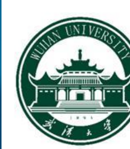

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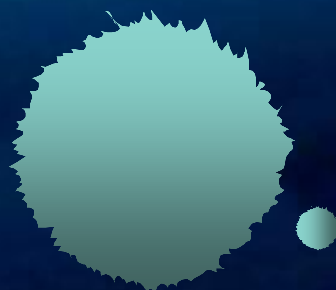
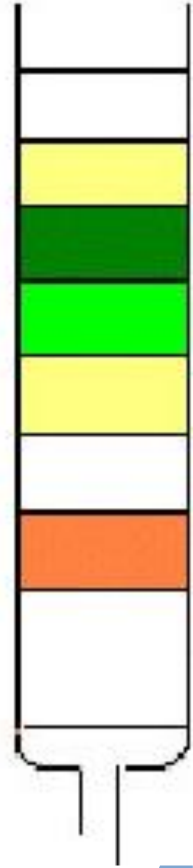
Chromatography

层析/色谱



Chromatography

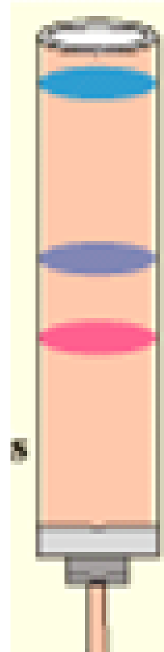
- Is a technique used to separate and identify the components of a mixture
- The separation is based on their differences of physical and chemical properties including absorption capacity, solubility, molecular shape and size, molecular polarity and binding affinity for a particular ligand.



Principle of Chromatography

There are two phases for different types of chromatography, stationary phase and mobile phase.

- ***Stationary phase*** – be kept stationary state, the part of the apparatus that does not move with the sample;
- ***Mobile phase*** – refers to a phase which flows over or through the stationary phase, gas or liquid that carries the components.



Components distributing preferentially in the mobile phase will move faster than those distributing preferentially in the stationary phase.

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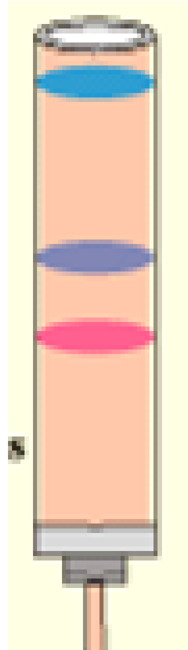
For example:

- When a protein is applied to the column, it **equilibrates** between the stationary phase and the mobile phase as it passes through the column.



Proteins that are more tightly bound to the stationary phase require a stronger mobile phase to elute them from the column.

The classification

Physical nature of the mobile phase
- *gas C*
- *liquid C*

Mode of the stationary phase
- *column C*
- *thin layer C*
- *paper C*
- *film C*

Distribution style

Principle of chromatography
- *adsorption C*
- *partition C*
- *Ion exchange C*
- *gel C*
- *affinity C*

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Chromatographic System

(an autosampler with a pump)

Buffer Reservoir

Column (containing stationary phase)

computer / Recorder

(detector) UV monitor

Fraction Collector

Basic components of column chromatography

Types of chromatography

--on the basis of interaction of the analyte with stationary phase

Adsorption C – solute adsorbed on surface of stationary phase; for polar non-ionic compounds

Ion Exchange C– attraction of ions of opposite charges; for ionic compounds anions or cations

Partition C – based on the relative solubility of an analyte in mobile and stationary phases

Size Exclusion (gel filtration, gel permeation) – separates molecules by size; sieving - not real interaction

Affinity C – specific interactions, like a particular antibody to protein

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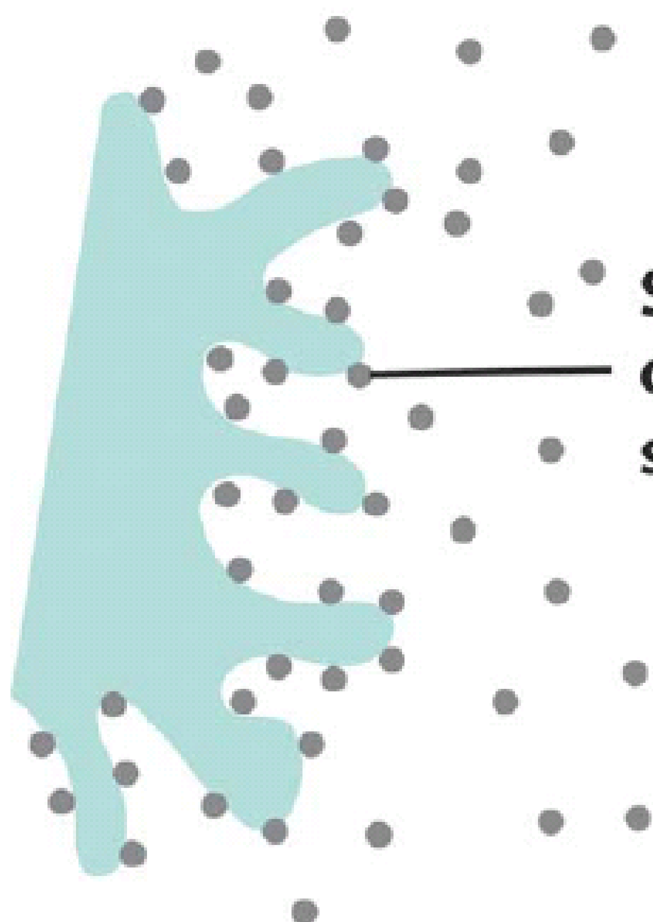
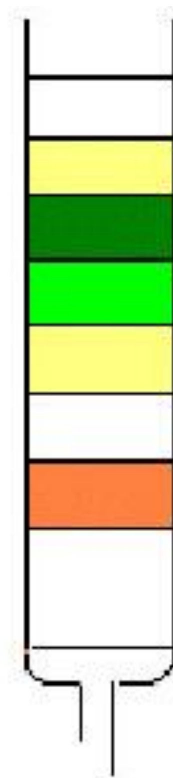


Solute adsorbed
on surface of
stationary phase



1. 吸附剂
2. 叶黄素
3. 叶绿素
4. 浅绿色色素
5. 叶黄素
6. 吸附剂
7. 橙黄色色素
8. 吸附剂

叶绿素通过碳酸钙管柱所形成
（淋洗液：石油醚）

Adsorption chromatography

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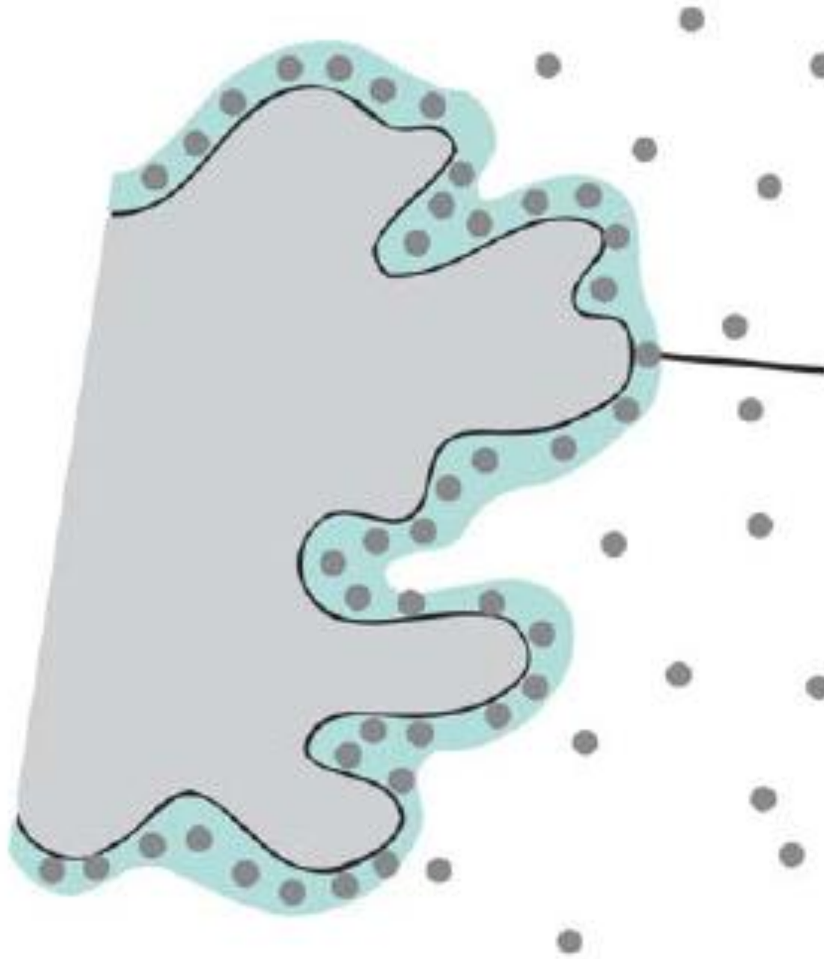
The molecule with higher kd retain more in the stationary phase and flow slowly.



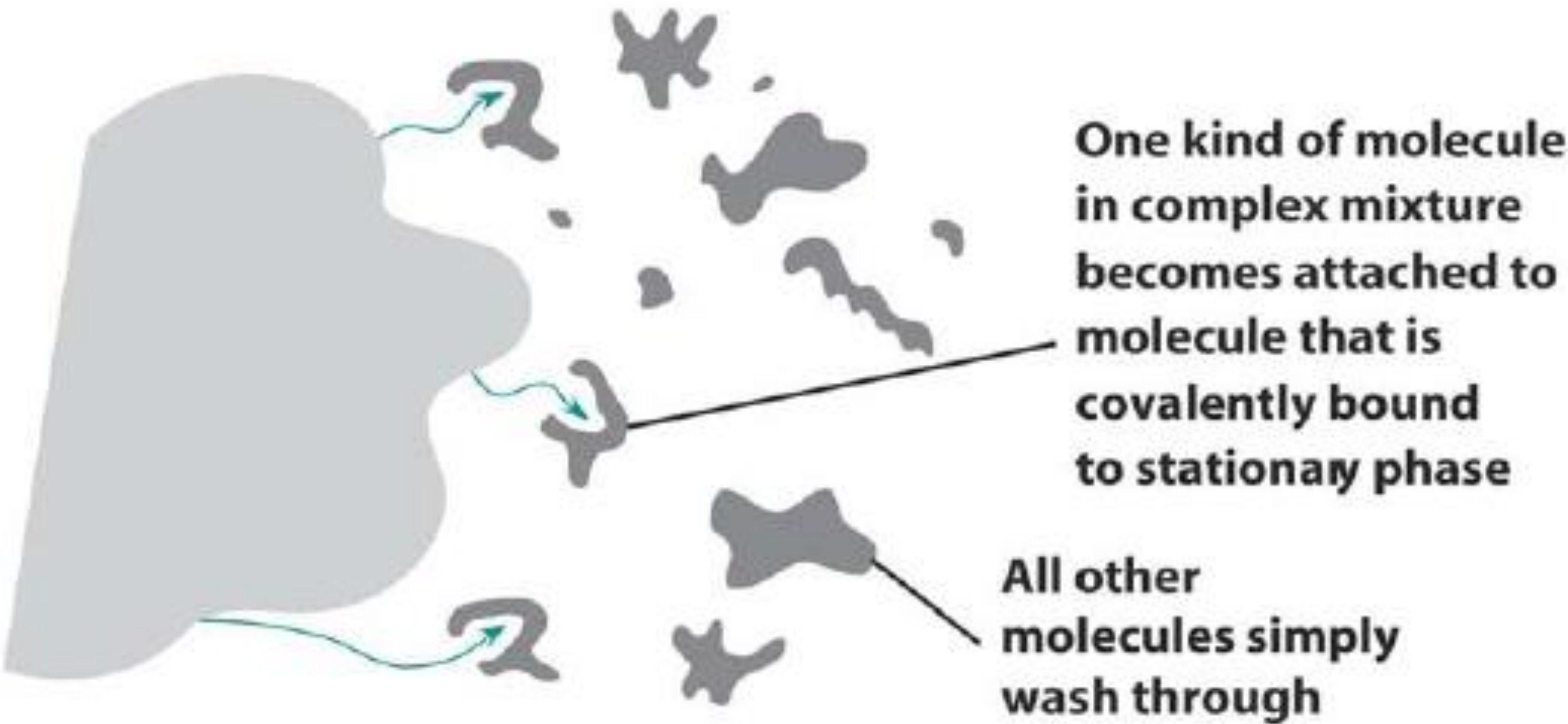
Partition coefficient K_D

$$K_D = \frac{C_s}{C_m}$$

C_s: concentration in stationary phase

C_m: concentration in mobile phase

There is a specific binding capacity between E and substrate, Antigen and Ab, avidin and biotin, hormone and its receptor, RNA and its cDNA.



Mobile anions held near cations that are covalently attached to stationary phase

Anion-exchange resin; only anions can be attracted to it

Ion-exchange chromatography

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Ion exchange Chromatography

离子交换层析

- A separation based on charge. The charge on the protein affects its behavior.
- *The stationary phase* has either positively or negatively charged species immobilized on its surface.
- *The mobile phase*： electrolyte solution (pH and ionic strength)
- Proform in a Column format
- Elution process： pH and ionic strength

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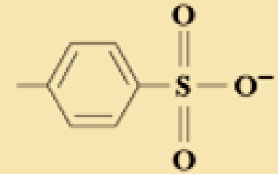
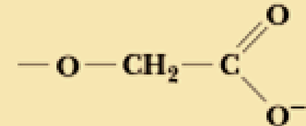
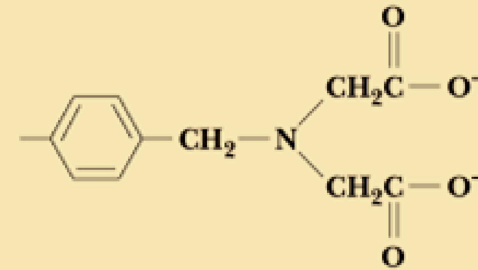
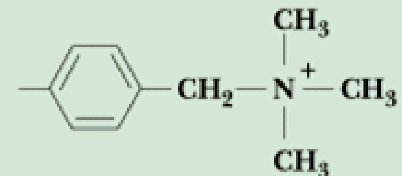
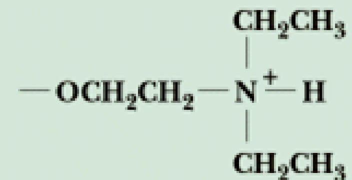
Ion Exchanger

Different types of ion exchange resins

(a) Cation exchanger

(b) Anion exchanger.

(a) Cation Exchange Media	Structure
Strongly acidic, polystyrene resin (Dowex–50)	
Weakly acidic, carboxymethyl (CM) cellulose	
Weakly acidic, chelating, polystyrene resin (Chelex–100)	

(b) Anion Exchange Media	Structure
Strongly basic, polystyrene resin (Dowex–1)	
Weakly basic, diethylaminoethyl (DEAE) cellulose	

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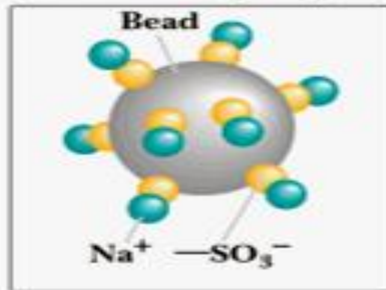
- The mobile phase in ion exchange chromatography of proteins consists of a buffer, salt and other additives such as preservatives.
- Initially a low salt mobile phase is used to allow the proteins to bind to the column. Then the salt concentration is increased to displace the proteins from the stationary phase and elute them from the column.
- In ion exchange chromatography, the mobile phase strength is increased by adding more salt.

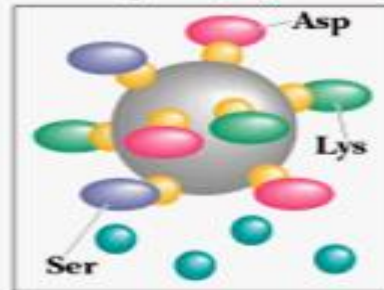
Cation Exchange Chromatography for Separation of amino acids (Asp, Ser and Lys)

Cation exchange bead before adding sample



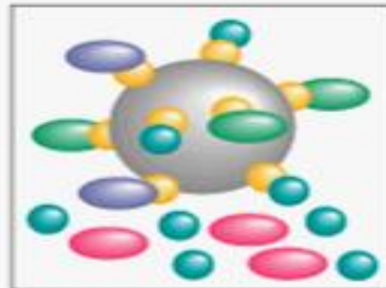
(a)

Add mixture of Asp, Ser, Lys



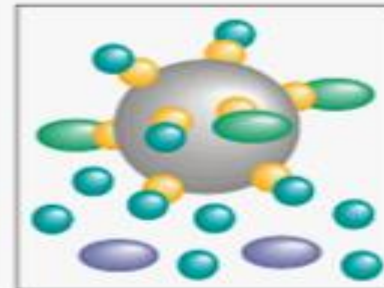
(b)

Add Na^+ (NaCl)



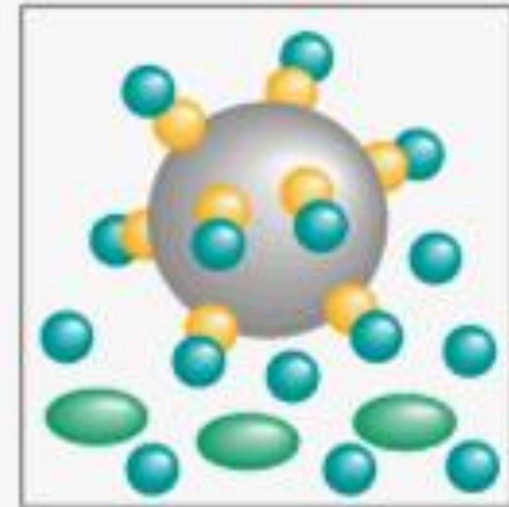
(c) Asp, the least positively charged amino acid, is eluted first

Increase $[Na^+]$



(d) Serine is eluted next

Increase $[Na^+]$



(e) Lysine, the most positively charged amino acid, is eluted last

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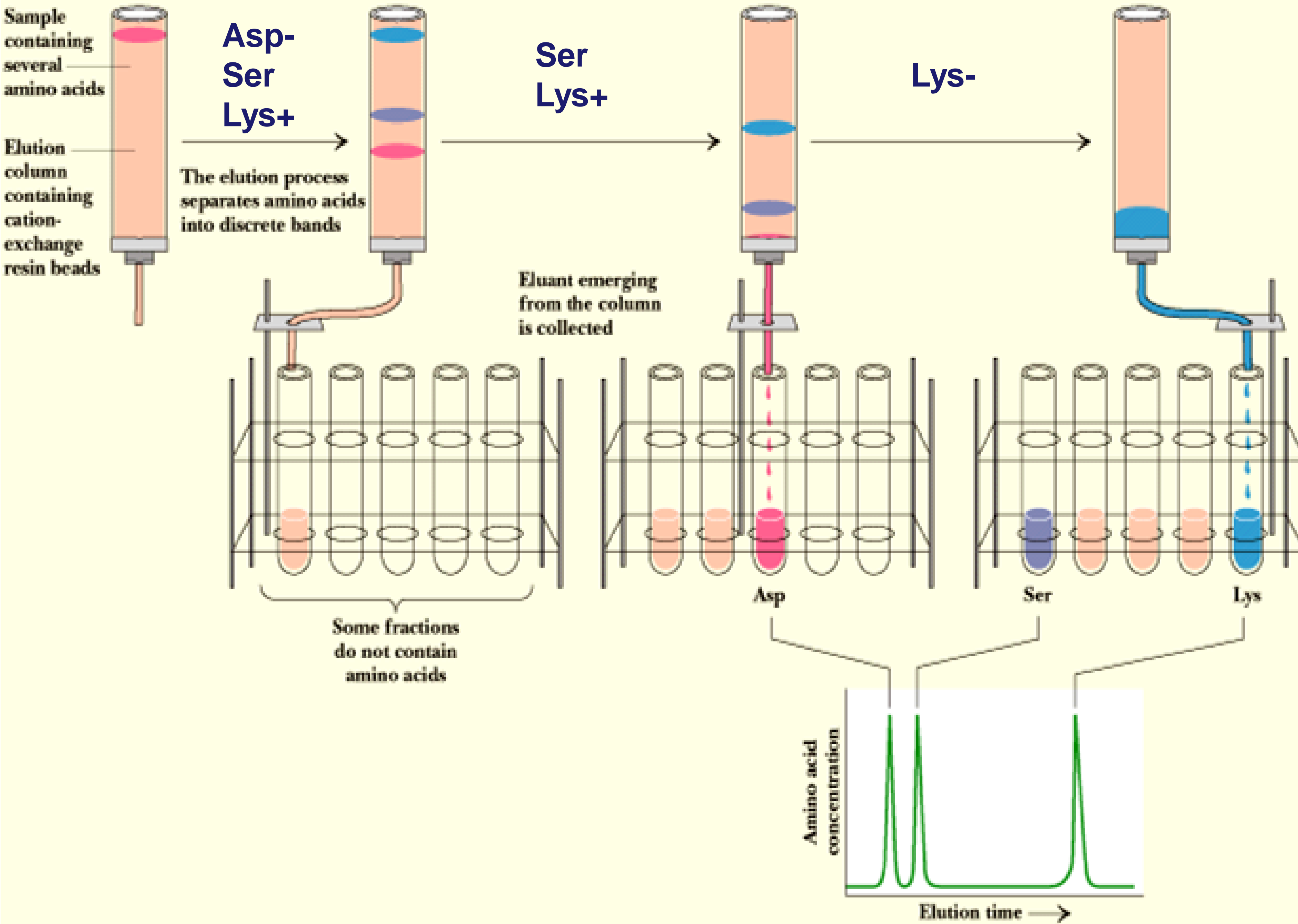


Sample containing several amino acids
Elution column containing cation-exchange resin beads
Asp-
Ser
Lys+
The elution process separates amino acids into discrete bands
Ser
Lys+
Lys-
Eluant emerging from the column is collected
Some fractions do not contain amino acids
Asp
Ser
Lys
Amino acid concentration
Elution time

Experiment 1

Separation of Mixed Amino Acids by Cation Exchange Chromatography

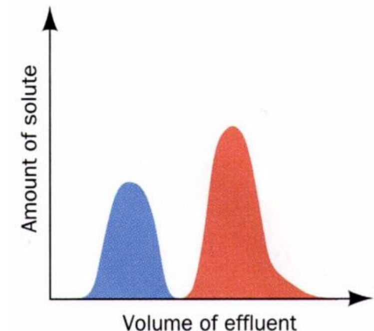
- Packing and equilibrium:
- Loading and elution：

load 0.5mL mixture of Asp (pI=2.97) and Lys (pI=9.74)

elute at 0.5 ml/min with pH 5.3 citric acid buffer, collect 5 tubes

elute at 0.5 ml/min with pH12 NaOH, collect 7 tubes (3.0 mL each)

- Amino acid detection: ninhydrin reaction
- Draw an elution curve
- Regeneration: pH5.3 citric acid buffer



Gel Filtration

- The molecular sieve effects of cancellated gel.
- Gel filtration can be used to separate proteins by molecular weight (size)

Applications

- Seperating the biomacromolecule
- Concentrating and desalting
- Removing pyrogenic substance
- Determining the protein molecular weight

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Principle

Gel filtration (molecular exclusion or gel permeation chromatography) is a separation based on molecular size.

The stationary phase consists of porous beads with a well-defined range of pore sizes.

The stationary phase for gel filtration has a fractionation range, meaning that molecules within that molecular weight range can be separated.

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The stationary phase of Gel filtration

- Native gel: Sepharose 2B, 4B, 6B/Biogel A
- Artificial synthesis gel:

  Dextrangel/Sephadex G–10,15,25,50,75,100,150,200;

  Polyacrylamide gel/ Biogel P-2,4,6,10,30,100,150,300

  Sephacryl S300, S400
- Select different gel depend on molecular weight of substrates

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Principle

- Proteins that are small enough can fit inside all the pores in the beads and are said to be included.
- These small proteins have access to the mobile phase inside the beads as well as the mobile phase between beads and elute last in a gel filtration separation
- Proteins that are too large to fit inside any of the pores are said to be excluded. They have access only to the mobile phase between the beads and, therefore, elute first.
- Proteins of intermediate size are partially included - meaning they can fit inside some but not all of the pores in the beads. These proteins will then elute between the large ("excluded") and small ("totally included") proteins.

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Principle Image

Stokes radius

分子大小、形狀

Small molecules

Sample

Mobile phase

固定相

Large molecules

Small molecules elute later

large molecules elute first

Gel Filtration for Separation of Hb and protamine

- **Sample:** Hemoglobin (Hb, red, MW 64.5 kD)
  protamine (yellow, MW 2～10 kD)
- **Packing:** Sephadex G-50
  No bubbles; NEVER let the column dry
- **Loading**
- **Elution:** distilled water (dH_2O)
- **Observe and collect the colored bands.**

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(a)
Gel bead
Gel matrix

(b)
Solvent
Gel beads

(c)

(d)
Small molecules
Large molecules

(e)
Amount of solute
Volume of effluent

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