9. Separation by Adsorption – Affinity Techniques

1. Principles of Affinity Chromatography

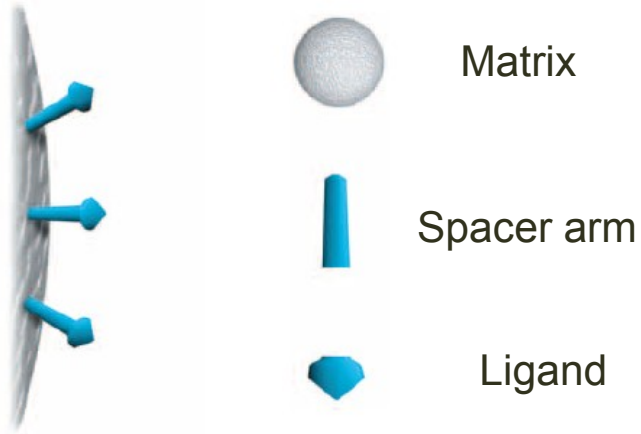
Affinity Chromatography

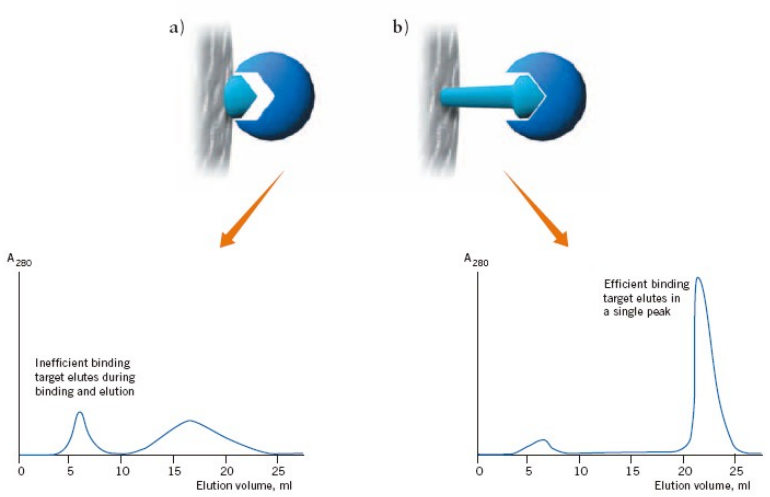
- **Principles of affinity chromatography**
  - Based on specific binding of natural ligand to protein's binding site
  - Examples
    - Enzyme: substrate analogue, inhibitor, cofactor
    - Antibody : antigen
    - Lectin : polysaccharide, glycoprotein
    - Nucleic acid : complementary sequence, nucleic acid binding protein
    - Hormone, vitamin : receptor, carrier protein
  - Pseudo affinity or biomimmetic
    - Interaction of unnatural compound to protein's binding site
  - More general meaning of 'affinity'
    - Includes interaction with non-natural ligands

- **Applications of affinity techniques**
  - Chromatography
  - Precipitation
  - Electrophoresis
  - Liquid phase partitioning

Components of affinity adsorbents

- **Matrix**
  - Requirements
    - Porosity for large surface area
    - Rigidity
  - Types
    - Agarose, synthetic organic or inorganic material
- **Spacer arm**
  - Improves ligand-protein interaction by overcoming steric hindrance
  - Provides additional hydrophobic interaction with target protein
- **Ligand**
  - Molecule that bind reversibly to a specific target





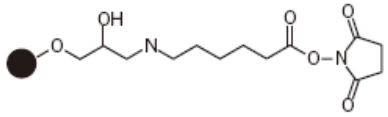
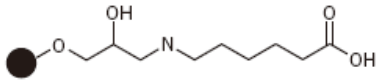
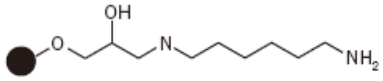
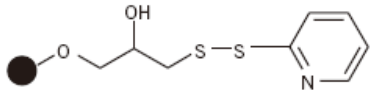
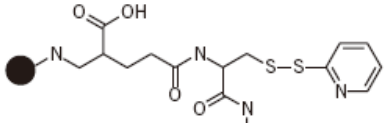
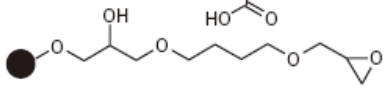
Synthesis of Affinity Adsorbents

- **Requirements for affinity adsorbent**
  - Attachment of ligand without disturbing ligand-protein interaction
  - Prevent nonspecific interactions
  - Stable linkage of ligand

- **Procedures**
  - Activation of matrix → binding of spacer arm → binding of ligand
  - Binding of spacer arm with ligand → binding to matrix

- **Activation of matrix**
  - Generation of electrophiles that will react with amines

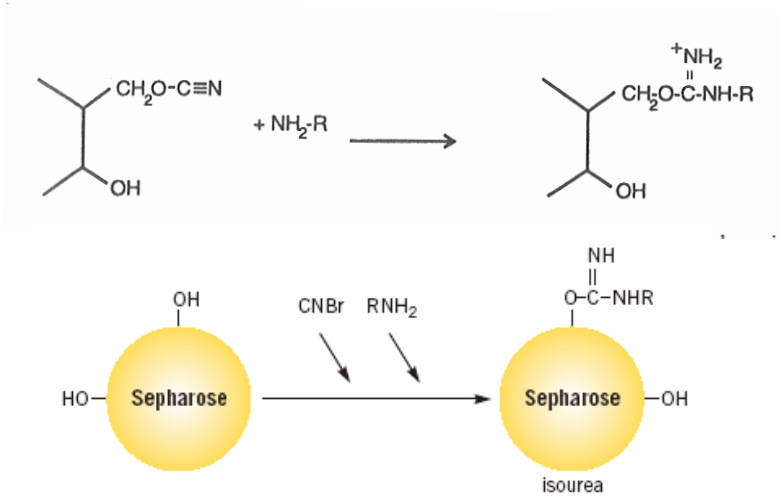
Types of Activated Matrix

Chemical group on ligand	Length of spacer arm	Structure of spacer arm	Product
Proteins, peptides, amino acids			
amino	10-atom		HiTrap NHS-activated HP NHS-activated Sepharose 4 Fast Flow
	None	–	CNBr-activated Sepharose 4B CNBr-activated Sepharose 4 Fast Flow
	10-atom		ECH Sepharose 4B
carboxyl	11-atom		EAH Sepharose 4B
thiol	4-atom		Thiopropyl Sepharose 6B
	10-atom		Activated Thiol Sepharose 4B
	12-atom		Epoxy-activated Sepharose 6B

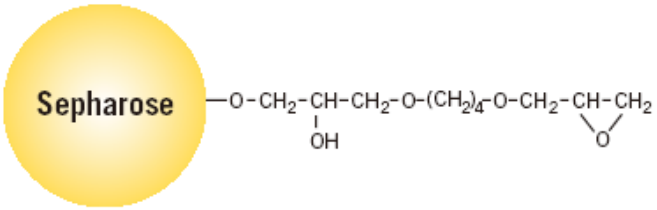
Amersham Biosciences

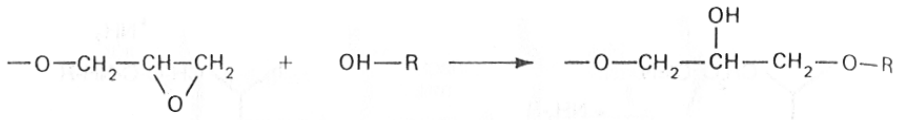
Types of Activated Matrix

- **Cyanogen bromide-activated matrix**
  - Complex reaction
  - Cyanate ester is the principal reactive component
    - React with primary amines in weakly alkaline conditions (pH 9-10)
      - Generation of isourea derivative



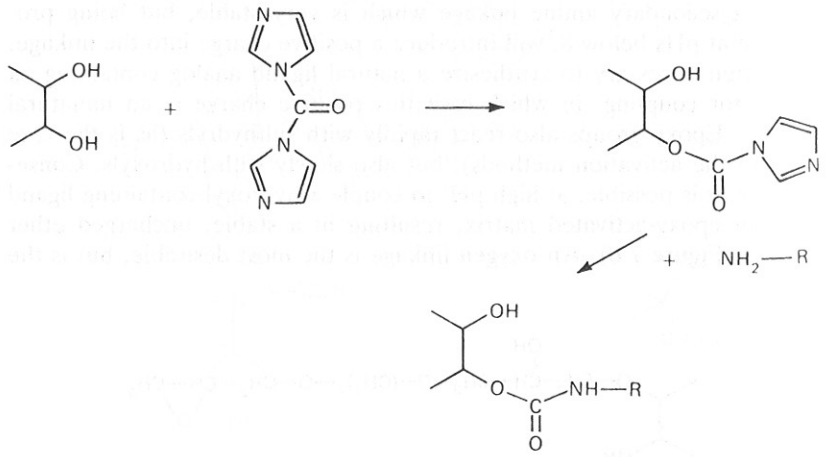
- **Epoxy-activated matrix**
  - Bisoxirane (1,4-butanediol diglycidyl ether)
    - 12-atom spacer arm
  - Reaction with
    - Primary amine → secondary amine linkage
      - Stable, protonated at < pH 8
    - Sulfhydryl (R-SH)
    - Hydoroxyl (R-OH) at high pH



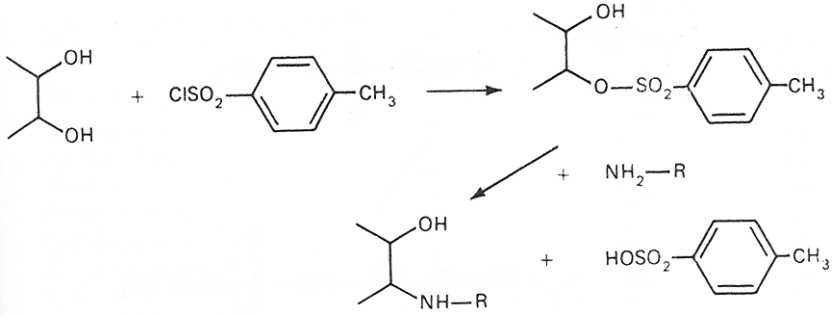


Types of Activated Matrix

- **Carbonyldiimidazole-activated matrix**
  - Reaction with primary amine
    - No generation of positive charge
    - Minimal spacing of urethane linkage
    - Less noxious than CNBr or epoxide

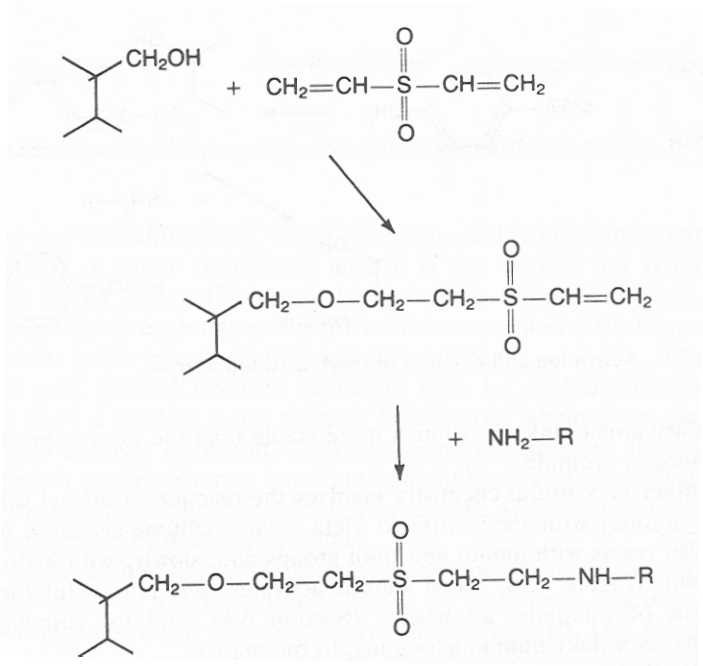


- **Tosyl (toluene sulfonyl chloride)-activated matrix**
  - Reaction with primary amine
    - Positive charge at neutral pH
    - No space introduction
    - Very stable linkage

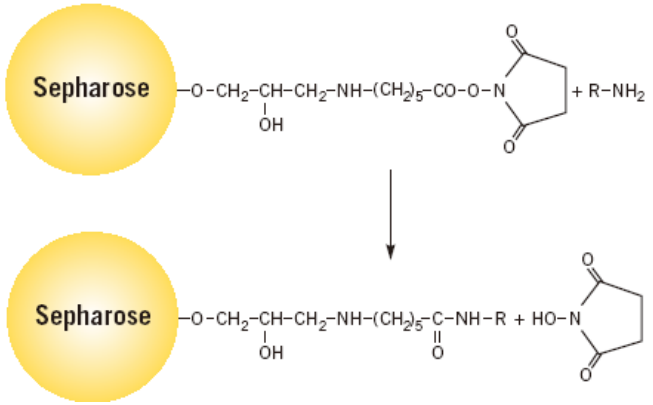


Types of Activated Matrix

- **Divinyl Sulfone**
  - Reaction with amino, thiol > hydroxyl group

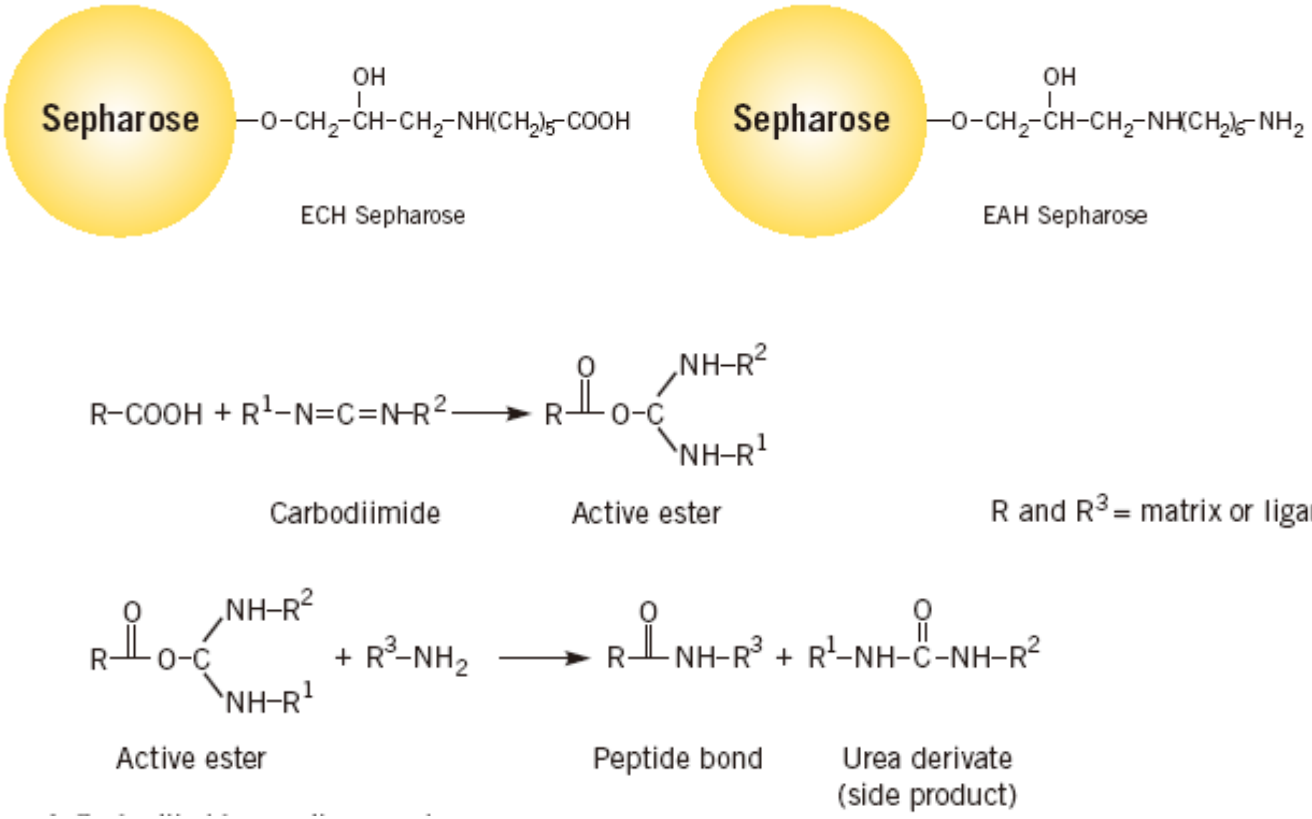


- **N-hydroxysuccinimide**
  - Reaction with primary amine
  - Very stable at high pH



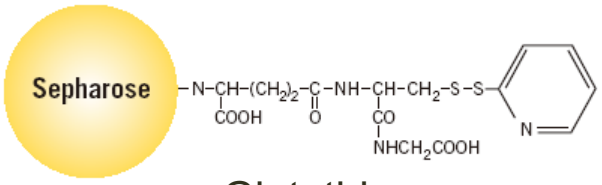
Types of Activated Matrix

- **EAH and ECH**
  - Reaction with carboxyl or amino group in the presence of coupling reagent carbodiimide

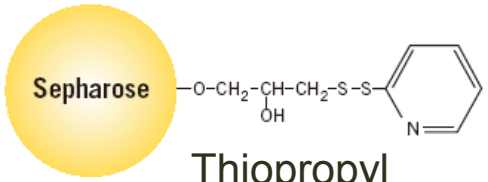


Types of Activated Matrix

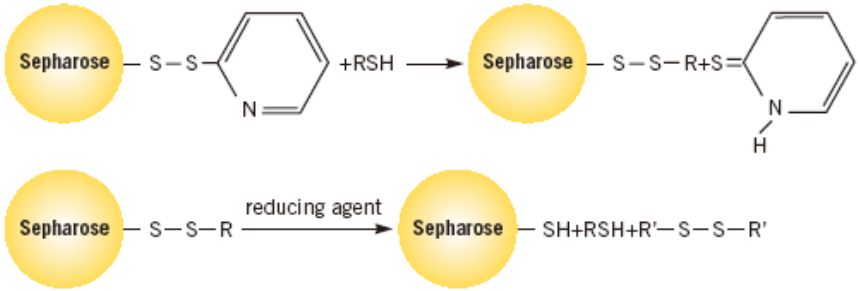
- **Thiolated matrix**
  - Binding to –SH by forming a mixed disulfide bond
  - Heavy metal ions and derivative → mercaptides
  - Alkyl or aryl halide ligands
  - Ligands containing C=O, N=N, and C=C udner certain conditions

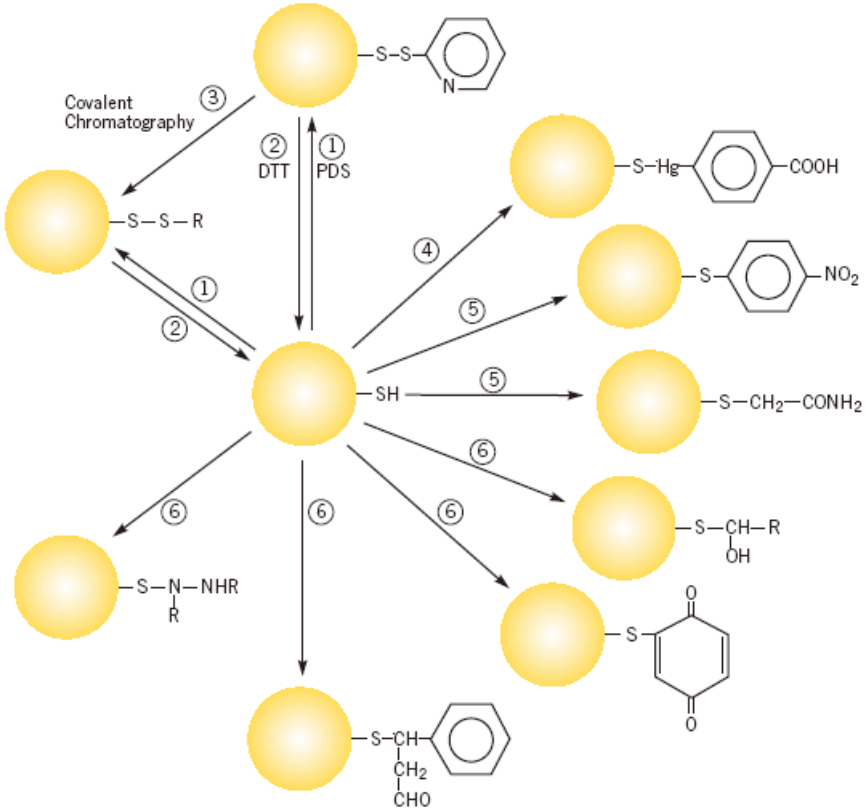


Glutathione



Thiopropyl



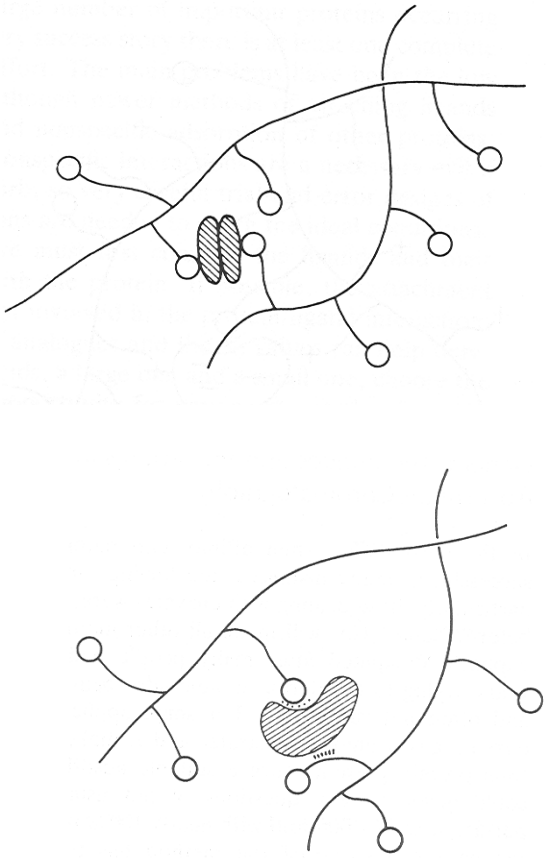


Coupling of Protein to Matrix

- **Through exposed Lys**
  - High pH (~9) ε-NH_2 of Lys to be nucleophilic
  - Should not inactivate protein
  - Vinyl sulfone-, tosyl-activated gels are fast acting in mild conditions
  - Should not use a buffer containing a primary amine (e.g. Tris buffer)
  - Use 0.1-0.2 M bicarbonate/carbonate buffer or borate buffer

Protein-Ligand Interaction in Affinity Chromatography

- **Interaction between protein and matrix**
  - Protein-ligand interaction
    - Mostly not strong enough for chromatography
    - Low-percentage occupancy
      - A small portion of the immobilized ligands are properly oriented
      - Exact spatial requirement for two point specific attachment to dimeric protein
      - Exact spatial requirement for additional nonspecific interactions
  - Nonspecific hydrophobic interaction
    - Spacer-protein

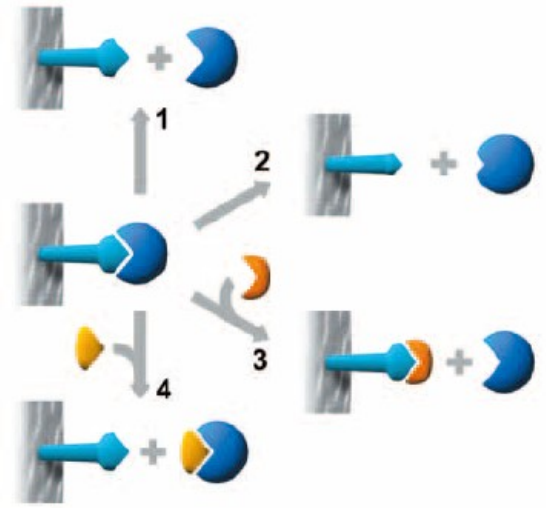


General Techniques and Procedures

- **Coupling of ligand to activated matrix**

Experimental condition	Recommended concentration for coupling
Readily available ligands	10–100 fold molar excess of ligand over available groups
Small ligands	1–20 μmoles/ml medium (typically 2 μmoles/ml medium)
Protein ligands	5–10 mg protein/ml medium
Antibodies	5 mg protein/ml medium
Very low affinity systems	Maximum possible ligand concentration to increase the binding

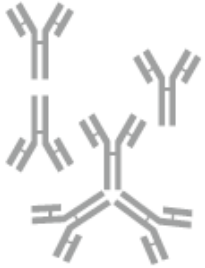
- **Application of sample**
  - Binding buffer
    - Charged affinity ligands
      - Use high ionic strength to prevent nonspecific ionic interaction
- **Elution**
  - Affinity elution
    - By free ligand or specific competitor
    - In combination with conditions reducing the binding affinity
  - Changing buffer composition
    - High salt
      - In some cases high salt can increase binding by increasing hydrophobic interaction
    - Extreme pH
    - Chaotropic agent : thiocyanate, urea
    - Dilution of the buffer



2. Immunoadsorbents

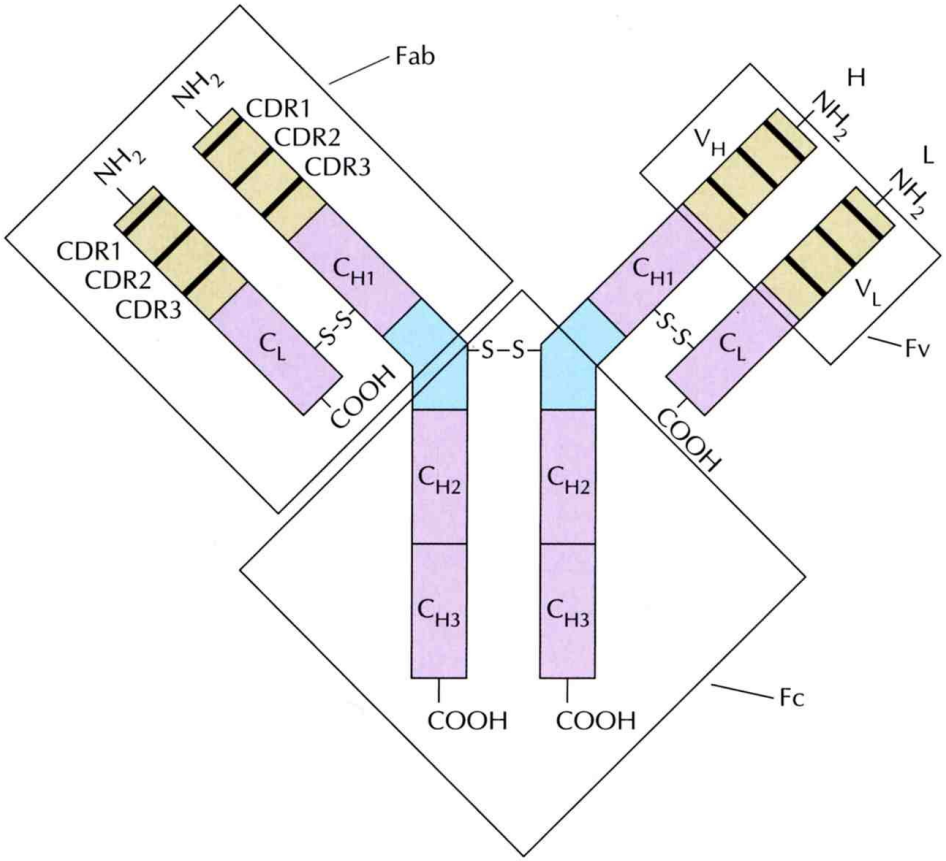
Structure of Antibody

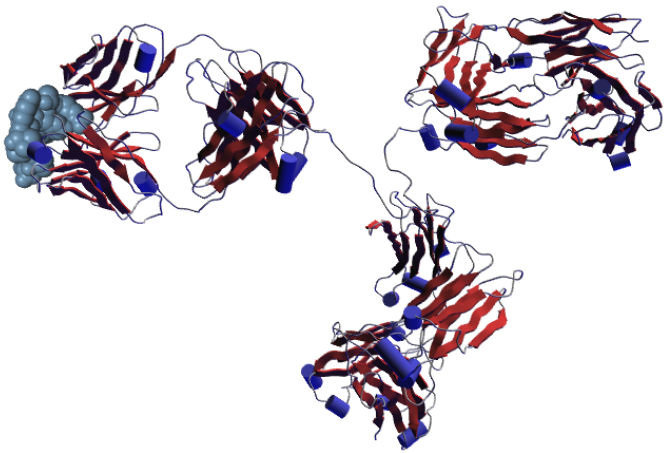
- **Immunoglobulin (150 kDa)**
  - Two heavy (H) chains and two light (L) chains
    - linked by H bond and disulfide linkage
  - Variable region (V_H and V_L)
    - Antigen recognition
    - Complementarity-determining regions (CDRs)
      - Recognition and binding antigens
      - The region with the greatest variability
  - Constant region (C_L and C_{H1}, C_{H2}, C_{H3})
- **Classes of antibody**

Characteristic	IgG	IgM	IgA	IgE	IgD
Heavy chain	γ	μ	α	ε	δ
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Y structure					

Structure of Antibody

- Proteolysis by papain
  - 2 Fab and Fc
- Fab (50 kDa) : L + V_H-C_{H1}
  - Fv (25 kDa) : N terminal part of Fab





Immunoadsorbents

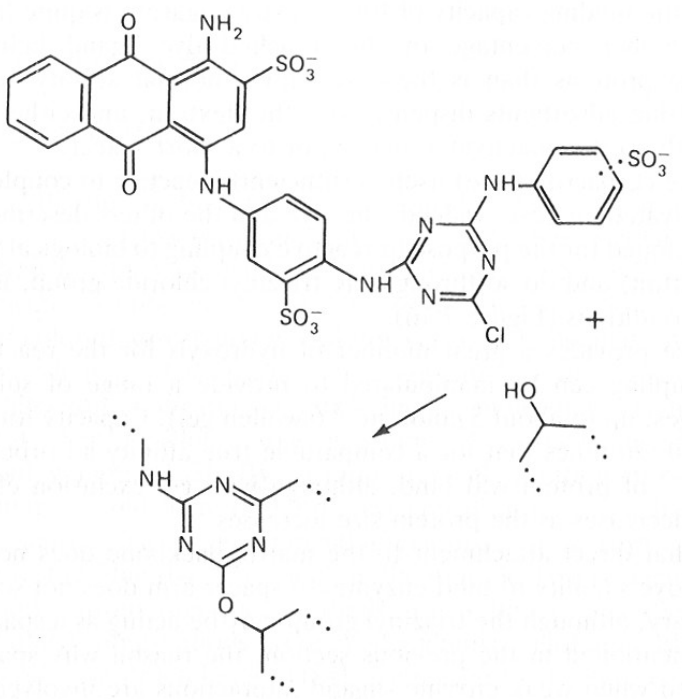
- **Principle**
  - Antibody as an adsrobent for a specific target protein
- **Procedure**
  - Binding of antibody to activated matrix
    - 1mg/ml capacity → only 0.1 mg/ml is effective for antigen binding
    - Slow Ag-Ab interaction
      - Use slow flow rate
  - Elution
    - Very tight binding → difficult to dissociate
      - K_p : 10^{-8}~10^{-12} M
    - Low pH : pH 2-3
    - Partial disruption of H-bonding and hydrophobic interactions
      - Urea or guanidine HCl
      - Organic solvents
      - Detergent
      - Ethylene glycol
      - Cold distilled water
    - Electrophoresis in solution
    - Cleavable spacer arm
      - Attachment of antibody through a phenyl ester linkage
      - Chemical cleavage with an imidazole-glycine buffer (pH 7.4)

3. Dye Ligand Chromatography

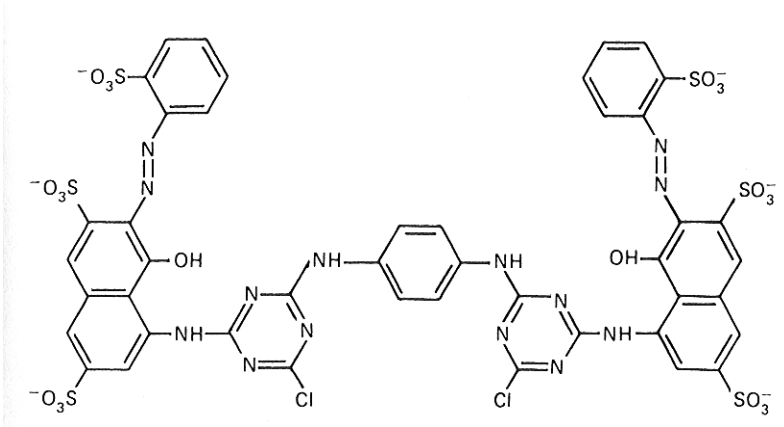
Dye Ligands

- **Dye ligand**
  - Pseudo affinity adsorbent
  - Aromatic ring and conjugated double bonds
  - Binding to targets
    - Specific : binding to natural ligand binding site with high affinity
    - Nonspecific
- **Types**
  - Tiazinyl chloride type
    - Cibacron Blue F3G-A
      - Binding to nucleotide fold
        - » AMP, IMP, ATP, NAD, NADP binding site
        - » High binding affinity : 10^{-6} M
          - » No need to additional interaction provided by spacer arm
      - Highly reactive
        - » Can be coupled directly to nonactivated agarose
      - Very high capacity
        - » 5 μmol/ml of dye ligand
        - » Up to 30 mg/ml protein binding
    - Procion Red H-E3B
      - Selective binding to NADP-binding proteins
    - And many others
  - Vinyl sulfone type

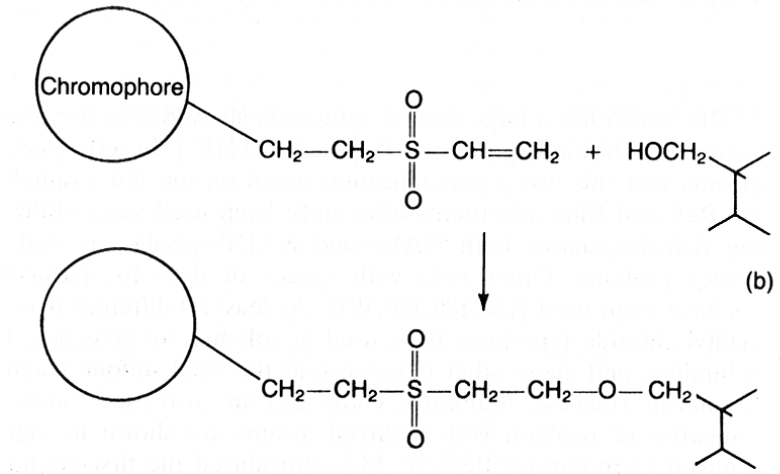
Types of Dye Ligands



Cibacron Blue F3G-A



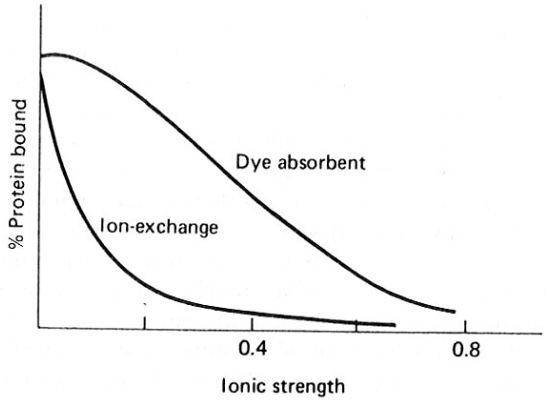
Procion Red H-E3B



Vinyl sulfone dye

Dye-Protein Interaction

- **Specific**
- **Nonspecific**
  - Electrostatic interaction of cation exchange type
    - Higher binding at low pH
      - Proteins are not necessarily to be basic to bind to the column
      - Interaction of protein to Π electrons in conjugate structures of the dye
    - Higher binding at 0-0.1M ionic strength compared with ion exchangers
  - Hydrophobic interaction
  - Hydrogen bonding



- **Other factors affecting binding affinity**
  - Phosphate
    - Sulfonate group binds to phosphate binding site of protein
      - Decrease in interaction in the presence of phosphate ion
  - Divalent metal ions
    - Increase in binding affinity
    - Cu, Ni, Co, Mn, Zn > Mg

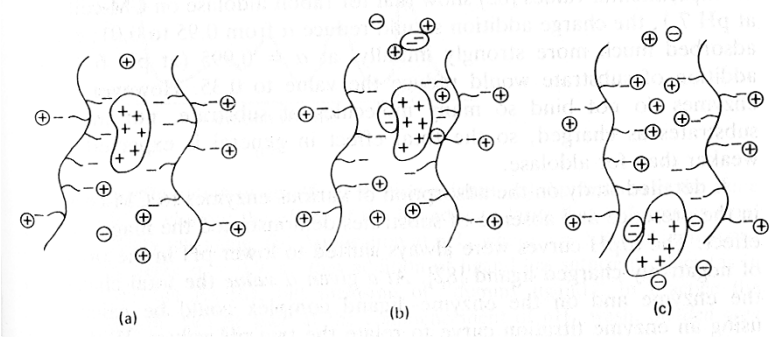
Dye Ligand Chromatography

- **Screening to obtain a suitable adsorbent**
  - Negative column
    - Binds many proteins but not the target protein
  - Positive column
    - Binds the target protein but little other proteins
  - Differential chromatography
    - Tandem application of negative and positive columns
- **Elution of proteins**
  - Affinity elution
    - Elution using specific ligand
  - Changing buffer conditions
    - Increasing salt concentration
    - Increasing pH
    - Other less popular methods
      - Decreasing hydrophobic interaction by solvent
      - Nonionic detergent
      - Changes in temperature

4. Affinity Elution from Ion Exchangers and Other Adsorbents

Affinity Elution from Ion Exchangers

- **Principle**
  - Binding of charged ligand to target protein
    - Decrease in net charge binding affinity of the protein to ion exchanger
  - Effect of ligand binding
    - Masking charges in the active site
      - Could be more effective than random masking of charges
    - Conformational change of protein



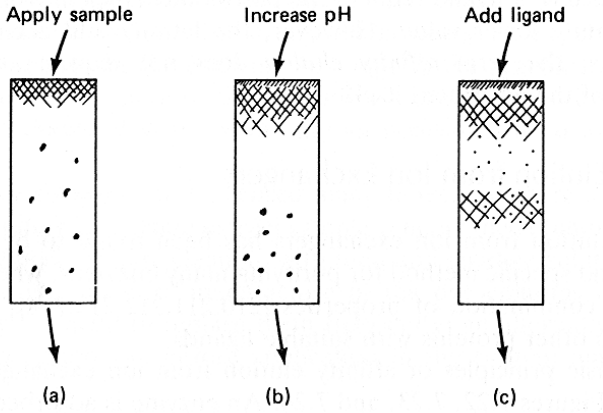
- **Requirements**
  - Adsorbents and ligand (mostly negative charge) should have the same charge
  - Ligand should be able to bind to the protein at the operating pH
  - The number of charges added by the ligand /100 kDa protein : > 4
  - Dissociation constant for the ligand : < 10^{-3} M, the smaller the better

Affinity Elution from Other Adsorbents

- **Phosphocellulose column**
  - Binding to nucleic acid-binding proteins
    - tRNA synthetase, hexokinase etc.
  - Affinity elution using phosphocellulose
- **Dye ligand column**
  - Elution by natural ligand
    - 10x excess of K_L
      - 1mM ATP or NAD^+, 0.2 mM NADH or $NADP^+$ for Cibacron Blue F3G-A column
  - Elution by other ligands
    - Usually charged ligands
- **Hydrophobic column**
  - Elution of lipid-binding protein by relevant lipid

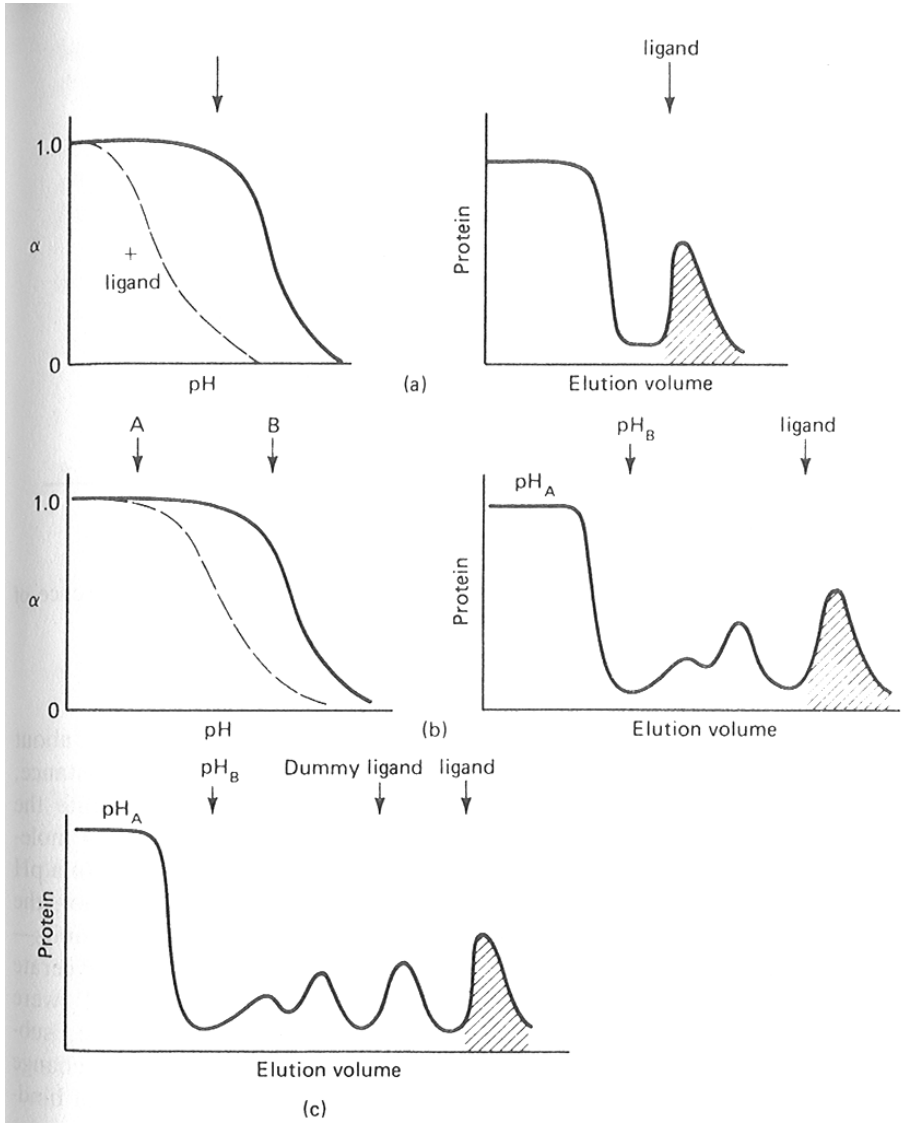
Practice and Theory of Affinity Elution

- **Affinity elution after reducing α**
  - Changing pH
  - Changing salt concentration



- **Dummy ligand**
  - Similar propertied to the real ligand
  - Elution of proteins which can be eluted by changing the buffer conditions (ionic strength, surface tension, pH) during elution by real ligand
  - Ligand :1 mM trivalent anion → dummy ligand: 1 mM EDTA, or sodium citrate

Affinity Elution from Ion Exchangers



Elution by

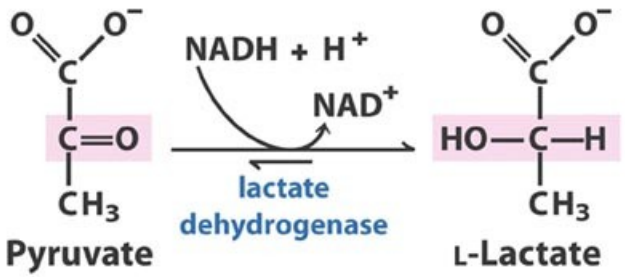
Ligand

Ligand
+ pH change

Ligand
+ dummy ligand
+ pH change

Affinity Elution of Lactase Dehydrogenase

- **Lactase dehydrogenase**
  - Tetramer
  - Binding of lactate or pyruvate only in the presence of NAD^+ of NADH

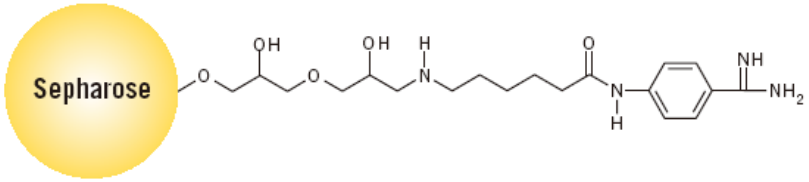


- **AMP affinity column**
  - Elution by low concentration (0.1 mM) of NAD^+ and 1 mM sulfite (SO_3^{2-}) (pyruvate analogue)
- **Oxamate (pyruvate analogue) affinity column**
  - Binding in the presence of NAD^+
  - Elution by removing NAD^+
    - Negative affinity elution
    - Elution in the absence of ligand

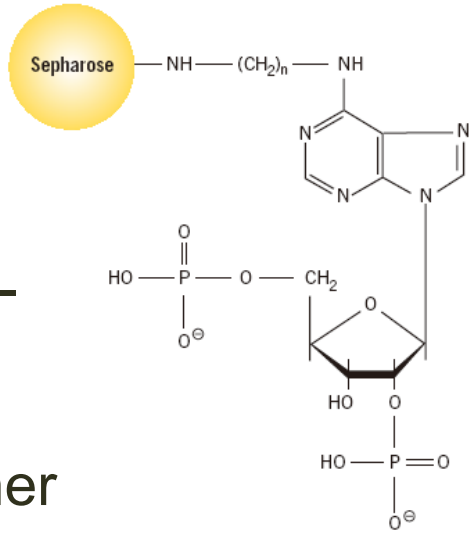
5. Commonly Used Affinity and Pseudo-Affinity Adsorbents

Small Ligands

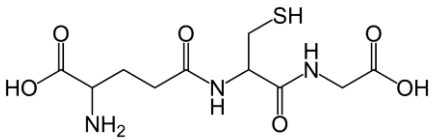
- **Bensamidine**
  - Potent inhibitor of trypsin
  - Purification of trypsin, trypsin-like protease, zymogens



- **5’ AMP,**
  - NAD^+-dependent dehydrogenase and ATP-dependent kinases
- **2’5’ ADP**
  - $NADP^+$-dependent dehydrogenase and other enzyme with affinity for $NADP^+$

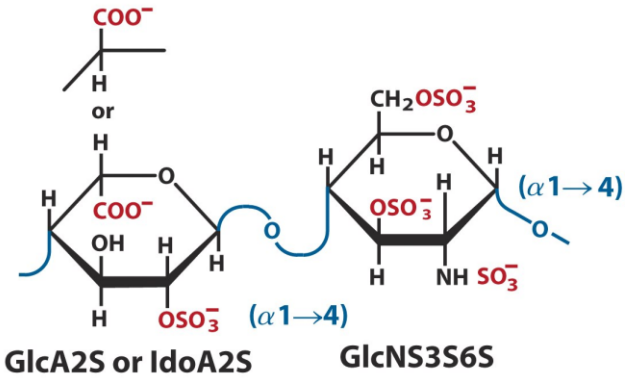


- **Glutathione**
  - Recombinant protein containing GST (glutathione S-transferase) tag



Biopolymer Ligands

- **Protein A and G**
  - Purification of antibody
- **Heparin**
  - Sulfate polysaccharides
  - Purification of DNA binding proteins
- **Lectins**
  - Purification of glycoproteins
  - e.g. Concanavalin A
- **DNA**
  - Purification of DNA binding protein
  - e.g. Calf thymus DNA, specific DNA sequence



Heparin, 15-90