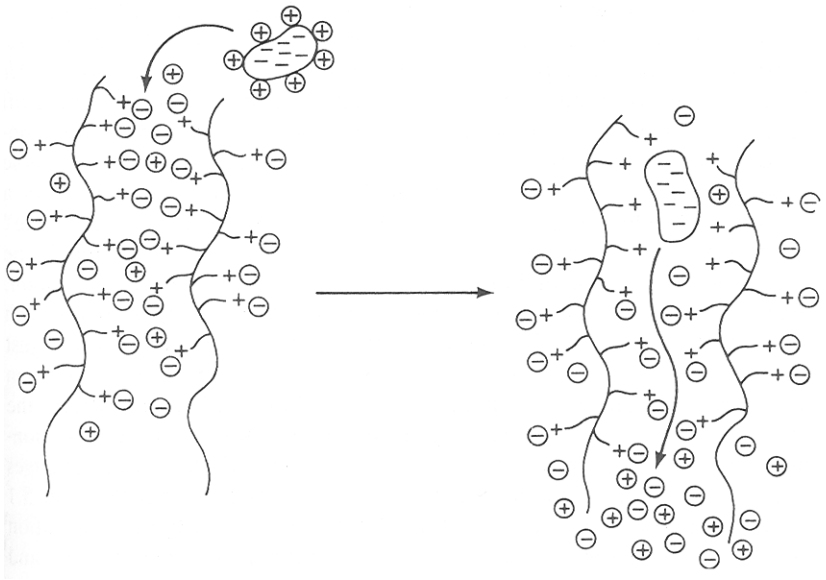
Separation by Adsorption II: Ion Exchangers and Nonspecific Adsorbents

1. Ion Exchangers-Principles, Properties, and Uses

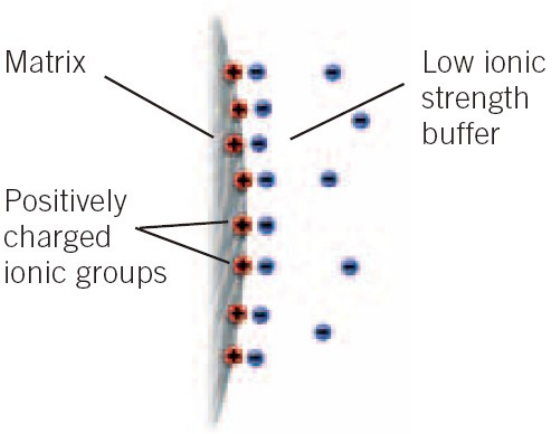
General Principles

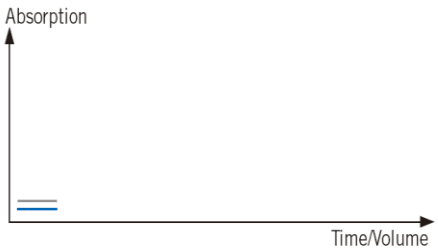
- **Ion exchangers**
  - Substitution level
    - 0.5 mmol/cm^{-3} ≈ 0.5 M of charged group
    - 15 nm distance between each charged group if the distribution is even
      - Diameter of 30 kDa protein ≈ 4 nm
  - Charges of adsorbents and proteins are balanced by couterions
    - Displacement of ions by charged protein → ion exchange



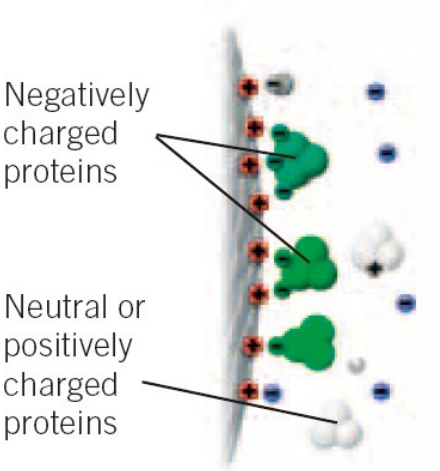
Principles of an Anion Exchange Separation

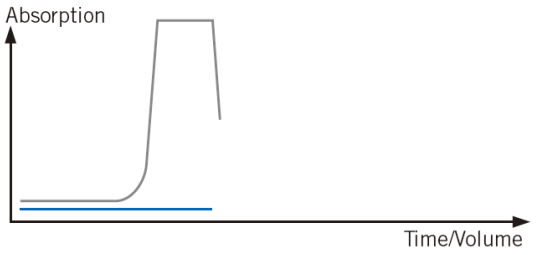
Equilibration



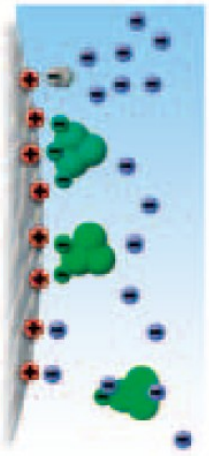


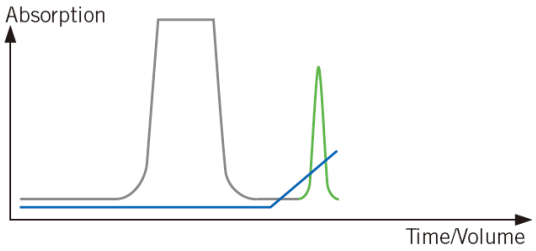
Sample application



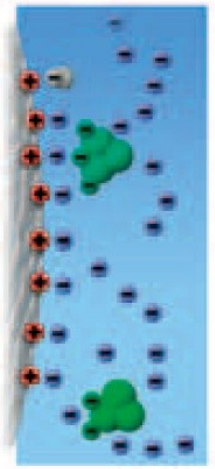


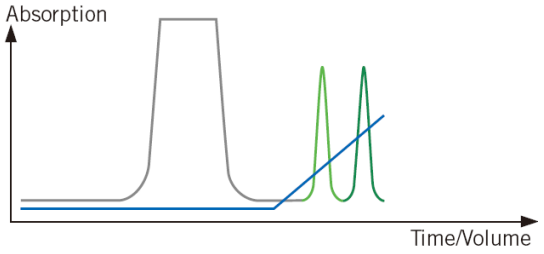
Elution 1





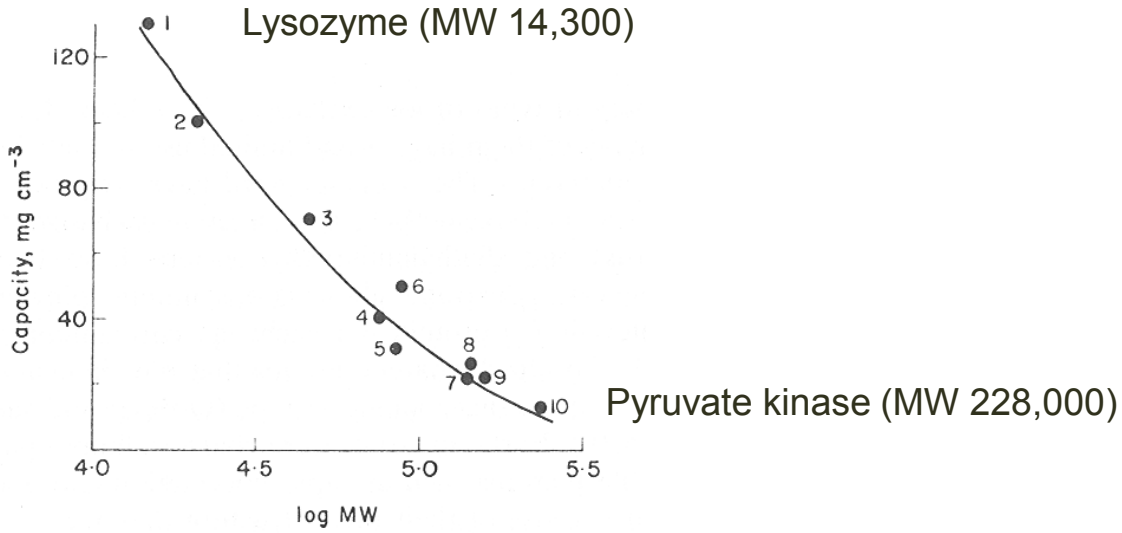
Elution 2





Adsorptive Capacities of Ion Exchangers

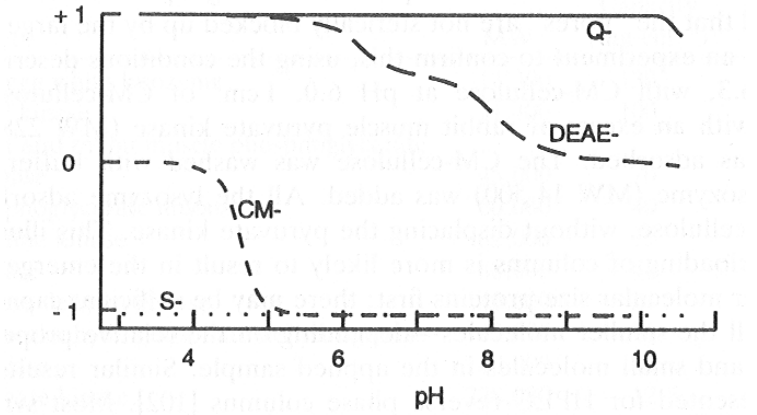
- **Capacities of ion exchangers**
  - Very high
  - Higher capacity for smaller proteins
  - Large molecules
    - Can bind only to the surface
    - MW> 10^6 : excluded from most cellulose-based ion exchangers



Capacity of CM-celluose

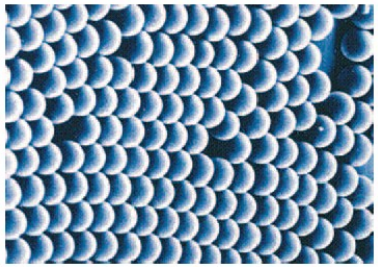
Types of Ion Exchangers

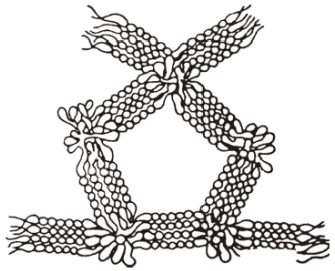
- **Charge**
  - Plus or minus
  - The nature of the group
    - Cation exchangers
      - Carboxylmetyl (CM): weak
      - Sulfopropyl (S-, SP): strong
    - Anion exchangers
      - DEAE : weak
      - Quaternaty amino group (Q-): strong



- **The nature of the matrix particles**
  - Bead size, flow rate required under pressure, capacity, cost

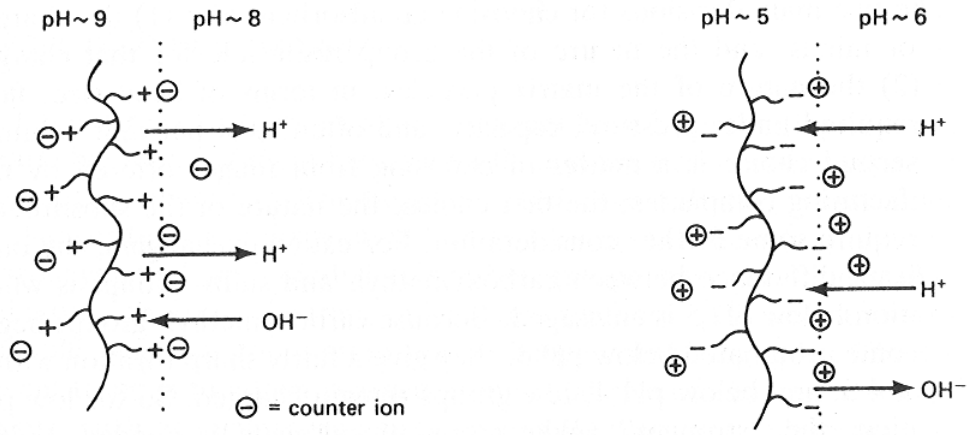
	Form	Mean particle size
MiniBeads™	Polystyrene/divinyl benzene	3 µm
MonoBeads™	Polystyrene/divinyl benzene	10 µm
SOURCE 15	Polystyrene/divinyl benzene	15 µm
SOURCE 30	Polystyrene/divinyl benzene	30 µm
Sepharose High Performance	Agarose 6%	34 µm
Sepharose Fast Flow	Agarose 6%	90 µm
Sepharose 4 Fast Flow	Agarose 4%	90 µm
Sepharose XL	Agarose 6%, dextran chains coupled to agarose	90 µm
Sepharose Big Beads	Agarose 6%	200 µm





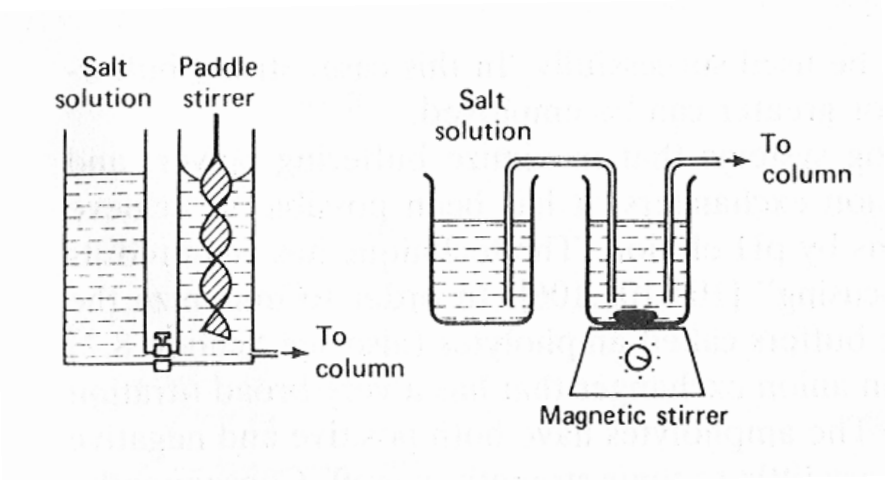
pH and Donnan Effects

- **Donnan effects**
  - Change in pH in the microenviroment of an ion exchanger
    - Anion exchanger
      - Increase in pH
    - Cation exchanger
      - Decrease in pH
  - Larger difference for buffer with lower ionic strength
  - Can affect stability of pH-sensitive proteins
    - Maximize buffering power to prevent this effect



Elution of Adsorbed Protein

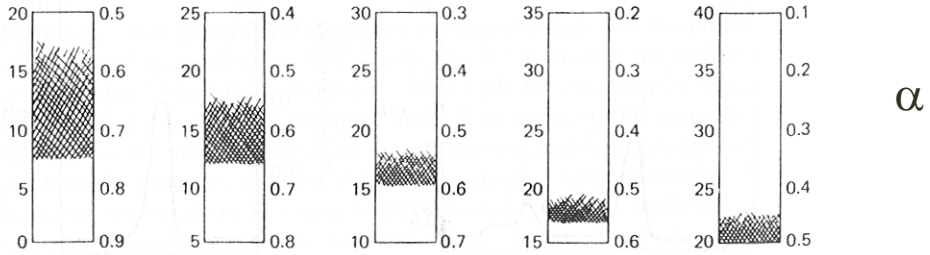
- **Changing buffer pH**
  - Reduce binding by changing the net charge on a protein
    - Lower pH for an anion exchanger
    - Higher pH for an cation exchanger
  - Can involve working near the pI of the target protein
    - Protein can be aggregated
  - Generally not very successful
- **Salt gradient**
  - Weakening electrostatic interaction between protein and adsorbent by increasing ionic strength
  - Linear KCl or NaCl gradient upto 1M



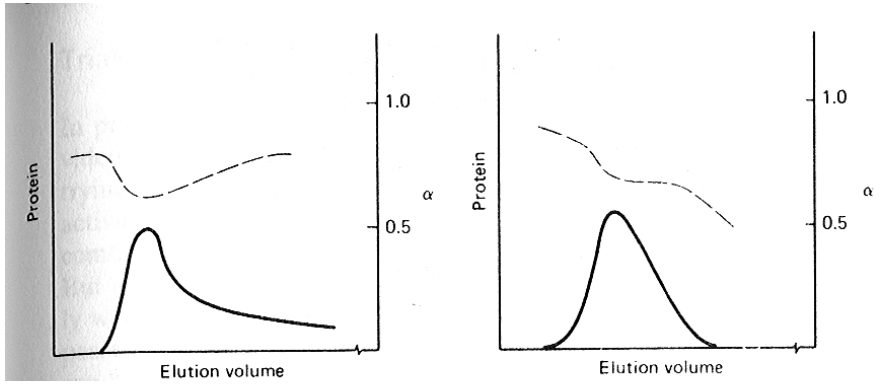
Elution by Salt Gradient

- **Band sharpening by salt gradient**
  - Higher salt concentration → lower α → faster movement along the column
  - Faster mobility at the rear of the band → band sharpening

Salt conc. (mM)



α



No gradient

With gradient

2. Ion Exchange Chromatography- Practical Aspects

Charged Groups in a Protein

- **Positive charge**
  - N-terminal NH^{3+}
  - Arg, Lys (pH< 8.5)
  - His : $pK_R = 6$
- **Negative charge**
  - C-terminal COO^-
  - Asp, Glu
  - Cys (pH>8)
- **Histidine**
  - Mainly responsible for the pH-dependent variation of protein net charge in the normal pH range (pH 5-9)
  - Protein with few His
    - Little variation over a wide pH range
  - Protein with high His content
    - Large pH-dependent variation

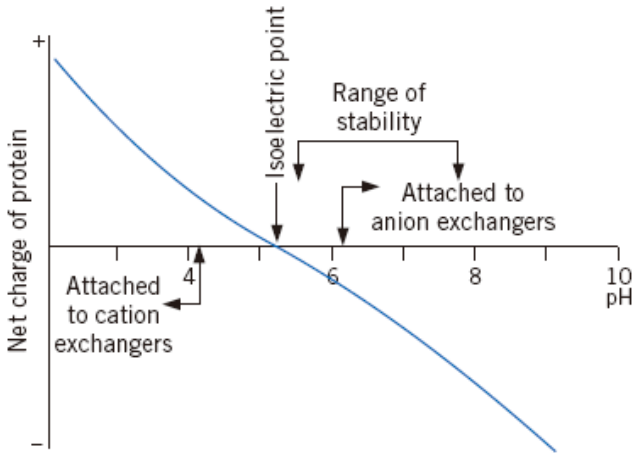
TABLE 3–1 Properties and Conventions Associated with the Common Amino Acids Found in Proteins

Amino acid	Abbreviation/ symbol	M_r*	pK_a values: pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R (R group)	pI	Hydropathy index†	Occurrence in proteins (%)‡
Nonpolar, aliphatic R groups								
Glycine	Gly G	75	2.34	9.60		5.97	−0.4	7.2
Alanine	Ala A	89	2.34	9.69		6.01	1.8	7.8
Proline	Pro P	115	1.99	10.96		6.48	1.6	5.2
Valine	Val V	117	2.32	9.62		5.97	4.2	6.6
Leucine	Leu L	131	2.36	9.60		5.98	3.8	9.1
Isoleucine	Ile I	131	2.36	9.68		6.02	4.5	5.3
Methionine	Met M	149	2.28	9.21		5.74	1.9	2.3
Aromatic R groups								
Phenylalanine	Phe F	165	1.83	9.13		5.48	2.8	3.9
Tyrosine	Tyr Y	181	2.20	9.11	10.07	5.66	−1.3	3.2
Tryptophan	Trp W	204	2.38	9.39		5.89	−0.9	1.4
Polar, uncharged R groups								
Serine	Ser S	105	2.21	9.15		5.68	−0.8	6.8
Threonine	Thr T	119	2.11	9.62		5.87	−0.7	5.9
Cysteine§	Cys C	121	1.96	10.28	8.18	5.07	2.5	1.9
Asparagine	Asn N	132	2.02	8.80		5.41	−3.5	4.3
Glutamine	Gln Q	146	2.17	9.13		5.65	−3.5	4.2
Positively charged R groups								
Lysine	Lys K	146	2.18	8.95	10.53	9.74	−3.9	5.9
Histidine	His H	155	1.82	9.17	6.00	7.59	−3.2	2.3
Arginine	Arg R	174	2.17	9.04	12.48	10.76	−4.5	5.1
Negatively charged R groups								
Aspartate	Asp D	133	1.88	9.60	3.65	2.77	−3.5	5.3
Glutamate	Glu E	147	2.19	9.67	4.25	3.22	−3.5	6.3

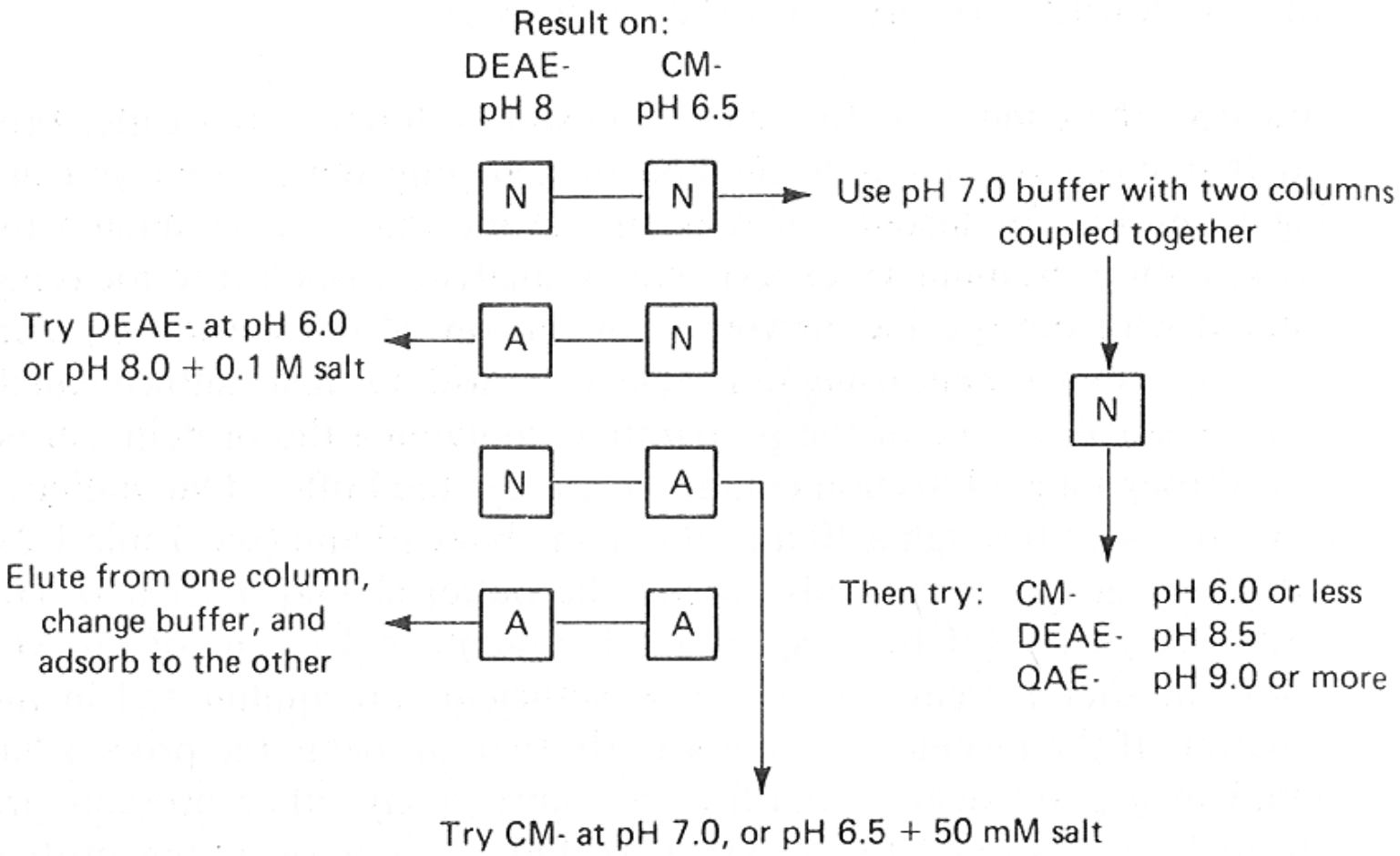
Choice of Ion Exchanger

- **pH-related features of enzyme**
  - Isoelectric point
  - Optimum pH for activity
  - pH stability range
- **Choice of buffer pH**
  - Anion exchanger
    - 0.5 to 1.5 pH unit above the pI of the target protein
  - Cation exchanger
    - 0.5 to 1.5 pH unit below the pI of the target protein
  - Not all protein follow the rule
    - Non-electrostatic interaction
    - Distribution of the charged group can affect binding affinity

pI	Ion exchange	Buffer pH
8.5	Cation	< 7.0
7.0	Cation	< 6.0
	Anion	> 8.0
5.5	Anion	> 6.5



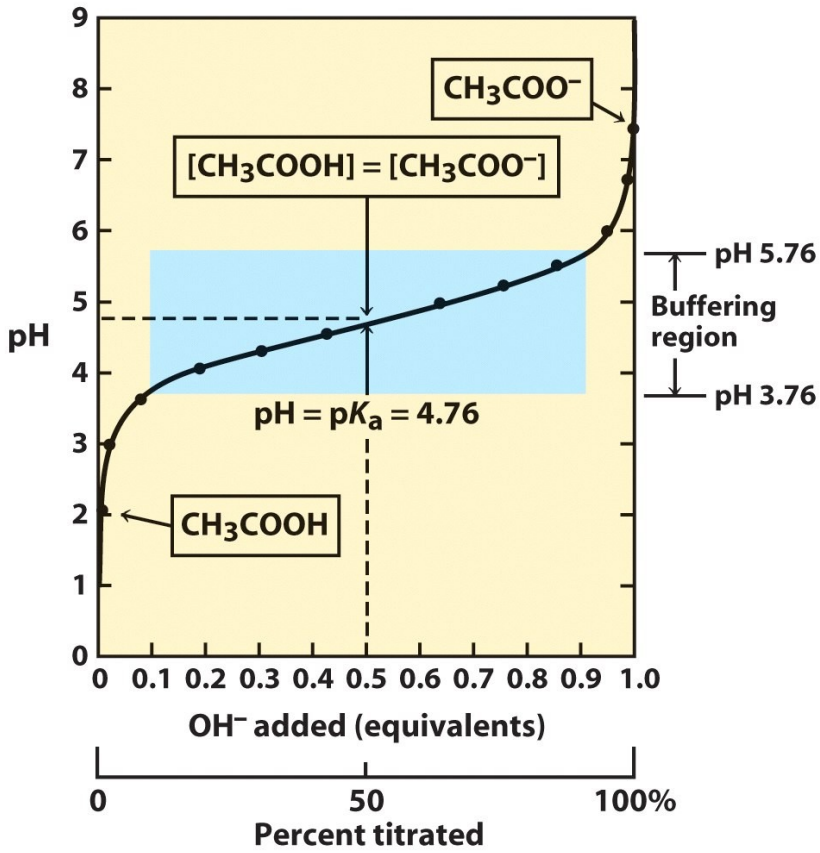
Trials to Determine Ion-Exchange Behavior



Buffers for Use in Ion-Exchange Chromatography

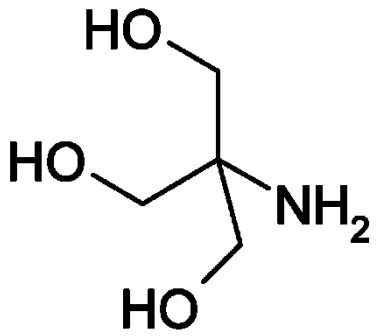
- **Do not use buffering ions interacting with adsorbents**
  - Charged form of buffer with the same sign of the adsorbents
  - Anion exchangers
    - Cl^-, acetate
    - EDTA (polyanionic complex) may compete with protein binding
  - Cation exchangers
    - K^+, Na^+, Mg^{2+}, $HTris^+$ for pH<7

- **Maximizing buffering power at the least possible ionic strength**
  - To ensure adsorption of a weakly binding protein
  - Rules
    - pK_a of the buffer : should not be 0.5 (preferably 0.3) unit away from the pH being used
    - One of the buffering species should be uncharged
      - No contribution to ionic strength



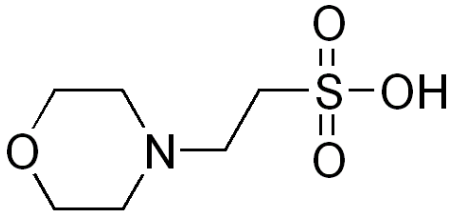
Buffers for Use in Ion-Exchange Chromatography

- **For anion exchangers at pH 8.0**
  - Tris-HCl buffer : Good
    - pK_a of Tris : 8.1 at 25 ^{o}C
    - Couterion : Cl^-
    - Buffering species
      - $HTris^+$ (noninteractive) and Tris (neutral)
  - Phosphate buffer : Nod recommended
    - Acting as counter ions
    - Buffering species
      - HPO_4^{2-} and $H_2PO_4^-$ (not neutral)



Tris(hydroxymethyl) aminomethane

- **For cation exchangers at pH 6.5**
  - K-Mes buffer
    - pK_a : 6.2
    - Counterion: K^+
    - Buffering species
      - HMes (neutral) and Mes^-



2-(*N*-morpholino)ethanes ulfonic acid

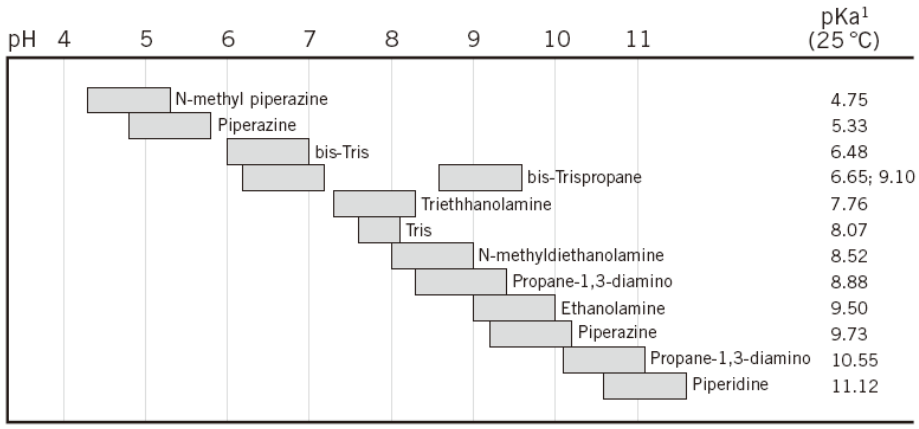
Buffering Power

- **Buffering power**
  - The amount of acid (for pH<pK_a) or alkali (for pH>pK_a) required to completely titrate the buffering species (unit: mM)
  - Buffering power / ionic strength
    - Monovalent > divalent
- **pK_a is dependent on temperature and ionic strength**

- z = charge on a given species
- $n = 2z\text{-}1$, z =charge on the acidic buffer form
- I, ionic strength = $\frac{1}{2}\sum c_i(z_i)^2$

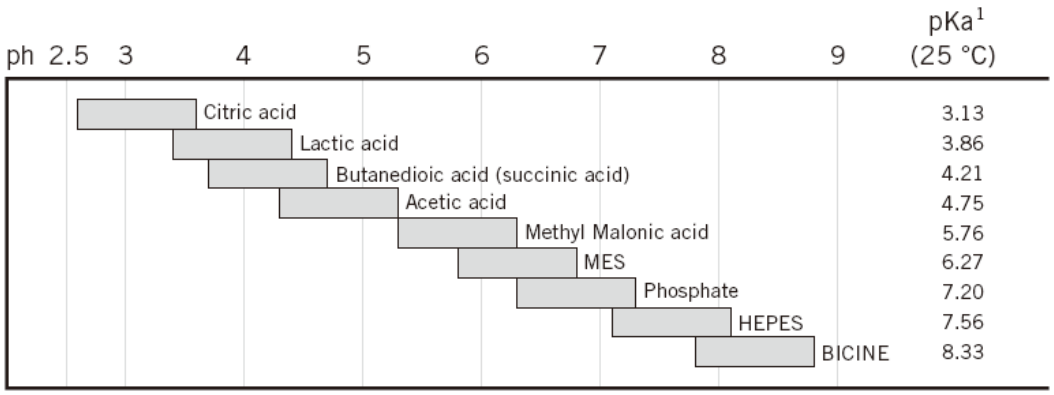
Buffer	pH	Buffering power for $I = 0.01$	Total buffer concentration for $I = 0.01$	Buffering power per mM of buffer
Acetate	4.2	10	42	0.24
	4.4	10	30	0.33
$n = -1$	4.7[a]	10	20	0.50
	5.0	5	15	0.33
	5.2	3	13	0.24
Imidazole	6.5	3	13	0.24
	6.7	5	15	0.33
$n = +1$	7.0[a]	10	20	0.50
	7.3	10	30	0.33
	7.5	10	42	0.24
Phosphate	6.5	1.7	6.7	0.24
	6.7	2	6	0.33
$n = -3$	7.0[a]	2.5	5	0.50
	7.3	1.4	4.2	0.33
	7.5	0.9	3.0	0.24
Tris	7.6	3	13	0.24
	7.8	5	15	0.33
$n = +1$	8.1[a]	10	20	0.50
	8.4	10	30	0.33
	8.6	10	42	0.24

Buffers for Anion Exchangers



pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl^-	6.04	
6.0–7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Tris propane	20	Cl^-	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	-0.020
7.6–8.6	Tris	20	Cl^-	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4	Cl^-	8.88	-0.025
		50 at pH 8.8			
8.4–9.4	Propane 1,3-Diamino	20	Cl^-	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2–10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0–11.0	Propane 1,3-Diamino	20	Cl^-	10.55	-0.026
10.6–11.6	Piperidine	20	Cl^-	11.12	-0.031

Buffers for Cation Exchangers



pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
1.4–2.4	Maleic acid	20	Na^+	1.92	
2.6–3.6	Methyl malonic acid	20	Na^+ or Li^+	3.07	
2.6–3.6	Citric acid	20	Na^+	3.13	-0.0024
3.3–4.3	Lactic acid	50	Na^+	3.86	
3.3–4.3	Formic acid	50	Na^+ or Li^+	3.75	+0.0002
3.7–4.7; 5.1–6.1	Succinic acid	50	Na^+	4.21; 5.64	-0.0018
4.3–5.3	Acetic acid	50	Na^+ or Li^+	4.75	+0.0002
5.2–6.2	Methyl malonic acid	50	Na^+ or Li^+	5.76	
5.6–6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7–7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0–8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8–8.8	BICINE	50	Na^+	8.33	-0.0180

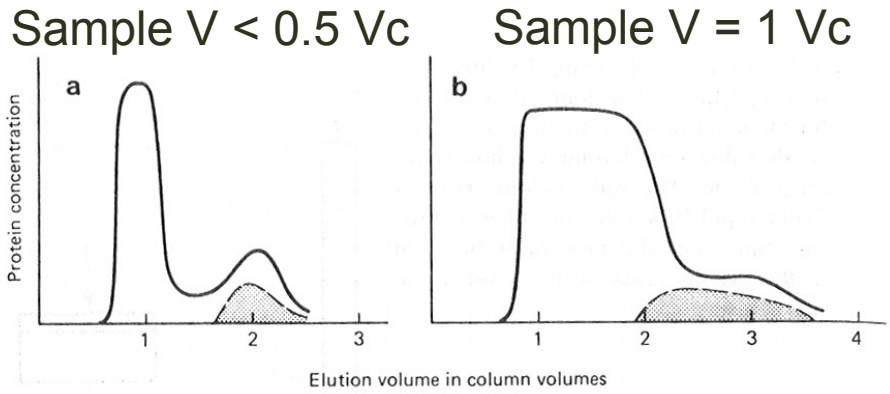
Conditions of Adsorption

- **Sample application**
  - Adjusting the sample (pH, ionic strength) to the start buffer equlibrating the column
    - Dialysis
    - Desalting by gel filtration
  - Protein concentration
    - Should not exceed 50-70 mg/ml
    - Apply 30% of binding capacity
      - Capacity is dependent on various factors including molecular weight of proteins
- **Size and dimensions of the column**
  - Example
    - 1g protein in 100 ml buffer → apply to 100-200 ml column
  - Column shape
    - For high loading, column shape does not affect plate number
    - Long, thin column
      - Slow flow rate, even out uneven flow
    - Squat column
      - Fast flow rate, flow should be even for high resolution
      - Useful for scale-up

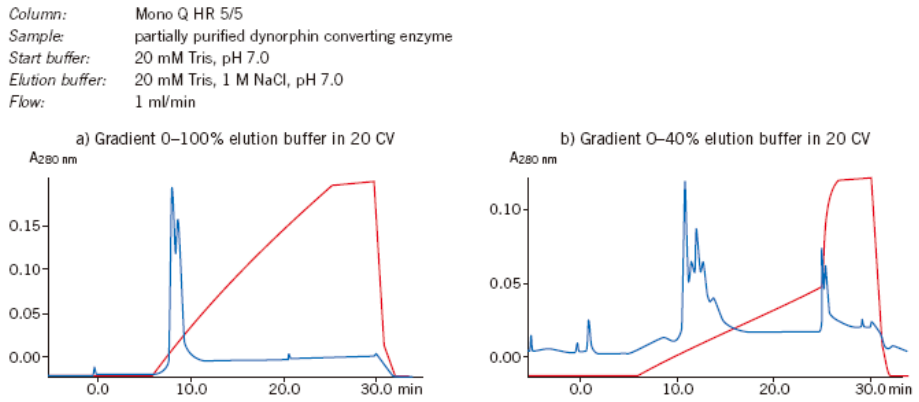
Elution

- **α=0**
  - Collected in the flow through fraction
- **0.5< α<0.9**
  - Can be collected without elution step

α=0.5



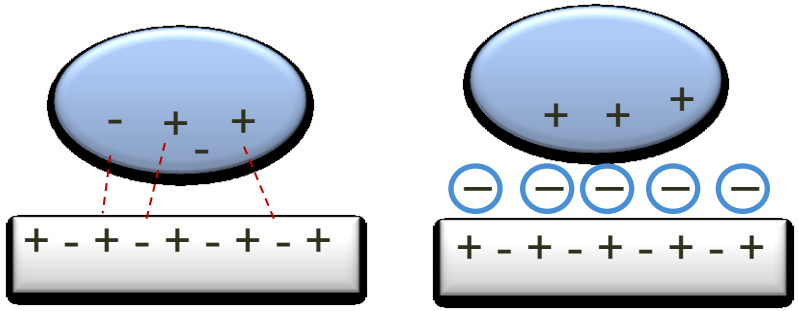
- **α> 0.9**
  - Elution by salt gradient
    - Anion exchangers
      - Upto 0.5 ~ 1 M
    - Cation exchangers
      - Upto 0.5 M is enough
  - Stepwise elution
    - Long tailing



3. Inorganic Adsorbents

Inorganic Adsorbents

- **Principle**
  - Binding to protein by polar dipole-dipole interaction
    - Adsorption to protein between pH 6 and 9
  - In the presence of phosphate buffer
    - Masking of positively charged surface
    - Similar behavior to ion exchanger
- **Types**
  - Alumina gel Cγ (gel and crystalline)
  - Bentonite (silicaceous powder)
  - Calcium phosphate
    - Aged gel
    - Hydroxyapatite
  - Titanium oxide
  - Zinc hydroxide gel
- **Advantage**
  - Cheap



Hydroxyapatite and Calcium Phosphate Gels

- **Calcium phosphate gel**
  - Mixing calcium chloride with tribasic sodium phosphate
  - Washing the gelatinous precipitate with water
  - Age for several months
  - Useful for batch method
- **Hydroxyapatite**
  - 0.5 M $CaCl_2$ + Na_2HPO_4 + 1M NaCl

  → Brushite : $CaHPO_4 \cdot 2H_2O$

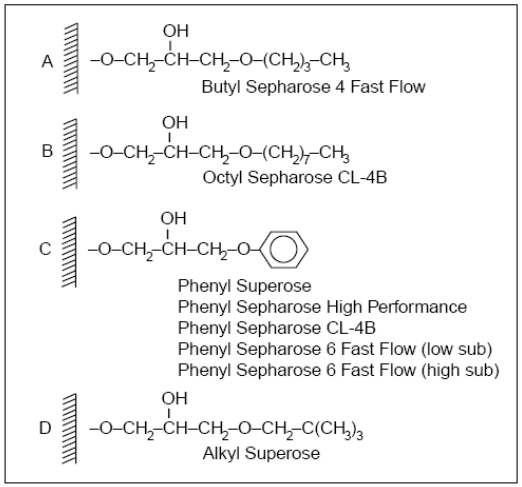
  → boiling with NaOH

  → hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$
  - No protein penetration
    - Limited capacity: ~ 0.1 mg/cm^{-3} on the surface of 0.1 mm diameter particle
  - Elution
    - High phosphate concentration
    - KCl , NaCl or $CaCl_2$ for basic proteins
      - Acidic protein: binding to Ca^{2+}
      - Basic protein: binding to PO_4^{n-}

4. Hydrophobic Adsorbents

Hydrophobic Adsorbents

- **Types of adsorbents**
  - Linear aliphatic chains
    - C6, C8, C10
  - Phenyl group
  - Chains containing a terminal amino group
    - Originally designed for affinity ligand attachment
    - Additional interaction with protein

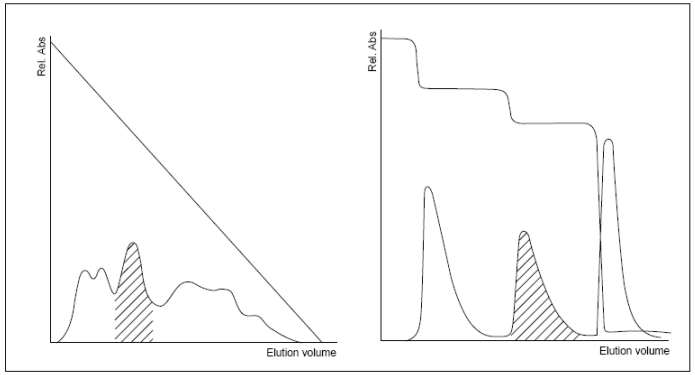


- **Principle**
  - Hydrophobic interaction in the presence of high salt
    - Similar to salting out
  - Low resolution
    - No sharp change in α depending on conditions
    - Slow association and dissociation : not in equilibrium mode
    - Similar proteins have tendency to aggregate
  - Advantages
    - High capacity : 10 to 100 mg/ml
    - Binding at high salt concentration : addition of enough salt to the sample without changing the buffer
    - Good recovery because of stabilizing effect of salt

Application of Sample

- **Application of sample**
  - Sample preparation
    - Add high salt : ammonium sulfate (0.5 M) of chloride salts (1 to 2 M)
  - Adjusting binding affinity
    - Salt concentration
    - Different adsorbents
    - Lowering pH increases binding affinity
      - Groups with π electron orbitals (aromatic group)

      : stronger interaction with positive charged region of protein



- **Elution**
  - Lowering salt concentration
    - Gradient or step-wise elution
  - Decreasing binding affinity
    - Lowering temperature
    - Inclusion of organic solvent : 30% i-propanol
    - Inclusion of polyols : ethylene glycol
    - Inclusion of detergent : 1% Triton X-100
    - Increasing pH

Reverse Phase Chromatography

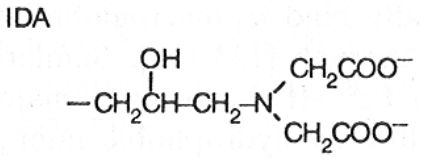
- **Adsorbents**
  - Aliphatic chains C_8-C_{18}
- **Sample application**
  - In an aqueous solvent, often a dilute acid
  - Denaturation of protein on adsorption
- **Elution**
  - Gradient of miscible organic solvent
    - Methanol, acetonitrile
- **Advantage**
  - Very high resolution
  - Useful for peptide purification

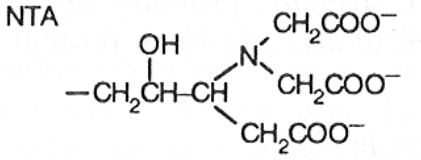
5. Immobilized Metal Affinity Chromatography (IMAC)

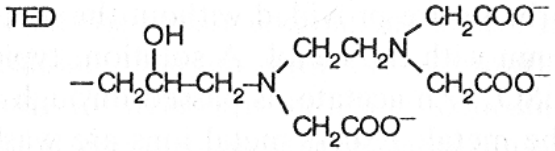
IMAC

- **Principle**
  - Formation of weak coordinate bonds between immobilized metal ions and basic groups on protein (His>> Trp, Cys)
  - Acts like affinity chromatography
  - Useful for purification of recombinant protein tagged with His

- **Adsorbents**
  - Metal chelators
    - Imino diacetate structure (IDA)
    - Binding to ions of the transition metals
      - – Divalent: Fe, Co, Ni, Cu, Zn
      - – Trivalent: Fe, Al
        - » Fe^{3+}
        - selective for phosphorylated proteins







Operating Conditions for IMAC

- **Loading up the column with metal**
  - Saturation of the column with 50 mM metal salt ($CuSO_4$, Zn acetate etc.)
  - Washing out unbound metals
    - 1-10 mM imidazole or 0.5 M glycine
- **Operation at high salt (1M NaCl) to prevent ion exchange effect**
  - Provide additional hydrophobic interaction
- **Elution**
  - By stronger complexing agent : imidazole, EDTA
  - Lowering pH of the buffer
    - Protonation of His
- **Stripping**
  - With strong EDTA solution