Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2018

Lecture 19:

Introduction to Separation Methods and Thiol-disulfide Chemistry

13 March 2018

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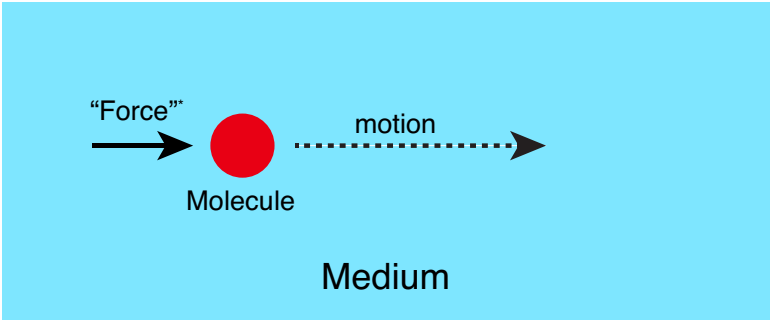
Separation Techniques in Biochemistry

- Isolation of pure components
- Analysis of complex mixtures
- Can be the basis of enzyme assays
- Physical characterization:

  Separation methods generally depend on differences in physical properties of molecules, such as size, shape and charge.

Separation Methods: The General Idea



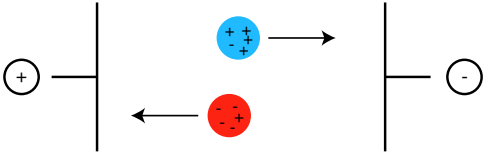
- Something (a "force"*) causes molecules to move through a medium.
- The rate of motion depends on the strength of the force and the interactions of the molecules with the medium.
- Different kinds of molecules move at different rates, allowing them to be separated.

* "Force" is used rather loosely here to describe anything that causes motion of the molecules.

Two Biochemical Separation Methods that We Will Use

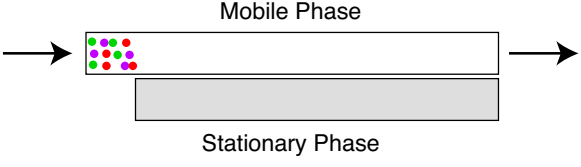
- Electrophoresis

  Charged molecules are subjected to an electric field and move through a medium.

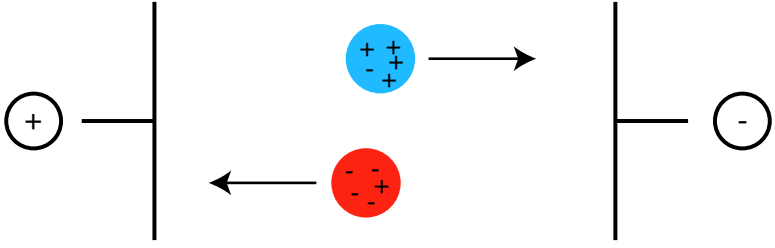


- Chromatography

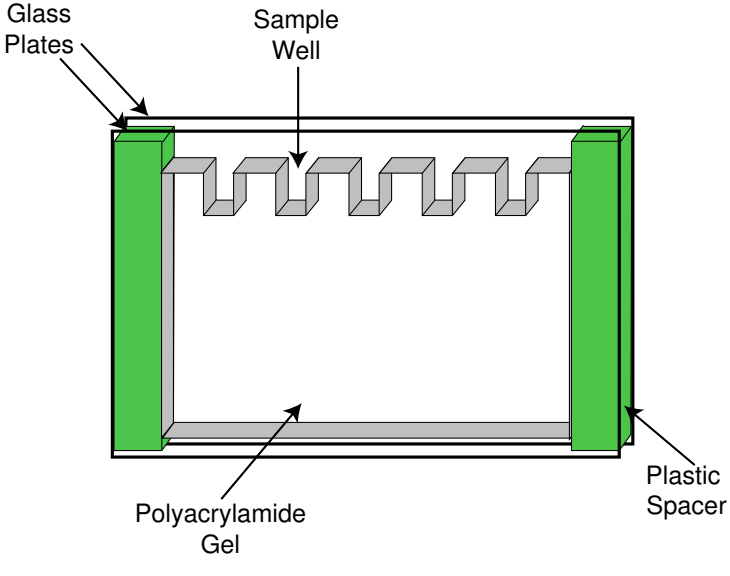
  Molecules are carried by flow of medium in one phase past a second, stationary phase.



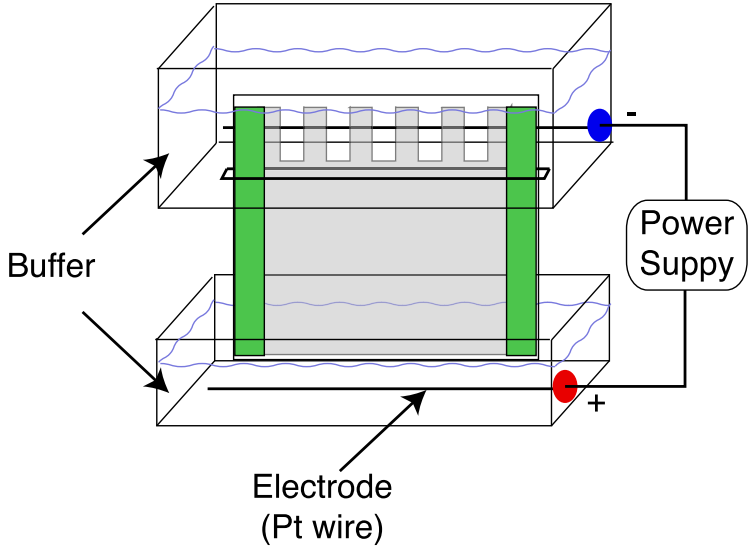
Electrophoresis:
Separation based on movement in an electric field



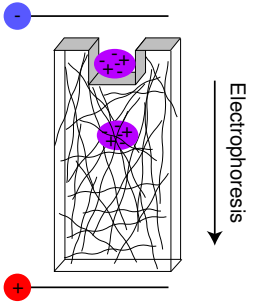
A Gel “Sandwich” for Electrophoresis



Apparatus for Gel Electrophoresis



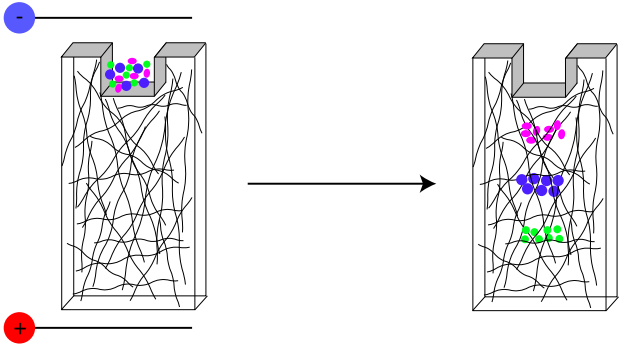
Electrophoresis Through a Gel



Rate of migration through the gel depends on:

- Net charge of protein
- Size and shape of protein.
- Density of gel matrix

Separation of Proteins by Electrophoresis

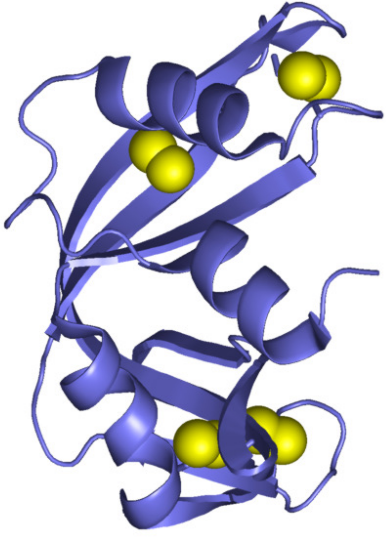


- Proteins with different mobilities migrate as “bands” in the gel.

Two Major Variants of Gel Electrophoresis for Proteins

1. Non-denaturing (“Native”) electrophoresis.
   - Carried out in the absence of denaturants, though sometimes relatively low or high pH values are used.
   - Protein migrates through the gel on the basis of its net charge, size, shape and sieving effect of the gel.
2. SDS gel electrophoresis
   - Proteins are denatured in the presence of sodium dodecyl sulfate (SDS), a detergent that denatures proteins and complexes.
   - Mobilities reflect molecular weights of polypeptide chains.
   - Very useful for analyzing complex samples and macromolecular complexes composed of multiple polypeptides (*e.g.,* viruses, organelles, membranes).

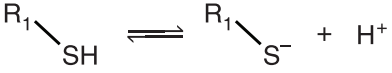
Ribonuclease A: A “Classic” Protein Stabilized by Disulfide Bonds



- Hydrolyzes RNA, much as trypsin hydrolyzes proteins.
- Like trypsin, made in pancreas.
- A favorite protein for chemical, enzymatic and structural studies in the 1950s and 1960s. Two Nobel prizes (4 awardees).
- Produced in large quantities (kilogram) by the Armour Meat Packing Company after World War II, and provided free and without constraints to scientists.
- Close relatives are cytotoxic and are being explored as anti-cancer agents.
- Presence of 4 disulfide bonds allows some neat chemical manipulations of the protein.

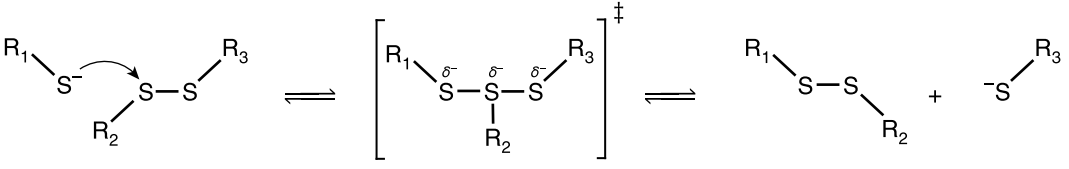
Thiol-disulfide Exchange Chemistry

- Reactive species is the ionized thiol group, a thiolate:



  - Thiol pK_as are about 8–9.
  - Un-ionized thiol is not very reactive.
  - Ionized Cys is the most reactive of all amino-acid side chains.

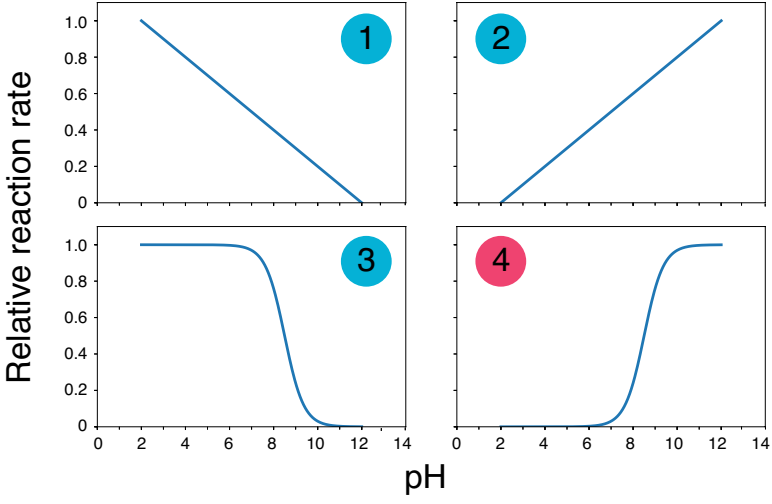
- Exchange reaction:



  - Reaction is an SN2 nucleophilic substitution.
  - For the ionized thiolate, the second-order rate constant is about $20\,s^{-1}M^{-1}$

Clicker Question #1

How does the reaction rate change with pH? (All answers count for now!)

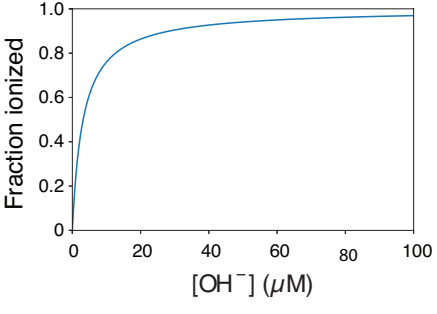


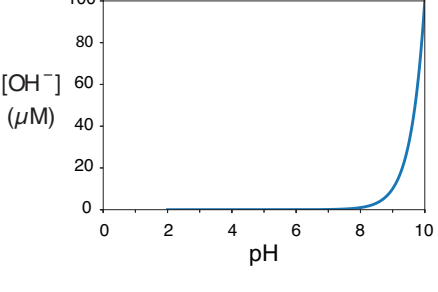
Why Does the Reaction Rate vs. pH Curve Have the Shape it Does?

- The reaction rate is proportional to the fraction of molecules in which the thiol is ionized.

$$f_{\text{ion}} = \frac{[\text{R}-\text{S}^-]}{[\text{R}-\text{S}^-] + [\text{R}-\text{SH}]}$$

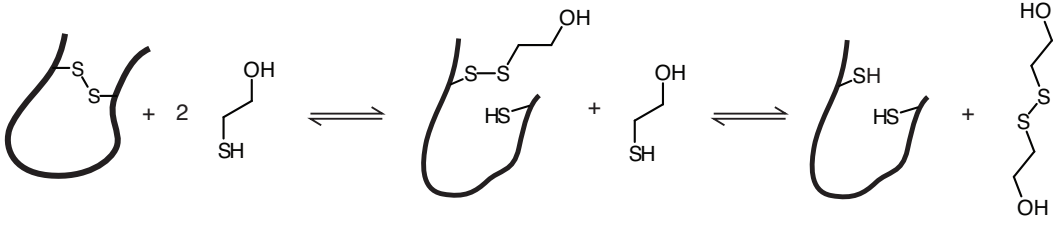
- The fraction ionized increases as the pH increases, and as the concentration of OH^- increases.
- The concentration OH^- increases in proportion to 10^{pH}
- The two curves combine to give a curve that represents the fraction ionized as a function of pH.



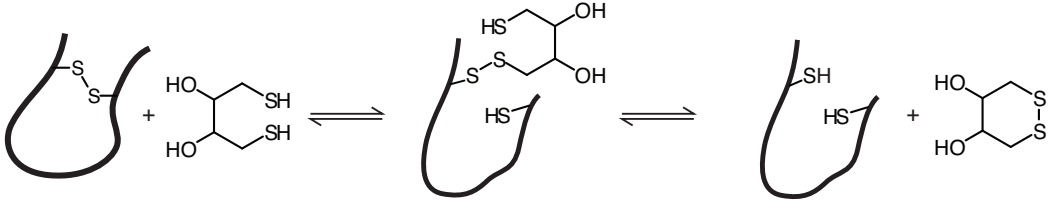


Reduction of Protein Disulfides by Thiol-Disulfide Exchange

- By 2-mercaptoethanol (β-mercaptoethanol, BME)



- With dithiothreitol (DTT, Cleland's reagent)



- Which reaction is faster, at equal reagent concentrations?

Reformation of Disulfides Can be Prevented by Alkylating the Cys Thiols

- Reaction with iodoacetamide

Protein thiol | Iodoacetamide | Carboxyamidomethylated protein thiol

- Reaction with iodoacetic acid

Protein thiol | Iodoacetic acid | Carboxymethylated protein thiol

- Reactions are essentially irreversible.
- Reactive species is ionized thiol (thiolate).

Three Forms of RNase A for Electrophoresis Experiment

1. Native RNAse A (N). Compact, net positive charge.
2. Reduced and carboxyamidomethylated (RCAM). Less compact than native, same net charge as native.
3. Reduced and carboxymethylated (RCM). Less compact than native, decreased positive charge.

How will they behave upon electrophoresis?

Outline of Experiment 5

- Day 1:
  1. Preparation of RCAM and RCM RNase
- Day 2:
  1. Non-denaturing gel electrophoresis of N, RCAM and RCM RNase
  2. Trypsin treatment of RNase A forms
- Day 3:
  1. SDS gel electrophoresis of trypsin-treated RNase A samples.
  2. Image capture of non-denaturing gel
- Day 3+1 (first day of experiment 6):
  1. Image capture and quantitation of SDS gel