# Chapter 2 : Protein Electrophoresis

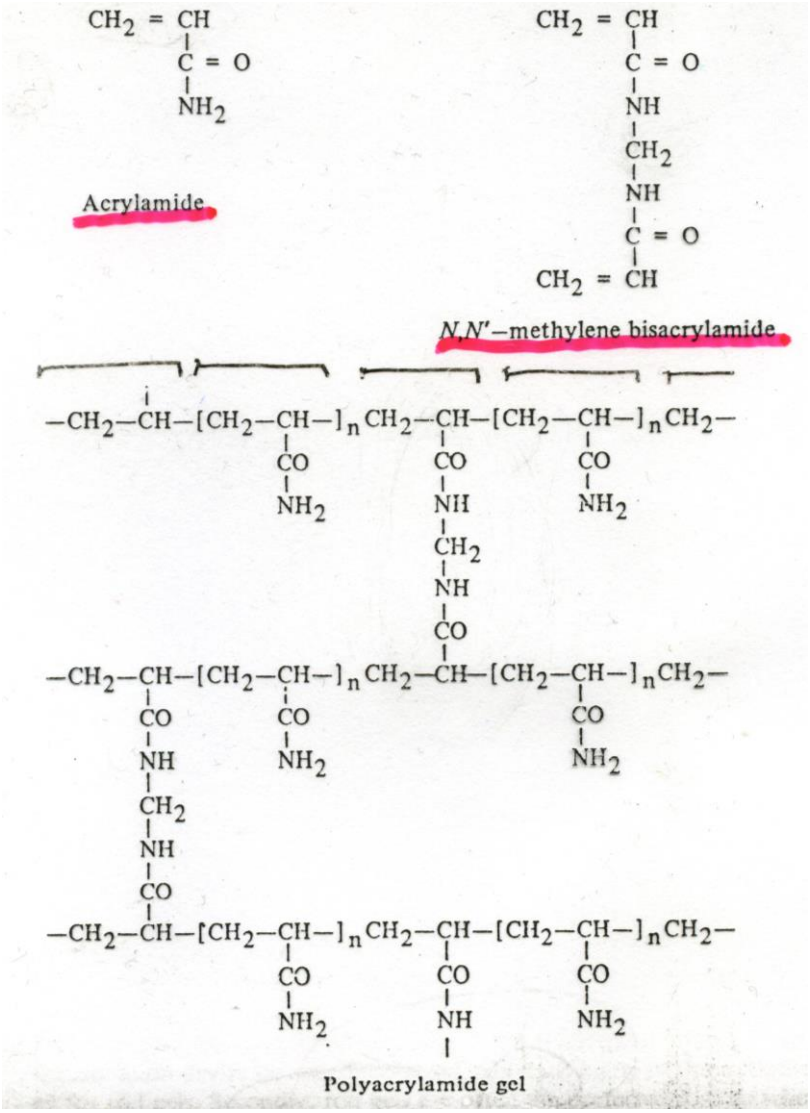
## I. Introduction and properties of polyacrylarnide gel

### Chemical structure and polymerization

*Polyacrylamide gel results from the polymerization of **acrylamide** monomer into long chains and cross-linking of these by bifunctional compounds such as N, N'-methylene bisacrylamide reacting with free functional groups at chain termini.

*Polymerization of acrylamide is initiated by the addition of **ammonium persulfate**. In addition, N,N,N\N'-tetramethylethylenediamine (**TEMED**) is added as an accelerator of the polymerization process.

*Oxygen inhibits polymerization and so gel mixtures are usually degassed prior to use.



**Effective of pore size**

*The effective pore size of polyacrylamide gels decreases as acrylamide concentration increases. When bisacrylamide is included in the polymerization mixture, the proportion of crosslinker is increased, the pore size decreases.

**Rod or slab gels**

*Originally analytical zone electrophoresis in polyacrylamide made use of cyclindrical rod gels in glass tubes but now flat slab gels, 0.5-1.5 mm thick, are usually preferred.

*The advantages of slab gels are:

1) many samples, including marker proteins can be electrophoresed under identical conditions in a single gel such that the band patterns produced are directly comparable.
2) any heat produced during electrophoresis is more easily dissipated by the standard slab gel than the thick rod gels, thus reducing distortion of protein bands due to heating effects.
3) they can be easily dried for storage or autoradiography.
4) less time is required for the preparation of gels for a large number of samples to be electrophoresed under identical conditions.

**Dissociating or non-dissociating system**

*The vast majority of studies empolying electrophoresis of proteins in acrylamide gel use a buffer system designed to dissociating all proteins into their individual polypeptide subunits.

*The most common dissociating agent used is the ionic detergent, **sodium dodecyl sulfate (SDS)**. Proteins from almost any source are readily solubilized by SDS. The protein sample is denatured by heating at 100℃ in the presence of excess SDS and a thiol reagent (to cleave disulfate bonds).

*Polypeptides are enclosed by SDS so that the **intrinsic charges of the polypeptide are insignificant** compared to the negative charges provided by the bound detergent. As a result, the SDS-complexes have essentially identical charge densities and migrate in polyacrylamide gels of the correct porosity strictly according to protein **size**.

*Urea has also been used as a dissociating agent and works by disrupting hydrogen bonds. High **urea concentrations (~ 8M)** are necessary, a **thiol reagent** is also required for complete denaturation of proteins containing disulfate bonds, and urea must present during electrophoresis to maintain the denatured state.

*The advantage of **urea** for some application is that it does not affect the intrinsic charge of proteins and so separation of the constituent polypeptides will be **on the basis of both size and charge**.

*The disadvantage of using urea as a dissociating agent is that:

1) the combination of size and charge fractionation prevents accurate molecular weight determinations.
2) urea is not as good as SDS in dissociating proteins; up to 50% of a complex protein mixture may fail to enter the gel whereas at least 90% of even crude-cell lysates will enter the gel if SDS is the dissociating agent used.

*Electrophoresis of native proteins under **non-dissociating buffer systems** is designed to fractionate a protein mixture in such a way that **subunit interaction**, n**ative protein conformation, and biological activity are preserved**. Separation of the native proteins occurs on the basis of **size, shape and charge**.

**Continuous or discontinuous (multiphasic) buffer system**

*Continuous buffer system is the electrophoretic systems in which the same buffer ions are present throughout the sample, gel, and electrode vessel reserviors at constant pH. In contrast, **discontinuous** (or **multiphasic**) buffer systems employ different buffer ions in the gel compared to those in the electrode reserviors. Most discontinuous buffer systems have discontinuities of both buffer composition and pH. The **Laemmli SDS-discontinuous system** refers to a discontinuous buffer system with SDS added to all buffers that originally described by Laemmli.

*The major advantage of these discontinuous buffer systems over continuous buffer systems is that relatively large volumes of dilute protein samples can be applied to the gels but good resolution of sample components can still be obtained.

**Choice of gel concentration**

*No gel concentration will give maximal separation of all the components from each other in a complex protein mixture. Fractionation limits of uniform concentration gels:

| | |
|---|---|
| 5% gels | 25 kDa |
| 10% gels | 15 kDa |
| 15% gels | 12 kDa |

*__Gradient gels__ have two considerable advantages over uniform concentration gels.

1) Fractionate proteins over a wider range of molecular weights than any uniform concentration gel.
2) the gradient in pore size causes significant sharpening of protein bands during migration. A useful gradient gel for initial SDS-PAGE analysis is a 5-20% or 6-18% linear gradient slab gel.

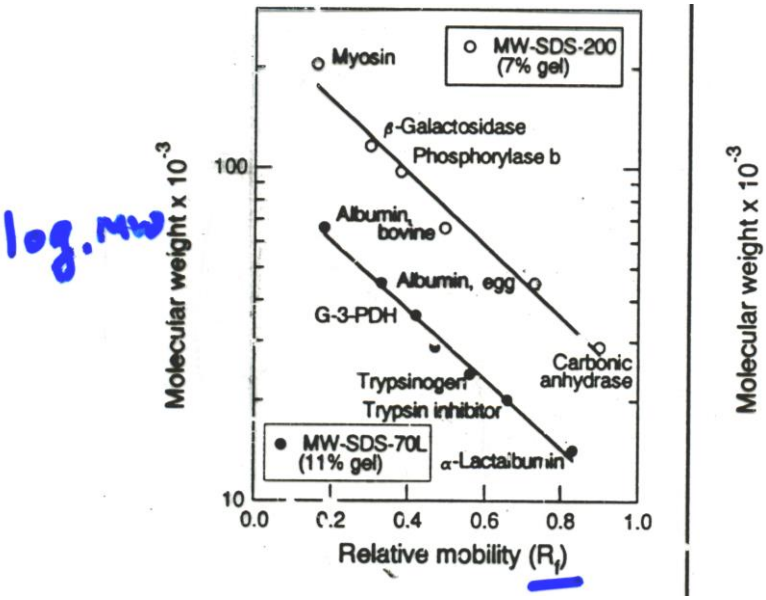
**Determination of molecular weight of a protein**

*The approach is to electrophorese a set of **marker protein** of known molecular weight and use the distance migrated by each to construct a standard curve from which the molecular weight of the sample polypeptides can be calculated based on their mobility under the same electrophoretic conditions.

*The relative mobility, $\mathbf{R_f}$, refers to the mobility of the protein of interest measured with reference to a marker protein or to a tracking dye where:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

*For any given gel concentration the relationship between $\log_{10}$ molecular weight and relative mobility is linear over only a limited range of molecular weight. As a general guide, a linear relationship holds true for 15% acrylamide gels with mol wt range from12 to 45 kDa.



## II. Preparation of SDS-PAGE

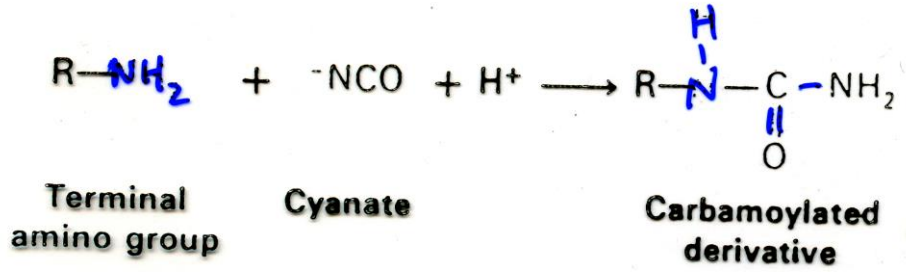
### Reagents

*Both acrylamide and bisacrylamide are **neurotoxins**. Therefore, disposable plastic gloves should be worn when handling all solutions containing these reagents. Polyacrylamide itself is not toxic but usually contains some unpolymerized monomer which is.

*SDS: **highly purified** grades should be used. Many problems in SDS-PAGE can be traced to the purity of the SDS used.

*Urea: When urea is used, the main problem is the accumulation of **cyanate ions** in stock solutions as a result of chemical isomerization. The cyanate reacts with amino groups to form stable **carbamylated** derivatives thus altering the charge of the proteins. If this reaction does not go to completion, several artifactual species of proteins with differing charges will result.

1) use **fresh urea** solutions and, where appropriate, to buffer the solutions with **Tris,** the free amino groups of which neutralize the cyanate ions.
2) Since cyanate ion formation is accelerated with increasing temperature, heating of urea-containing solutions should be avoided.
3) Add **lysine** in the extraction buffer.




### Gel mixture preparation

*Higher concentration gels usually polymerize more rapidly than lower concentration gels at any given TEMED concentration.

*The acrylamide concentration of the **stacking gel** (4.5%) for the SDS-discondnuous system is constant irrespective of the acrylamide concentration chosen for the resolving gel.

**Preparation of slab gels**

*Disposable plastic gloves should be worn during slab gel preparation to prevent contamination of clean glass plates with skin proteins.

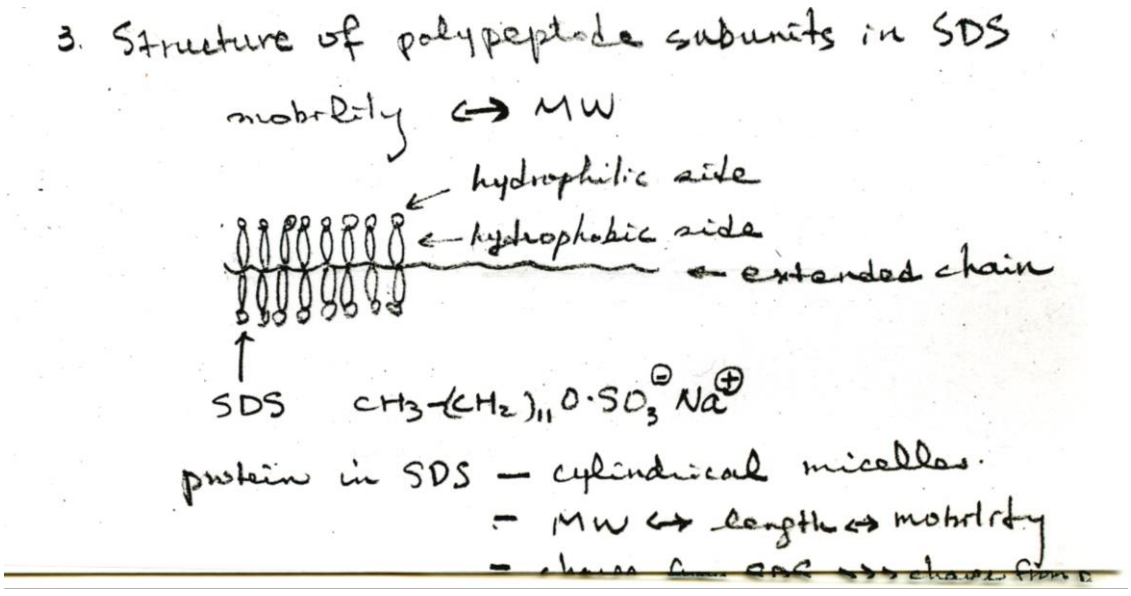
**Sample preparation**

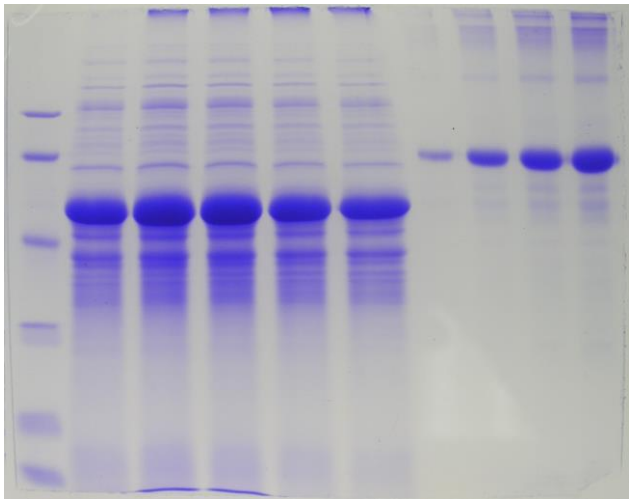
*Determine sample protein concentration before electrophoretic analysis is performed. An overloaded sample in one track can also distort the electrophoretic pattern of band in adjacent tracks. For total protein about 30-60 μg is usually sufficient for Coomassie blue staining.

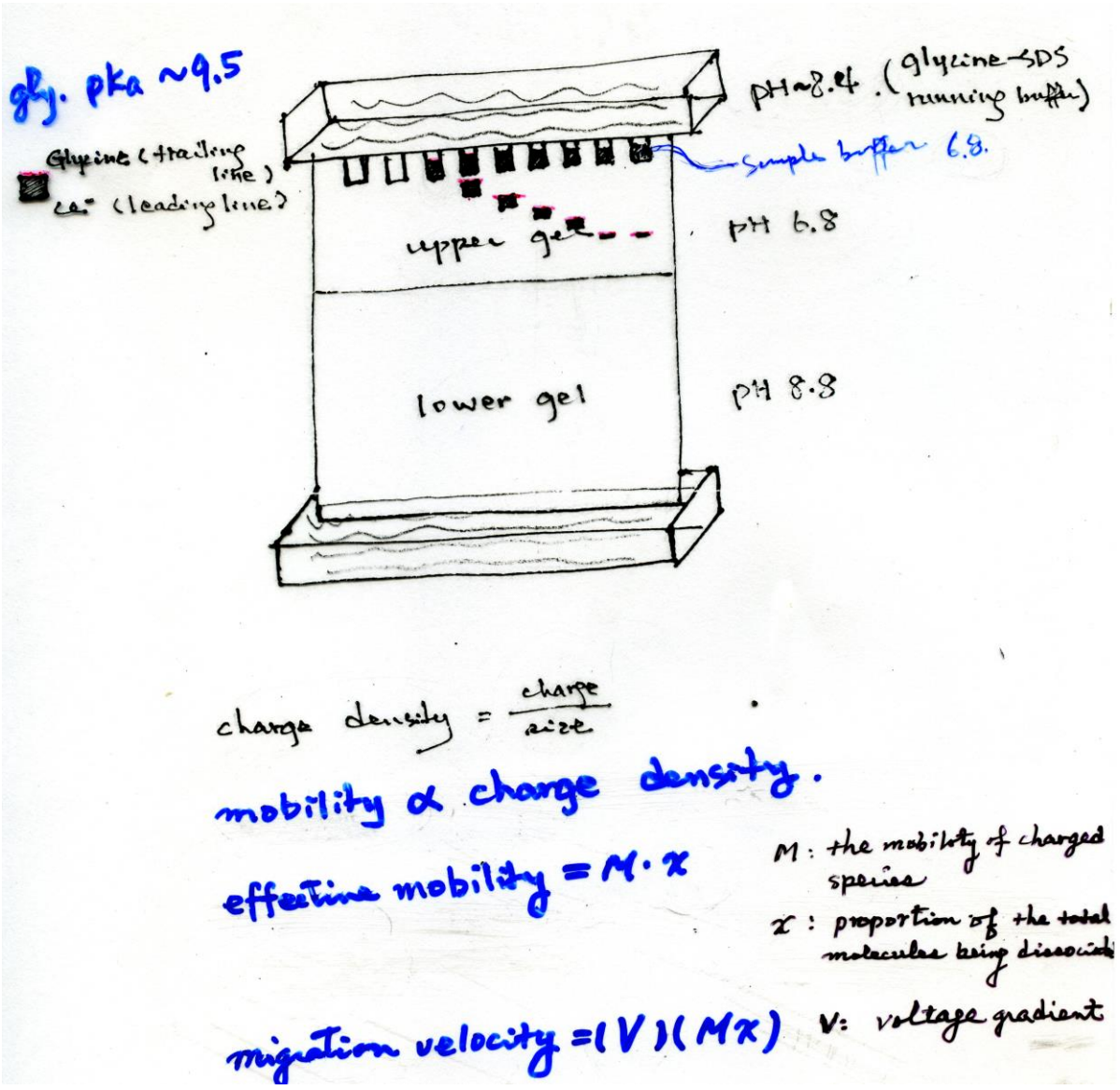
*A number of methods can be used for concentrating protein samples too dilute for immediate electrophoretic analysis. These include lyophilization, **precipitation** (by TCA, acetone, or ammonium sulfate).

*Prior to electrophoresis, samples for SDS-PAGE are heated in a boiling water-bath for 1-3 min. This ensures denaturation of the protein. It is of crucial importance that **any insoluble material should be removed by centrifugation** (10,000 *x g* for 5 min) or this will cause protein streaking during gel electrophoresis.

*lf samples containing urea must be heated prior to loading, the sample should contain Tris as the buffer to minimize cyanate modification of proteins. The heating temperature is 50℃ instead of 100℃






gly. pka ~9.5
Glycine (trailing line)
Cl⁻ (leading line)
pH 8.4 (glycine-SDS running buffer)
sample buffer 6.8.
upper gel
pH 6.8
lower gel
pH 8.8
charge density = charge / size
mobility ∝ charge density.
effective mobility = M · x
M: the mobility of charged species
x: proportion of the total molecules being dissociated
migration velocity = (V)(Mx)
V: voltage gradient

**Tricine-SDS-PAGE**

*Tricme-SDS-PAGE is employed for the separation of low molecular weight of proteins or peptides below 40 kDa. Even 5 kDa proteins or below are easily separated by this system.

*Tricine, used as a trailing ion, allows a better resolution of small proteins than in glycine-SDS-PAGE systems. Peptides and proteins less than 10 kDa can be separated on this type of gel.

*At the usual pH values between 6.8 and 8.8, Tricine migrates much faster than glycine in a stacking gel despite its higher molecular mass, because much more Tricine was in a the migrating, anionic form.

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# Tricine–Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa

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Tricine gel Glycine/Tris gel.

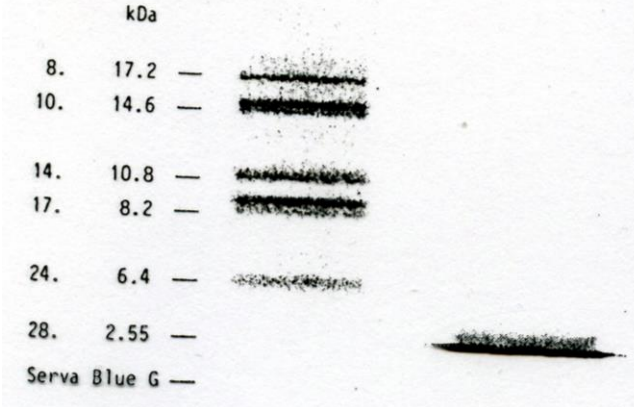



FIG. 5. Resolution of the PMW standard protein kit in 10% T, 3% C gels achieved with our method (lane 1), compared to that of the Laemmli system (lane 2).

9.7 % acrylamide gel

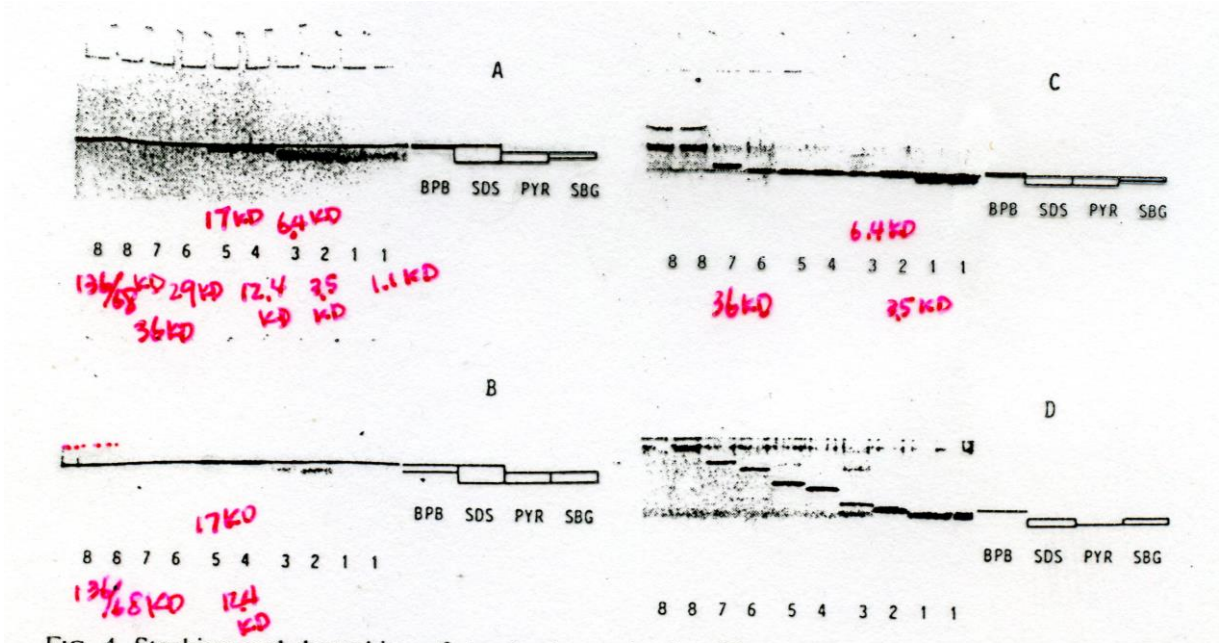



FIG. 4. Stacking and destacking of standard proteins in different 4% T, 3% C gels. (A) Stacking gel according to Laemmli (3), i.e., 125 mM Tris–HCl, pH 6.8; glycine cathode buffer according to Laemmli. (B) Stacking gel as described in Table 2, i.e., 0.75 M Tris–HCl, pH 8.45; glycine cathode buffer according to Laemmli. (C) Same stacking gel as in (B), but Tricine cathode buffer as described in Table 1. (D) Same gel as in (C) combined with an additional 10% T, 3% C "spacer" gel. The filling height of the sample was 5 mm. The standard proteins applied were lane 1, Bradykinin (1.06 kDa); lane 2, glucagon (3.5 kDa); lane 3, subunit 11 of $bc_1$ complex (6.4 kDa); lane 4, cytochrome *c* (12.4 kDa); lane 5, myoglobin (17.0 kDa); lane 6, carboanhydrase (29 kDa); lane 7, glyceraldehyde-3-phosphate-dehydrogenase (36 kDa); lane 8, dimeric and monomeric bovine serum albumin (136/68 kDa). Additionally, the positions of bromphenol blue (BPB), Serva blue G (SBG), Pyranin (PYR), and SDS are indicated.

I

## III. Analysis of gels following electrophoresis

### Protein Staining

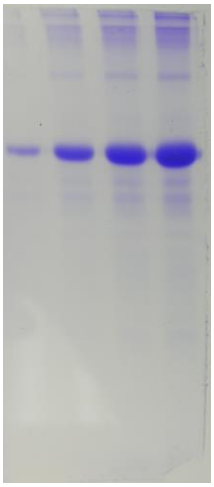
#### 1. Coomassie Blue staining

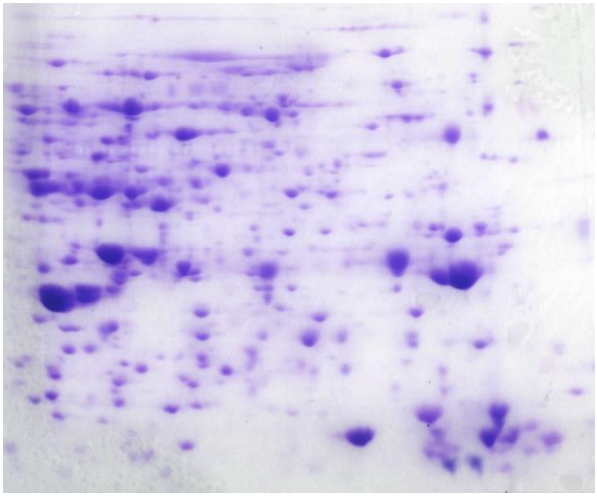
*Coomassie Blue staining requires an acidic medium for the generation of an electrostatic attraction between the dye molecules and the amino groups of the proteins. The ionic attraction, together with van der Waals forces, binds the dye-protein complex together.

*Coomassie Blue stains exhibit three times the staining intensity of Fast Green and six times the intensity of Amido Black. Usually **0.2-0.5 µg of any protein** in a sharp band can be detected using Coomassie Blue staining.

*Since Coomassie Blue is predominantly non-polar, it is usually used in methanolic solution and excess dye removed from the gel later by destaining.

*Stacking gels are usually discarded before staining the resolving gels. Gel slabs are placed in the staining solution and are stained fully within 4-6 h at room temperature if only 1.5 mm thick.





**2. Silver staining**

*Silver staining is **50-100 x** sensitive than Coomassie Blue staining.

*The principal reactive groups are the free amines and the sulfur groups (the basic and sulfur-containing amino acids) contained on the proteins.

*Most proteins stain with monochromatic brown or black colors. Lipoproteins tend to stain blue while some glycoproteins appear yellow, brown or red.

*Artifactual bands with molecular weights ranging from 50 kDa to 68 kDa have been commonly observed in silver-stained gels. Evidence has been presented indicating that these contaminating bands are due to keratin skin proteins.

## IV. Preparation of 2D-PAGE

### Introduction

Two-dimensional PAGE allows the resoludon of a complex protein mixture into more discrete components than ID-PAGE since it separates on the basis of protein charge and molecular weight. The first-dimension is usually isoelectric focusing (IEF) in which proteins are separated by charge. The second-dimensional gel is SDS-PAGE in which proteins are separated by molecular weight.

### First-dimensional gels

*The first-dimensional runs are usually carried out in gel rods using a simple apparatus which can take 8 - 20 gels. The final quality of the two-dimensional separation is very dependent on the degree of resolution obtained in the first dimension.

*A typical recipe of the first-dimensional gel:

| | |
|---|---|
| 9.2 M urea | ----denaturant |
| 6% acrylamide | ----carrier |
| 2% Triton X-100 | ----increase solubility |
| 2% ampholine | ----form pH gradient |
| ammonium persulfate | |
| TEMED | |

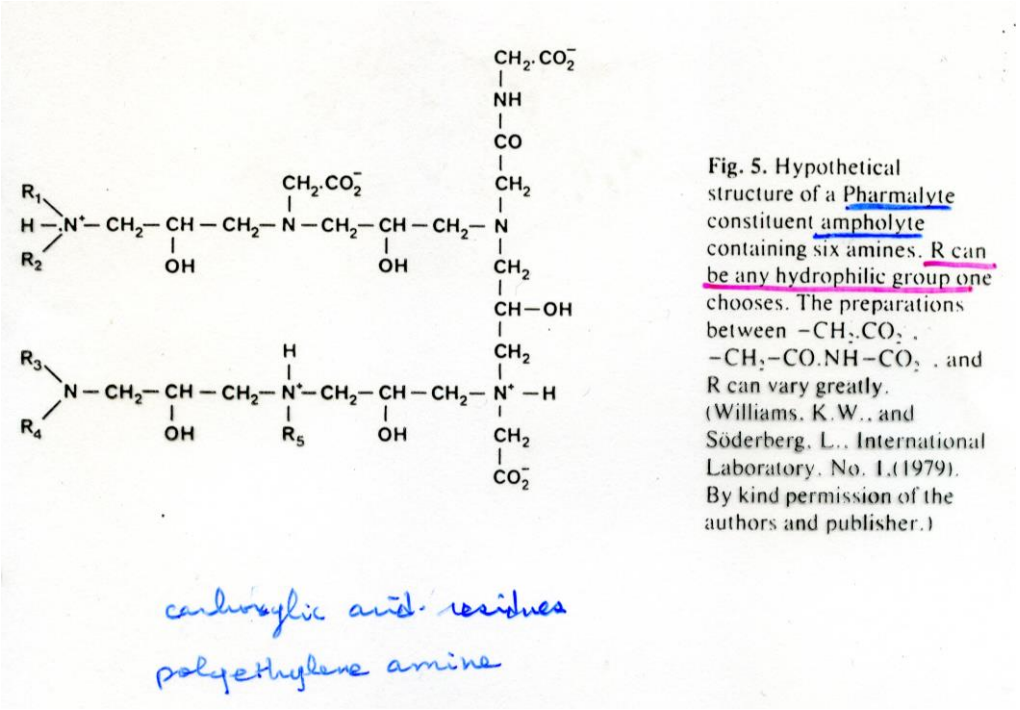


Fig. 5. Hypothetical structure of a Pharmalyte constituent ampholyte containing six amines. R can be any hydrophilic group one chooses. The preparations between $-CH_2.CO_2^-$, $-CH_2-CO.NH-CO_2^-$, and R can vary greatly. (Williams, K.W., and Söderberg, L., International Laboratory, No. 1,(1979). By kind permission of the authors and publisher.)

carboxylic acid residues

polyethylene amine

*ln the presence of urea, some authors have reported the benefits of adding **lysine** to compete for the reaction of isocyanate with proteins. Thus the carbamylation of proteins which leads to a row of artifactual additional spots can be avoided.

***Complete disaggregation and solubilization of protein samples are necessary** to ensure complete penetration of the proteins into the gel, and good resolution. The aggregation of proteins leads to artefactual spots and a decrease in the intensity of the actual polypeptide spots. Progressive dissociation of aggregates during IEF leads to streaking.

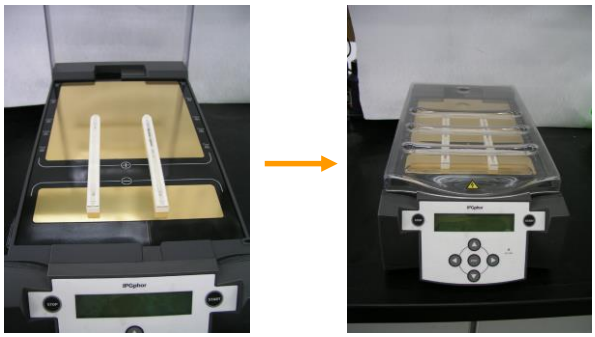
*After electrophoresis, the gel is removed from the tube by extruding it under gentle pressure. The gels can be stored in the deep freeze, **at -80"C preferred**.

*Before ready for the second dimension, the gels have to equilibrate in the buffer used for running second-dimension. The total equilibration time recommended in various methods varies from 5 min to 2 h. The loss of polypeptides during equilibration process can be as high as 25%.

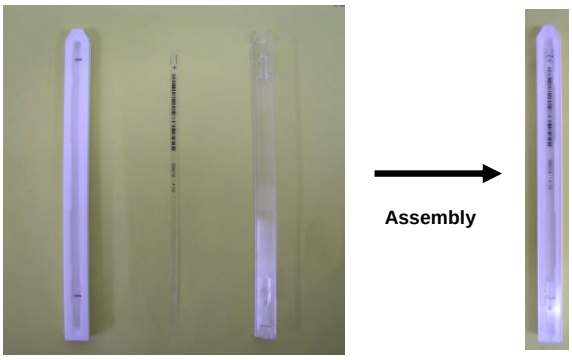
*An alternative first dimension: non-equilibrium pH gradient electrophoresis (**NEPHGE**) is particularly useful for basic proteins.

***IPG (Immobilized pH gradient) gel** is claimed to be more stable than traditional IEFgel.

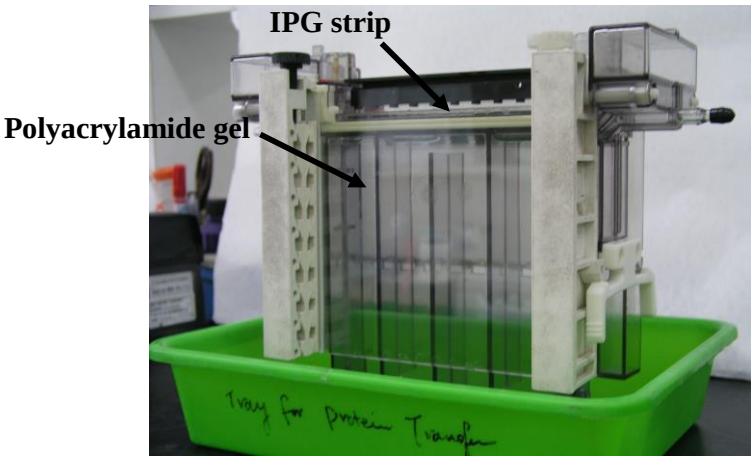
IPGphor



# First dimension - IPG




# Second dimension – SDS-PAGE




**Second-dimensional gels**

*The second-dimensional separations are always carried out in slab gels. The polyacrylamide slab gel is set up in the standard way.

*The first-dimensional gel is usually placed in the top of the slab gel plates and sealed in place with 1% agarose although the exposure of the gel to hot agarose may adversely affect the proteins being separated. Ensure the first-dimensional gel as straight as possible and that no air bubbles between it and the surrounding gel.

*The popularity and advantage of 2-DE in the proteomic analysis is that

1) a 2-DE gel can easily resolve 1000 and as many as 10,000 different proteins at one time. No other technique or combination of techniques has to date been

developed that can deliver this level of detail in a similar time frame and with similar amounts of starting material.

2) 2-DE gels are also quantitative.

**Application of 2D-PAGE**

*2D-PAGE is presently the most powerful analytical tool to achieve manifold protein separation and is applied in a broad range of biological fields, ranging from developmental and physiological studies to genetics.

1) Monitor protein accumulation during development
   examples: Soluble proteins of cotton cotyledon during embryogenesis; Total protein accumulation in developing lily anthers
2) Comparisons between differentiated organs and tissues
   2D-PAGE is used to reveal different states of genome expression that can be related to the specialization of organs or tissues.
3) Comparisons of genetic variability within and between species.
4) Detection of protein synthesis effected by various environmental stimuli such as: growth substances (ABA, GA), abiotic stresses (high temperature, low temperature, drought, anaerobiosis and salt), and pathogenic attack.

## V. Proteomics versus genomics

The term proteome was first discussed in print by Wasinger et al. in 1995 who defined it as the "**total protein complement of a genome**". Similar definitions of the proteome, such as "**the set of proteins encoded by a genome**" may frequently be found in the literature. These description of proteome are not exactly right because

1) The genome does **not explicitly encode the full structure and diversity** of the proteins present in an organism. The expression level of individual mRNAs often does not reflect the level of expression of their protein products. In addition, the level of expression of an individual protein within a cell, organism or tissue does not necessarily reflect the activity of the protein.
2) The proteome is **highly cell specific**: different cells express different subsets of the total protein complement of an organism.
3) The proteome is **highly dynamic**, with the subset of proteins expressed changing from moment to moment according to the developmental and physiological state of the cell.

## VI. Mass spectrometry-based methods for protein identification

Mass spectrometry has been a significant driving technology in the formation of the field of proteomics.

*The accurate mass of a molecule is particularly attractive because it is highly constraining and can be determined with great accuracy, rapidly and sensitively by mass spectrometry.

*MS became more compatible with the analysis of biopolymers, proteins, nucleic acids and carbohydrates with the introduction of two ‘soft’ ionization methods: **electrospray ionization** (ESI) and **matrix-assisted laser desorption/ionization** (MALDI). These methods are referred to as “soft” because they do not degrade the molecule during the ionization process.

*Mass spectrometers can be described as consisting of three components: (1) an ionization source such as MALDI or electrospray; (2) a mass filter such as time-of-flight (TOF), quadrupole, or ion trap (IT); and (3) a detector. The three commercially available types of instruments which are most commonly used for protein and peptide analysis are the MALDI-TOF mass spectrometer, the ESI-triple quadrupole (TQ) mass spectrometer and the ESI-IT mass spectrometer.

*There are **two main approaches** to the integration of mass spectrometry into a proteomics program:

1) **Peptide fingerprinting** using MALDI-TOF mass (matrix-assisted laser desorption/ionization-time of flight) spectrometry is approached for established databases of well-known species.
2) The **amino acid sequence** must be determined in order to identify proteins that are not present in the genomic and proteomic databases. To accomplish this, researchers have been using quadrupole or electrospray **tandem mass spectrometry** to identify the amino acid sequence of individual peptides.