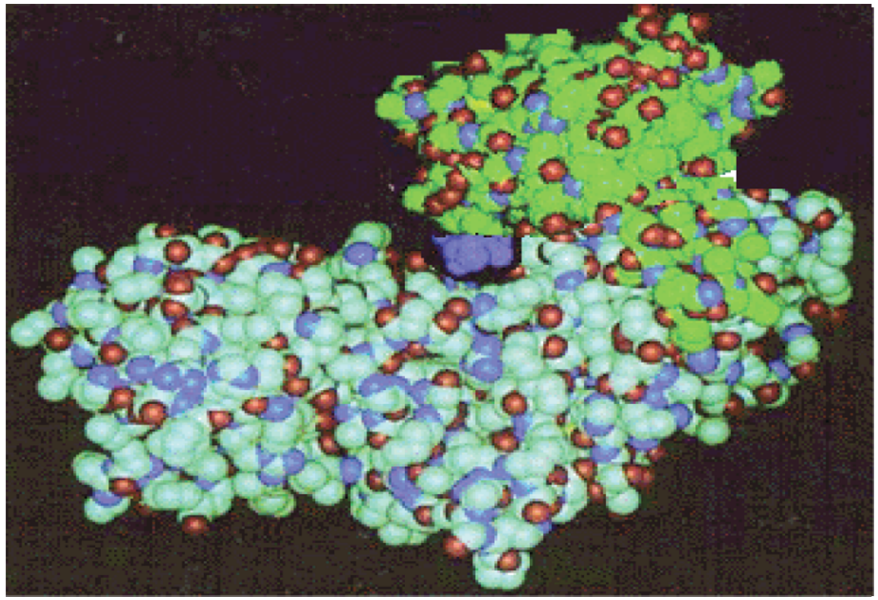
Protein Purification Methods

- Separating the Components in the Extract
  - Chromatography
    - **Affinity chromatography** relies on the ability of proteins to bind specifically and reversibly to uniquely shaped compounds called *ligands*

Affinity chromatography

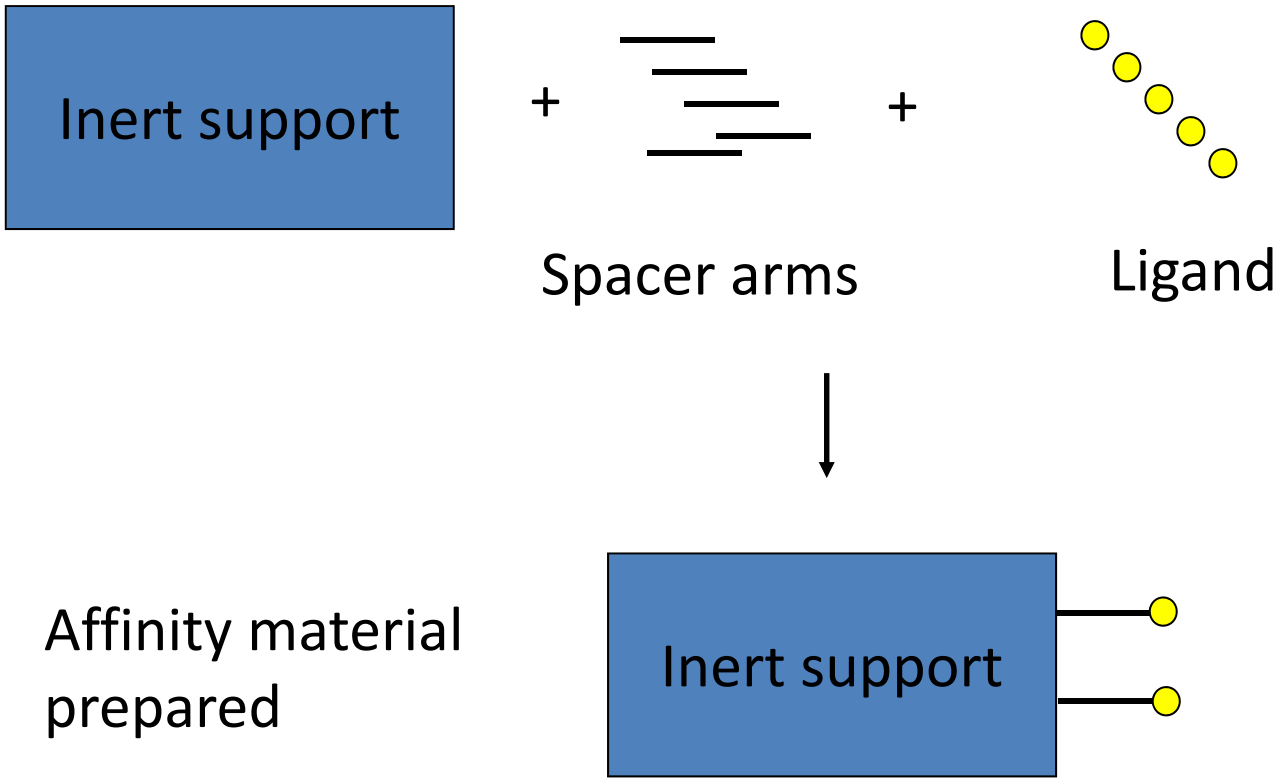
- Many proteins can bind specific molecules very tightly but noncovalently.
- We can use this to our advantage with **affinity chromatography**.



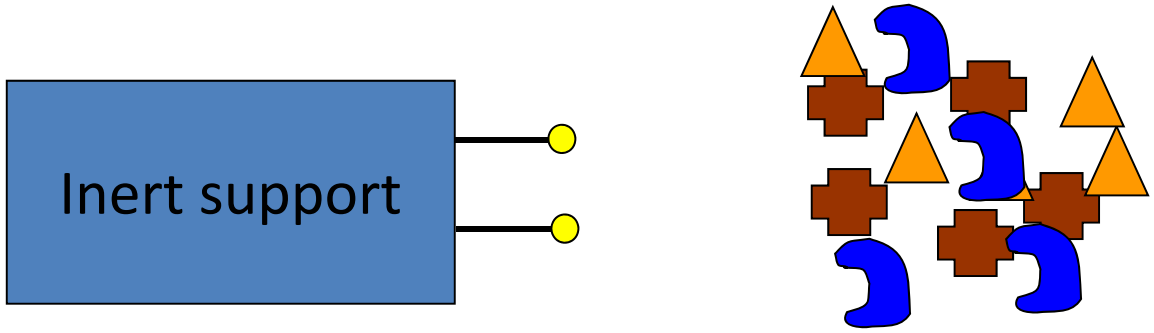
Glucose (small dark blue molecule) binding to hexokinase.
The enzyme acts like a jaw and clamps down on the substrate (glucose)

Affinity chromatography

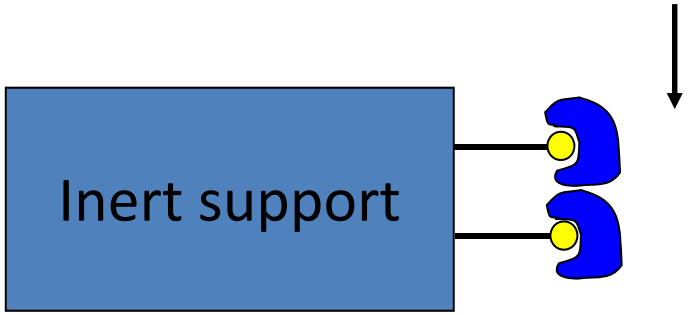
- How does it work?
- **Ligand** - a molecule that specifically binds to the protein of interest.



Affinity chromatography

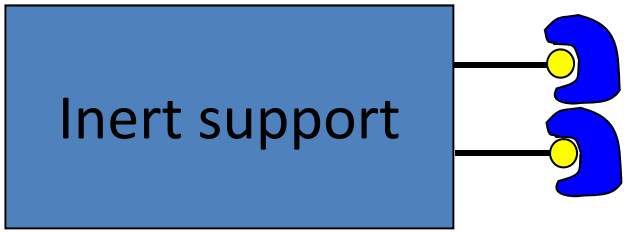


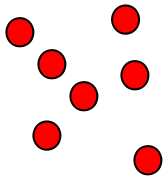
Mixture of proteins



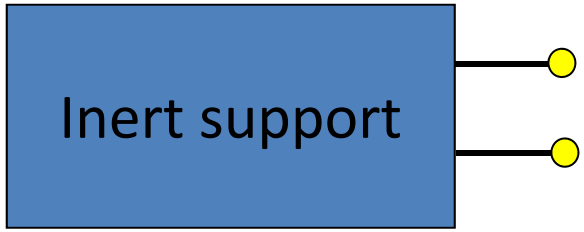
Unwanted proteins

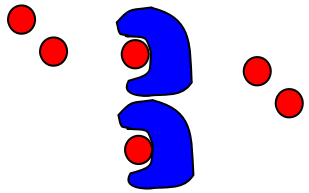
Affinity chromatography





Elute with competitive ligand.





Remove from competitive ligand by dialysis.

Affinity chromatography

- To remove the protein of interest from the column, you can elute with a solution of a compound with higher affinity than the ligand (competitive)
- You can change the pH, ionic strength and/or temperature so that the protein-ligand complex is no longer stable.

Immunoaffinity chromatography

- Monoclonal antibodies can be attached to the column material.
- The column only binds the protein against which the antibody has been raised.
- 10,000-fold purification in a single step!
- Disadvantges
  - Difficult to produce monoclonal antibodies (expensive $$!)
  - Harsh conditions to elute the bound protein

Protein purification by chromatography

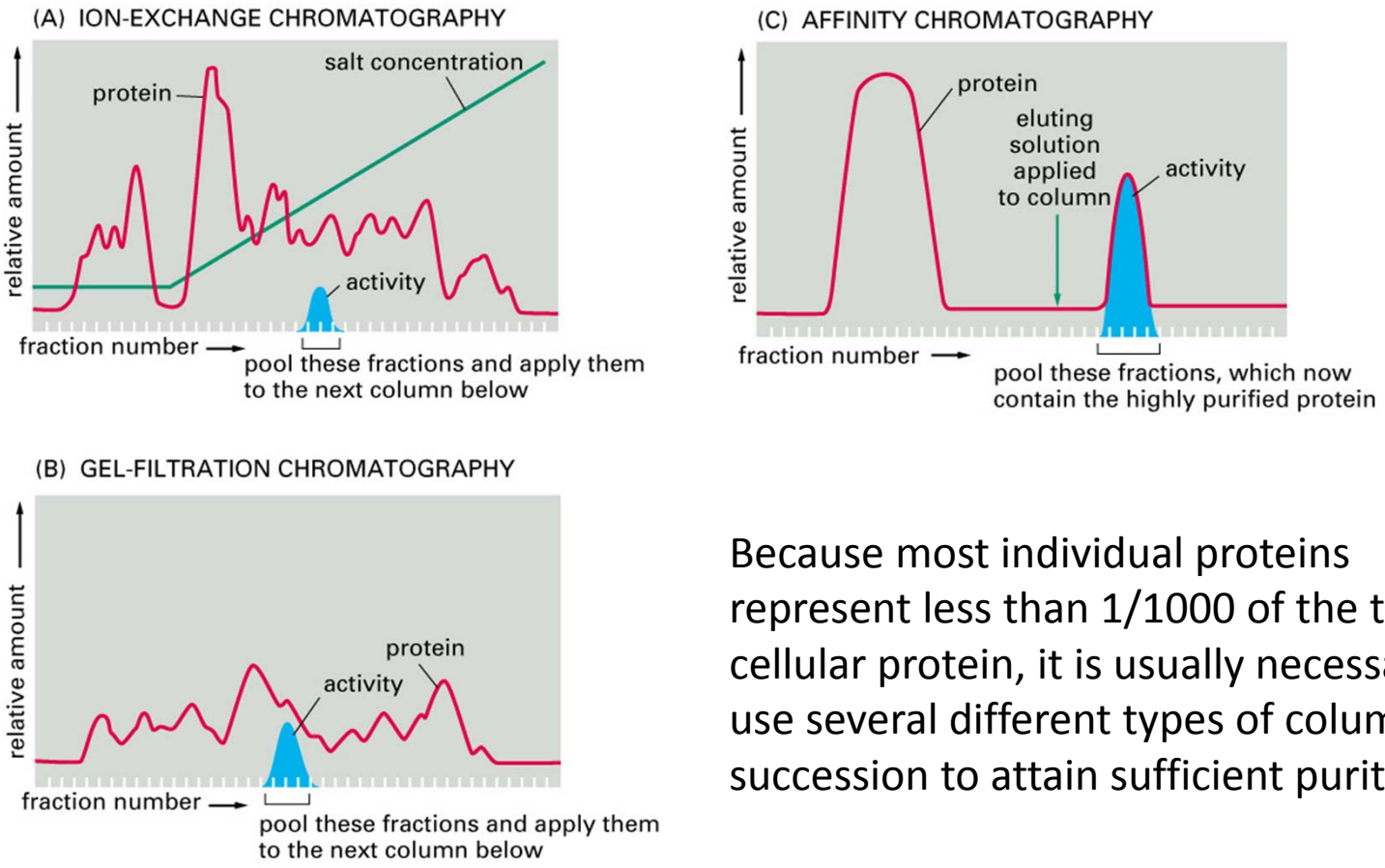


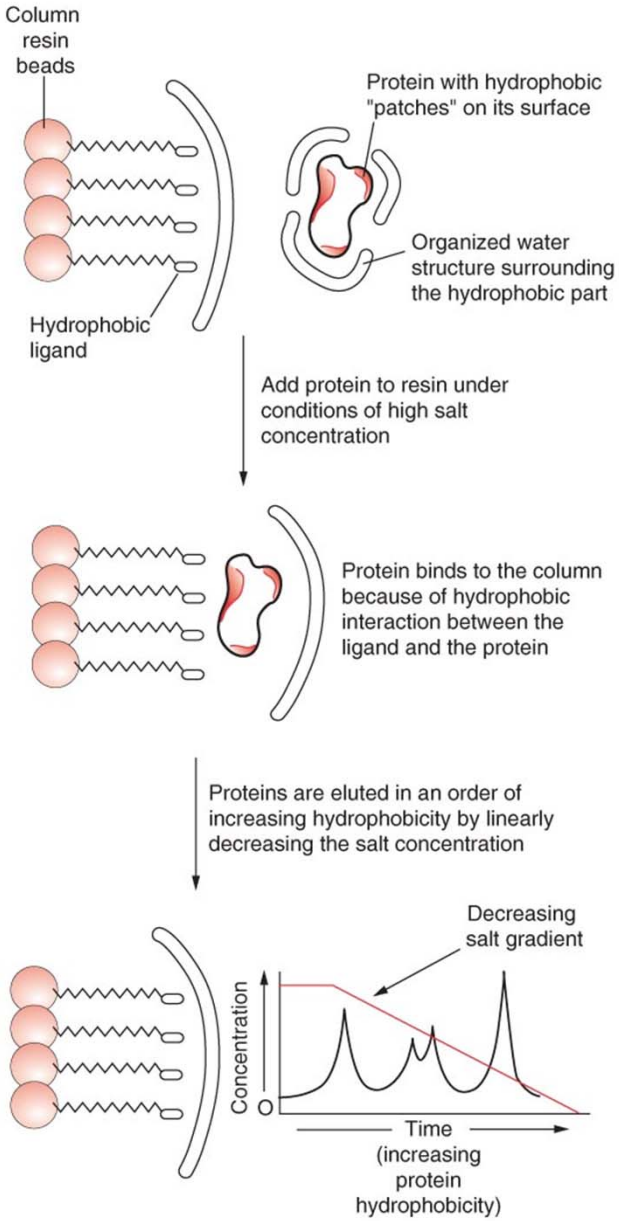
Figure 8–12 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Because most individual proteins represent less than 1/1000 of the total cellular protein, it is usually necessary to use several different types of column in succession to attain sufficient purity.

Protein Purification Methods

- Separating the Components in the Extract
  - Chromatography
    - **Hydrophobic interaction chromatography (HIC)** sorts proteins on the basis of their repulsion of water
    - When a hydrophobic region of a biopolymer binds to the surface of a mildly hydrophobic stationary phase, hydrophilic water molecules are effectively released from the surrounding hydrophobic areas causing a thermodynamically favorable change in **entropy.**

Protein Purification Methods



Protein Purification Methods

- Separating the Components in the Extract
  - **Iso-electric focusing** used in QC to identify two similar proteins that are difficult to separate by any other means
    - Each protein has a specific number of charged amino acids on its surface in specific places
    - Creates a unique electric signature known as its iso-electric point (IEP) where charges on the protein match the pH of the solution

Charges on proteins

- Different proteins have different native charges.
- The overall charge on a protein will depend on:
  - The sequence
  - The pH

What is pI?

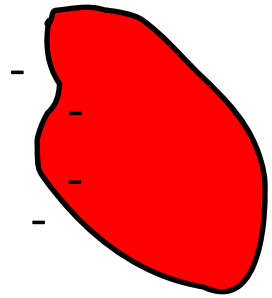
The **isoelectric point** (**pI**), sometimes abbreviated to **IEP**, is the **pH** at which a particular molecule or surface carries **no net electrical charge**.

Determining the *pI* of a protein

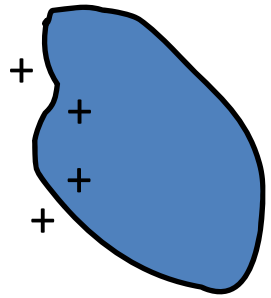
- It can be predicted from the difference between the sum of the **acidic side chains** (asp + glu) and the sum of the **basic side chains** (lys + arg + his).
- It is determined experimentally by techniques such as **isoelectric focusing**. The protein is placed in a pH gradient and subjected to an electric field. The protein moves to its pI.

Determining the *pI* of a protein

- Those proteins with more acidic residues will have a lower pI
- Those proteins with more basic residues will have a higher pI.



More acidic residues
Lower pI

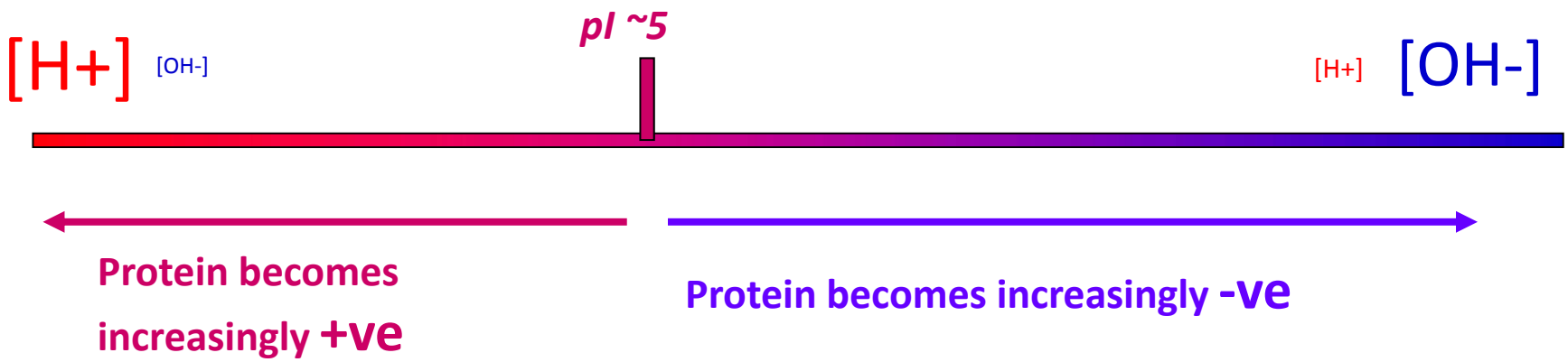


More basic residues
Higher pI

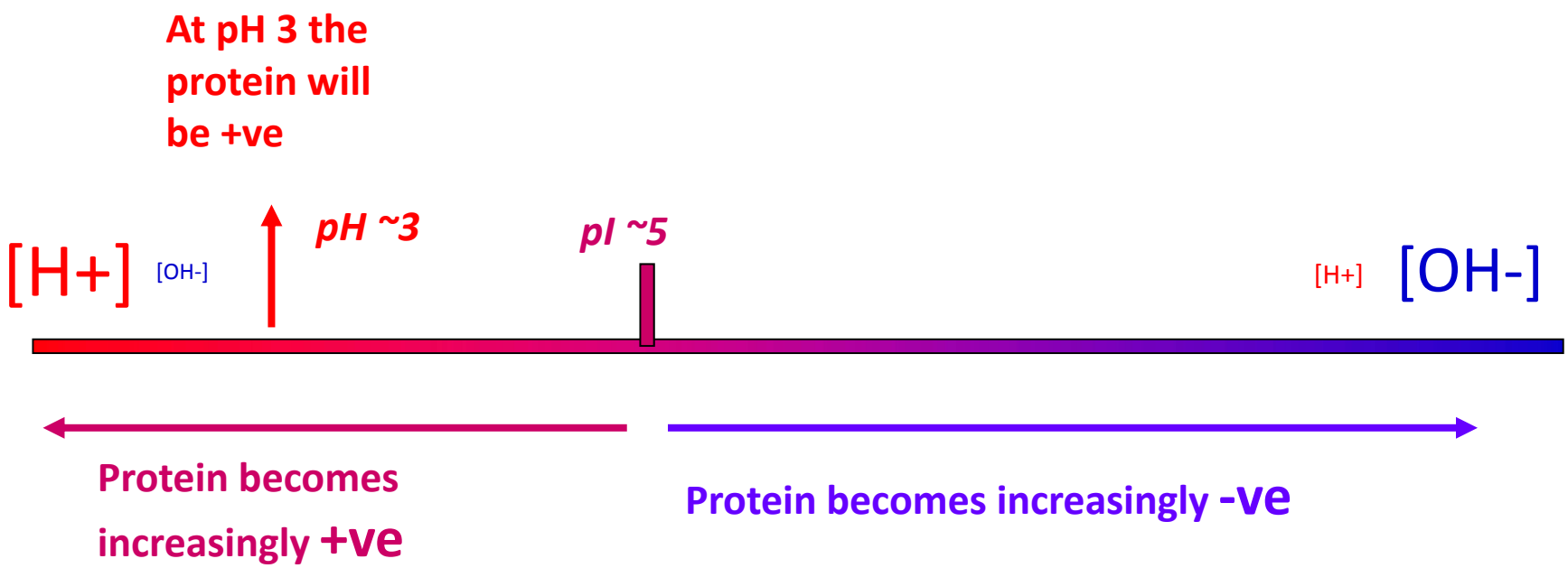
Estimating the charge of a protein

- What we really want to know is the charge of a protein at a particular pH, like 7.
- How do we use pI data to predict the charge of our protein?
- Acidic residues lower the pI
- Basic residues raise the pI.

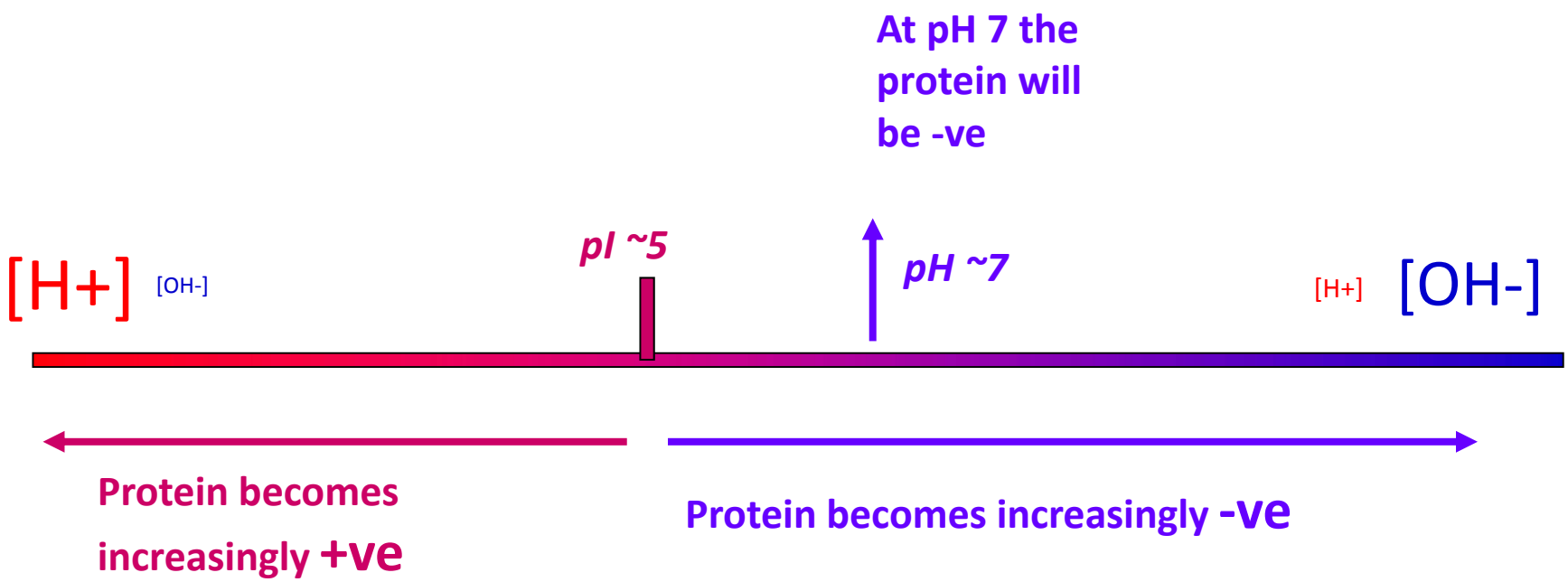
Estimating the charge of a protein



Estimating the charge of a protein



Estimating the charge of a protein



At a particular pH..

- If the pH of the environment is below (more acidic >[H+]) the pI then the protein will be positive (+ve)
- If the pH of the environment is above (more basic >[OH-]) the pI then the protein will be negative (-ve).

Native Gel Electrophoresis

- Proteins with pIs < the pH of the buffer will be negatively charged and will move to the anode (+ve), the red electrode!!
- Proteins with pIs > the pH of the buffer will be positively charged and will move to the cathode (-ve), the black electrode!!

Isoelectric Focusing

- A pH gradient is set up along the length of the gel
- An electric field is applied
- Proteins move to the point where they no longer have a charge i.e. their pI
- A mixture of proteins can be electrophoresed through a solution having a stable pH gradient from the anode to the cathode and each protein will migrate to the position in the pH gradient according to its isoelectric point. This is called **isoelectric focusing.**
- Used as the first dimension of **2D gel electrophoresis**

Isoelectric Focusing

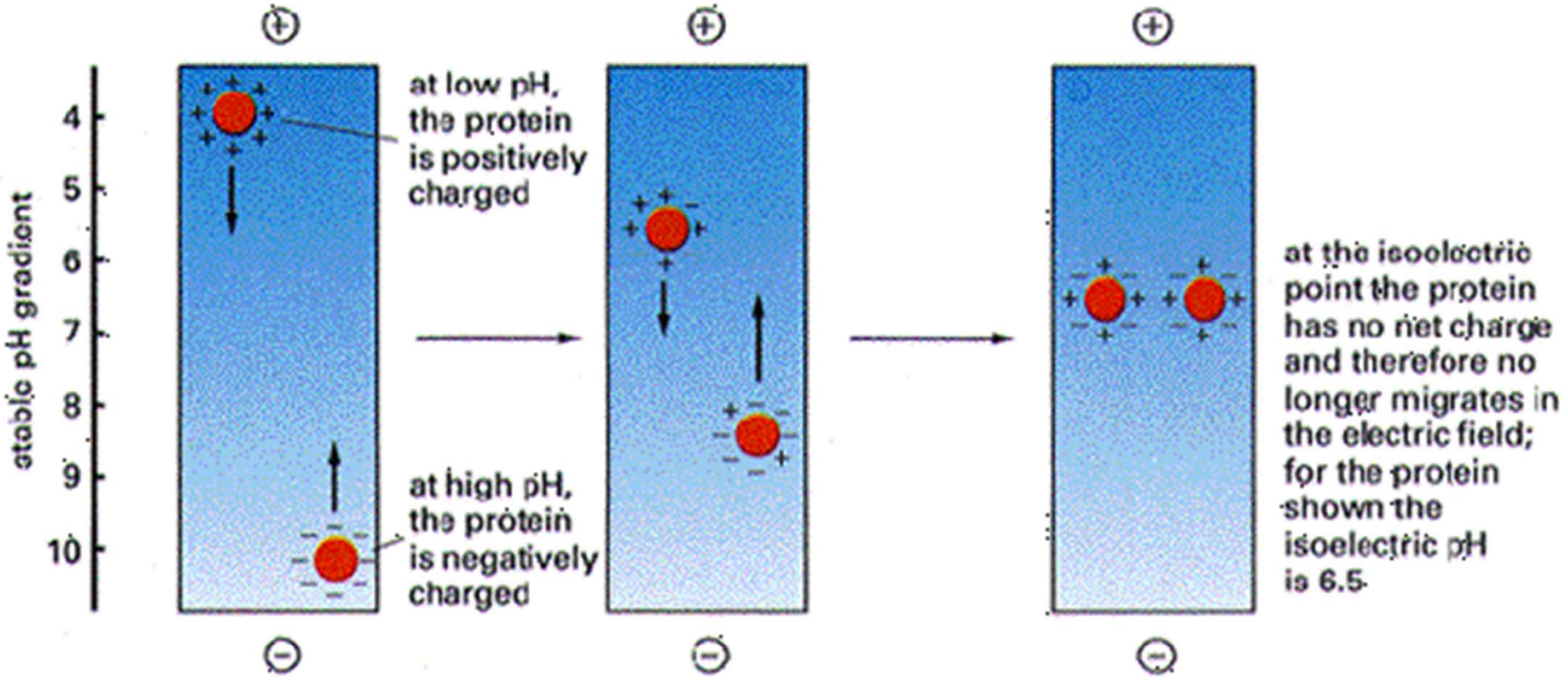


Figure 4-44. Alberts et al. Molecular Biology of the Cell, 3rd edn.

Electrophoresis

- The migration of ions in an electric field to separate molecules.
- Many forms of electrophoresis-we will focus on **polyacrylamide gel electrophoresis (PAGE)**.
- PAGE techniques are often used determine the purity of proteins.
- Polyacrylamide works like agarose except the matrix has smaller pores and so polyacrylamide gels separate smaller molecules (like proteins).
- Agarose is used for much larger molecules such as DNA and RNA.

Sodium dodecyl sulfate (SDS) PAGE

- Unlike DNA and RNA, proteins do not have a nice constant charge to mass ratio and can have any charge at a given pH, depending on their sequence, hence pI.
- To overcome this problem proteins are coated with a detergent, SDS, which makes them negatively charged.
- They then separate by molecular weight.

Sodium dodecyl sulfate (SDS) PAGE

- The SDS will disrupt the secondary, tertiary and quaternary structure so the subunits will separate. For this reason SDS-PAGE separates by subunit molecular weight.

SDS-PAGE

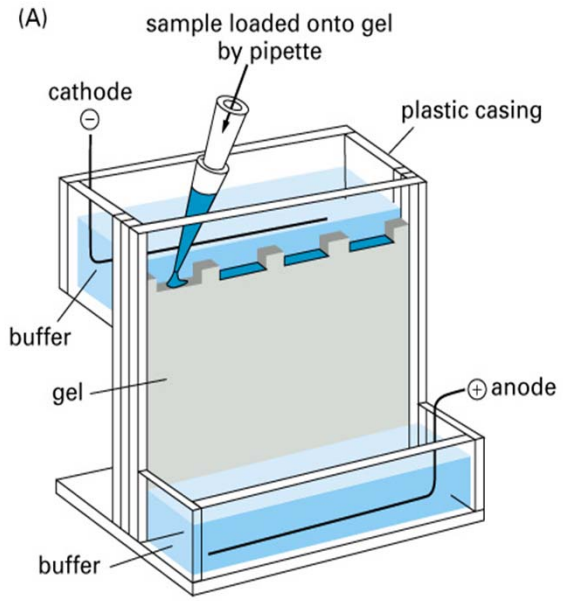


Figure 8–14 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

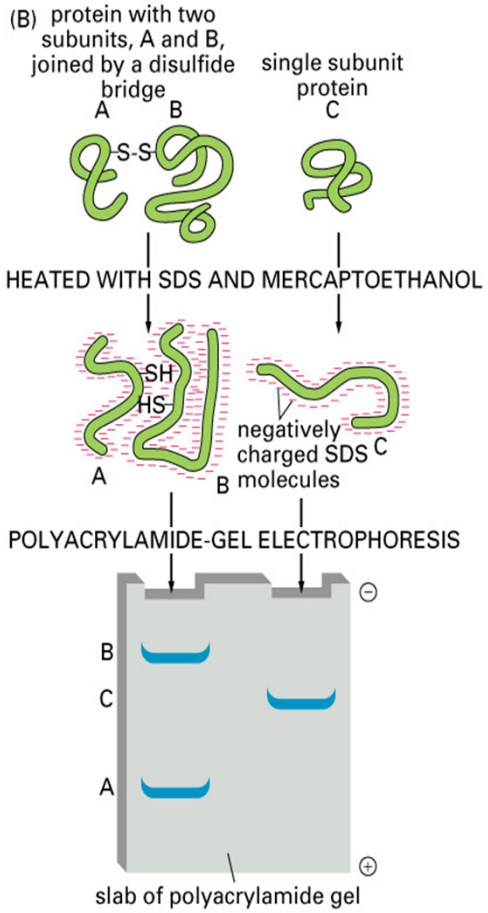
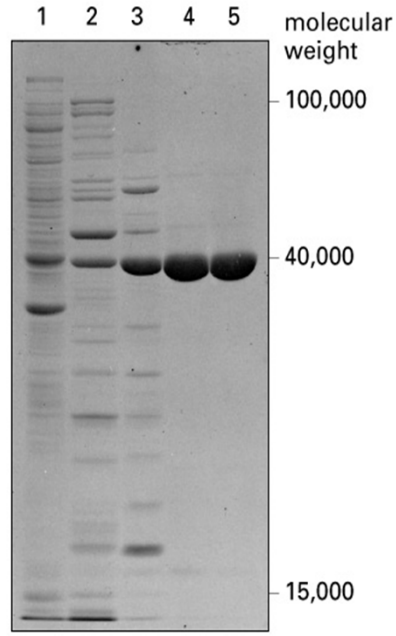


Figure 8–14 part 2 of 2. Molecular Biology of the Cell, 4th Edition.

Coomassie Blue/
Silver Staining



- Since SDS is an anionic detergent it imparts a negative charge to all the proteins in your sample. More importantly, these charges swamp the inherent charge of the proteins and give every protein the same charge-to-mass ratio. Because the proteins have the same charge-to-mass ratio, and because the gels have sieving properties, mobility becomes a function of molecular weight.

- The velocity of a charged particle moving in an electric field is directly proportional to the field strength and the charge on the molecule and is inversely proportional to the size of the molecule and the viscosity of the medium. Adding a gel with sieving properties (that is a gel where the resistance to the motion of a particle increases with particle size) increases the differences in mobility between proteins of different molecular weights. This is the basis of separation.

Two-dimensional Gel Electrophoresis
2DGE

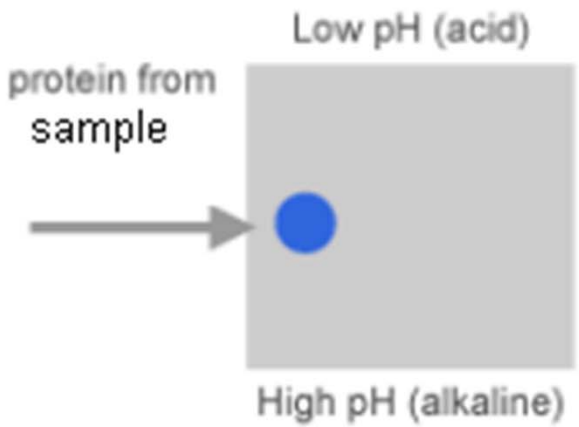
What is it?

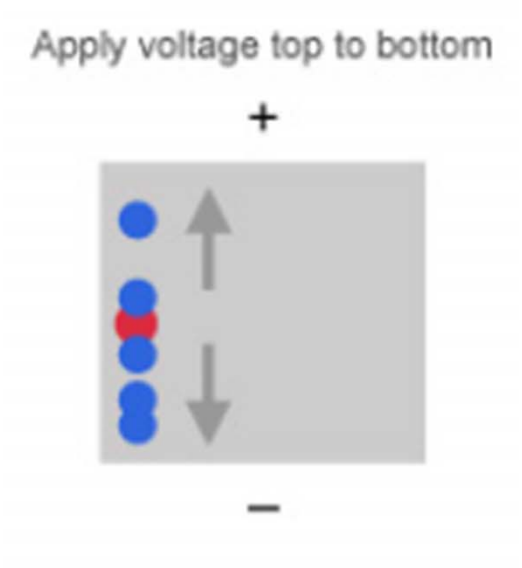
a method for **separating** and **identifying** the proteins in a sample by displacement in **2 dimensions** oriented at right angles to one another

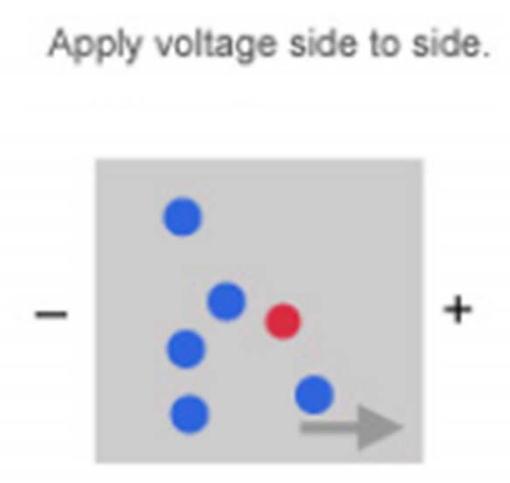
Combines IEF and gel electrophoresis

From Jefferies, *et al.*,
http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html#Section%201

2DGE







Load sample

Isoelectric focusing

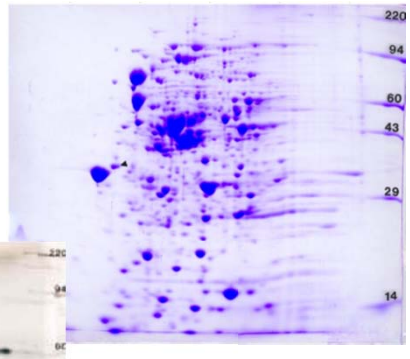
SDS-PAGE

Images retrieved from http://genome.wellcome.ac.uk/doc_wtd021045.html

Visualization of proteins

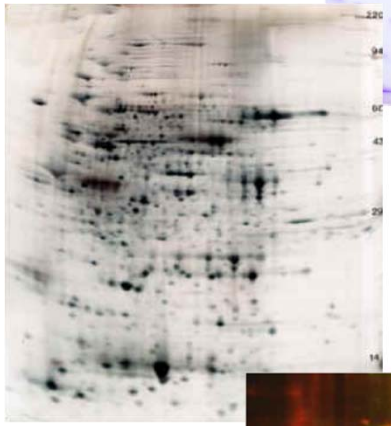
Coomassie blue staining

Detect 36-47ng



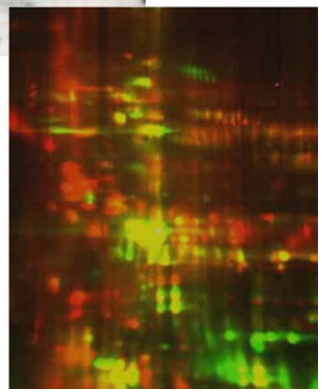
Silver staining

Detect 0.5-1.2ng



Fluorescent staining

Detect 1-2 ng



From Jefferies, *et al.*,
http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html#Section%201

Images from
http://www.kendricklabs.com/2d+CoomassieBlue.htm
http://www.unil.ch/dbcm/page48211_fr.html

2D-gel analysis

Advantages:

1) Very sensitive

2) High resolution

>10,000 different proteins

Limitations:

1) Lack of resolution of *all* proteins present

2) Irreproducibility of results

Fey, *et al.*, 2001

2D gel electrophoresis

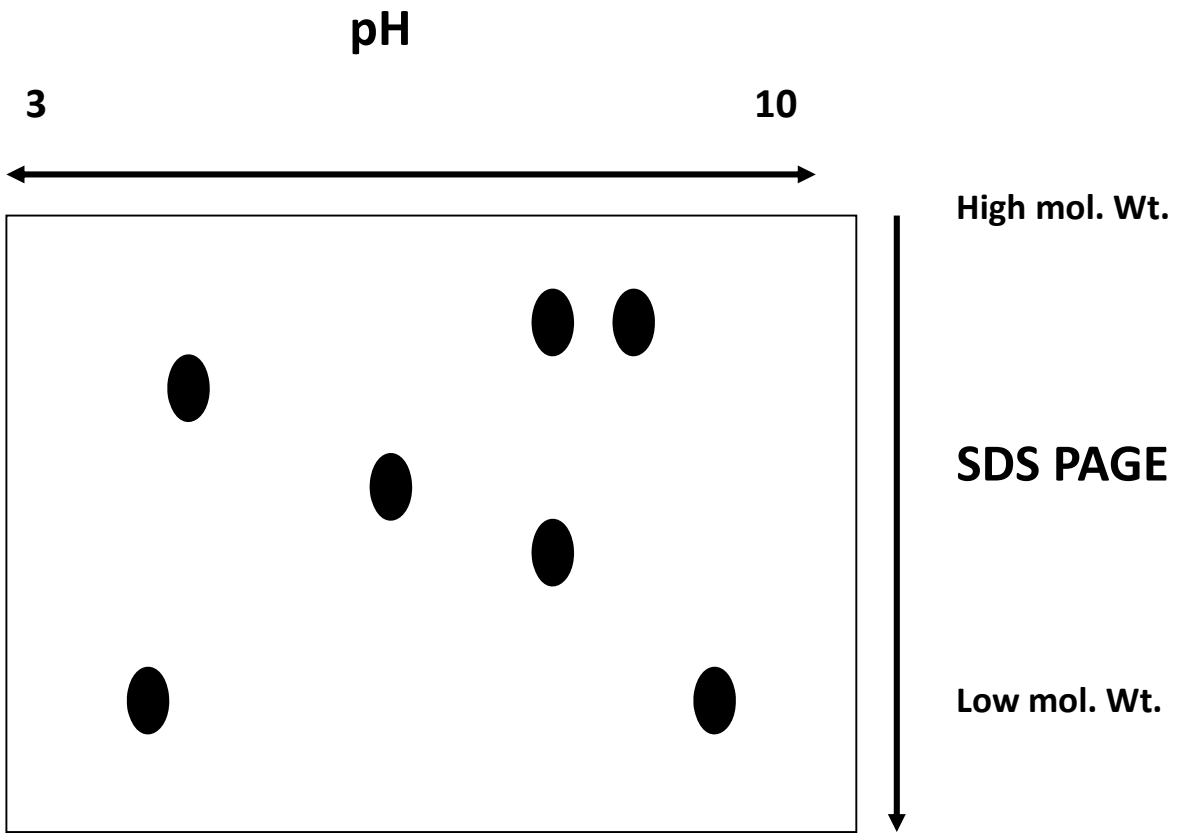
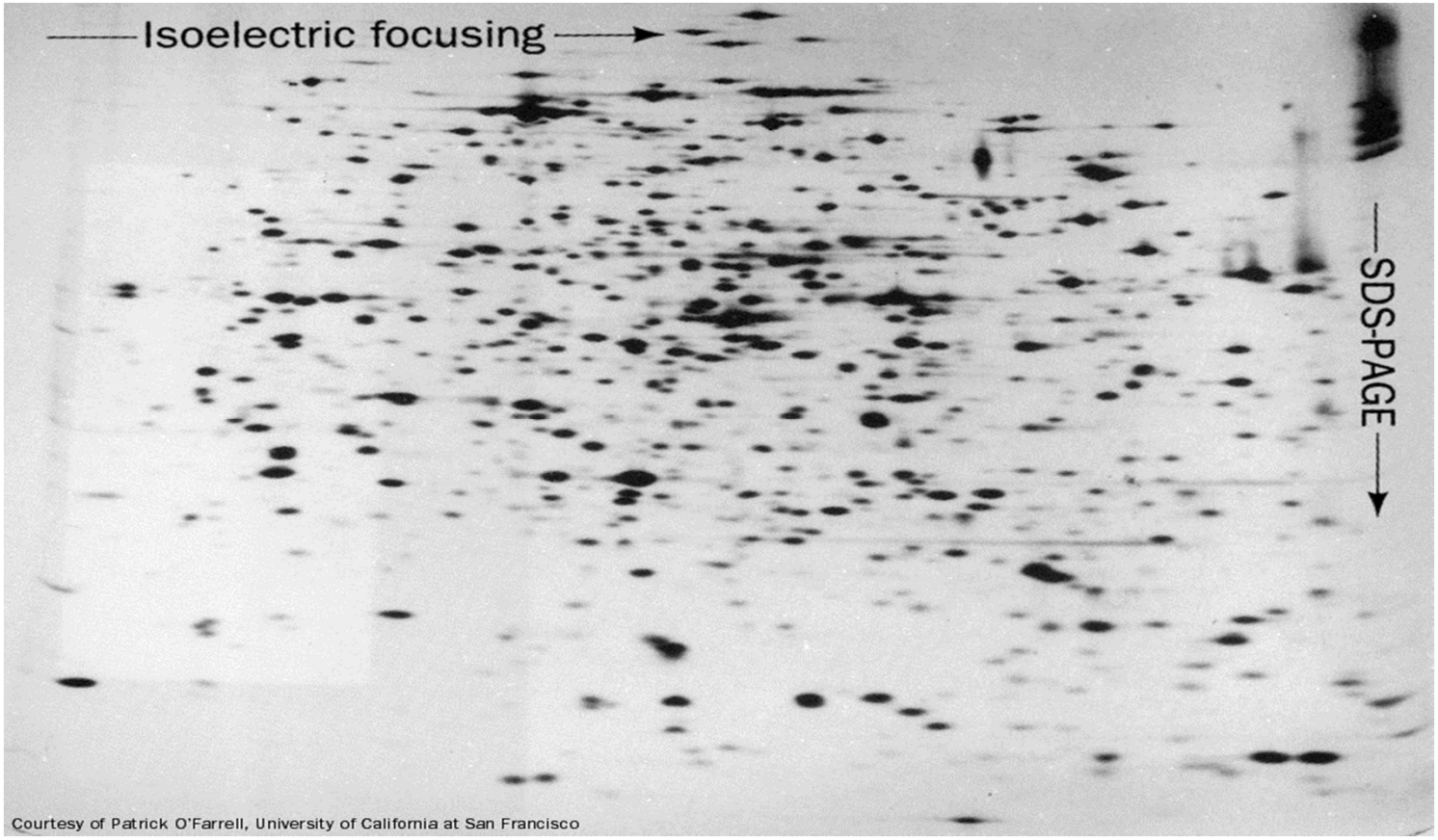


Figure 6-27 Two-dimensional (2D) gel electrophoresis.



Protein Purification Methods

- Separating the Components in the Extract
  - Analytic methods
    - High-Performance liquid chromatography (**HPLC**) – uses high pressure to force the extract through the column in a shorter time
    - However, less protein is separated

Protein Purification Methods

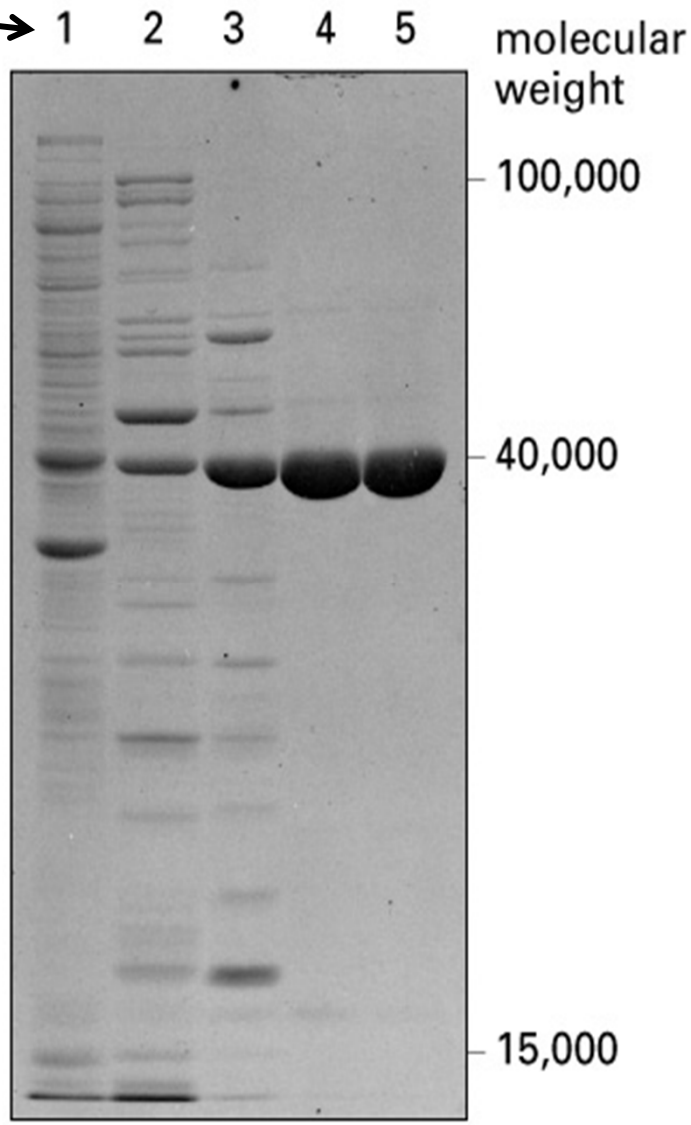
- Separating the Components in the Extract
  - Analytic methods
    - **Mass spectrometry** (mass spec) – highly sensitive method used to detect trace elements
      - Used to indicate the size and identity of most **protein fragments**
      - Samples in picograms can be analyzed
      - Widely used in protein sequencing
      - Three steps:
        - Suspend sample molecules into a charged gas phase
        - Separate molecules based on their mass-to-charge ratio by accelerating down a narrow tube
        - Detect the separated ions

Verification

- The presence and concentration of the protein of interest must be verified at each step of the purification process
  - SDS-PAGE (polyacrylamide gel electrophoresis)
  - Western blotting
  - ELISA

SDS PAGE

Different steps of purification

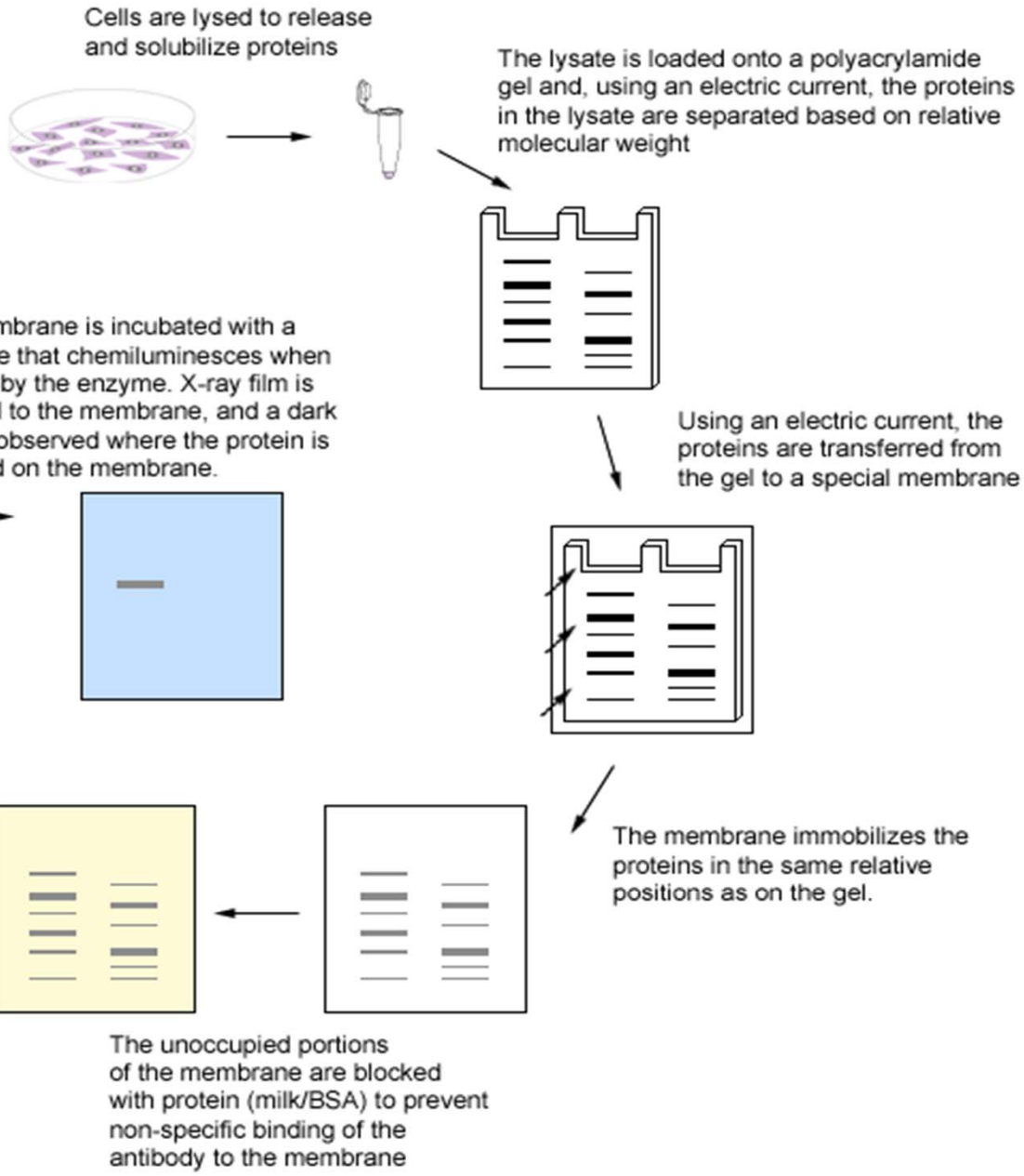


Coomassie Blue/ Silver Staining agents are used

Western blotting

- A technique in which proteins are separated by gel electrophoresis and transferred to a membrane sheet. A specific protein is then identified through its reaction with a labeled antibody.
- Proteins from cells are separated based on their size using an electric current. An antibody to the protein of interest is used to determine whether that protein is present in the cells.

Western Blotting



Preserving Proteins

- Lyophilization (freeze-drying)
  - Protein, usually a liquid product, is first frozen
  - A vacuum is used to hasten the evaporation of water from the fluid
  - Will maintain protein structure and can be stored at room temperature for long periods of time

Scale-Up of Protein Purification

- Protocols are usually designed in the laboratory on a small scale
- Must be scaled up for production
  - Process is approved by FDA so must make sure laboratory procedures can be scaled up

Postpurification Analysis Methods

- **Protein Sequencing**
  - Must determine the primary structure, the sequence of amino acids
- **X-ray Crystallography**
  - Used to determine the complex tertiary and quaternary structures