

Poly-acrylamide Gel Electrophoresis (PAGE)

PAGE is based upon the principle that a charged molecule will migrate in an electric field toward an electrode of opposite sign.

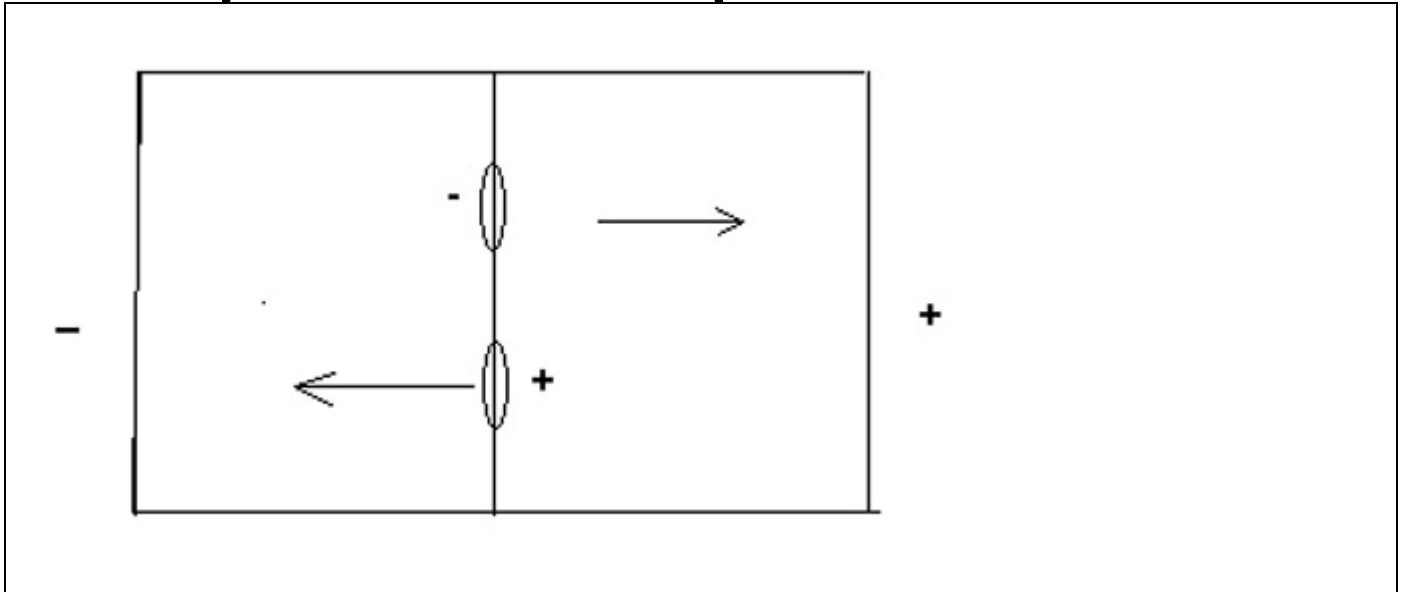
There are two types of PAGE experiments:

- **SDS-PAGE (99.9% of applications)**
- **Native PAGE (0.1% of applications)**

Uses of SDS-PAGE:

0. **Determine purity of proteins.**
0. **Molecular weight determination **NOT** dependent on shape {Stokes Radii} as is case for SEC.**
0. **Subunit composition (compare to native gel OR +/- BME).**
0. **Western Transfer always begins with SDS-PAGE.**
0. **Antibodies can be produced from proteins excised from gel.**
0. **Sometimes can reconstitute proteins.**
0. **Mass spectrometry and proteomic studies.**

Electrophoresis in Principle:



Separation of charged molecules in electric field is a function of:

- **Relative mobility of charged species (related to frictional resistance which is related to size).**
- **Charge on the species.**
- **If $\text{pH} < \text{or} > \text{pI}$ then proteins are charged.**
- **Will migrate toward cathode (-) or anode (+).**
- **Separation occurs due to different rates of migration due to magnitude of charge and frictional resistance (related to size).**

Mobility:

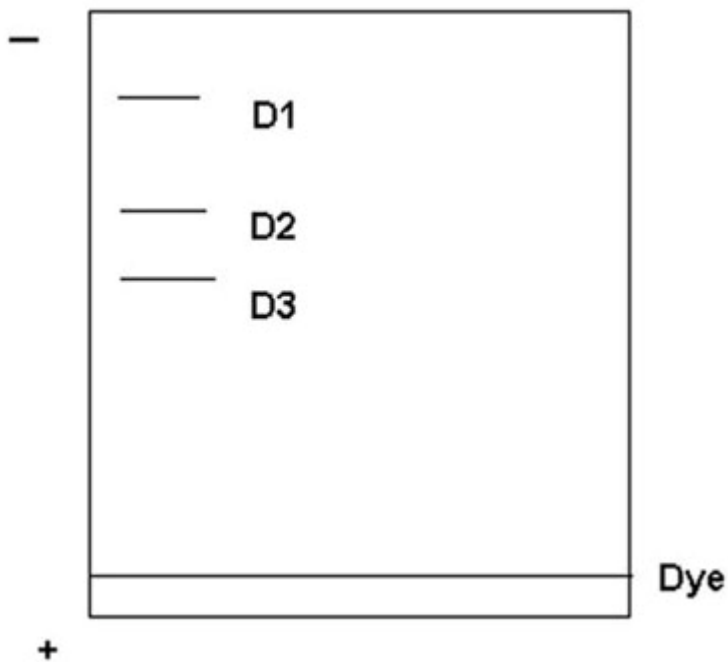
$$R_f = \frac{(Z E)}{f}$$

where

- Z = charge on molecule
- E = Voltage applied (driving force)
- f = frictional resistance

R_f is measured by:

$$R_f = \frac{\text{Distance protein band moves}}{\text{Distance dye front moves}}$$



$$R_f = \frac{D(1)}{D(\text{dye})} = \frac{Z(1) V}{f}$$

Factors influencing f:

- PAGE gel is a lattice or mesh with pores of defined size.
- Size of pore is inversely proportional to %acrylamide (the higher %acrylamide, the smaller the pore).
- Gel acts as a sieve.

Rate of migration $\sim 1/\text{molecular wt or mass of protein}$.

The larger the molecular, the slower it migrates in gel at constant voltage (opposite of behavior on SEC column!) and charge.

Problem is direction of movement is determined by Z:

if $Z < 0$, then $\rightarrow +$

if $Z > 0$, then $\rightarrow -$

if $Z = 0$, then no movement

How can you control Z?

- pH of the buffer (related to _____?).
- Uniformly coating the protein with negative charge using Sodium Dodecyl Sulfate (SDS).

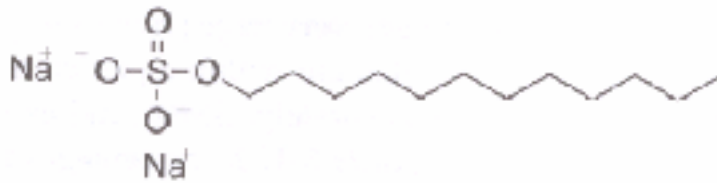


FIGURE 5-2 Structure of Sodium Dodecyl Sulfate (SDS).

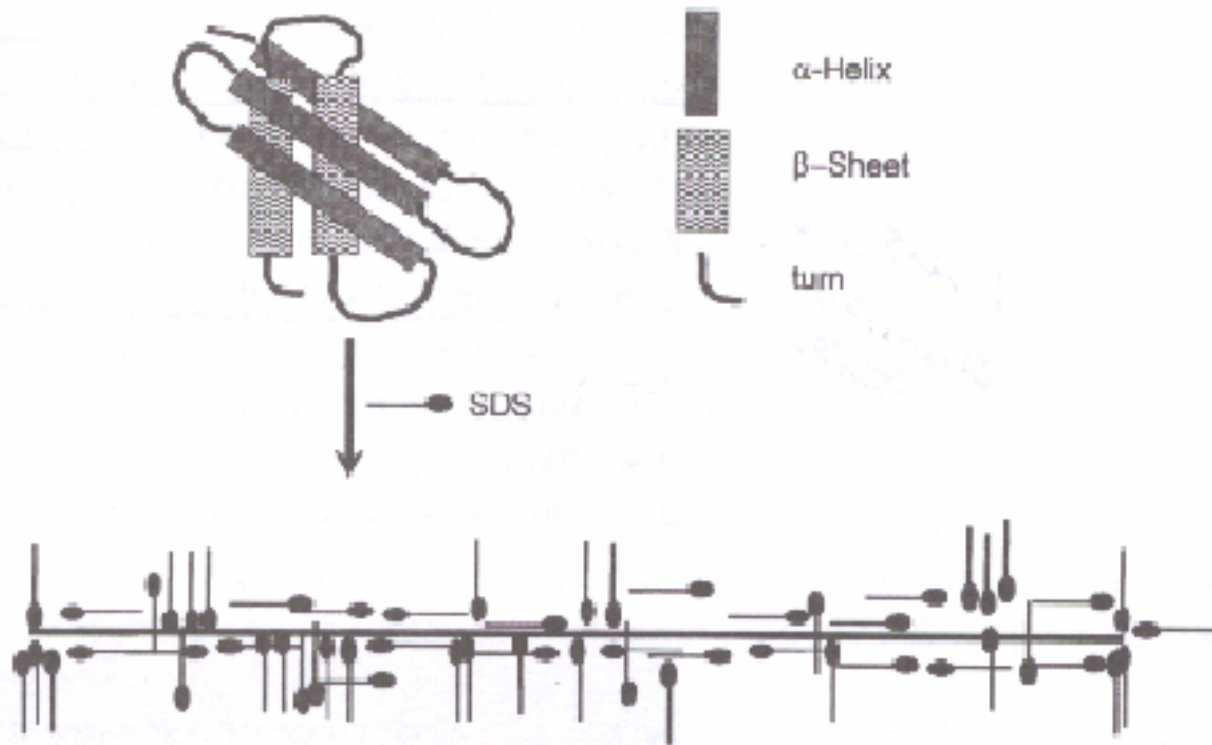


FIGURE 5-3 Denaturation of proteins by dodecyl sulfate. The native protein (*top left*) consists of two central β sheets surrounded by three α helices connected by turns. Amino acid side chains are not shown. The polar heads of the SDS molecules are represented by ●, while the hydrophobic tails are depicted as straight lines. When treated with SDS, the protein unfolds and the side chains are coated with SDS. Polar and nonpolar side chains and portions of the backbone result in an amalgam of different interactions with SDS.

In addition to coating the protein with negative charge, SDS also helps denature protein, exposing hydrophobic groups to solvent.

Statistically: 1 SDS / 2 amino acids.

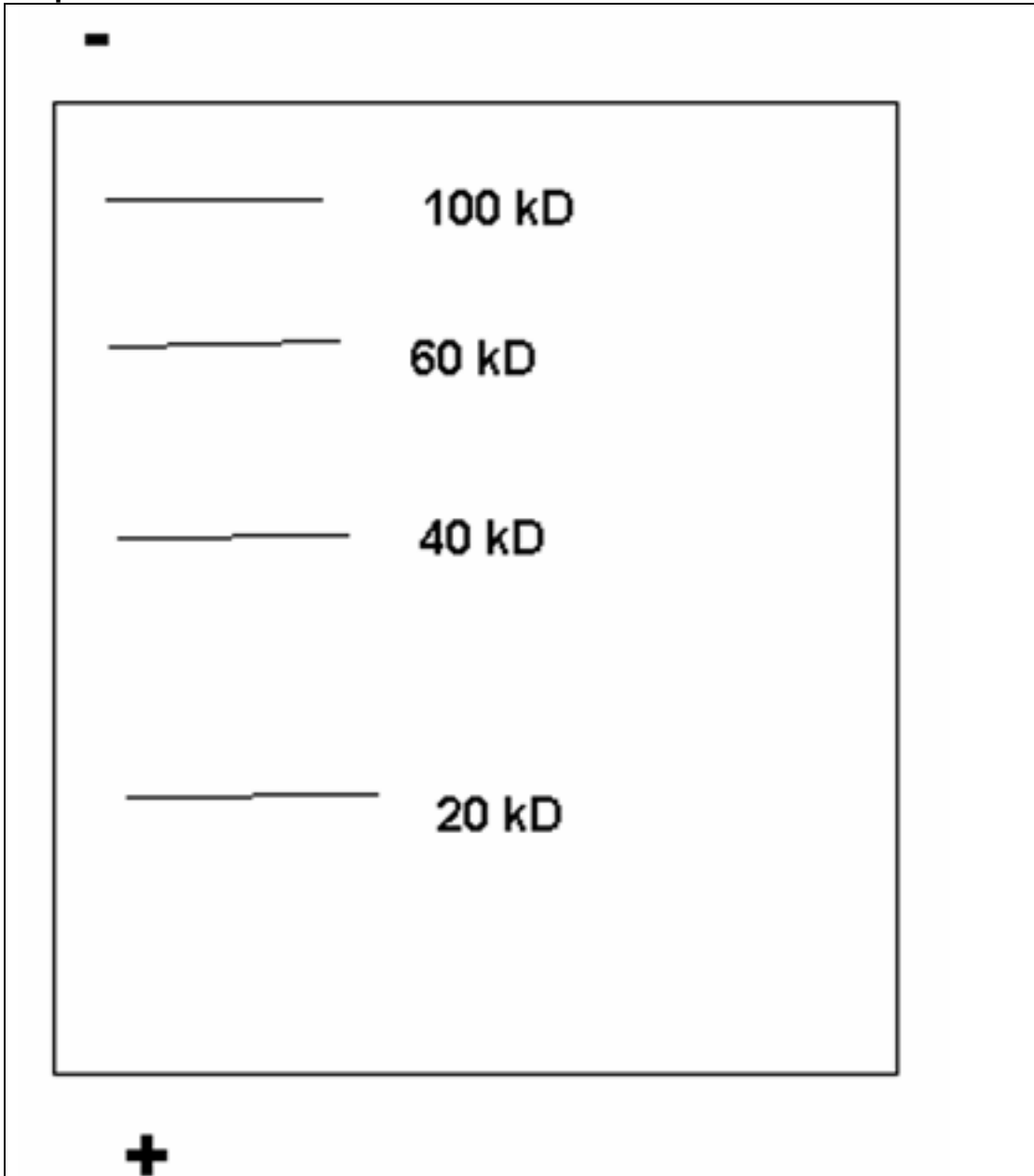
So, all proteins are negatively charged → they will migrate to ANODE (+)

AND

the (Z / mass) ratio for all proteins will be the same!!!!

Because of this, the R_f for proteins will only be dependent on the mass (f, frictional coefficient).

Remember $R_f \sim 1/\text{mass}$!



When the (Z / mass) ratio is the same (+SDS), the proteins separate **ONLY** based on **MASS**, geometry having no effect since the protein has been denatured.

Note: rate and order of migration is opposite that of SEC!!!!

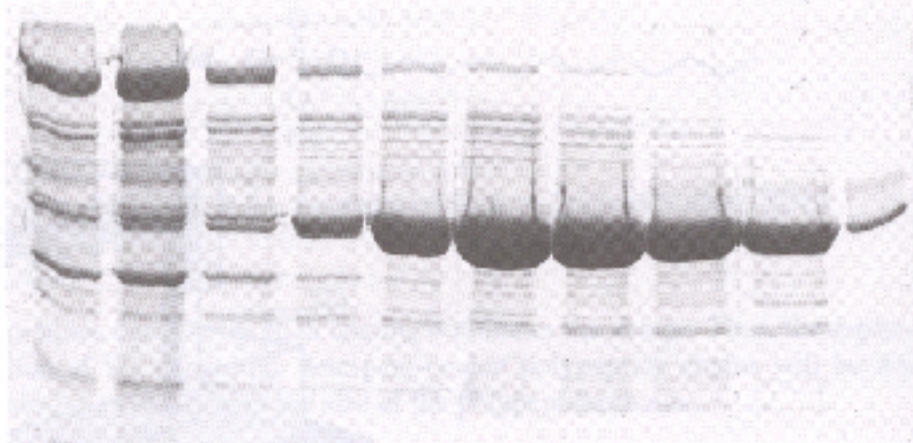


FIGURE 5-5 An example of a Coomassie-stained slab SDS-PAGE. This gel was used to scan fractions eluting from a Bio-Gel A-0.5M gel-filtration column for the presence of the protein NRI, a dimer of identical 51.5-kDa subunits. After allowing for the void volume of the column, an aliquot from every third fraction was run, from left to right on the gel. A prominent band corresponding to NRI is observed in samples 4-9. Gel courtesy of Quan Son.

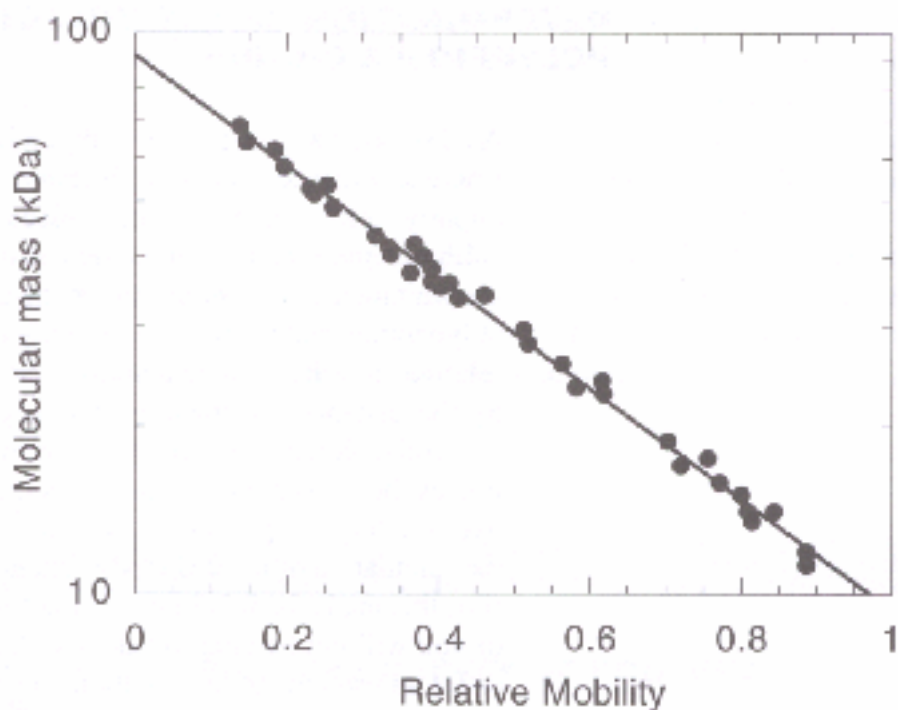


FIGURE 5-7 Plot of relative mobility versus log molecular mass in kilodaltons. Data obtained using a series of known proteins (Weber and Osborn, 1969).

The Chemistry of a PAGE gel:

- **APS (ammonium persulfate):** disproportionation leads to free radical species.
- **Acrylamide:** forms linear polymers.
- **Bis-acrylamide:** cross links linear polymers.
- **TEMED:** an amine catalyst, absolutely essential!

A stock 30% acrylamide soln (wt/vol) (often purchased) contains:

- 29.2% acrylamide
- 0.8% Bis-acrylamide

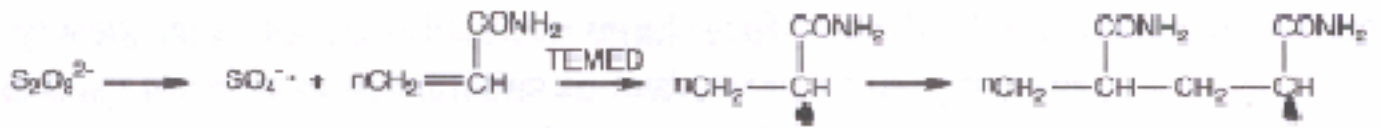
TABLE 5-1
Fractionation Range for Polyacrylamide Gels

PERCENT ACRYLAMIDE	OPTIMUM M_r RANGE
5-12	20,000-150,000
10-15	10,000-80,000
>15	<15,000

Polyacrylamide gels also contain a fixed proportion of bisacrylamide (5% of acrylamide).

Increasing the %acrylamide in gel decreases pore size, increasing f (frictional resistance).

A. Polymerization of Acrylamide



B. Reactions involved in cross-linking acrylamide chains

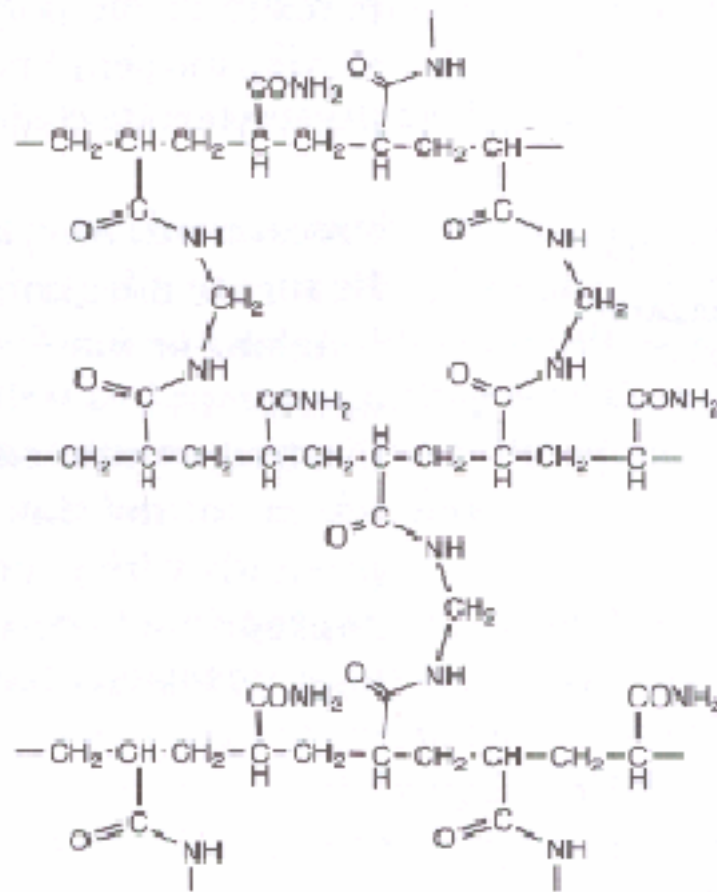
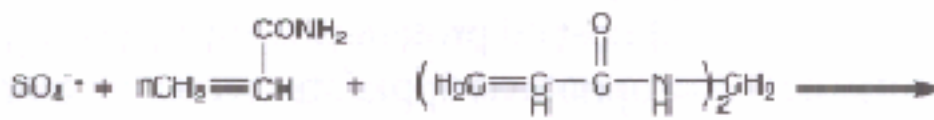
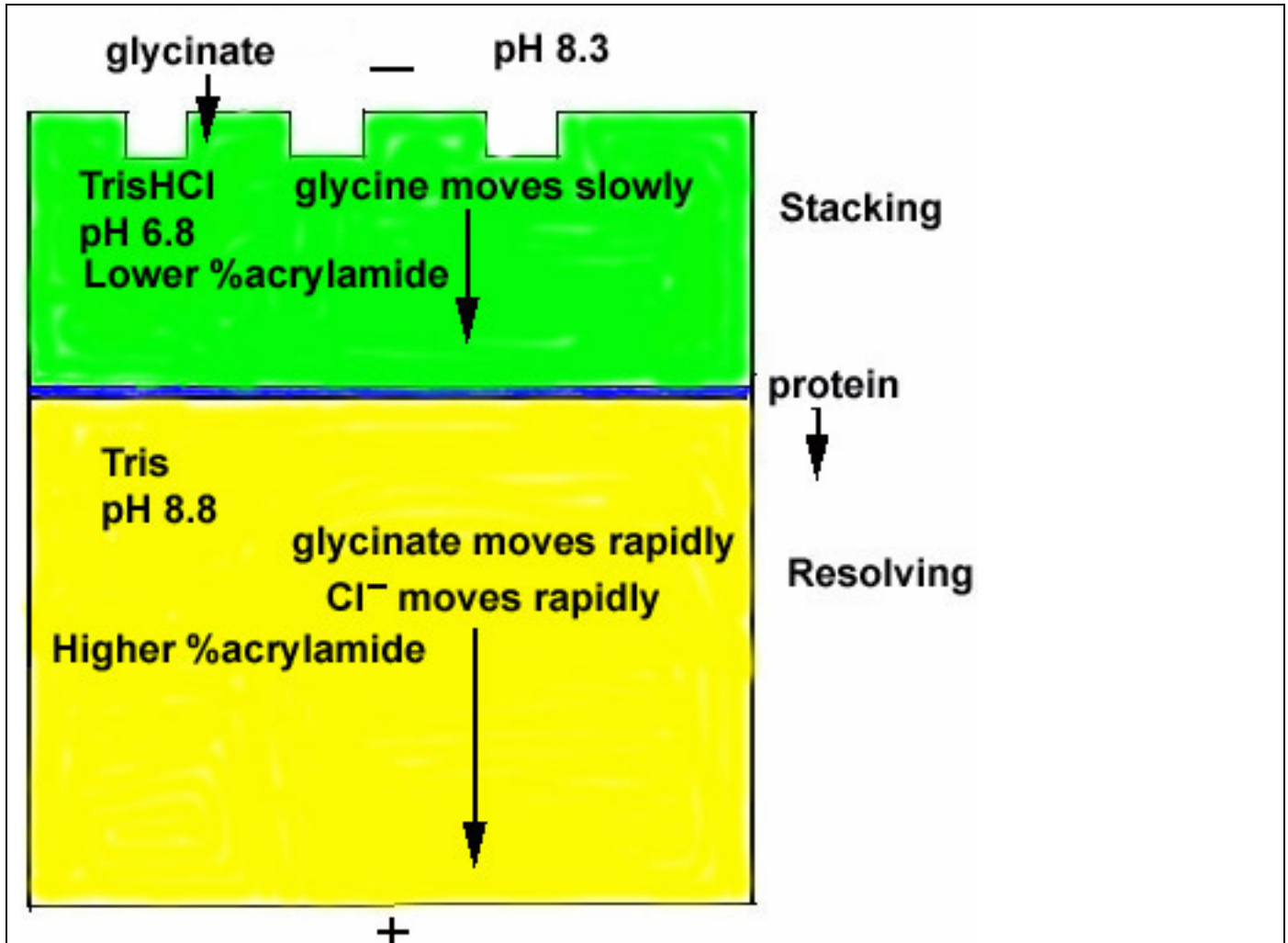


FIGURE 5-1 Polymerization of acrylamide and bisacrylamide.

Components of a Typical Discontinuous (Laemmli) SDS-PAGE Gel



There are two regions of an SDS-PAGE gel, the upper portion is called the **STACKING** gel where the protein bands get squeezed down to a very thin layer migrating toward the anode. Stacking occurs due to differential migration of ionic species that carry the electrical current through the gel.

Stacking Gel Interactions:

- **When an electrical current is applied to gel, ions carry the current to the anode (+).**
- **Cl⁻ ions, having the highest charge/mass ratio migrate faster, being depleted at cathode end and concentrated at anode end.**
- **glycine from electrophoresis buffer enters gel at pH 6.8 and becomes primarily zwitterionic moving slowly.**
- **protein, coated with SDS has a higher charge/mass ratio than glycine so moves fast, but slower than Cl⁻.**
- **when protein encounters resolving gel it slows down due to increased frictional resistance (smaller pore size), allowing following protein to “catch up” or stack.**
- **as protein is depleted from cathode end, glycine must carry current so begins to migrate behind protein, in essence concentrating the proteins further at stacking gel/resolving gel interface.**

Resolving Gel Interactions:

- when glycine reaches resolving gel it becomes anionic and migrates much faster than protein due to higher charge/mass ratio.
- now proteins are sole carrier of current and separate according to their molecular mass due to sieving effect of pores in gel.
- **NOTE:** in order for the proteins to behave in this manner, SDS performs two important functions: **Denaturing protein so geometry is not a factor AND coating the protein UNIFORMLY with negative charge!!!!!!**
- SDS is present in all of the buffers used AND is used to pretreat the protein prior to loading onto gel.

Loading Buffer (LB):

- tracking dye: 0.01% bromphenol blue
 - SDS
 - BME (reduces disulfide bonds)
 - glycerol (adds density)
 - stacking gel buffer
- protein is added to LB and boiled.**

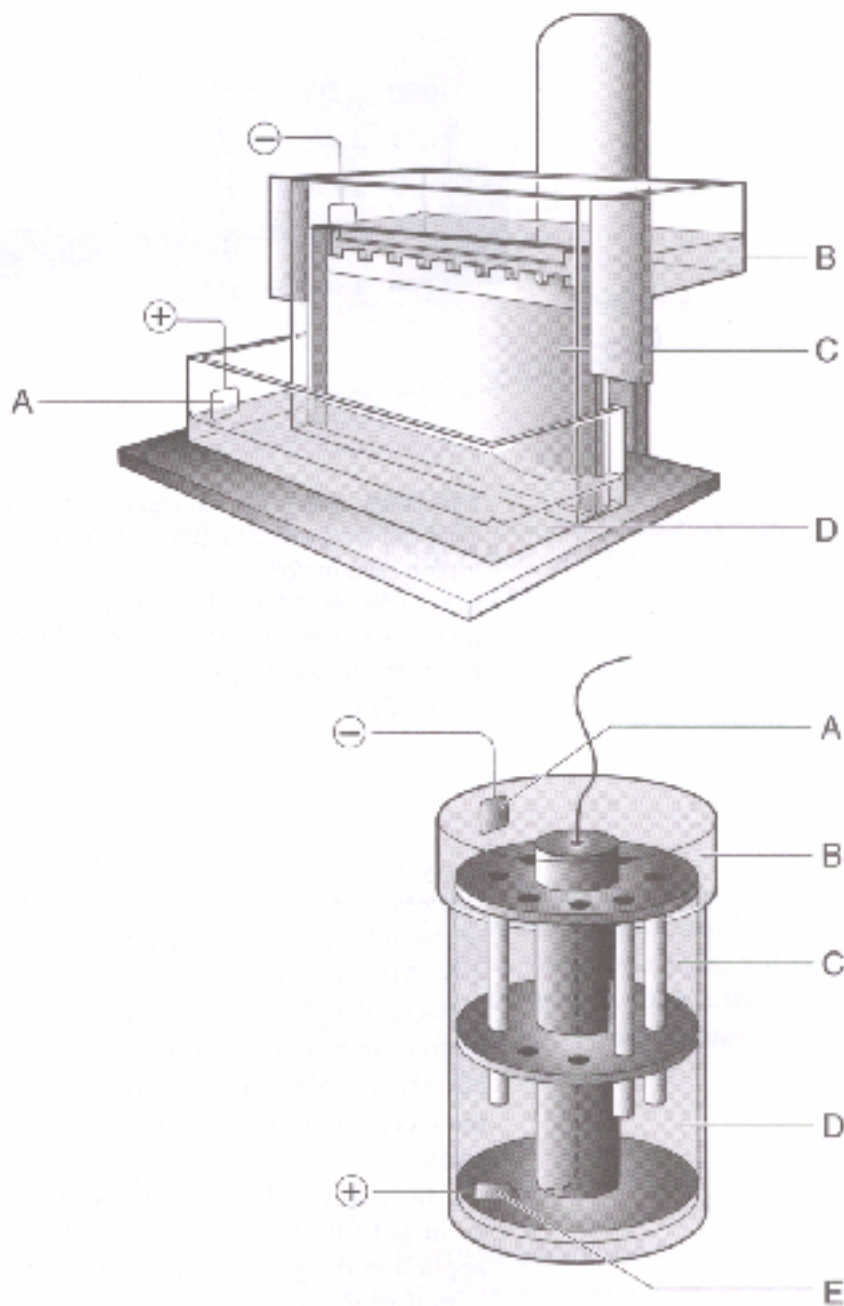


FIGURE 5-4 Formats for polyacrylamide electrophoresis. (Top) Slab gel apparatus. (A) Anode in the lower buffer chamber. (B) Upper buffer chamber with the cathode immersed (-). (C) Gel mounted vertically between glass plates. (D) Lower buffer chamber. The protein bands descend vertically and separate according to their mobility as shown in Fig. 5-5. (Bottom) Tube gel apparatus. (A) Cathode. (B) Upper buffer chamber. (C) Glass tubes containing the polyacrylamide gel and making contact with the two buffer chambers. (D) Lower buffer chamber. (E) Anode. This type of apparatus is also used for isoelectric focusing for the first direction in two-dimensional gels (Fig. 5-10).

Staining SDS-PAGE gels:

Coomassie stain: cheap, easy, common.

- Brilliant blue (stain)
- 50% MeOH
- 10% HOAc
- H₂O

During staining the MeOH/HOAc “fixes” protein bands in gel. Solution also tends to shrink gel because MeOH is hydroscopic.

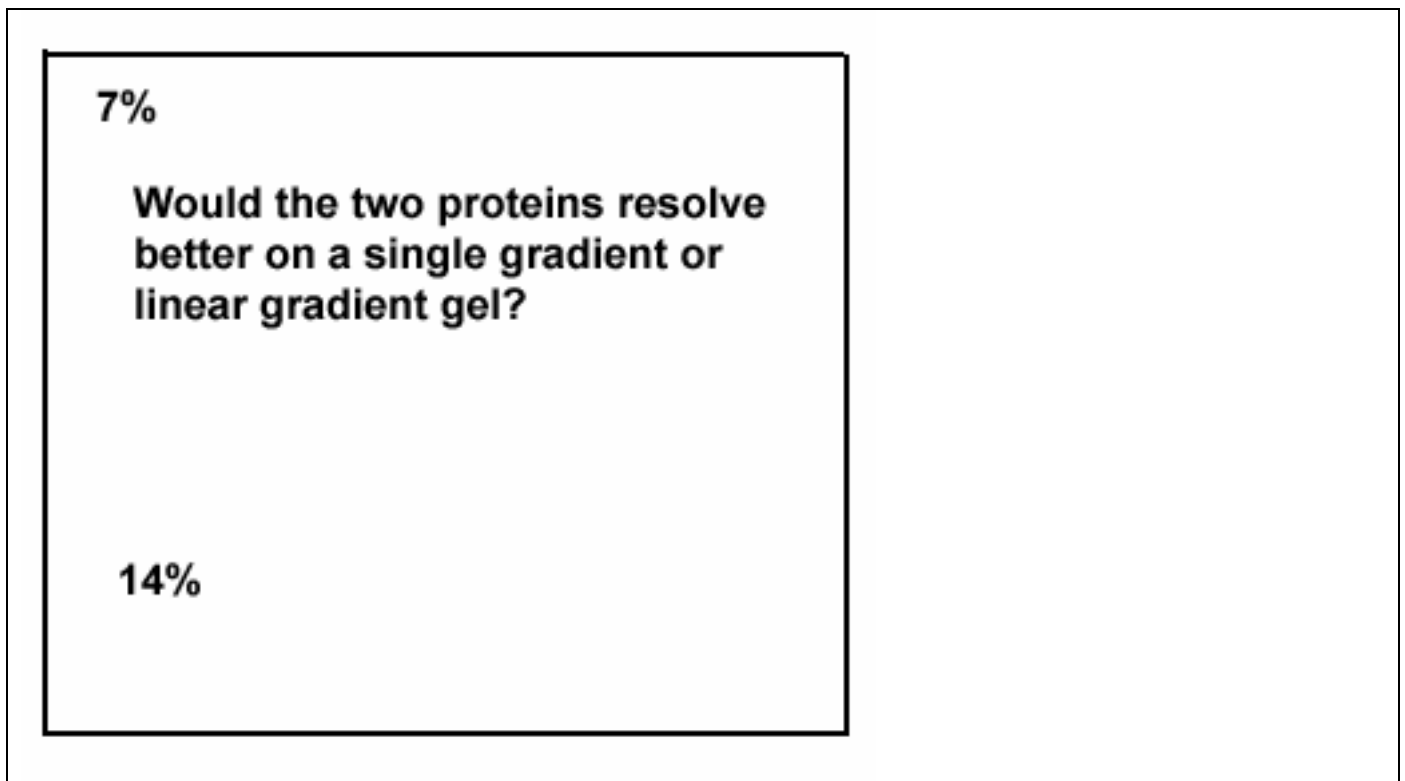
Destaining is done using a 50/10 solution followed by re-swelling using a 10/10 solution.

Ag⁺ stain: based on pptn of Ag⁺ ions in protein.

- more lengthy staining/destaining procedure.
- 100 – 1000X more sensitive than Coomassie.
- more expensive.

Single percentage gels vs. Gradient gels:

- for ease of preparation one often uses a single percentage gel, say either 7% or 14%.
- but, how fast would a low molecular wt protein migrate through the 7% gel vs. a 14% gel?
- how fast would a high molecular wt protein migrate through the two gels?
- suppose you had both proteins present, which gel would you use?



Although you can pour gradient gels, it is easier to buy them, more uniform!!!

Native PAGE

- **a rarely used technique, although it can be informative.**
- **proteins are not denatured as in SDS-PAGE.**
- **one can perform enzymatic assays on bands in gel as we shall do in this class.**
- **“primarily” separates based on mass of proteins, assuming low pI.**
- **is possible to get some idea of subunit composition by comparing to SDS-PAGE gel.**
- **can excise band from gel and extract in native state (useful in preparative gels).**
- **is necessary to run at high pH (~ 9) so most proteins will have negative charge (why?).**
- **Necessary to use cross-linked monomeric protein molecular weight standards which are expensive to determine molecular wt. of unknown proteins.**

Today's expt:

We will run a native gel containing both beta-galactosidase (b-gal) and alkaline phosphatase (AP).

We will then assay for both enzymes using different colorimetric assays.

beta-gal assay: same as before using ONPG as colorimetric substrate, which when hydrolyzed gives a yellow color.

AP assay: Fast Red Dye assay that contains 3-phospho-2 naphtholic acid and 2',4' dimethyl analide. When AP hydrolyzes the P_i group, the analide reacts giving a Red Azo complex.

Half the class will assay for beta-gal first, then AP.

Other half will assay for AP first, then beta-gal.

We will compare results and determine if order of assays influences the observed results and determine why.

BIOC 463A
Expt. 5: PAGE

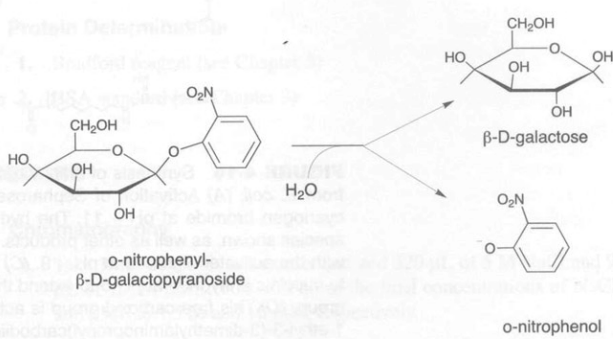


FIGURE 4-15 Reaction catalyzed by β-galactosidase used for spectrophotometric assay at 420 nm.

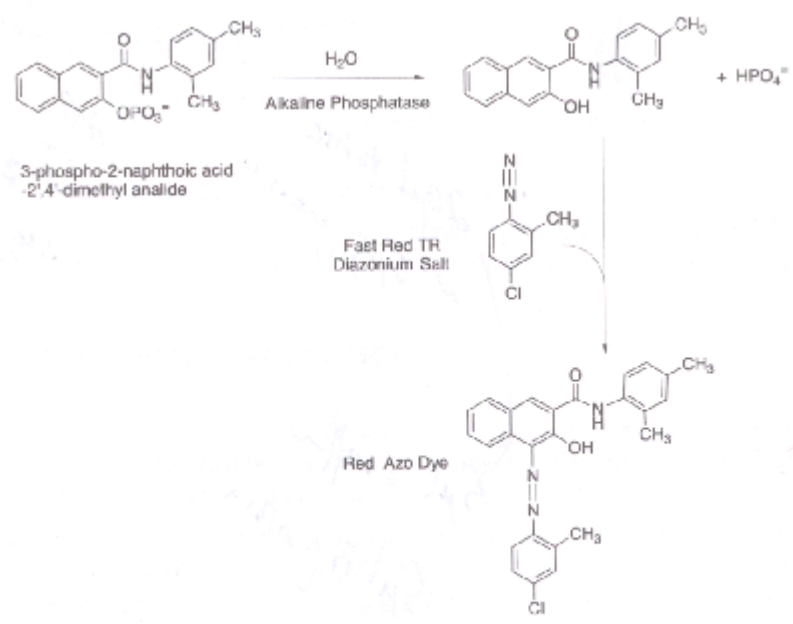


FIGURE 5-11 Reactions used for the staining of alkaline phosphatase in nondenaturing gels.