Protein Classification, Isolation & Purification

CHEM 420 – Principles of Biochemistry
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Chapter 6: Voet/Voet, *Biochemistry*, 2011
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TABLE 3–4 Conjugated Proteins

Class	*Prosthetic group*	*Example*
Lipoproteins	Lipids	β_1-Lipoprotein of blood
Glycoproteins	Carbohydrates	Immunoglobulin G
Phosphoproteins	Phosphate groups	Casein of milk
Hemoproteins	Heme (iron porphyrin)	Hemoglobin
Flavoproteins	Flavin nucleotides	Succinate dehydrogenase
Metalloproteins	Iron	Ferritin
	Zinc	Alcohol dehydrogenase
	Calcium	Calmodulin
	Molybdenum	Dinitrogenase
	Copper	Plastocyanin

Protein Purification: Laboratory Methods

Analytical
Preparative

Characteristic	Procedure
Solubility	1. Salting in 2. Salting out
Ionic Charge	1. Ion exchange chromatography 2. Electrophoresis 3. Isoelectric focusing
Polarity	1. Adsorption chromatography 2. Paper chromatography 3. Reverse-phase chromatography 4. Hydrophobic interaction chromatography
Molecular Size	1. Dialysis and ultrafiltration 2. Gel electrophoresis 3. Gel filtration chromatography 4. Ultracentrifugation
Binding Specificity	1. Affinity chromatography

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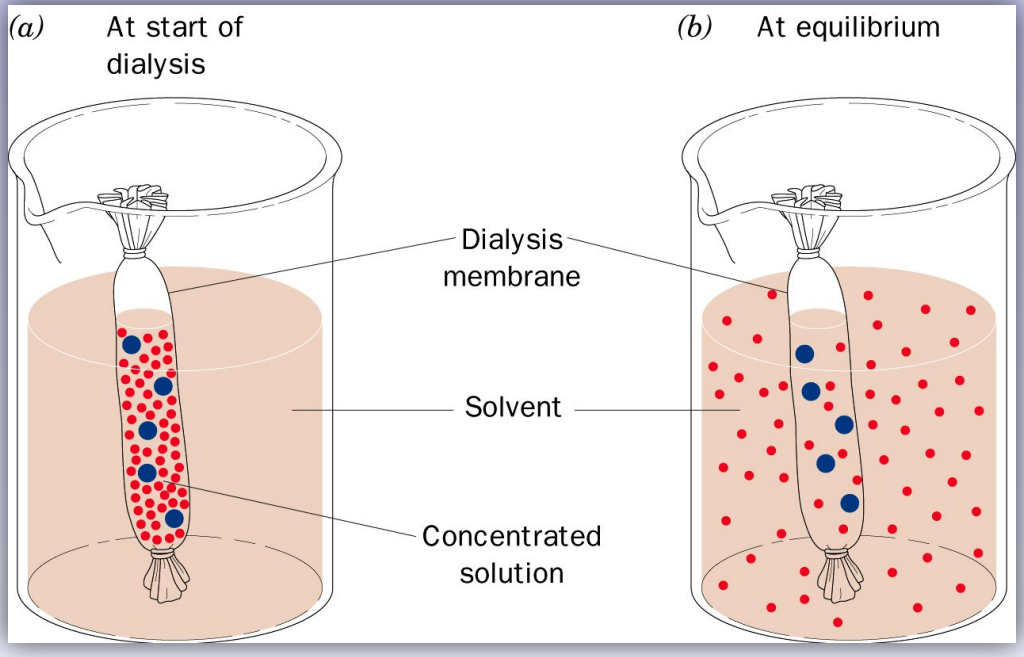
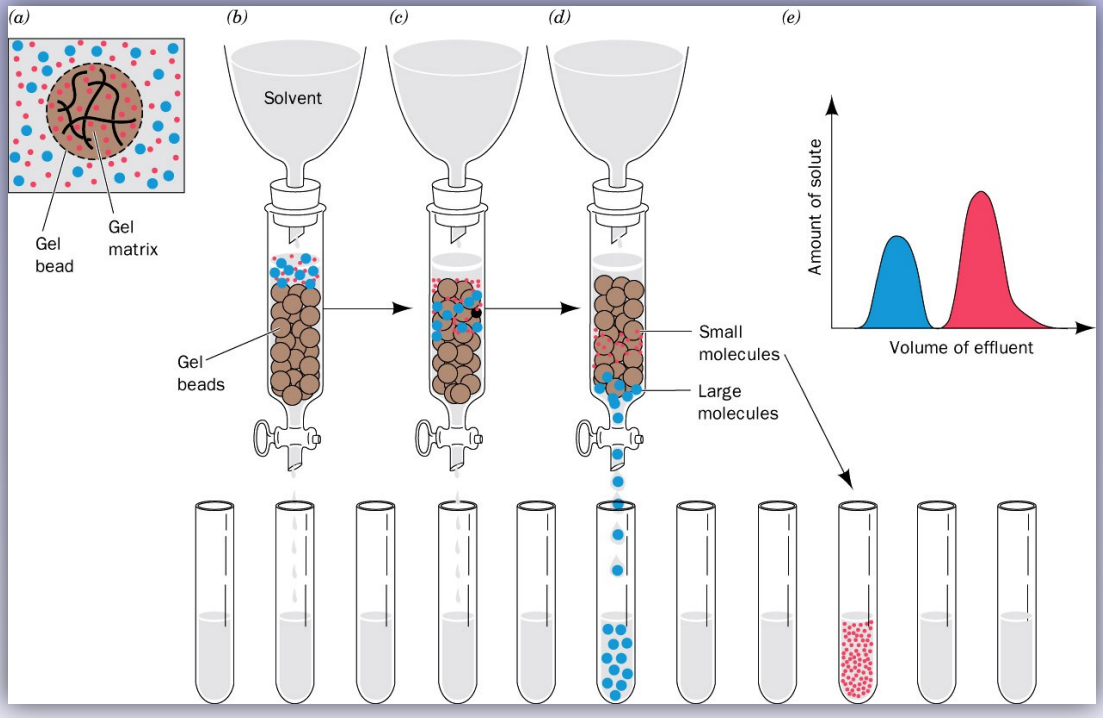
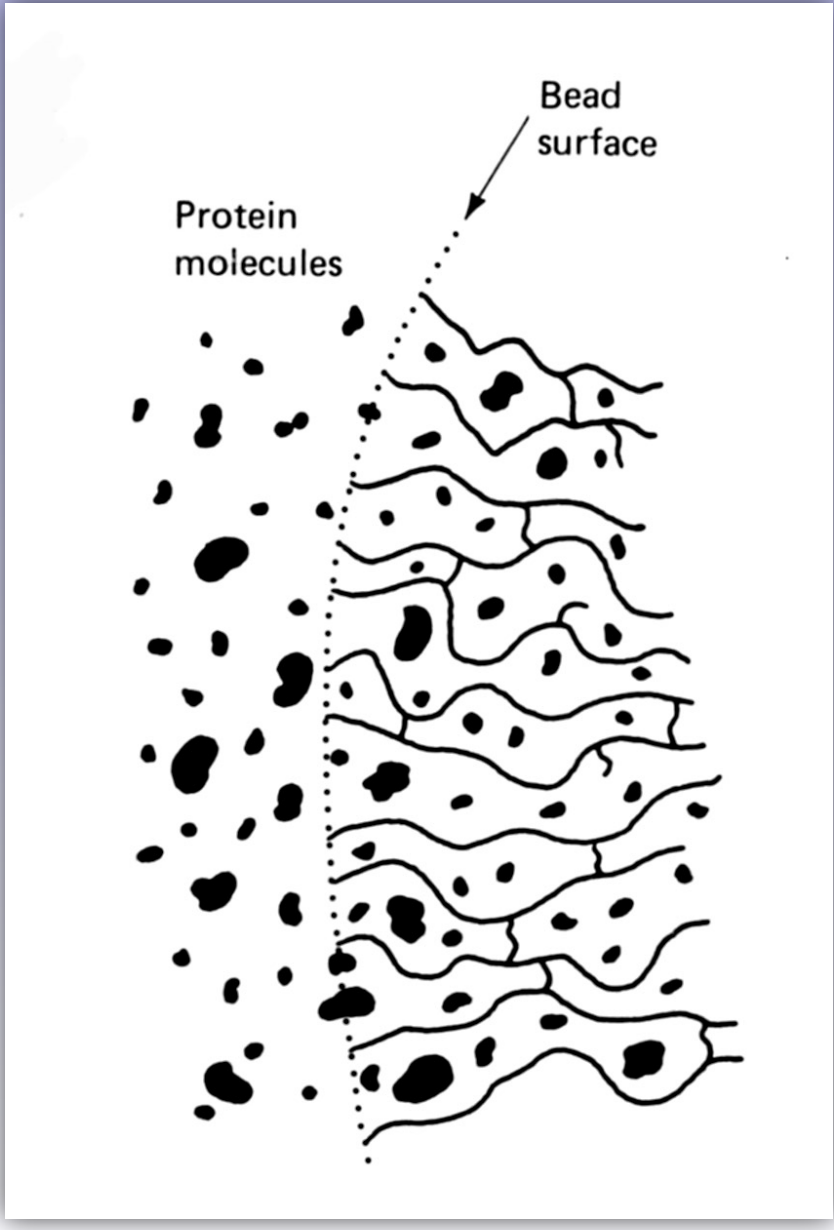
Membrane dialysis



Use of dialysis to separate small and large molecules; commonly used for desalting a protein solution; dialysis tubing can be purchased with different molecular weight cutoffs

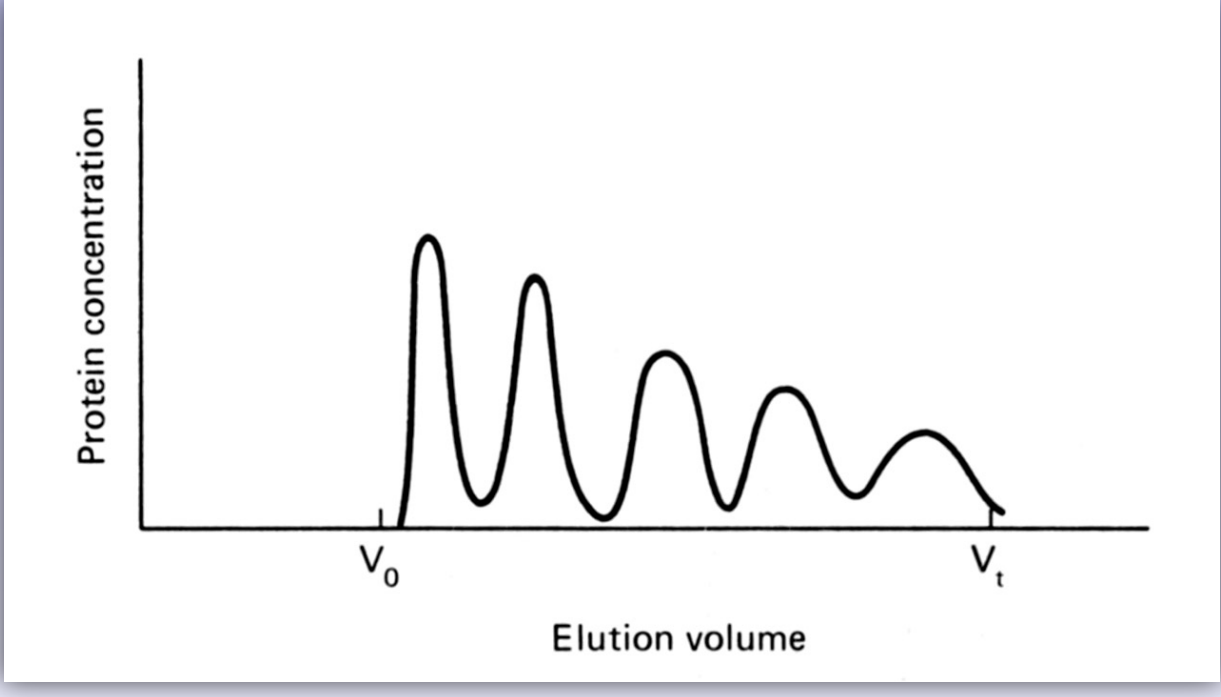


Gel filtration (size-exclusion) chromatography (GFC; SEC). Separation of proteins based predominantly on differences in **molecular mass** (size), with a secondary dependence on **shape**



In SEC, inaccessibility of solutes to the gel may be due to the fact that a particular pore is too narrow for the molecules to pass down, or that even if large enough, there is no channel from the surface by which it can be reached.

Relation between residence time and band width



Ideal separation of five proteins, molecular weights differing each by a factor of 2, on an SE column. The same amount of each protein was applied to the column; the peak heights decrease because of the greater diffusion of the smaller molecules.

SEC: Terms and definitions

exclusion limit: molecular mass of the smallest molecule unable to penetrate the pores of a given gel

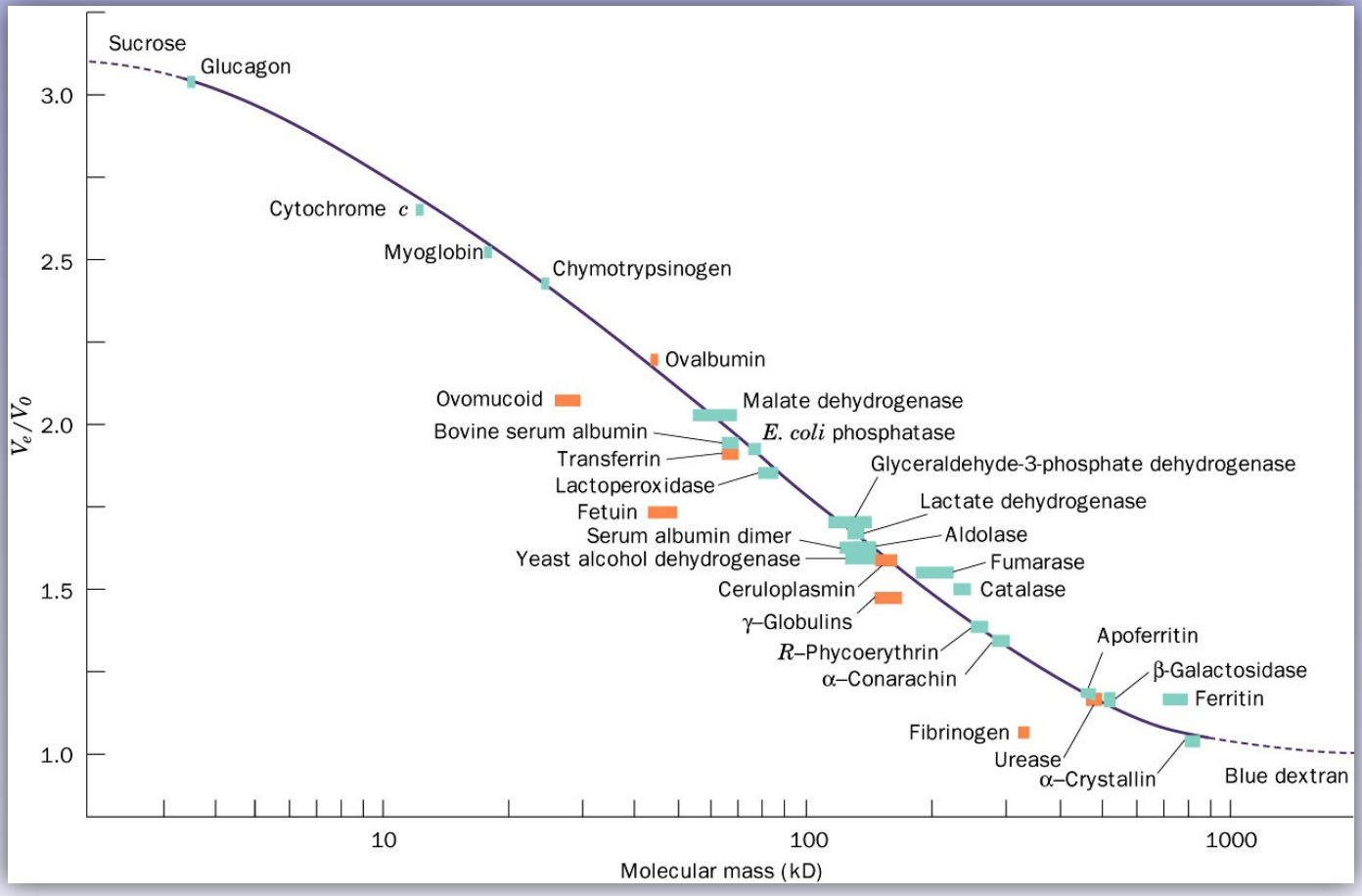
V_o: void volume; volume of solvent space surrounding the gel beads; determined experimentally using a very large standard molecule like blue dextran.

V_x: volume occupied by the gel beads

V_t: total bed volume; equal to $V_x + V_o$

V_e: elution volume; volume of solvent required to elute a solute from the column

V_e/V_o: relative elution volume; independent of the size of the particular column used



A molecular mass determination by SEC; plot of V_e/V_o against log *M* yields a highly "linear" standard curve; *M* is normally determined via HPLC using an array of standard proteins of known molecular mass

On the anomalous elution behavior of fibrinogen from a SE column

Fibrinogen elutes with a V_e/V_o ratio smaller than predicted by the standard V_e/V_o *vs* log *M* curve.

Fibrinogen behaves like a molecule having a higher molecular mass than it does.

Fibrinogen is not a compact, globular protein, but rather is rod-like; the longer dimension dictates its elution behavior. Thus, protein shape also affects elution volume.

Proteins with asymmetric shapes can exhibit anomalous elution behaviors in SEC.

Some commonly used gel filtration media

Table 6-3 Some Commonly Used Gel Filtration Materials

Name[a]	Type	Fractionation Range (kD)
Sephadex G-10	Dextran	0.05–0.7
Sephadex G-25	Dextran	1–5
Sephadex G-50	Dextran	1–30
Sephadex G-100	Dextran	4–150
Sephadex G-200	Dextran	5–600
Sephacryl S-100	Dextran, cross-linked	1–100
Sephacryl S-200	Dextran, cross-linked	5–250
Sephacryl S-300	Dextran, cross-linked	4–150
Sephacryl S-400	Dextran, cross-linked	20–8000
Bio-Gel P-2	Polyacrylamide	0.1–1.8
Bio-Gel P-6	Polyacrylamide	1–6
Bio-Gel P-10	Polyacrylamide	1.5–20
Bio-Gel P-30	Polyacrylamide	2.5–40
Bio-Gel P-100	Polyacrylamide	5–100
Sepharose 6B	Agarose	10–4,000
Sepharose 4B	Agarose	60–20,000
Sepharose 2B	Agarose	70–40,000

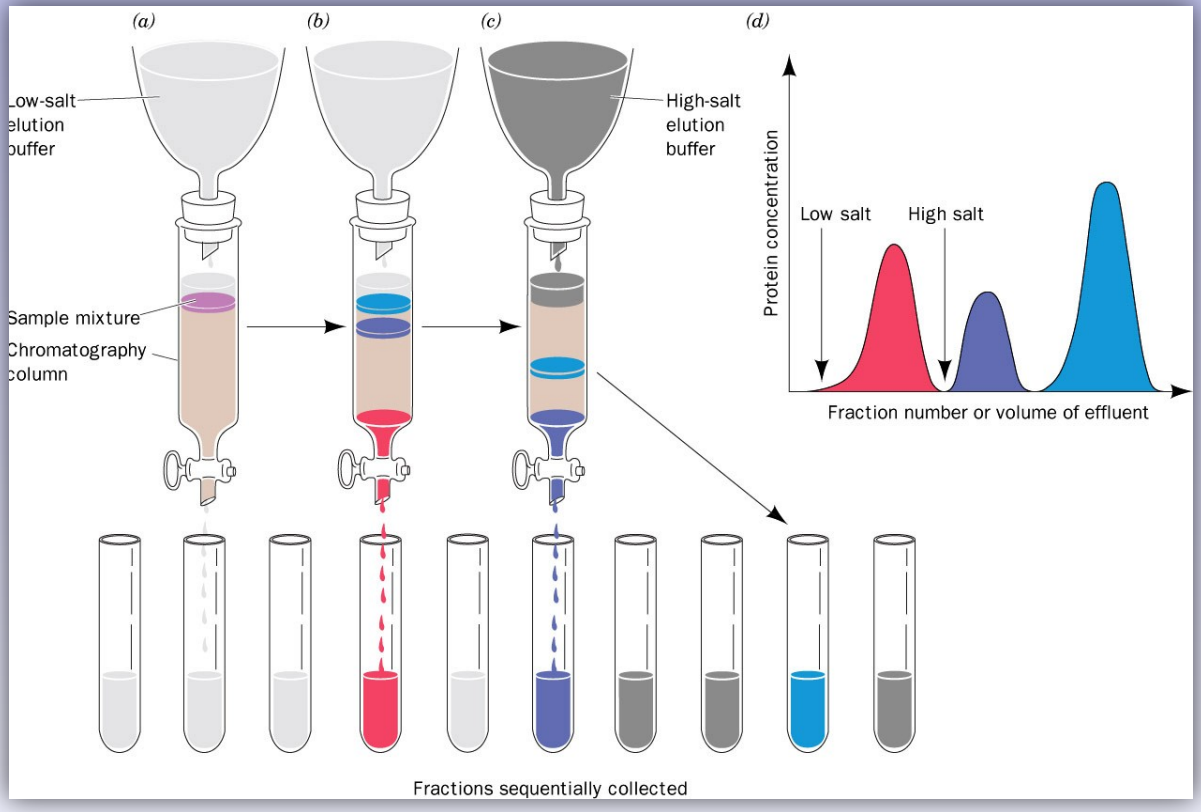
[a]Sephadex, Sephacryl, and Sepharose are products of GE Healthcare; Bio-Gel gels are products of BioRad Laboratories.

Table 6-3

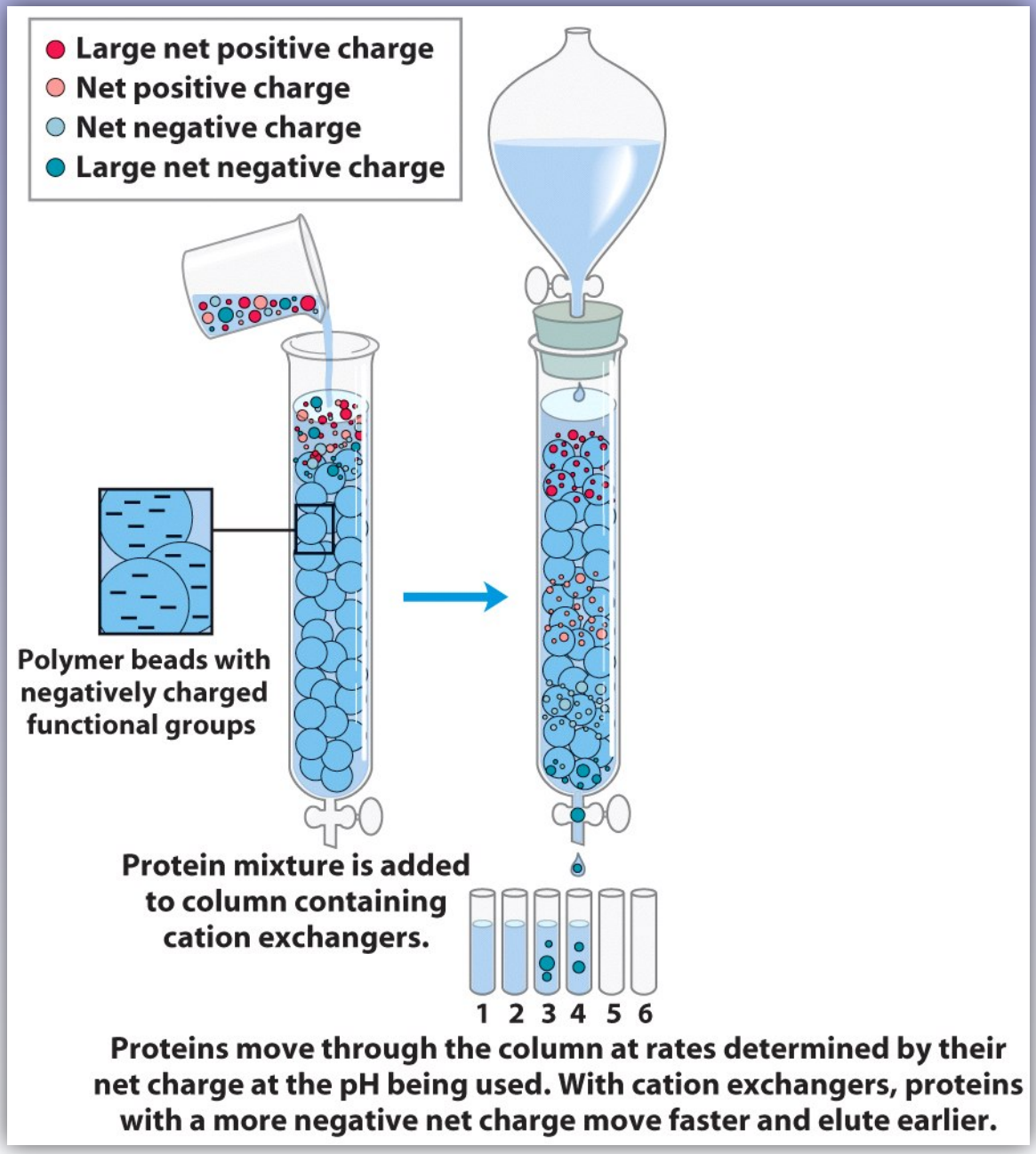
Table 6-1 Isoelectric Points of Several Common Proteins

Protein	Isoelectric pH
Pepsin	<1.0
Ovalbumin (hen)	4.6
Serum albumin (human)	4.9
Tropomyosin	5.1
Insulin (bovine)	5.4
Fibrinogen (human)	5.8
γ-Globulin (human)	6.6
Collagen	6.6
Myoglobin (horse)	7.0
Hemoglobin (human)	7.1
Ribonuclease A (bovine)	7.8
Cytochrome *c* (horse)	10.6
Histone (bovine)	10.8
Lysozyme (hen)	11.0
Salmine (salmon)	12.1

Table 6-1



Ion-exchange chromatography with stepwise elution. Separation of proteins based mainly on differences in their charge properties



Cation-exchange chromatography

The principle of ion-exchange chromatography as applied to a protein

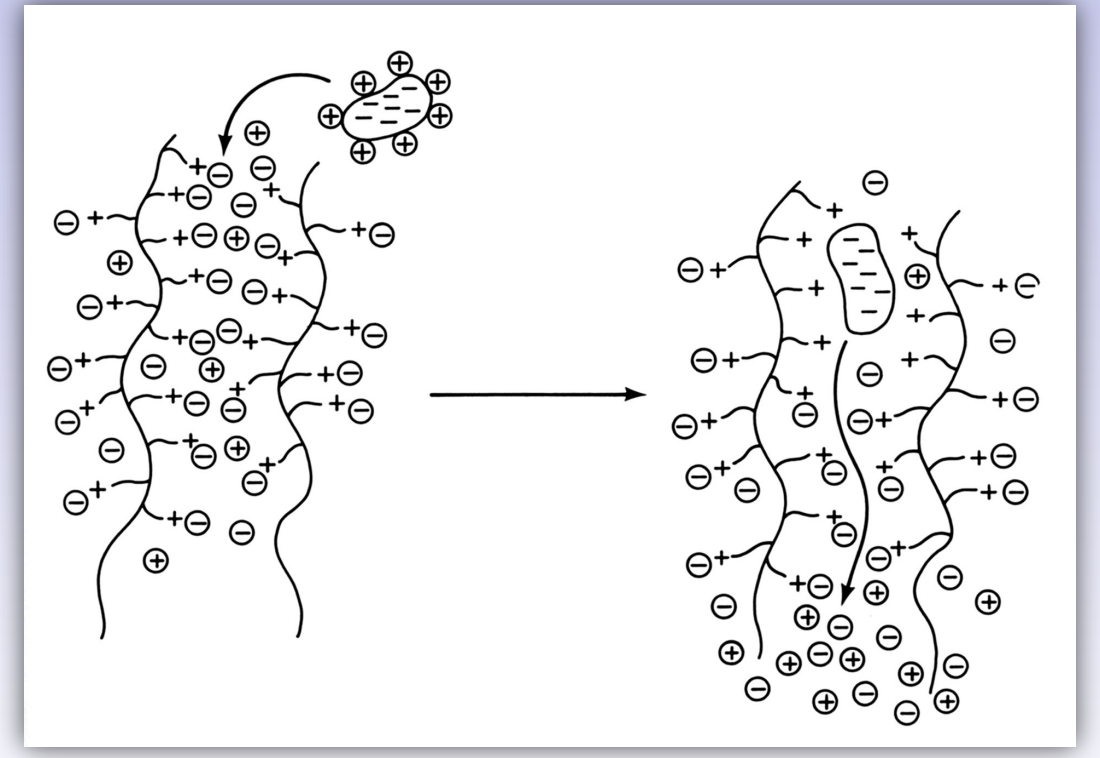
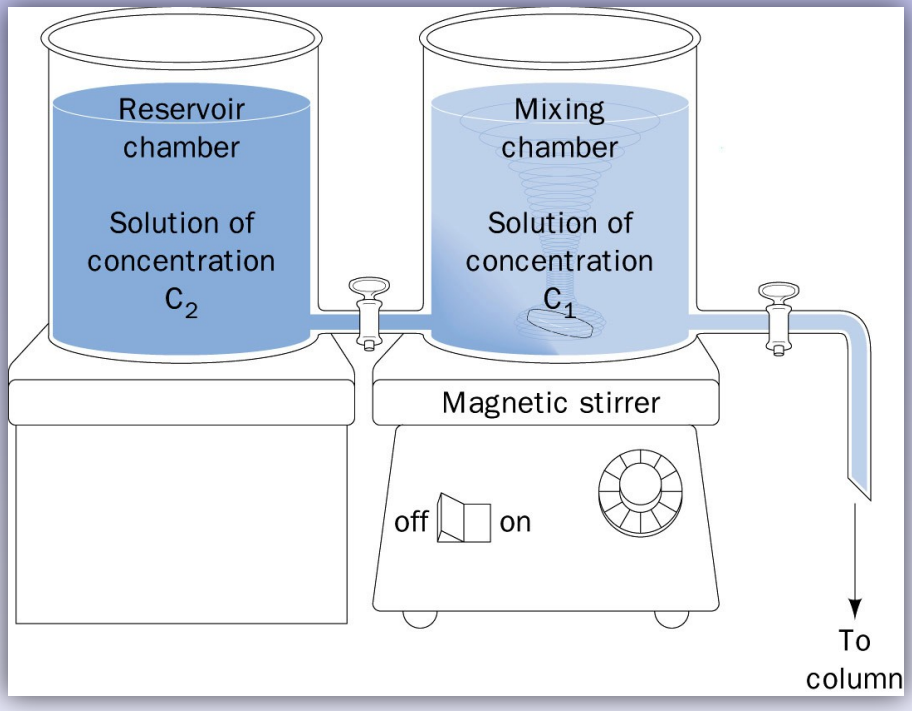
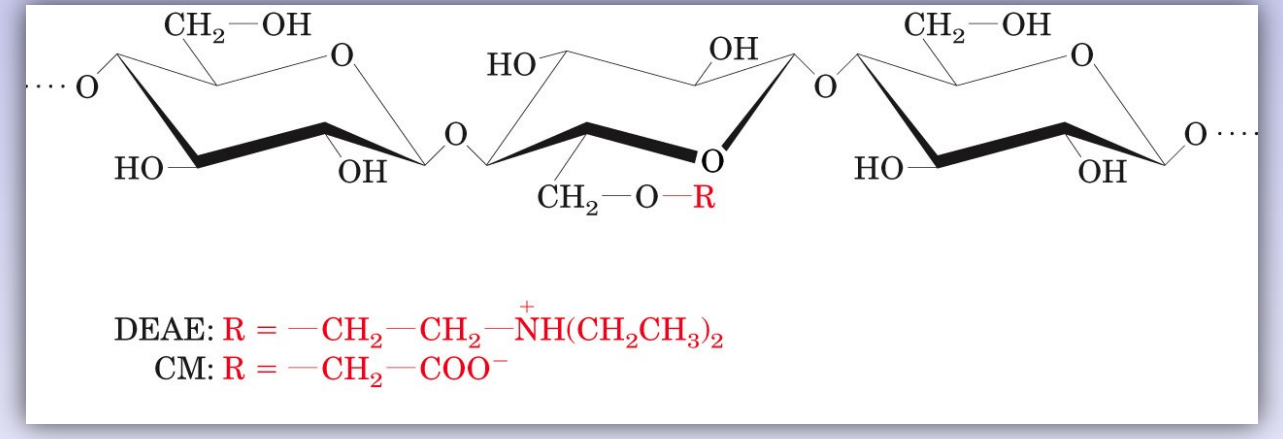


Illustration of the " ion-exchange" that occurs when a negatively charged protein adsorbs to an anion exchange resin. Seven positively charged ions (e.g., $HTris^{+}$) associated with the protein molecule are displaced, together with seven negative ions (Cl^{-}) from the exchange resin.



An alternative to stepwise elution. Experimental setup to generate a linear concentration gradient; non-linear and complex gradients can also generated, usually with an electronic gradient-maker.



Molecular formulas of cellulose-based ion exchangers; no molecular sieving properties

Some biochemically useful ion exchangers. Note combined ion-exchange/gel filtration mode of separation for gel-based exchangers

Name[a]	Type	Ionizable group	Remarks
DEAE-cellulose	Weakly basic	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	Used to separate acidic and neutral proteins
CM-cellulose	Weakly acidic	Carboxymethyl $-CH_2COOH$	Used to separate basic and neutral proteins
P-cellulose	Strongly and weakly acidic	Phosphate $-OPO_3H_2$	Dibasic; binds basic proteins strongly
Bio-Rex 70	Weakly acidic, polystyrene-based	Carboxylic acid $-COOH$	Used to separate basic proteins and amines
DEAE-Sephadex	Weakly basic cross-linked dextran gel	Diethylaminoethyl $-CH_2CH_2N(C_2H_5)_2$	Combined chromatography and gel filtration of acidic and neutral proteins
SP-Sepharose	Strongly acidic cross-linked agarose gel	Methyl sulfonate $-CH_2SO_3H$	Combined chromatography and gel filtration of basic proteins
CM Bio-Gel A	Weakly acidic cross-linked agarose gel	Carboxymethyl $-CH_2COOH$	Combined chromatography and gel filtration of basic and neutral proteins

[a]Sephadex and Sepharose gels are manufactured by Amersham Pharmacia Biotech, Piscataway, New Jersey; Bio-Rex resins and Bio-Gels are manufactured by BioRad Laboratories, Hercules, California.

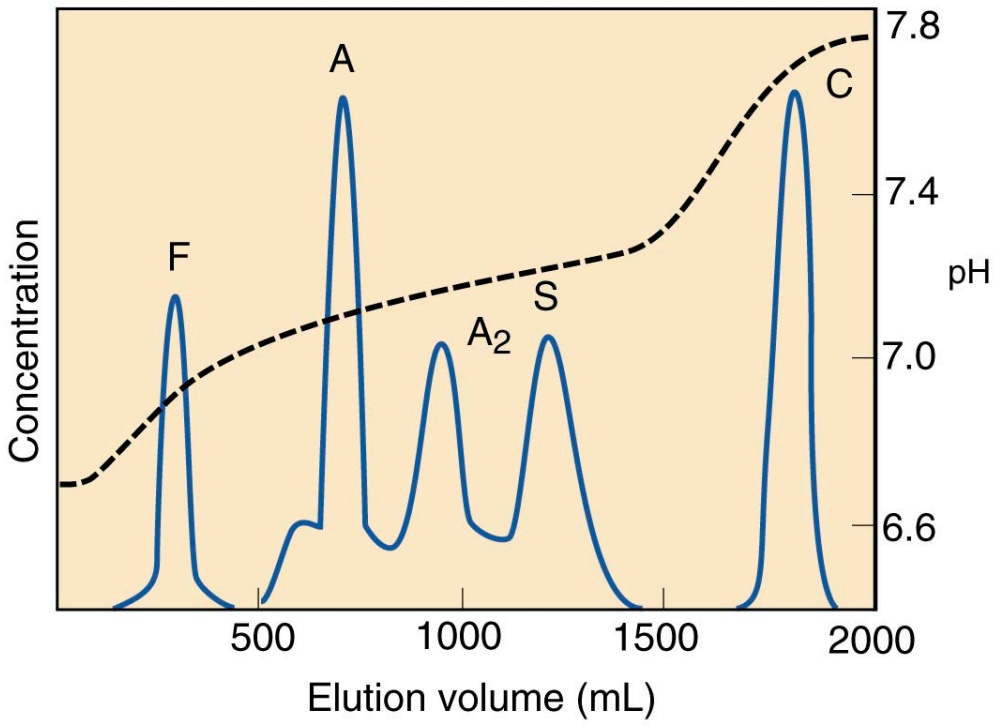
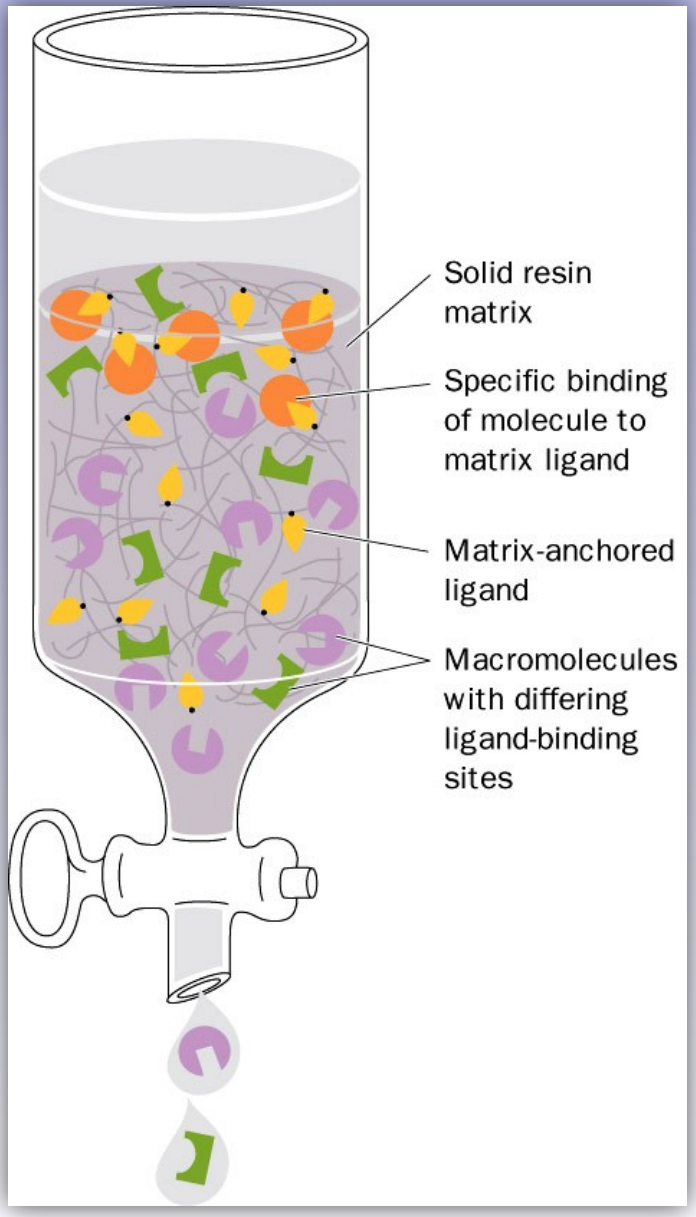
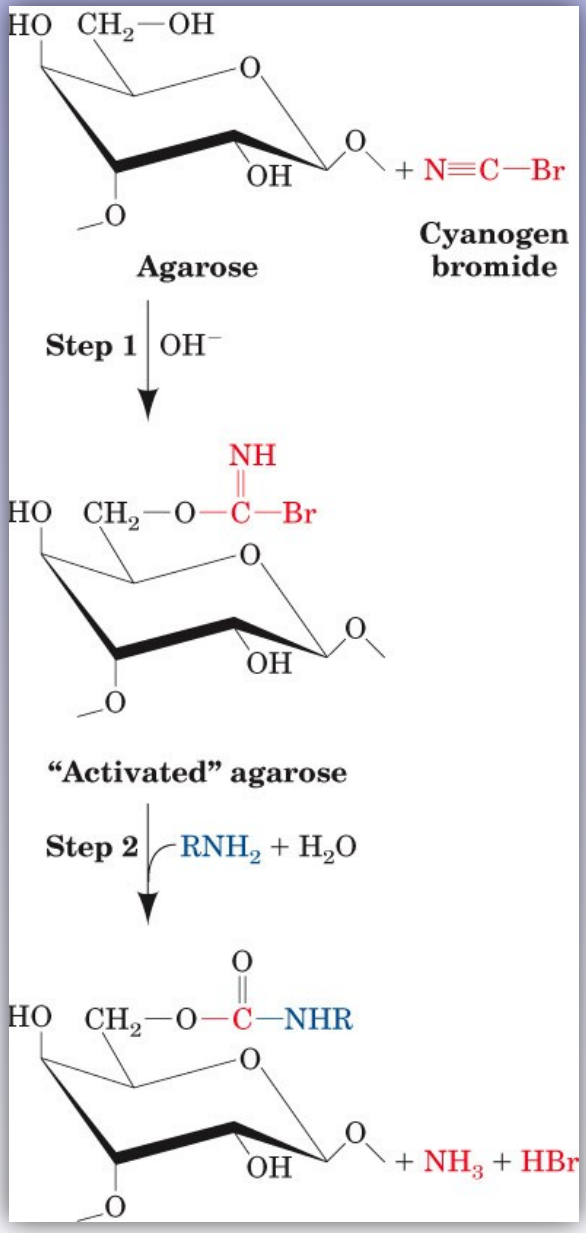


Figure 3.57. Example of ion-exchange chromatography. From Dozy, A. M. and Huisman, T.H. J. *J. Chromatogr.* 40:62, 1969.

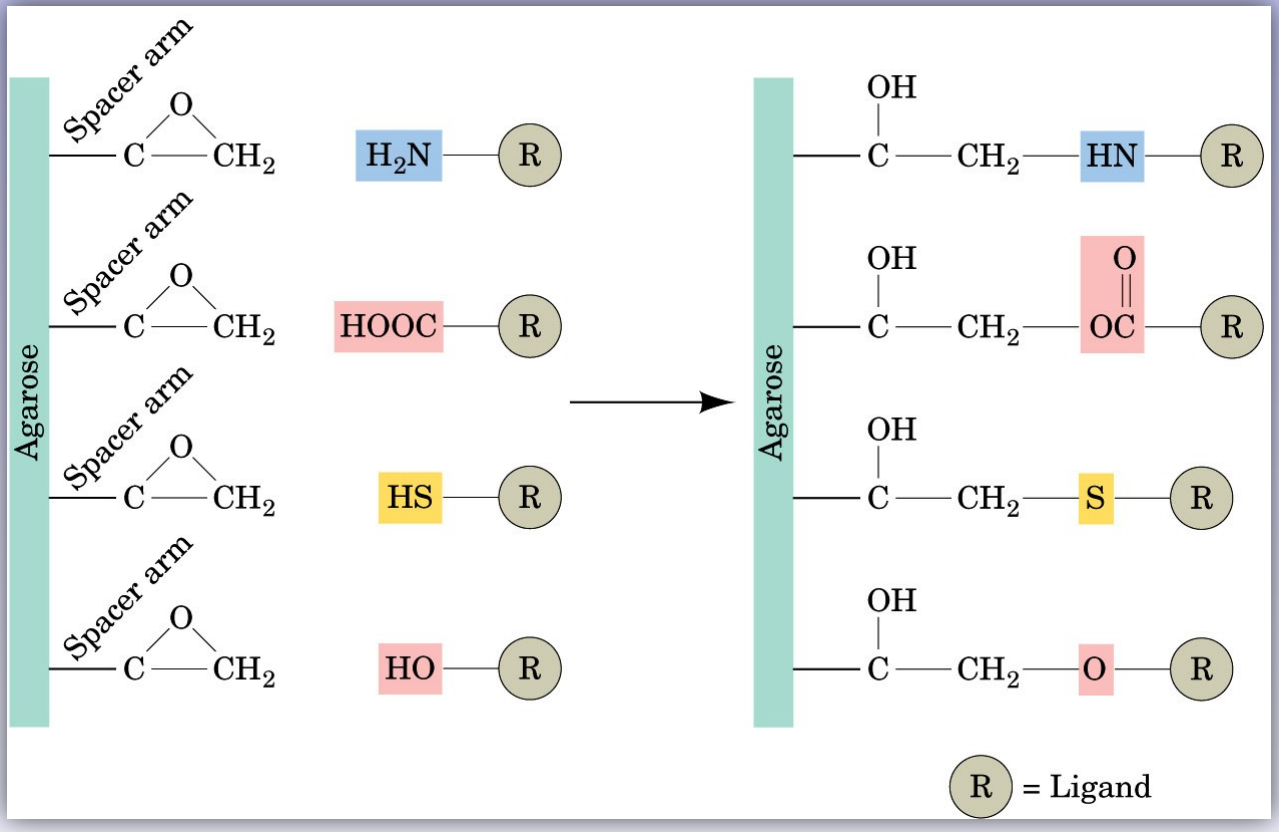
Elution profile:
Mixture of hemoglobin F, A_1, A_2, S and C separated on a carboxymethyl Sephadex C-50 column (cation exchanger with sieving properties) using a pH gradient



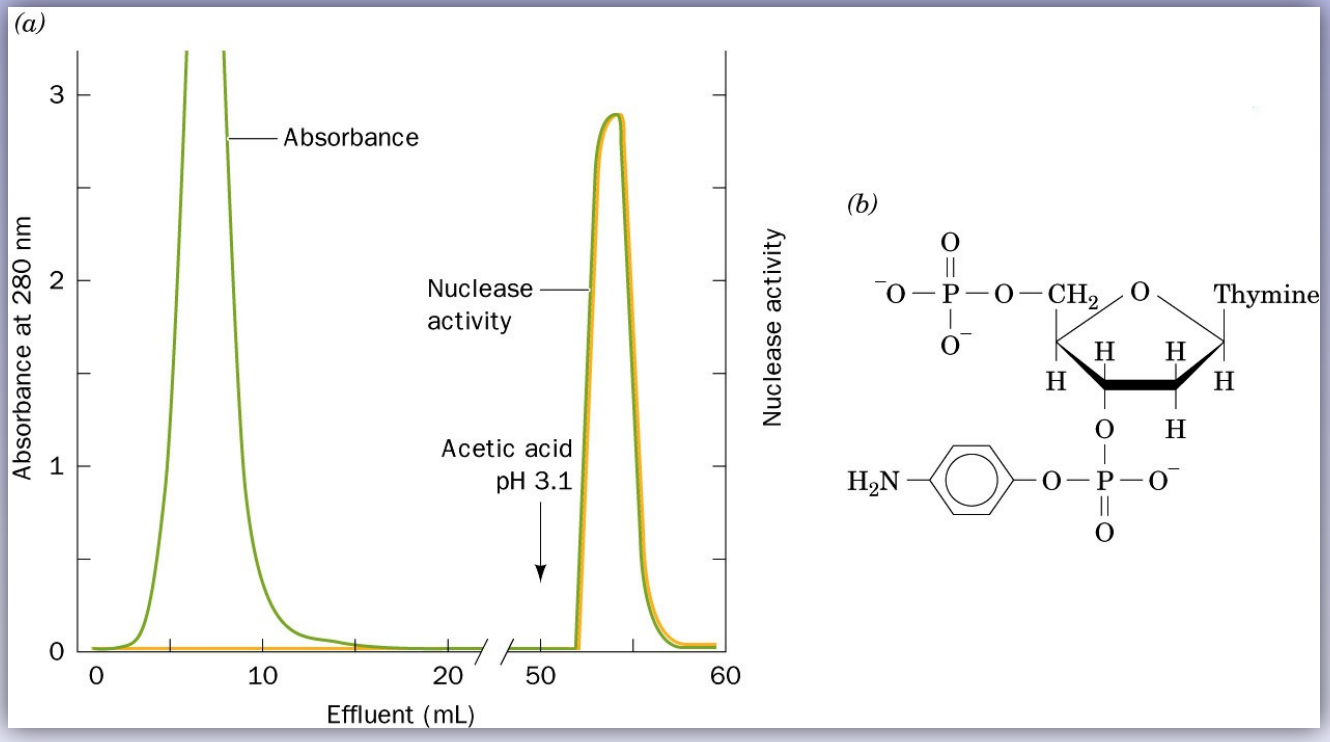
Affinity chromatography: the most powerful protein chromatographic purification method if an effective resin can be produced



Covalent linking of ligand to agarose using cyanogen bromide



Derivatization of epoxy-activated agarose



Purification of staphylococcal nuclease by affinity chromatography on bisphosphothymidine-linked agarose

A purification protocol to isolate/purify rat liver glucokinase

Stage	Specific Activity (nkat · g^{-1})[a]	Yield (%)	Fold[b] Purification
Scheme A: A "traditional" chromatographic procedure			
1. Liver supernatant	0.17	100	1
2. $(NH_4)_2SO_4$ precipitate	*c*	*c*	*c*
3. DEAE-Sephadex chromatography by stepwise elution with KCl	4.9	52	29
4. DEAE-Sephadex chromatography by linear gradient elution with KCl	23	45	140
5. DEAE-cellulose chromatography by linear gradient elution with KCl	44	33	260
6. Concentration by stepwise KCl elution from DEAE-Sephadex	80	15	480
7. Bio-Gel P-225 chromatography	130	15	780
Scheme B: An affinity chromatography procedure			
1. Liver supernatant	0.092	100	1
2. DEAE-cellulose chromatography by stepwise elution with KCl	20.1	104	220
3. Affinity chromatography[d]	**420**	**83**	**4500**

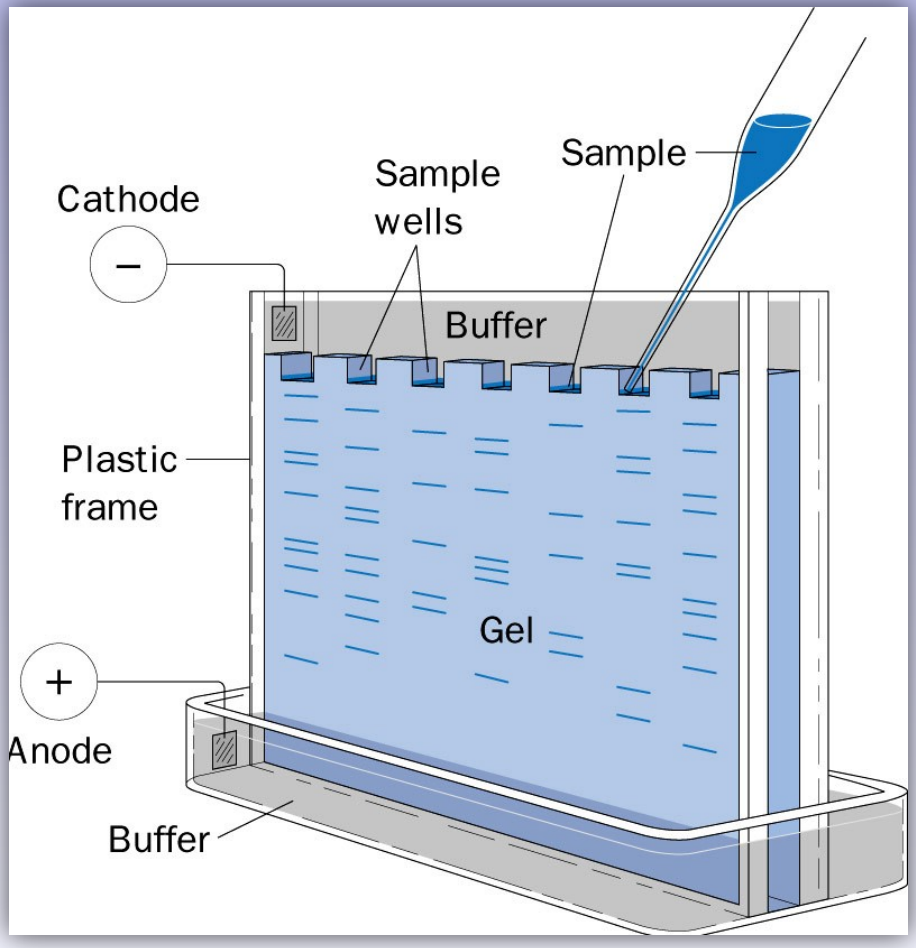
[a] A **katal** (abbreviation **kat**) is the amount of enzyme that catalyzes the transformation of 1 mol of substrate per second under standard conditions. One nanokatal (nkat) is 10^{-9} kat.

[b] Calculated from specific activity; the first step is arbitrarily assigned unity.

[c] The activity could not be accurately measured at this stage because of uncertainty in correcting for contamination by other enzymes.

[d] The affinity chromatography material was made by linking glucosamine (an inhibitor of glucokinase) through a 6-aminohexanoyl spacer arm to NCBr-activated agarose.

Source: Cornish-Bowden, A., *Fundamentals of Enzyme Kinetics, p.* 48, Butterworth (1979), as adapted from Parry, M.J. and Walker, D.G., *Biochem. J.* **99,** 266 (1966) for Scheme A and from Holroyde, M.J., Allen, B.M., Storer, A.C., Warsey, A.S., Chesher, J.M.E., Trayer, I.P., Cornish-Bowden, A., and Walker, D.G., *Biochem. J.* **153,** 363 (1976) for Scheme B.



Apparatus for slab gel electrophoresis

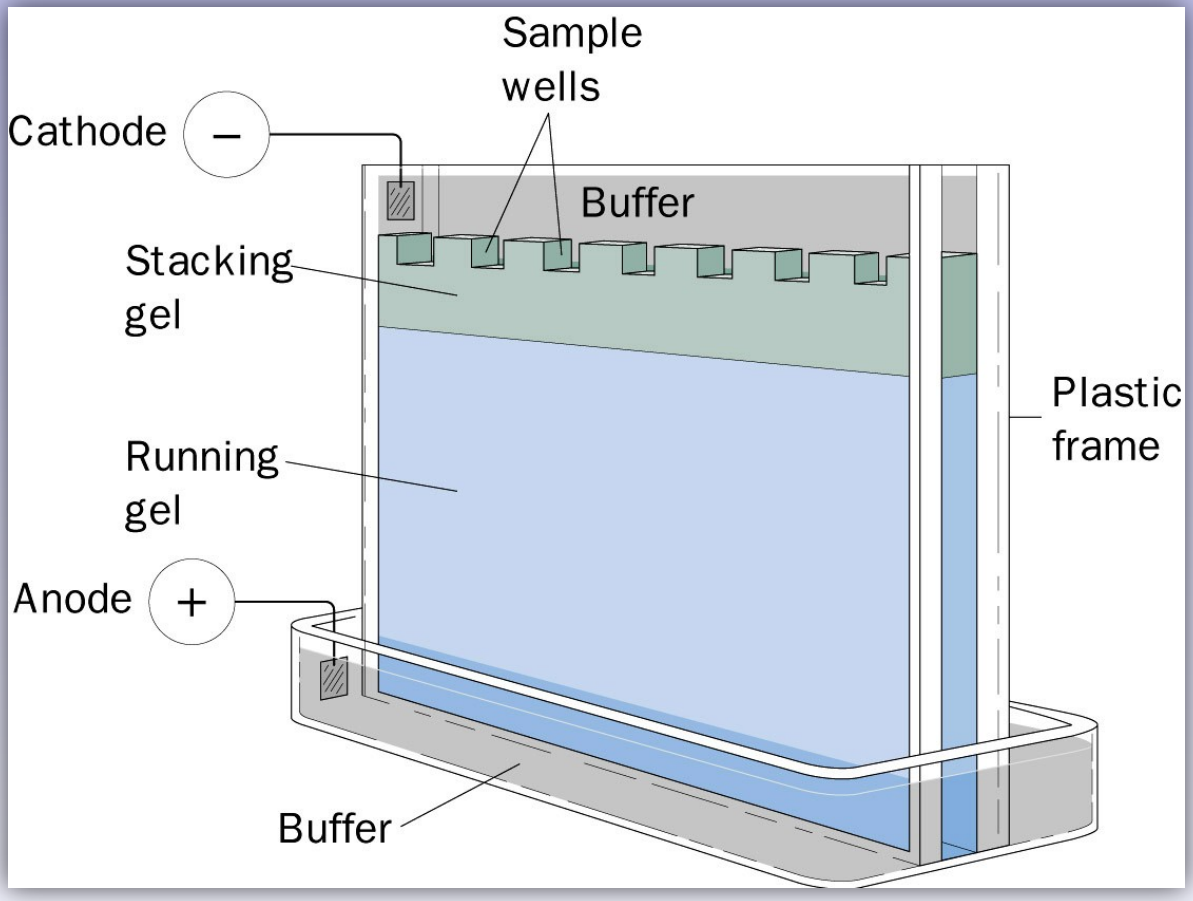
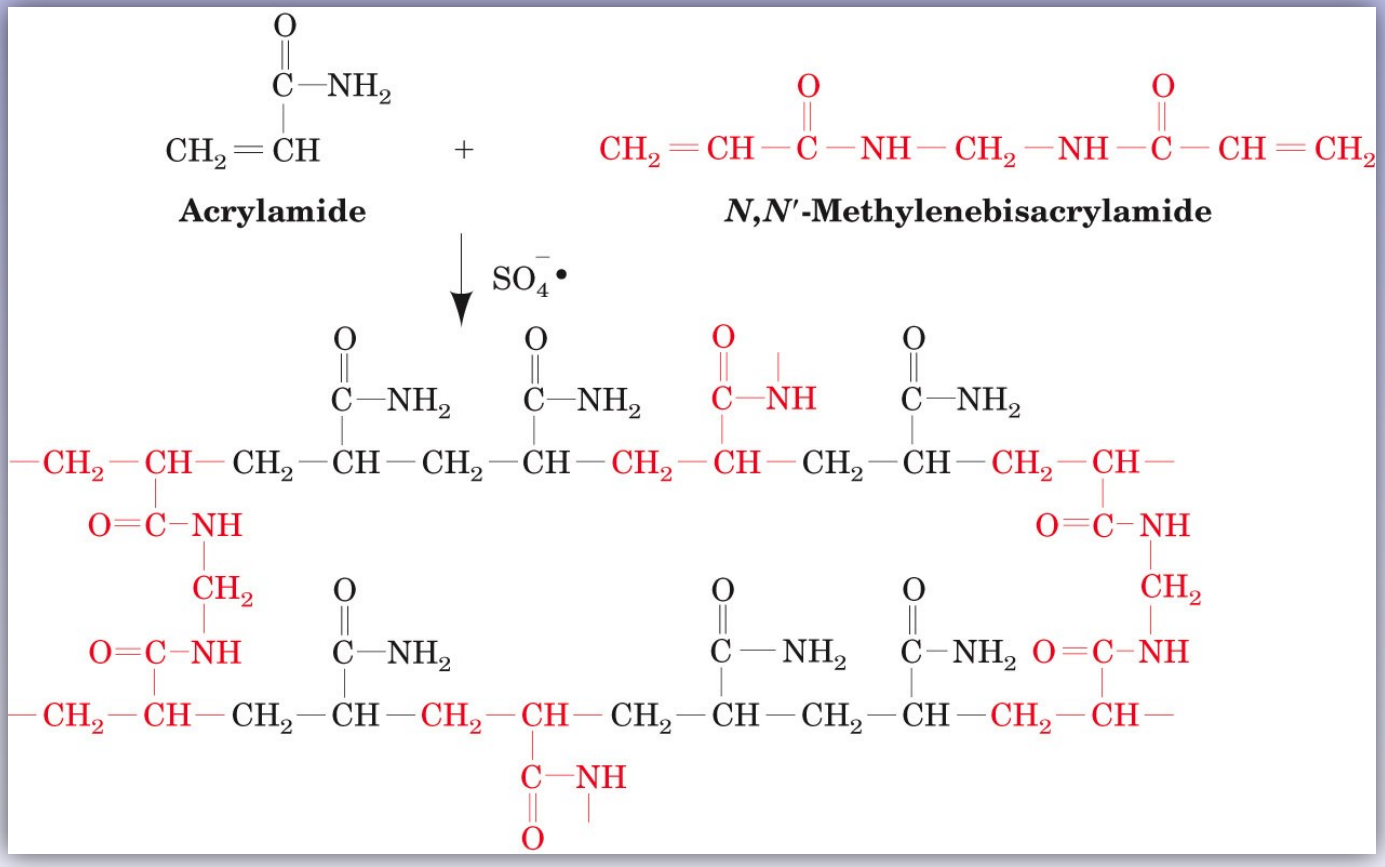


Diagram of a disc electrophoresis apparatus



Polymerization of acrylamide and *N*,*N*′-methylenebisacrylamide to form a cross-linked polyacrylamide gel for electrophoresis (PAGE)

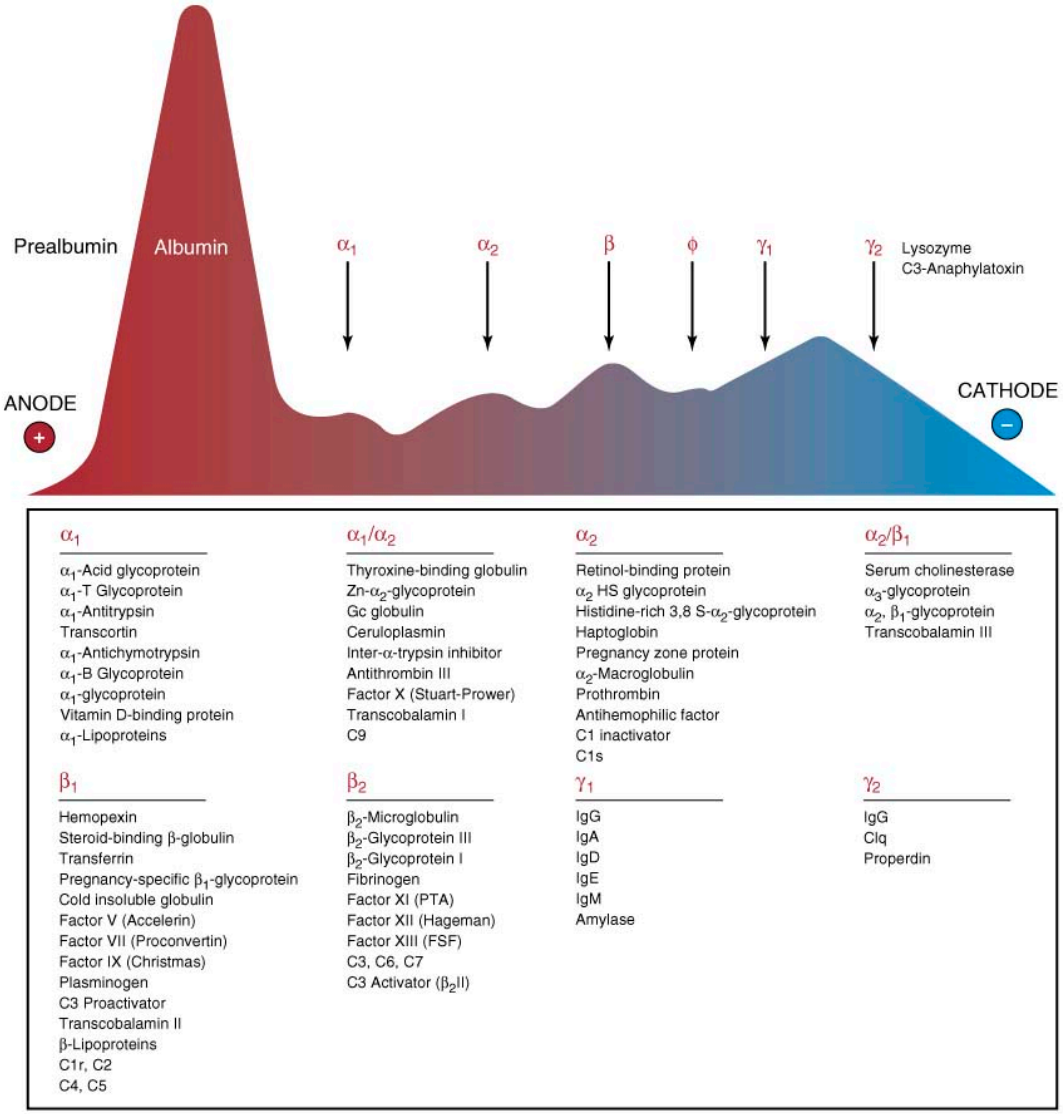
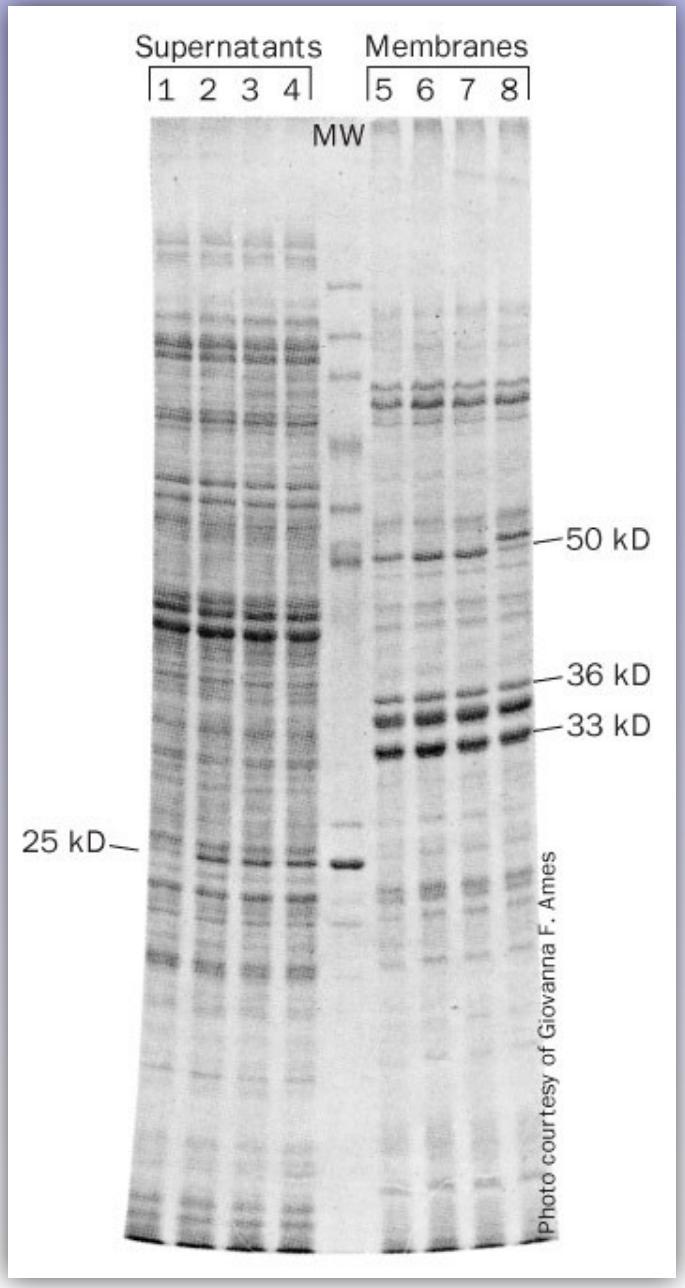


Figure 3.20. Electrophoresis pattern for plasma proteins at pH 8.6. Reprinted with permission from Heide, K., Haupt, H., and Schwick, H. G. In: F. W. Putnam (Ed.), *The Plasma Proteins*, Vol. III, 2nd ed. New York: Academic Press, 1977, p. 545.

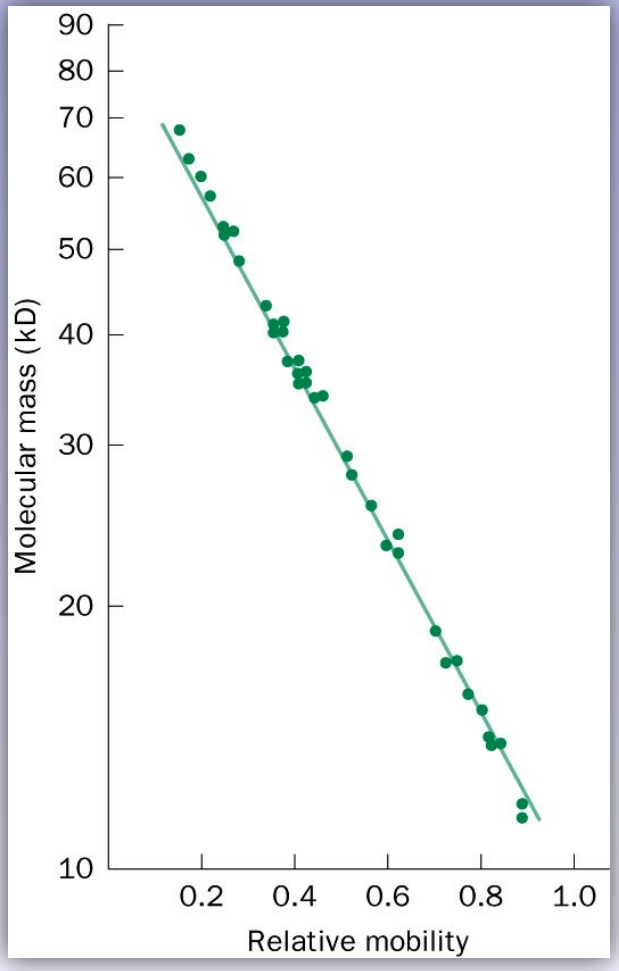


SDS-PAGE

$[CH_3\text{-}(CH_2)_{10}\text{-}CH_2\text{-}O\text{-}SO_3^-]Na^+$

sodium dodecyl sulfate (SDS)

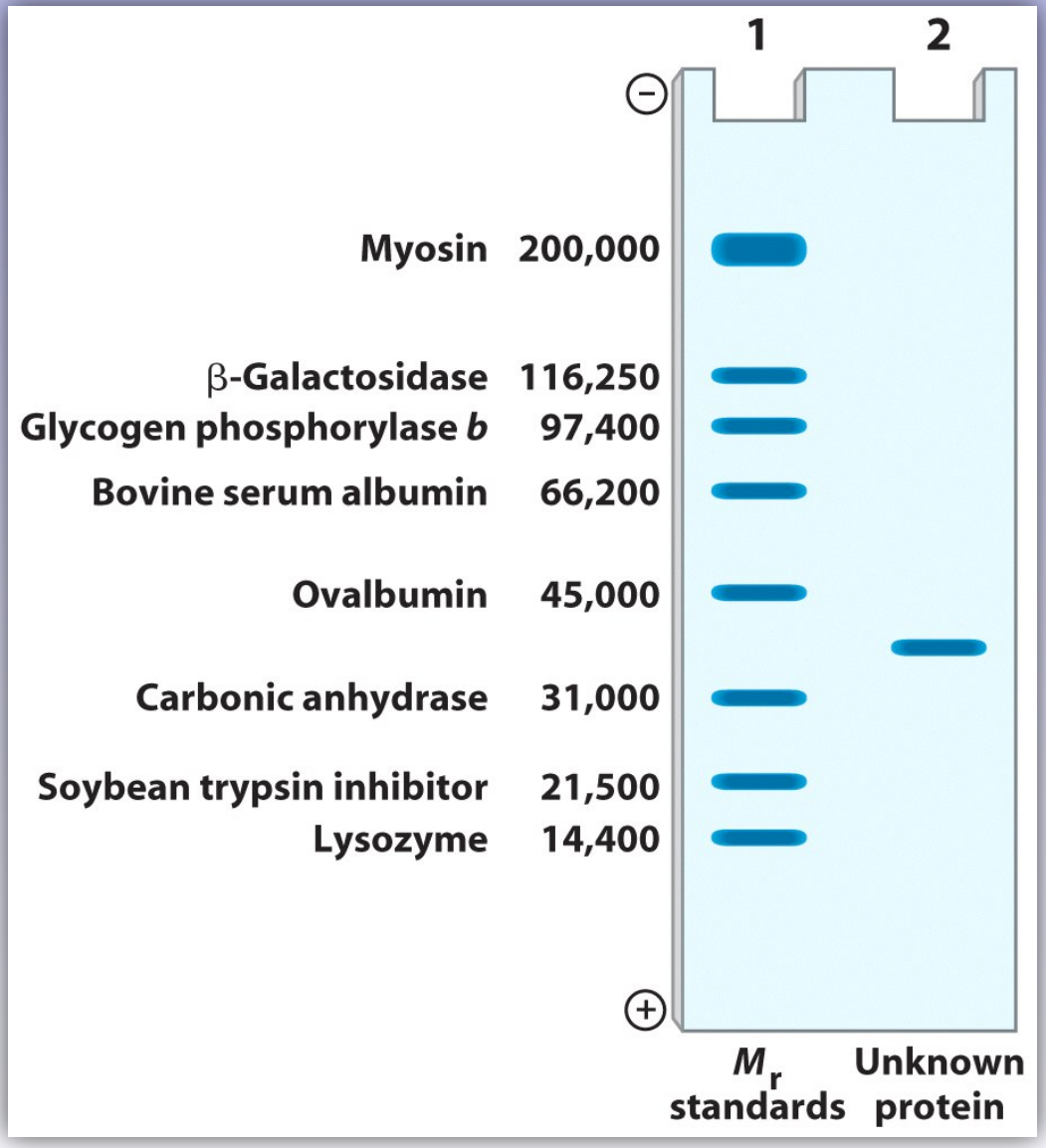
Most proteins bind SDS in the same ratio (1.4 g SDS per gram of protein; about 1 SDS for every 2 amino acid residues). The large (-) charge of SDS masks the protein's intrinsic charge. Thus, SDS-treated proteins have identical charge-to-mass ratios. Electrophoresis of proteins in an SDS-containing gel separates them in order of their molecular masses because of gel filtration effects.



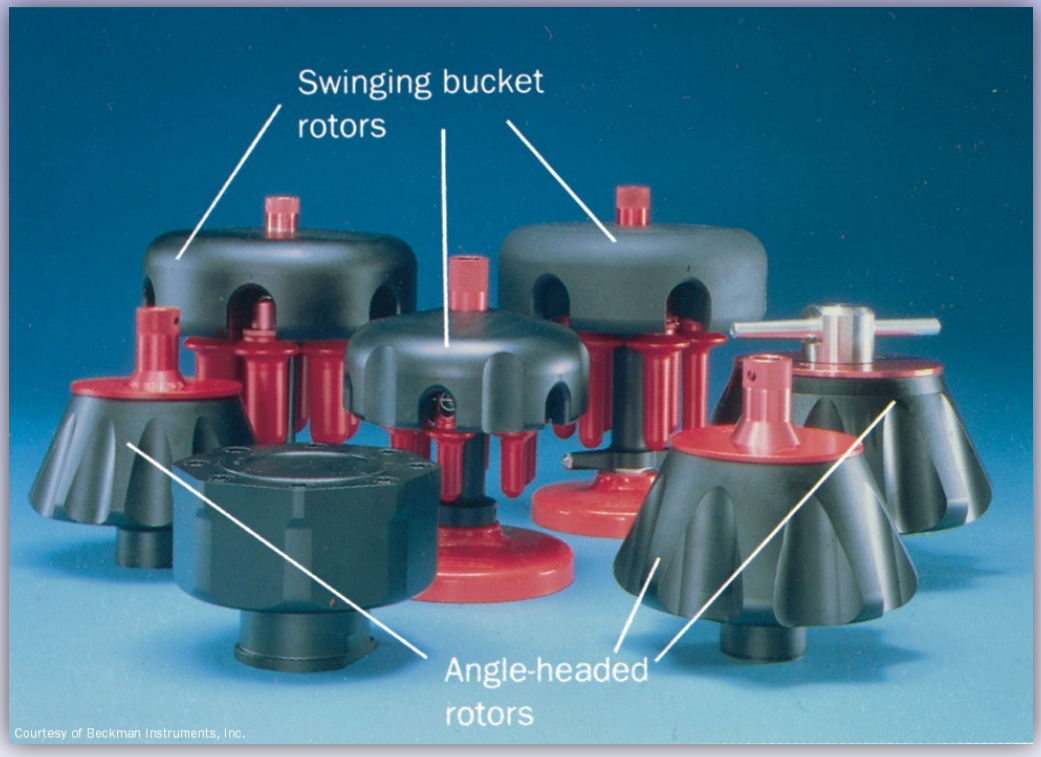
For multi-subunit proteins, SDS-PAGE gives the molecular masses of the protein's subunits rather than of the intact native protein; SDS disrupts non-covalent interactions between the subunits.

Logarithmic relationship between the molecular mass of a protein and its relative electrophoretic mobility in SDS-PAGE

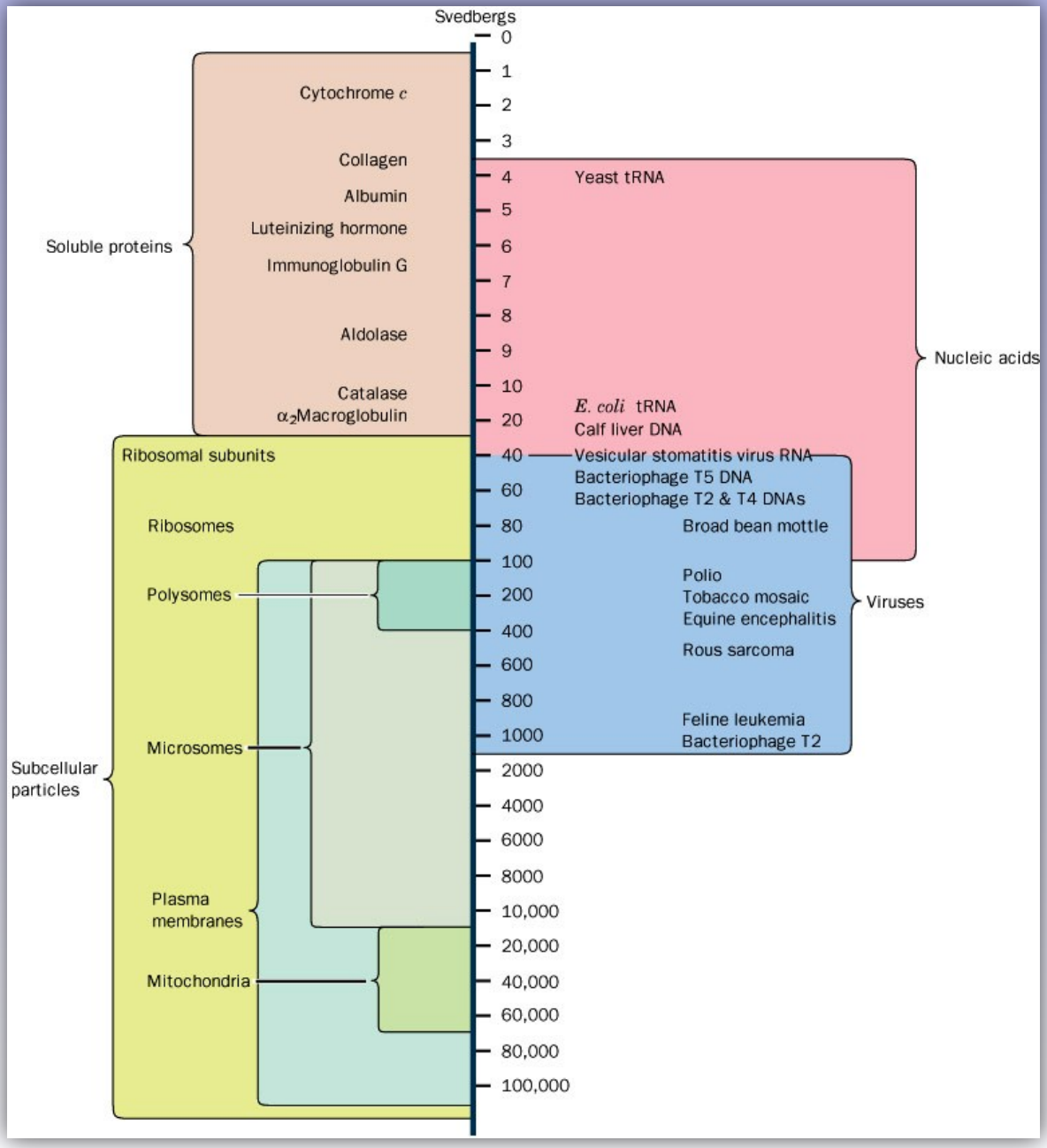
An example of SDS PAGE to determine polypeptide mass



Ultracentrifugation



A selection of preparative ultracentrifuge rotors

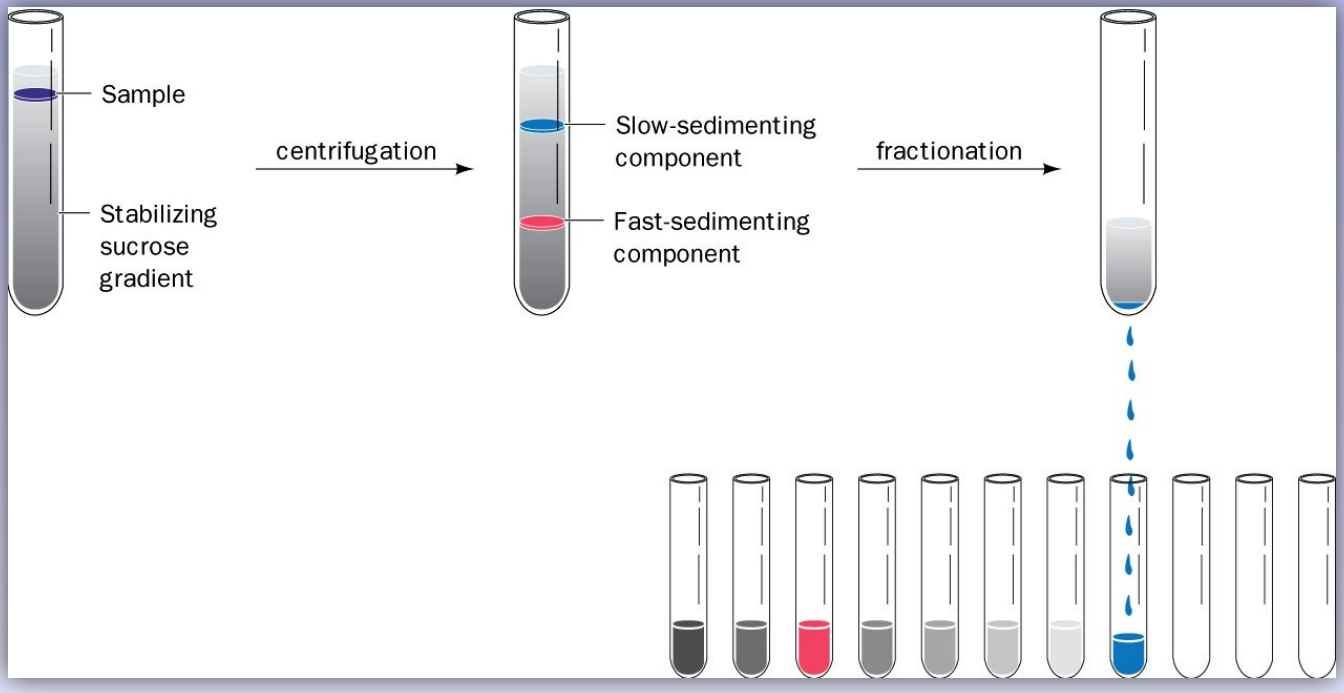


Sedimentation coefficients in Svedbergs (S) for some biological materials

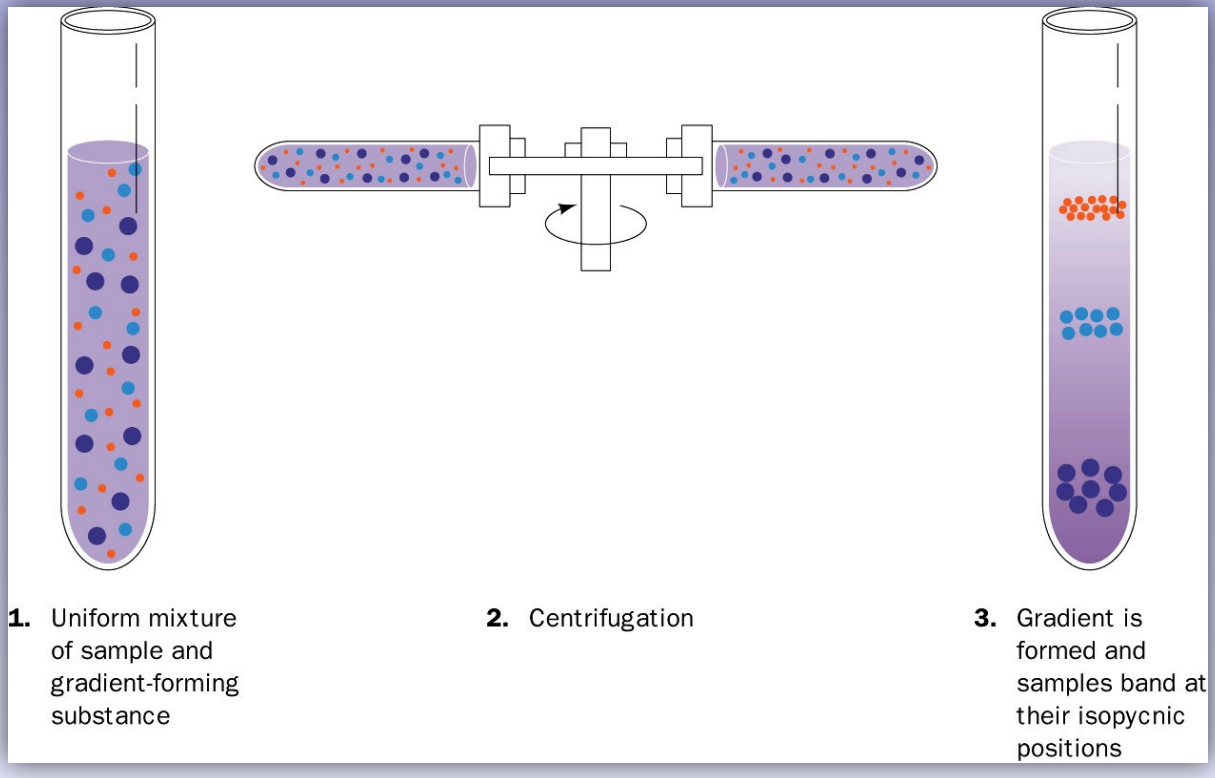
Physical constants of some proteins

Protein	Molecular Mass (kD)	Partial Specific Volume, $\bar{V}_{20,w}$ ($cm^3 \cdot g^{-1}$)	Sedimentation Coefficient, $s_{20,w}$ (S)	Frictional Ratio, f/f_0
Lipase (milk)	6.7	0.714	1.14	1.190
Ribonuclease A (bovine pancreas)	12.6	0.707	2.00	1.066
Cytochrome *c* (bovine heart)	13.4	0.728	1.71	1.190
Myoglobin (horse heart)	16.9	0.741	2.04	1.105
α-Chymotrypsin (bovine pancreas)	21.6	0.736	2.40	1.130
Crotoxin (rattlesnake)	29.9	0.704	3.14	1.221
Concanavalin B (jack bean)	42.5	0.730	3.50	1.247
Diphtheria toxin	70.4	0.736	4.60	1.296
Cytochrome oxidase (*P. aeruginosa*)	89.8	0.730	5.80	1.240
Lactate dehydrogenase H (chicken)	150	0.740	7.31	1.330
Catalase (horse liver)	222	0.715	11.20	1.246
Fibrinogen (human)	340	0.725	7.63	2.336
Hemocyanin (squid)	612	0.724	19.50	1.358
Glutamate dehydrogenase (bovine liver)	1015	0.750	26.60	1.250
Turnip yellow mosaic virus protein	3013	0.740	48.80	1.470

Source: Smith, M.H., *in* Sober, H.A. (Ed.), *Handbook of Biochemistry and Molecular Biology* (2nd ed.), *p.* C-10, CRC Press (1970).



Zonal ultracentrifugation: uses a preformed sucrose density gradient. This method separates similarly shaped macromolecules largely on the basis of their molecular masses (differing sedimentation coefficients).



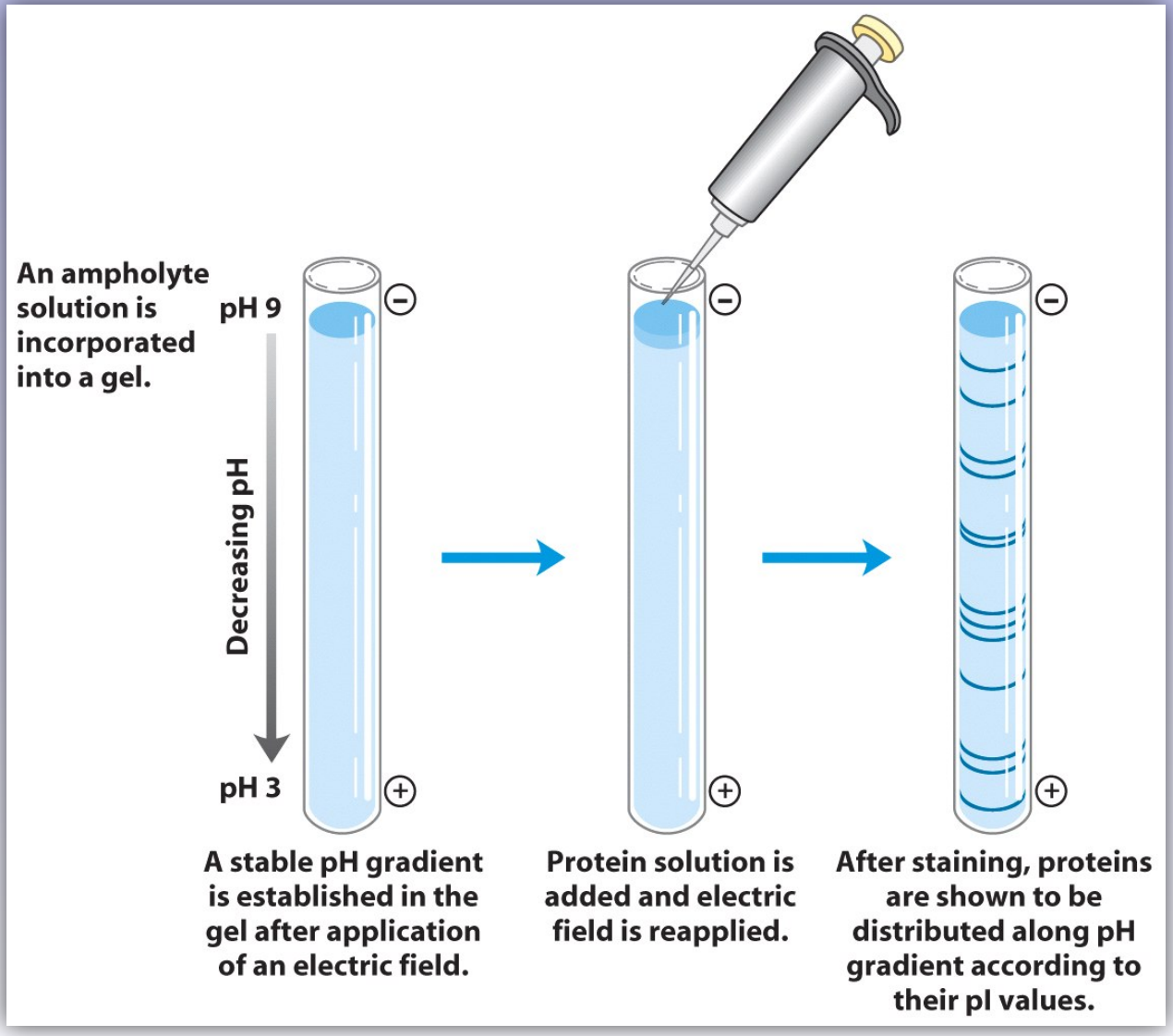
Isopycnic ultracentrifugation (equilibrium density gradient ultracentrifugation): Separates particles according to their densities. CsCl or Cs_2SO_4 solutions are spun at high speed to create a density gradient. Sample components band at positions where their densities equal that of the solution. Used for fractionation of subcellular organelles, not for fractionation of protein mixtures (proteins have similar densities)

Densities of biological material

Material	Density (g/cm^3)
Microbial cells	1.05 - 1.15
Mammalian cells	1.04 - 1.10
Organelles	1.10 - 1.60
Proteins	1.30
DNA	1.70
RNA	2.00

Other analytical techniques for protein purification and assays for homogeneity

Isoelectric focusing



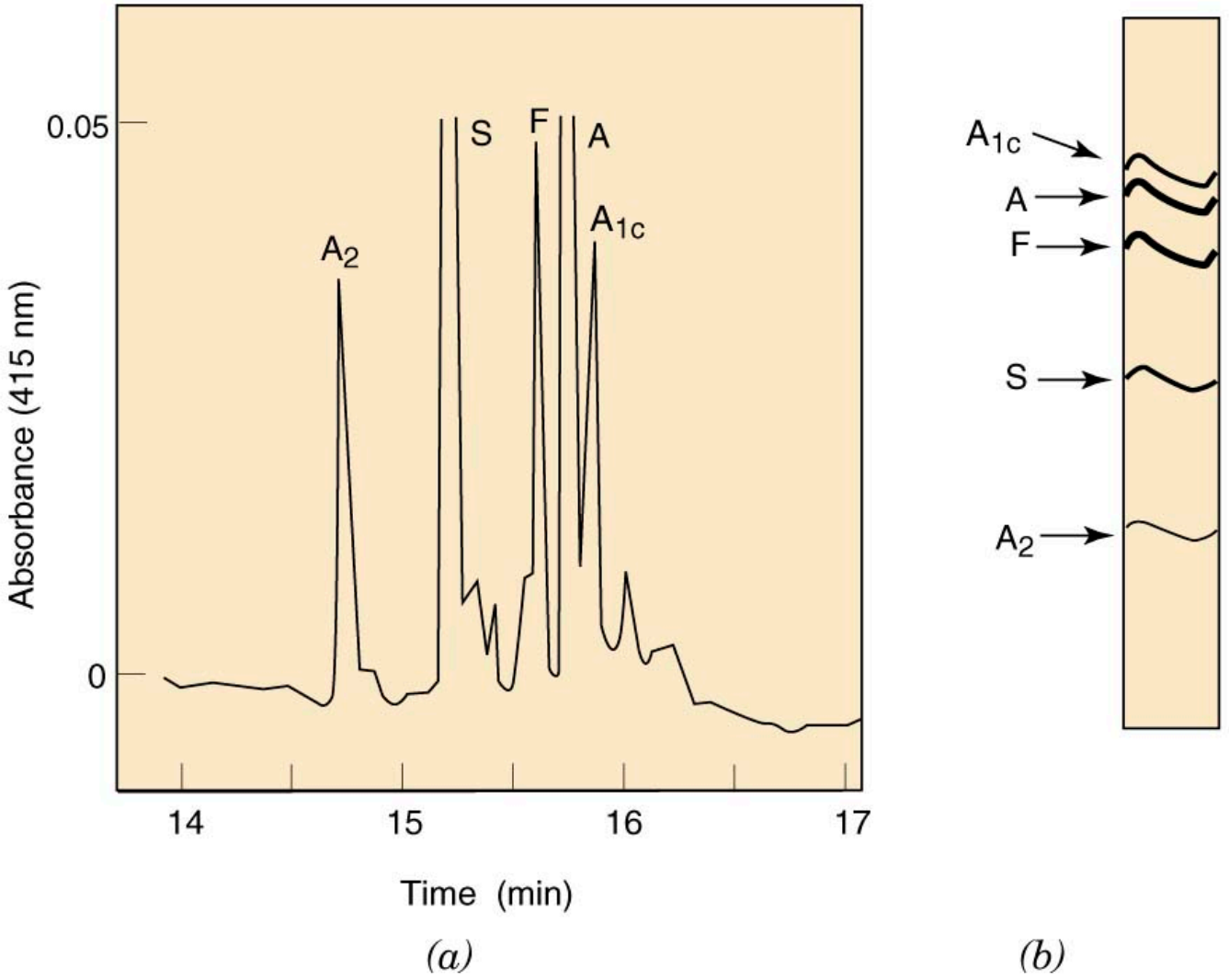
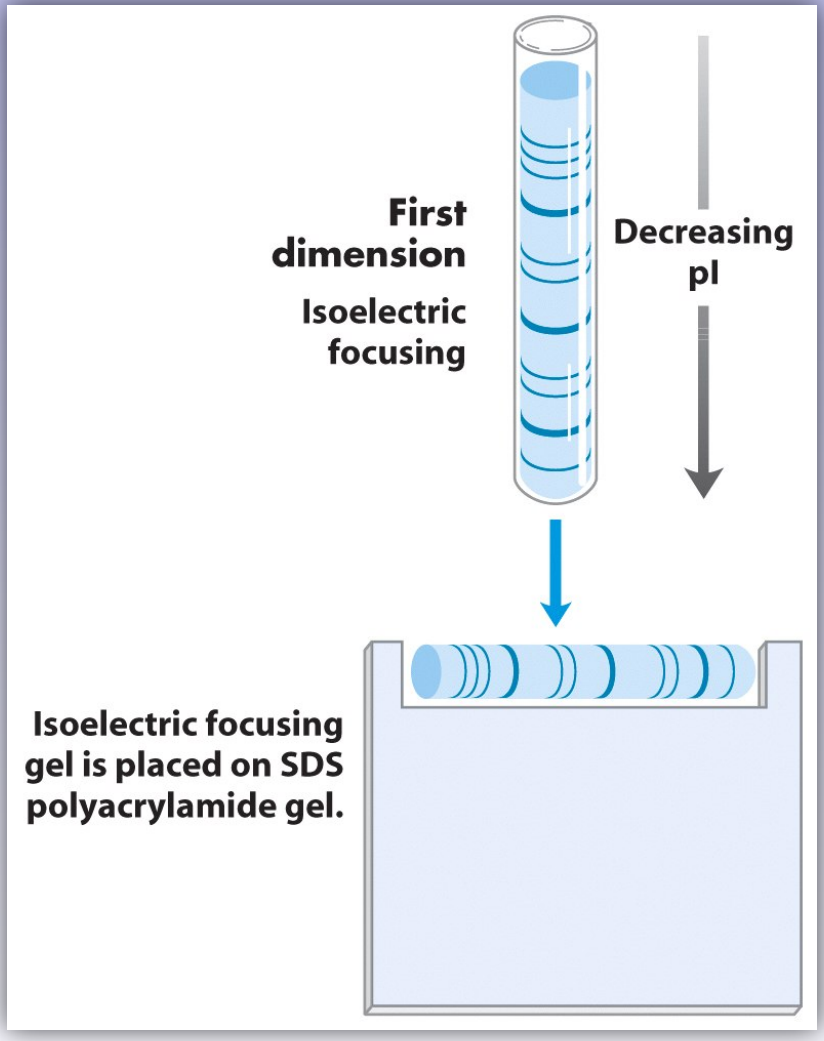
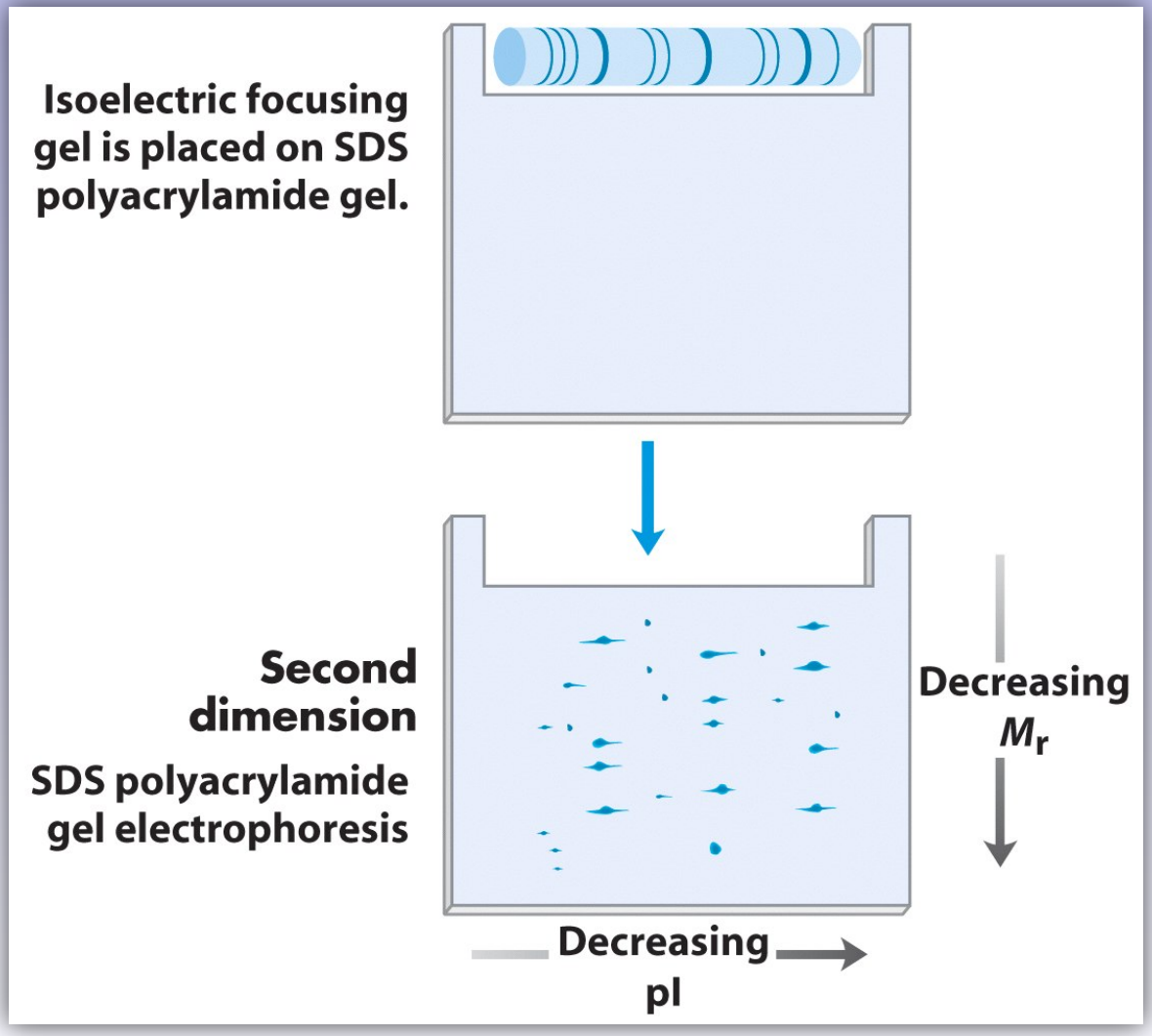


Figure 3.55. Isoelectric focusing of hemoglobins from a patient heterozygous for HbS and β-thalassemia. From Molteni, S., Frischknecht, H., and Thormann, W. *Electrophoresis* 15:22, 1994 (Figure 4, parts A and B).



Two-dimensional gel electrophoresis

1st dimension: isoelectric focusing



2nd dimension:
SDS-PAGE

General spectrophotometric properties of proteins

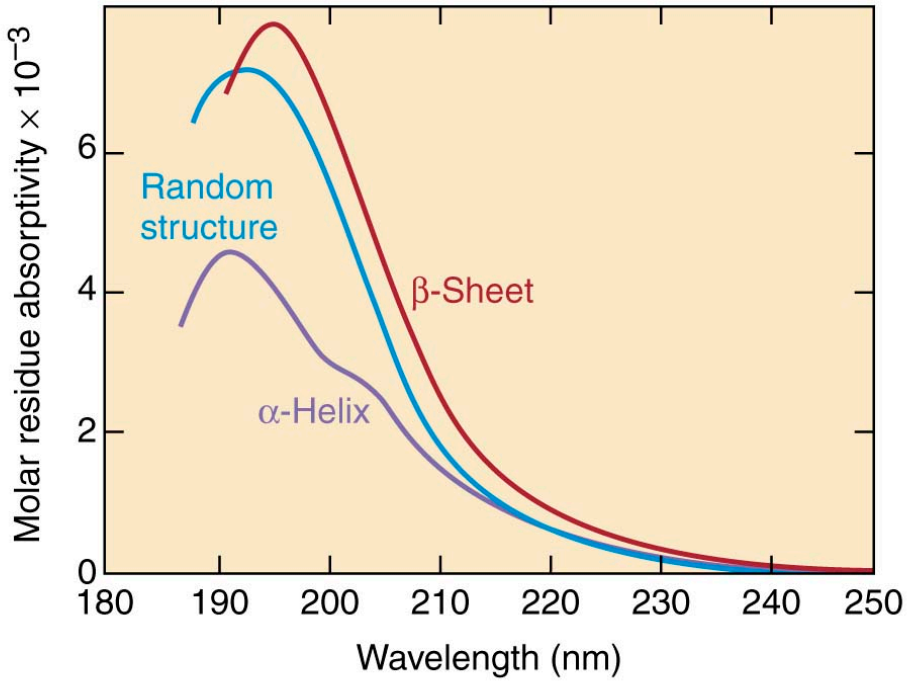
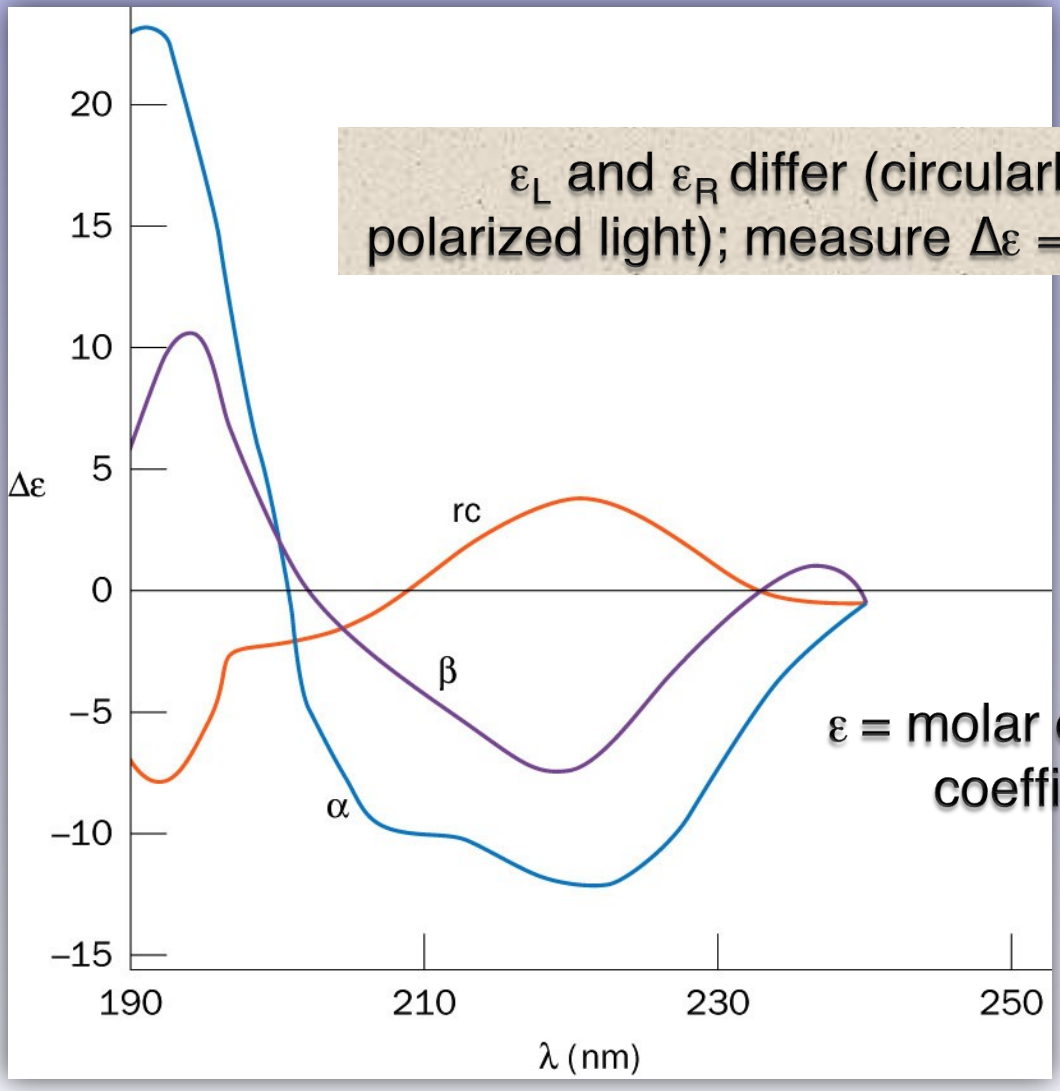


Figure 3.72. Ultraviolet absorption of the peptide bonds of a polypeptide chain in α-helix, random structure, and antiparallel β-sheet conformations. Redrawn from d'Albis, A. and Gratzer, W. B. In: A. T. Bull, J. R. Lagnado, J. O. Thomas, and K. F. Tipton (Eds.), *Companion to Biochemistry*. London: Longmans, 1974, p. 175.

Peptide bond UV absorption properties of polypeptides; recall that the aromatic sidechains of proteins (Phe, Tyr, Trp) absorb at ~ 280 nm.

Only chiral molecules give a CD spectrum.



ε_L and ε_R differ (circularly polarized light); measure $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$

ε = molar extinction coefficient

Circular dichroism (CD) spectra of polypeptides:
α = α-helix; β = β-sheet; rc = random coil