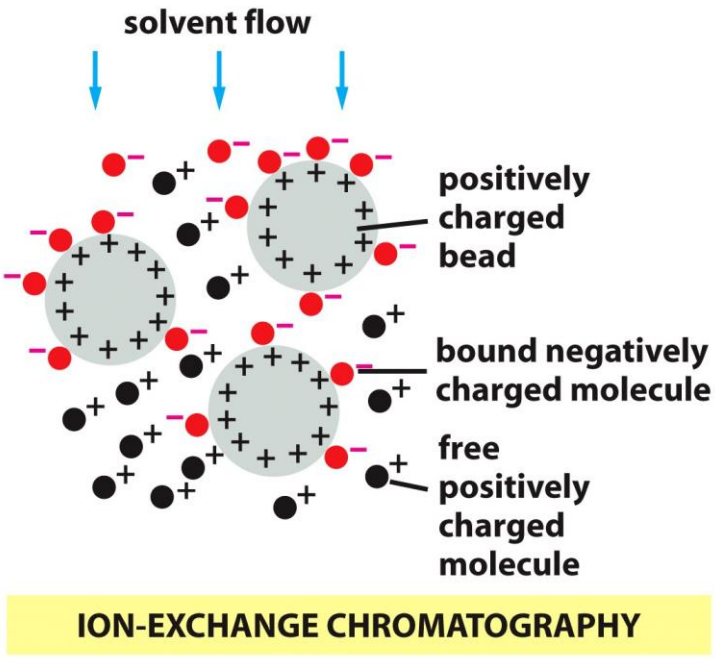
Depending on the **choice of matrix**, proteins can be separated according to:

- their charge (**ion-exchange chromatography**),
- their hydrophobicity (**hydrophobic chromatography**),
- their size (**gel-filtration chromatography**),
- their ability to bind to particular small molecules or to other macromolecules (**affinity chromatography**).



Proteins Can Be Separated by Chromatography

- **Ion-exchange columns** are packed with small beads that carry either a positive or a negative charge, so that proteins are fractionated according to the arrangement of charges on their surface.

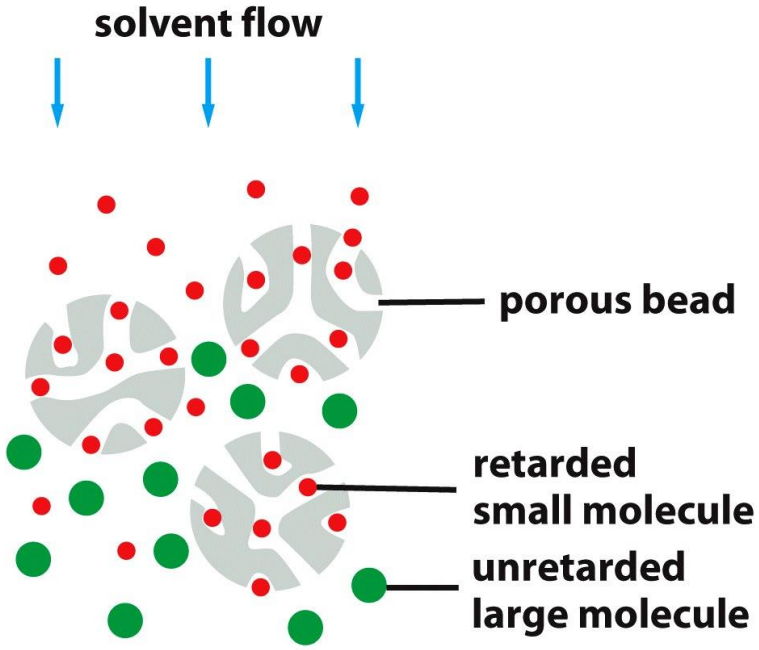


-Matrices used for separating proteins include **diethylaminoethylcellulose (DEAE-cellulose),** which is **positively** charged, and **carboxymethylcellulose (CM-cellulose)** and **phosphocellulose,** which are **negatively** charged.
-Analogous matrices based on agarose or other polymers are also frequently used.
-The **strength of the association** between the dissolved molecules and the ion-exchange matrix depends on both **the ionic strength** and **the pH of the solution that is passing down the column**, which may therefore be varied systematically to achieve an effective separation.

- **Hydrophobic columns** are packed with beads from which **hydrophobic side chains** protrude, selectively retarding proteins with exposed hydrophobic regions.

Proteins Can Be Separated by Chromatography

- **Gel-filtration columns**, which separate proteins according to their **size**, are packed with **tiny porous beads**: molecules that are small enough to enter the pores linger inside successive beads as they pass down the column, while larger molecules remain in the solution flowing between the beads and therefore move more rapidly, emerging from the column first.
- Besides providing a means of **separating molecules**, gel-filtration chromatography is a convenient way to **estimate their size**.



GEL-FILTRATION CHROMATOGRAPHY

-In gel-filtration chromatography, the small beads that form the matrix are **inert but porous**.

-Molecules that are small enough to penetrate into the matrix beads are thereby delayed and travel more slowly through the column than larger molecules that cannot penetrate.

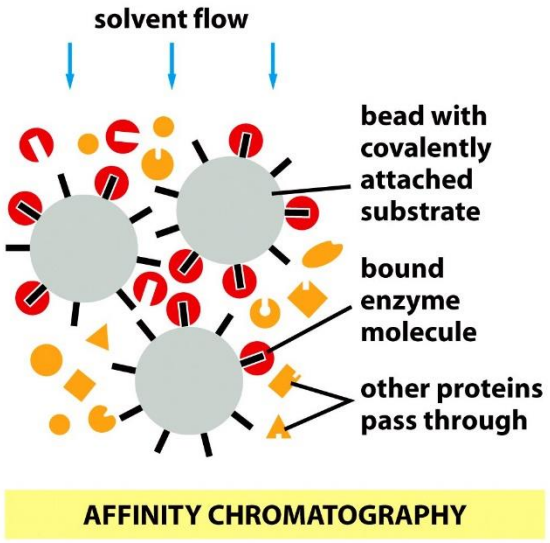
-Beads of **cross linked polysaccharide (dextran, agarose, or acrylamide)** are available commercially in a wide range of pore sizes, making them suitable for the fractionation of molecules of various molecular mass, from less than 500 daltons to more than 5×10^6 daltons.

Proteins Can Be Separated by Chromatography

- **Affinity chromatography** takes advantage of the biologically important binding interactions that occur on protein surfaces.

-If a **substrate** molecule is covalently coupled to an inert matrix such as a polysaccharide bead, the **enzyme** that operates on that substrate will often be specifically retained by the matrix and can then be eluted (washed out) in nearly pure form.
-Specific antibodies can be coupled to a matrix to purify protein molecules recognized by the antibodies.

Because of the great specificity of all such affinity columns, **1000- to 10,000-fold purifications** can sometimes be achieved in a single pass.l



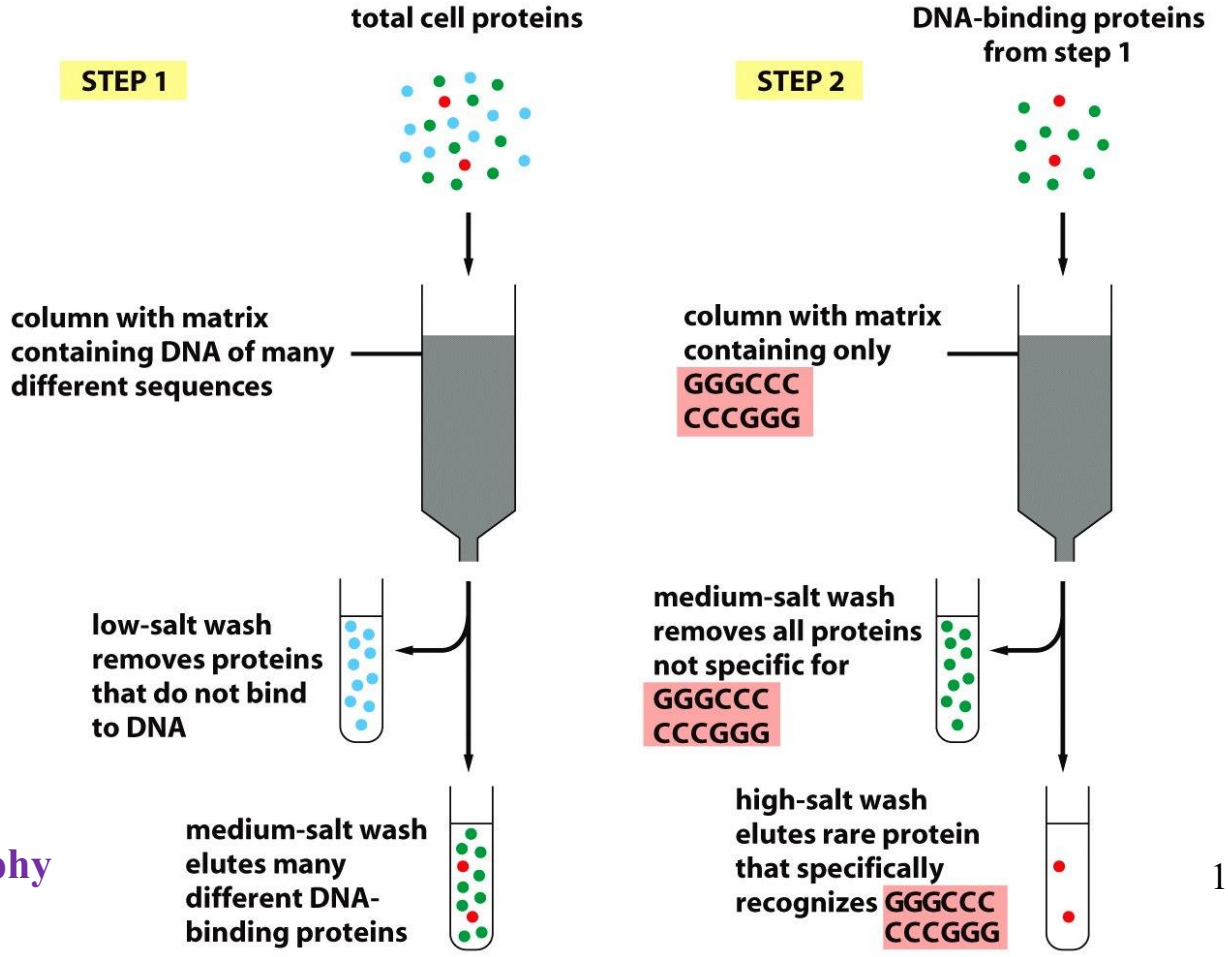
-Affinity chromatography uses an **insoluble matrix** that is **covalently linked to a specific ligand**, such as an antibody molecule or an enzyme substrate, that will bind a specific protein.

- ✓ Enzyme molecules that bind to immobilized substrates on such columns can be eluted with a **concentrated solution** of the free form of the substrate molecule.
- ✓ Molecules that bind to immobilized antibodies can be eluted by dissociating the antibody–antigen complex with **concentrated salt solutions** or **solutions of high or low pH.**

Proteins Can Be Separated by Chromatography

Affinity chromatography takes advantage of the biologically important binding interactions that occur on protein surfaces.

short DNA oligonucleotides of a specifically designed sequence can be immobilized in this way and used to **purify DNA-binding proteins** that normally recognize this sequence of nucleotides in chromosomes.

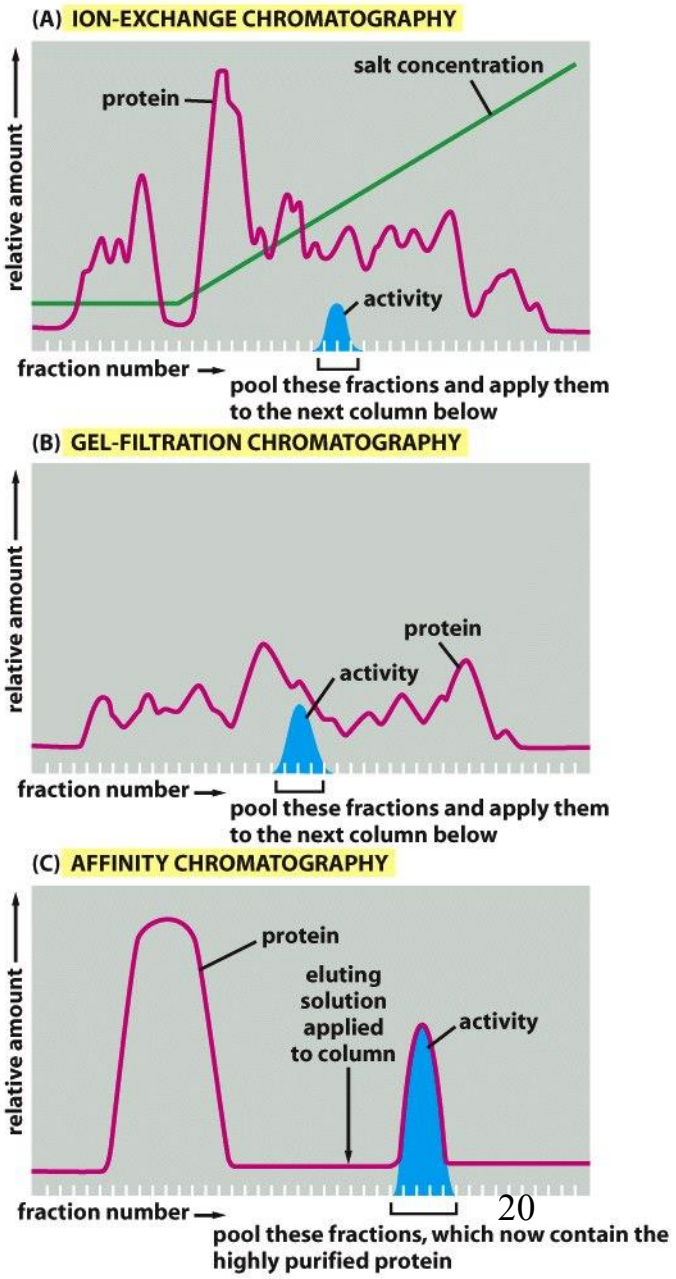


DNA affinity chromatography

Proteins Can Be Separated by Chromatography

- If one starts with a complex mixture of proteins, a single passage through an ion-exchange or a gel-filtration column does not produce very highly purified fractions, since these methods individually increase the proportion of a given protein in the mixture no more than twentyfold.

- Because most individual proteins represent less than **1/1000** of the total cell protein, it is usually necessary **to use several different types of columns in succession to attain sufficient purity, with affinity chromatography being the most efficient**



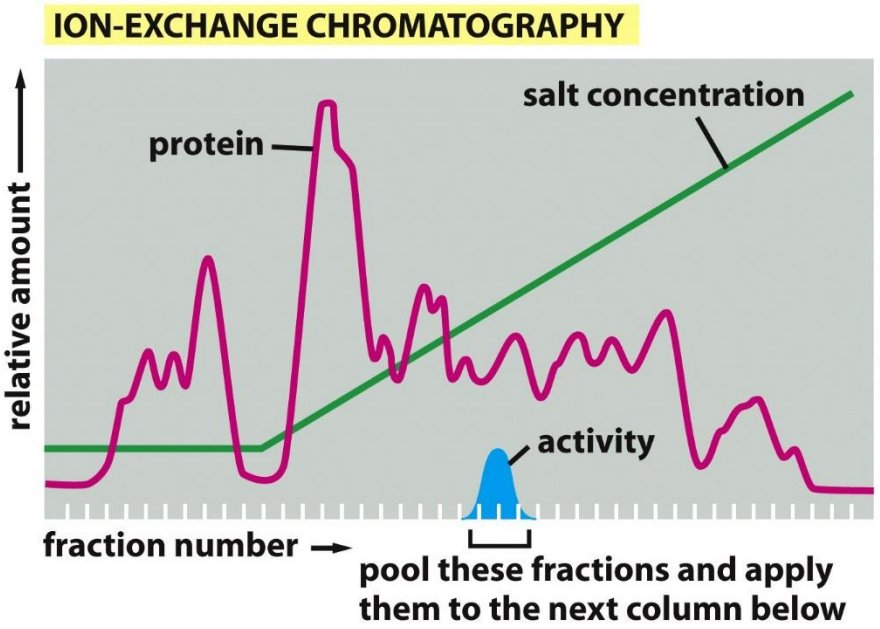
Protein purification by chromatography. Typical results obtained when three different chromatographic steps are used in succession to purify a protein.

Proteins Can Be Separated by Chromatography

In this example, a homogenate of cells was first fractionated by allowing it to percolate through an **ion-exchange resin** packed into a column (A).

The column was washed to remove all unbound contaminants, and the **bound proteins** were then eluted by pouring a solution containing a gradually increasing concentration of salt onto the top of the column.
Proteins with the lowest affinity for the ion-exchange resin passed directly through the column and were collected in the earliest fractions eluted from the bottom of the column. The remaining proteins were eluted in sequence according to their affinity for the **resin—those proteins binding most tightly to the resin requiring the highest concentration of salt to remove them**.

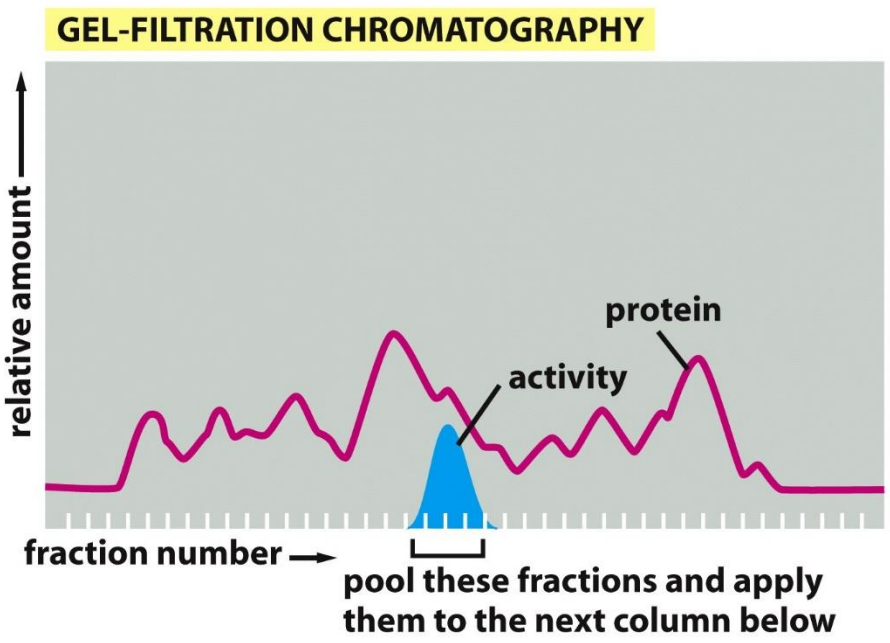
The protein of interest was eluted in several fractions and was detected by its enzymatic activity.

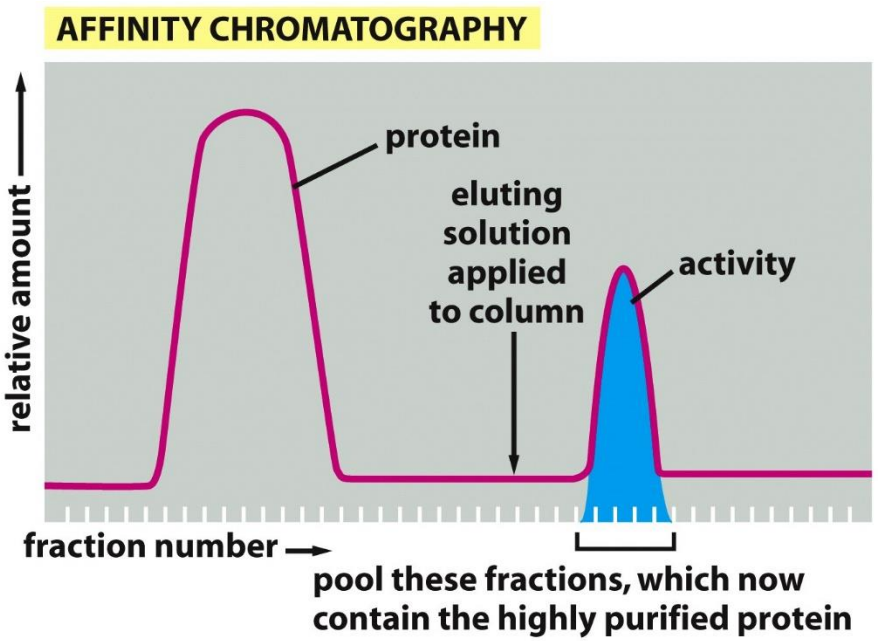


Proteins Can Be Separated by Chromatography

The fractions with activity were pooled and then applied to a **gel-filtration column** (B).

The elution position of the still-impure protein was again determined by its enzymatic activity, and the active fractions were pooled and purified to homogeneity on an **affinity column** (C) that contained an immobilized substrate of the enzyme.





Proteins Can Be Separated by Chromatography

Affinity purification of cyclin-binding proteins from S. cerevisiae, as analyzed by SDS polyacrylamide-gel electrophoresis.
Lane 1 is a total cell extract;
lane 2 shows the proteins eluted from an _ containing cyclin 82;
lane 3 shows one major Protein eluted from a cyclin 83 affinity column.
Proteins in lanes 2 and 3 were eluted from the affinity columns with salt, and the gel was stained with Coomassie blue.

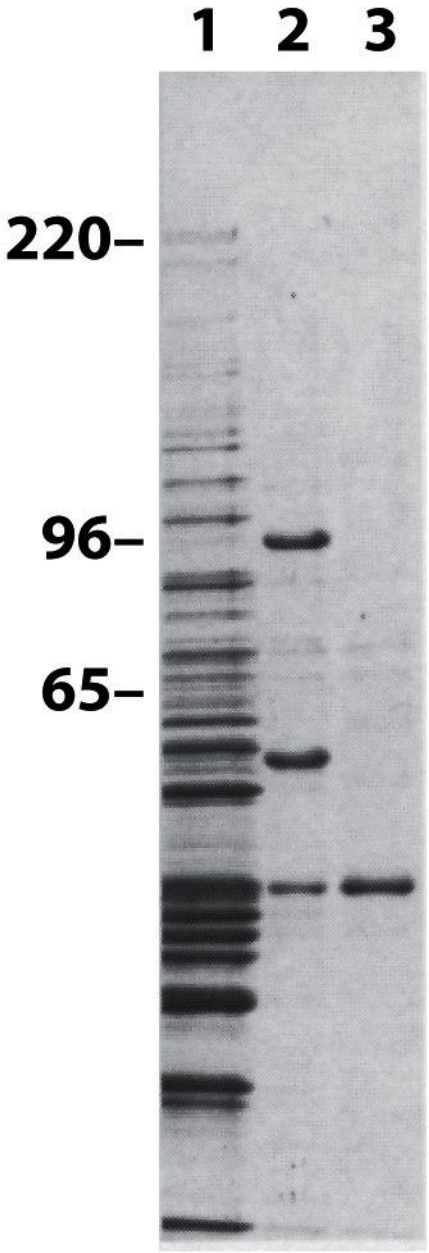


Figure 8-14d *Molecular Biology of the Cell* (© Garland Science 2008)

Proteins Can Be Separated by Chromatography

- **Inhomogeneities in the matrices** (such as cellulose), which cause an uneven flow of solvent through the column, limit the resolution of conventional column chromatography.

- Special chromatography resins (usually **silica-based**) composed of tiny spheres (3–10 μm in diameter) can be packed with a special apparatus to form a **uniform column bed**.

- Such **high-performance liquid chromatography (HPLC) columns** attain a high degree of resolution.

- In HPLC, the solutes equilibrate very rapidly with the interior of the tiny spheres, and so solutes with different affinities for the matrix are **efficiently** separated from one another even at very fast flow rates.

- HPLC is therefore the method of choice for **separating many proteins and small molecules**.

Immunoprecipitation Is a Rapid Affinity Purification Method

-Immunoprecipitation is a useful variation on the theme of affinity chromatography.

-Specific antibodies that recognize the protein to be purified are attached to small **agarose beads**.

-Rather than being packed into a column, as in affinity chromatography, a small quantity of the antibody-coated beads is simply added to a protein extract in a test tube and mixed in suspension for a short period of time— thereby allowing the antibodies to bind the desired protein.

-The beads are then collected by **low-speed centrifugation**, and the unbound proteins in the supernatant are discarded.

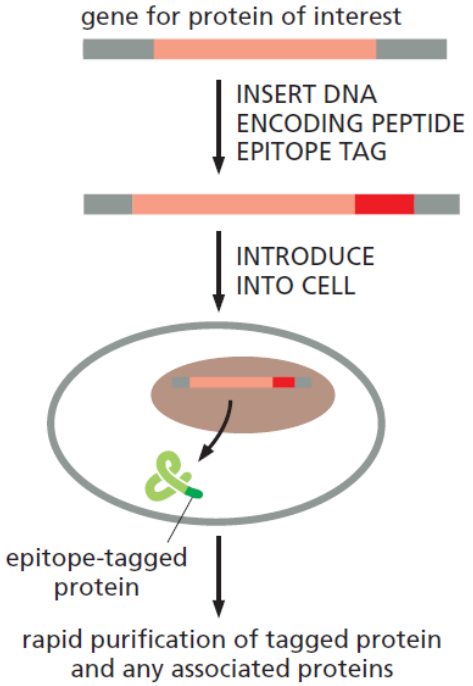
- **This method is commonly used to purify small amounts of enzymes from cell extracts for analysis of enzymatic activity or for studies of associated proteins.**

Genetically Engineered Tags Provide an Easy Way to Purify Proteins

- Using the recombinant DNA methods, any gene can be modified to produce its protein with a attached to it, so as to make subsequent purification of the protein simple and rapid.

- Often the recognition tag is itself an **antigenic determinant**, or **epitope**, which can be recognized by a highly specific antibody.

- The antibody can then be used to purify the protein by affinity chromatography or immunoprecipitation.



- Other types of tags are specifically designed for protein purification:

-For example, a repeated sequence of the **amino acid histidine binds to certain metal ions, including nickel and copper**.

-If genetic engineering techniques are used to attach a short string of **histidines** to one end of a protein, the slightly modified protein can be retained selectively on an affinity column containing immobilized nickel ions.

Metal affinity chromatography can thereby be used to purify the modified protein from a complex molecular mixture.

Genetically Engineered Tags Provide an Easy Way to Purify Proteins

- In other cases, an entire protein is used as the recognition tag:

-When cells are engineered to synthesize the small **enzyme glutathione S-transferase (GST)** attached to a protein of interest, the resulting **fusion protein** can be purified from the other contents of the cell with an affinity column containing **glutathione**, a substrate molecule that binds specifically and tightly to GST.

-As a further refinement of purification methods using recognition tags, an amino acid sequence that forms a **cleavage site for a highly specific proteolytic enzyme** can be engineered between the protein of choice and the recognition tag.

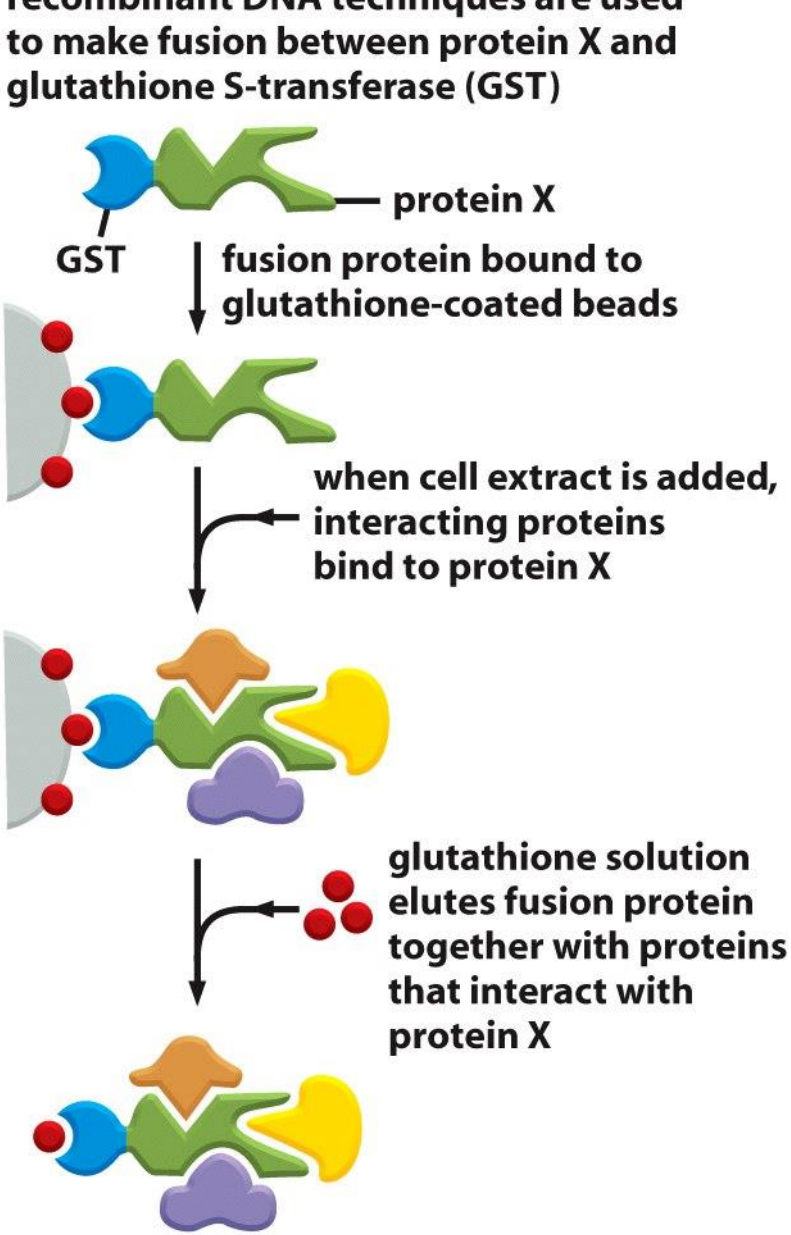
-Because the amino acid sequences at the cleavage site are very rarely found by **chance** in proteins, the tag can later be cleaved off without destroying the purified protein.

- **This type of specific cleavage is used in an especially powerful purification methodology known as tandem affinity purification tagging (TAP-tagging).**

-Here, one end of a protein is engineered to contain **two recognition tags** that are separated by a protease cleavage site. The tag on the very end of the construct is chosen to bind irreversibly to an affinity column, allowing the column to be washed extensively to remove all contaminating proteins. Protease cleavage then releases the protein, which is then further purified using the second tag.

-Because this twostep strategy provides an especially high degree of protein purification with relatively little effort, it is used extensively in cell biology.

-Thus, for example, a set of approximately 6000 yeast strains, each with a different gene fused to DNA that encodes a TAP-tag, has been constructed to allow any yeast protein to be rapidly purified.


recombinant DNA techniques are used to make fusion between protein X and glutathione S-transferase (GST)
protein X
GST
fusion protein bound to glutathione-coated beads
when cell extract is added, interacting proteins bind to protein X
glutathione solution elutes fusion protein together with proteins that interact with protein X