Separation and purification of proteins:

1- Crude extract preparation: break open tissue / cells to release proteins. Differntial centrifugation to isolate subcellualr fractions/ organelles.

2- Fractionation: treatment of extract to separate proteins into different fractions based on a property size/charge / differences in protein solubility at high salt conc.

3- Salting out: addition of a salt ammonium sulfate $(NH_4)_2SO_4$ in a right amount to selectively precipitate certain proteins while others remain in soln.

4- Dialysis : separate proteins from solvents depending on size in a bag/tube containing a semipermeable mb. which allows exchange of solvent and salt but not protein. (good to remove ammonium sulfate from separated protein)

5- Chromatography: depend on differences in protein, size, charge, binding affinity.

Column chromatography:

Composed of two phases

in a glass/plastic column

1- stationary phase:

Solid / porous matrix through

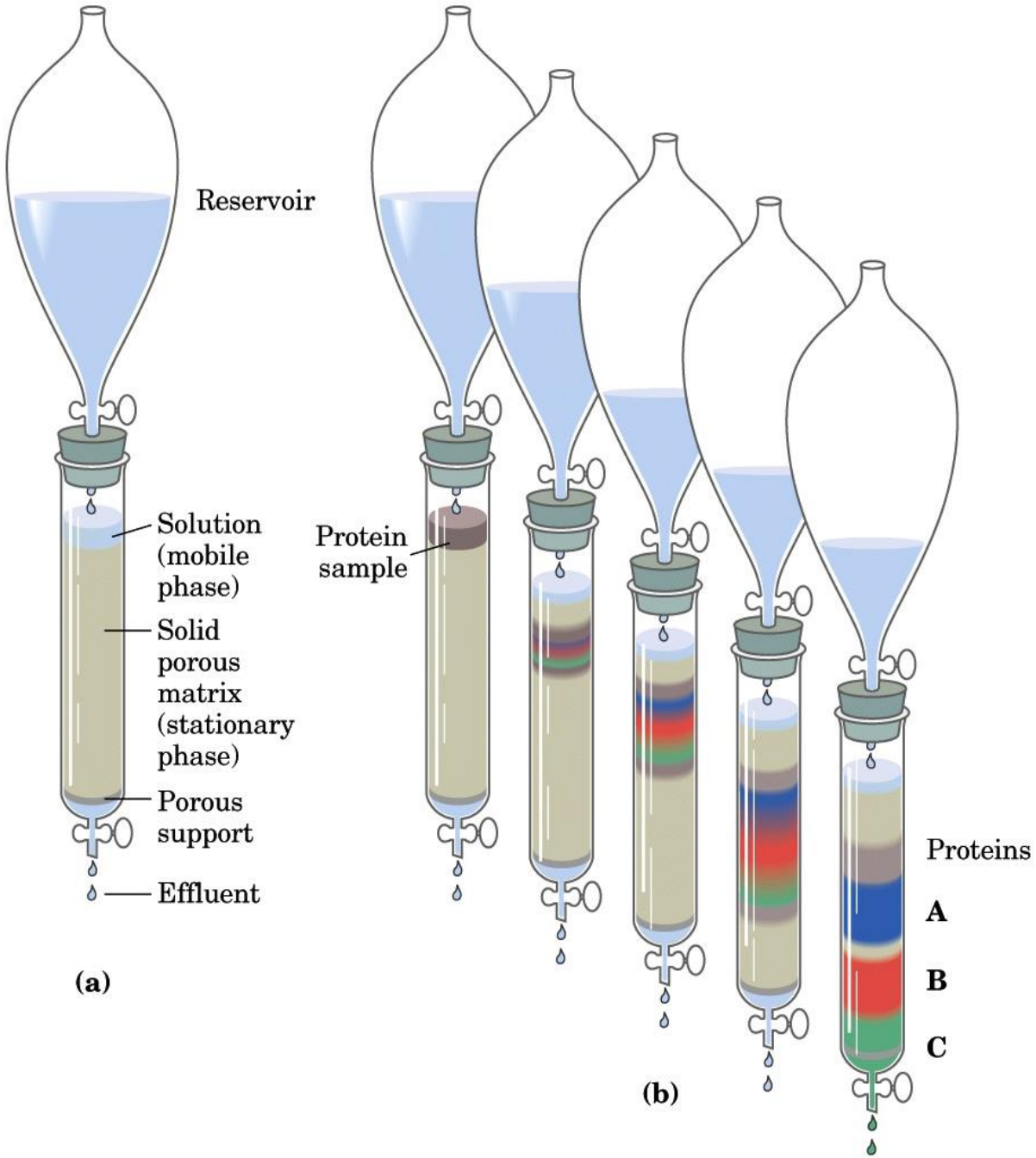
which flows the mobile

2- mobile phase: buffered soln

Rate of protein flow through

column ↓ with column length

and time spent ↑



Ion exchange chromatography:

Exploit differences in *sign* and *magnitude* of net electric charges of a protein.

Cation-exchangers:

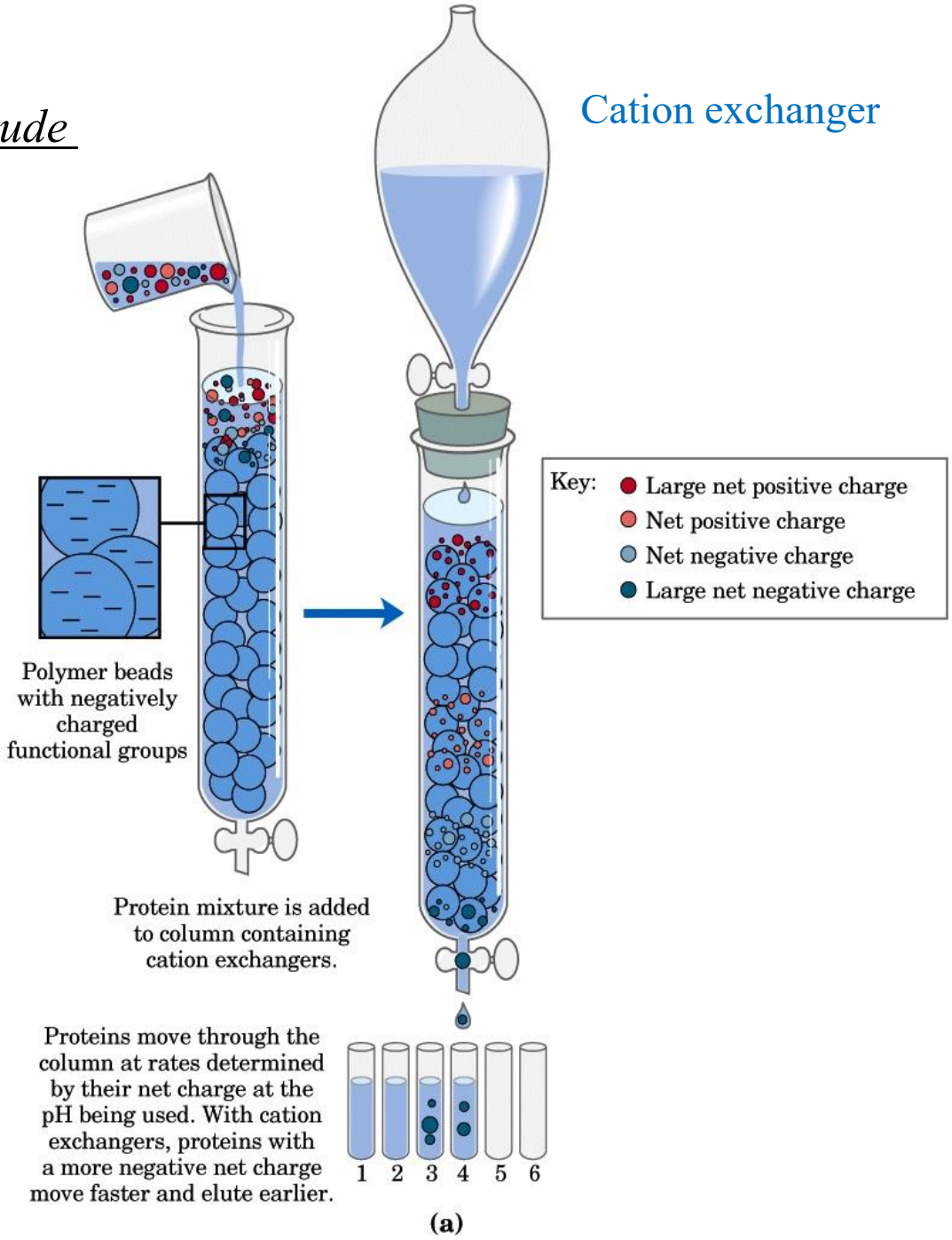
Solid matrix has –ve charge groups

Anion+exchangers:

Solid matrix has +ve charge groups

Note:

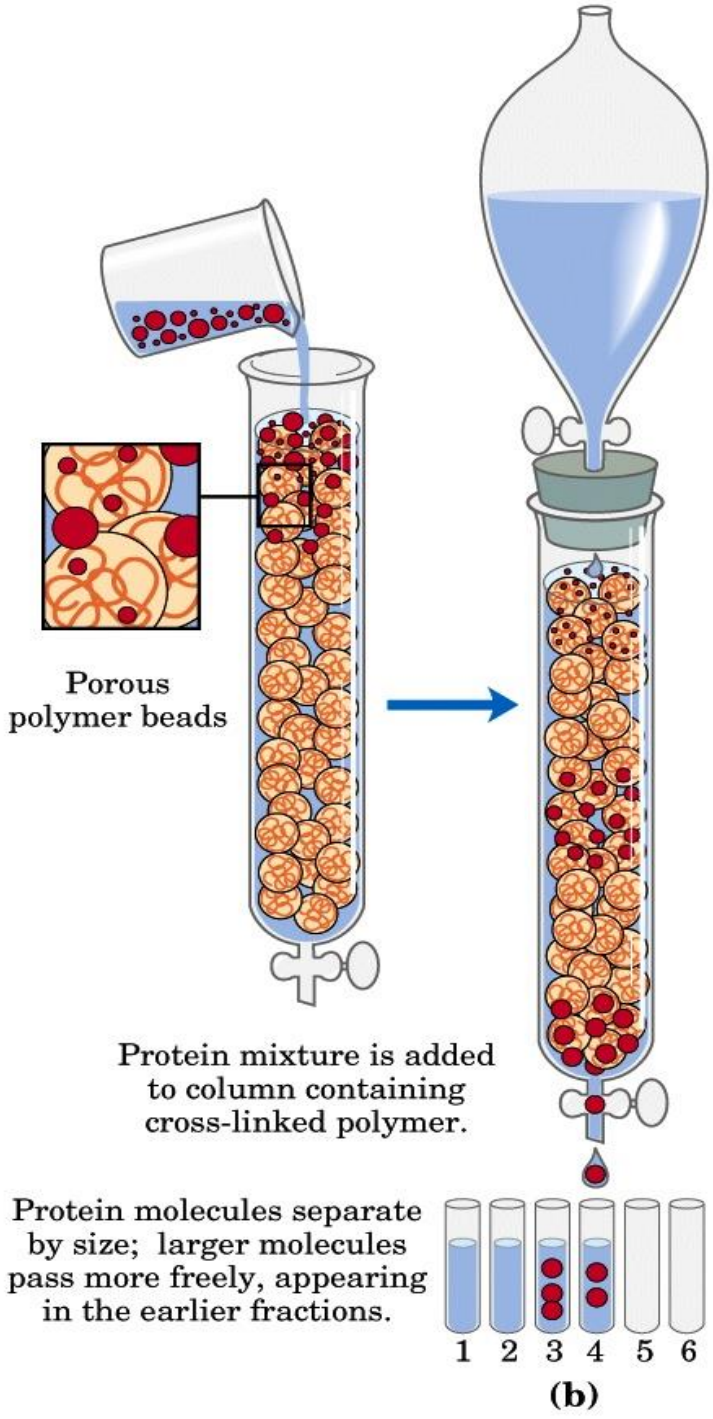
Elution: washing out of the column



Size exclusion chromatography:

Solid phase: beads with pores / cavities.

Large proteins emerge sooner than small ones since they cant enter cavities so they take short rapid path around the beads through column.

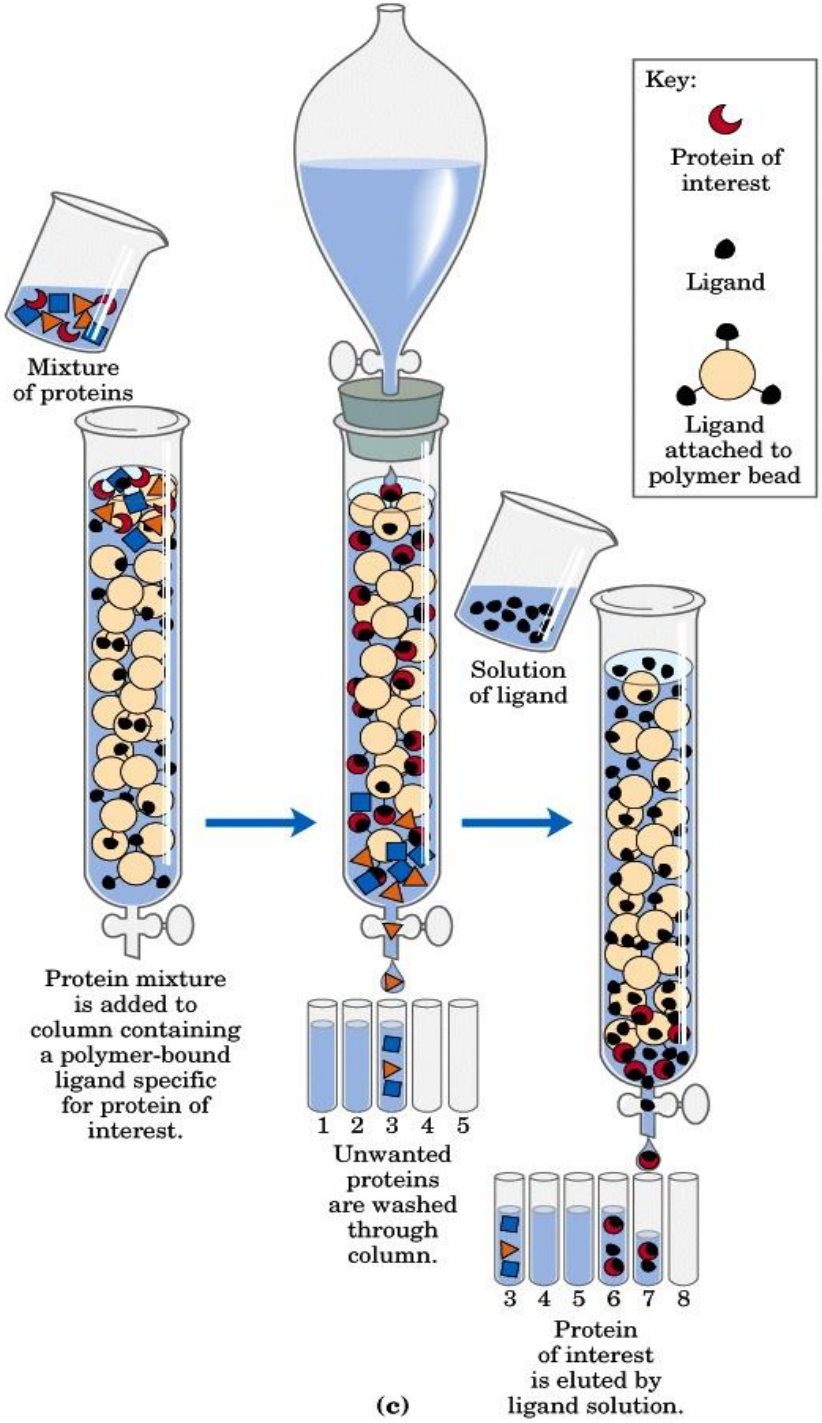


Affinity chromatography:

Based on binding affinity of a protein

Beads have a covalently attached chemical group = ligand

A protein with affinity to this ligand binds the beads other proteins flow through the column



Note:

Ligand: a group / molecule that binds a macromolecule such as a protein.

A refinement in chromatographic methods:

HPLC =High Performance Liquid Chromatography

Speeds the rate of protein flow through the column using:

1) high pressure pumps.

2) high quality beads that can withstand crushing due to high pressure.

Protein separation:

thousands of published protocols → start with inexpensive protocol.

table 5–5

A Purification Table for a Hypothetical Enzyme*

Procedure or step	Fraction volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
1. Crude cellular extract	1,400	10,000	100,000	10
2. Precipitation with ammonium sulfate	280	3,000	96,000	32
3. Ion-exchange chromatography Expensive	90	400	80,000	200
4. Size-exclusion chromatography	80	100	60,000	600
5. Affinity chromatography	6	3	45,000	15,000

*All data represent the status of the sample *after* the designated procedure has been carried out. Activity and specific activity are defined on page 137.

Separation and characterization of proteins by electrophoresis:

Migration of charged proteins in an electric field.

Advantage:

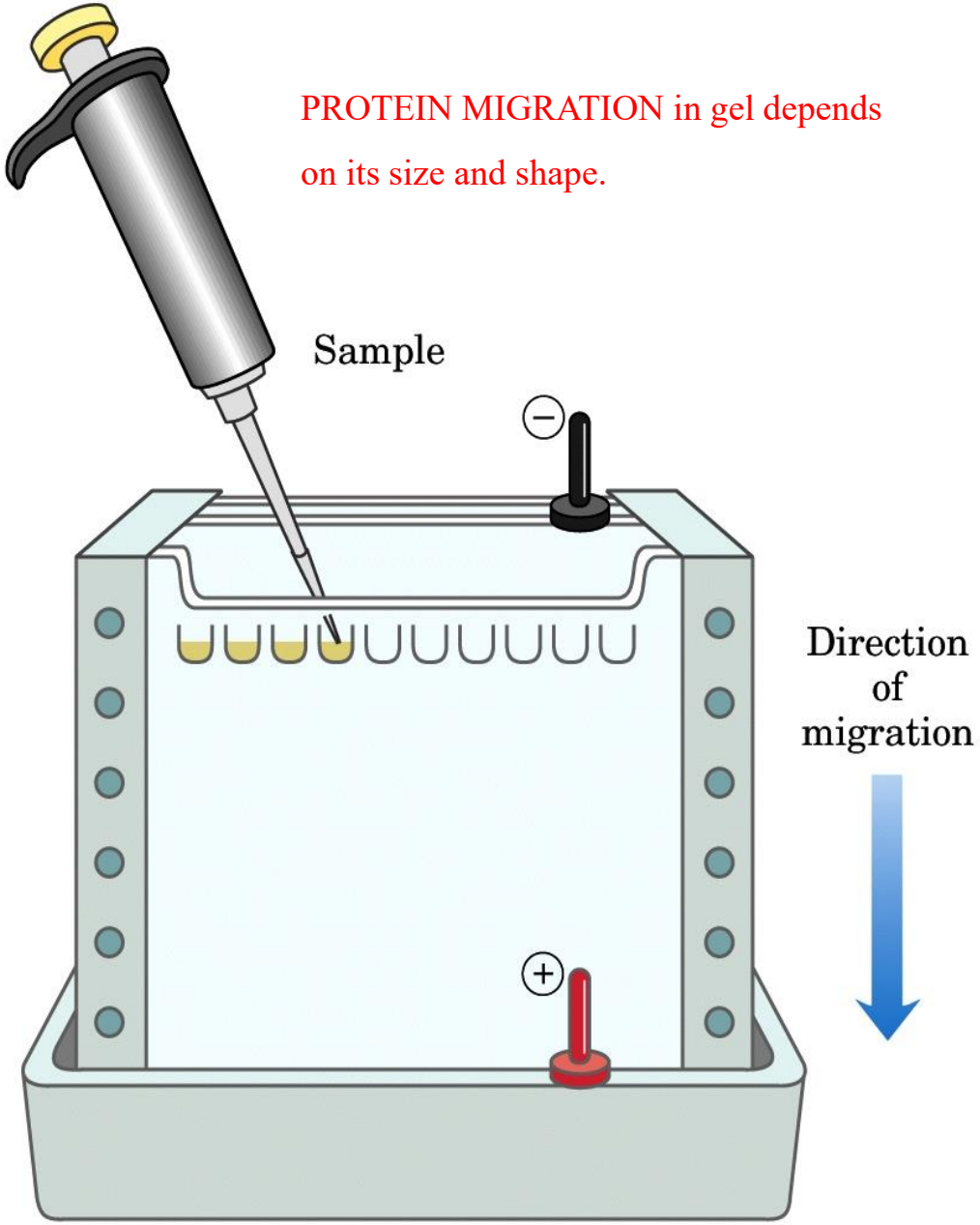
1) a protein can be visualized as well as separated.

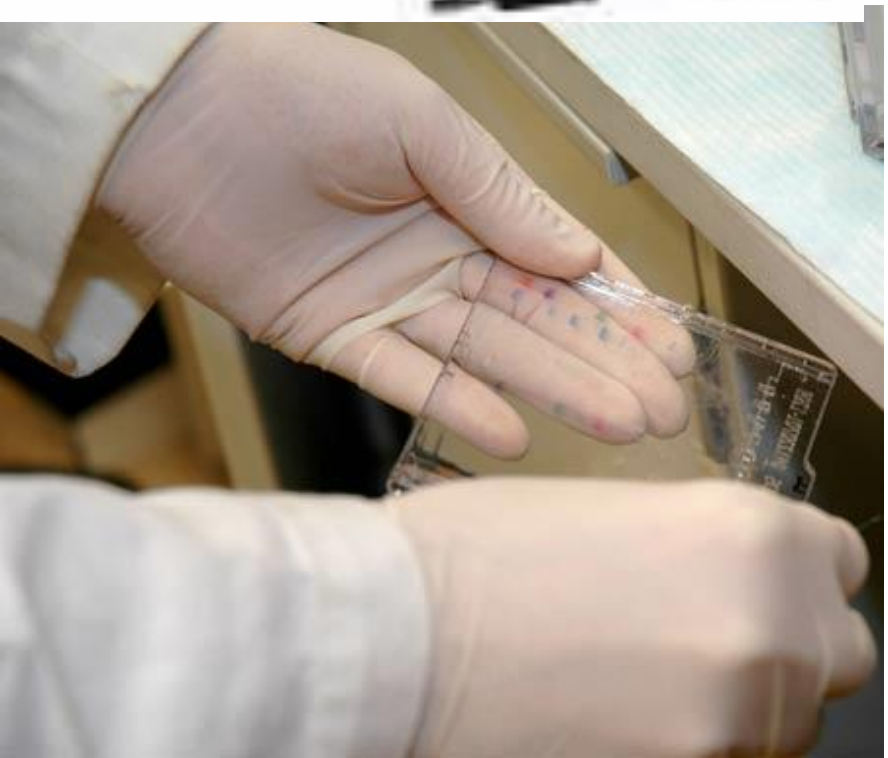
2) allows determination of isoelectric point (IP) and molecular weight (Mwt).

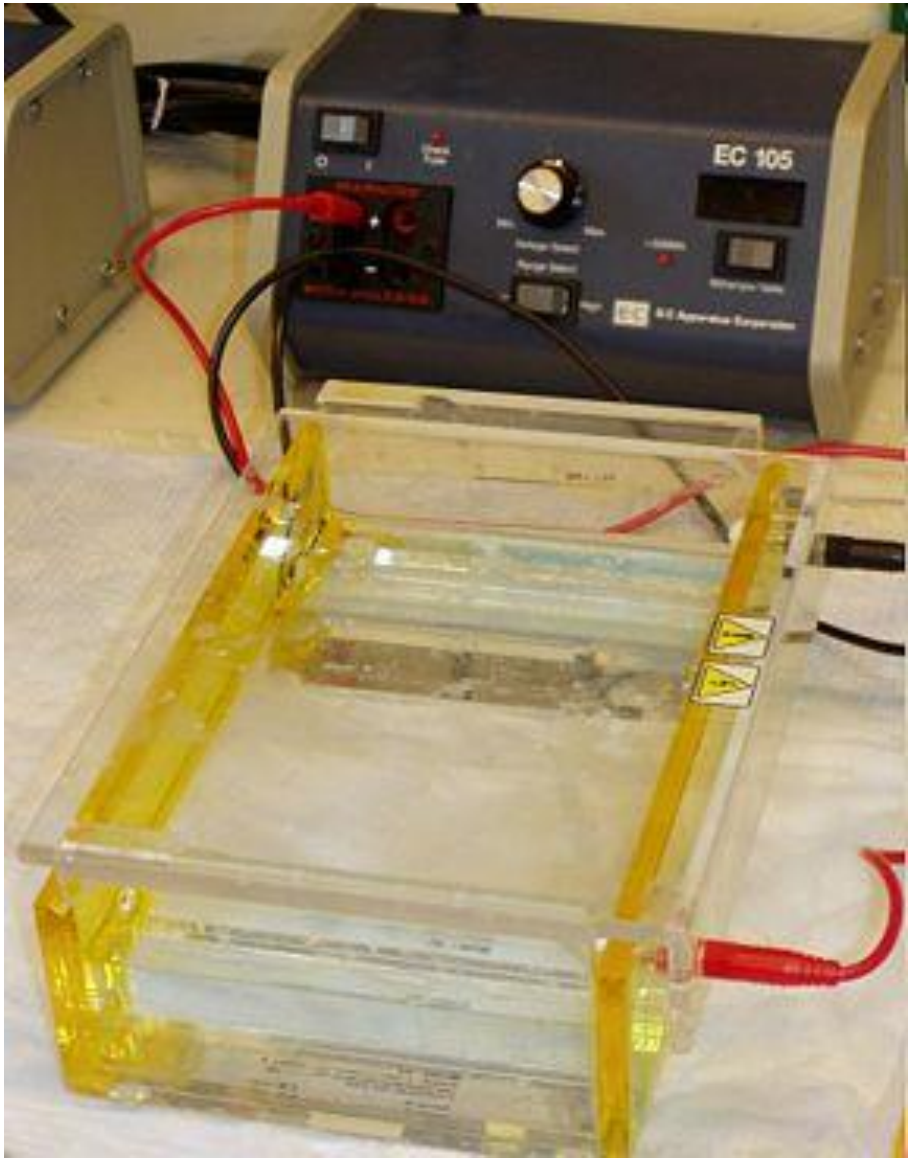
3) Degree of purity of protein sample.

Gels made of crossed linked polymer polyacrylamide . Acts as a molecular sieve. Slows proteins migration in proportion to their charge/ mass ratio

PROTEIN MIGRATION in gel depends on its size and shape.

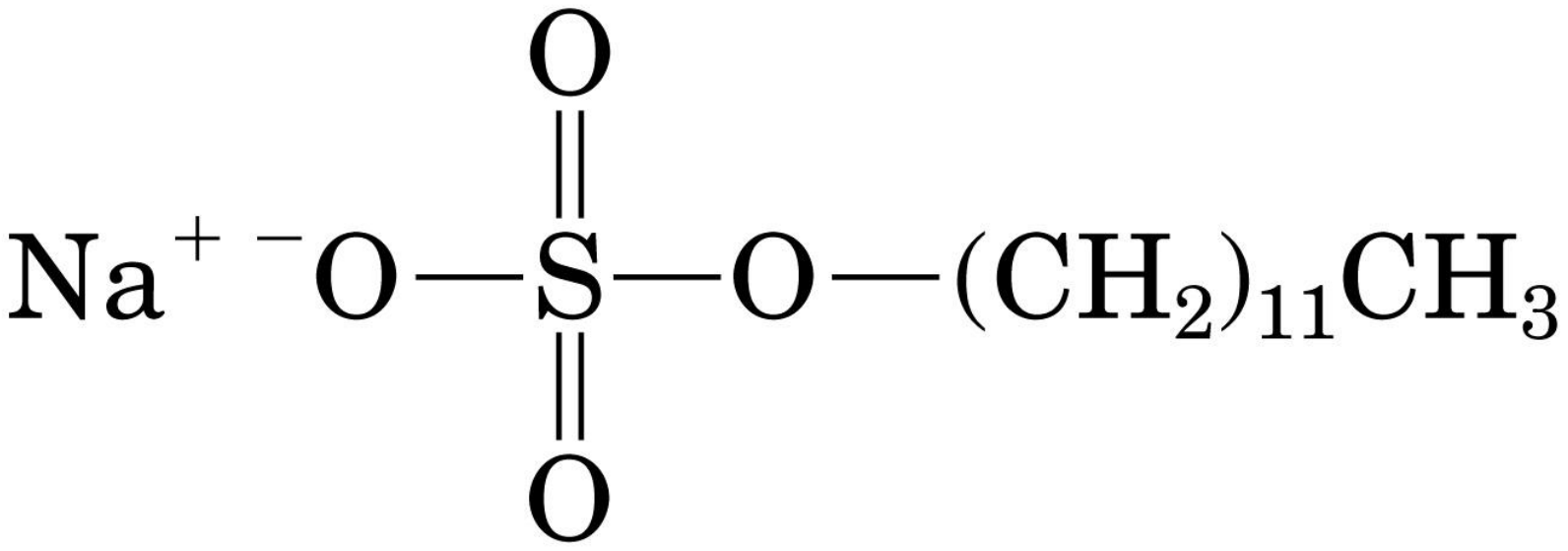





EC 105

A detergent binds to protein in proportion to its M wt. :
1molecule SDS / 2 a.a.

SDS separates proteins relative to mass.



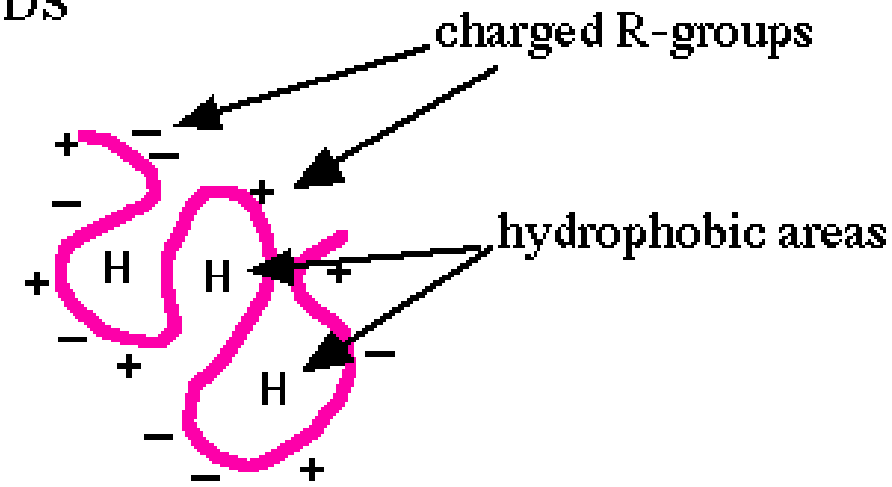
Sodium dodecyl sulfate
(SDS)

The end result :

all proteins contain only

primary structure

BEFORE SDS



AFTER SDS

denatured protein (reduced to its primary structure) and lenearized

Gel staining:

After electrophoresis proteins visualized by treating the gel with a stain/dye Coomassie blue which binds proteins not the gel.

Useful to monitor the purification steps:

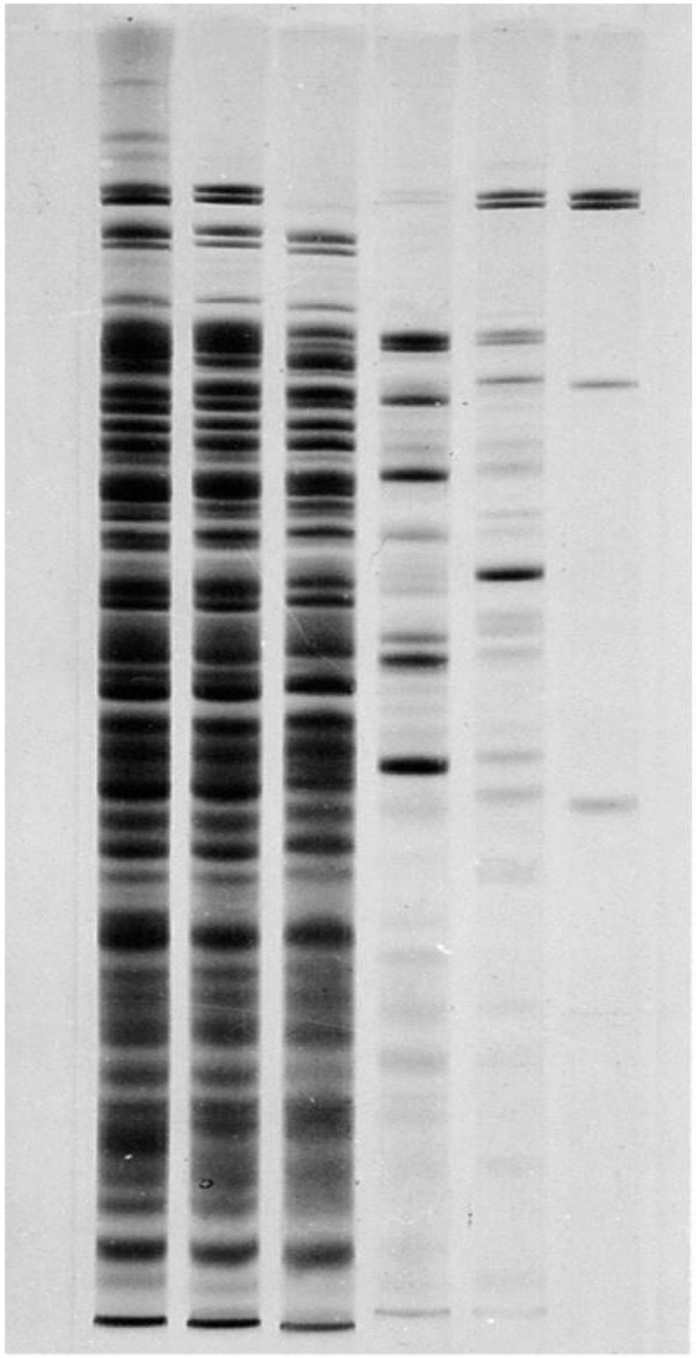
Estimate no. of different proteins, Mwt. when purification ended.

Figure on the right: RNA pol (E coli)

First lane: proteins in crude extract

Successive lanes: proteins extracted after each purification step.

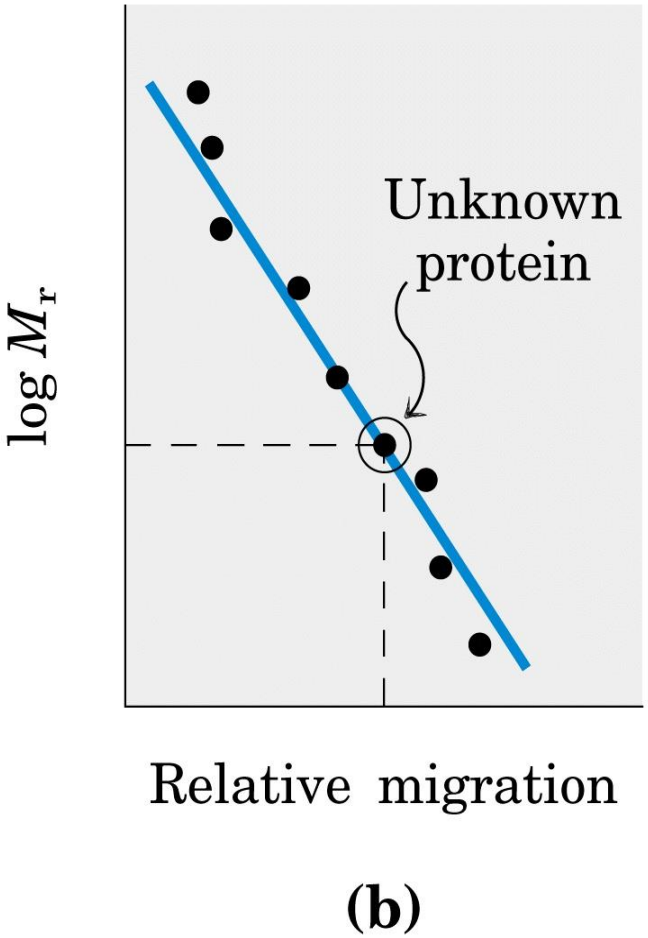
Last lane :purified protein contains 4 subunits

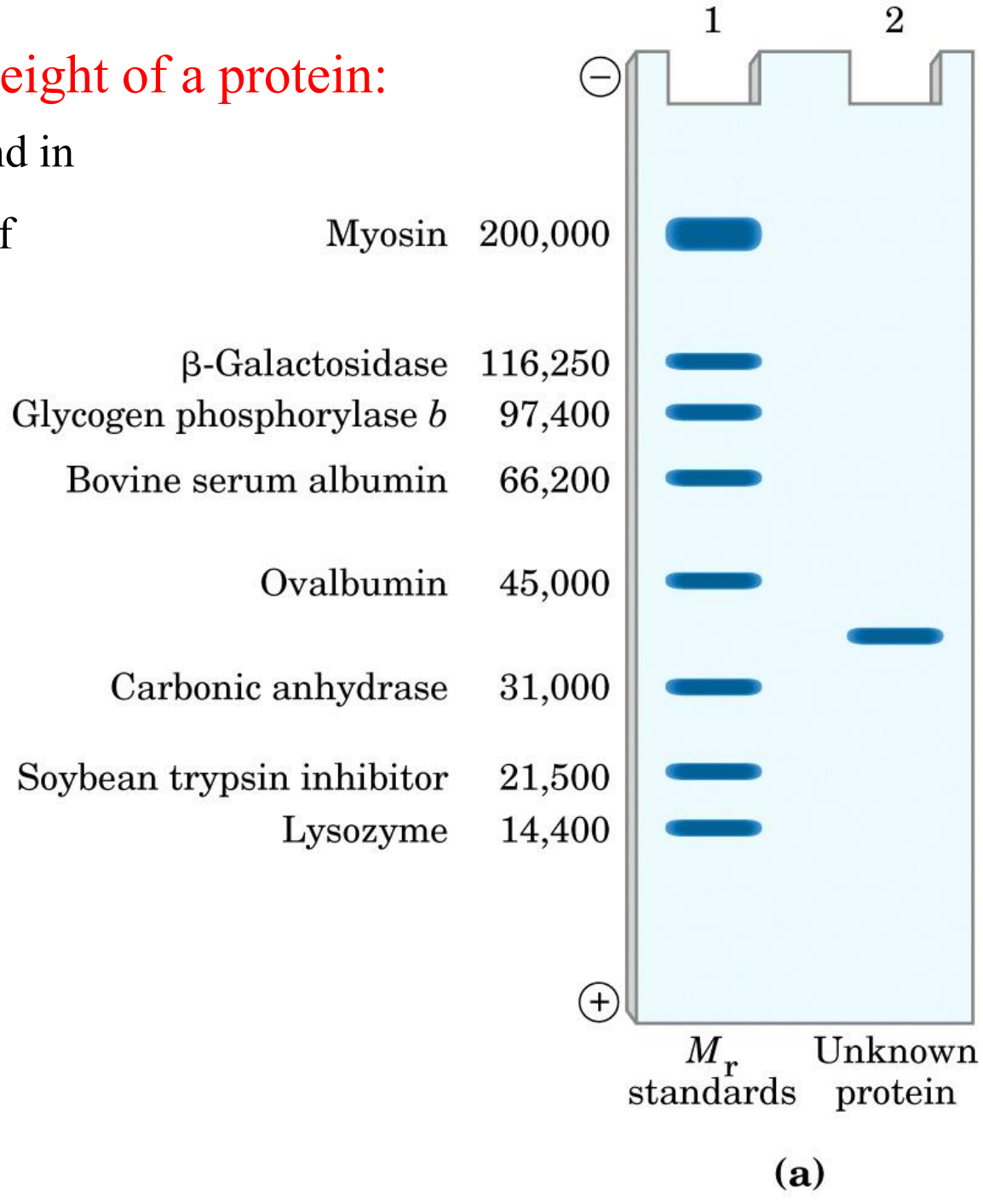


(b)

Estimating molecular weight of a protein:

The position of a protein band in the gel provides a measure of its M wt.





pI = pH the net charge of a protein = zero

table 5–6

The Isoelectric Points of Some Proteins

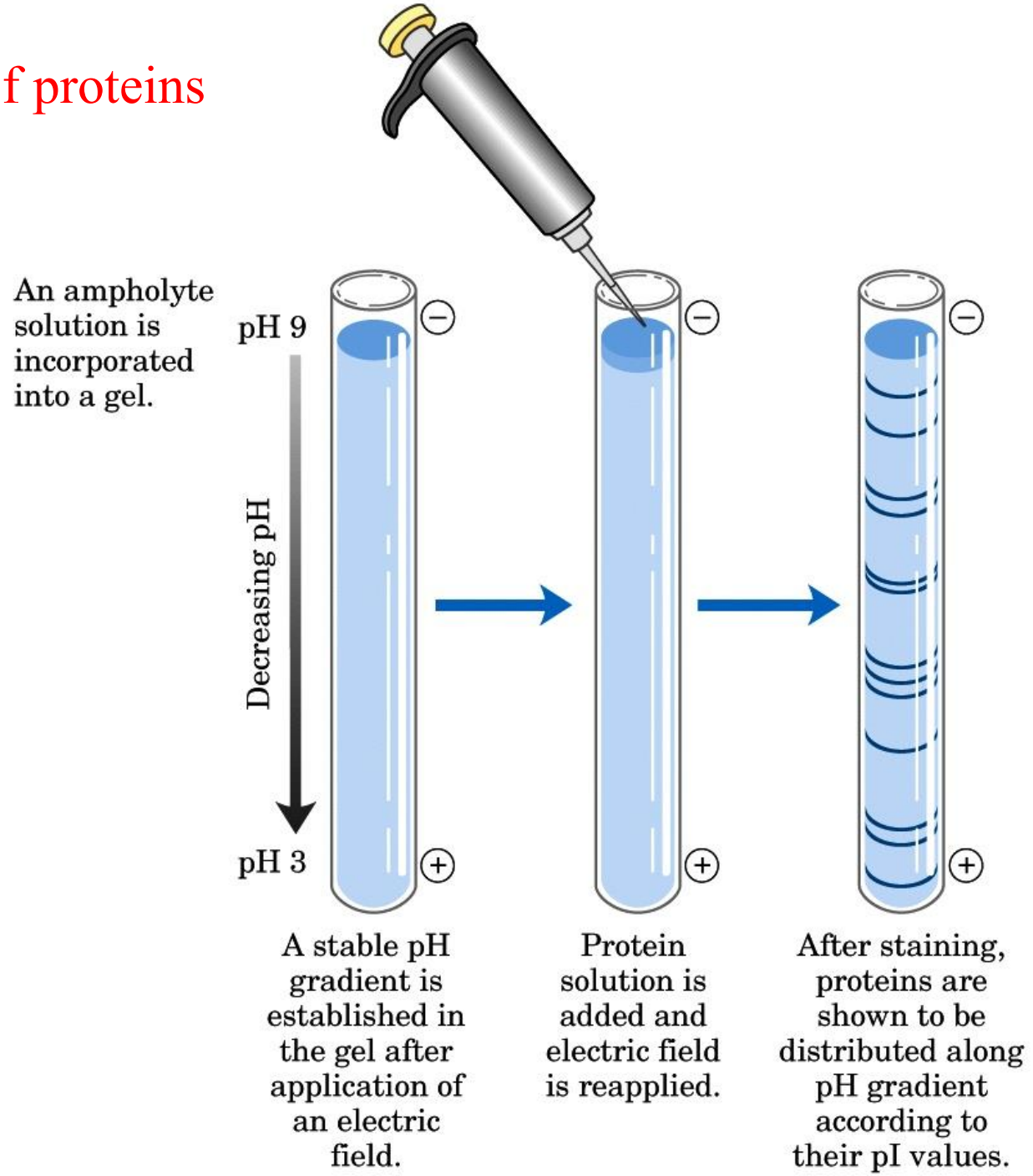
Protein	pI
Pepsin	~1.0
Egg albumin	4.6
Serum albumin	4.9
Urease	5.0
β-Lactoglobulin	5.2
Hemoglobin	6.8
Myoglobin	7.0
Chymotrypsinogen	9.5
Cytochrome *c*	10.7
Lysozyme	11.0

Determination of the IP of proteins by isoelectric focusing:

1- preparation of a pH gradient by ampholytes addition in cylindrical gel.

2- addition of protein mix.

3- migration of proteins until each reaches a pH equivalent to its PI

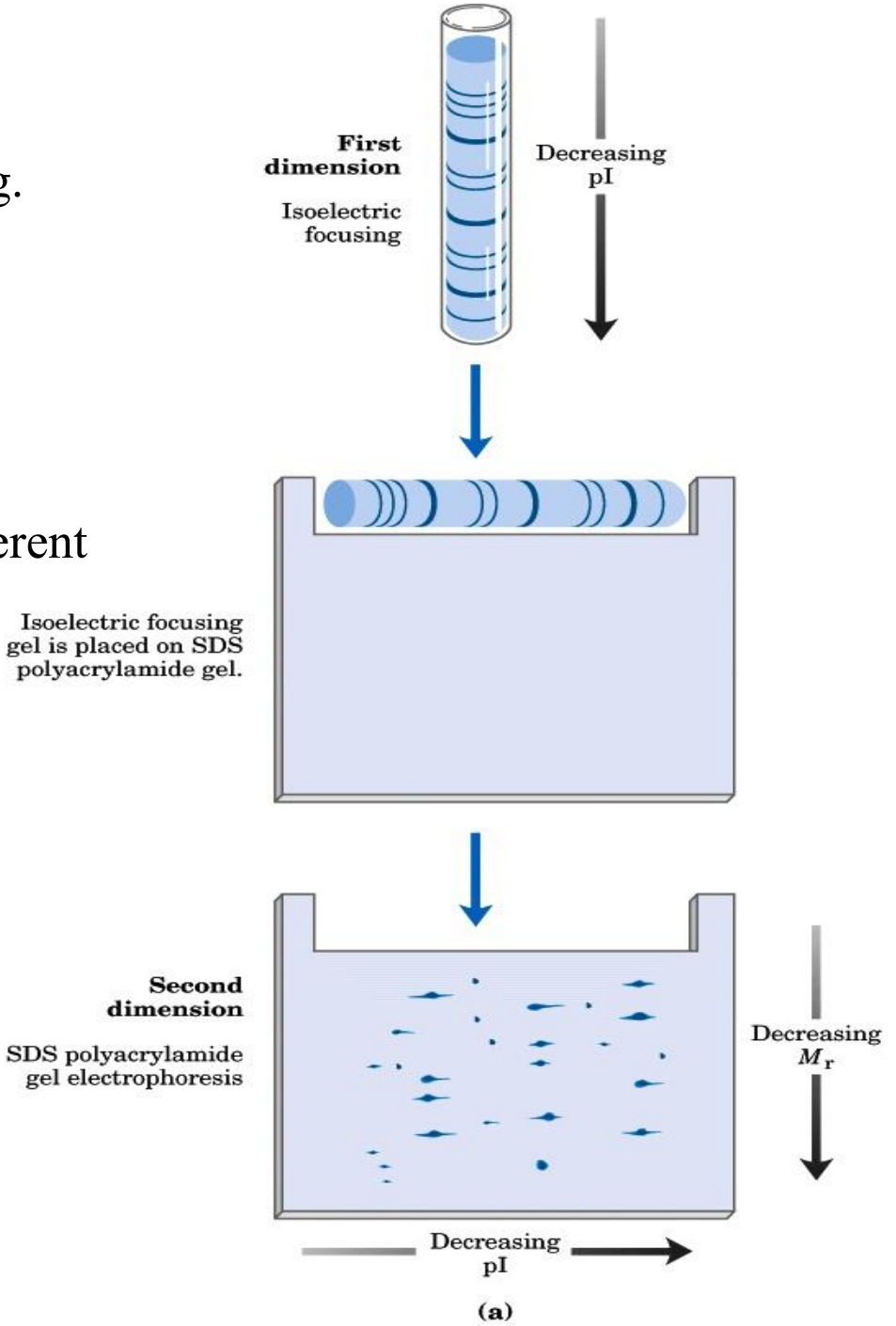


Two dimensional electrophoresis:

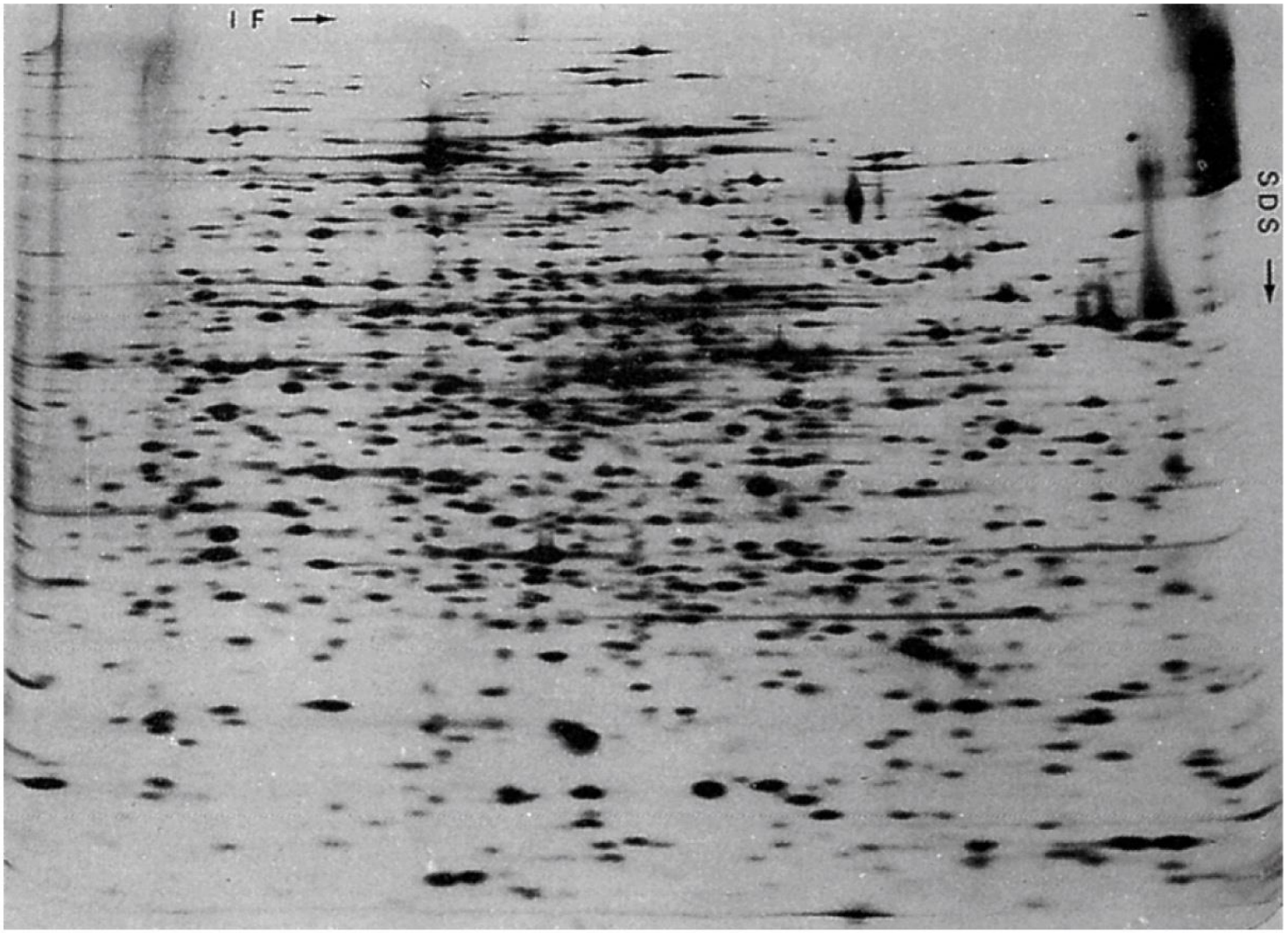
SDS polyacrylamide + isoelectric focusing.

More sensitive analytical method than any alone.

Separates proteins with similar PI but different M wt. , or vice versa.



Two dimensional electrophoresis > 1000 protein separated from E.coli



(b)

Separation of a protein that is an Enzyme:

The amount in a given soln. / tissue extract expressed as enzyme activity=

The rate at which the substrate is converted →rxn products.

So we need to know the following:

1) The overall equation of rxn.
2) Analytical procedure for product appearance / substrate disappearance.
3) Requirement for cofactors metal ions/ coenzymes.
4) Dependence on substrate conc.
5) Optimum pH .
6) Temperature zone at which enzyme is stable and active, usually (25-38 ^{0}C).

Activity versus specific activity:

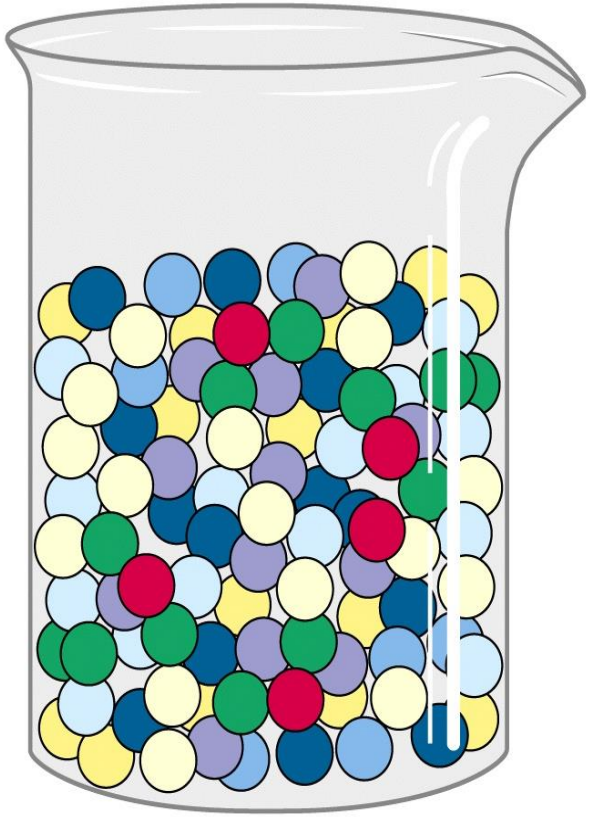
1 unit of E activity= amount of E transforming1.0 umol substrate / min. at 25°C

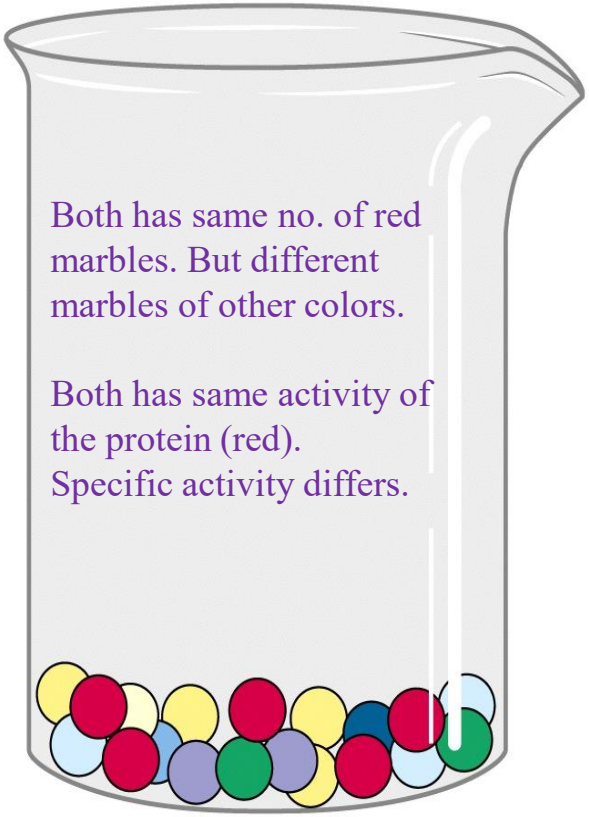
Activity = total unit of E in a soln.

Specific activity = no. E units / mg total protein .

Specific activity measure of E purity, ↑ during purification procedures.

Maximal +constant when E is pure.





The primary structure of a protein determines how it folds into its three-dimensional structure → determines protein function.

Relationship between a.a sequence and function:

1) Proteins with different functions has different a.a sequence.
2) Human genetic: diseases linked to defective proteins (in one third of these cases a single a.a is changed).
3) Functionally similar proteins from different species have similar a.a sequences.

   e.g. ubiquitin (76 a.a. protein) identical fruit flies →humans.

The a.a sequence is not completely fixed, but flexible.

20-30% of human proteins are polymorphic = proteins that contain a.a variations in human population.

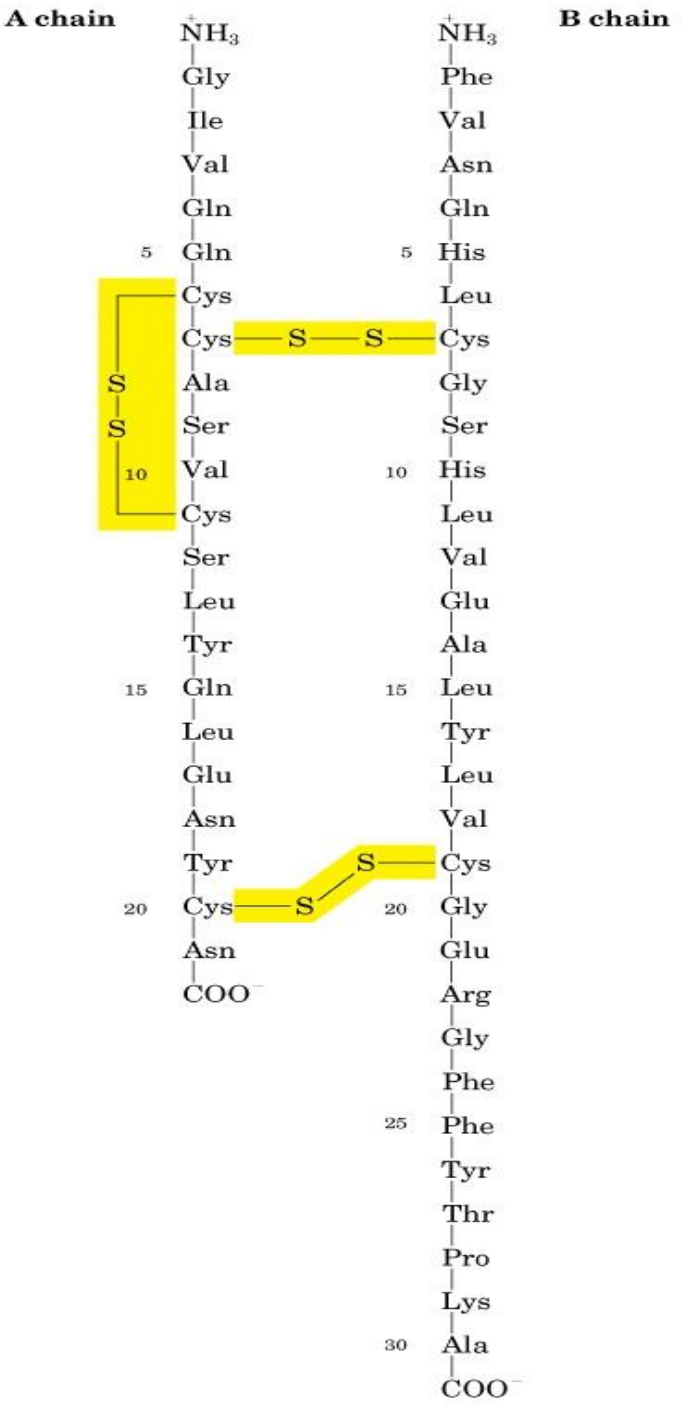
Proteins contain crucial regions with conserved sequences essential to their function.

Two major discoveries in biochem history 1953:

1) Watson Crick and DNA double helix.
2) Frederick Sanger and insulin sequence.

Two polypeptide chains joined by disulfide linkage.

Similarity in sequence bw. human, dog, horse, cow, pig, and other animals.



Polypeptide sequencing of primary structure:

Labeling and identification of the N-terminal a.a residue:

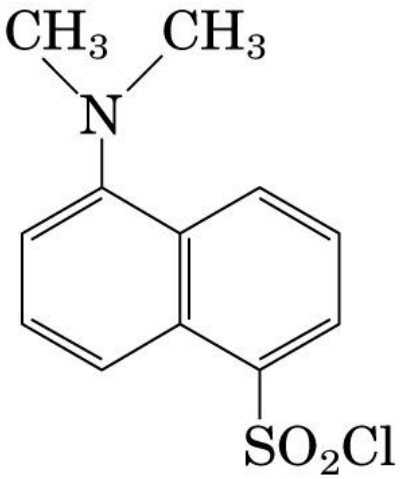
Sanger developed (FDNB) 1-fluro-2,4-dinitrobenzene.

Other reagents: dansyl chloride, dabsyl chloride.

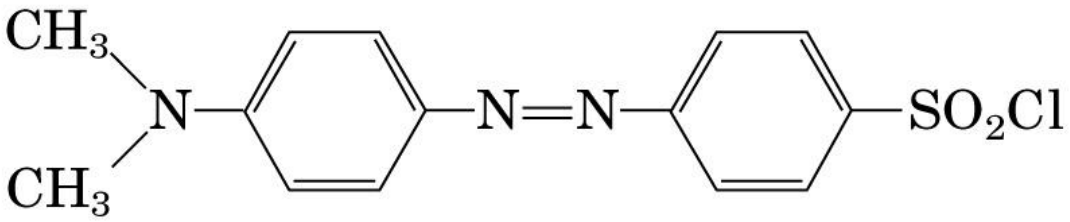
All label the N-terminal residue.

To identify *only* this residue the protein is hydrolyzed → a.a and is destroyed

No. of polypeptides identified in protein e.g. phe and Gly for insulin.



Dansyl chloride



Dabsyl chloride

Dansyl / dabsyl chloride

- a reagent that reacts with primary amino groups in a.a. → blue or blue green fluorescent sulfonamide adducts.

- Dansyl chloride is widely used in protein sequencing and amino acid analysis.

Edman degradation: To sequence the entire polypeptide:

Labels and removes only the amino terminal residue from a peptide, leaving all other bonds intact.

1) The peptide + phenylthiocarbamoyl (PTC) in alkaline conditions.

2) Cleavage of peptide bond next to PTC in trifluoroacetic acid with removal of terminal a.a in acidic conditions.

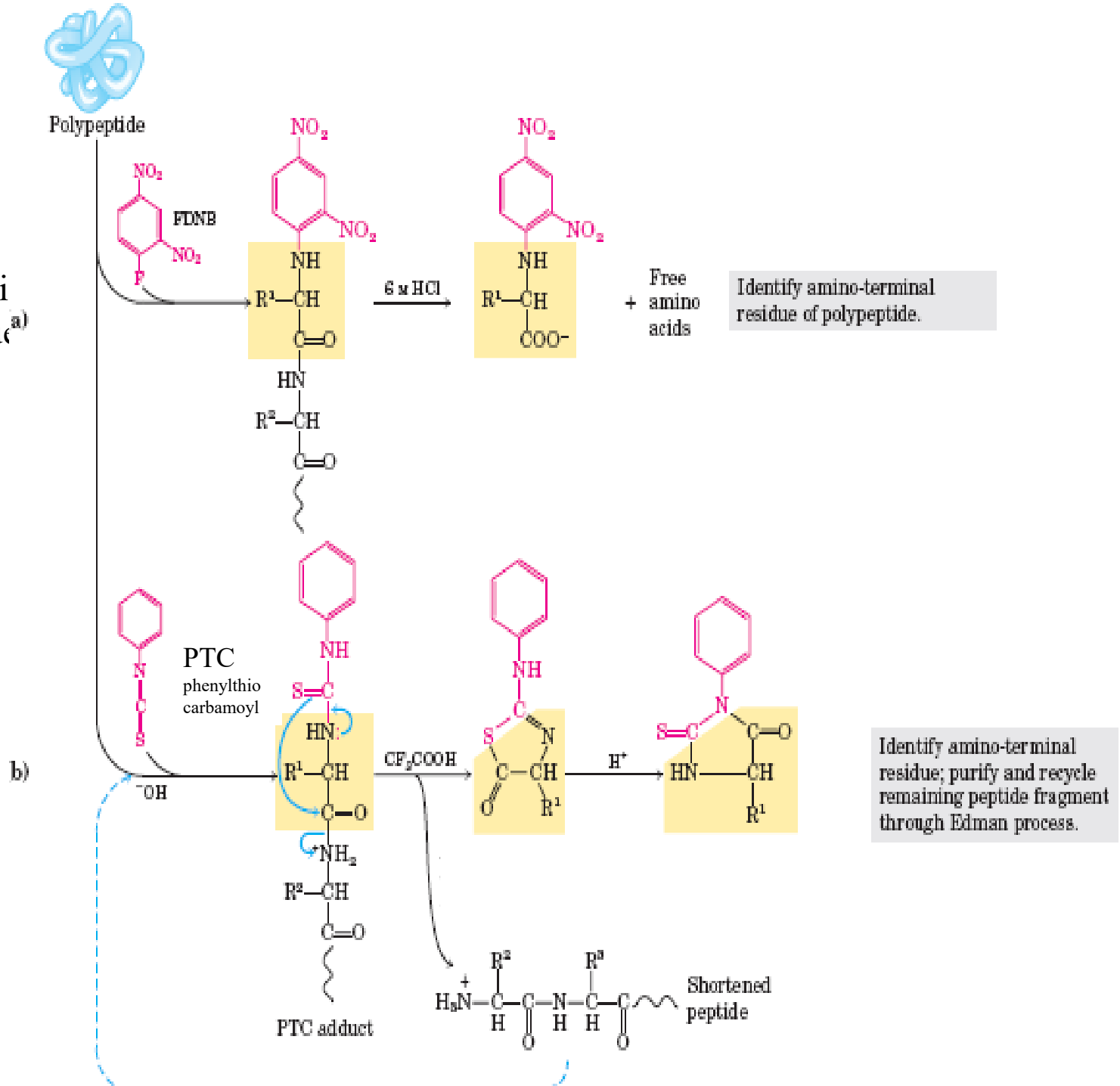
Each a.a is labeled, removed and identified through this sequence of rxns.

The procedure is repeated until the entire sequence is determined.

-Edman degradation carried out in a machine called Sequencer mixes reagents in proper proportions , separates products, identifies and records the results. (from few micrograms of protein)

Sangers method identi
amino terminal residue

Edman degradation reveals the entire sequence of peptide



Sequencing of Large polypeptides:

1) Disulfide bonds cleaved.
2) Protein cleaved by chemical /enzymatic methods.
3) Each fragment purified and sequenced by Edman procedure.
4) Peptide fragments ordered.
5) Disulfide bonds located.

Cleaving polypeptide chain:

1) Proteases: catalyze hydrolytic cleavage of peptide bonds.

The no. of smaller peptides produced by trypsin can be predicted from no. of total Lys and Arg residues in original polypeptide.

2) Chemicals :

can function in a specific manner

table 5–7

The Specificity of Some Common Methods for Fragmenting Polypeptide Chains

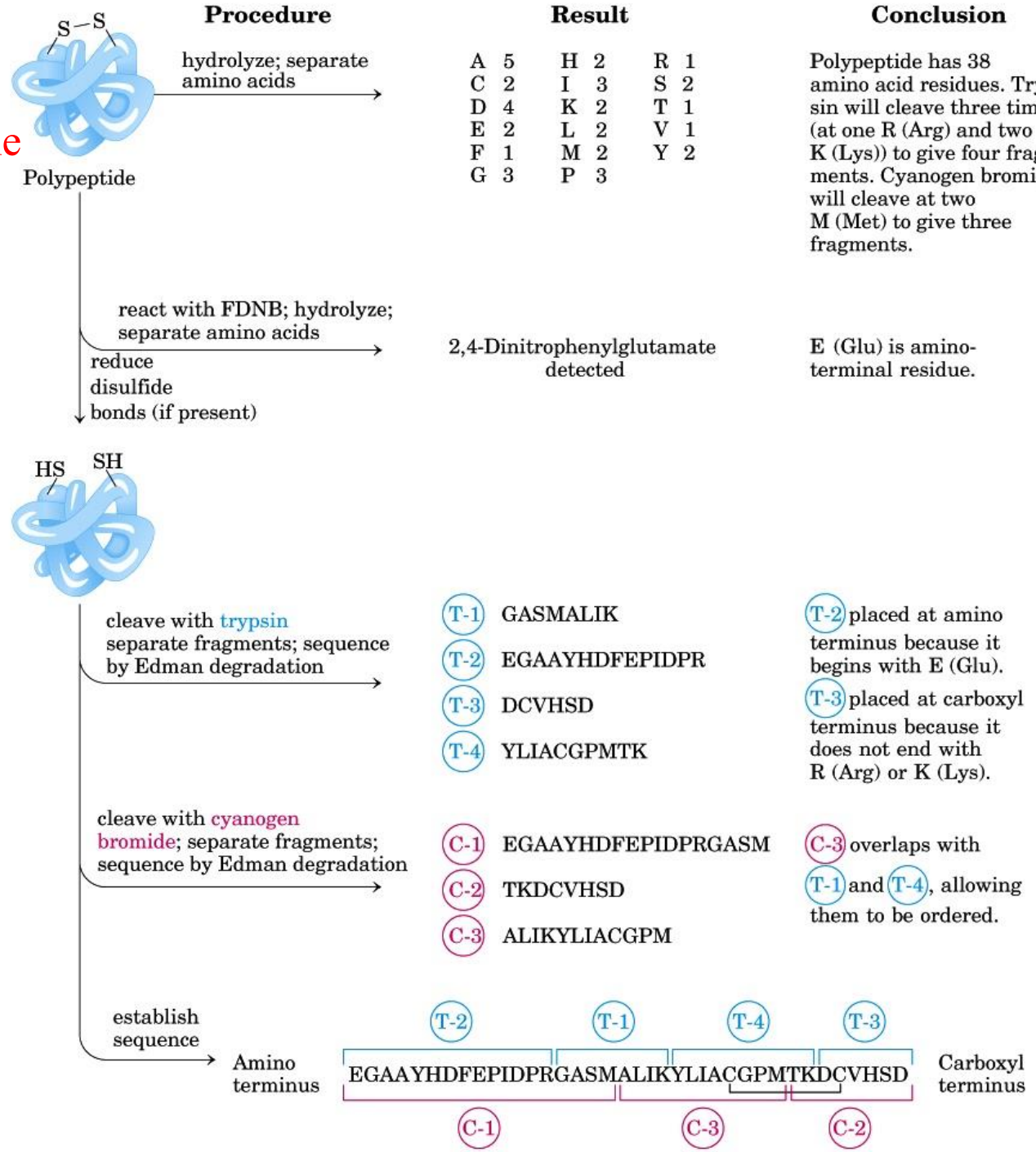
Treatment*	Cleavage points†
Trypsin	Lys, Arg (C)
Submaxillarus protease	Arg (C)
Chymotrypsin	Phe, Trp, Tyr (C)
Staphylococcus aureus V8 protease	Asp, Glu (C)
Asp-*N*-protease	Asp, Glu (N)
Pepsin	Phe, Trp, Tyr (N)
Endoproteinase Lys C	Lys (C)
Cyanogen bromide	Met (C)

*All except cyanogen bromide are proteases. All are available from commercial sources.

†Residues furnishing the primary recognition point for the protease or reagent; peptide bond cleavage occurs on either the carbonyl (C) or the amino (N) side of the indicated amino acid residues.

Cleaving protein and sequencing and ordering the peptide fragments:

1) Determine the a.a composition and terminal residue.
2) Break disulfide bonds
3) Fragmentation by two methods.
4) Overlap sequences.



Determination of the polypeptide sequence from DNA sequence:

Development of DNA sequencing methods.

When the gene is available easier to sequence DNA than protein.