酵素純化與分析

Enzyme Purification and Analysis

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Professor RH Juang, Institute of Microbiology and Biochemistry

酵素純化與分析

B2

- 酵素純化方法 Enzyme purification methods
- 酵素分析方法 Enzyme analysis methods
- 問題集 Problems

酵素純化方法 Enzyme purification methods

1. 酵素純化實驗室
   Enzyme purification laboratory
2. 蛋白質抽取 Protein extraction
3. 色層分析法 Chromatography
4. 其它純化或分離方法 Other methods
5. 純化策略 Purification strategy

1 酵素純化實驗室 Enzyme purification laboratory

- 1.1 儀器 Instruments 儀器正確使用及保養
- 1.2 小型器具 Small tools 注意準確度
  消耗品 Consumables 供應正常
- 1.3 藥品 Reagents 藥品的貯藏溫度 正確使用
- 1.4 個人用品 Personal utilities 管好自己的用具

Sigma 目錄的試劑說明 Sigma catalogue

FOB 航空運送　品名 包裝　別名　純度

新品

目錄編號

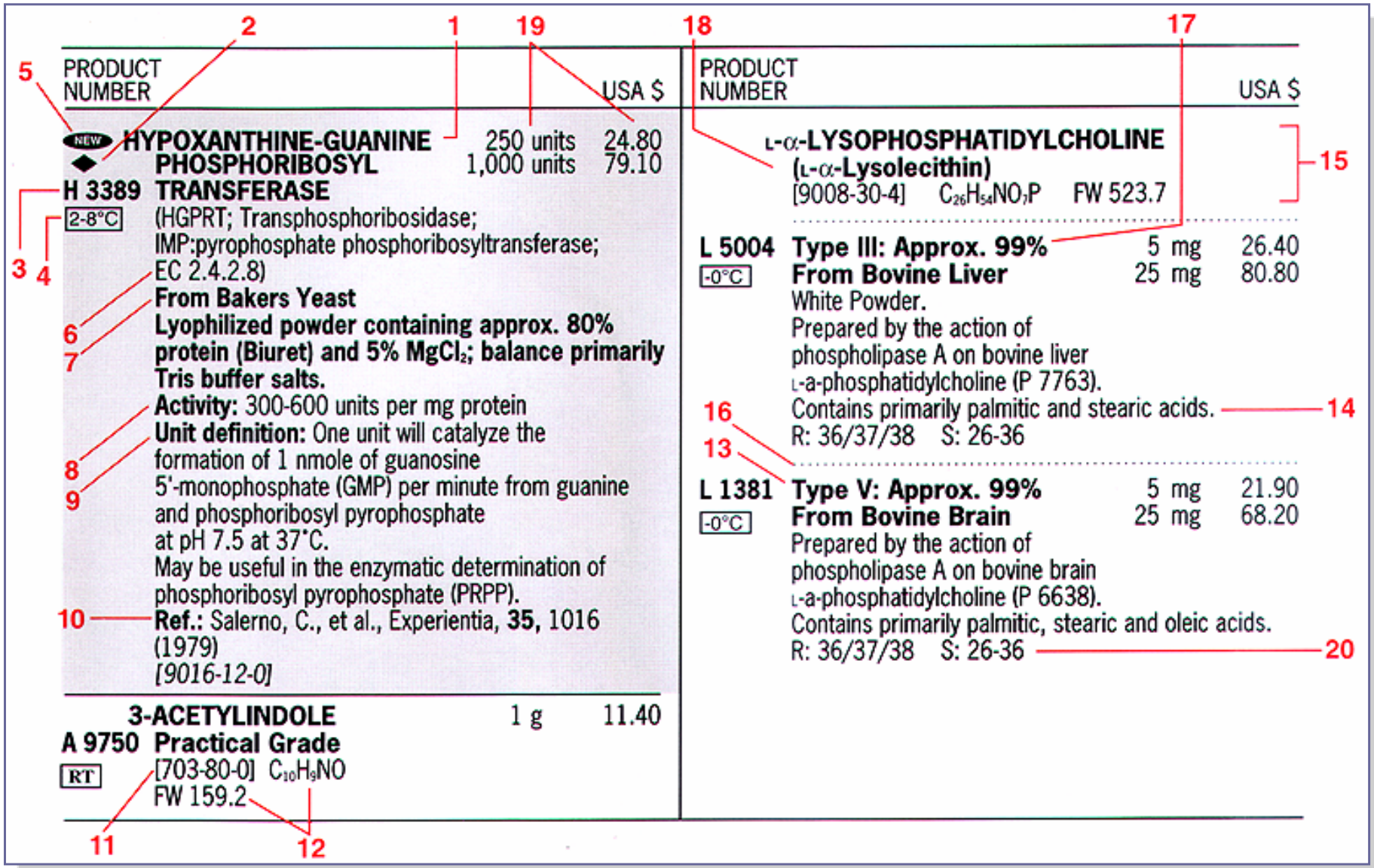
貯藏溫度

EC 編號

材料來源

活性定義

參考文獻



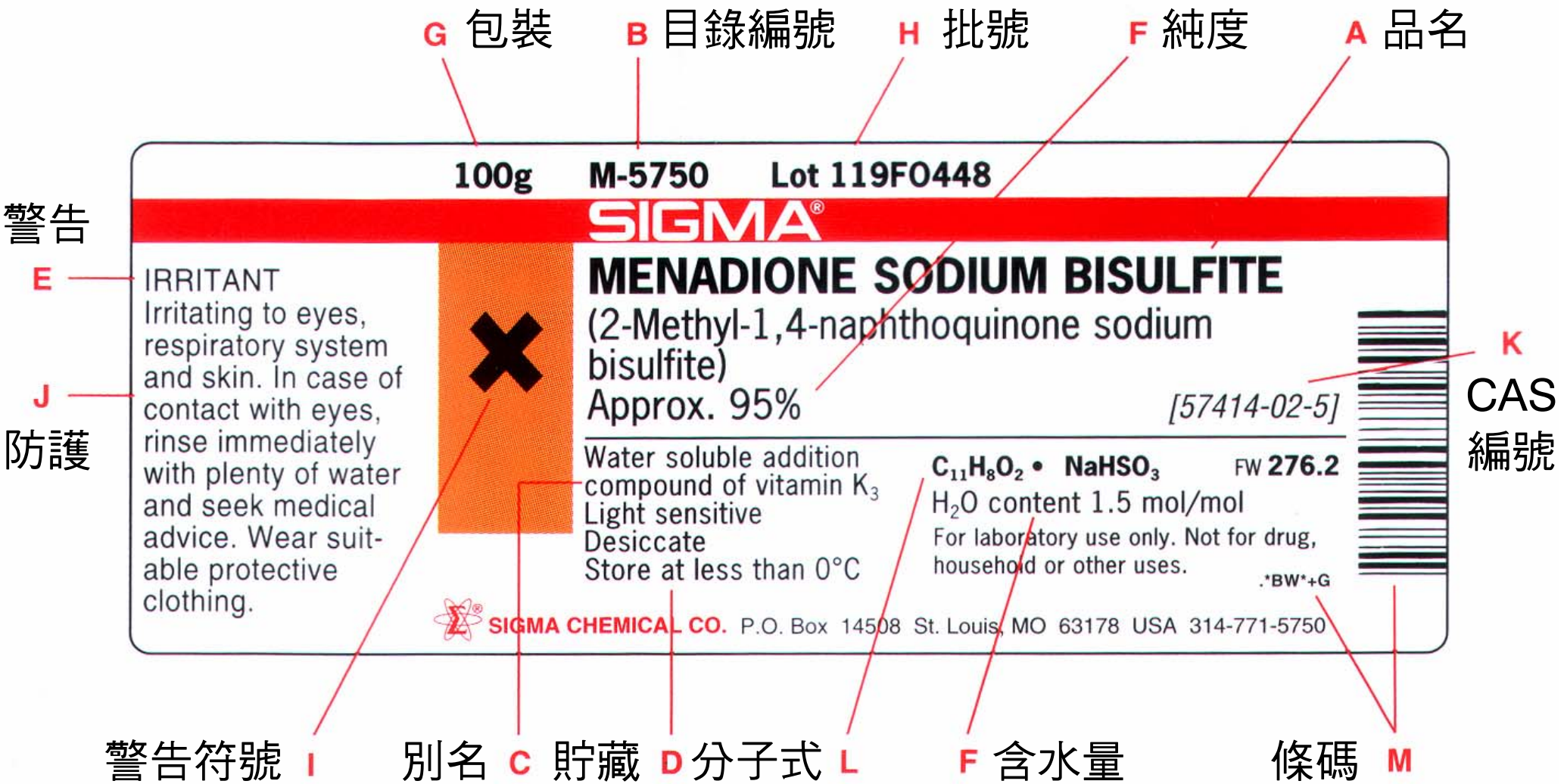
說明

型號

RS 編號

CAS 編號　分子式

Sigma 目錄的試劑標籤 Sigma reagent label



Sigma Catalogue

常見警告符號 Common chemical warning signs

爆裂物

Explosive

氧化劑

Oxidizing

易燃物

Highly Flammable or
Extremely Flammable

毒藥

Toxic or
Very Toxic

Harmful or
Irritant

刺激性

Corrosive

腐蝕性

Biohazard

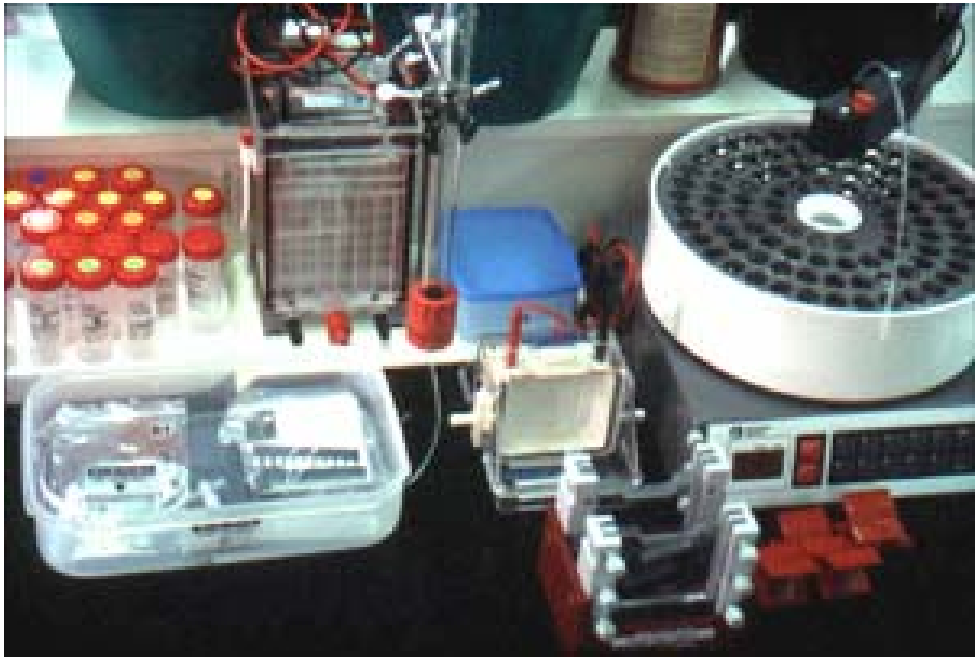
生物傷害

Dangerous for
the Environment

環境傷害

Sigma Catalogue

寫好你的標籤紙 Label your reagents well



濃度　試劑名稱　酸鹼度

1 M Tris-HCl *p*H 7.4

JRH　050825Thu

姓名縮寫　日 期

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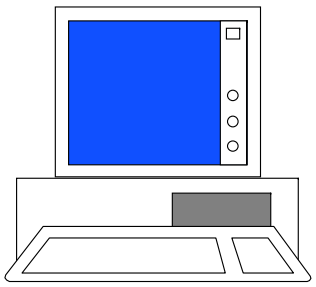
文獻查詢及管理 Manage your reference well



電腦網路資料庫

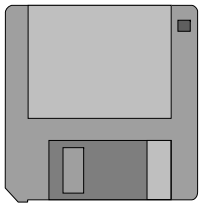
列印

Reference List

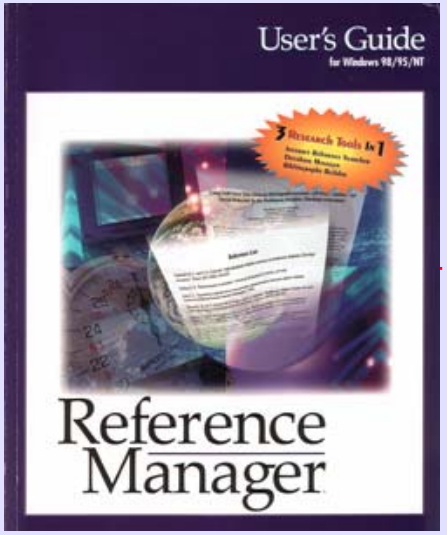
PDF

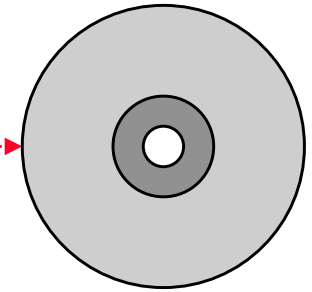
Science

查閱 Original Paper

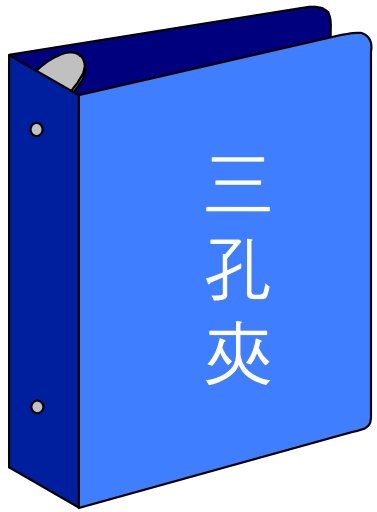


Data.TXT





Database.RM



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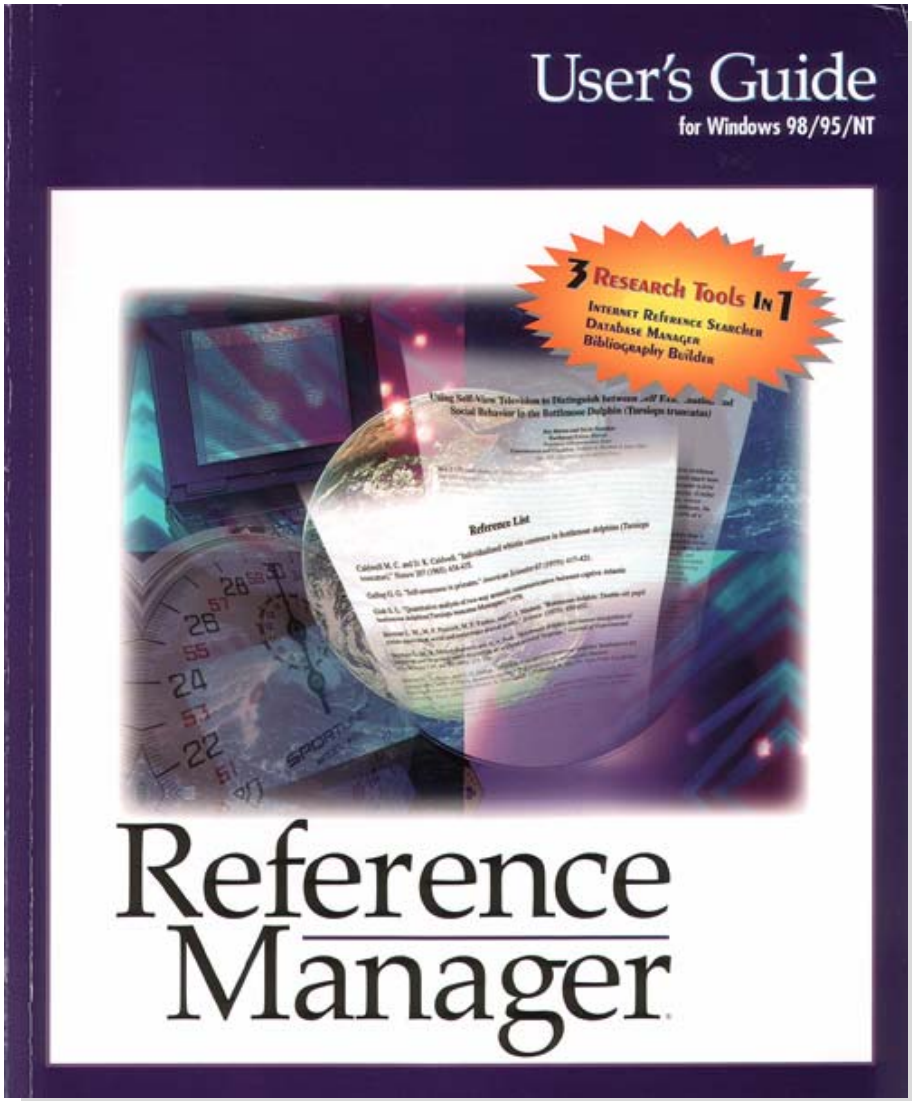
文獻查詢及管理 Create your reference list



- **Build up your database**

  Search and store your reference using RefMan

- **Insert references in your article**

  Insert {reference ID} in your Word article

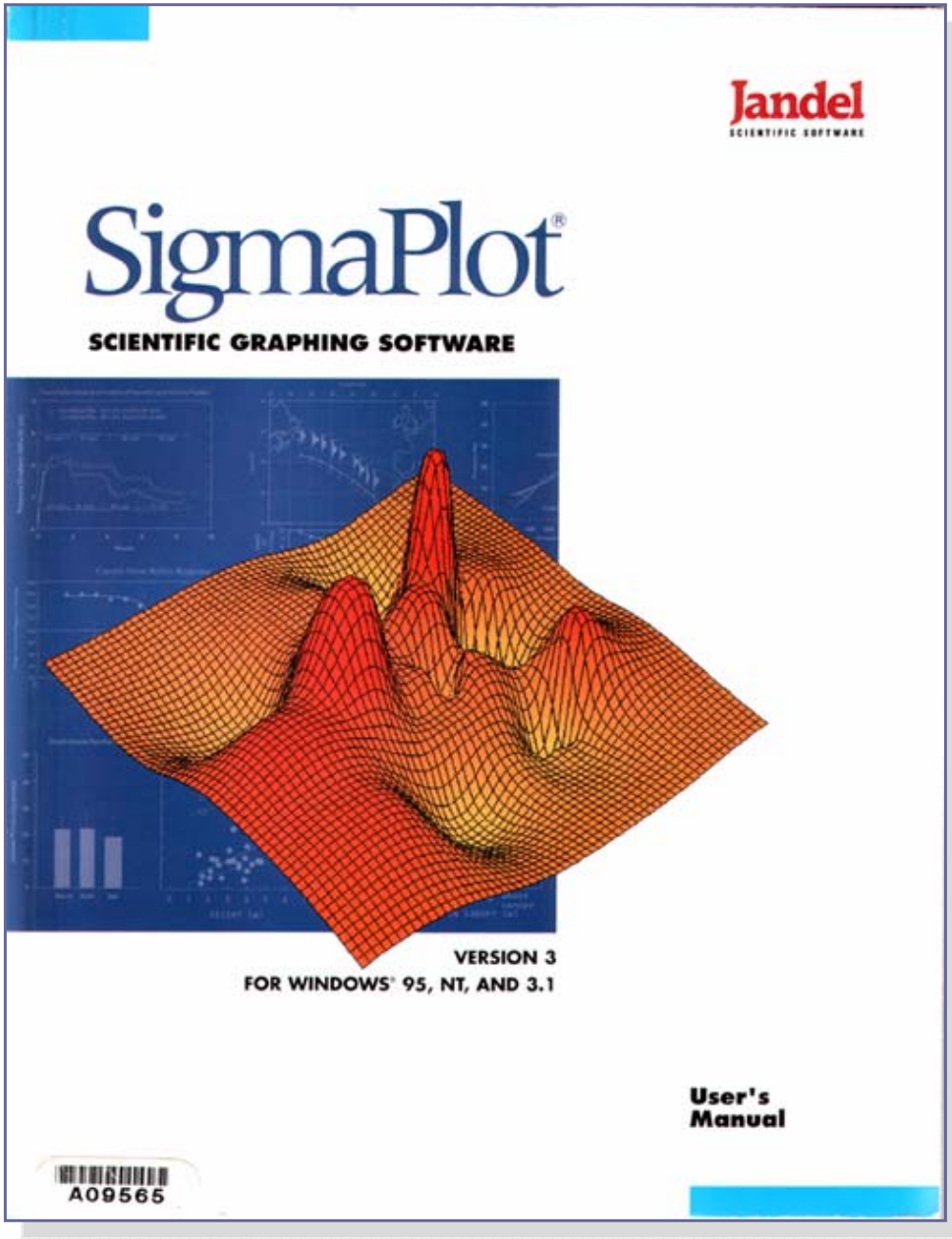
- **Create reference list**

  You may use any preferred format of reference list

- **Citation will be inserted in article automatically**

  Every {ID} is changed to citation

必備製圖軟體 SigmaPlot for scientific graph



- **Key in your data**

  Any kind of (X, Y) data

- **Choose preferred graph**

  Useful graph types available

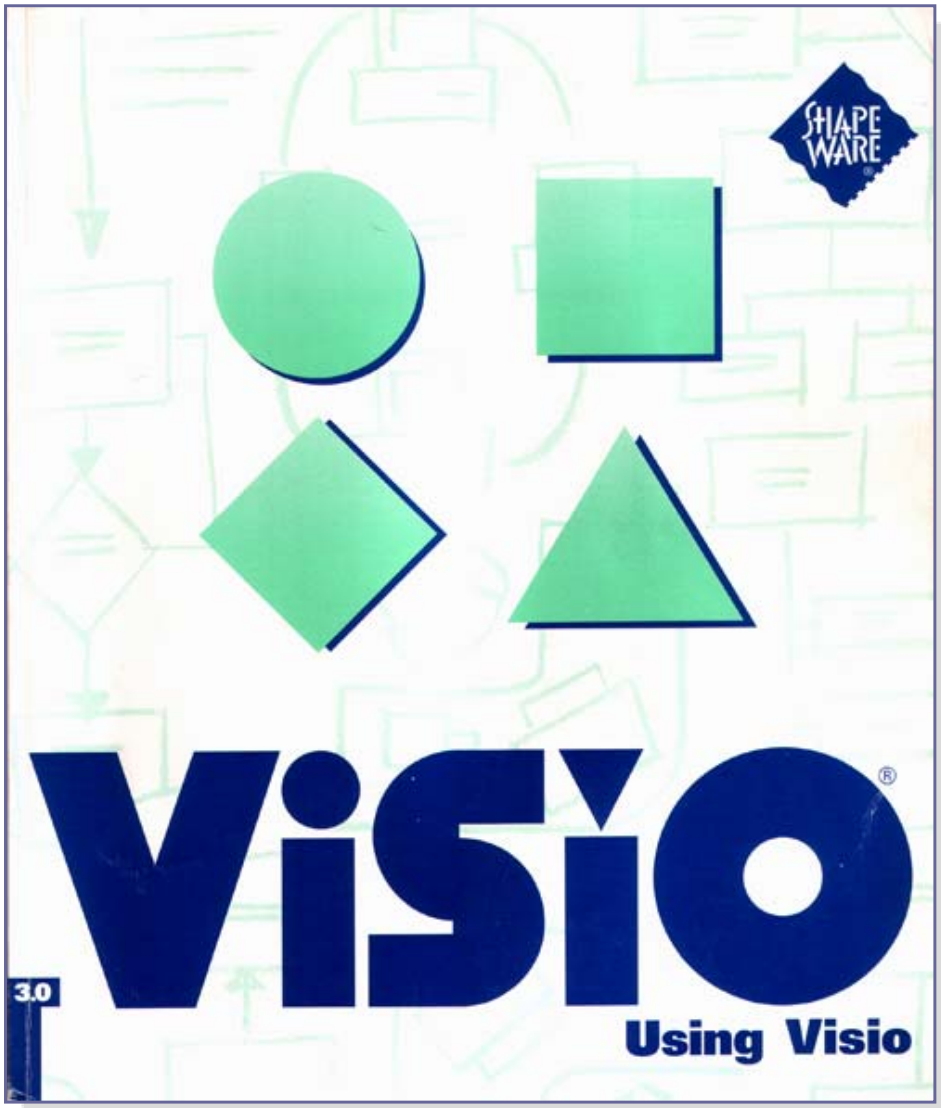
- **Create the graph**

  Graph is created automatically

- **Modify title or legend**

  You can modify graph freely

流程軟體 Visio creates professional flow charts



Drag plates to create tasks

All tasks are put on various types of plates (different shapes)

Link plates with lines

Connecting line indicates the relationship between tasks

Paste your own graph

Make your own plates with special graph of figure

Useful in many jobs

Experimental flow chart, maps

流行的製圖軟體 CorelDraw produces cool graphs

Create vector-based graphs

Experimental graph

Maps and indications

Molecular formula

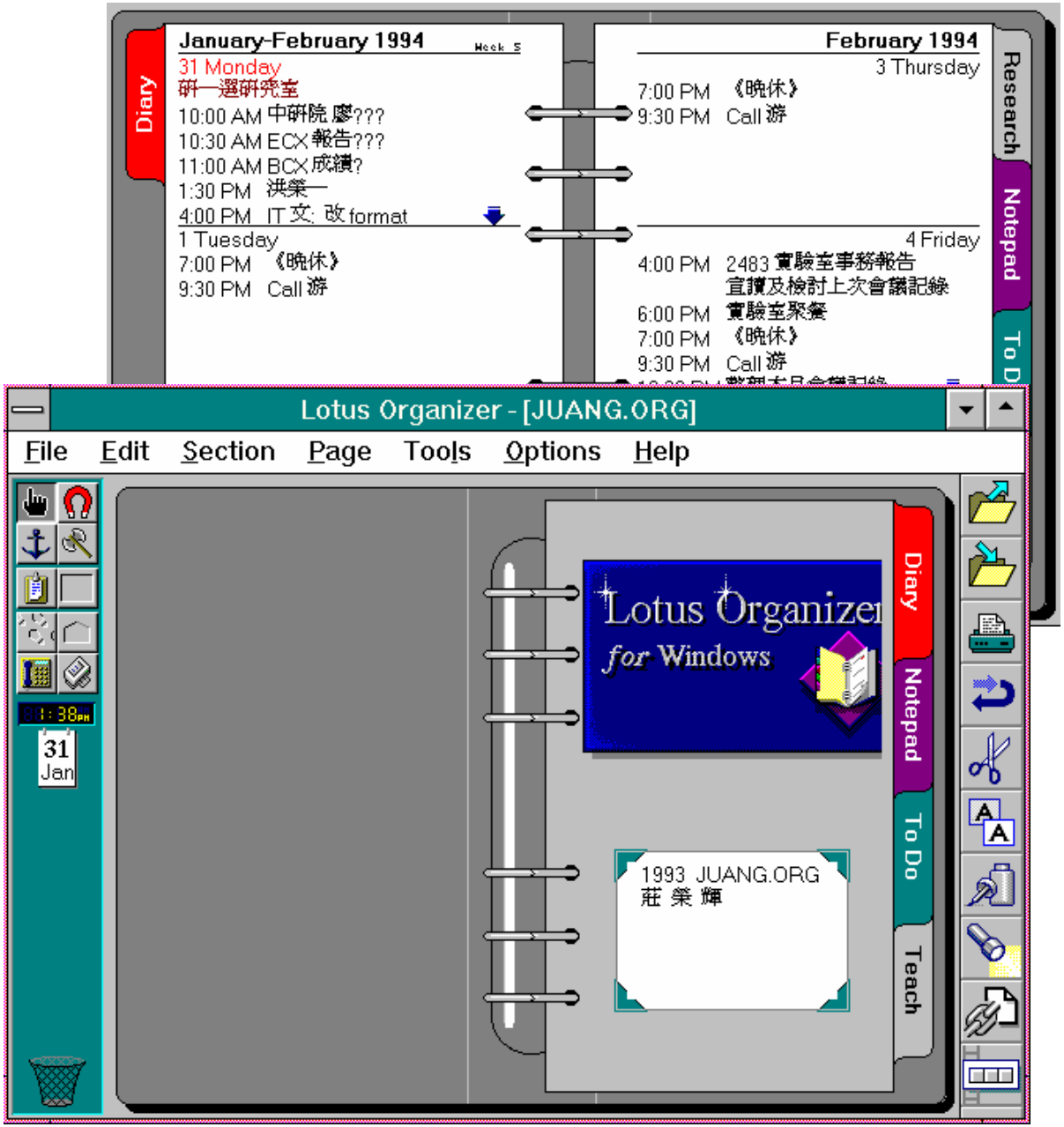
DNA sequence alignment

Amino acid sequences

Bitmap also editable

Put labels on any bitmap (for a electrophoresis gel)

每日生活排程軟體 Organizer organize you life



- Personal information management
  - Diary
  - To do list
  - Note pad
  - Address book
- A dynamic notebook
  - Week view or month view
  - Convenient linking
  - No page limitation

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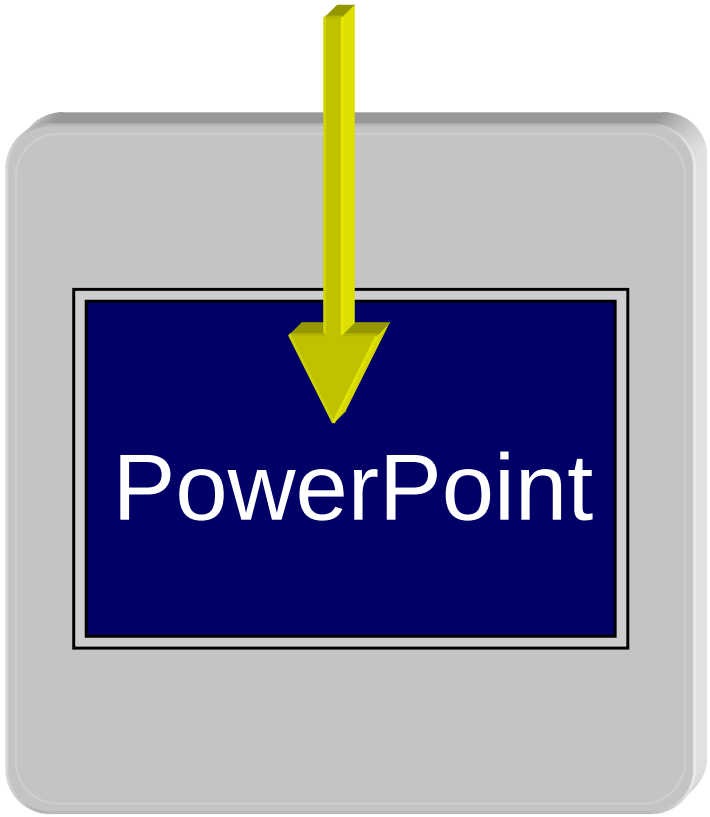
展示成果 PowerPoint demonstrates your result

- Introduction
- Flow chart
- Results
- Graph
- Photo
- Gels



Write on the storyboard

Turn your report into scenario

Design every slide exhaustively

Clean, clear, correct

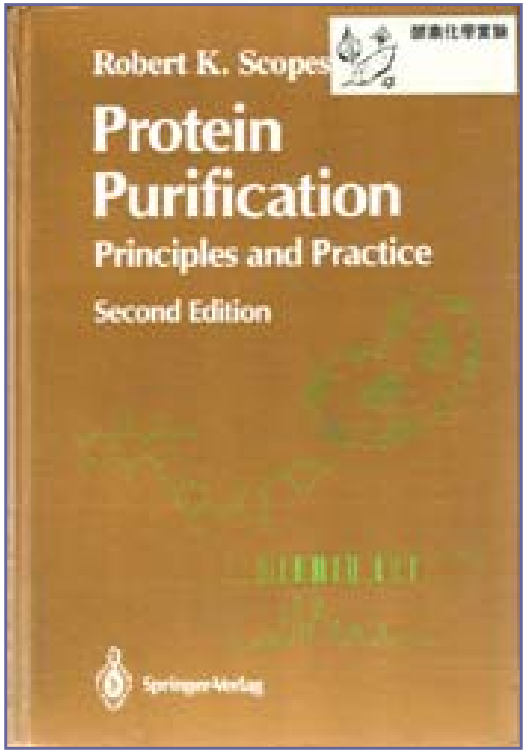
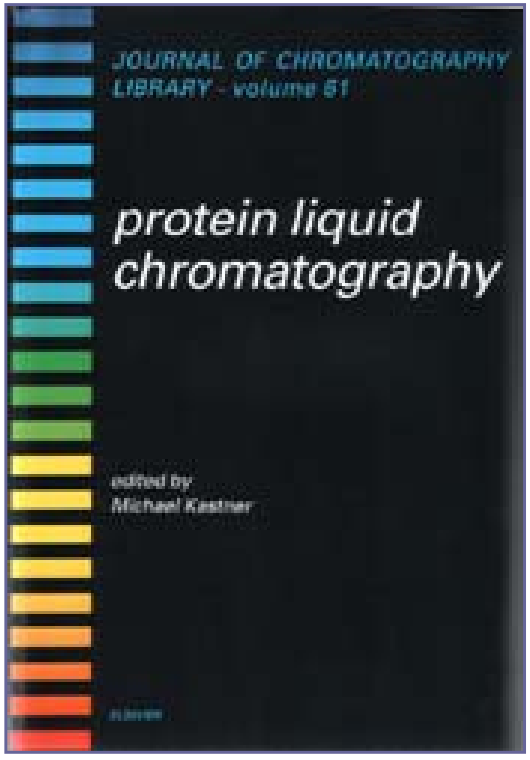
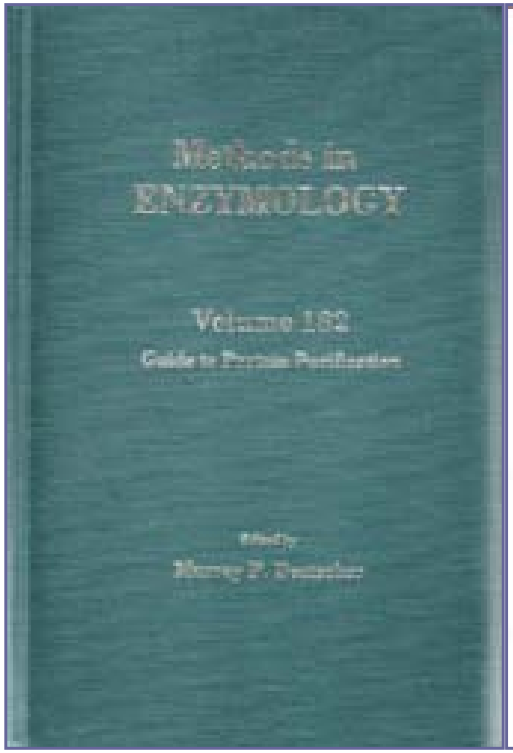
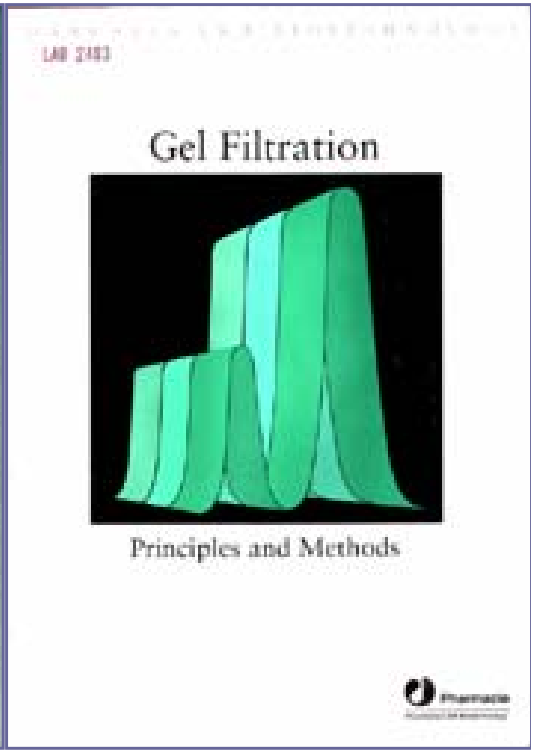
Rehearsal is very critical

At least five formal rehearsals for an important talk

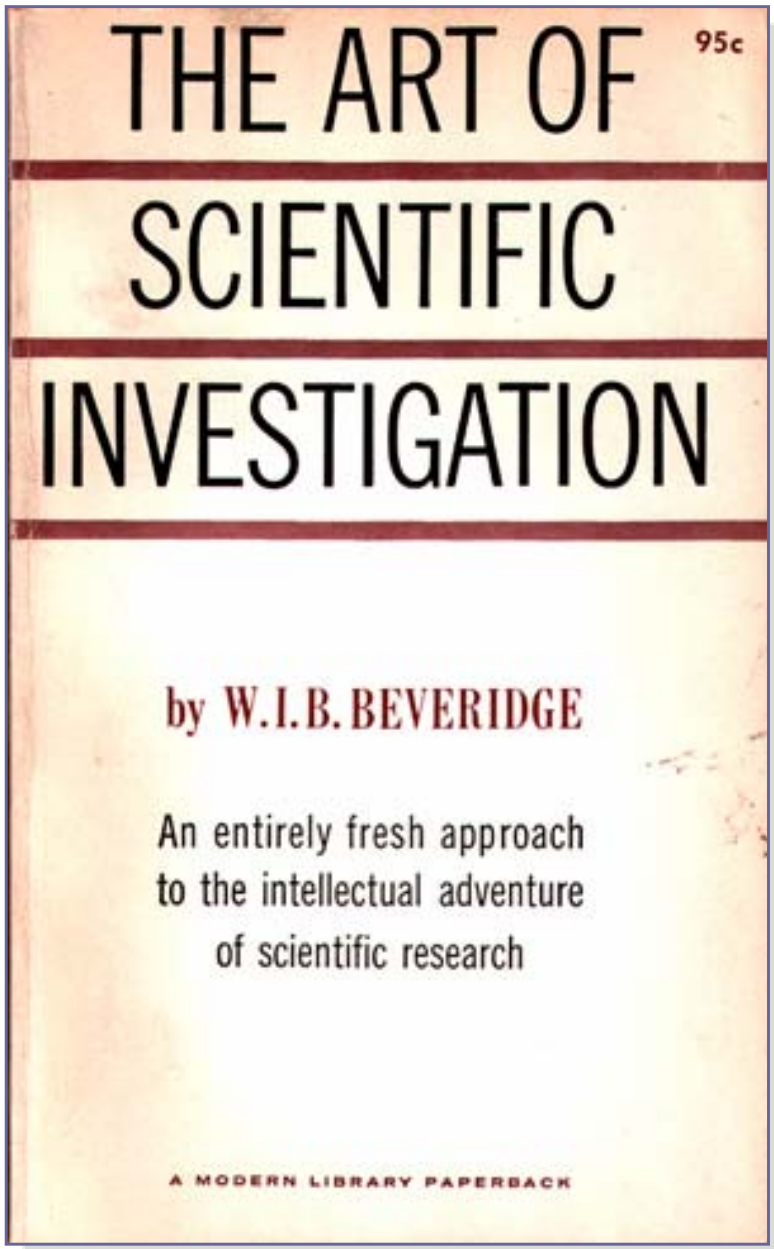
Do your best when talk

Talk enthusiastically and answer the questions frankly

酵素純化工具 References for enzyme purification

Protein Purification	Protein Liquid Chromatography	Methods in Enzymology	Pharmacia Handbooks
			
有第三版	最新最完全	酵素研究寶藏	完全操作手冊

科學之路 The philosophical aspects for science



構成我們學習上最大障礙的是已知的東西，而不是未知的東西。

達爾文：『大自然一有機會就要說謊』

很多具有時代意義的發現先前都被提出過，只是未能繼續發展研究，直到適逢其人才得以正確展開。

測定智力技能的最佳標準，可能是檢測其放棄謬誤的速度。

人們最出色的工作往往是在處於逆境的情況下做出來的。

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2 蛋白質抽取 Protein extraction

- 2.1 如何開始？ How to start?

  5W principles 基本原則

- 2.2 材料來源 Materials & sources

  材料取得與保存

- 2.3 均質及抽取 Homogenization & extraction

  確實做好第一步

- 2.4 鹽析及沉澱法 Salting-out & precipitation

  最經濟方便的方法

如何開始？ How to start?

先考慮以下諸點：　　**5W**

a. 要純化那一個蛋白質？　What ?

b. 為何要純化此蛋白質？　Why ?

c. 由何種材料純化？　Where, from ?

d. 由那一個生長期？　When ?

e. 如何純化此蛋白質？　How ?

酵素純化過程的三個階段 Three stages

(1) 粗蛋白 (crude protein)：

採樣 → 均質打破細胞 → 抽出全部蛋白，多用鹽析沉澱法。

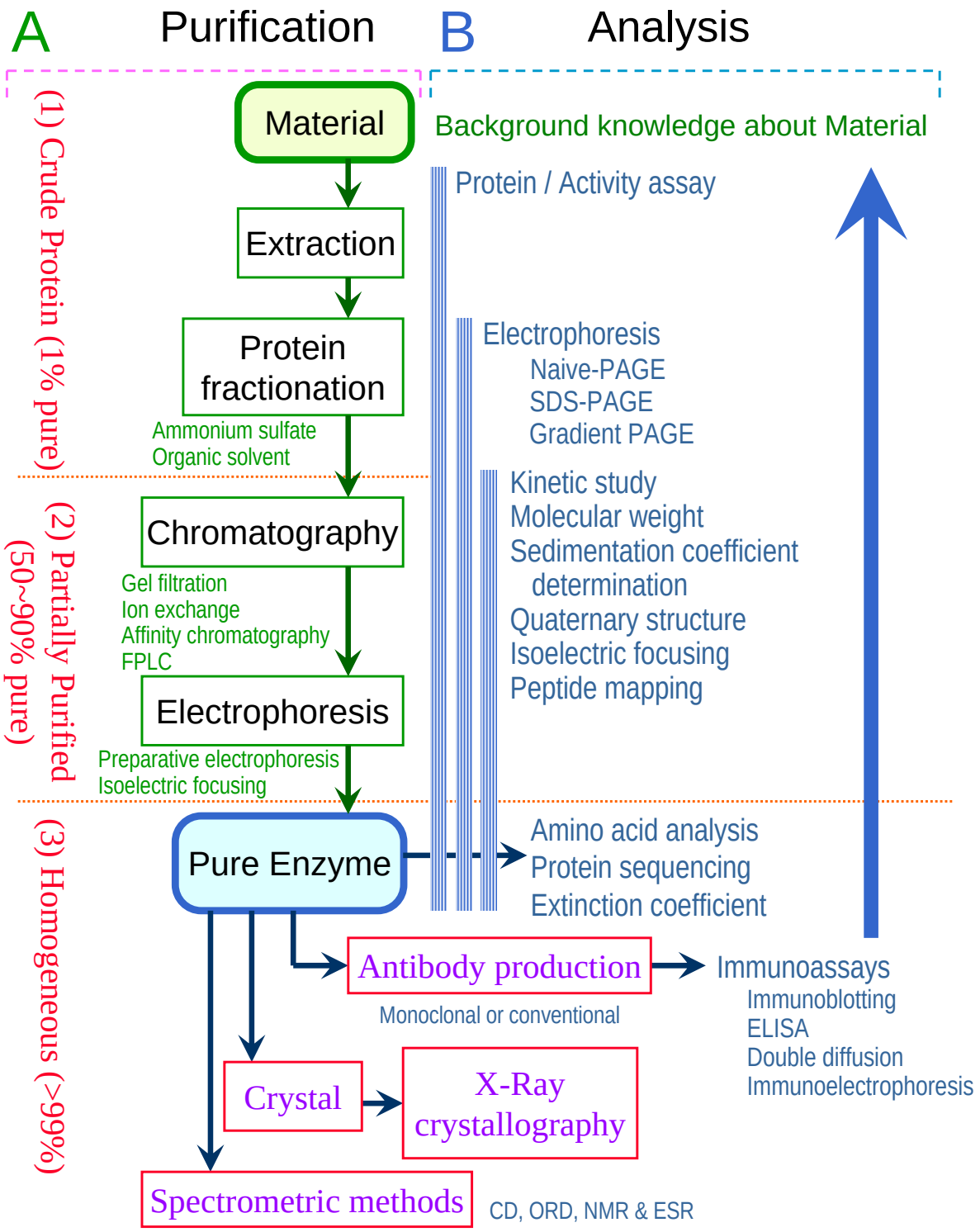
(2) 部分純化 (partially purified)：

初步的純化，使用各種管柱層析法。

(3) 均質酵素 (homogeneous)：

目標酵素的進一步精製純化，可用製備式電泳或 HPLC 等。

酵素純化階段及分析方法



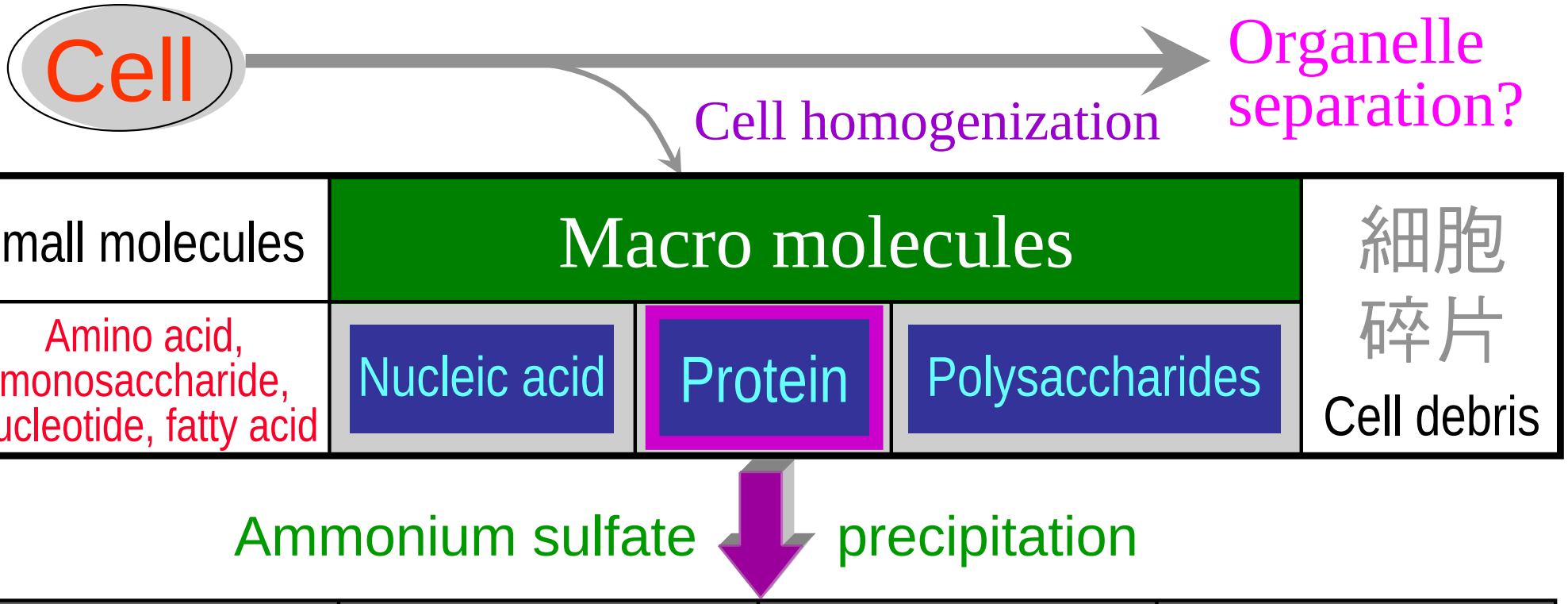
(1) 粗蛋白
Crude protein

(2) 部分純化
Partially purified

(3) 均質酵素
Homogeneous

Juang RH (2005) EPA

各種純化或分析方法的原理



Ammonium sulfate precipitation

Molecular size	Molecular charge	Molecular polarity	Affinity
Gel filtration, SDS-PAGE, Ultrafiltration	Ion exchange, Chromatofocusing, Disc-PAGE, Isoelectric focusing	Reverse phase chromatography, HIC, Salting-out	Affinity chromatography, Hydroxyapatite

Basic protein properties useful for planning separation steps

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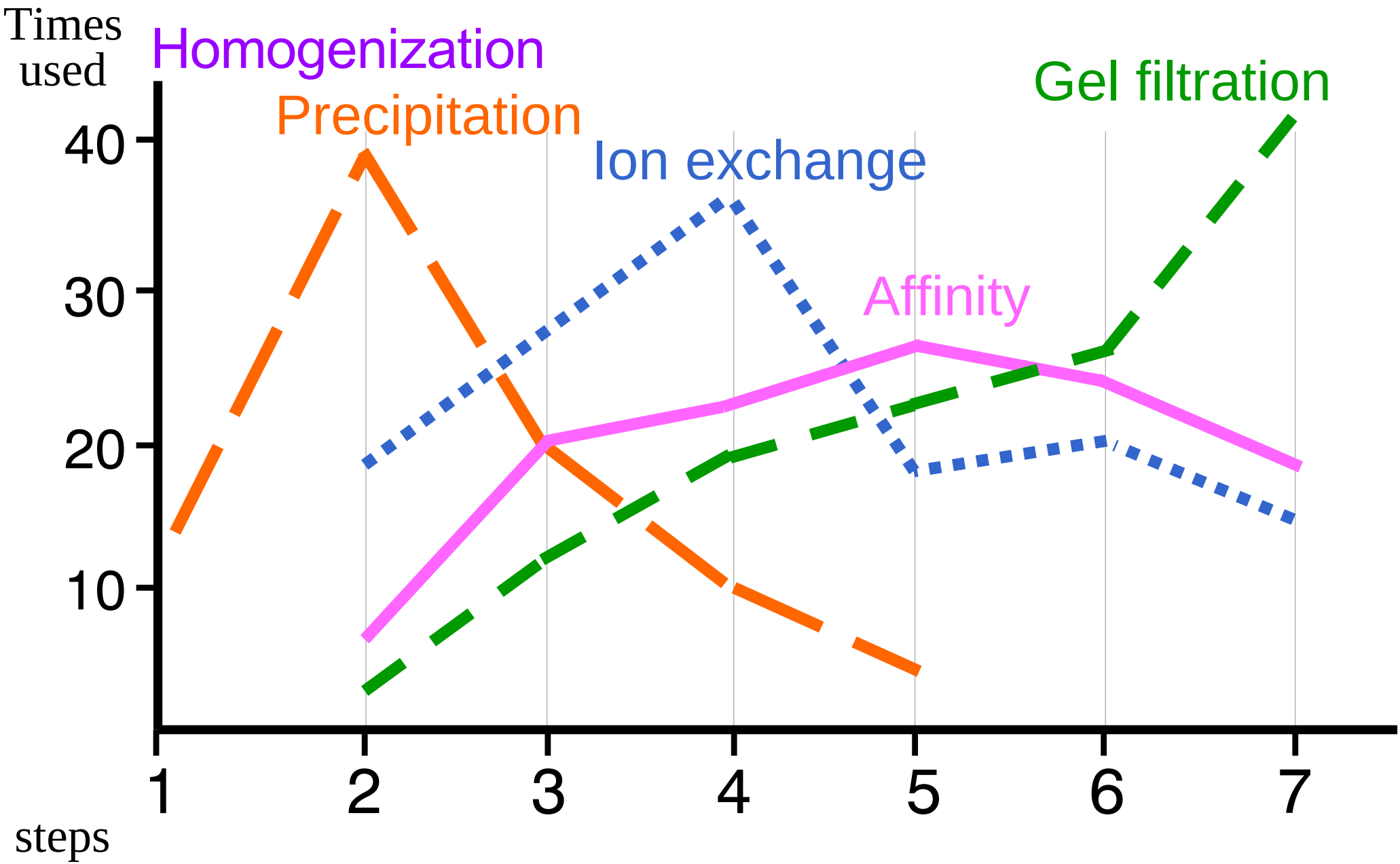
各種純化方法的應用次序 Which step first?



Adapted from Bonnerjera et al. (1986) Bio/Technology, 4:954-958

2 蛋白質抽取 Protein extraction

- 2.1 如何開始？ How to start?
  5W principles 基本原則
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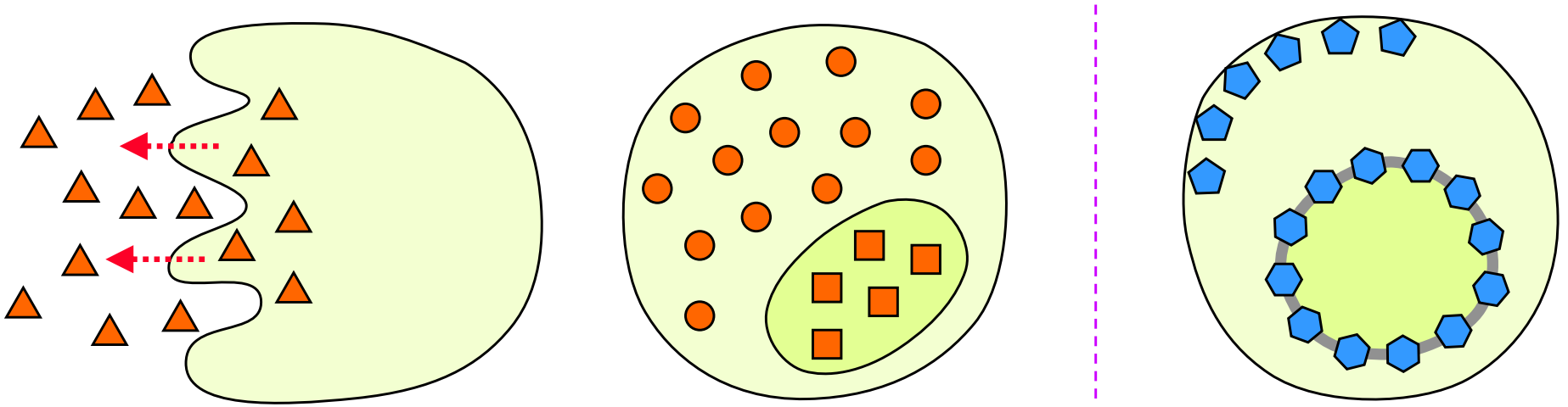
- 2.3 均質及抽取 Homogenization & extraction
  確實做好第一步
- 2.4 鹽析及沉澱法 Salting-out & precipitation
  最經濟方便的方法

目標材料之選擇 What's your starting material?

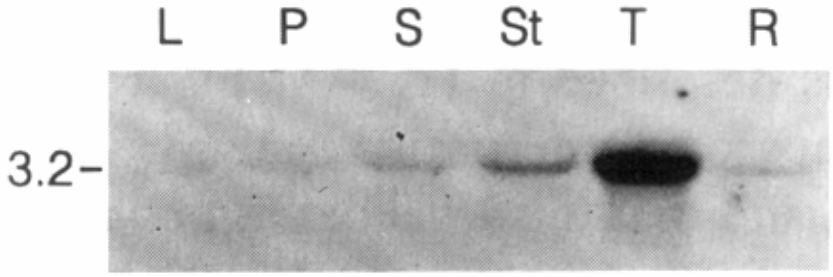
- Which organism? 動物、植物、微生物
- Which tissue? 根莖葉花果或組織培養
- Which organelle? 細胞核、液泡、葉綠体
- Secreted enzyme? 有關酵素的穩定性
- Membrane protein? 影響抽取策略的設計



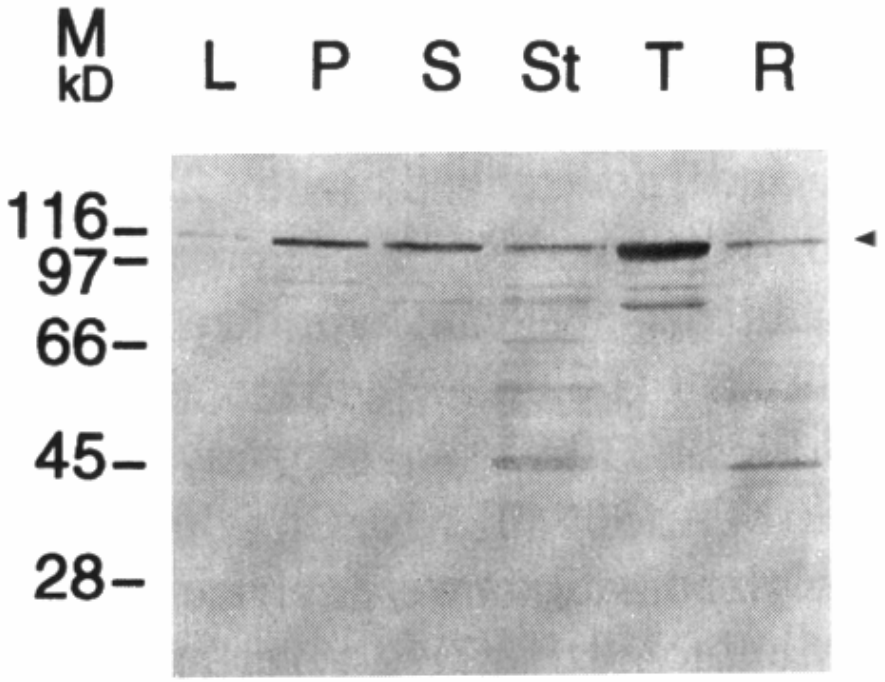
Juang RH (2005) EPA

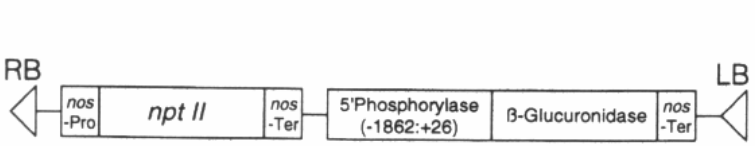
St Pierre,B.; Bertrand,C.; Camirand,A.; Cappadocia,M.; Brisson,N. (1996) Plant Molecular Biology 30: 1087-1098
The starch phosphorylase gene is subjected to different modes of regulation in starch-containing tissues of potato

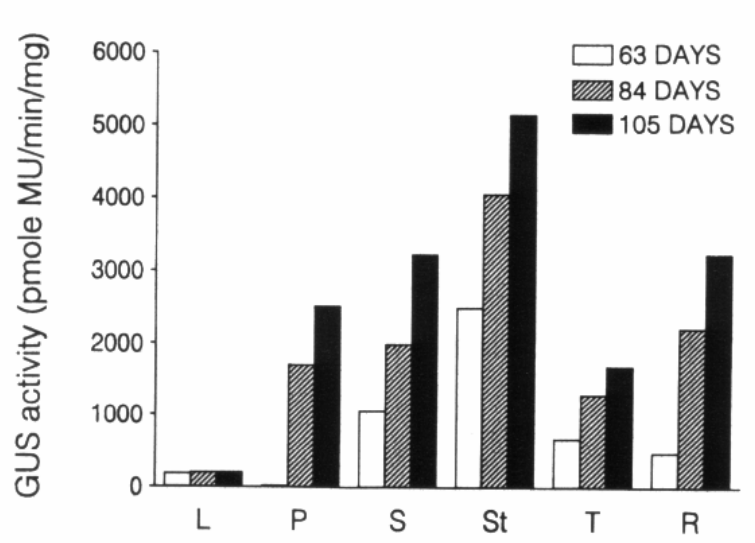
SP RNA expression (potato tissues)

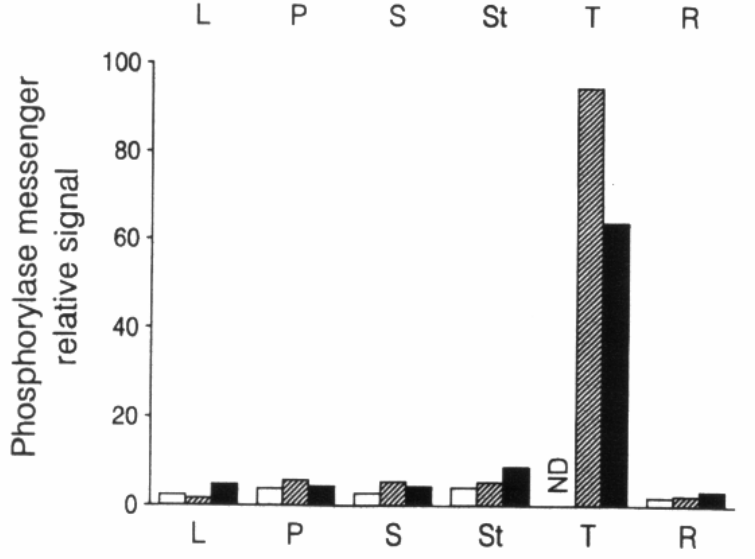


SP protein expressed (potato tissues)

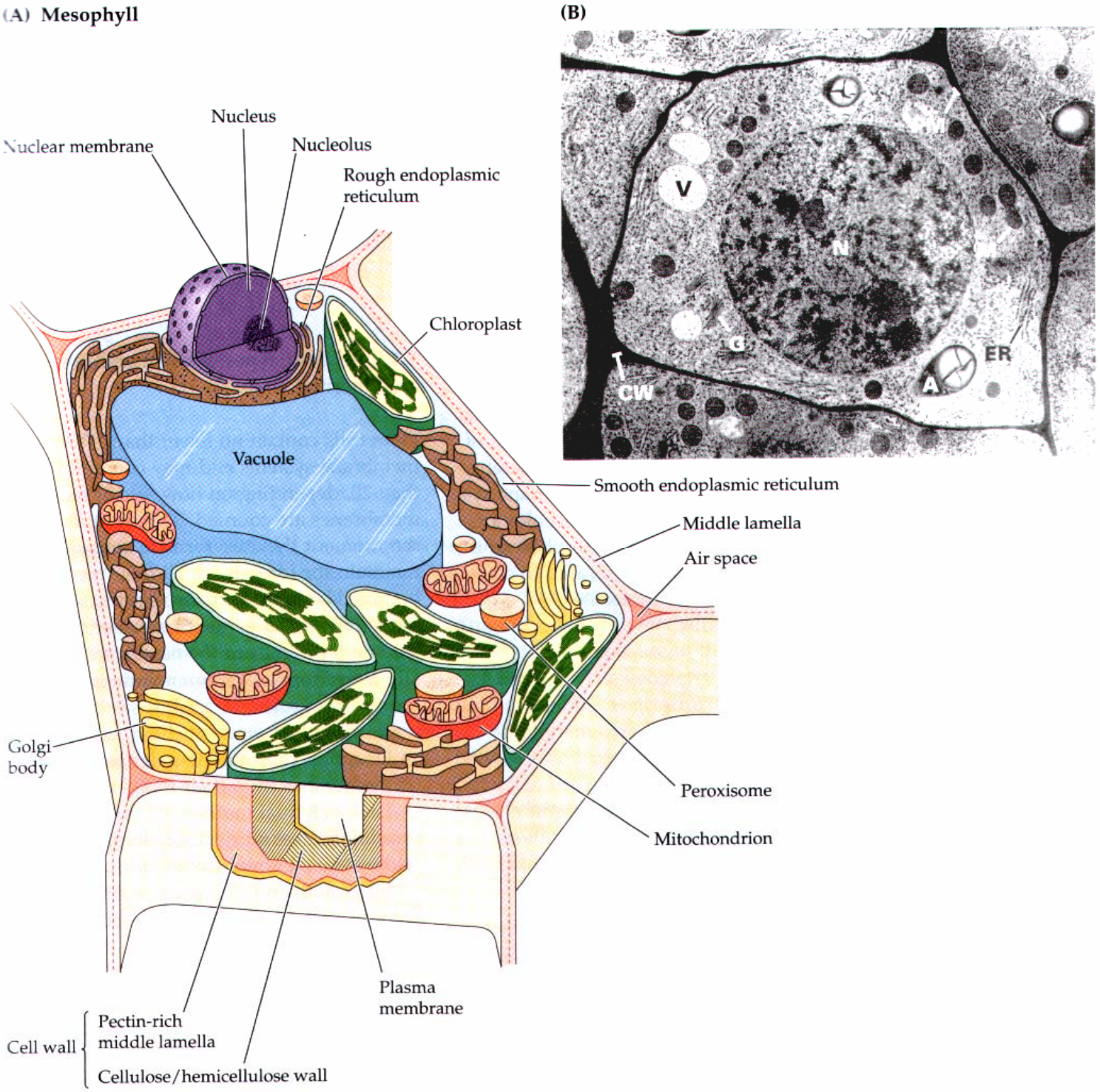








一個典型植物細胞

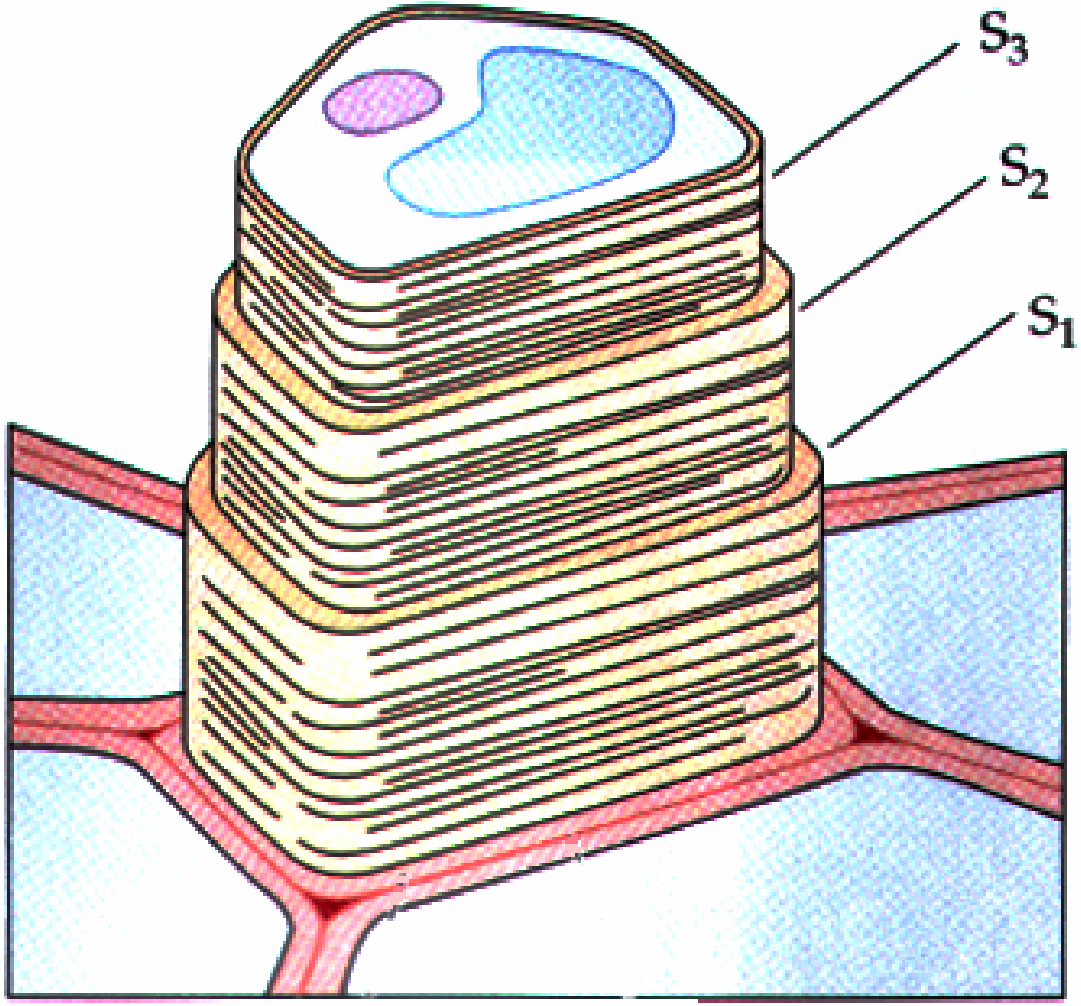


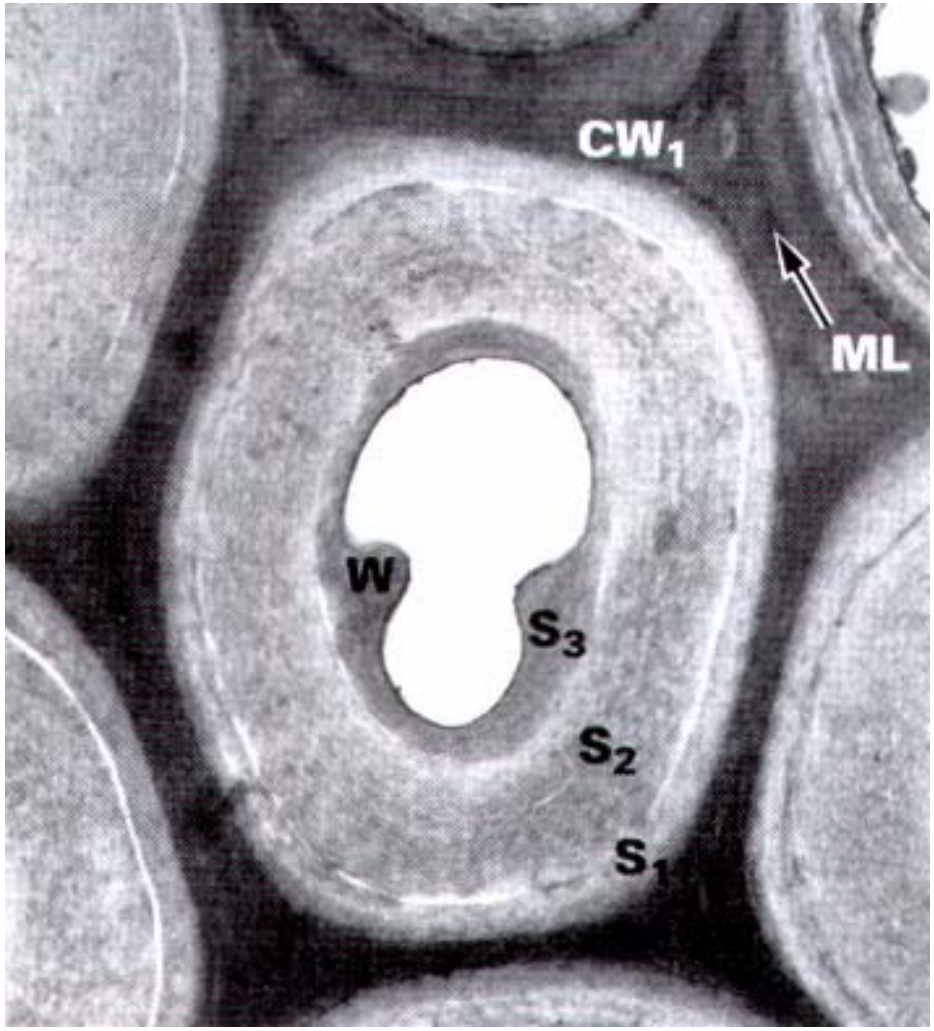
A typical plant cell

Buchanan et al (2000) Biochemistry and Molecular Biology of Plants p.3

植物的細胞壁 Plant cell wall is a barrier

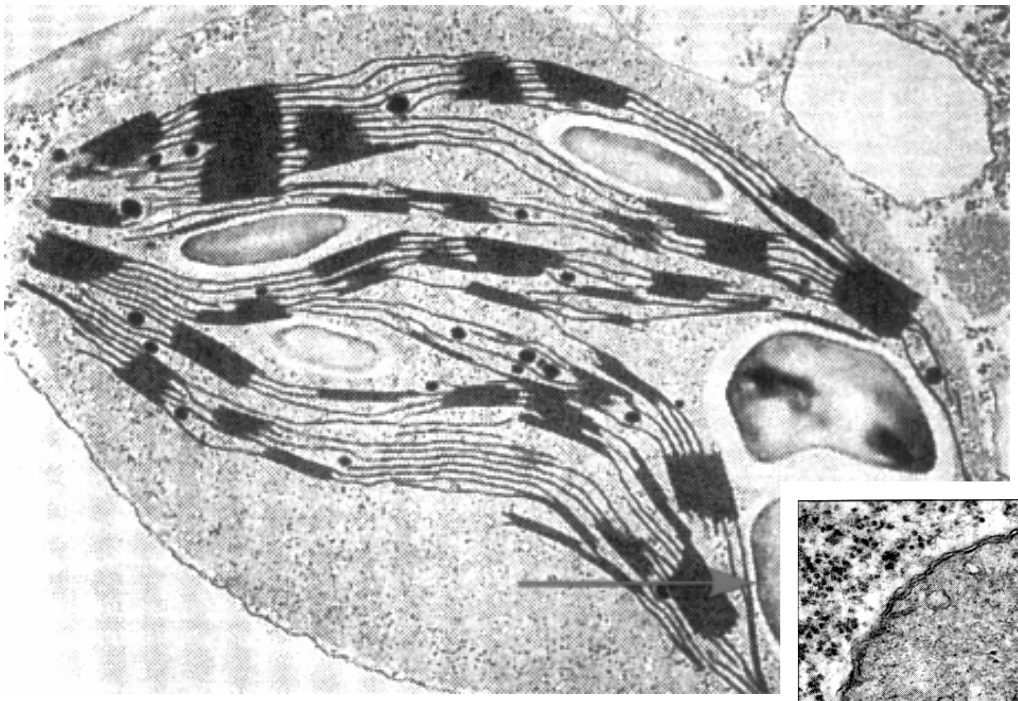
- Old plant cell wall is very tough to break

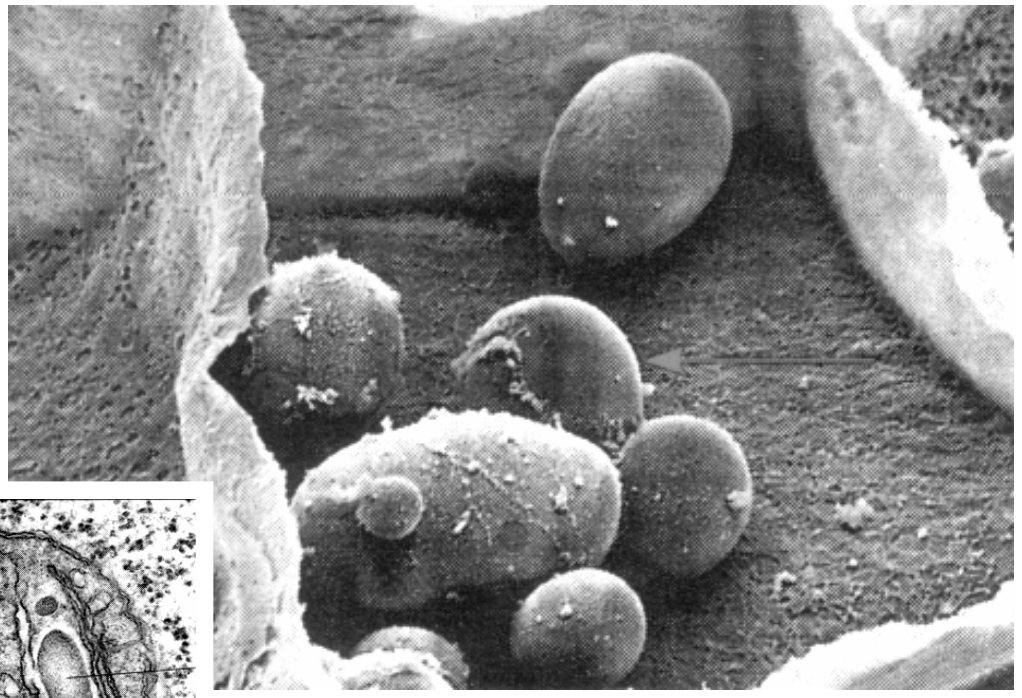


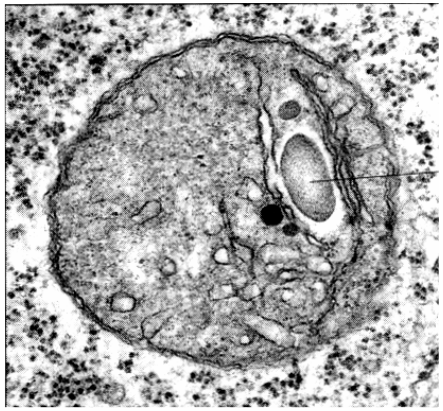


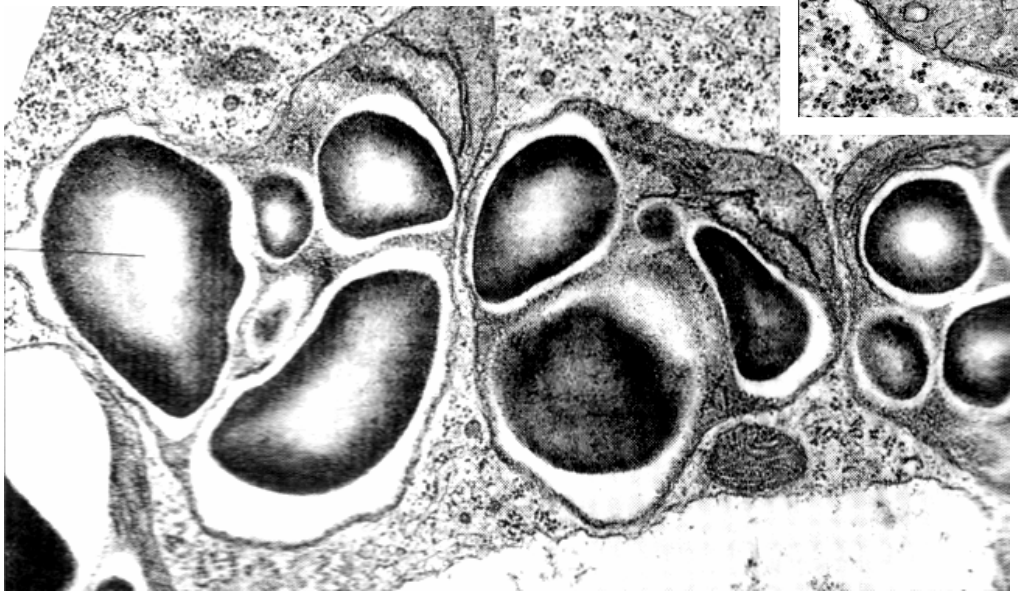
Buchanan et al (2000) Biochemistry and Molecular Biology of Plants p.54

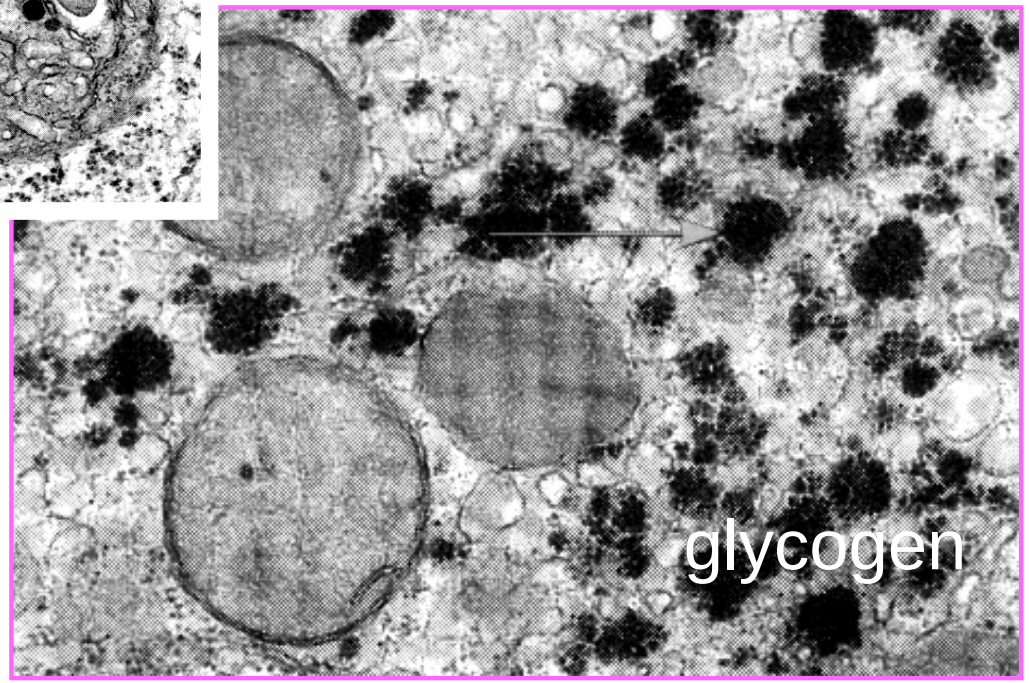
Transformed from proplastid to amyloplast and chloroplast











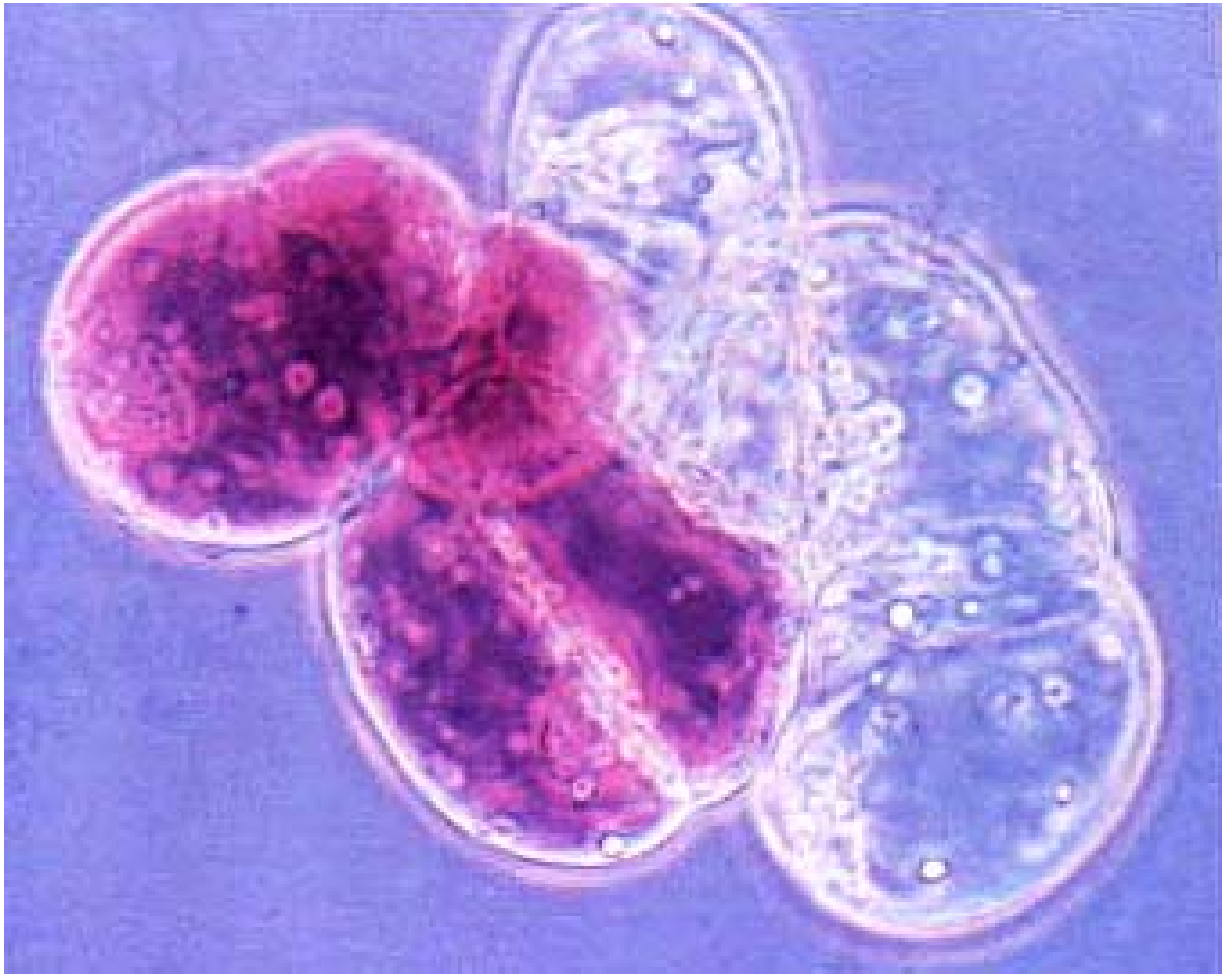
Alberts et al (2002) Molecular Biology of the Cell (4e) p.794

Mathews et al (1999) Biochemistry (3e) p.299

植物色素在純化上的問題 Pigment is a problem

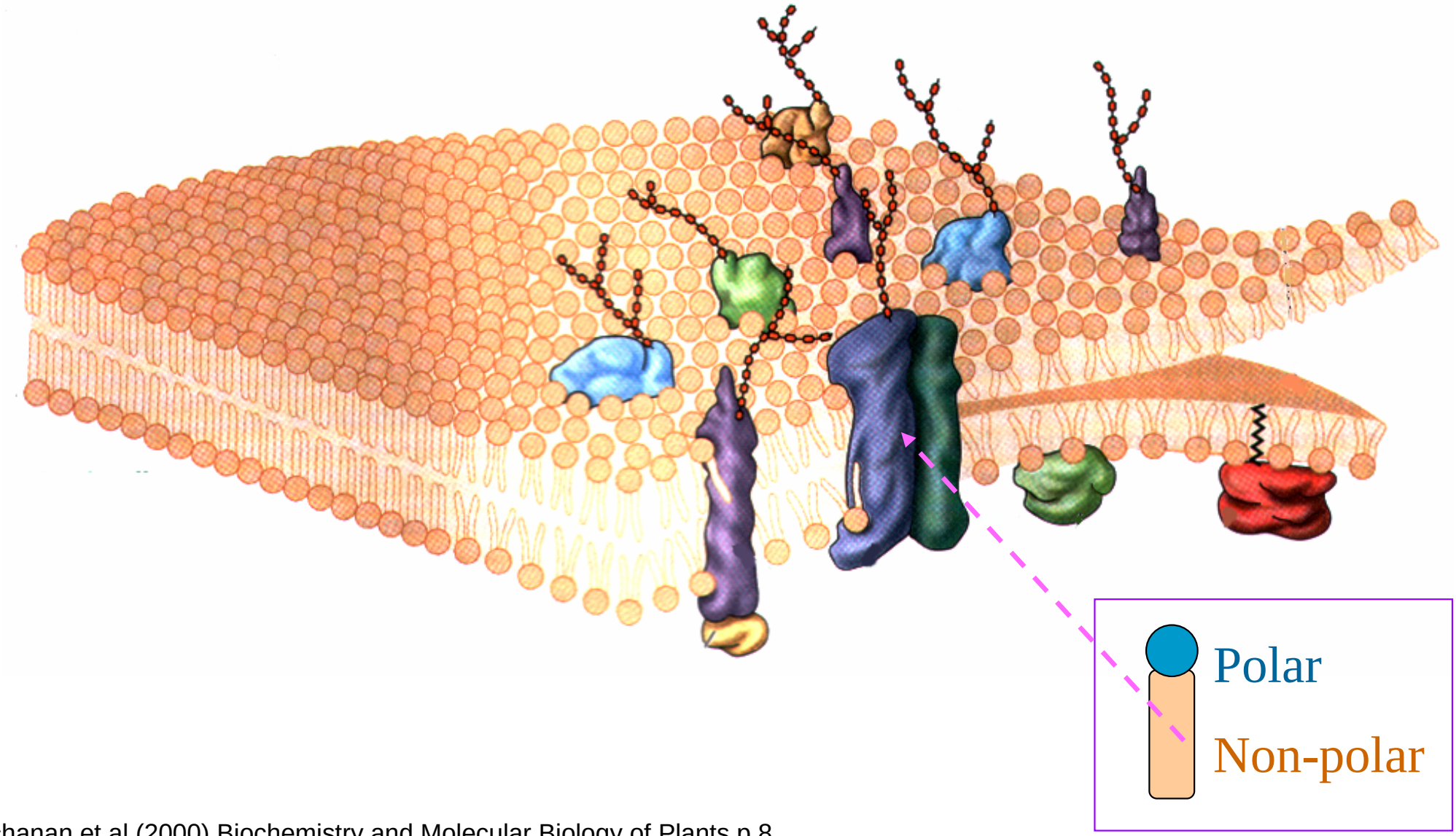
- Pigment stains the gel during purification

Scientific American



細胞膜蛋白質抽取較困難 Membrane proteins

- Detergent (Triton X) is used to solubilize membrane proteins



Buchanan et al (2000) Biochemistry and Molecular Biology of Plants p.8

植物材料問題 Special problems for plant materials

- 細胞壁 Cell wall 較難打破
- 葉綠体 Chloroplast 特有的酵素
- 液 泡 Vacuole 有許多干擾物質

蛋白酶 (proteases)

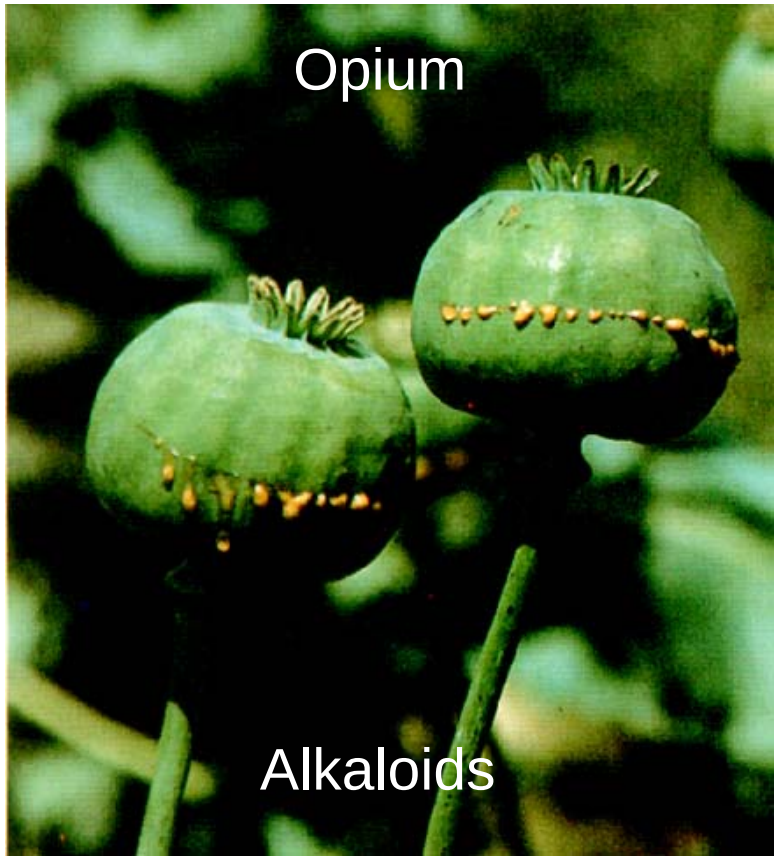
多酚化合物 (polyphenols):

Alkaloid 生物鹼

Flavonoid 類黃酮

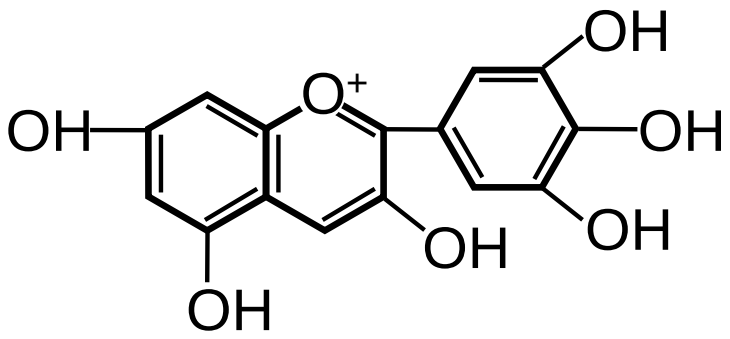
Tannin 單寧

植物二次代謝物有重要藥理作用



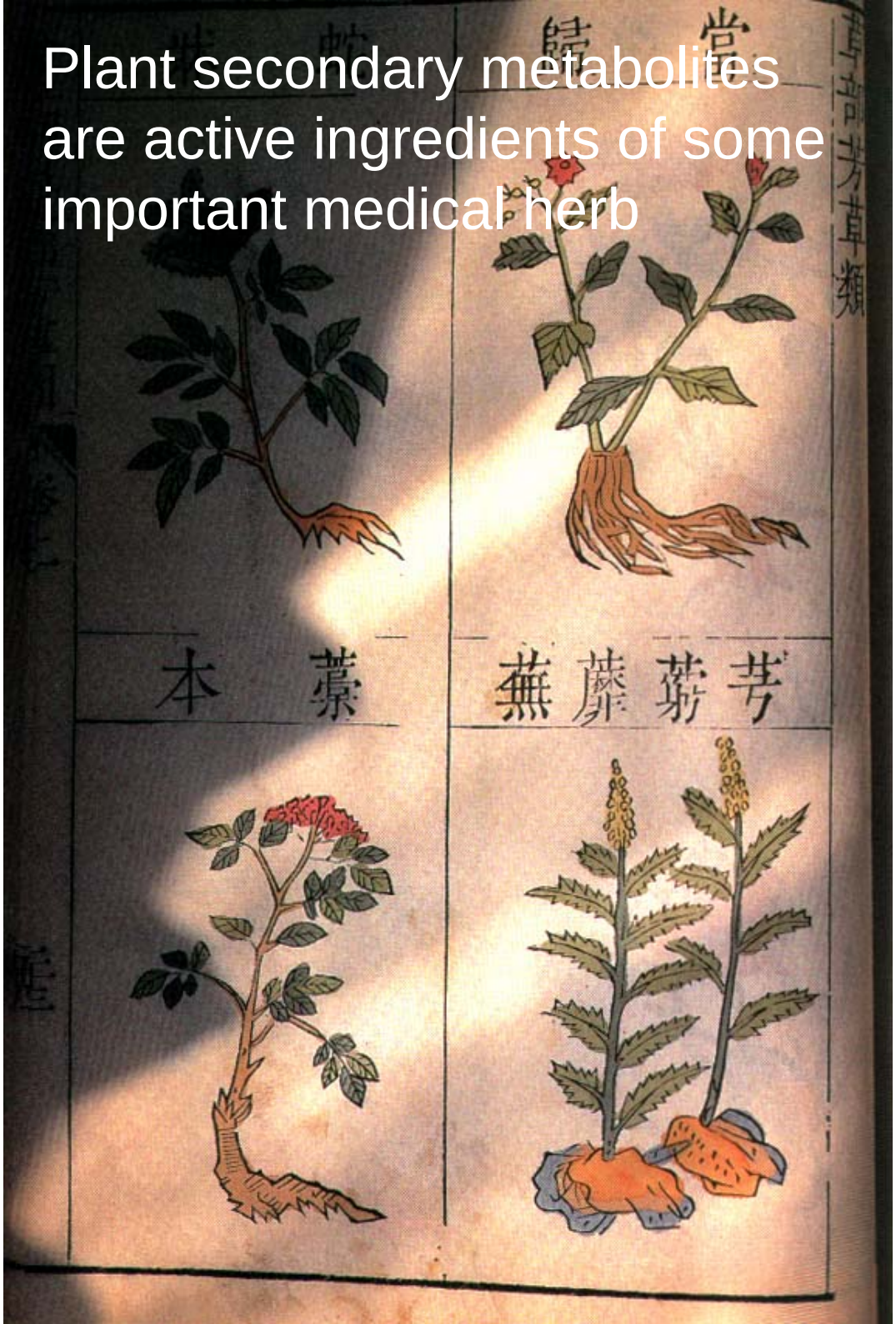
牛頓雜誌 (1991) 第 164 期, p.48-49

Polyphenolic compounds

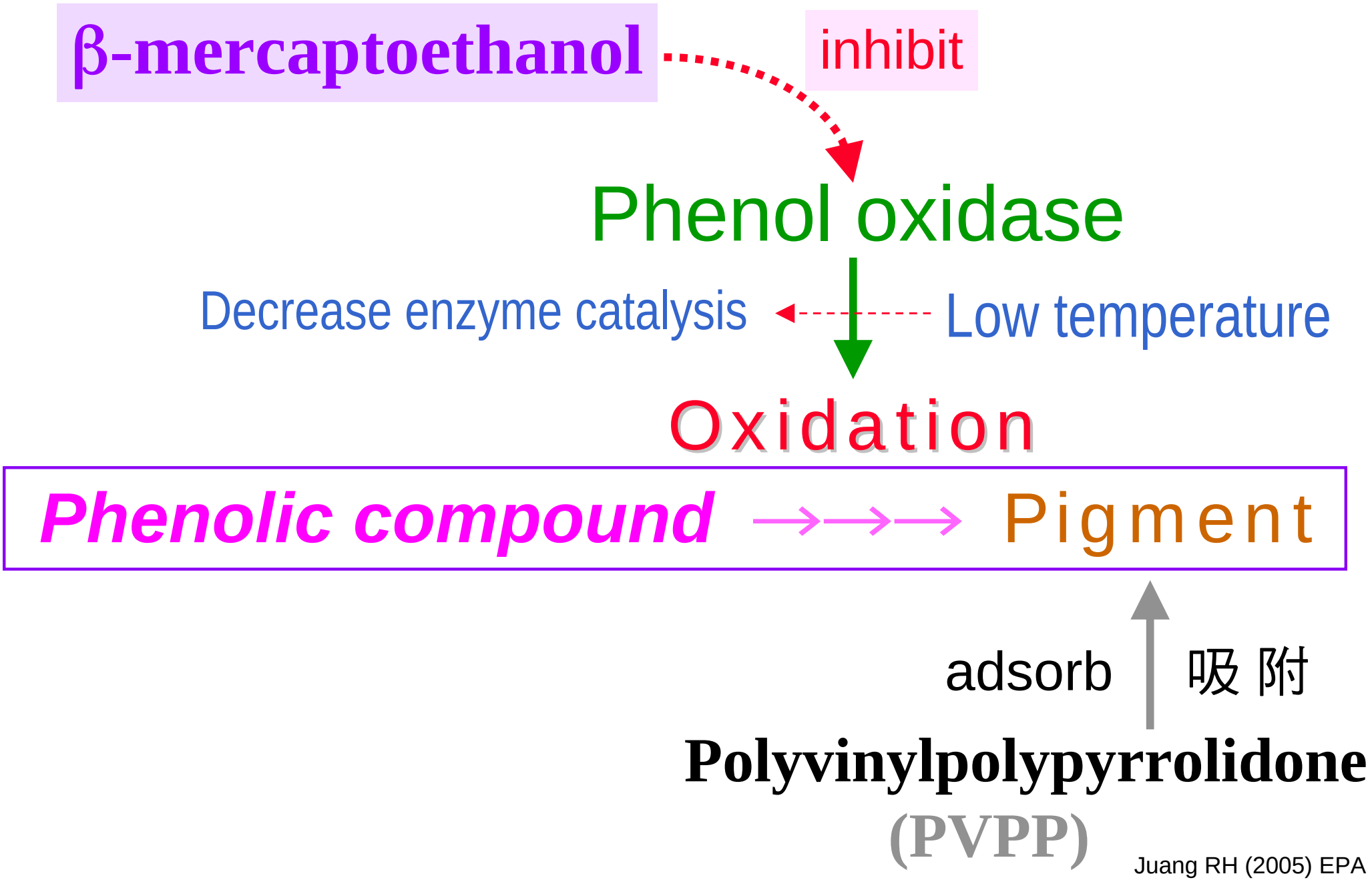


Everyday Science Explained

Plant secondary metabolites are active ingredients of some important medical herb



植物色素之產生與去除 How to remove pigment



Juang RH (2005) EPA

2 蛋白質抽取 Protein extraction

- 2.1 如何開始？ How to start?

  5W principles 基本原則

- 2.2 材料來源 Materials & sources

  材料取得與保存

- 2.3 均質及抽取 Homogenization & extraction

  確實做好第一步

- 2.4 鹽析及沉澱法 Salting-out & precipitation

  最經濟方便的方法

打破細胞的方法 101 ways to break the cell

Dry way:

液態氮研磨 (grinding in liquid nitrogen), 磨粉機 (coffee grinder), 球磨機 (ball mill)

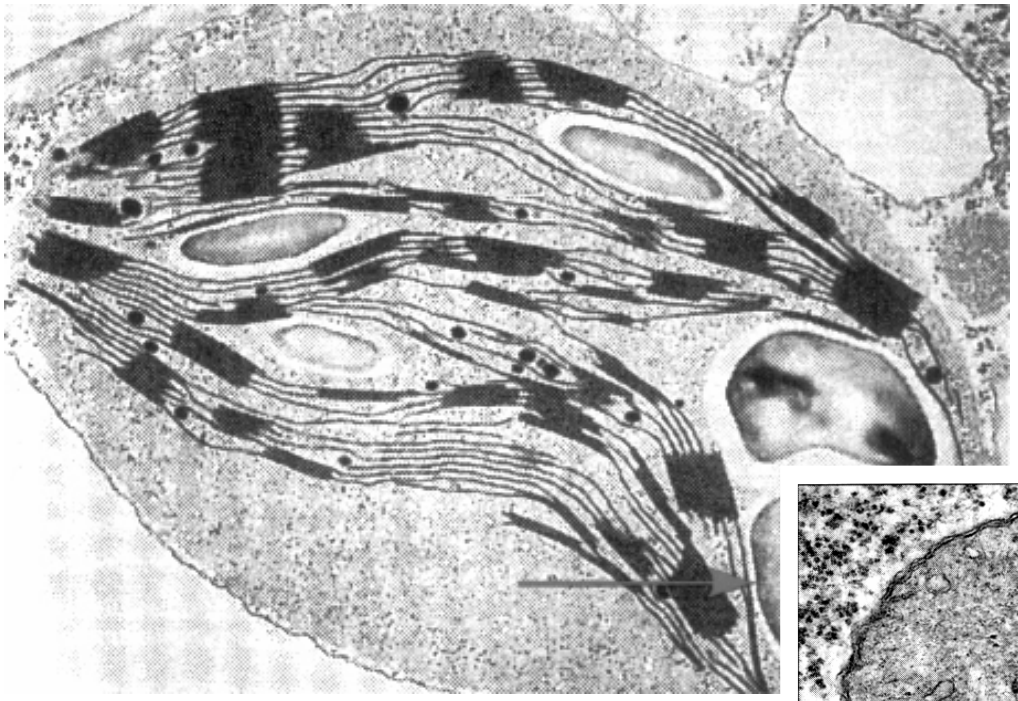
Wet way:

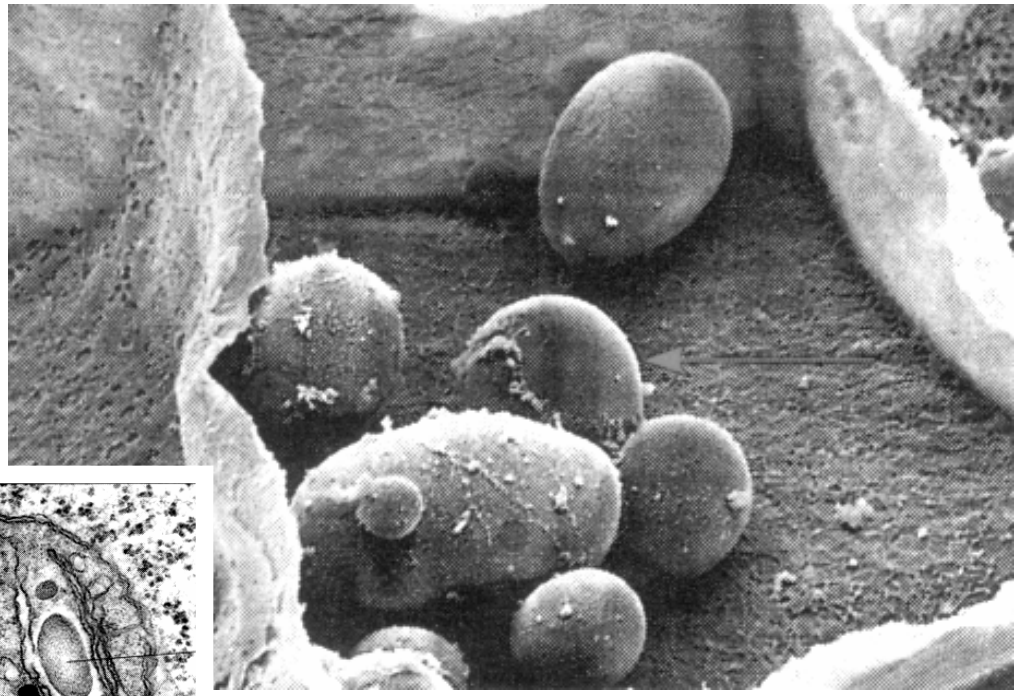
均質器 (homogenizer), 果汁機 (Waring blender), Polytron, 研砵 (mortar), 玻璃球 (glass bead mill), 超音波震盪 (ultrasonication), French press

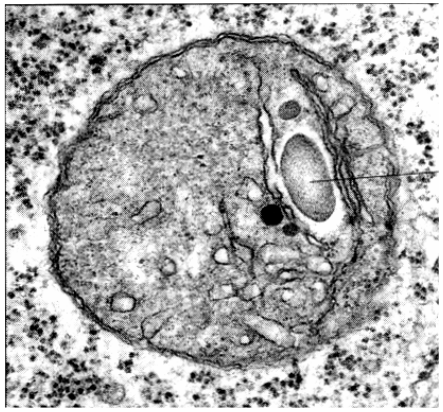
Is your target protein released from the cell?

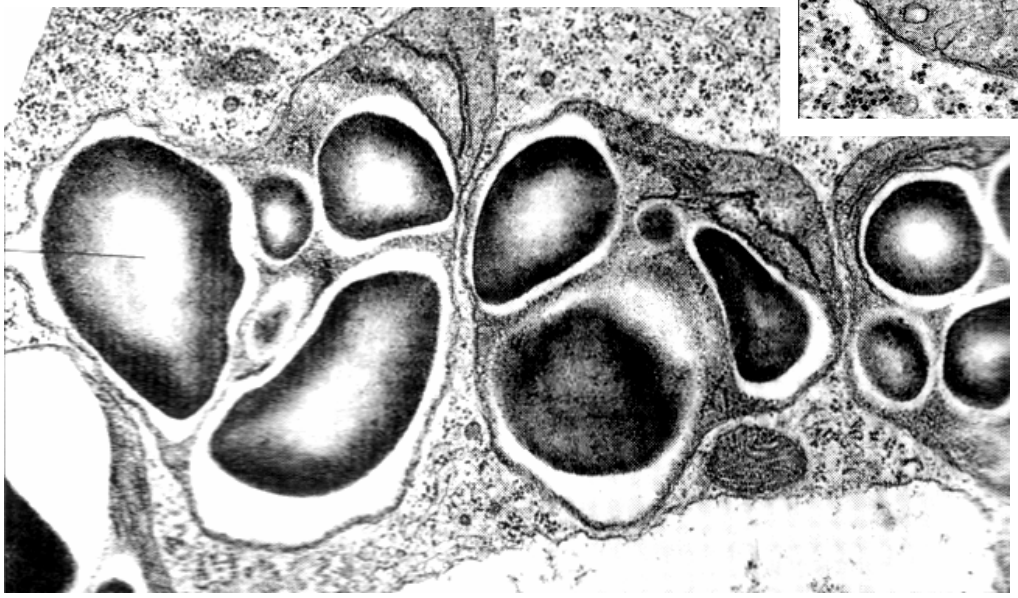
Mathews et al (1999) Biochemistry (3e) p.299

● 某些胞器的分離相當困難 Some organelles are difficult to isolate

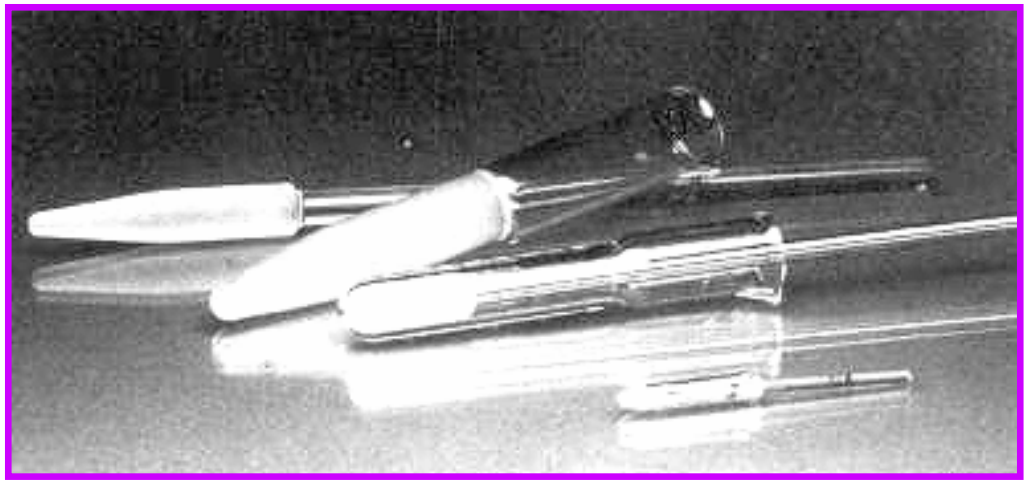






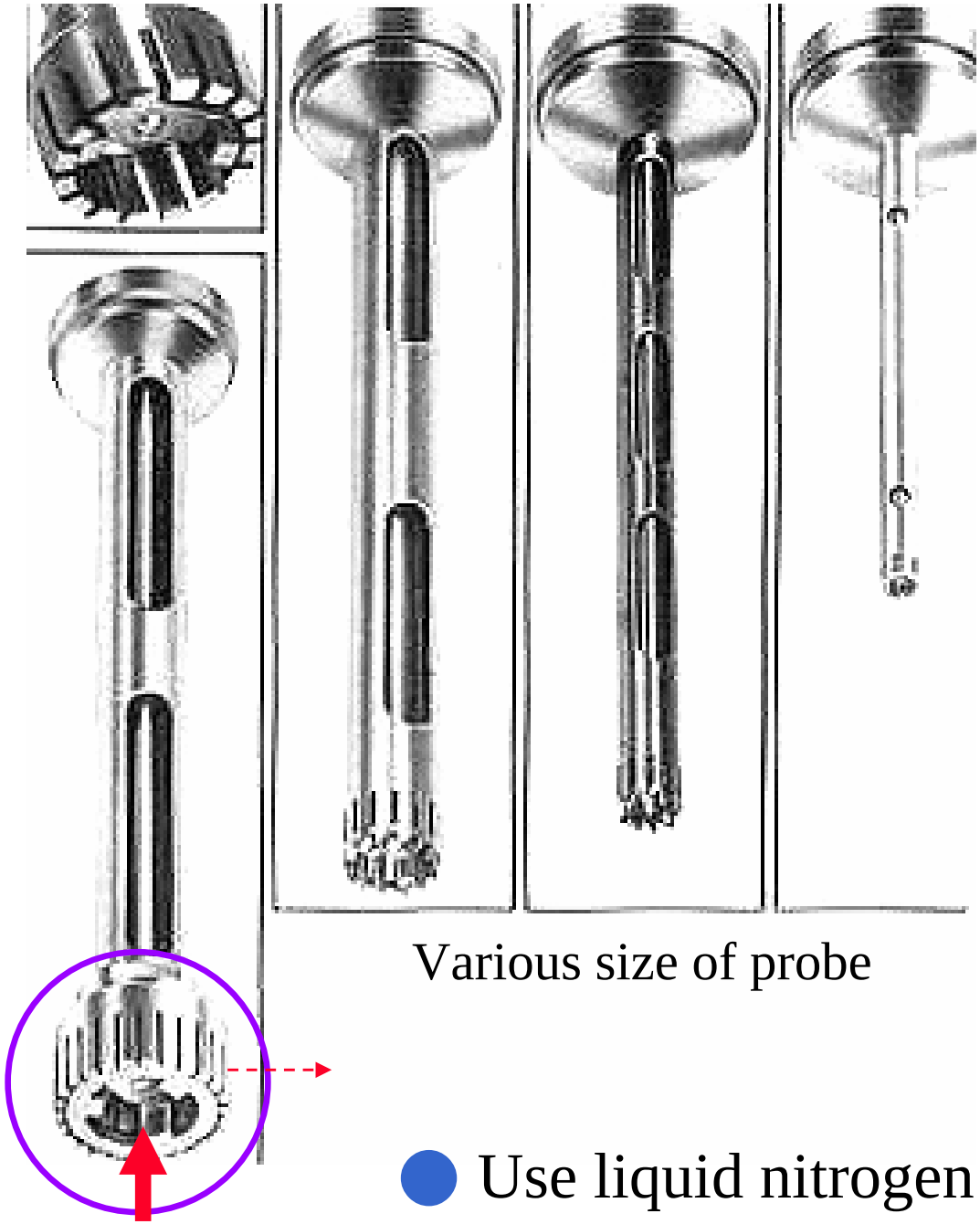


▼Homogenizer for breaking the cell but leave organelles intact



Alberts et al (2002) Molecular Biology of the Cell (4e) p.794

研磨樣本常用方法 Two popular methods

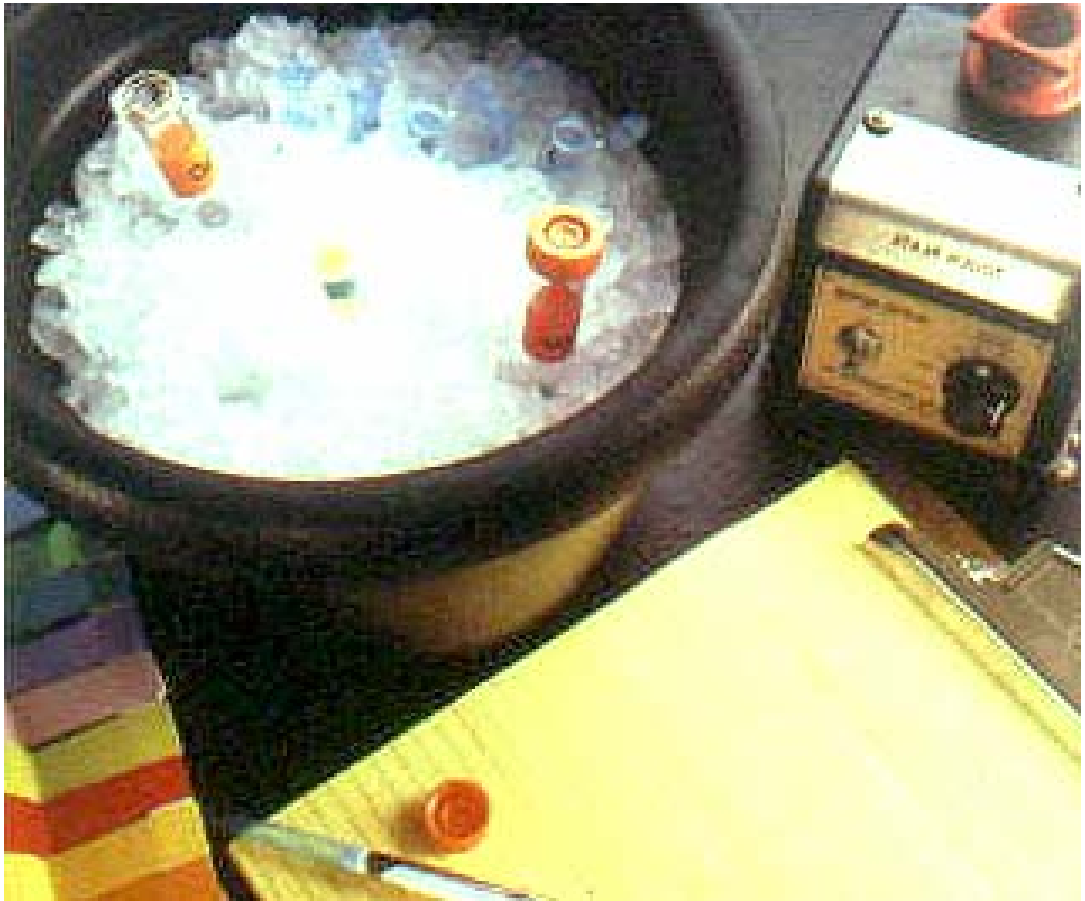


Various size of probe

- Use Polytron

- Use liquid nitrogen

細胞打破之後 After breaking the cell…..



(1) 降低溫度
Keep temperature low

(2) 儘速純化
Purify as soon as possible

(3) 避免氧化
Avoid oxidation

(4) 避免吸著
Avoid adsorption by flask

(5) 避免污染
Avoid contamination

Juang RH (2005) EPA

2 蛋白質抽取 Protein extraction

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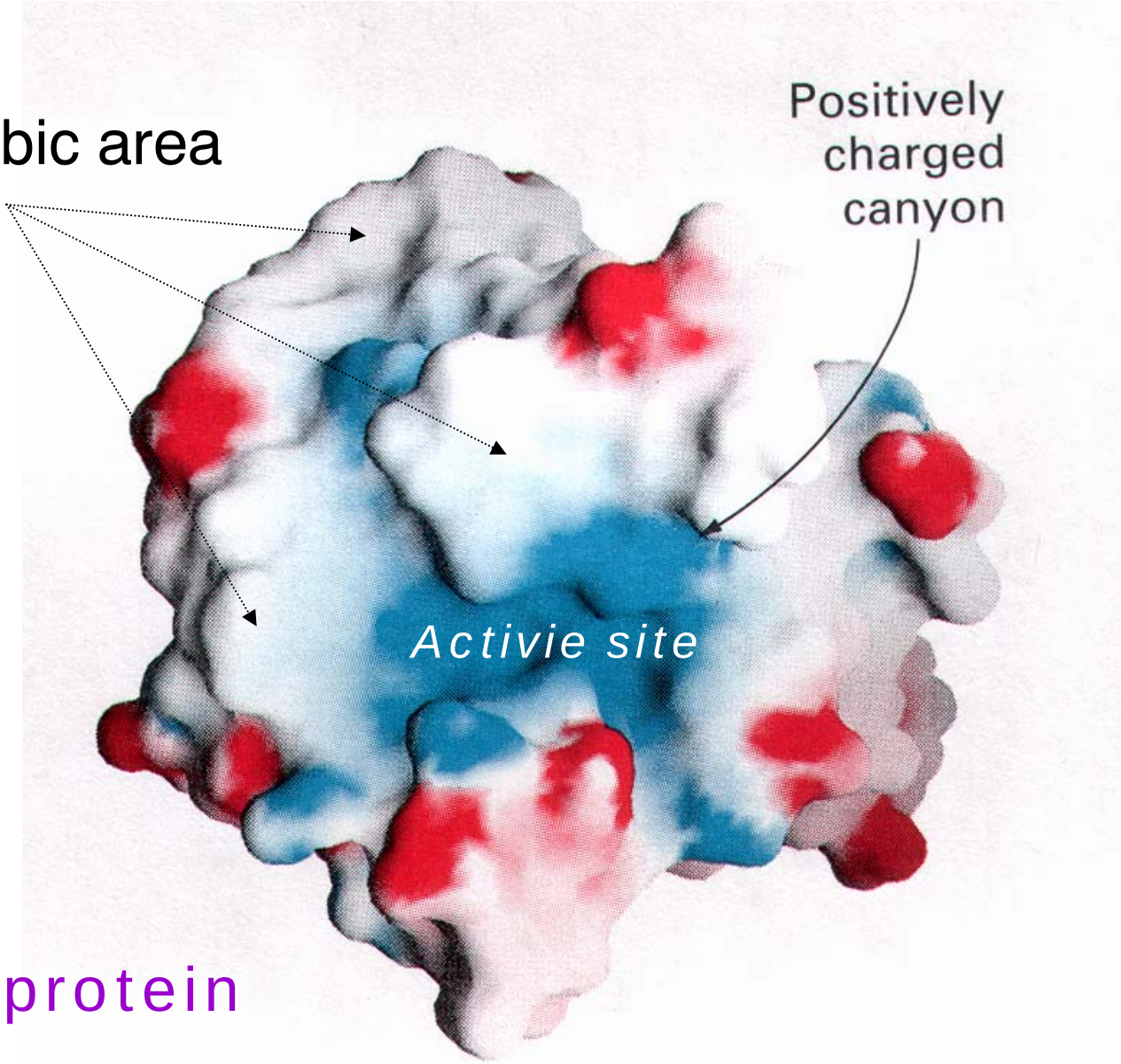
  最經濟方便的方法

蛋白質表面的極性或非極性分布



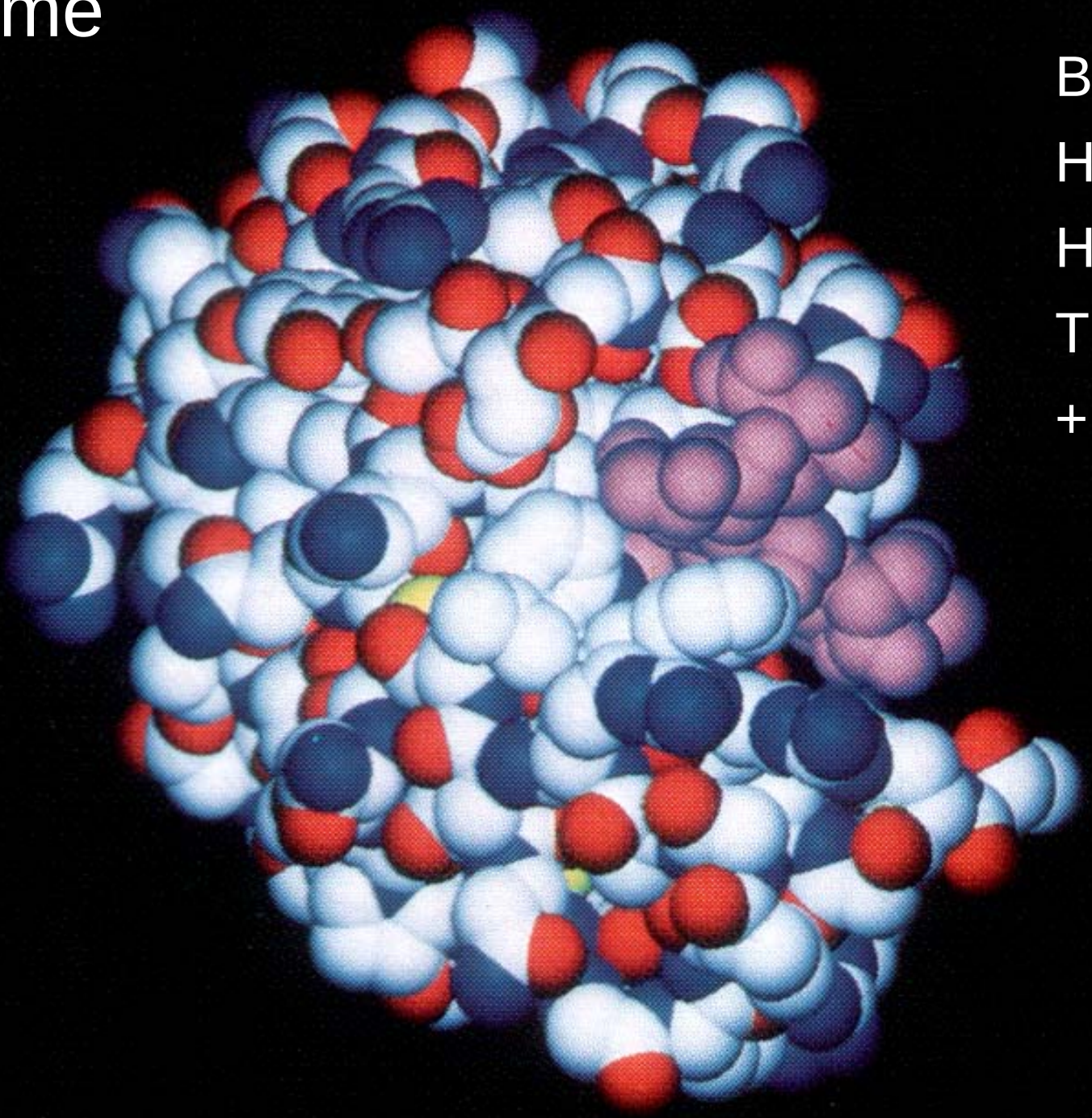
A typical protein

Superxoide dismutase (SOD)

Protein surface has both polar and non-polar patches.

Stryer L (1995) Biochemistry 4/e Fig. 21-36

Lysozyme

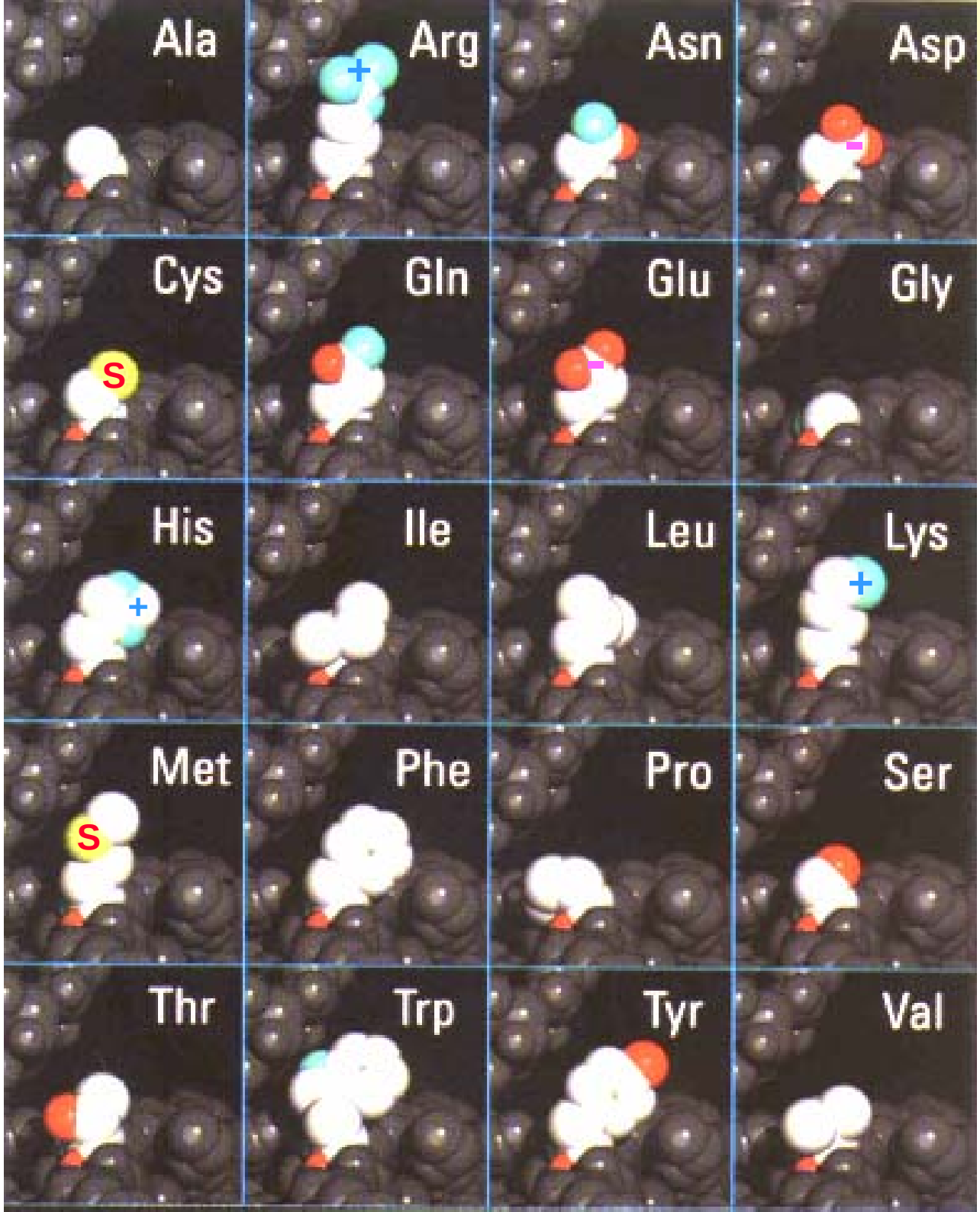


Backbone
Hydrophobic
Hydrophilic
Total
+ substrate

Watson et al. (1987) Mol Biol Gene, Plate 3

二十種胺基酸的特徵

Structural features of 20 amino acids



Side chains

有大有小

Large or small

有正有負

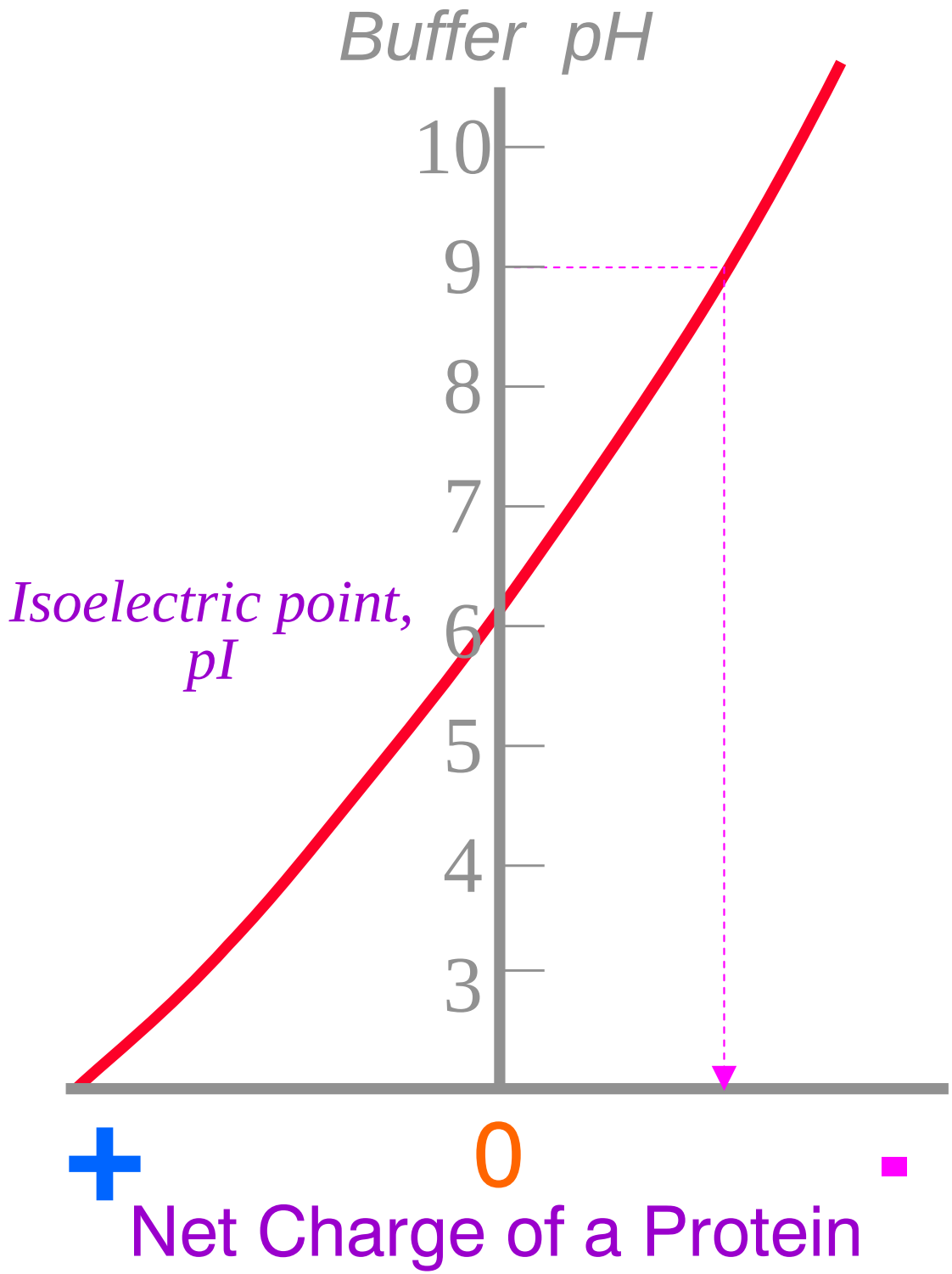
Positively or negatively charged

有極性

非極性

Polar or non-polar

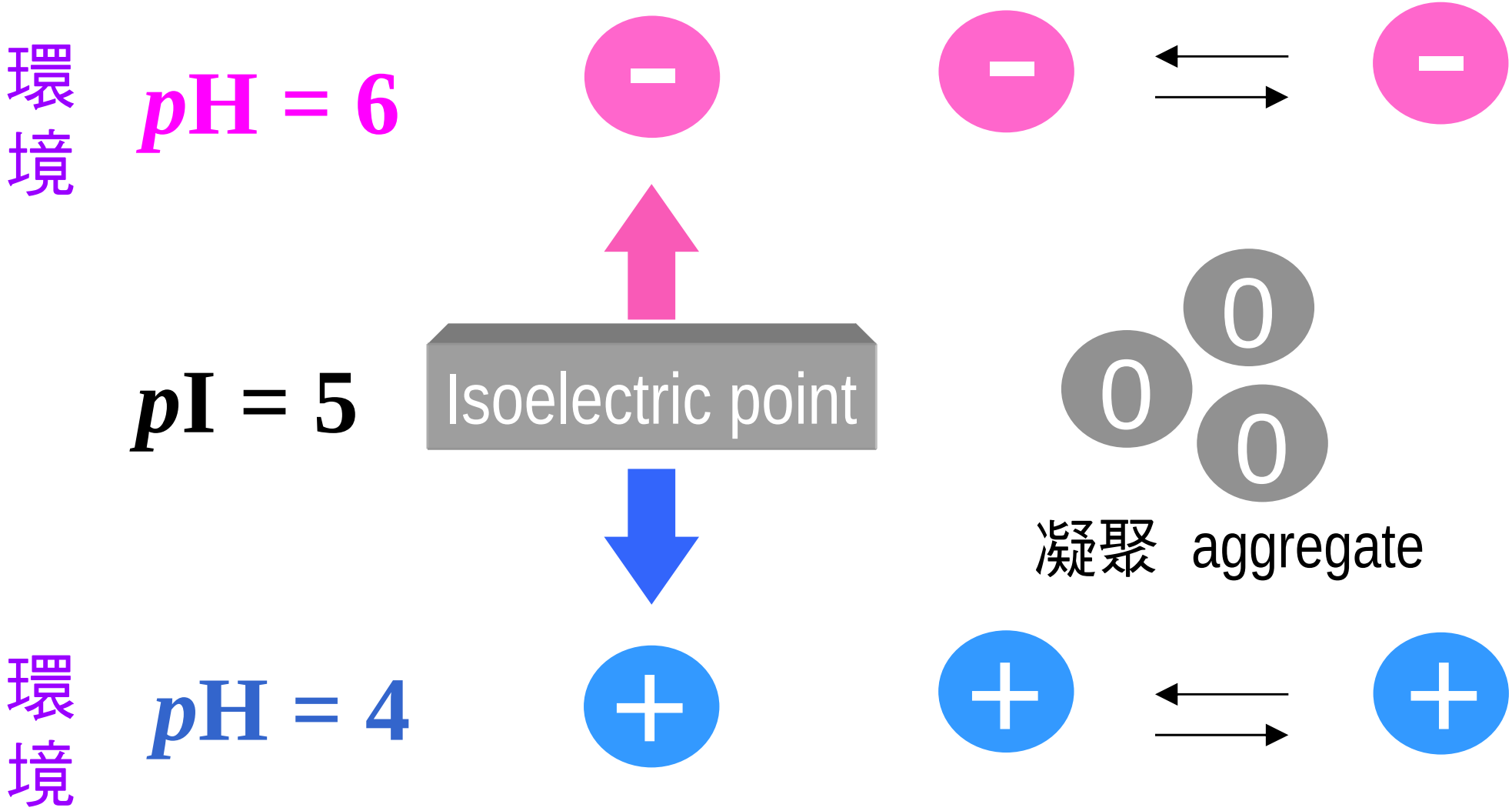
環境影響分子的帶電性質



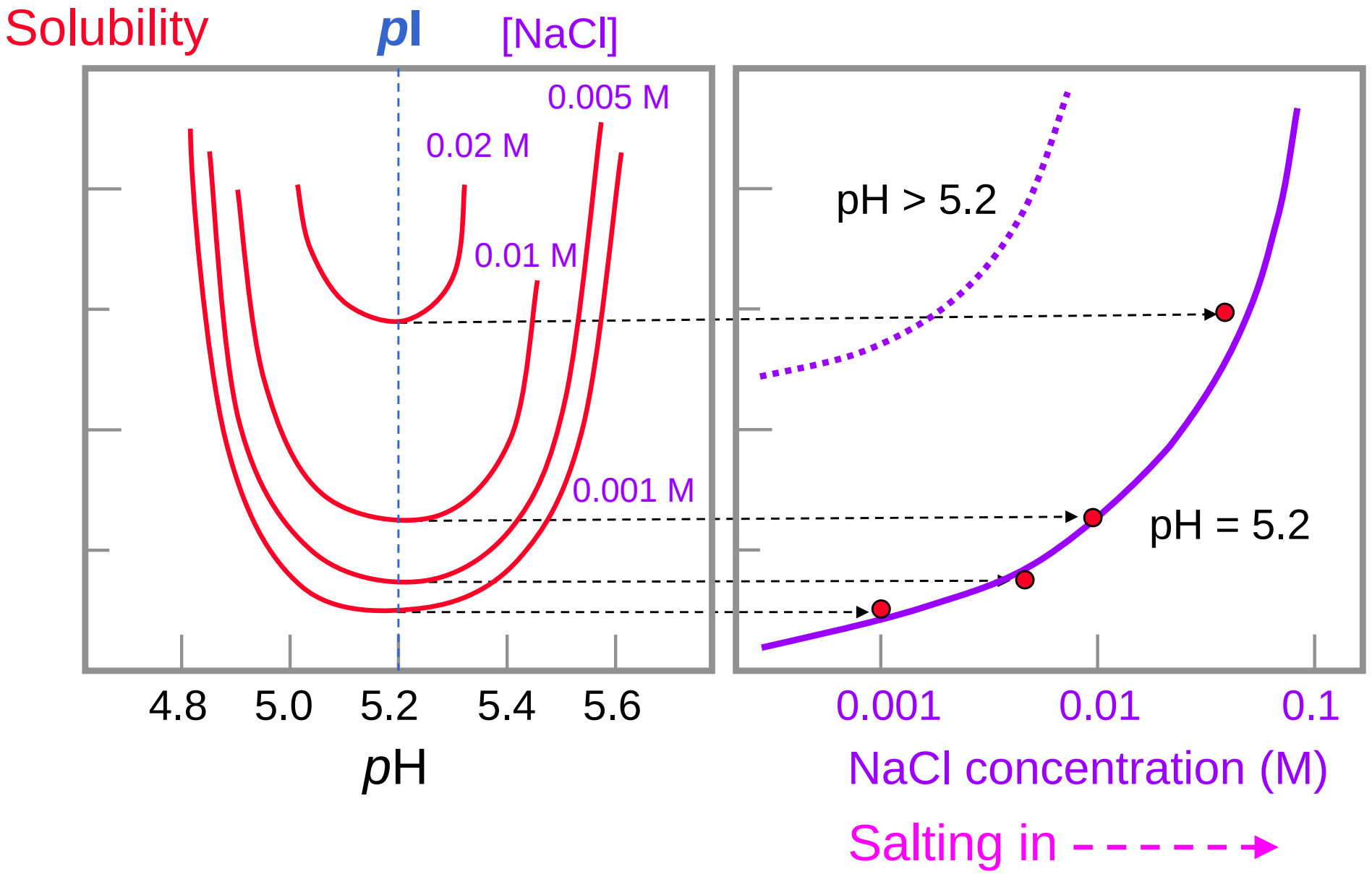
Environmental pH effects the charge properties of a protein

Juang RH (2005) EPA

等電點與環境 *p*H 的關係 pI versus pH



提高鹽濃度增加蛋白質溶解度

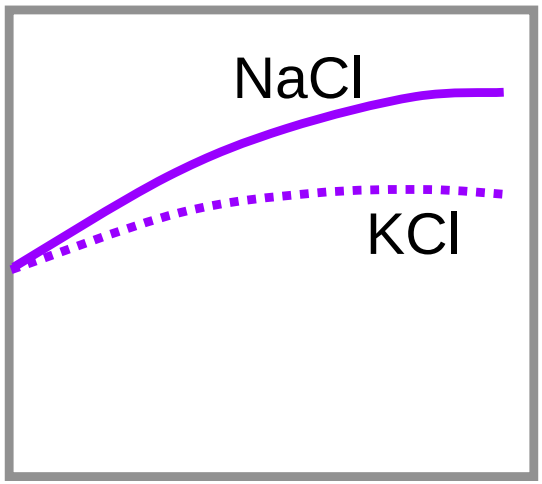


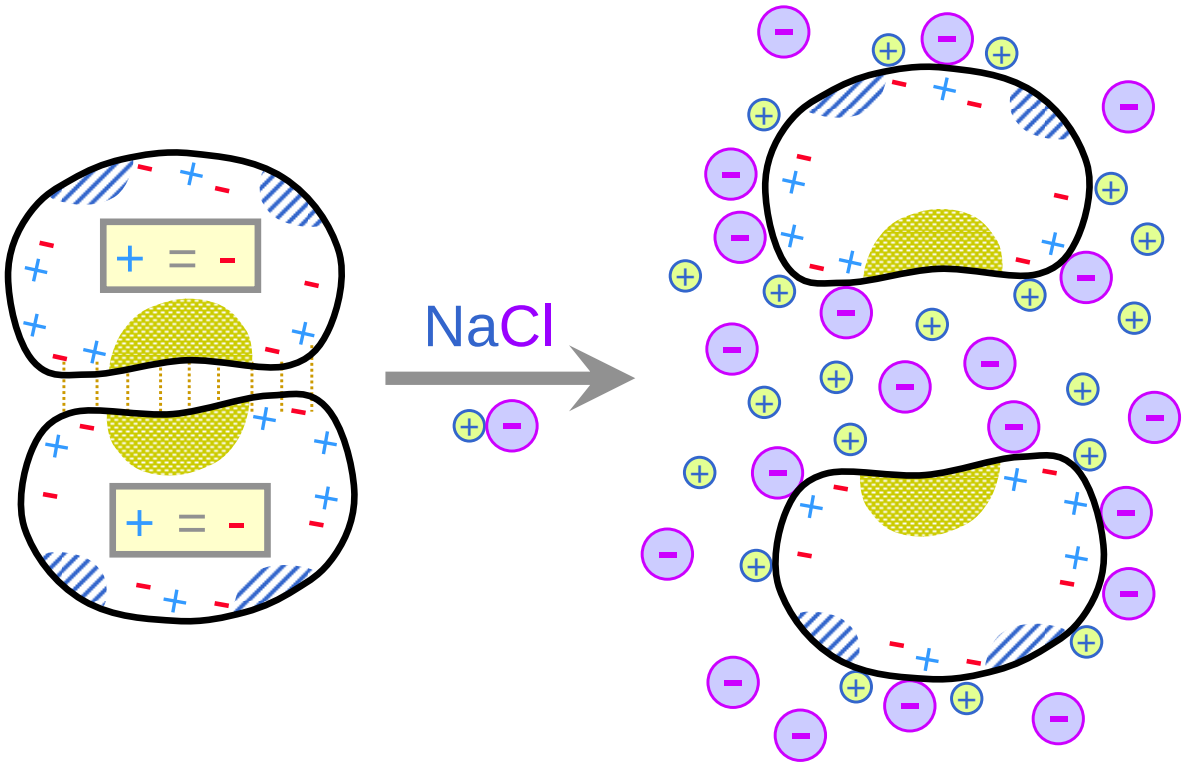
Higher salt concentration increases the solubility of a protein

Juang RH (2005) EPA

鹽溶 Salting-in effect

Solubility



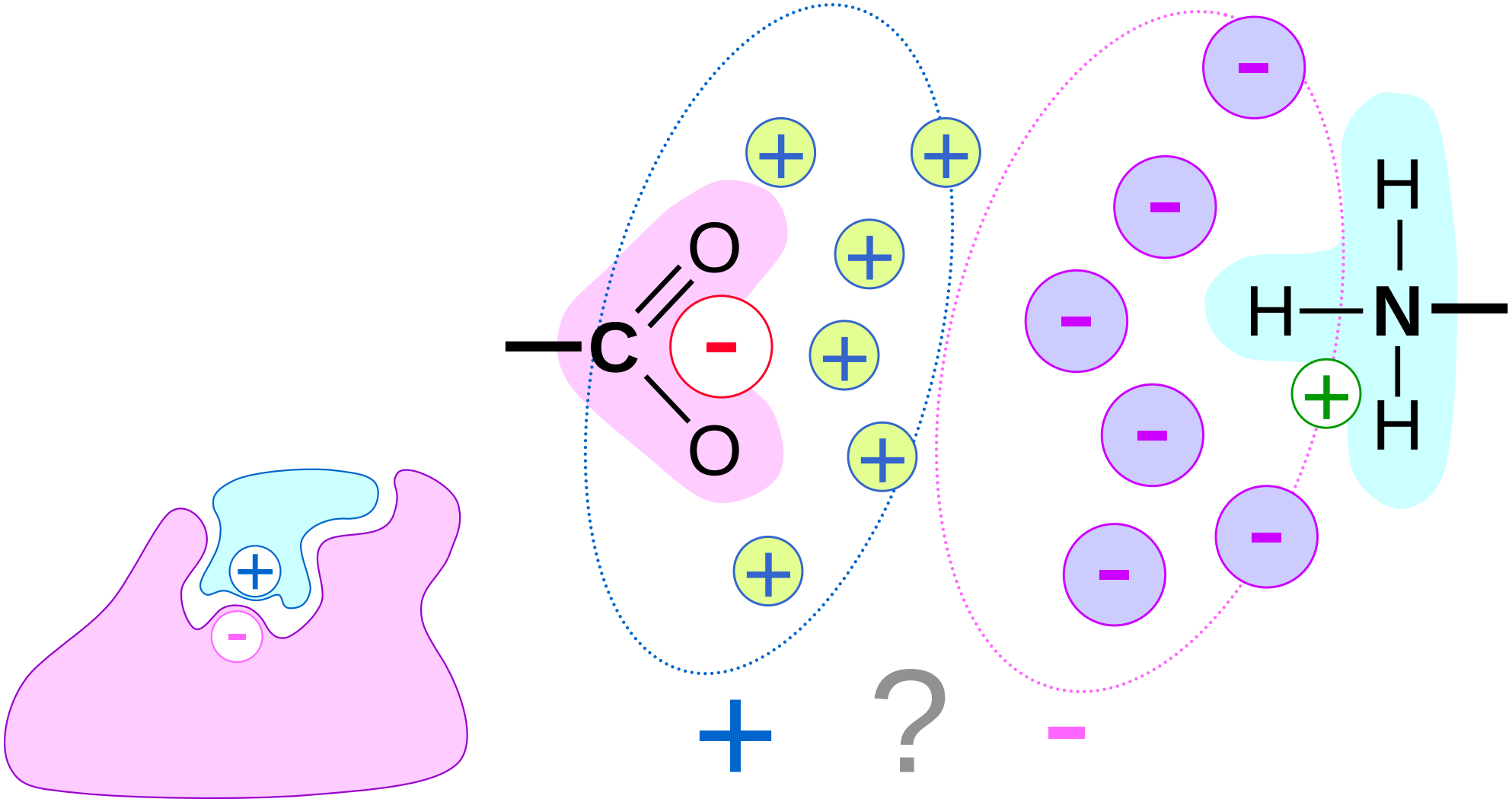


分子在其等電點時，容易互相吸引，聚合成沈澱；加入鹽離子會破壞這些吸引力，使分子散開，溶入水中。

Juang RH (2005) EPA

離子鍵在鹽溶液中不易形成

Adapted from Alberts et al (2002) Molecular Biology of the Cell (4e) p.115



- 水中的酵素與基質還是可以生成離子鍵

But the enzyme-substrate binding can still depend on ionic interactions (why?)

Ionic bonding is not stable in water solution

鹽影響蛋白質溶解度 Salt effects protein solubility

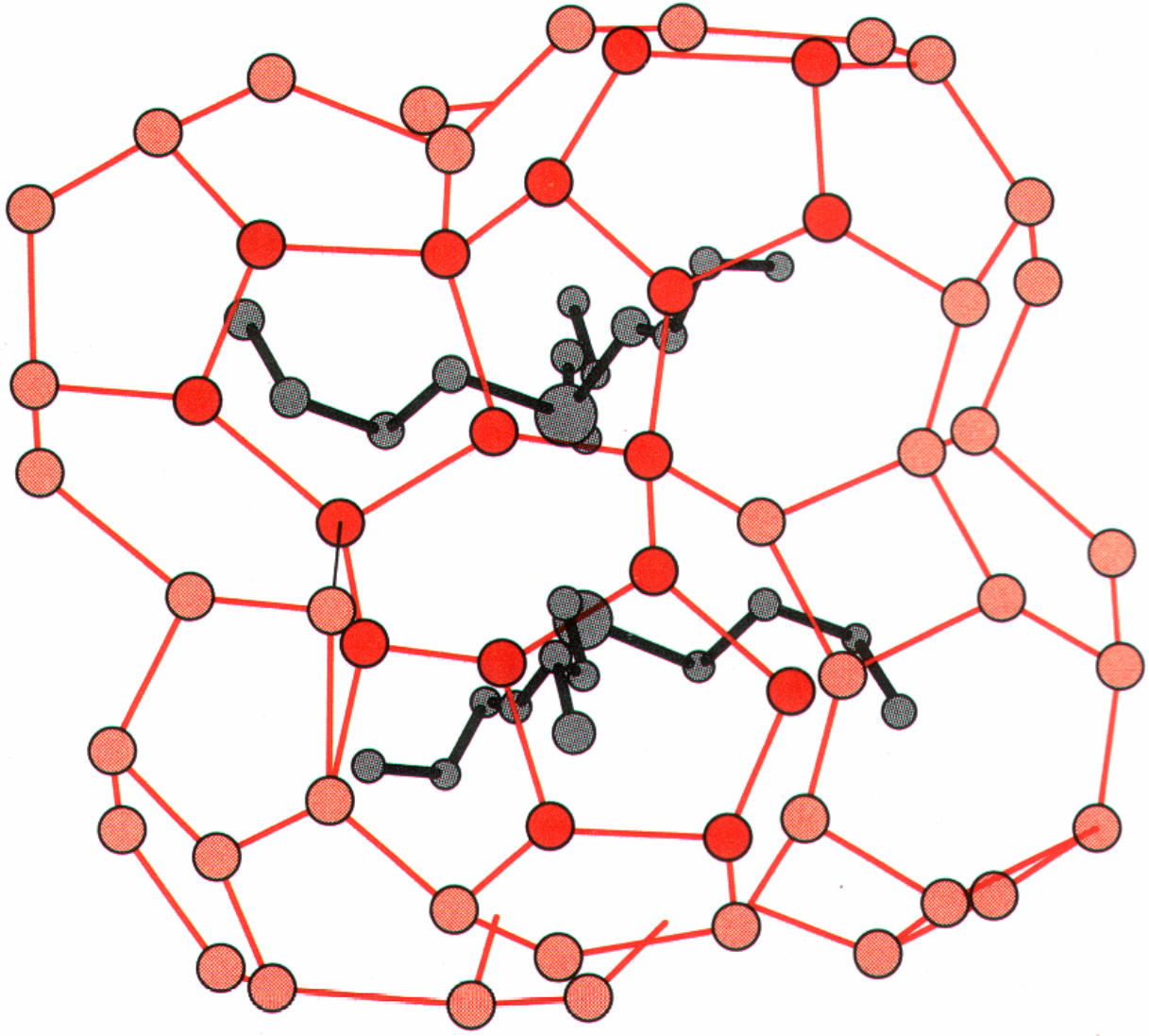
- 鹽溶 Salting-in:

  加鹽使蛋白質溶入水溶液中

- 鹽析 Salting-out:

  加鹽使蛋白質由水溶液中沉澱出來

疏水性物質間的親和力 水籠 Clathrate

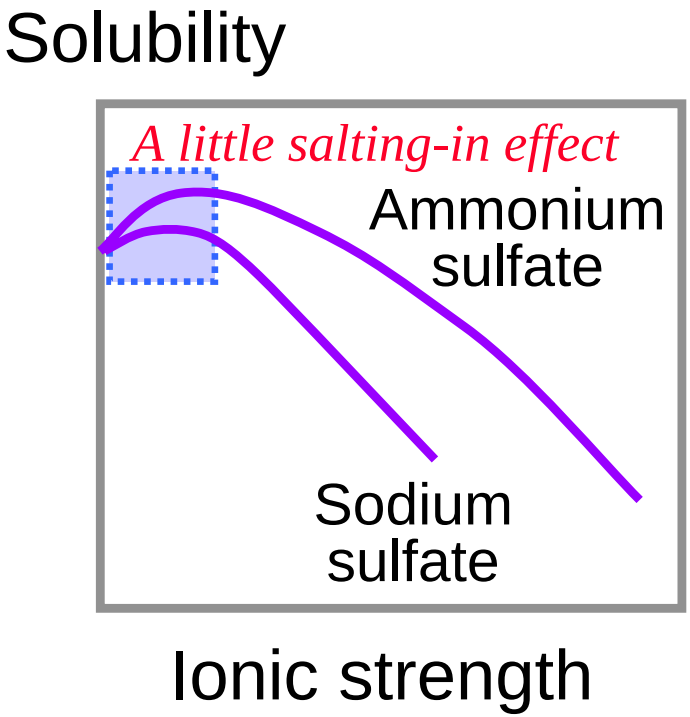


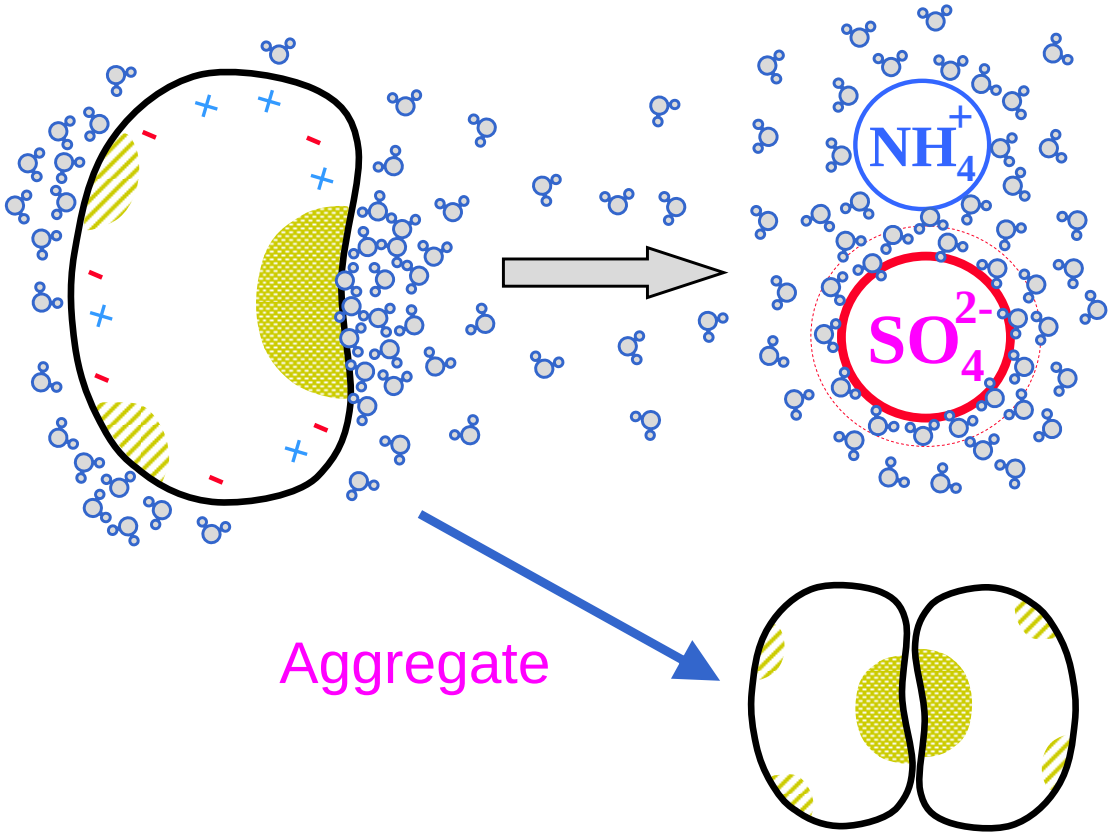
- 水分子會包圍在非極性分子四周，形成類似竹籠的構造，隔離非極性分子，水分子本身的流動性因此而降低。

Water molecules surrounding the outer surface of non-polar molecules are “immobilized” and form a “cage of water” to isolate these non-polar interface

Alberts et al (1994) Molecular Biology of the Cell (3e) p.49

鹽析 Salting-out





蛋白質分子表面的疏水性區域，都聚集許多水分子，當鹽類加入時，這些水分子被抽出，以便與鹽離子進行水合，暴露出來的疏水性區域互相結合，形成沈澱。

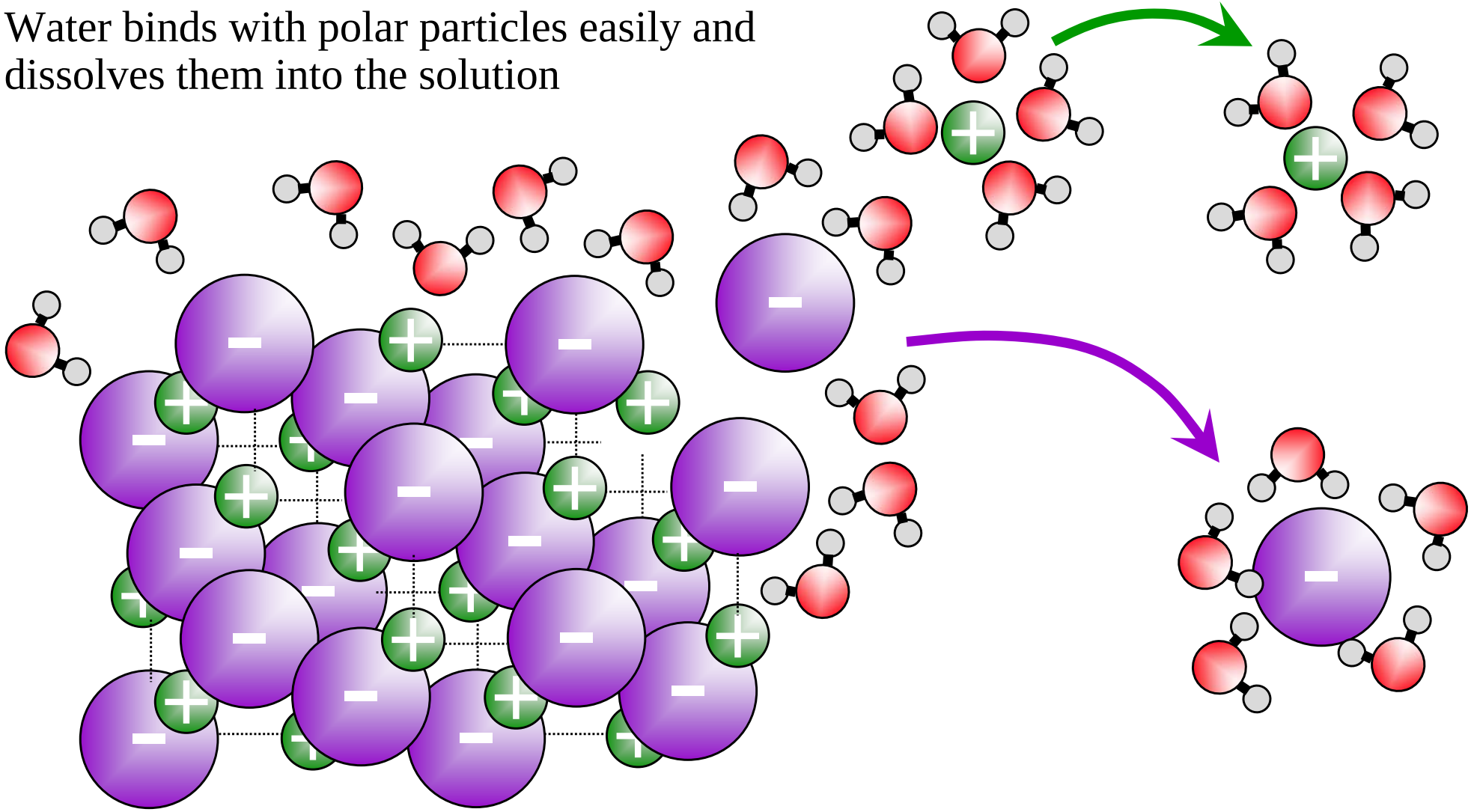
= hydrophobic

Juang RH (2005) EPA

無所不在的水合作用 Hydration is everywhere

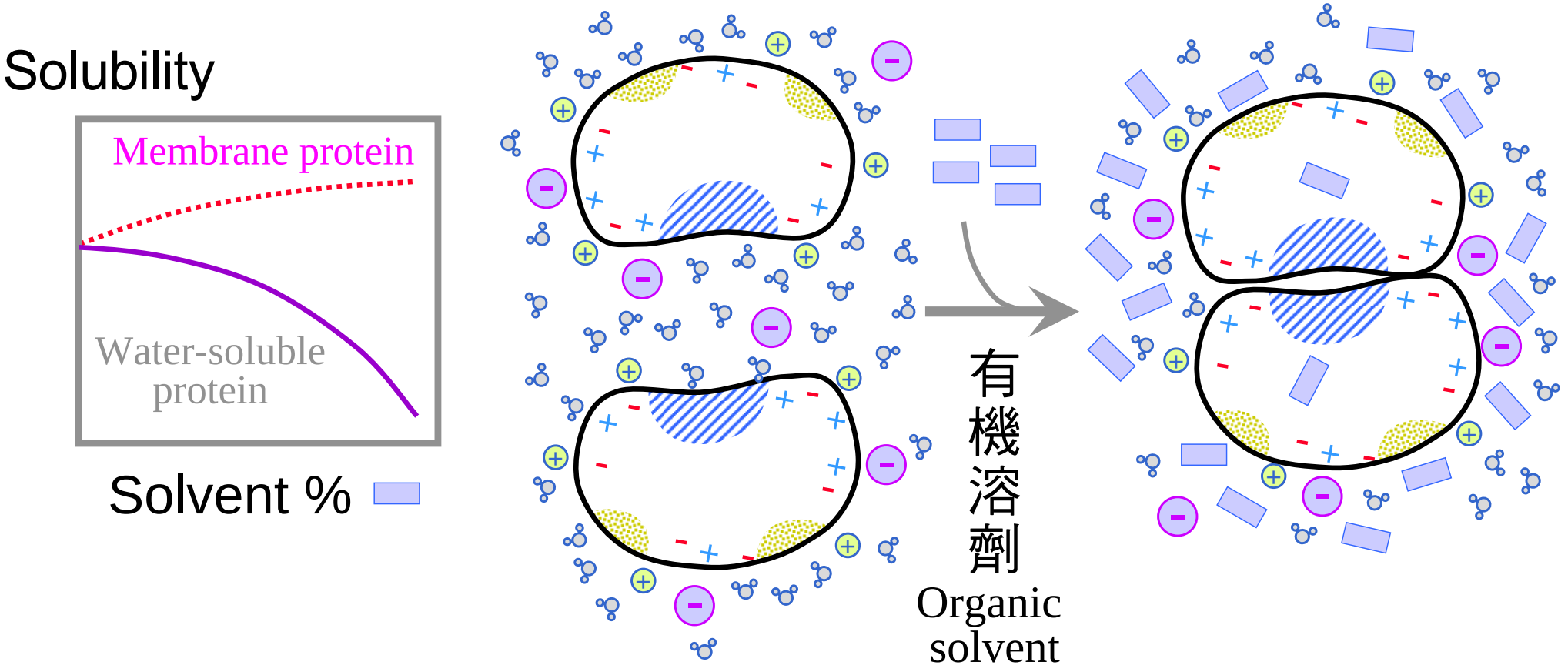
- 水分子易與極性粒子發生水合

Water binds with polar particles easily and dissolves them into the solution



Adapted from Nelson & Cox (2000) Lehninger Principles of Biochemistry (3e) p.87

有機溶劑沈澱法 Precipitation by organic solvent



降低水活性，使溶液的介電常數下降，增加蛋白質溶質分子之間的作用力，因而聚集在一起。

= hydrophilic

Juang RH (2005) EPA

各種鹽析沉澱法比較 Comparison of methods

	Salting-in 鹽溶	Salting-out 鹽析	Organic solvent
Factors	Ionic interactions on protein surface	Non-polar area of protein surface	All interaction forces on protein surface except hydrophobic
Reagents	NaCl (monovalent)	$(NH_4)_2SO_4$ (divalent)	Methanol, acetone
Mechanism	Protein has no net charge at its pI, that leads to the binding between proteins via ionic interactions, and precipitation. Salt can interfere these ionic interactions and separate bound protein molecules.	Big divalent ions attract water molecules immobilized on the protein surface, expose the non-polar surface, which then interacts with other proteins to form precipitate.	Organic solvent decreases the water activity and the dielectric constant of the solution, which then decreases the solubility of the protein and precipitates it.
Fig	Fig 2.3	Fig 2.4	Fig 2.5
Remarks	The reverse process of salting-in is not salting-out, it is the dialysis process against a dilute solution.	1) Non-polar proteins will be precipitated earlier. 2) Protein is very stable in ammonium sulfate.	1) Some proteins might be denatured by heat produced. 2) Factors facilitate precipitation: larger protein, pH close to protein pI. 3) Lipophilic protein might be dissolved more readily.

3 色層分析法 Chromatography

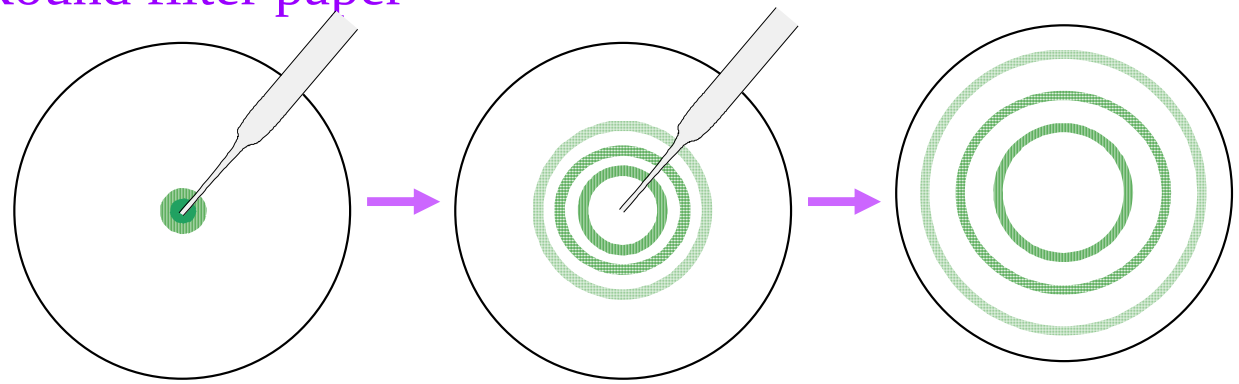
- 3.1 色層分析原理 Basic principles
  極性不同的分子在兩相中有不同分佈比例
- 3.2 膠体過濾法 Gel filtration
  依樣本分子量的不同來做分離純化
- 3.3 離子交換法 Ion exchange
  利用樣本分子的表面帶電性質不同來進行分離
- 3.4 親和層析法 Affinity chromatography
  利用分子間的親和性大小不同來進行分離
- 3.5 HPLC 及 FPLC
  改善介質的材質及吸附容量可增加速度及解析力

色析法演進過程 Historical review

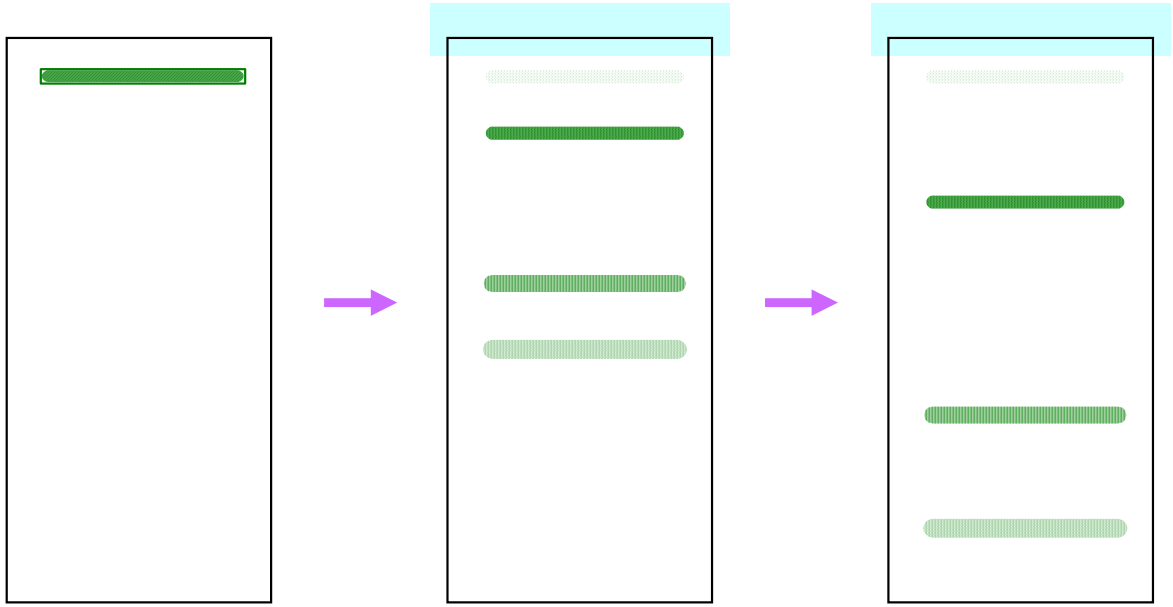
Martin, Synge (1952)

Paper partition chromatography (PPC)

Round filter paper

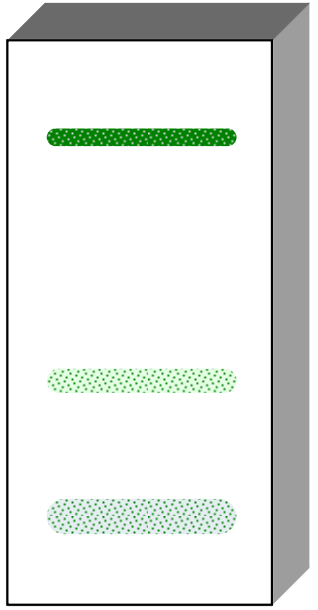


Development



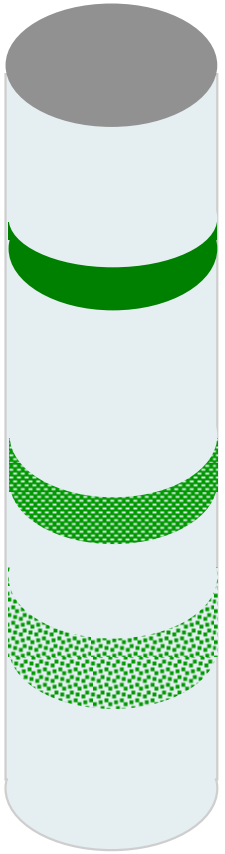
Long rectangle paper

Thin layer (TLC)



Sample capacity increased

Column



Larger capacity

Adapted from Scope RK (1987) Protein Purification – Principles and Practice p.9

色層分析法的基本機制 Essential mechanism

Like
Dissolves
Like

- 極性相似的兩分子間，其親和力較強。

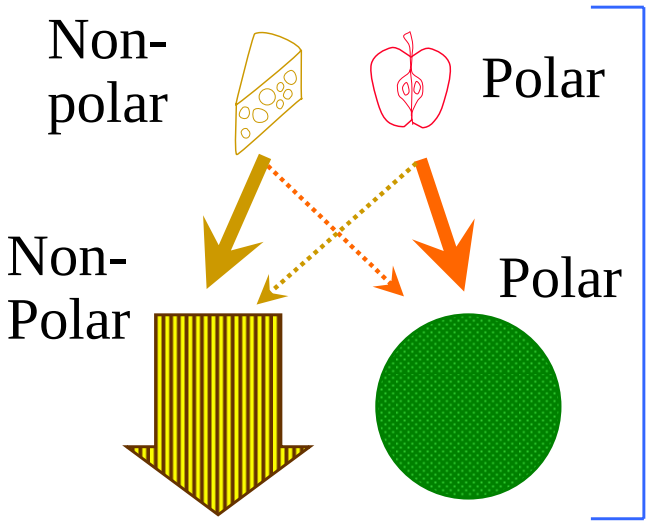
Polar → Polar

Non-polar → Non-polar

色層分析法原理 Two-phase separation system

A

Two-phase



流動相 固定相

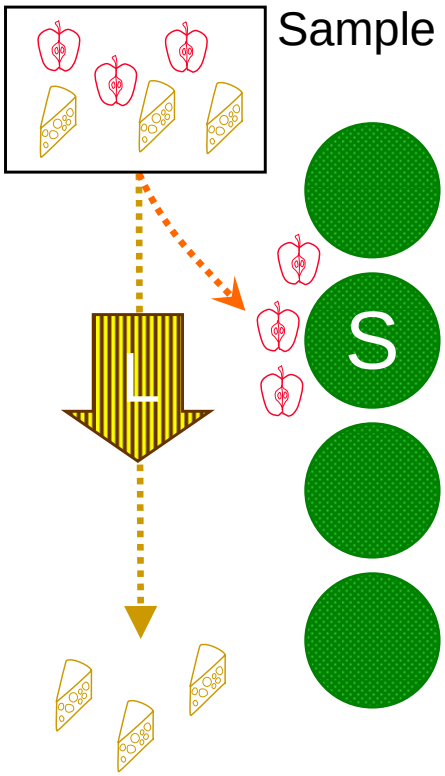
樣本分子依其分子極性
選擇親和兩相之一

每次分離樣本
都要做一次選擇

One Plate of Separation

Theoretical plate number

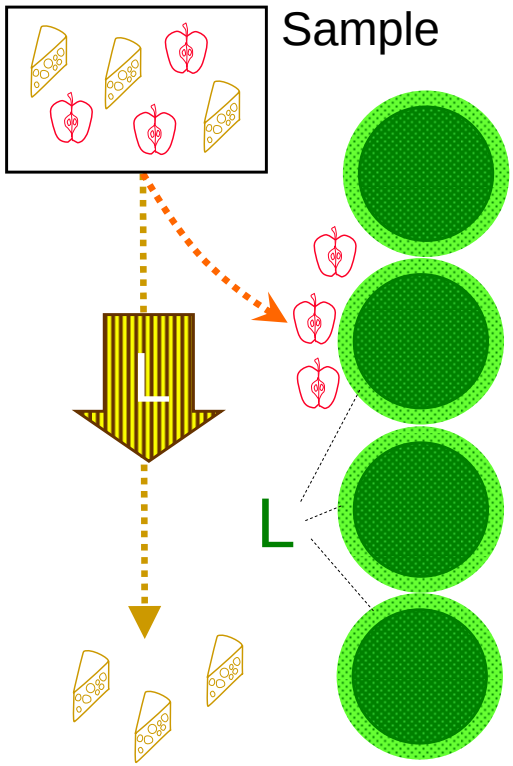
B



ADSORPTION

Liquid - Solid

C

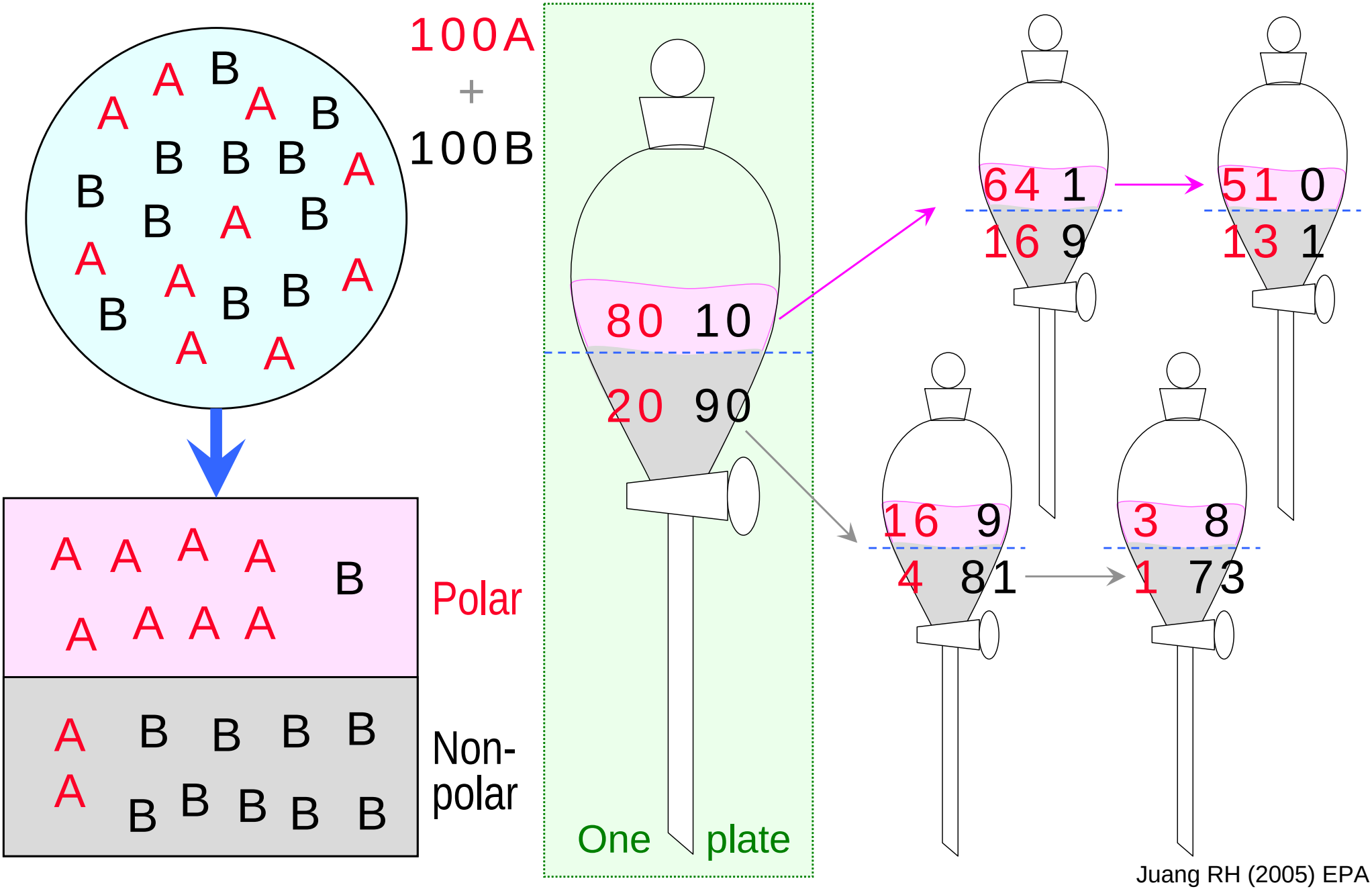


PARTITION

Liquid - Liquid

Juang RH (2005) EPA

層析法的板數概念 One separation, one plate



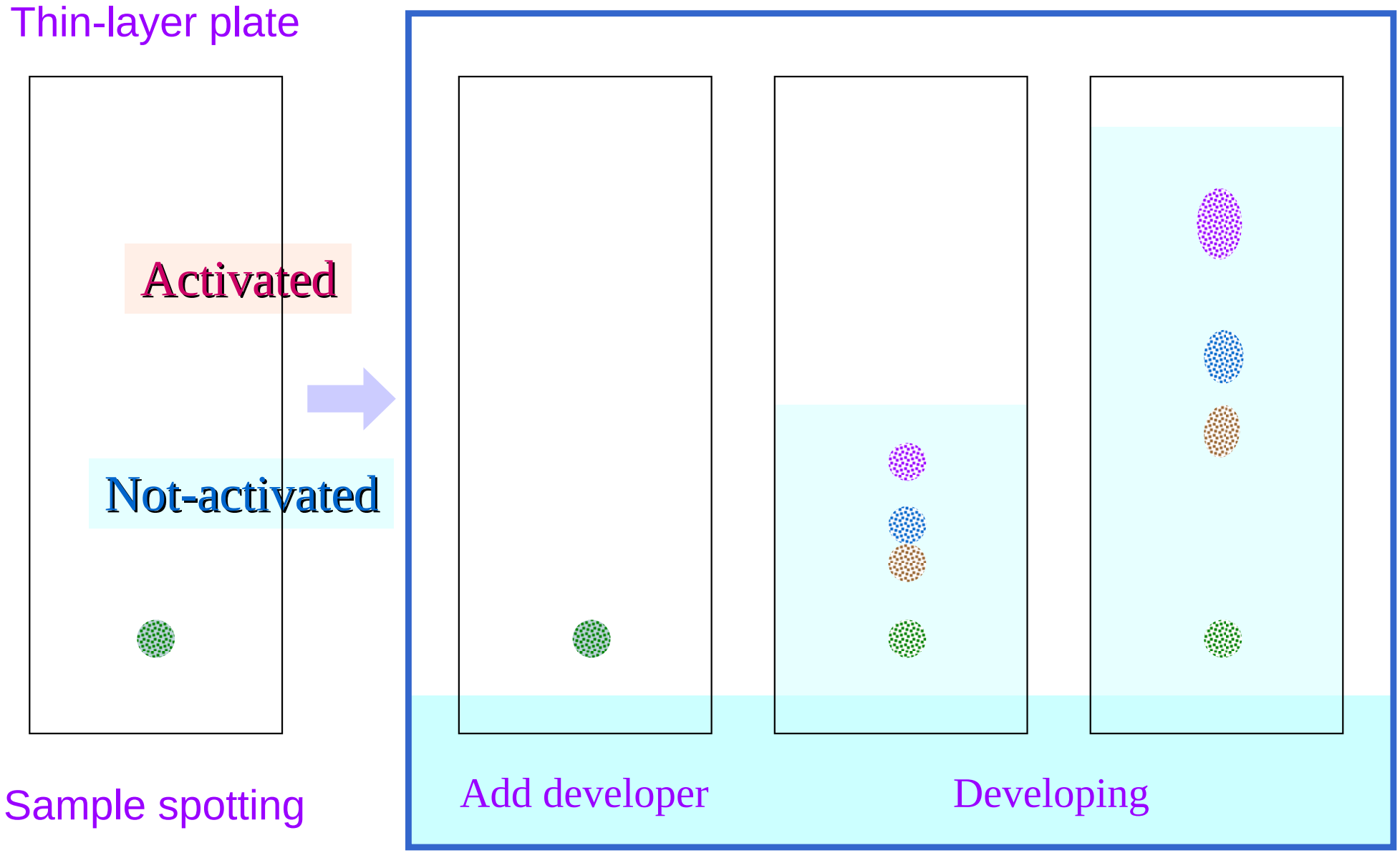
Juang RH (2005) EPA

常用層析法 Common chromatographic methods

	Partition	Adsorption
Large molecules	Gel filtration Reverse phase chromatography	Ion exchange Affinity chromatography Hydrophobic interaction Hydroxyapatite
Small molecules	PPC, TLC, GC	TLC, GC

Juang RH (2005) EPA

薄層層析法操作 Thin-layer chromatography



Juang RH (2005) EPA

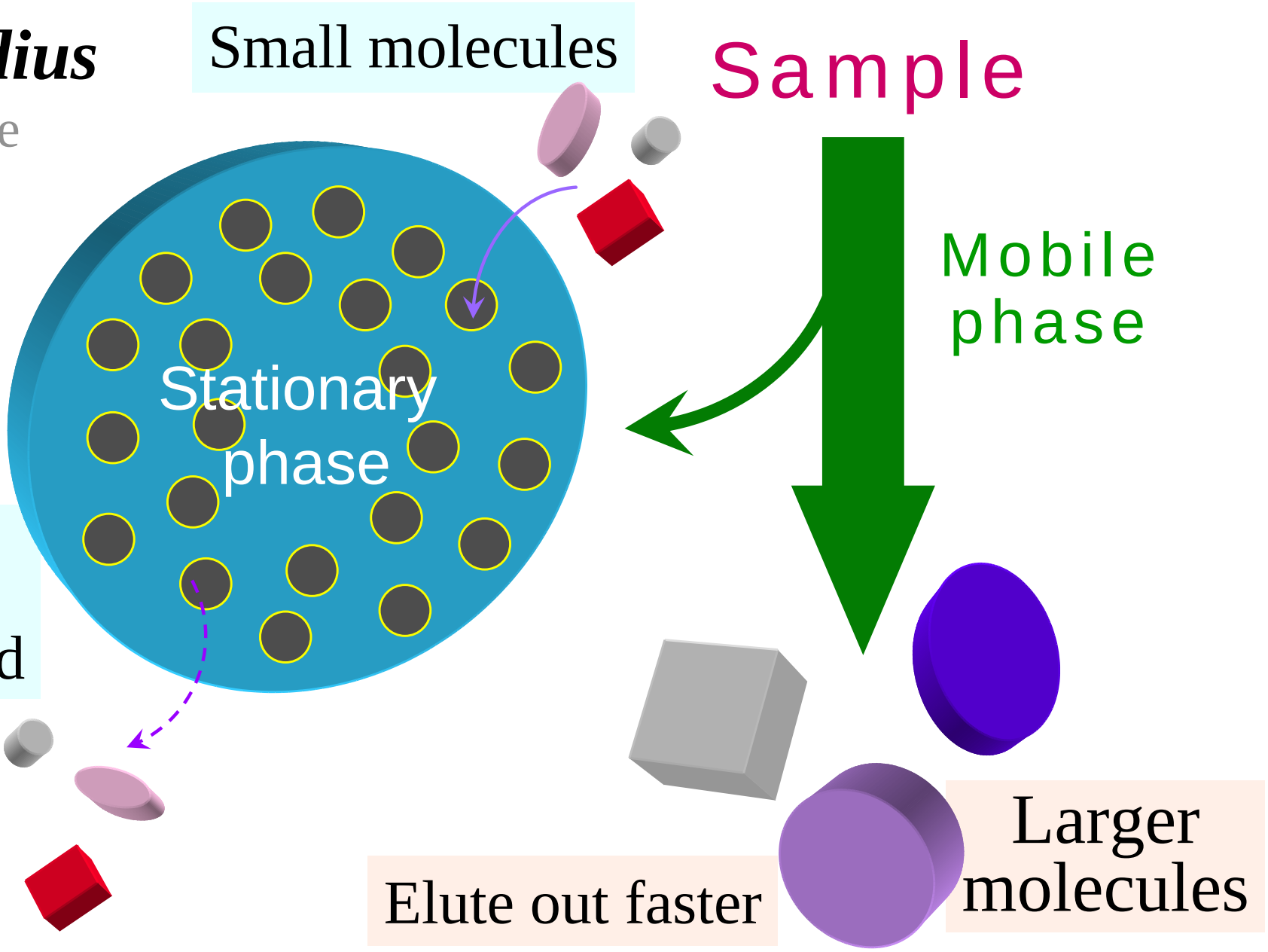
3.2 膠体過濾法 Gel filtration

- 3.2.1 原理概述 Basic principles
  是一種 partition 層析法
- 3.2.2 膠体介質 Gel materials
  是一種長鏈的大分子聚合物
- 3.2.3 膠体管柱 Gel and column
  管柱性質、影響因素及管柱系統
- 3.2.4 管柱操作 Column operation
  裝填並操作一支膠体過濾管柱
- 3.2.5 問題及解決 Problem and solution
  常見的錯誤要先避免之

膠体過濾法是一種 Partition 層析法

Stokes radius

Molecular size
and shape



Gel filtration is a partition type chromatography

Juang RH (2005) EPA

各種色析膠體

Pharmacia

Sephadex	glucose (dextrose)
Sepharose	agarose
Sephacryl	glucose + acrylamide
Sephacel	cellulose

FPLC

Superose, Superdex

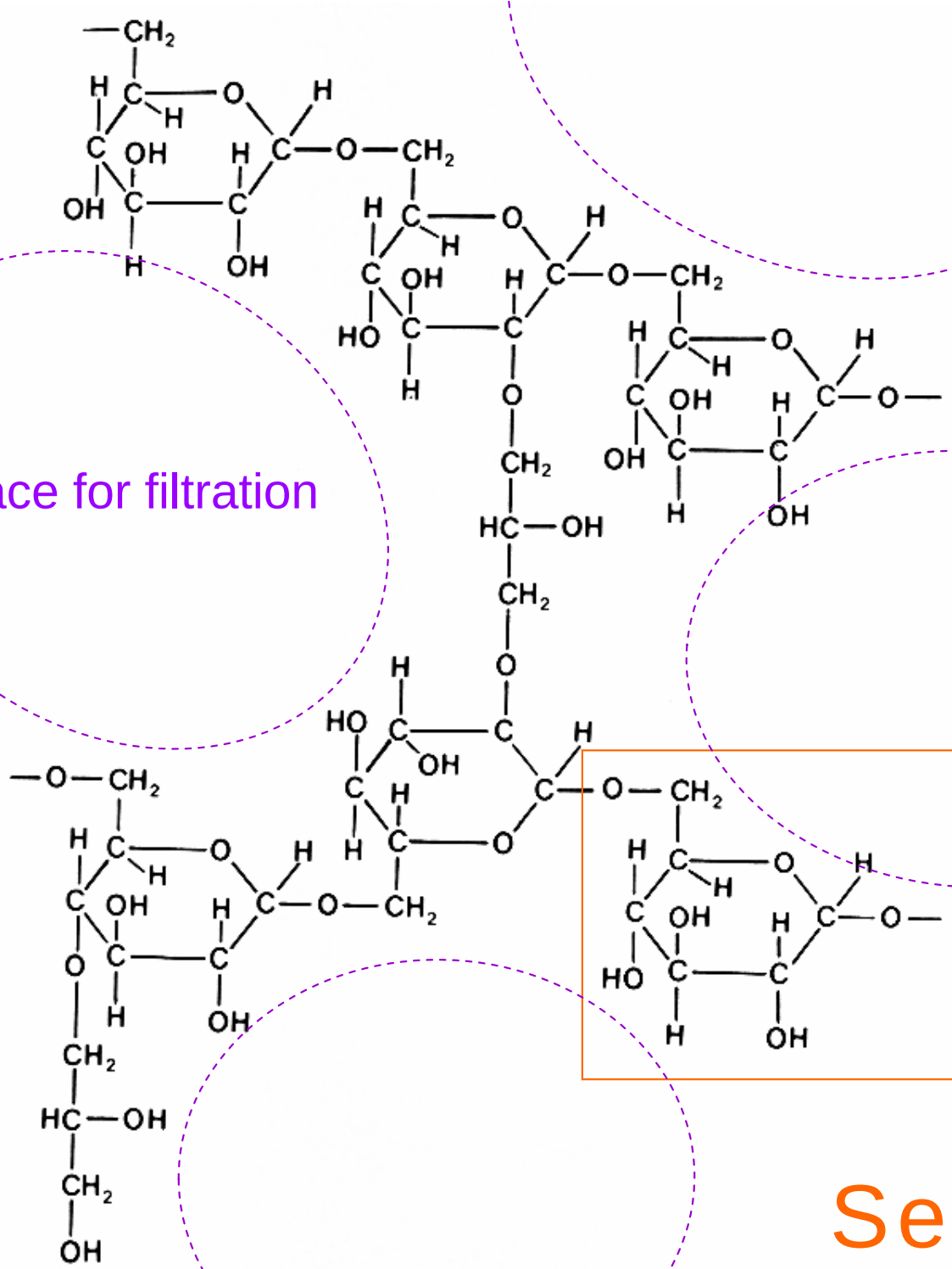
Mono Q, Mono S

Bio-Rad

BioGel P	acrylamide
BioGel A	agarose

Gel material is a polymer of carbohydrate or acrylamide

膠體的構成

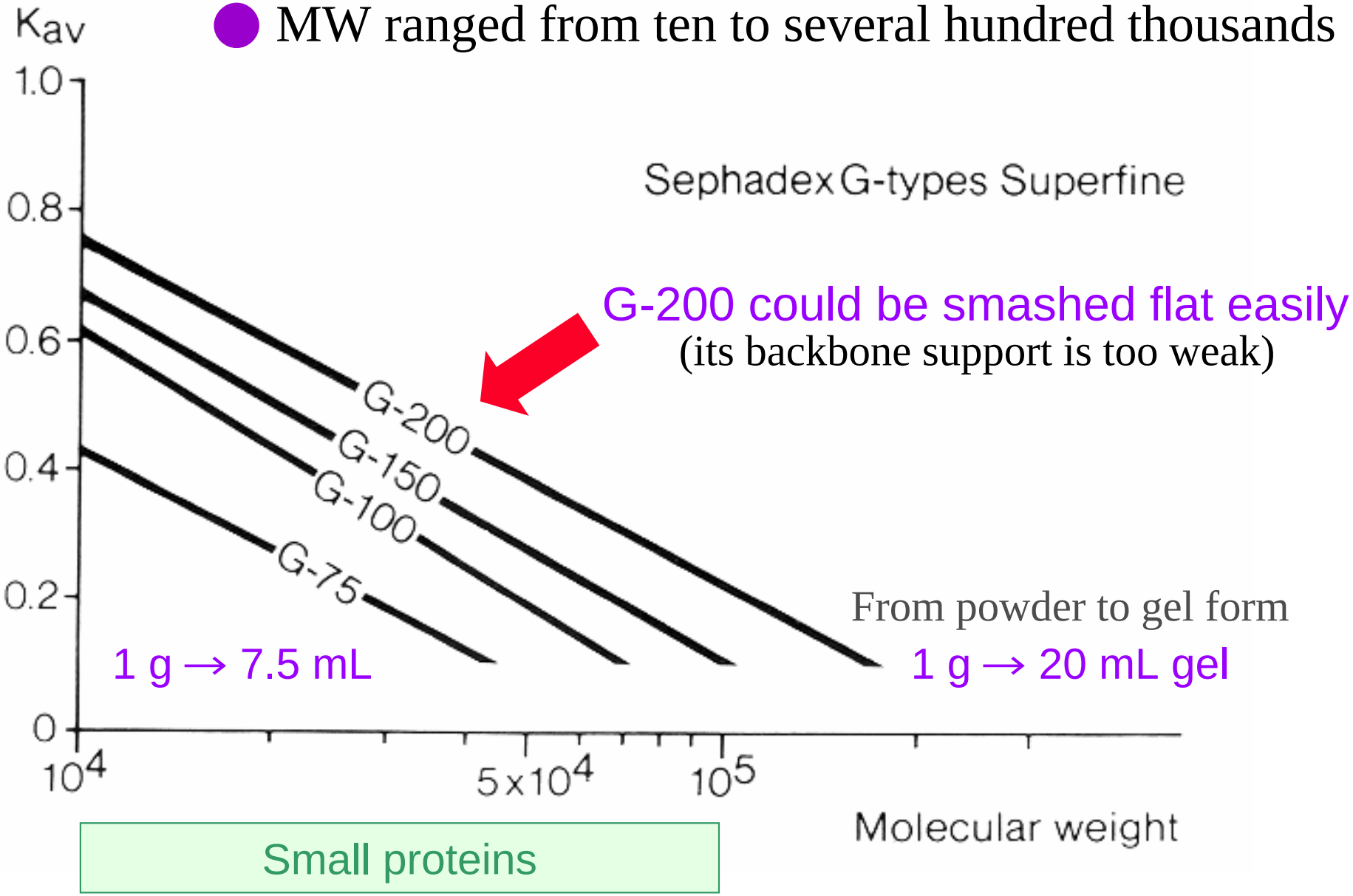


Sephadex

Sephadex is the polymer of glucose with chemical cross-linking

膠體的使用範圍 Sephadex G

- MW ranged from ten to several hundred thousands



G-200 could be smashed flat easily
(its backbone support is too weak)

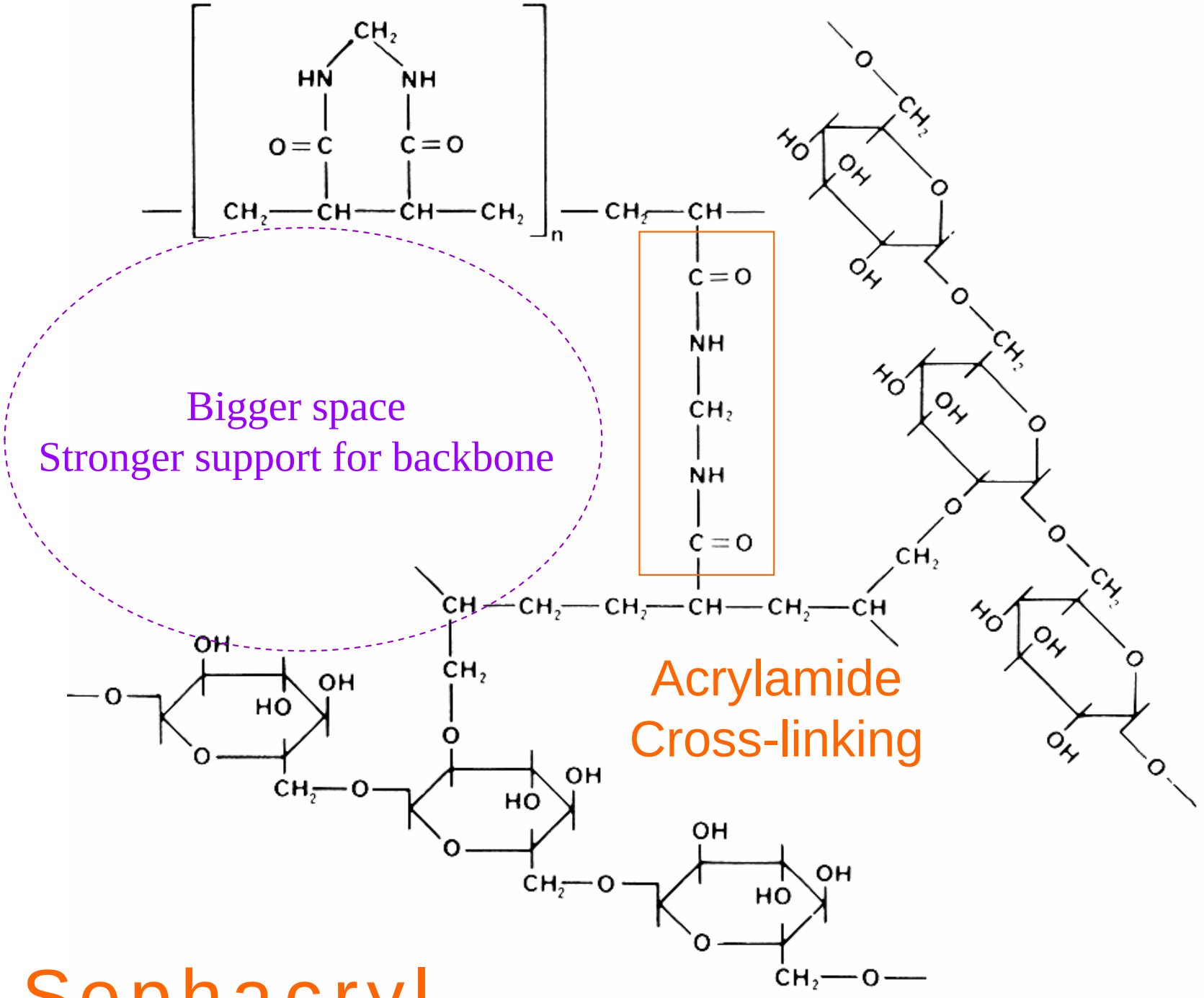
From powder to gel form
1 g → 20 mL gel

1 g → 7.5 mL

Small proteins

Pharmacia (1991) Gil Filtration – Principles and Methods p.37

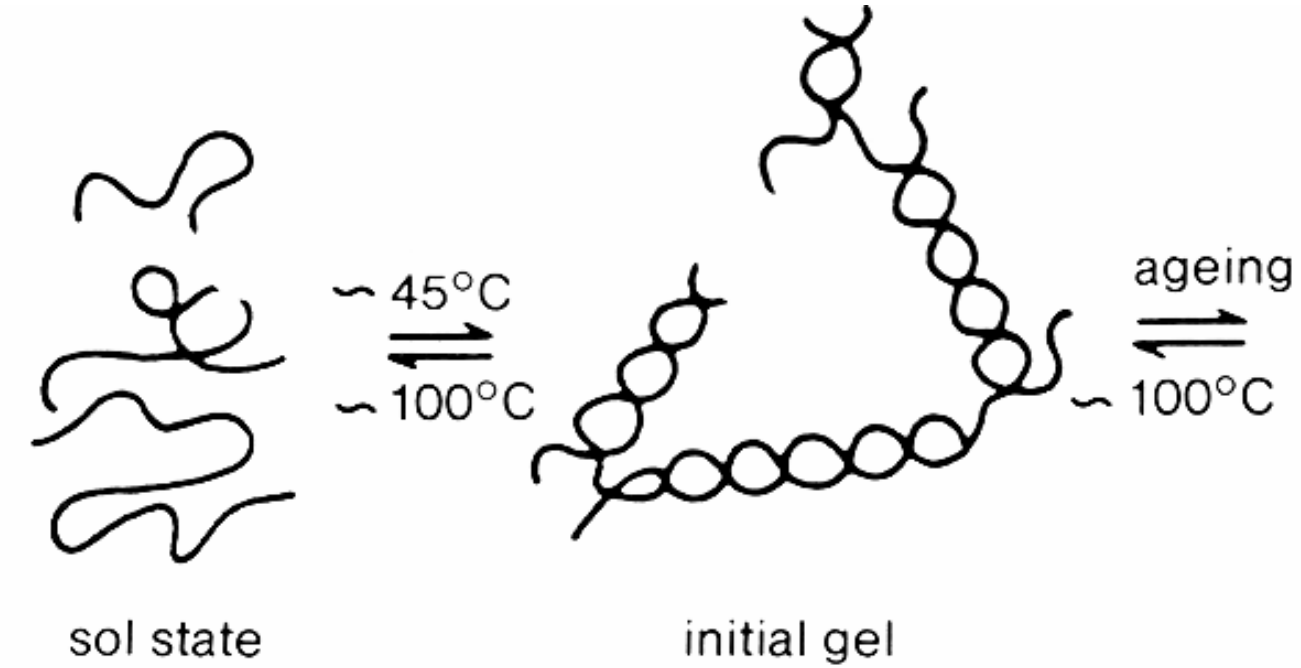
膠體的構成

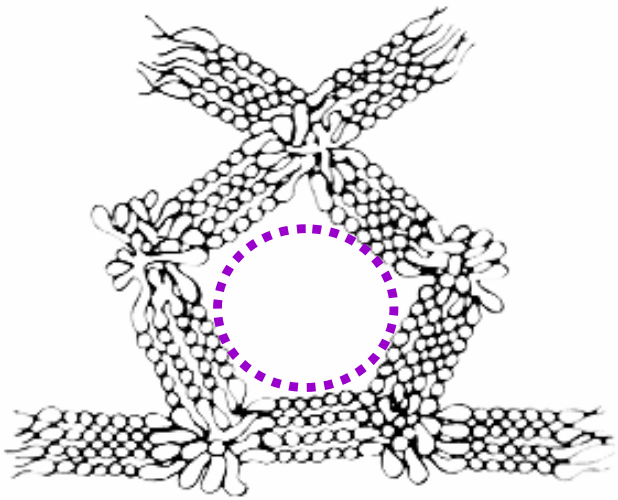


Sephacryl

Pharmacia (1991) Gil Filtration – Principles and Methods p.22

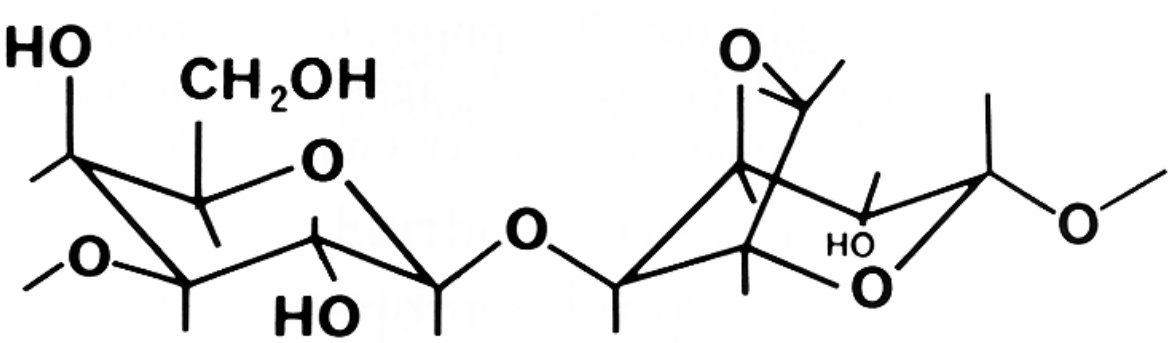
洋菜膠體的成膠反應 Agar gel formation





Even stronger backbone

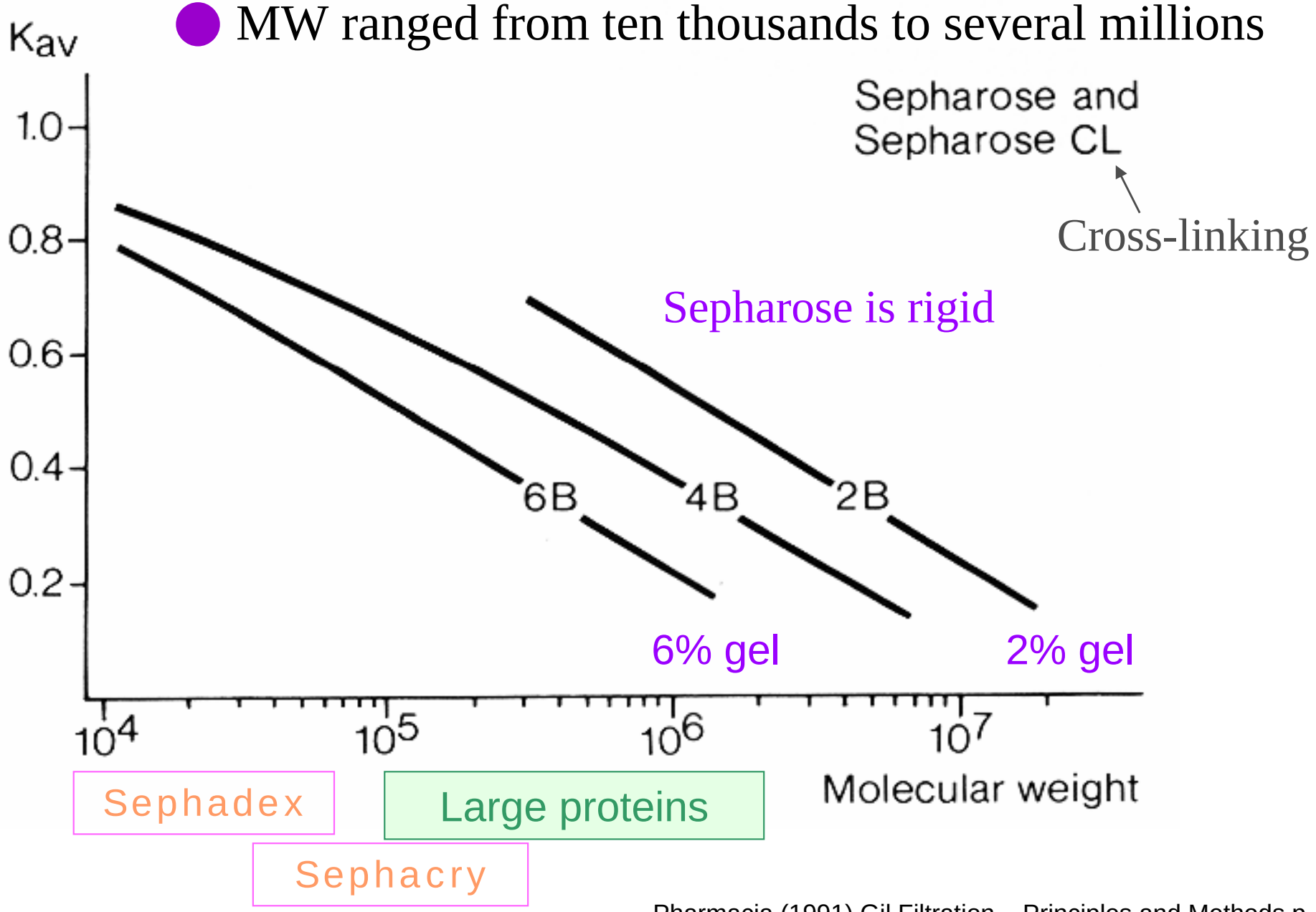
Much bigger space



Pharmacia (1991) Gil Filtration – Principles and Methods p.38, 39

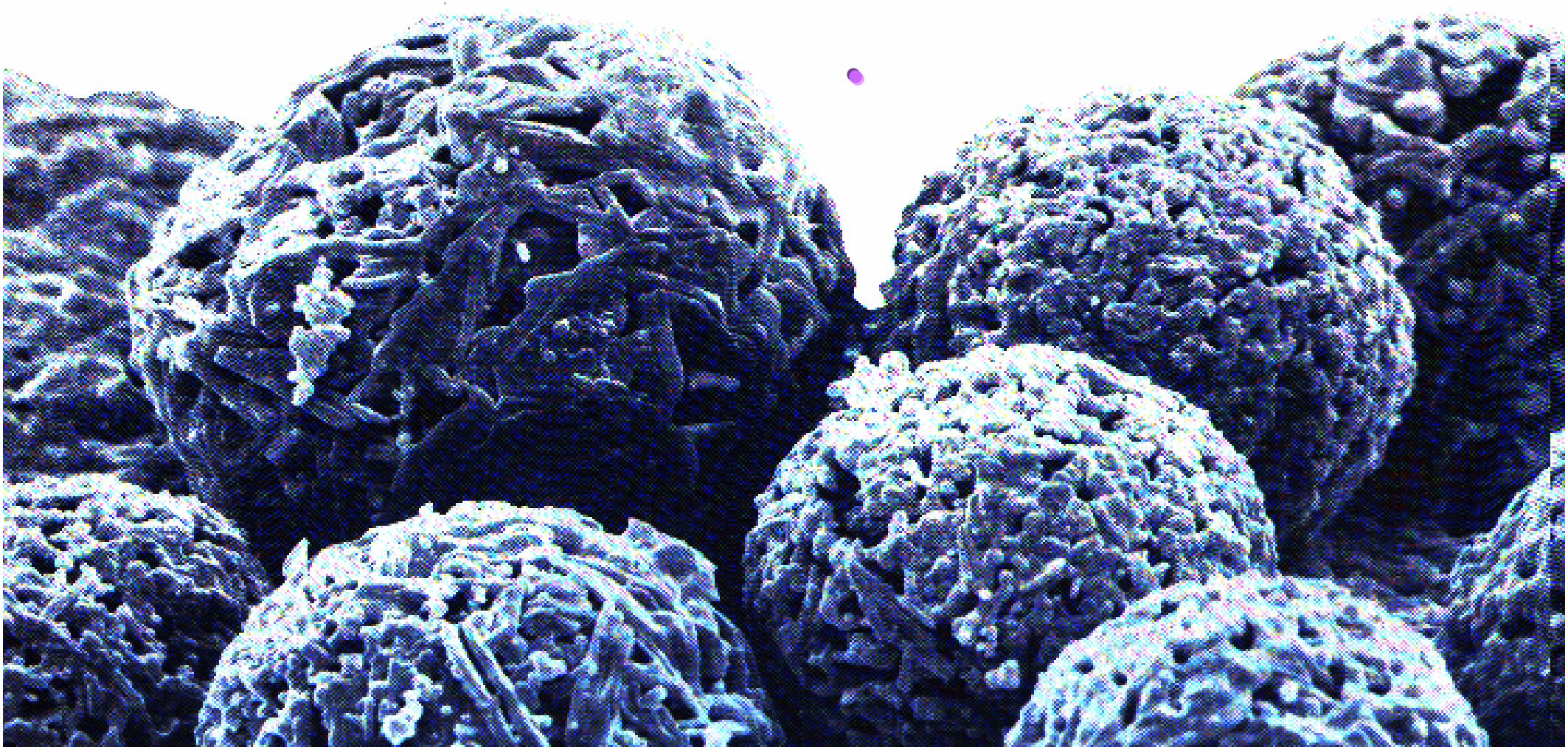
膠體的使用範圍 Sepharose

- MW ranged from ten thousands to several millions

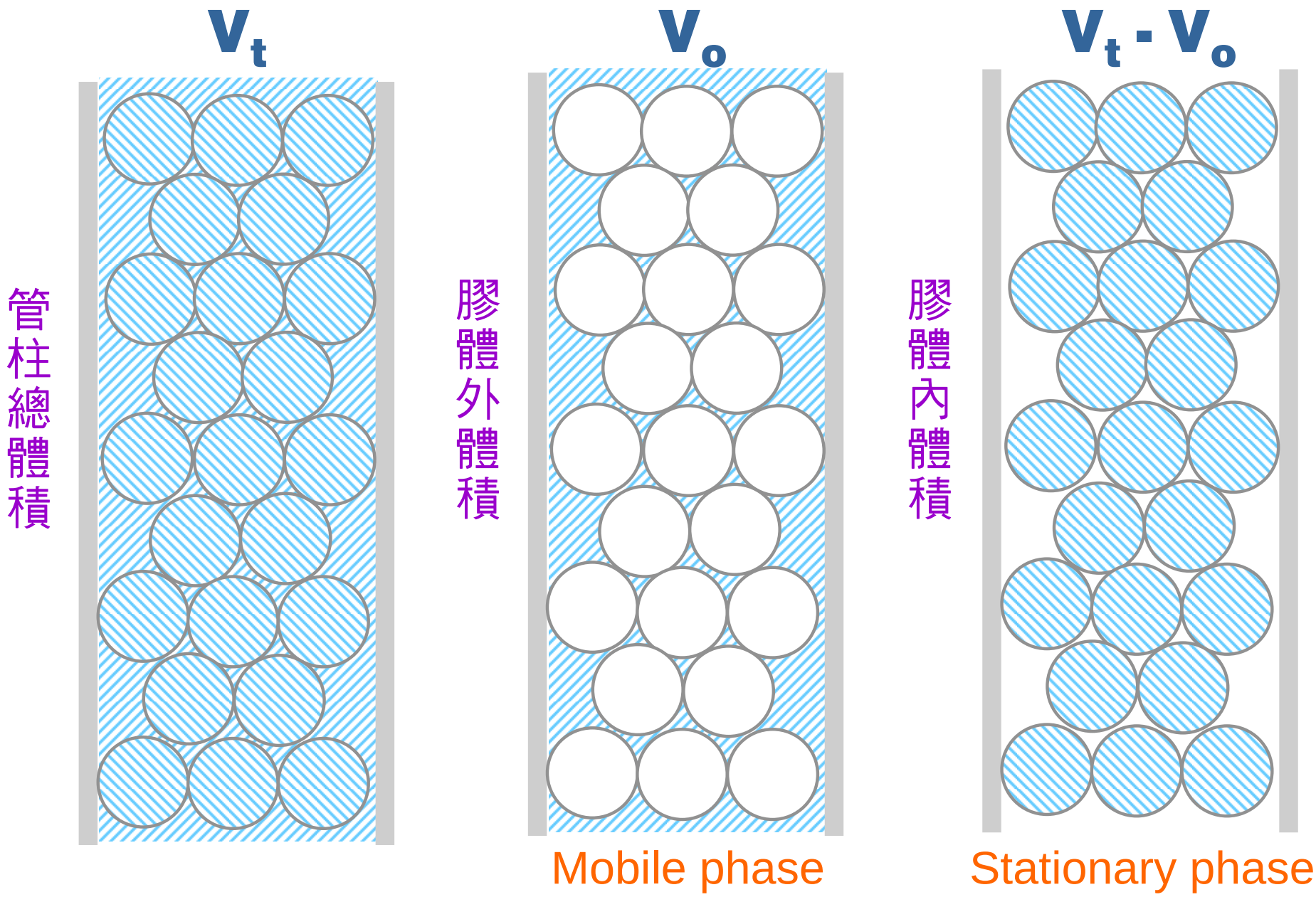


Pharmacia (1991) Gil Filtration – Principles and Methods p.40

膠体過濾法的膠球 Gel filtration beads

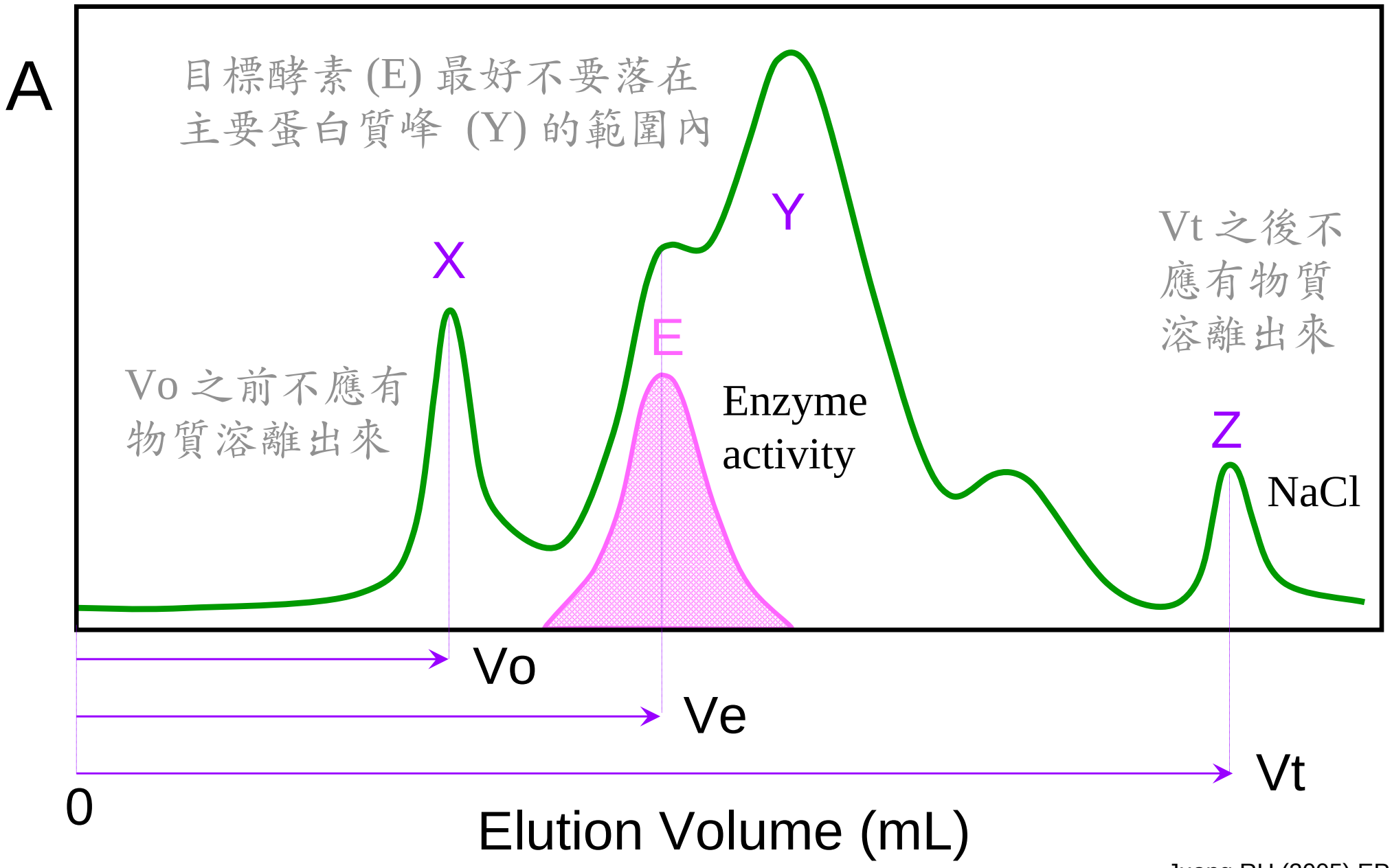


管柱內膠體的組成區隔 Spaces in a column



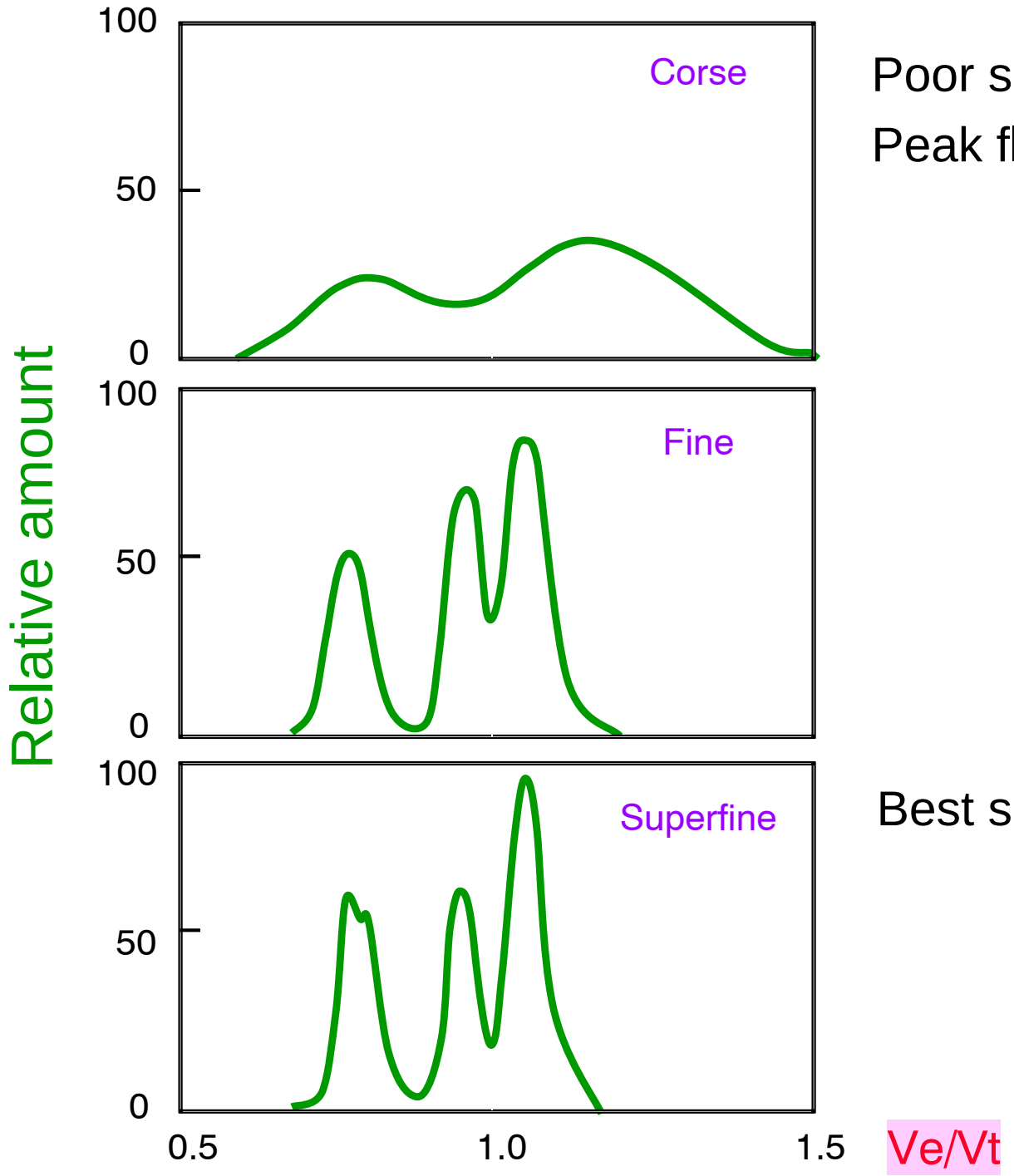
Adapted from Pharmacia (1991) Gil Filtration – Principles and Methods p.11

膠体過濾的溶離圖譜 A typical chromatogram



Juang RH (2005) EPA

介質的粗細影響解析力



Poor separation

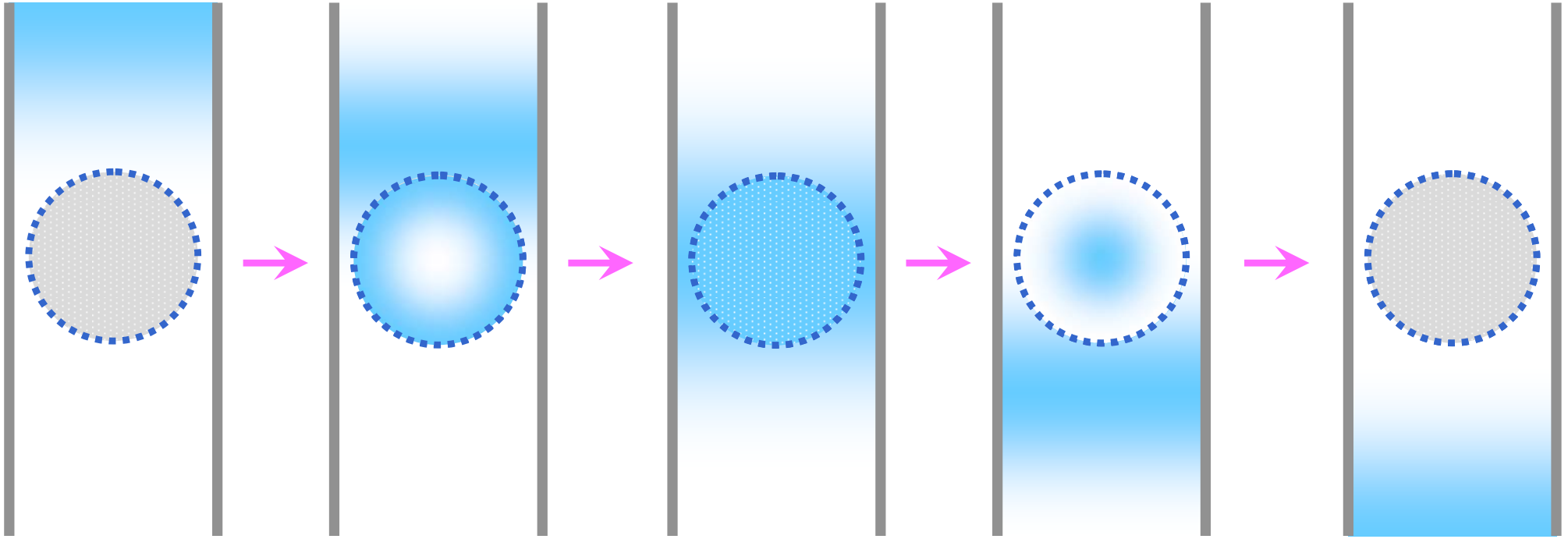
Peak flattened

Best separation

Bead size is critical to the resolution of gel chromatography

Adapted from Pharmacia: Gil Filtration – Principles and Methods

溶離液擴散進出膠球 Diffuse in and out bead

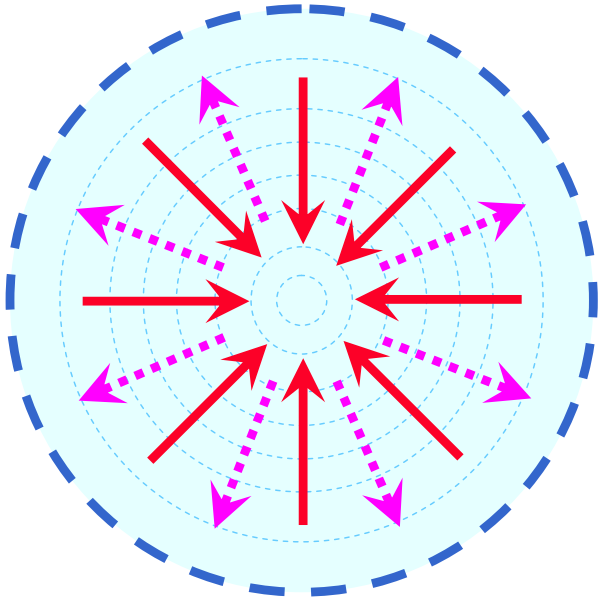


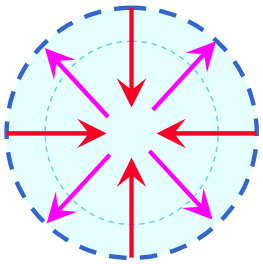
溶離液或樣本分子，由膠體粒子外圍均勻向內或外擴散。

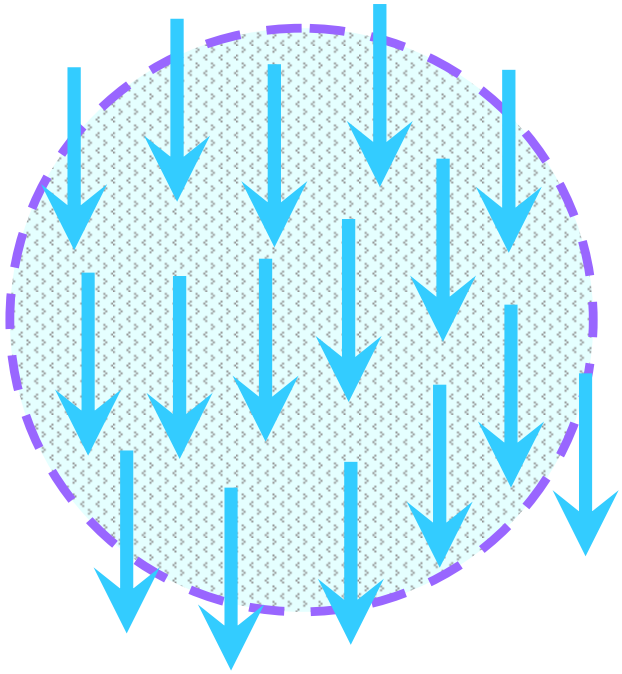
Sample or buffer is diffusing in and then out of the gel particle.

Diffusion → Dispersion

溶離液對膠球的擴散或瀰散 Two types of gel







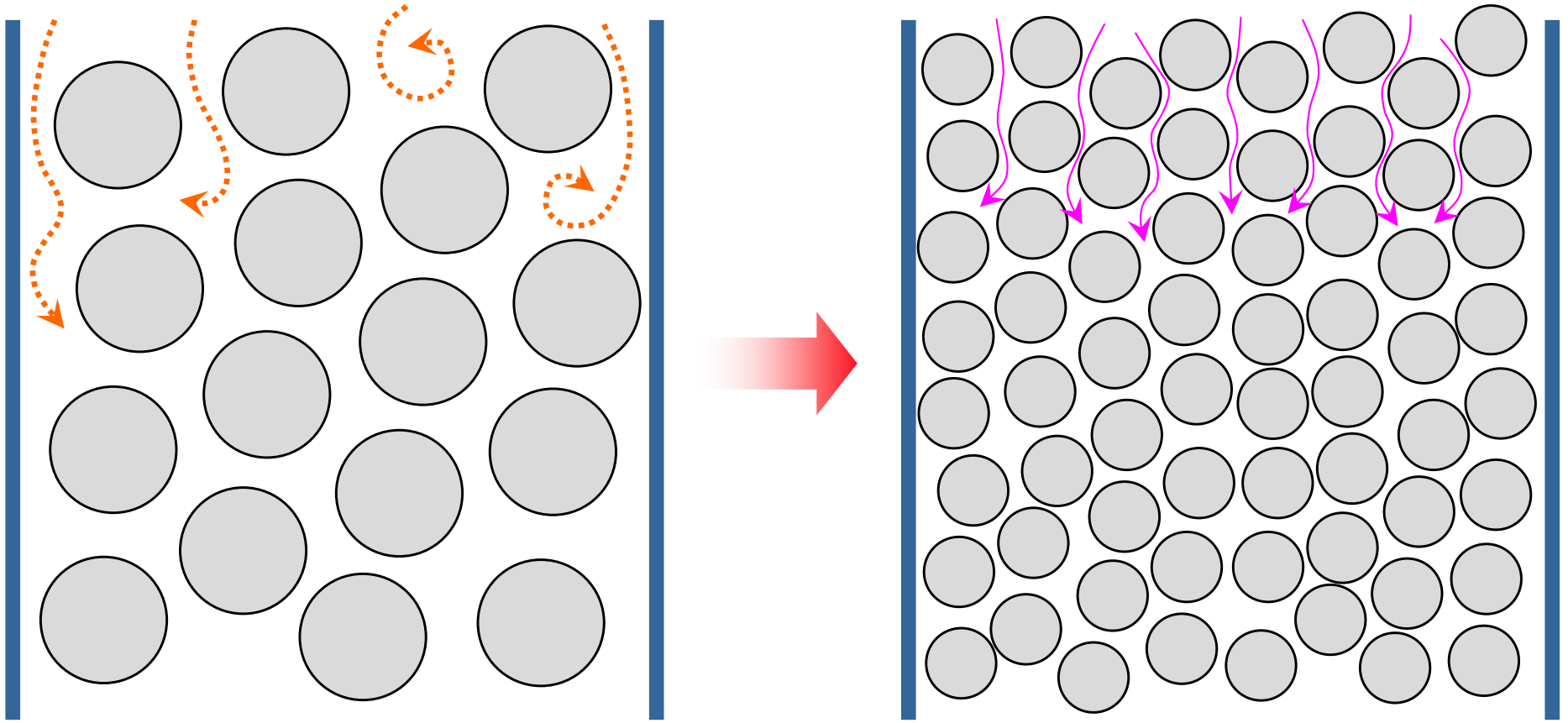
若膠體粒子太大，則由外圍擴散到內部的距離長，通過粒子所需要的時間拉長，降低分離效果。

Small particle size reduces the diffusion time and increases the resolution of the gel

Dispersion (瀰散) 方式的膠體因通透性佳，溶離液可直接流入膠體，不再靠擴散作用。

Dispersion type gels let the sample molecules flow directly through the gel body, and have better resolution

粒子粗細影響溶離液流動 Bead size vs flow rate

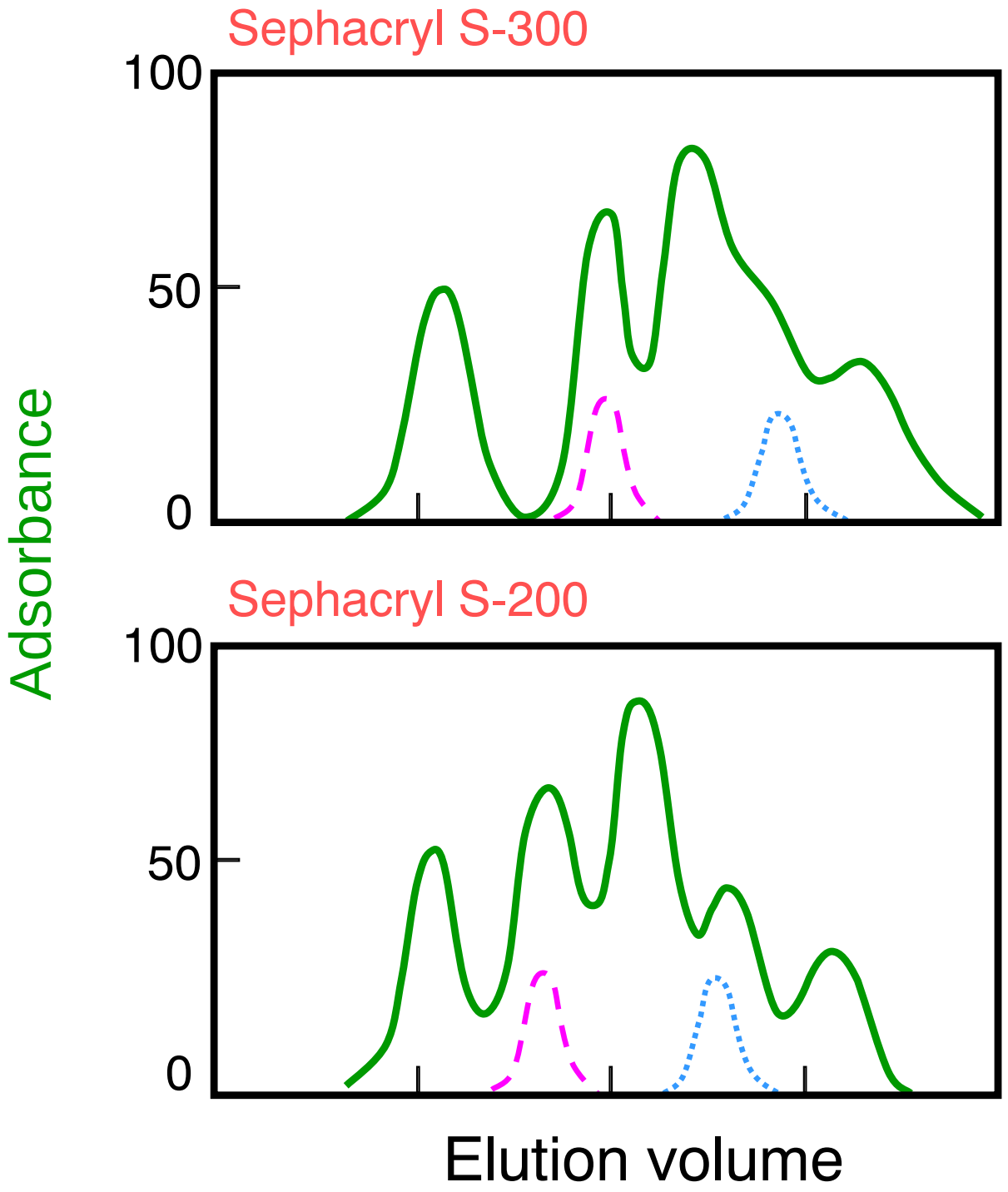


膠體的顆粒越小 其解析力越大 但流速變慢

Smaller particles also reduce the space between the beads, and prevent the turbulence as the buffer flows, but the flow rate might be decreased

Adapted from Scope RK (1987) Protein Purification – Principles and Practice p.192

不同介質的運用選擇



高分子量處
分離較好
色帶都較晚
溶離出來

低分子量處
分離較好
色帶都較快
溶離出來

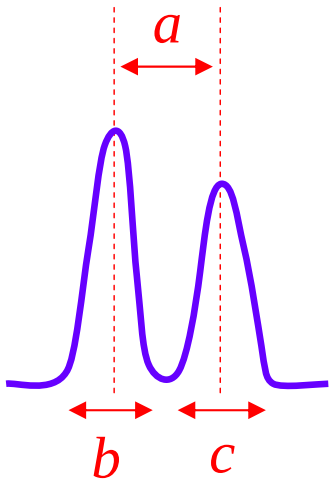
Choose the gel which brings your target protein out of the column earlier

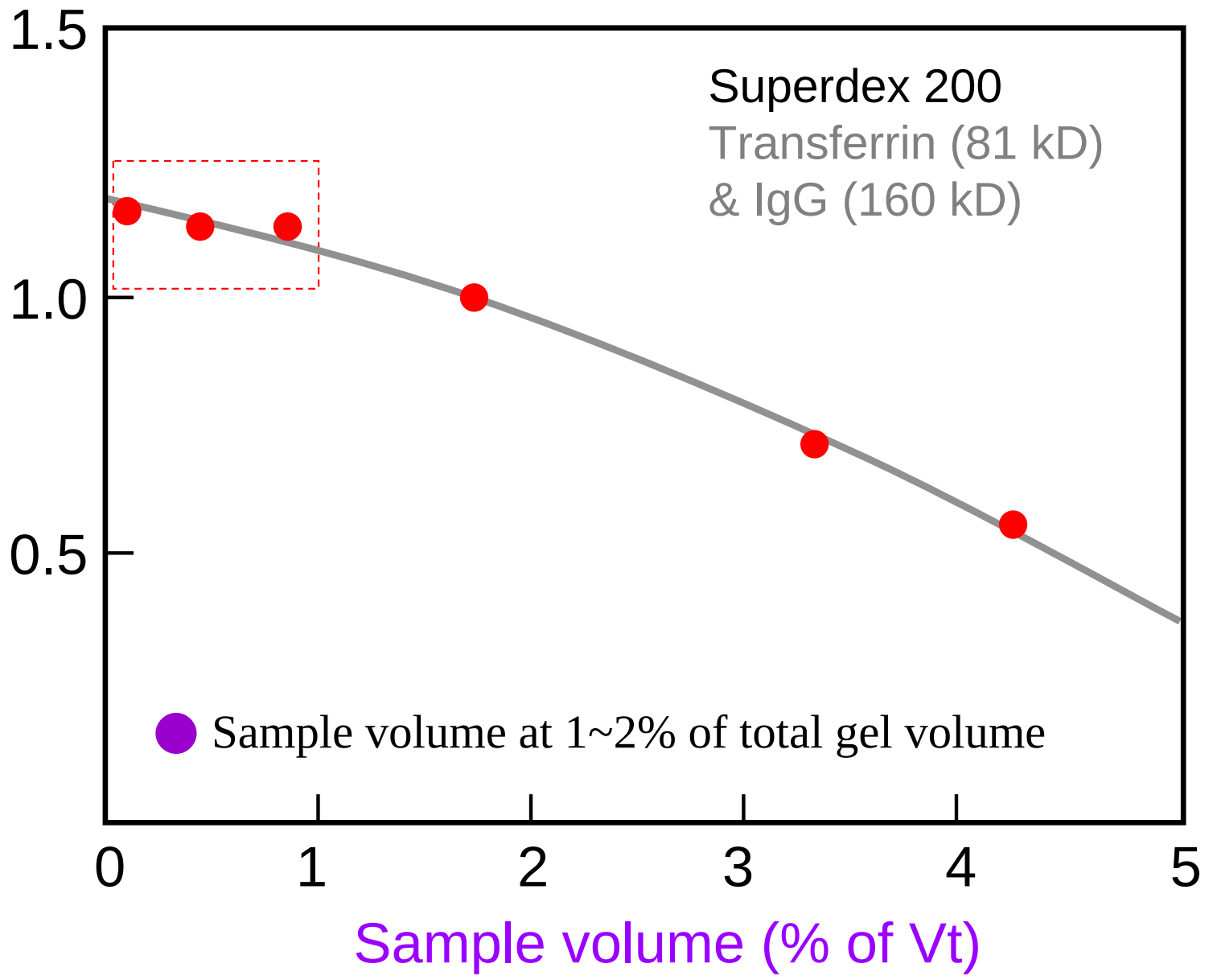
Adapted from Pharmacia: Gil Filtration – Principles and Methods

樣本體積的影響 Sample volume at 1% of Vt

Resolution

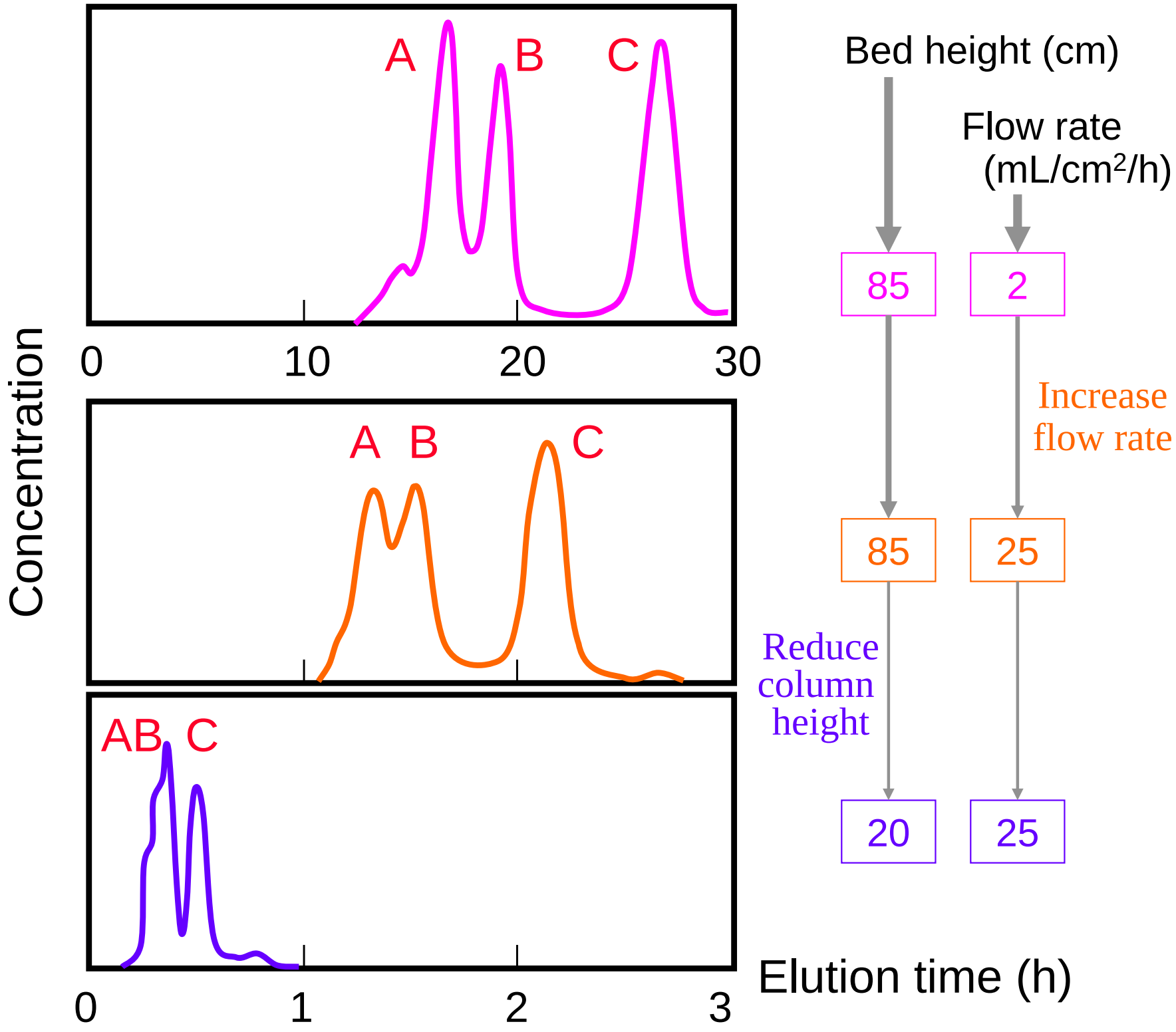
$$R = \frac{a}{(b+c)/2}$$





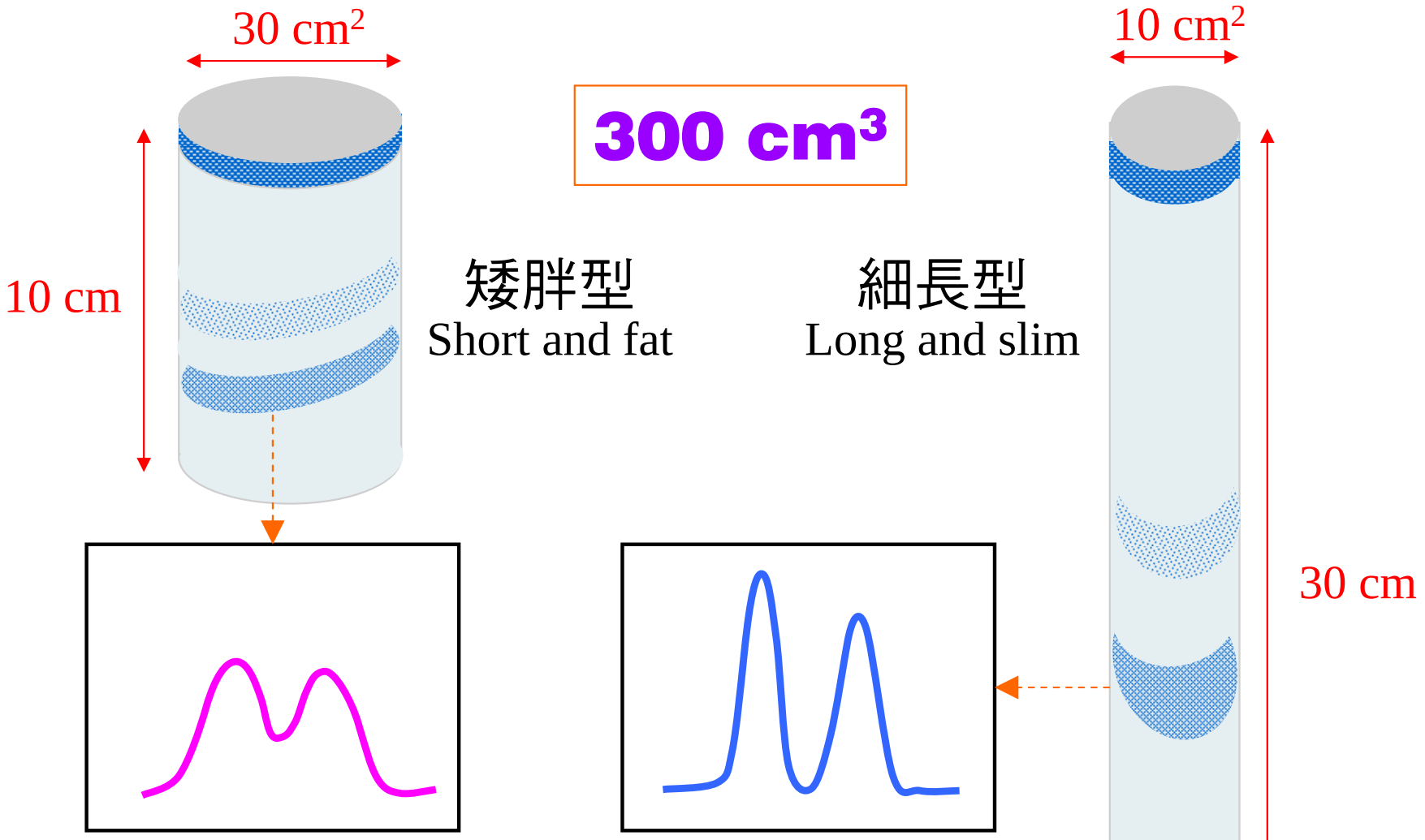
Adapted from Pharmacia (1991) Gil Filtration – Principles and Methods p.46

膠體管柱的最佳化調整



Adapted from Pharmacia (1991) Gil Filtration – Principles and Methods p.59

色析管柱的形狀

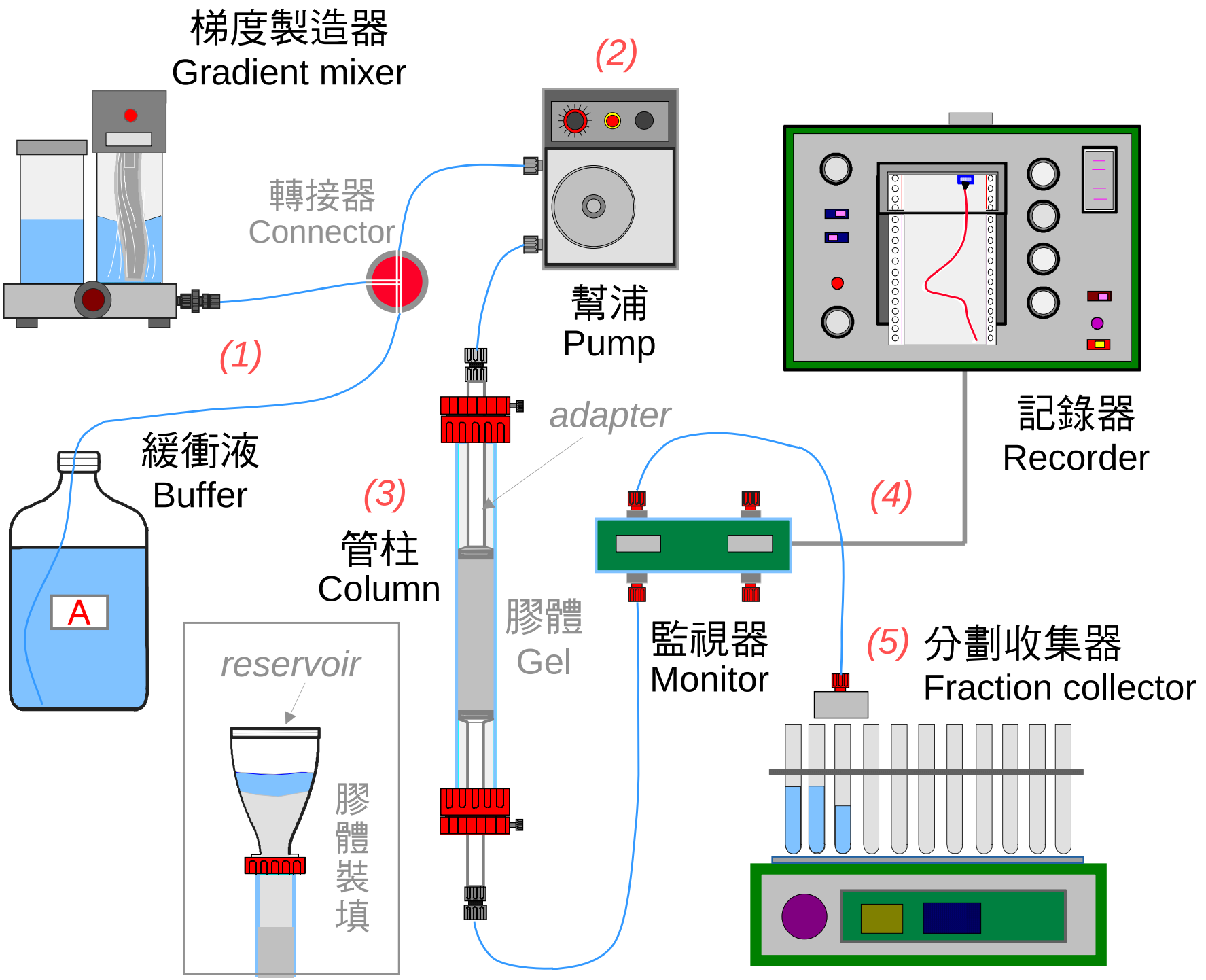


- 矮胖型管柱不能容忍分離不佳
  Fat column cannot tolerate poor separation

但其流速及容量均較大
But it has better flow rate and higher capacity

Adapted from Scope RK (1987) Protein Purification – Principles and Practice p.195

液相層析管柱系統



The whole family of liquid chromatography apparatus

Juang RH (2005) EPA

膠柱裝填方法 Packing column step by step

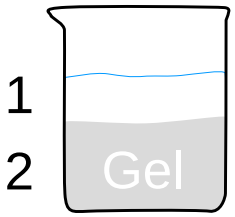
清洗膠體
Wash gel well

預估體積
Estimate
gel volume

緩衝液
平衡
Equilibrated
in buffer

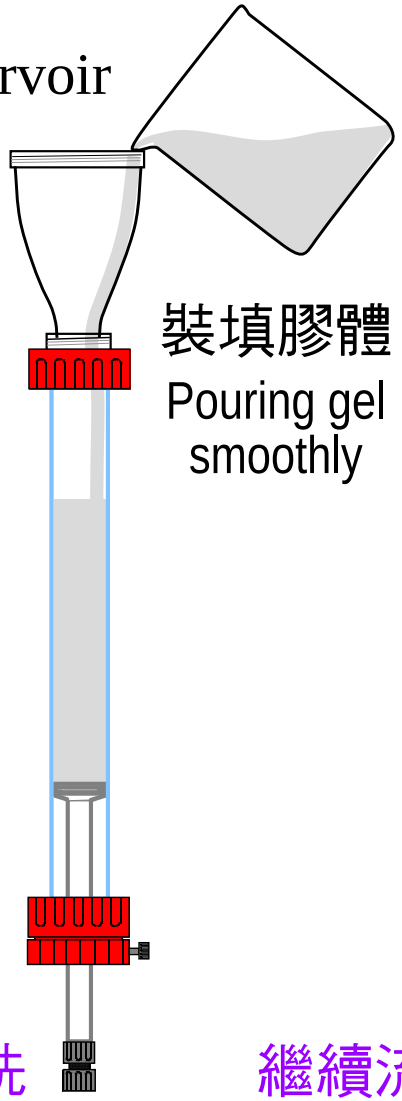
靜置膠體
Gel stands o/n

溫度平衡
Temperature
equilibrated



檢查管柱
是否暢通
Check flow
rate of empty
column

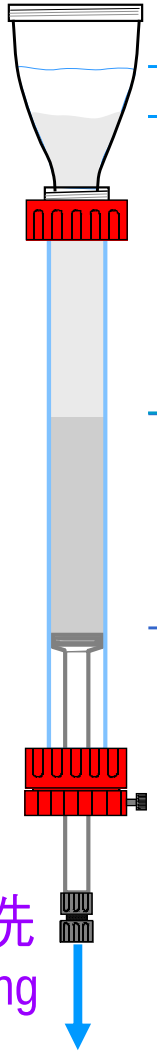
Put on reservoir



裝填膠體
Pouring gel
smoothly

暫停流洗
Stop elution

X



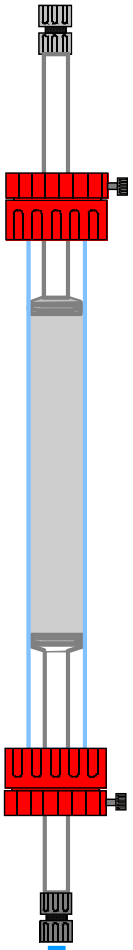
繼續流洗
Keep eluting

上清
Supernatant

沈積中
In progressing

已堆積
Sediment

Put on adaptor

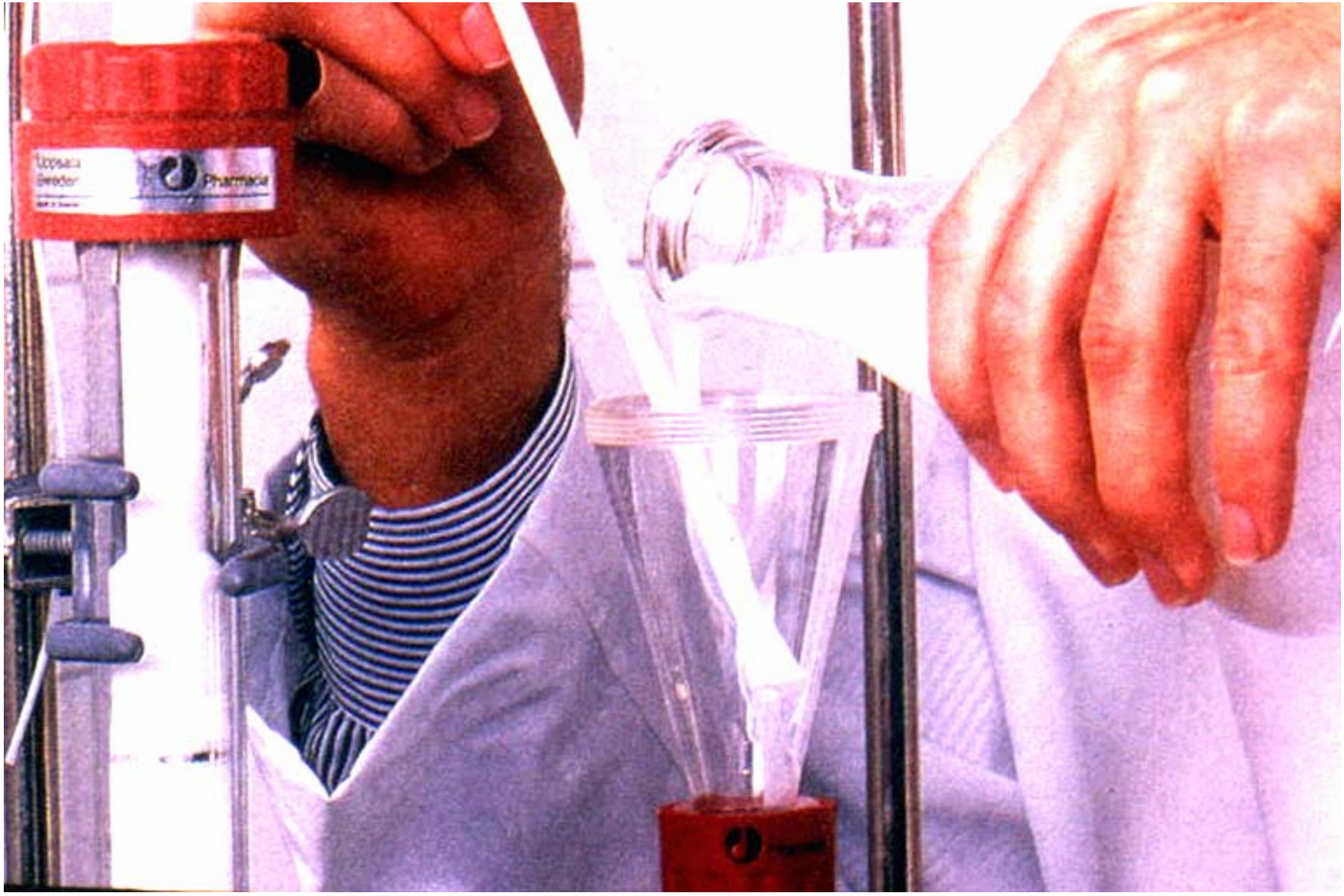


加壓流洗
Elute under
High pressure

緊密堆積

Gel should be packed tightly

色析膠體的裝填 Packing column



一口氣倒入膠體，勿陷入氣泡。

Pour gel slurry smoothly (non-stop), avoid trapping any bubble

Pharmacia (1991) Gil Filtration – Principles and Methods

General principle for column chromatography

Gel selection	Make target protein elute out column earlier
Bead size	Finer bead has better resolution, slower flow rate
Column size	Use larger column size but consider practical need
Column shape	Slim column for gel filtration, fat column for others
Pack tightly	Pack the gel tightly for better resolution
Flow rate	Fast flow reduces resolution, slow
Sample volume	Apply 1% of total gel volume for sample

3.3 離子交換法 Ion exchange

- 3.3.1 原理概述 Basic principles

  離子與固相擔体帶電基團間的爭奪戰 (Ion wars)

- 3.2.2 交換介質 Exchange materials

  是帶有電荷基團的多醣長鏈聚合物 (膠球)

- 3.3.3 緩衝液與層析系統 Buffer system

  緩衝液的影響極大

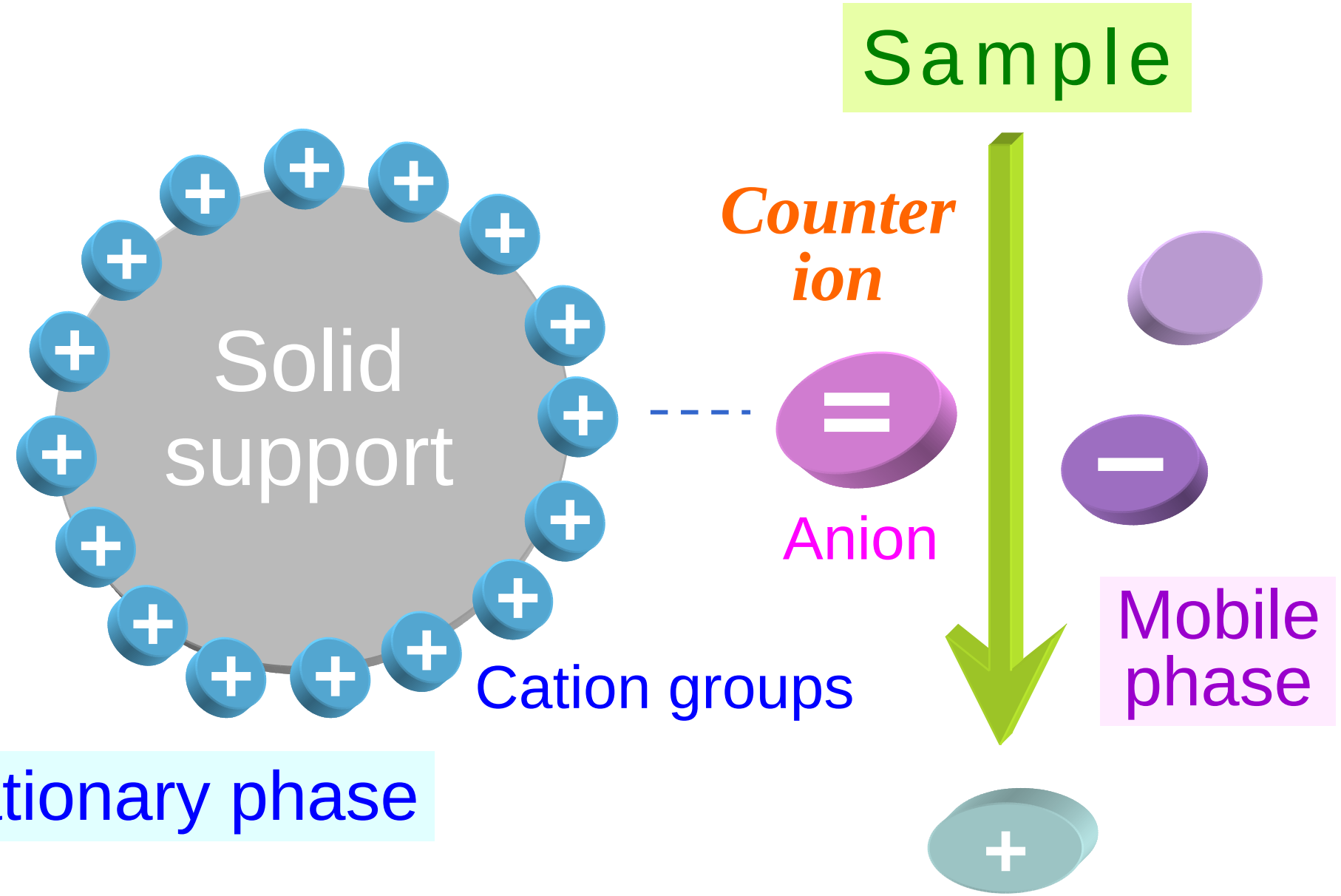
- 3.3.4 管柱操作方法 Column operation

  如何操作一支離子交換管柱

- 3.3.5 色層焦集法 Chromatofocusing

  依蛋白質等電點之差異來進行分離

陰離子交換法 Anion Exchange

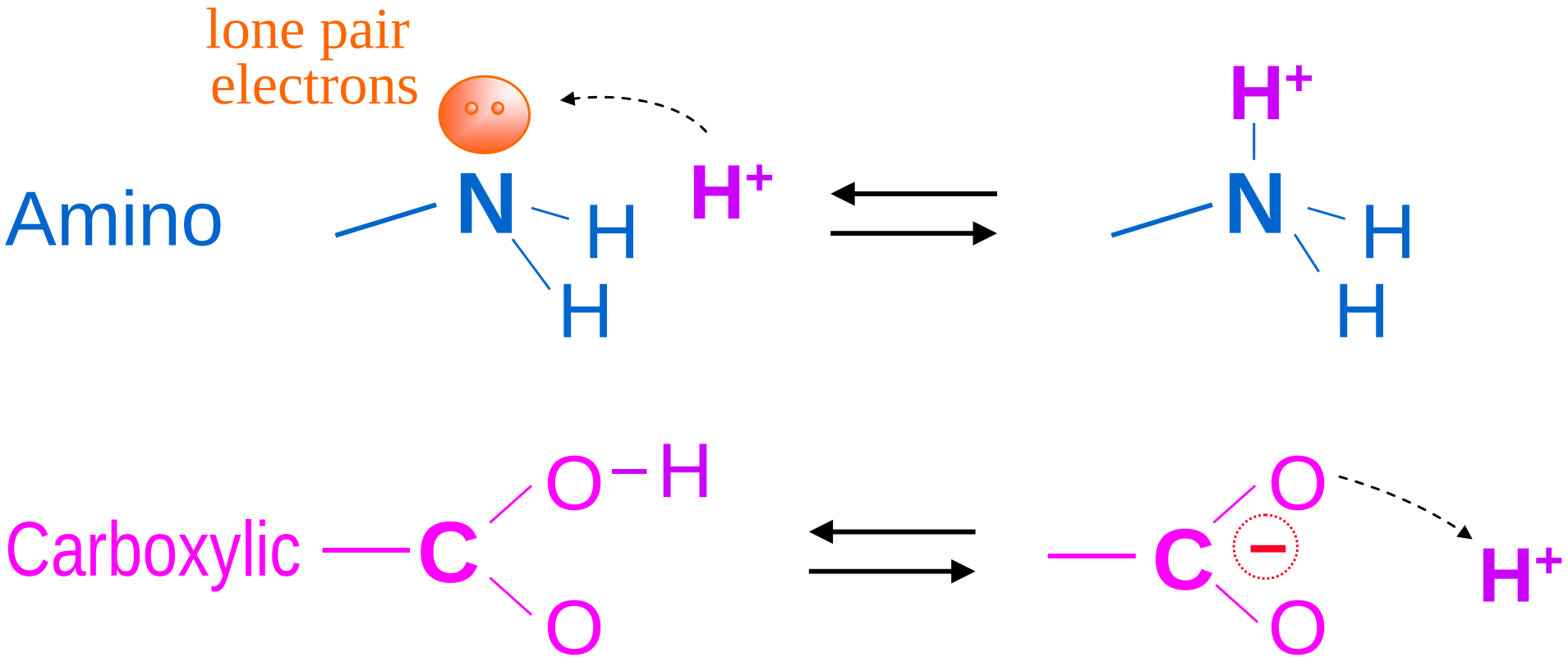


Ion exchange is an adsorption chromatography

Juang RH (2005) EPA

質子可以吸著或脫離一基團

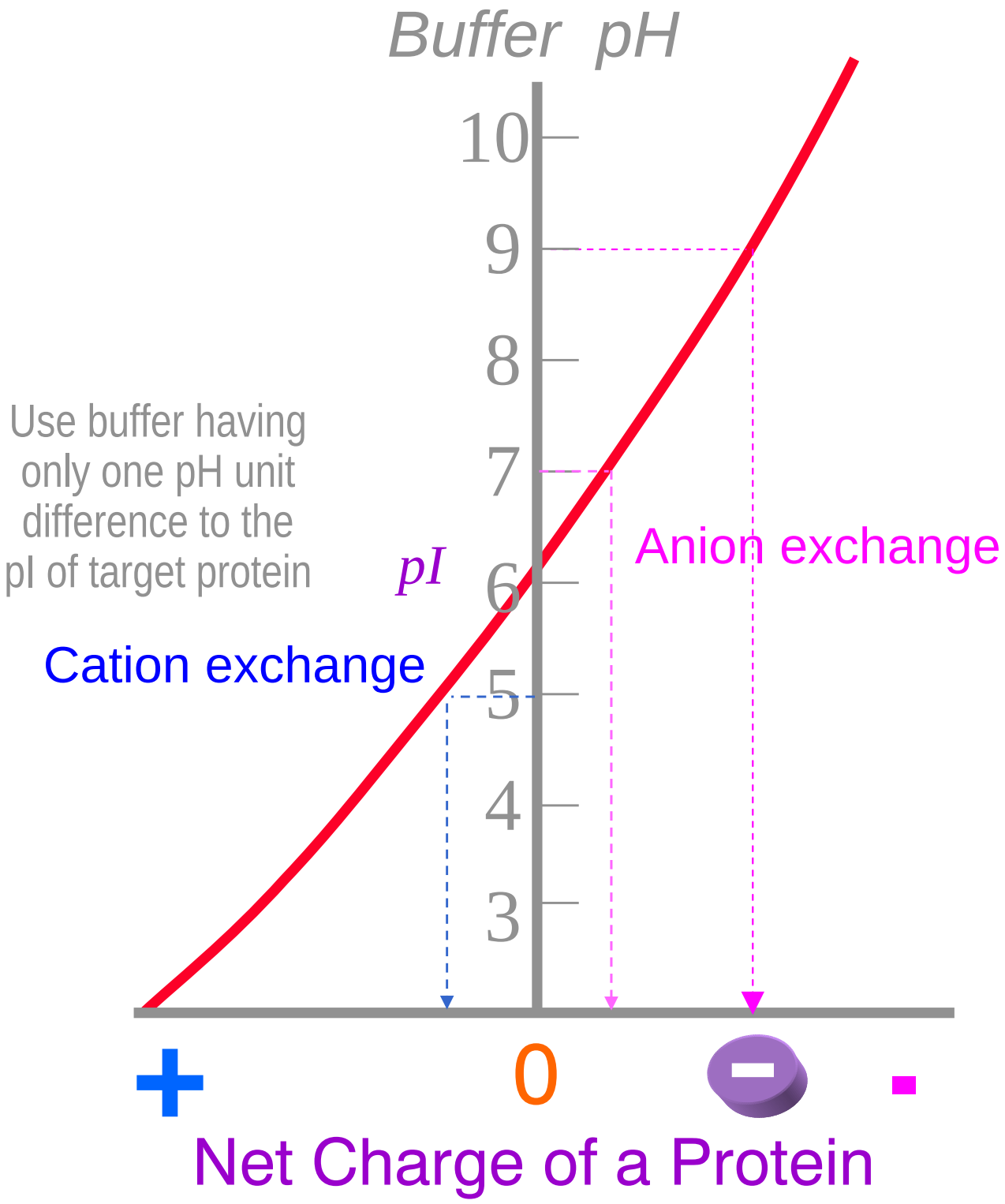
Proton： the smallest and most abundant particle in the living cell controlling the pH and the charge property of a molecule



Ampholyte: a molecule contains both positively and negatively charged groups

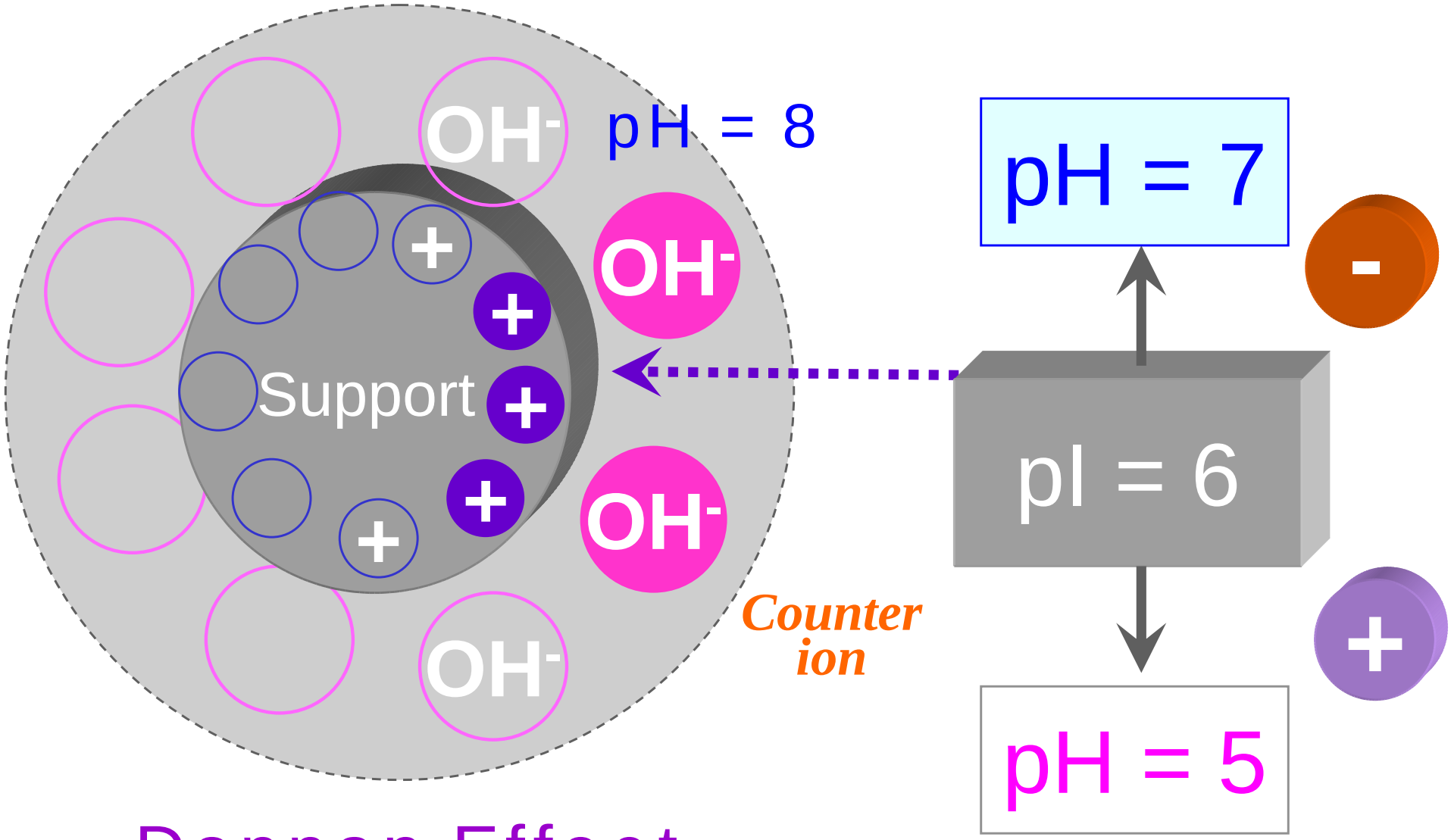
Proton can enter or leave a functional group relatively freely

選擇離子交換介質



Juang RH (2005) EPA

陰離子交換膠體的 pH 變化

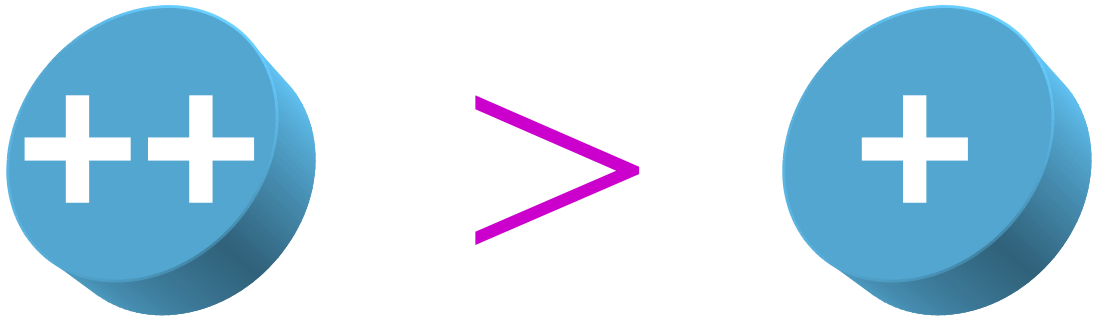


The pH change of the microenvironment surrounding the ion exchange gel particle

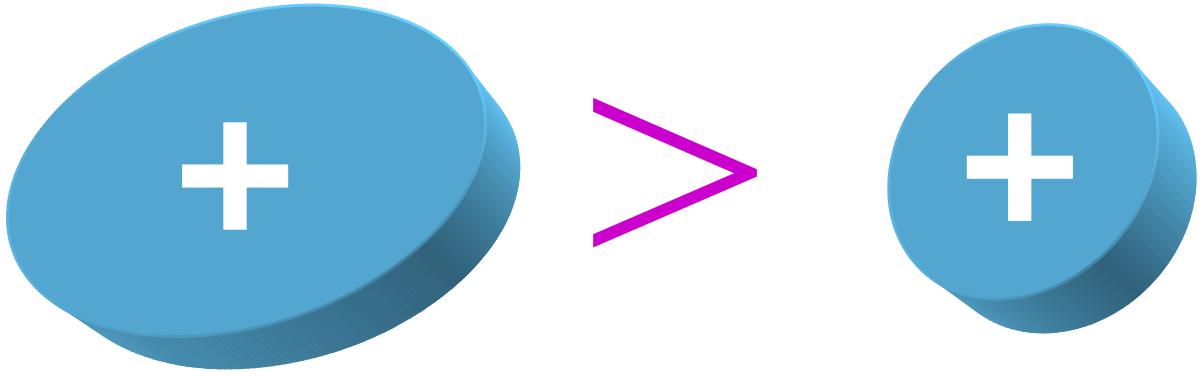
Juang RH (2005) EPA

離子取代優先順序 Displacing order of ions

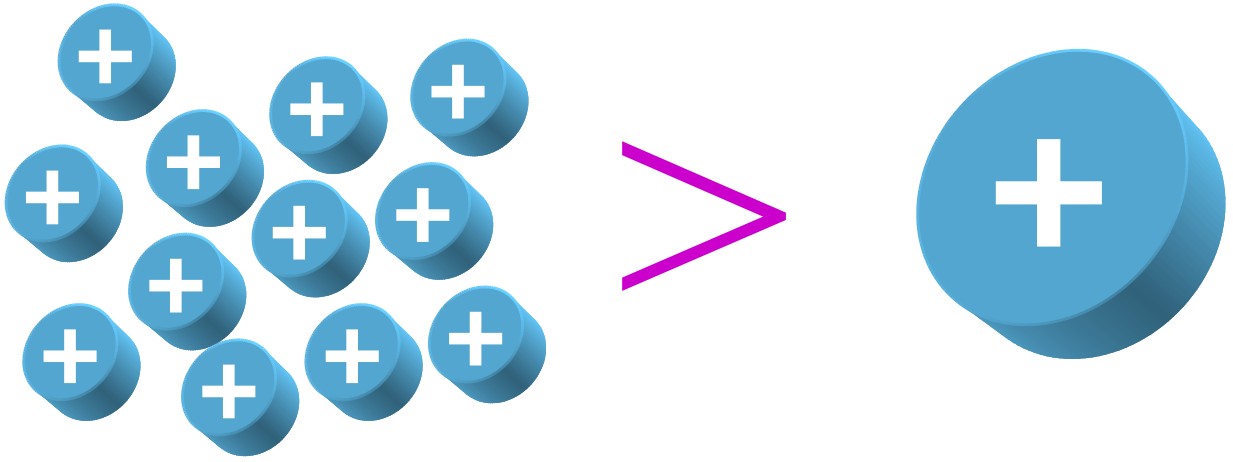
電荷高者
(higher charge)



離子大者
(larger ion)



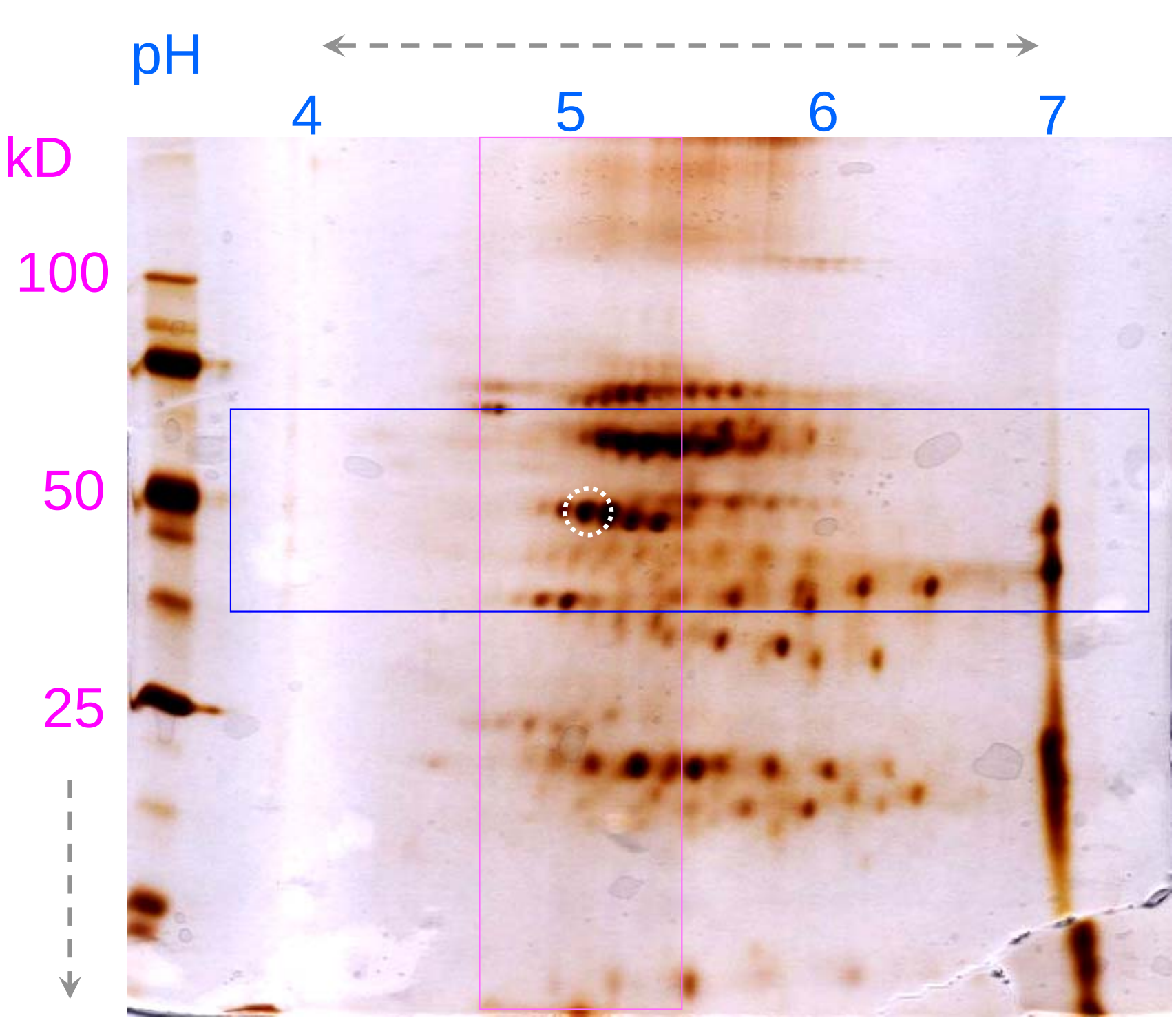
濃度大者
(higher concentration)
change pH, NaCl gradient



Key properties determining the order of ion replacement

Juang RH (2005) EPA

陰離子交換法的分離範圍



The approximate range of proteins isolated by chromatography

Juang RH (2005) EPA

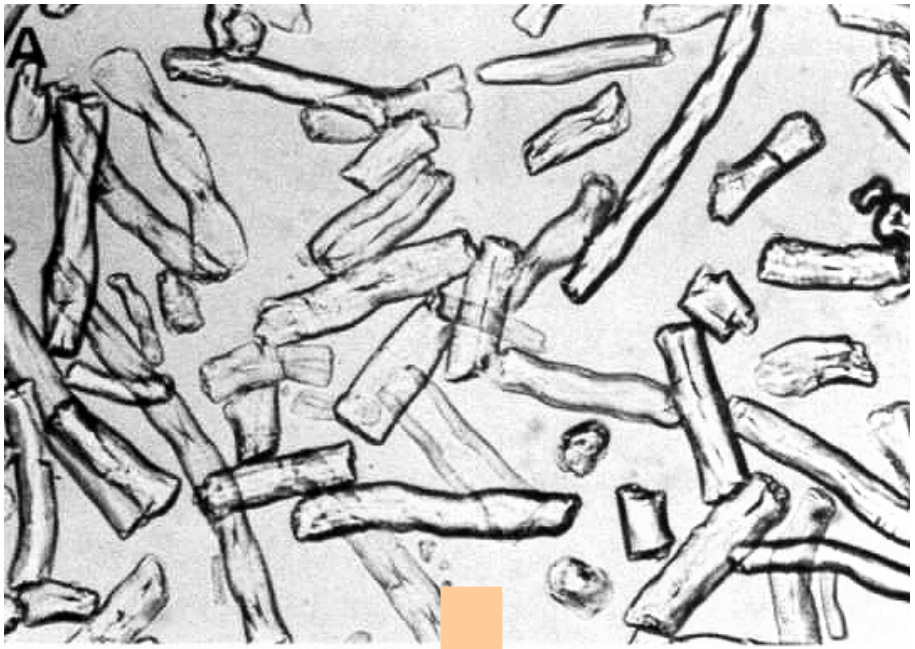
離子交換介質 Common ion exchange materials

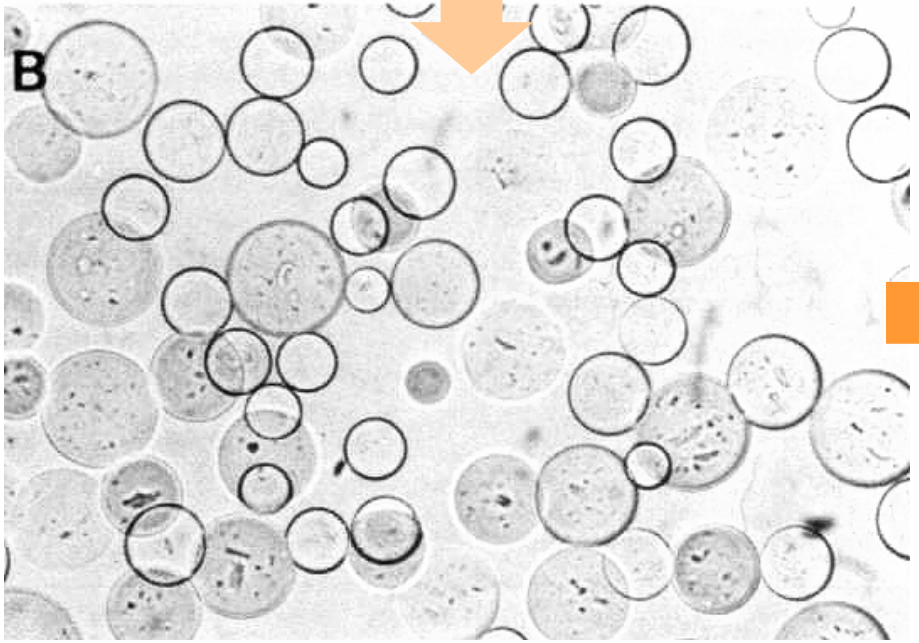
Classification		Resin / Polystyrene		Glycan / Cellulose = X		Mono bead
Anion Exchanger	Strong	Dowex-1 Dowex-2	$-\overset{+}{N}R_3$	TEAE-X (QAE-X)	$-\overset{+}{N}R_3$	Q
	Weak	Dowex-3 IR-45	$-\overset{+}{N}HR_2$	DEAE-X	$-OCH_2CH_2\overset{+}{N}HR_2$	
Cation Exchanger	Strong	Dowex-50	$-SO_3^-$	Phospho-X	$-PO_4^{2-}$	S
	Weak	IRC-150	$-COO^-$	CM-X	$-OCH_2COO^-$	

X = Sephadex, Sepharose, Sephacel or cellulose

Juang RH (2005) EPA

膠體的組成與外型 Support material and bead





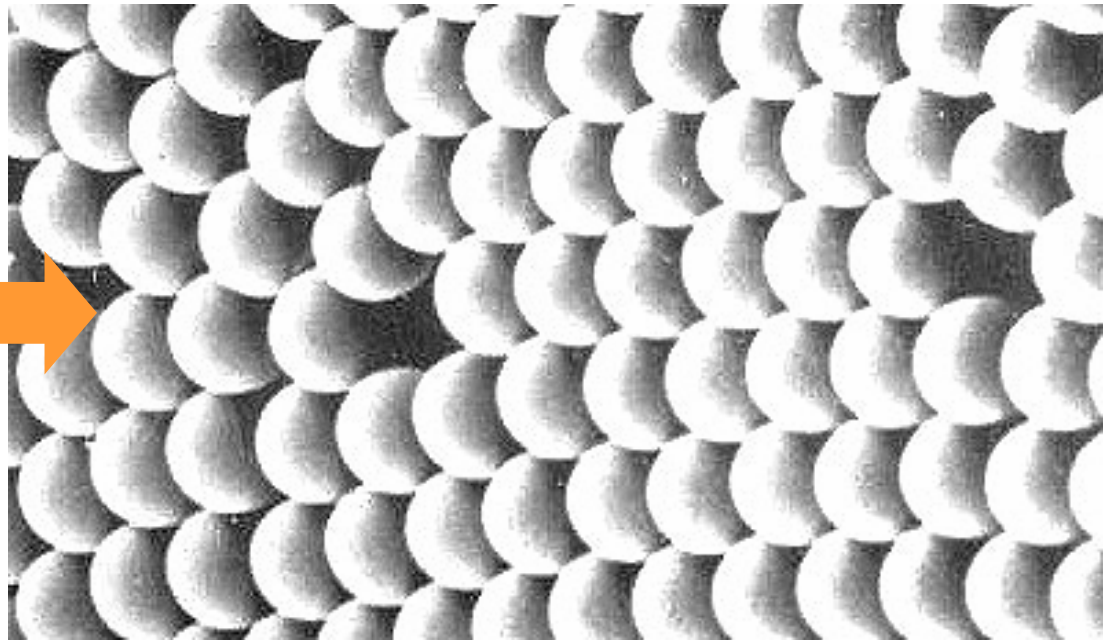
Pharmacia (1980) Separation News: 5

Cellulose

Sephacel

Monobead

- Homogeneous bead shape is critical to its resolution and flow rate



Pharmacia (1991) Ion Exchange Chromatography – Principles and Methods p.24

不同膠體的吸著容量有很大差異

Adapted from Pharmacia (1991) Ion Exchange Chromatography – Principles and Methods p.64

	Lactalbumin	Albumin	Ferritin
DEAE-Sephadex A-25	191	31	2
DEAE-Sephadex A-50	10	102	1
DEAE-Sepharose CL-6B	45	115	4.3
DEAE-Sephacel	38	86	8.6

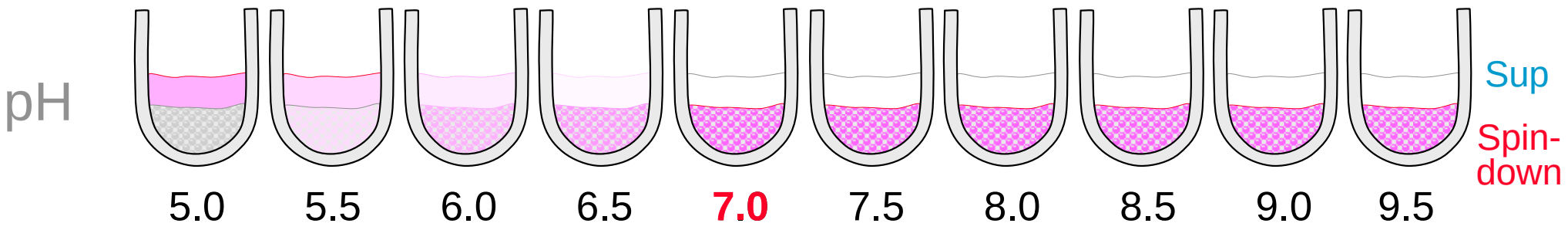
Buffer: 0.01 M Tris-HCl, pH 8.0

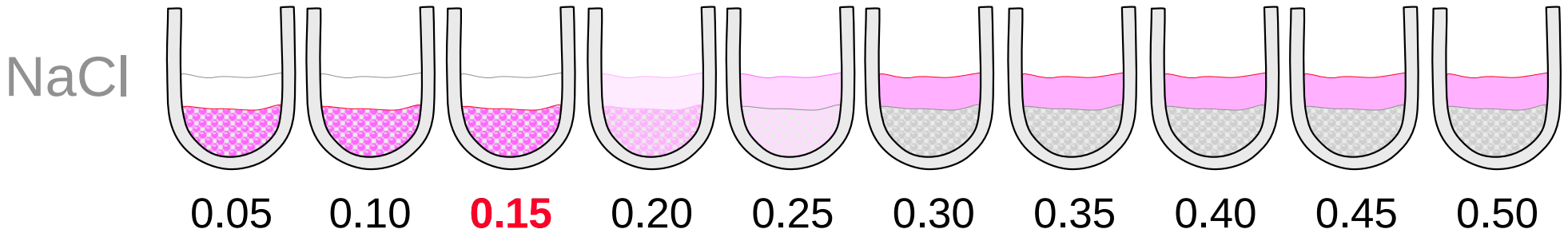
mg / mL gel

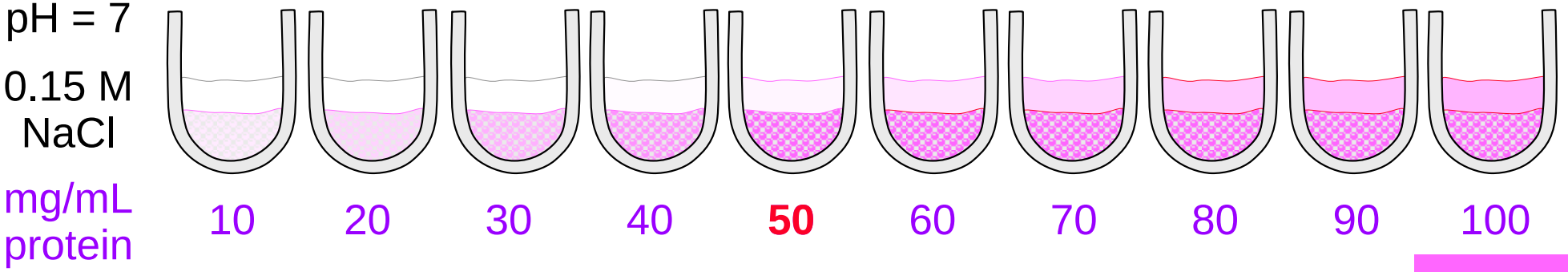
Each gel has different adsorption capacity toward different target proteins

離子交換法預備試驗 Determine the conditions

Enzyme + Buffer + Gel → Incubation → Spin → Sup + Spindown







Capacity

Adapted from Pharmacia (1991) Ion Exchange Chromatography – Principles and Methods p.70

膠柱裝填方法 Packing column step by step

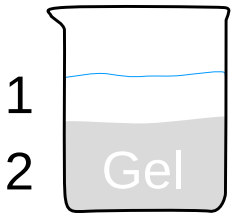
清洗膠體
Wash gel well

預估體積
Estimate
gel volume

緩衝液
平衡
Equilibrated
in buffer

靜置膠體
Gel stands o/n

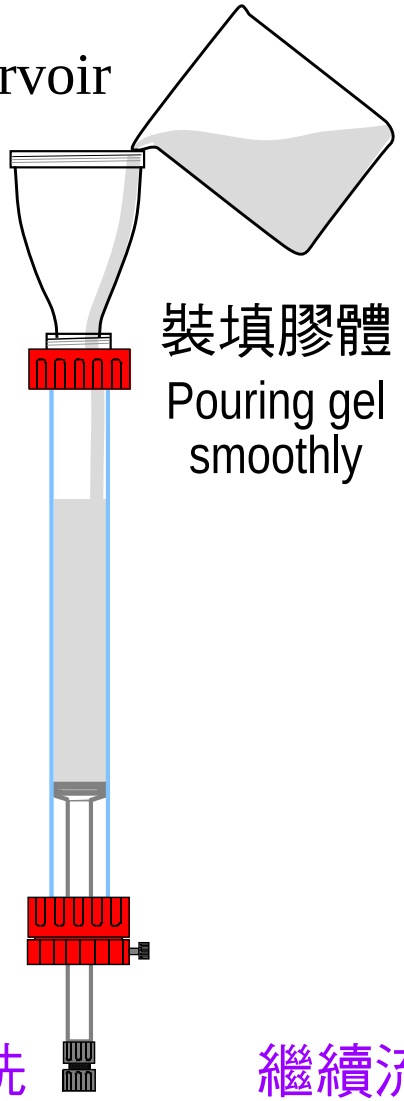
溫度平衡
Temperature
equilibrated



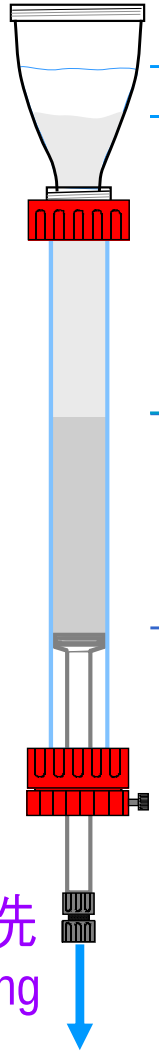
Put on reservoir

檢查管柱
是否暢通
Check flow
rate of empty
column

裝填膠體
Pouring gel
smoothly

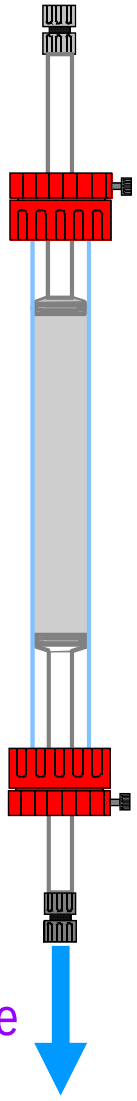


暫停流洗
Stop elution
X



繼續流洗
Keep eluting

Put on adaptor



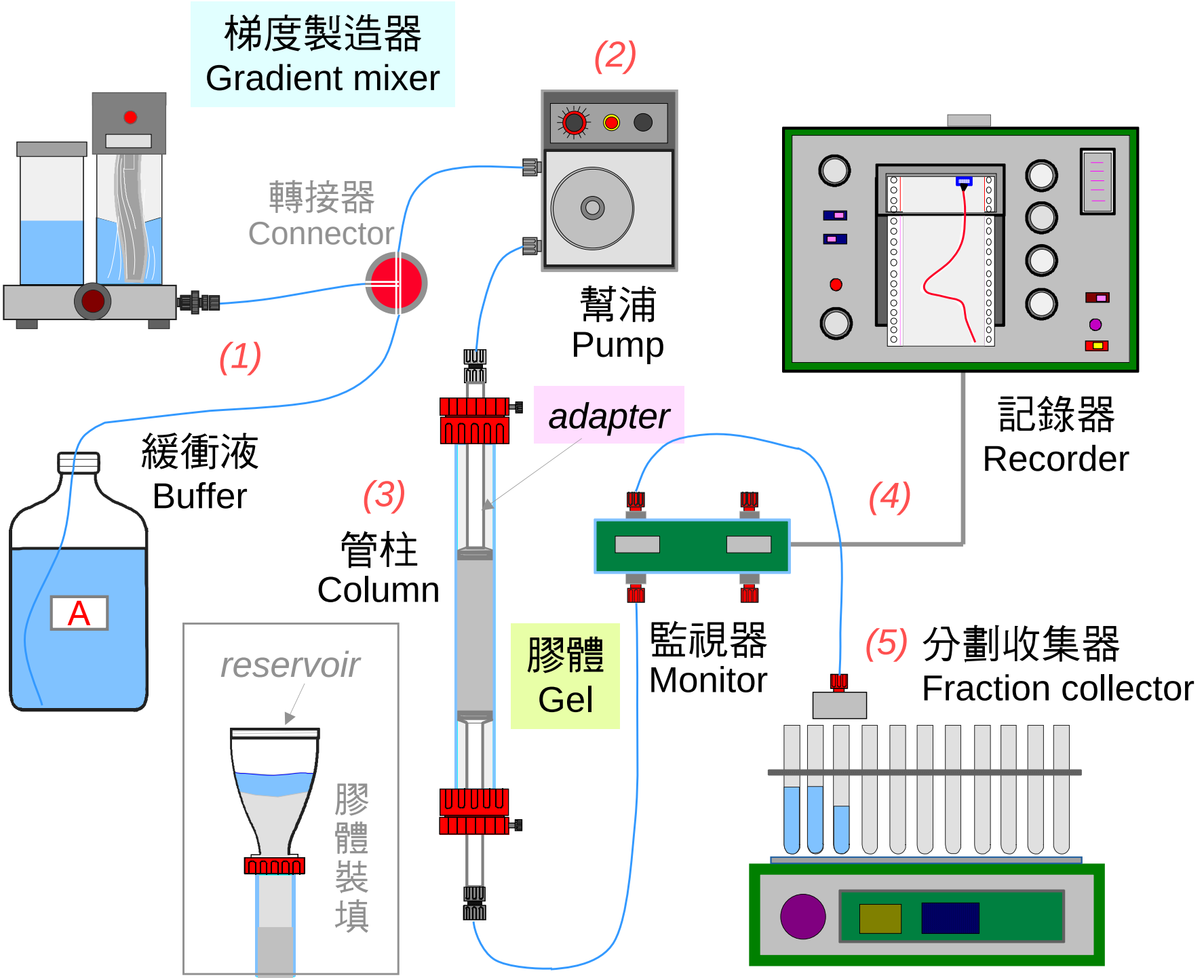
緊密堆積

加壓流洗
Elute under
High pressure

Gel should be equilibrated completely before packing

Juang RH (2005) EPA

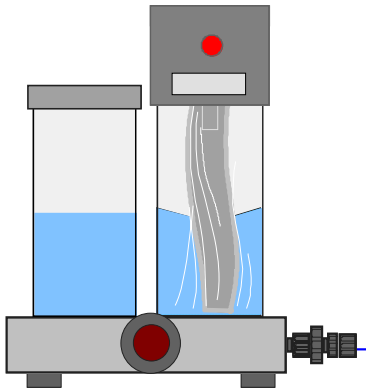
液相層析管柱系統



The whole family of liquid chromatography apparatus

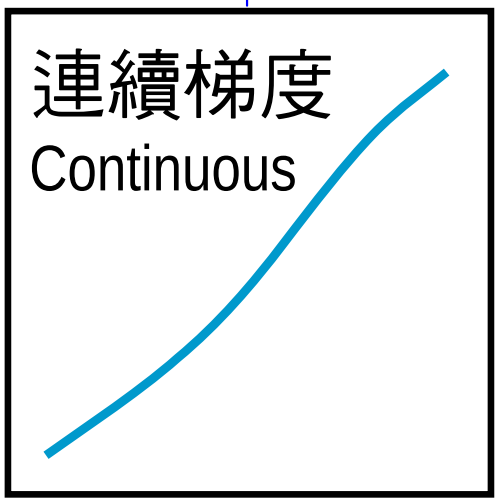
Juang RH (2005) EPA

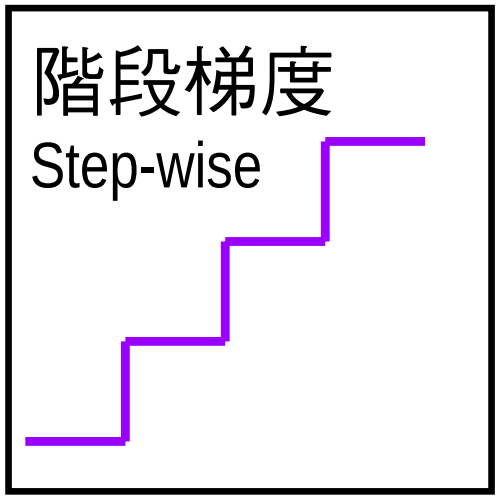
鹽梯度的兩種方式 Two ways for making gradient

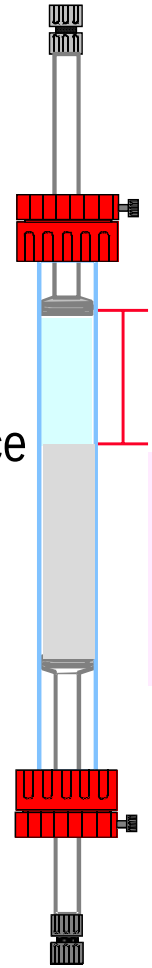


高限溶液 低限溶液

Upper-limit Lower-limit







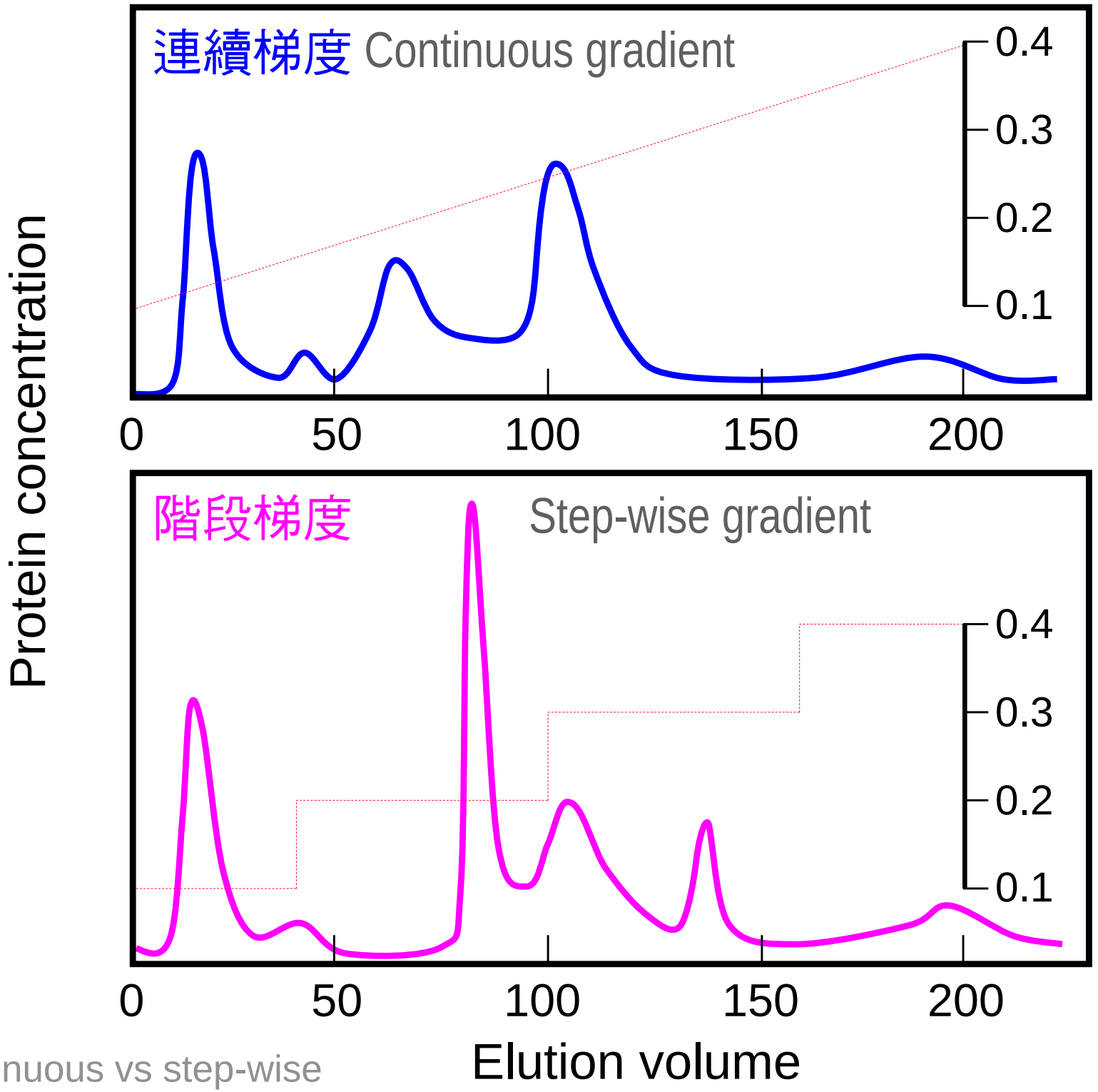
Pharmacia

兩種方式均可，各有特點。

Both methods have their specific applications

Juang RH (2005) EPA

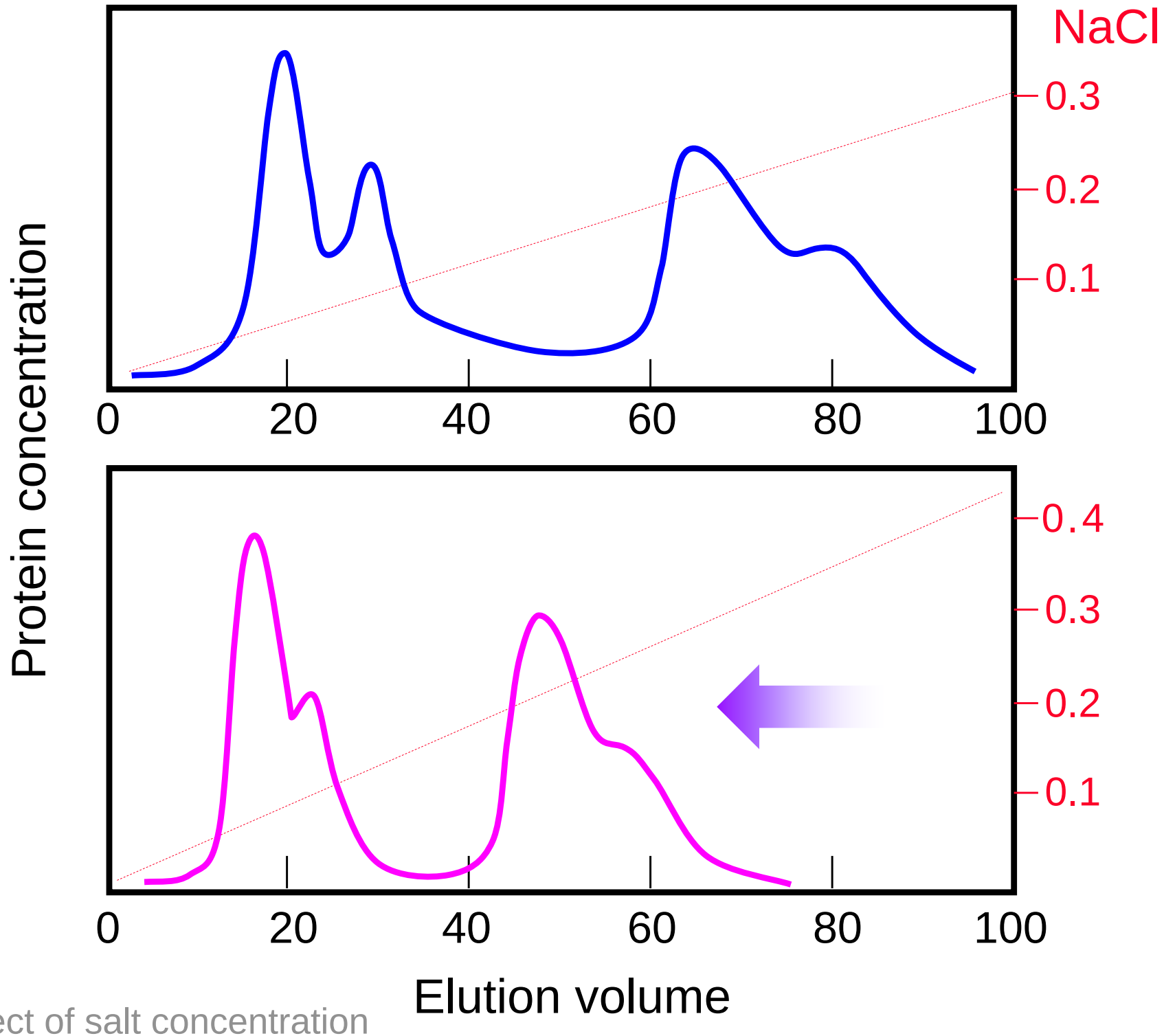
連續與階段梯度比較



Continuous vs step-wise

Adapted from Pharmacia: Ion Exchange Chromatography – Principles and Methods

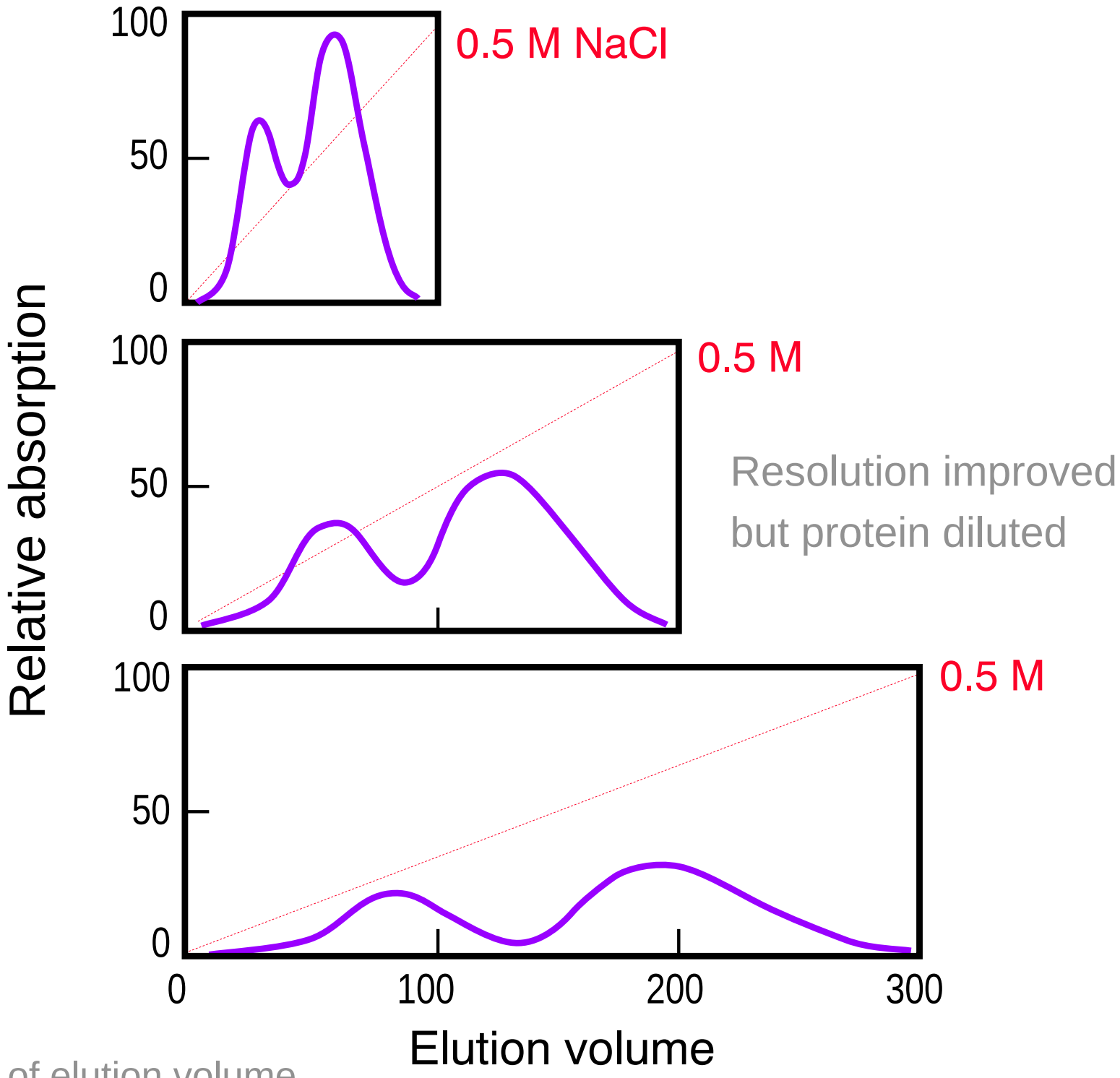
鹽濃度比較



Effect of salt concentration

Adapted from Pharmacia: Ion Exchange Chromatography – Principles and Methods

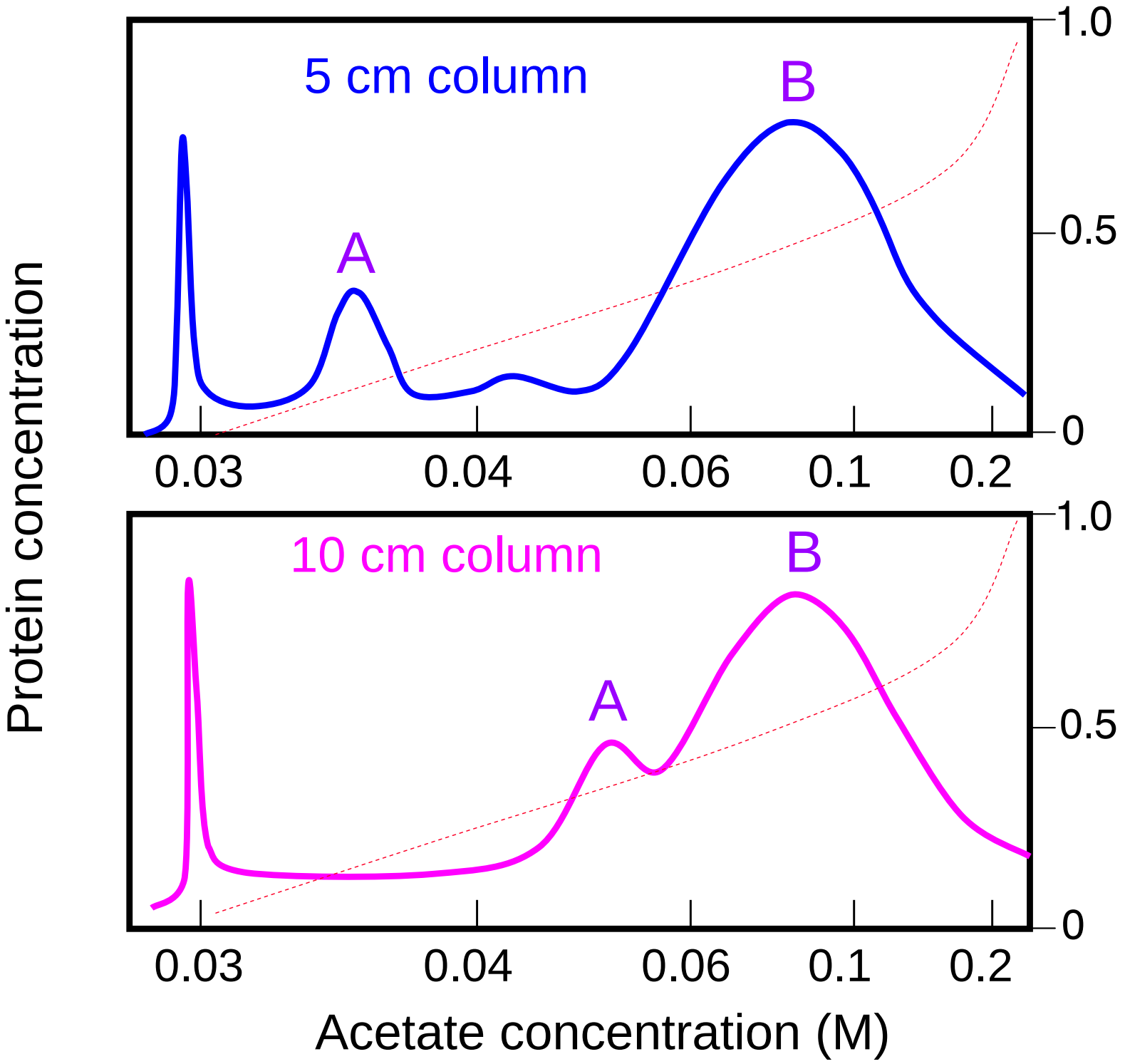
溶離體積會影響解析度



Effect of elution volume

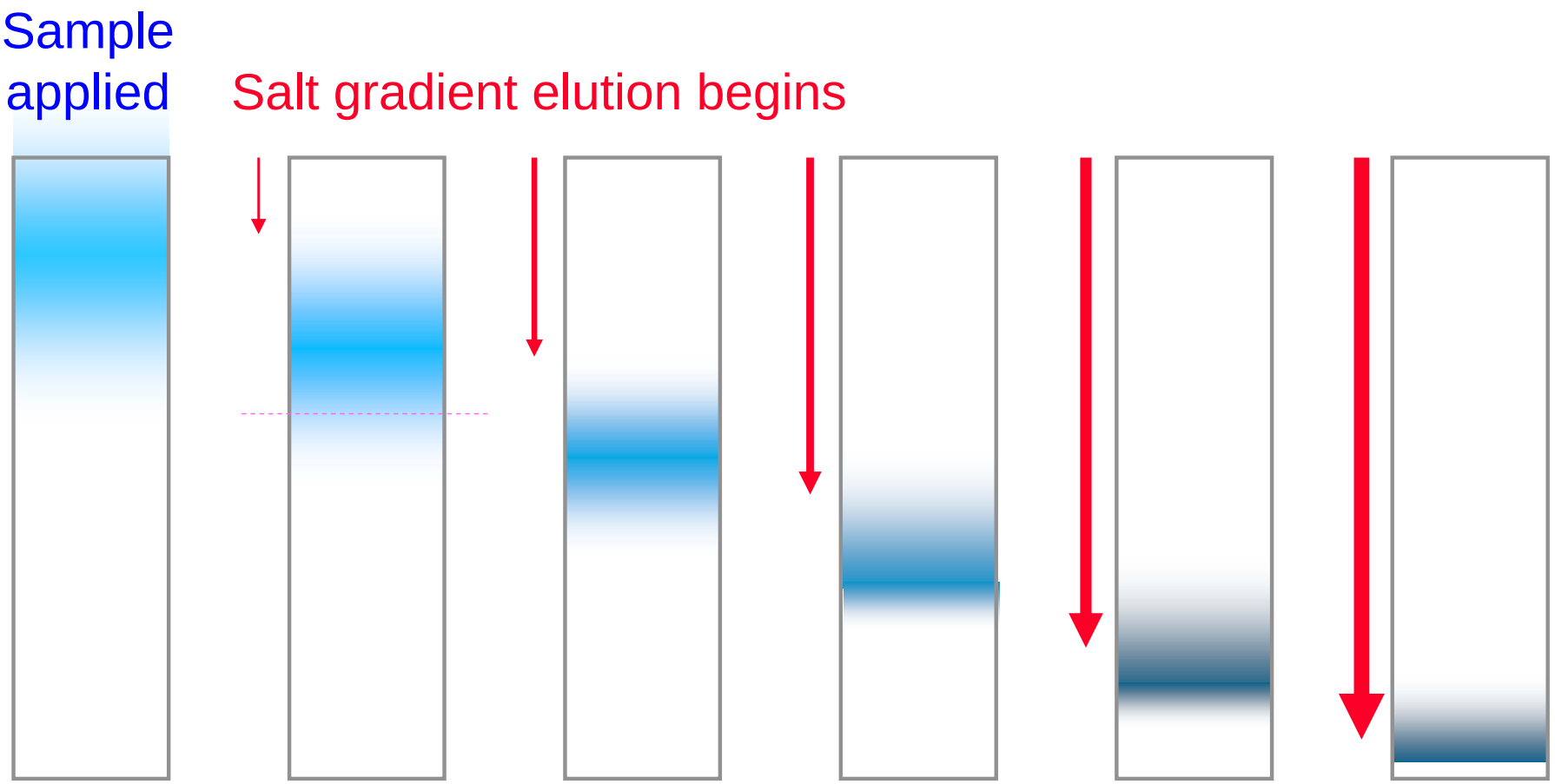
Adapted from Pharmacia: Ion Exchange Chromatography – Principles and Methods

管柱長短的影響



Effect of column length

離子交換的梯度溶離有濃縮效果



Salt elutes and brings the proteins moving forward,
but proteins are adsorbed again in the front and retarded

Salt gradient of ion exchange can concentrate the proteins in sample elution

Adapted from Scope RK (1987) Protein Purification – Principles and Practice p.112

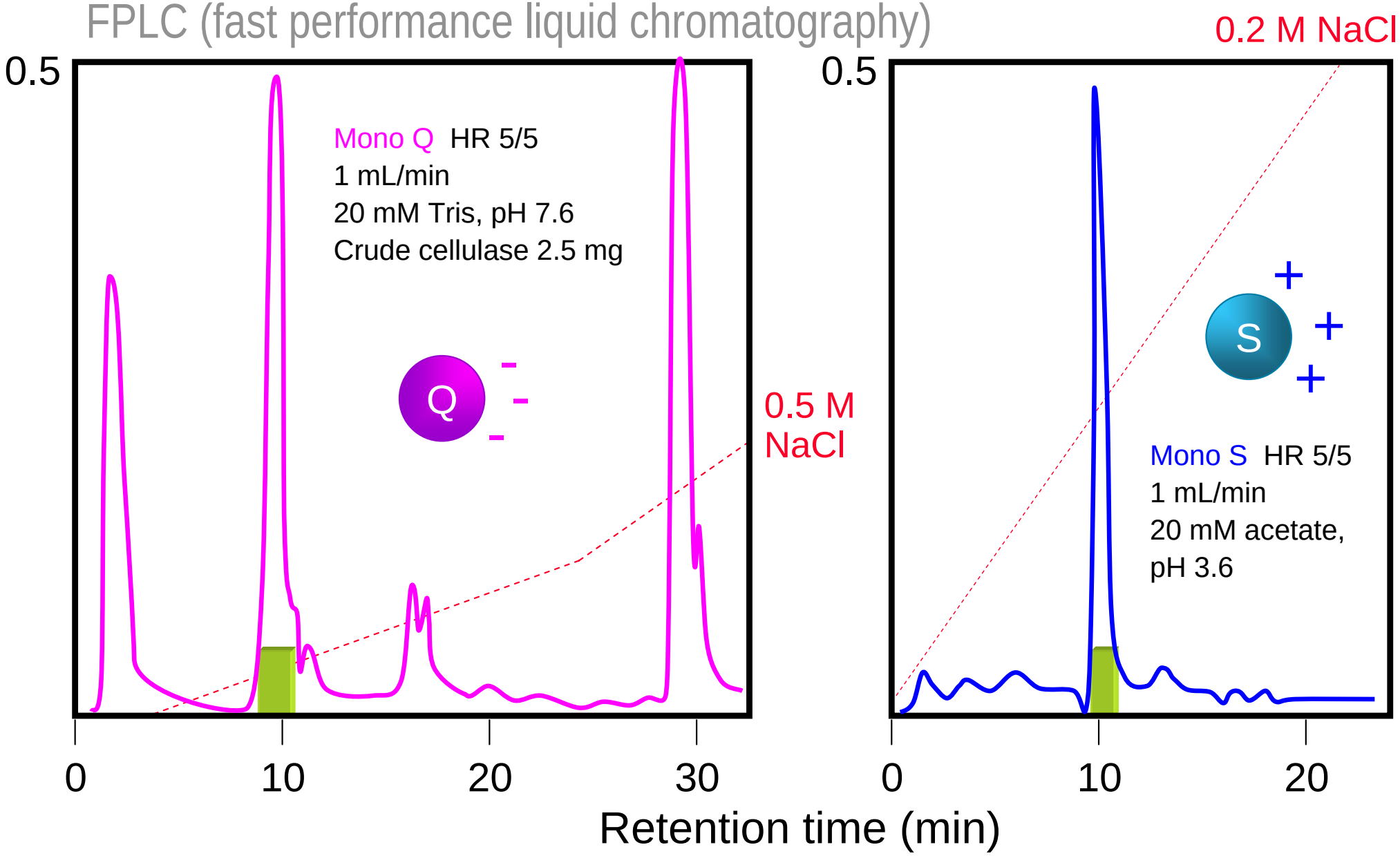
離子交換法操作要點 Summary on operation

Equilibration	Used gel should be regenerated and equilibrated well
Elution	Eluted with NaCl in continuous or step-wise gradient
Gradient	Use proper concentration and volume in elution
Buffer pH	Keep target proteins in weak charged state
Non-specific	Wash out contaminants with low NaCl concentration
Sample	Equilibrated in buffer, don’t overload column capacity
Elute through	Flow target protein through and adsorb others
Dead volume	Eliminate any dead volume in the column

Juang RH (2005) EPA

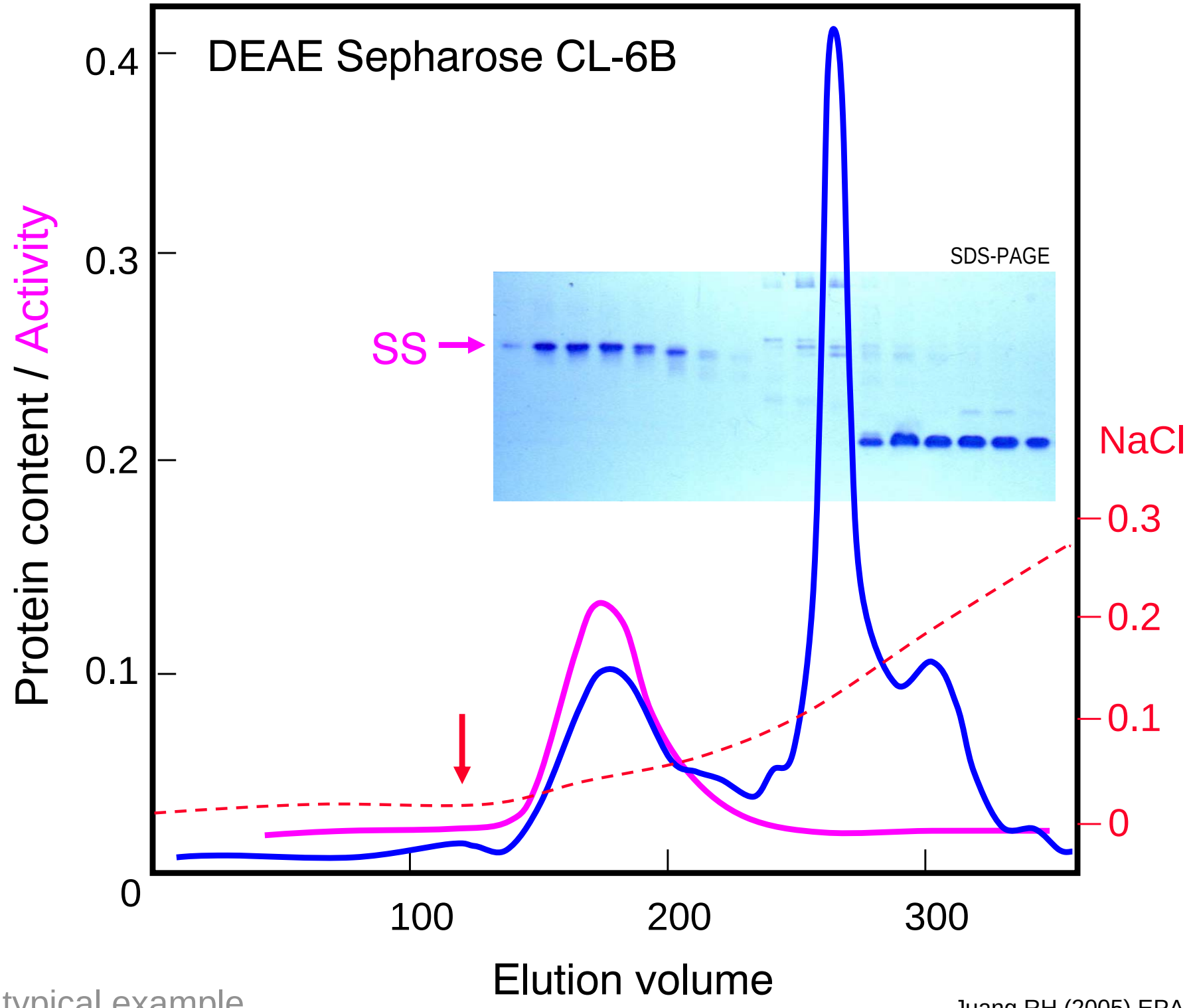
離子交換法實例 Two-step cellulase purification

FPLC (fast performance liquid chromatography)



Adapted from Pharmacia (1991) Ion Exchange Chromatography – Principles and Methods p.127

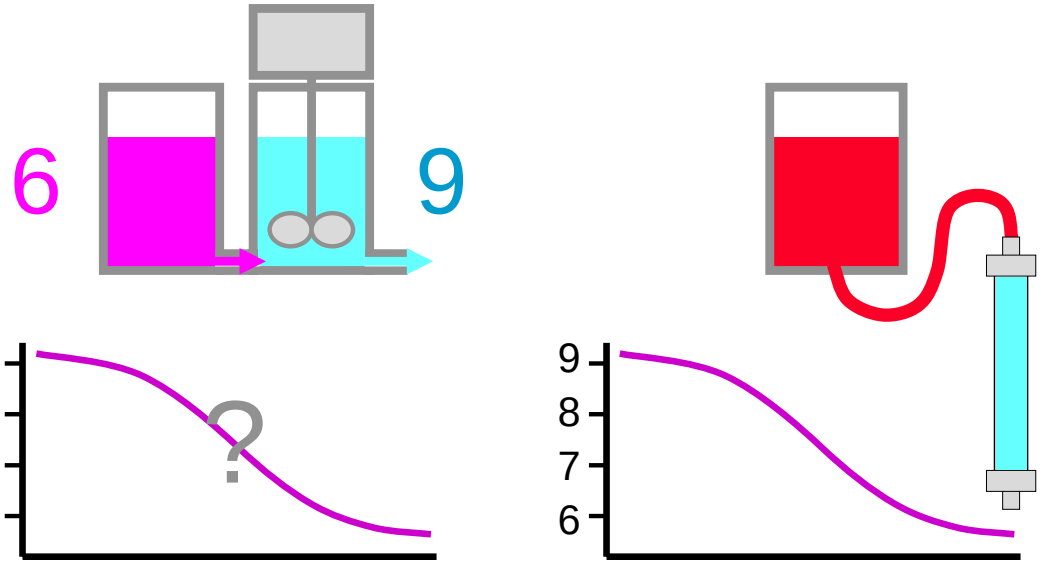
離子交換法實例



A typical example

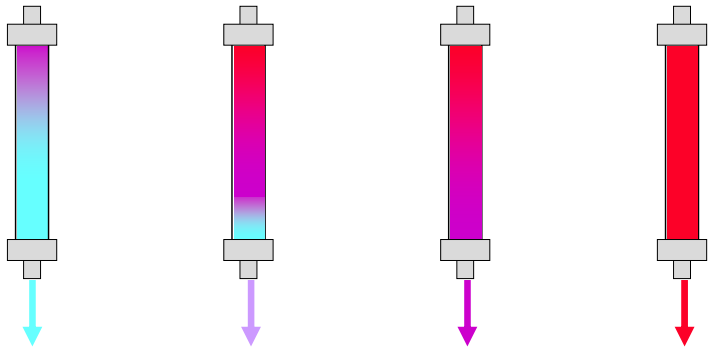
Juang RH (2005) EPA

色層焦集法如何拉出 pH 梯度

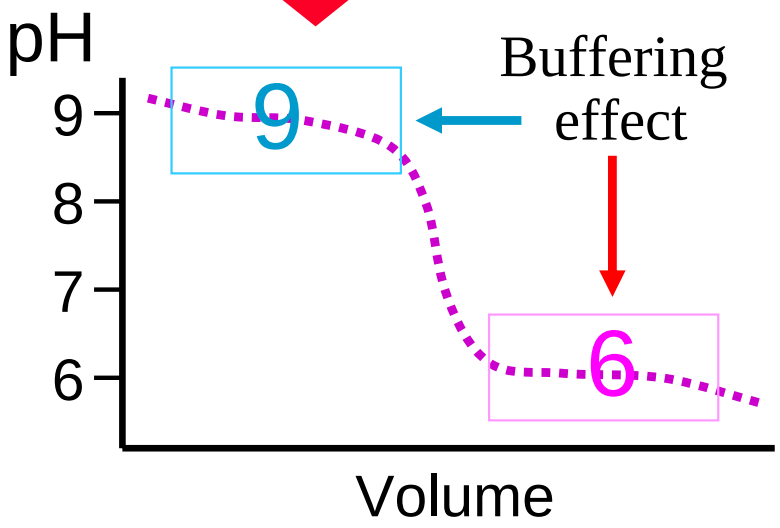


● Polybuffer 含有連續 pK_a 的緩衝分子 (ampholyte) 可拉出連續 pH 梯度

● Polybuffer contains *ampholyte* which is a mixture of chemicals having continuous pK_a

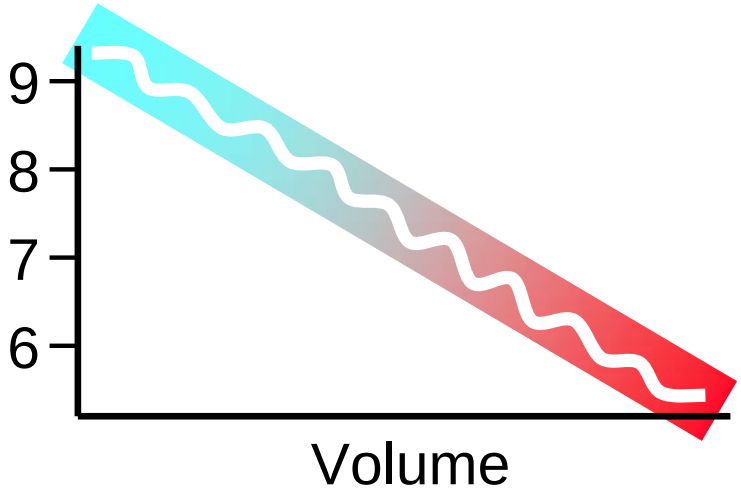


實際結果 ➡ 無法拉出 pH 梯度



假如含有許多緩衝分子 ➡

If there are many buffering molecules in the buffer…..



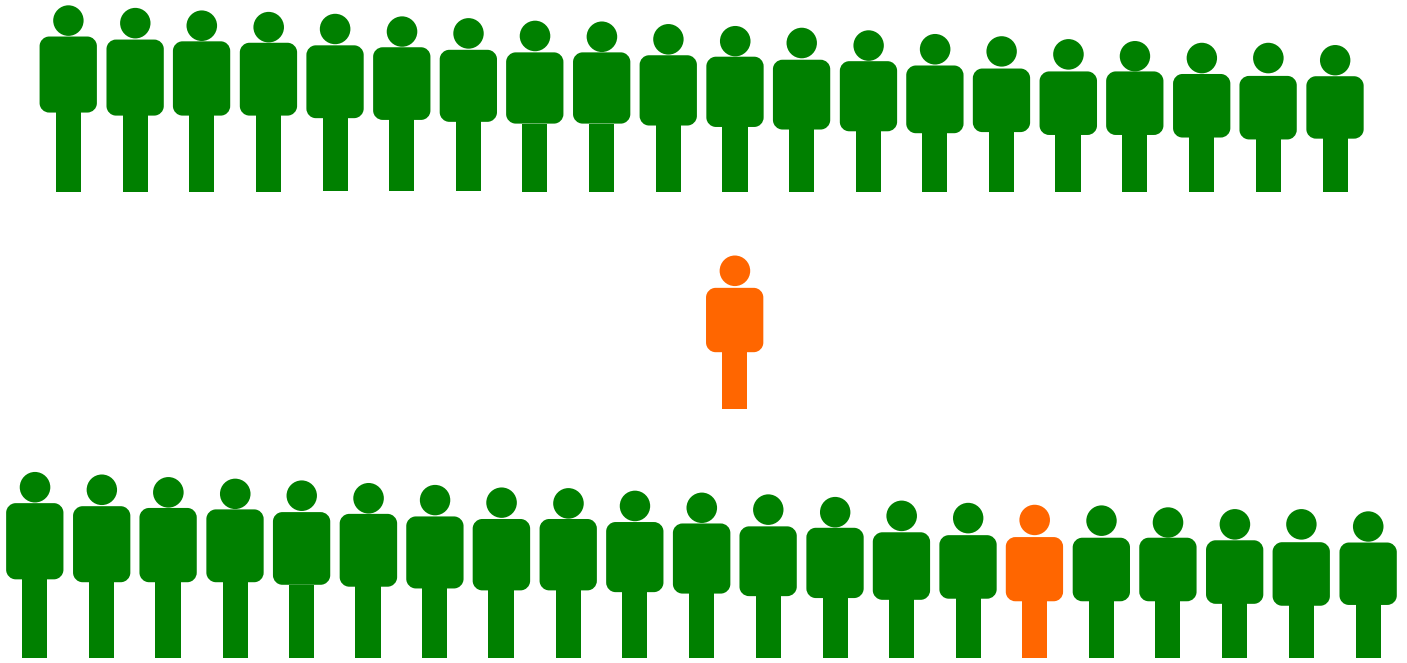
How chromatofocusing creates its pH gradient

Juang RH (2005) EPA

3.3.5 色層焦集法 Chromatofocusing

其介質也是一種離子交換介質

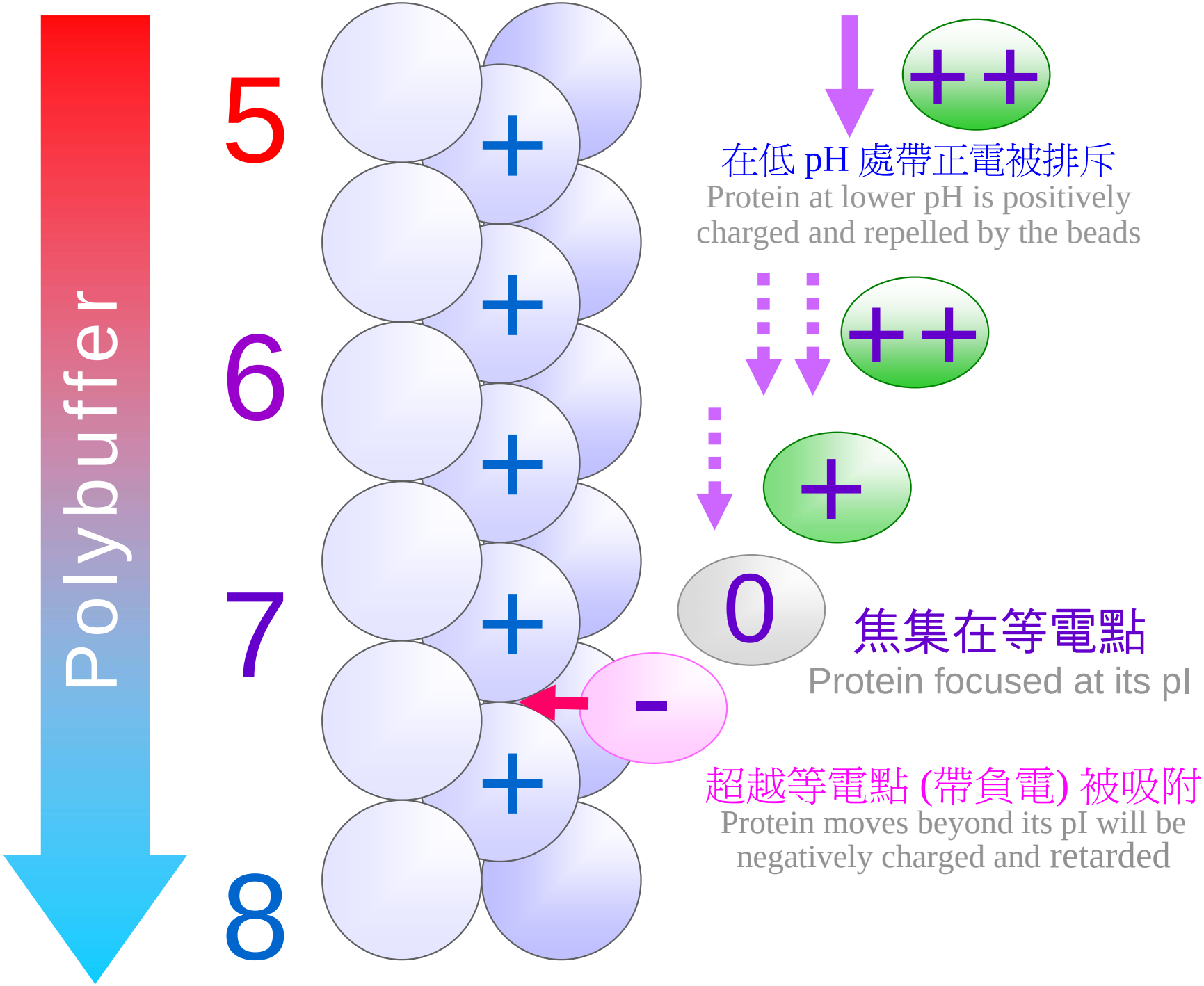
但所使用的 Polybuffer 可拉出穩定的 pH 梯度



Chromatofocusing using ion exchange bead, but eluted with Polybuffer

Juang RH (2005) EPA

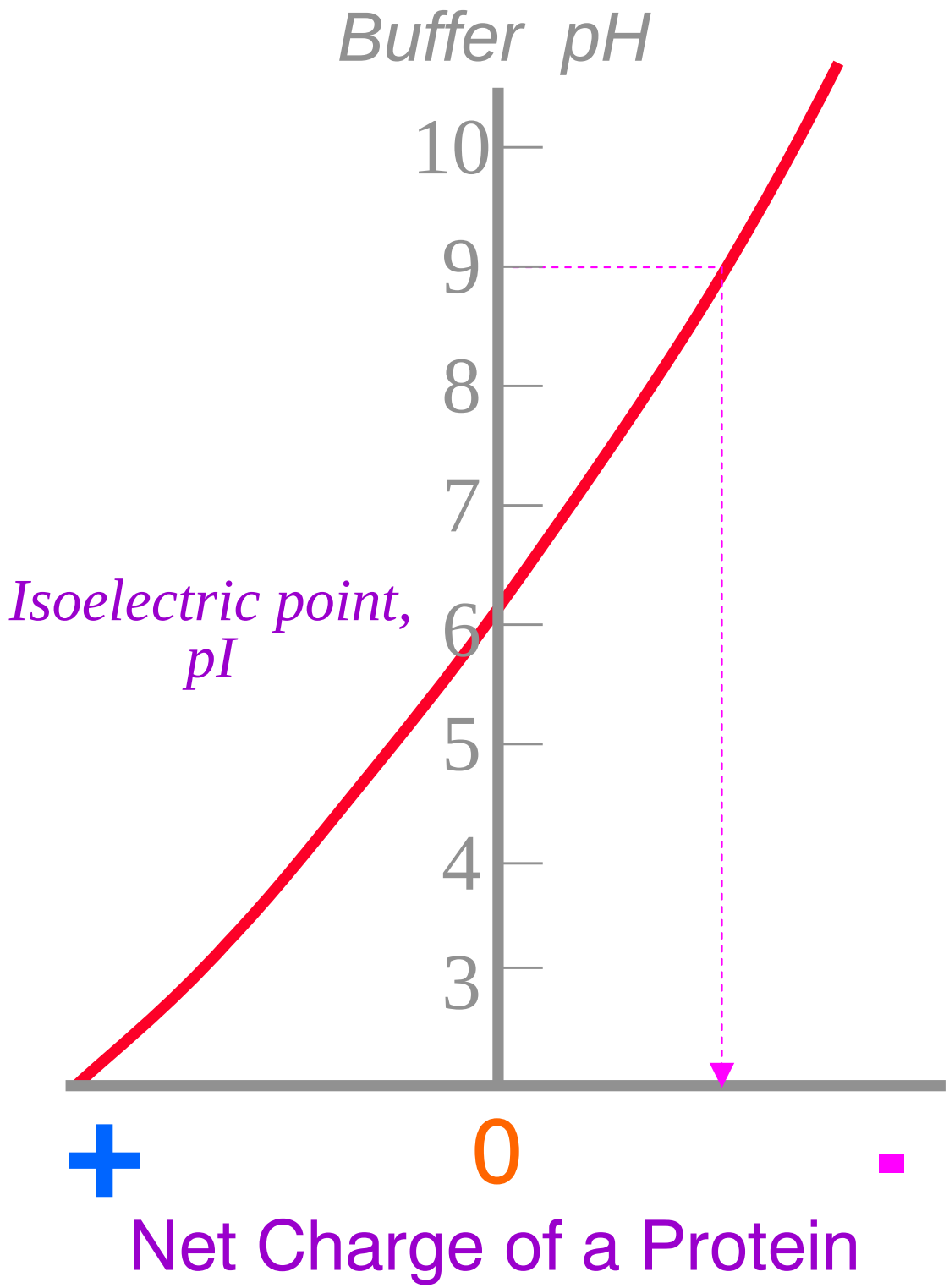
色析焦集法的焦集機制



Focusing mechanism in chromatofocusing

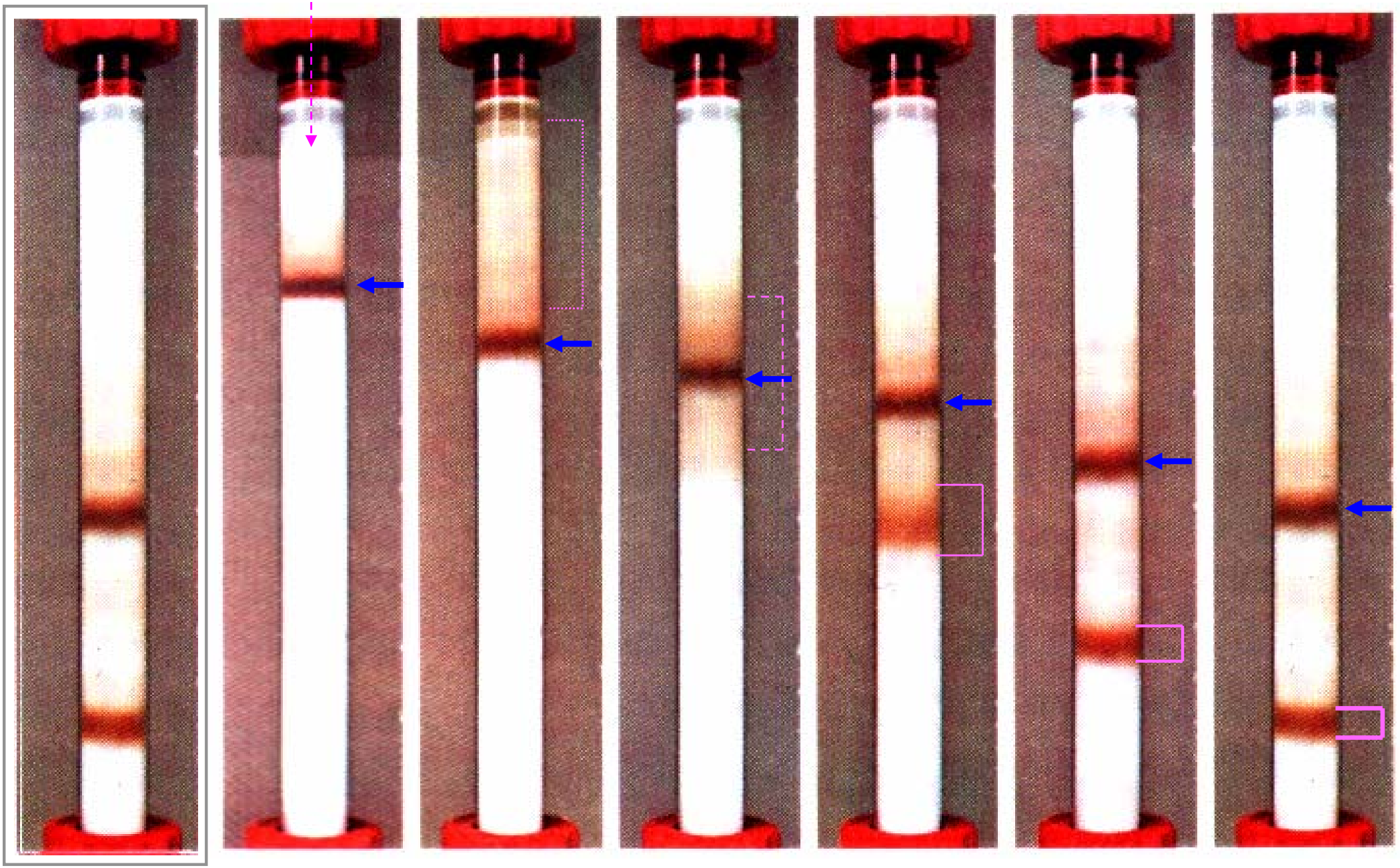
Juang RH (2005) EPA

環境影響分子的帶電性質



Juang RH (2005) EPA

● 抹香鯨肌紅蛋白 (pI = 8.2) 追過 馬肌紅蛋白 (pI = 7.4)



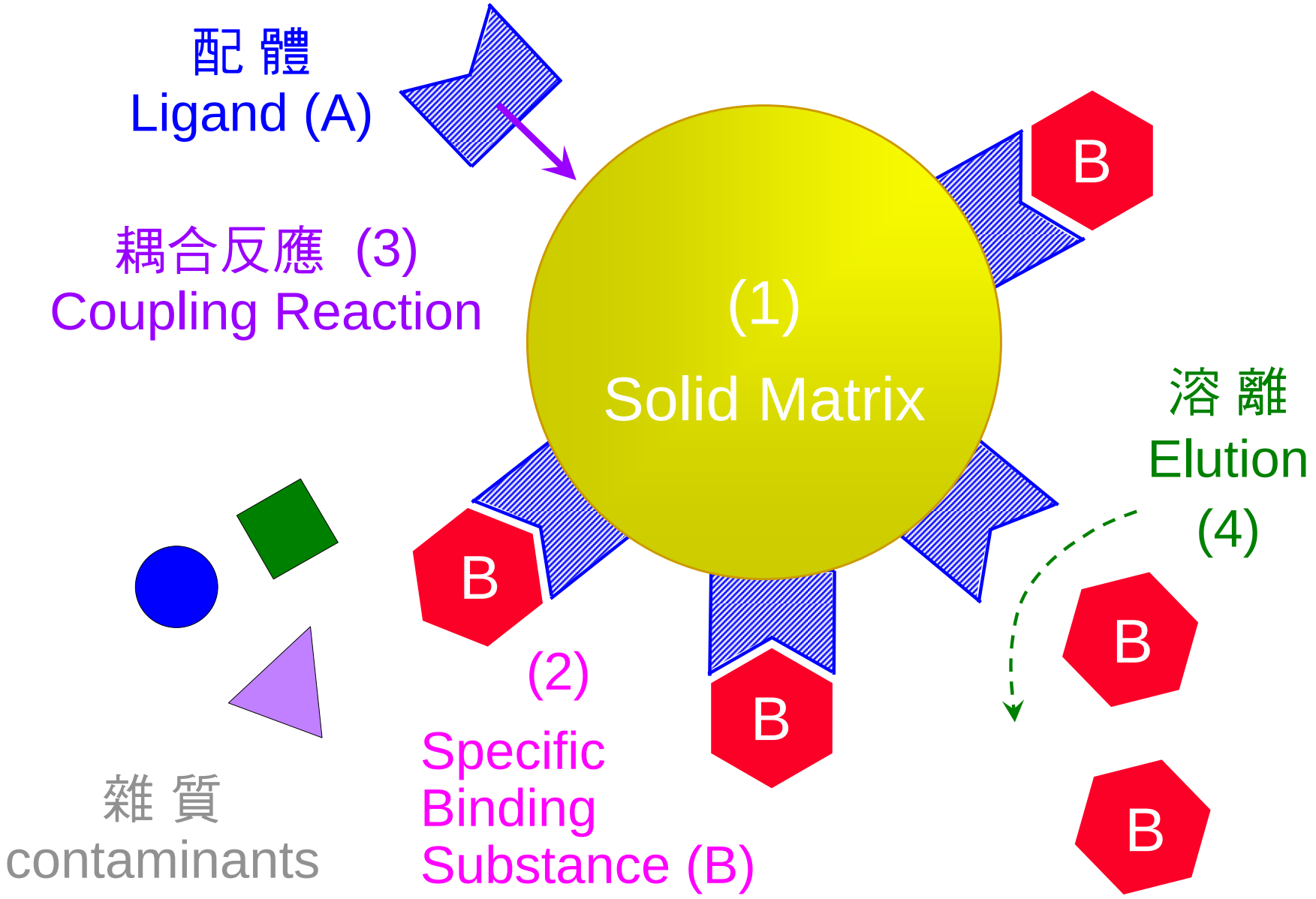
A molecule might be caught up by another protein in chromatofocusing

Pharmacia

3.4 親和層析法 Affinity chromatography

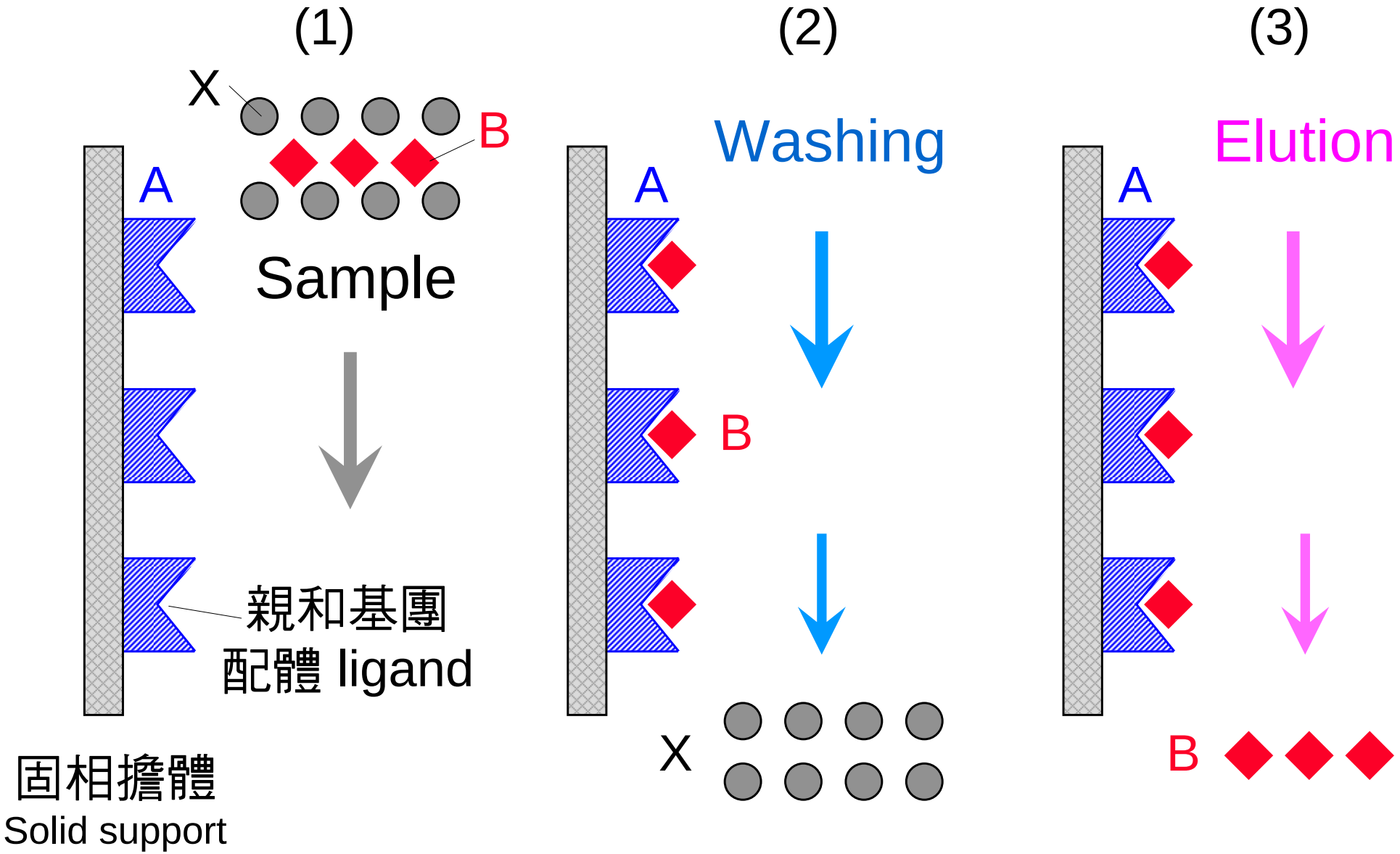
- 3.4.1 原理概述 Basic principles
  要有一對具有高親和性的分子
- 3.4.2 親和吸著劑 Affinity adsorbent
  有很多親和性吸著劑可利用
- 3.4.3 金屬螯合層析法 Metal chelating
  利用分子與金屬的吸引力
- 3.4.4 疏水性層析法 Hydrophobic interaction
  利用分子間的疏水性引力
- 3.4.5 液相分配 Liquid partition
  分子在兩液相間的分配比例不同

親和層析法的四項要素 Four essential factors



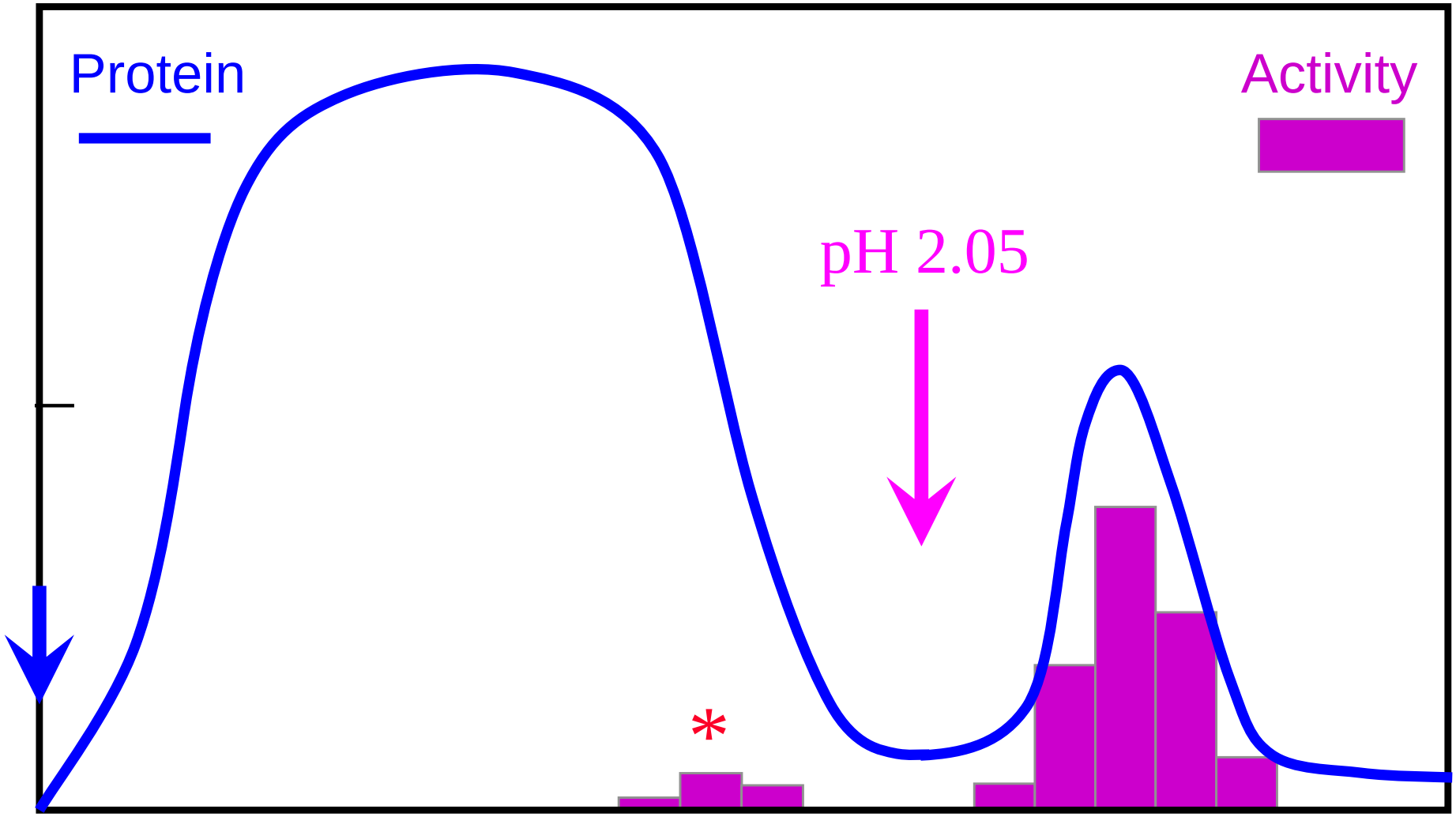
Juang RH (2005) EPA

親和層析法的作用機理 How it works



Juang RH (2005) EPA

典型的親和層析操作 Typical elution pattern



Juang RH (2005) EPA

專一性結合力量的構成因素 Specific interactions

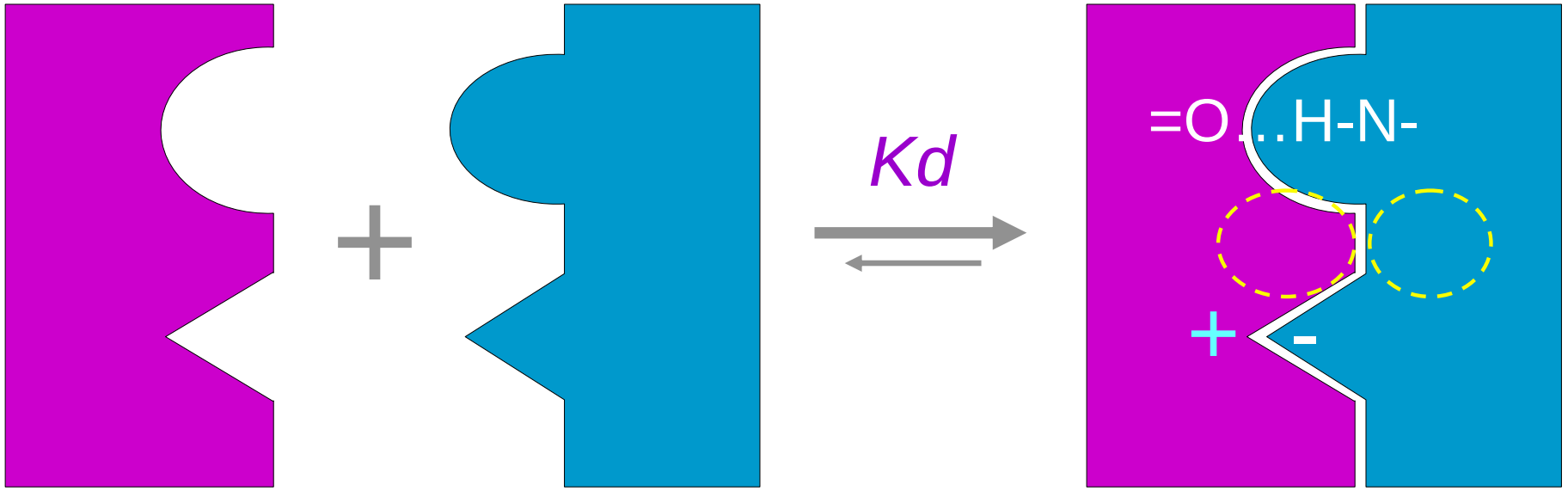
I. Conformational Match:

Van der waals interaction

兩分子間因構形互補所造成的
吸引力是由凡得瓦爾力所貢獻

II. Interaction Forces:

(1) Hydrogen bond
(2) Hydrophobic interaction
(3) Electrostatic interaction
(4) Van der waals interaction

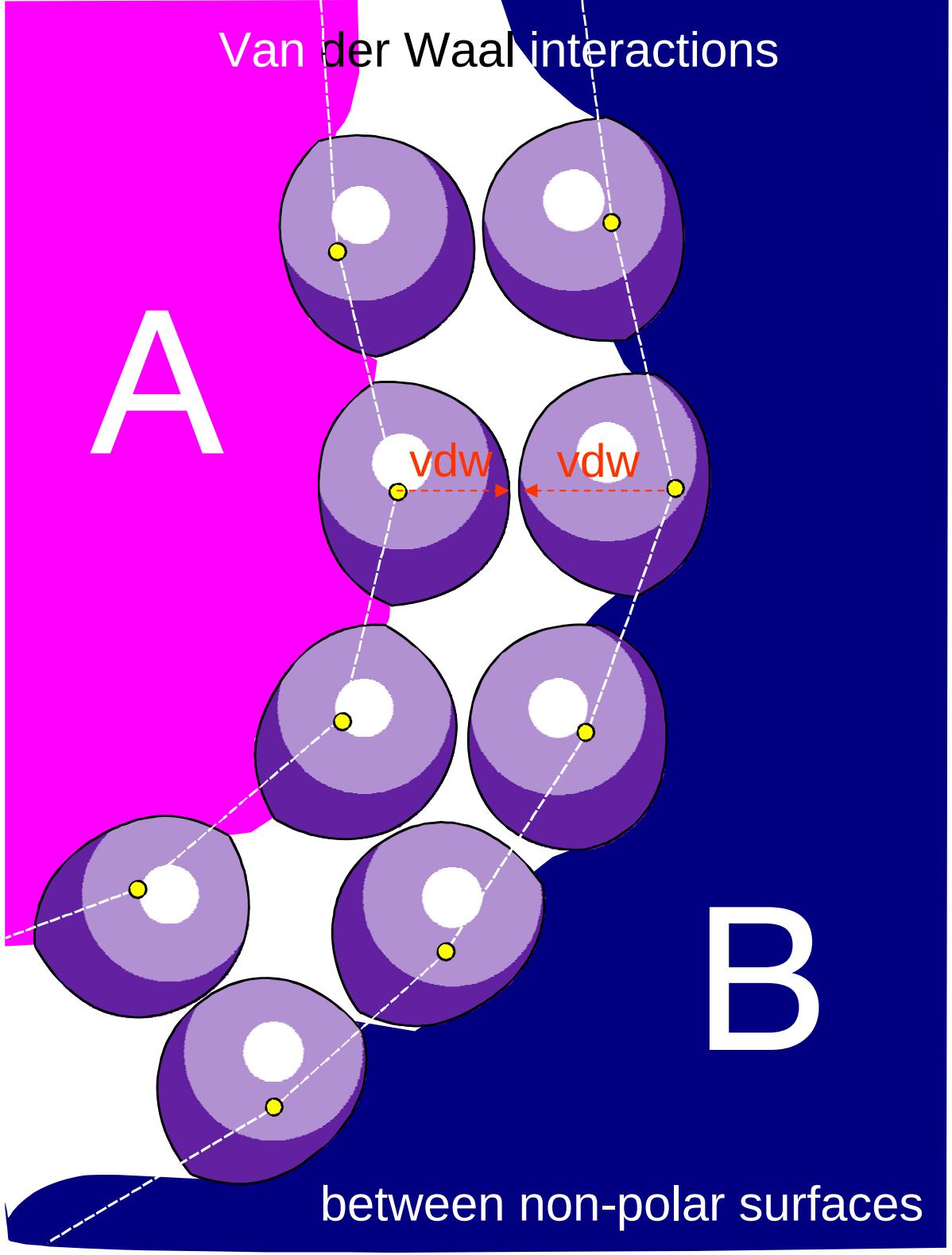


Two types of forces contribute to the specific affinity between proteins

Juang RH (2005) EPA

構形互補所造成的吸引力

凡得瓦爾鍵數目夠多即足以造就親和力



Juang RH (2005) EPA

生化分子的反應基團 Some functional groups

氰 基	-C≡N			●●●●●
醛 基	-HC=O			●●●
酸 基	-COOH	胺 基	$-NH_2$	●●
醇 基	-OH	硫醇基	-SH	●
醚	-O-			●
烷 基	$-CH_3$			X

The functional groups in a living cell are mild in reactivity

Juang RH (2005) EPA

可與各種配体基團反應的介質

Pharmacia

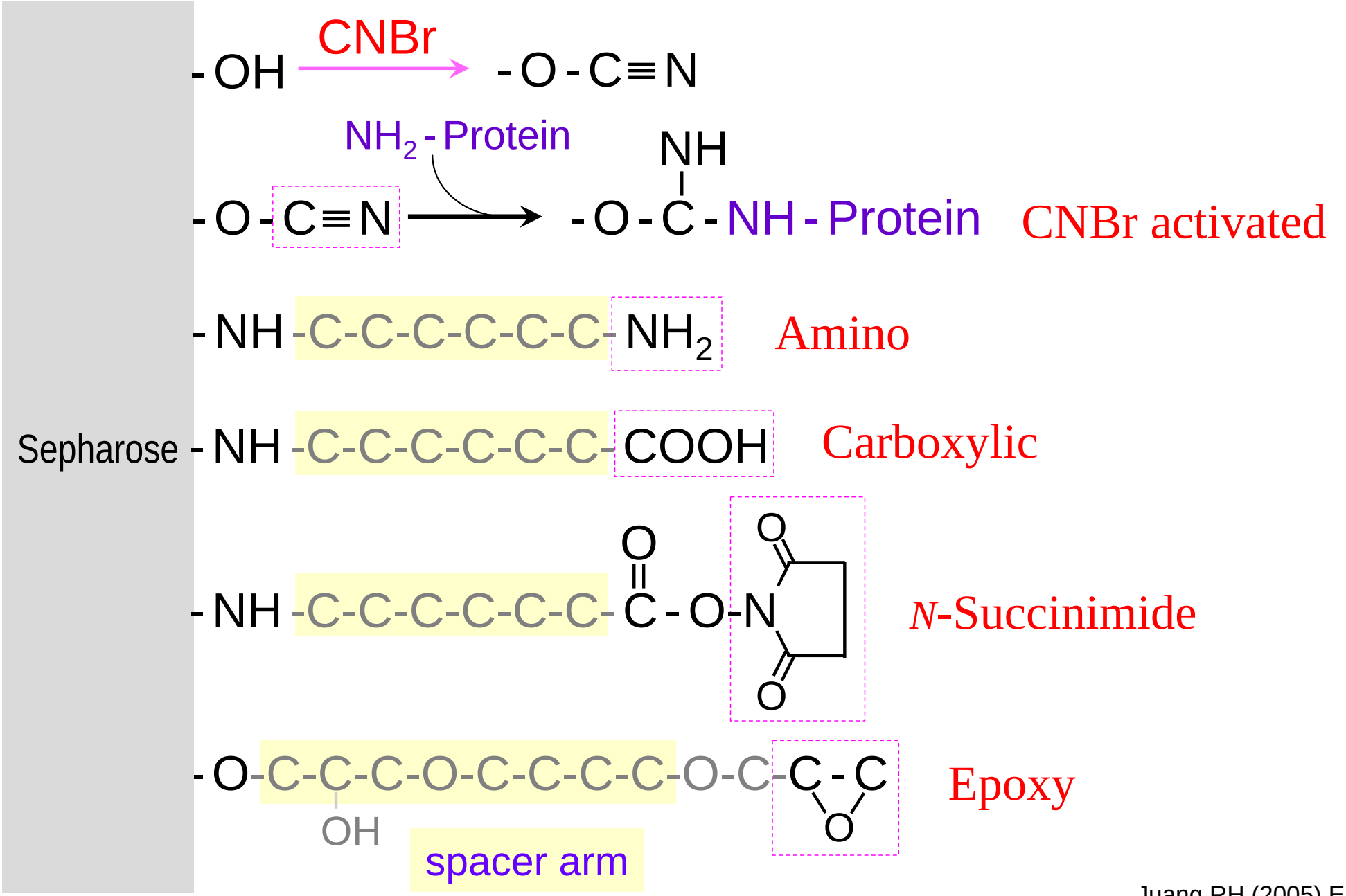
Ligand	Affinity materials	Reaction	How
$-NH_2$	CNBr-activated Sepharose 4B	-C≡N	Directly
	CH-Sepharose 4B 或活化型	-COOH	+ EDC*
		N-OH-succinimide	Directly
	Epoxy-activated Sepharose 4B	oxirane	Directly
-COOH	AH Sepharose 4B	$-NH_2$	+ EDC*
-OH	Epoxy-activated Sepharose 4B	oxirane	Directly
-SH	Epoxy-activated Sepharose 4B	oxirane	Directly
	Thiopropyl-Sepharose 4B	-S-S-R	+ DTT
	Activated thio-Sepharose 4B	-G-S-S-R	Directly

* EDC = *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide HCl

Affinity materials are designed to react with functional groups on ligands

Juang RH (2005) EPA

親和性介質的反應基團 Functional groups



Juang RH (2005) EPA

各種親和性介質及其專一性基團

Pharmacia

Ligand	Target	Remarks
Antibody	Specific antigen	Immunosorbent (custom made)
Substrate/inhibitor	Specific enzyme	Enzyme-substrate binding
Protein A	Part of IgG	mAb purification
Con A	Glycoprotein	Specific to α-D-glc and mannose
Heparin	Fibrin et al	Heparin Sepharose CL-6B
Oligo (dT)	mRNA	Oligo (dT)-cellulose
Cibacron-Blue	$NAD(P)^+$ binding	Blue Sepharose CL-6B
AMP, ADP	$NAD(P)^+$ binding	5'AMP-, 2', 5'ADP-Sepharose 4B
Oligosaccharides	Lectin	Purify lectins

(There are more other affinity adsorbents from commercial sources)

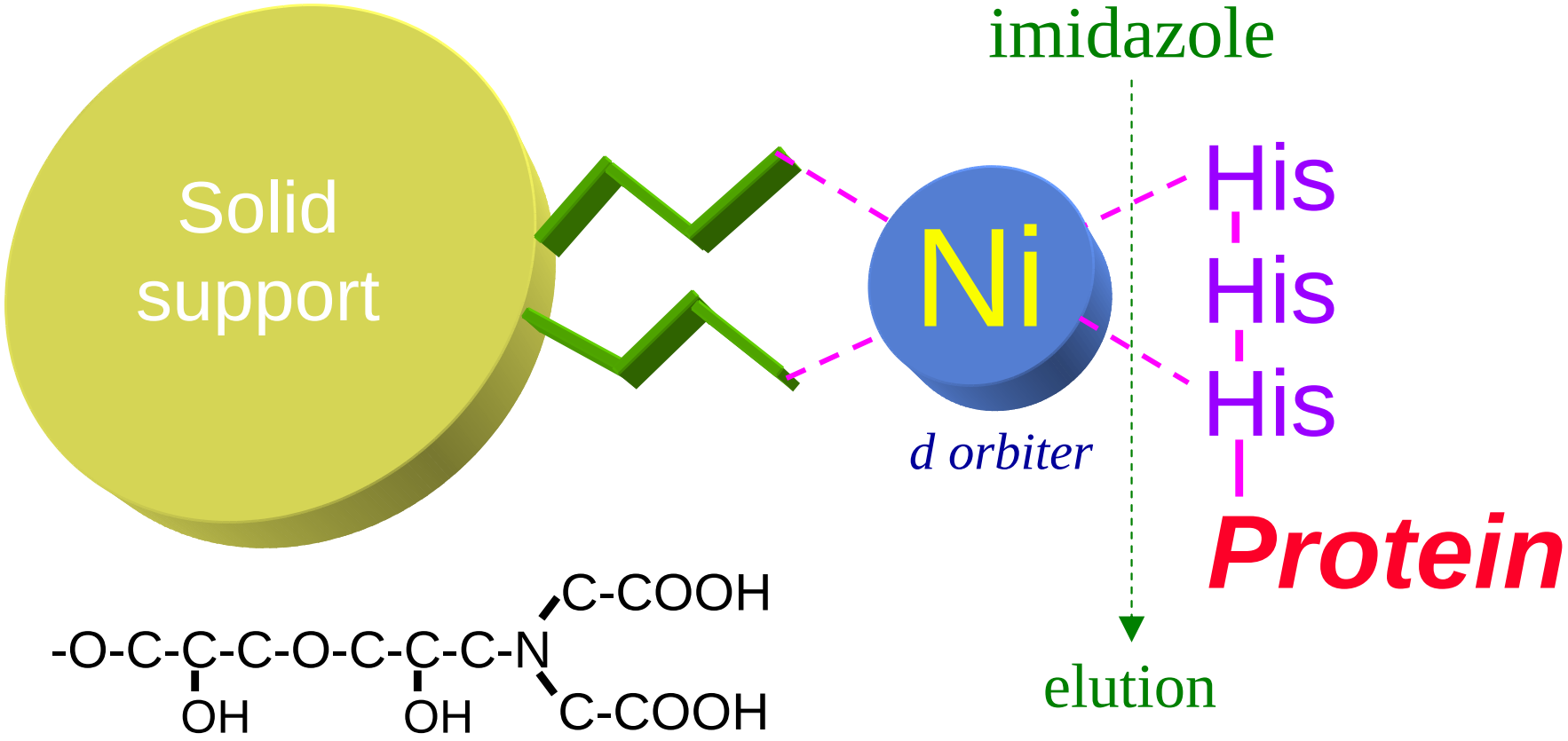
Affinity adsorbents with special affinity ligands

Juang RH (2005) EPA

金屬螯合層析法 Using metal-chelating affinity

Transition metals 過渡元素

Tag

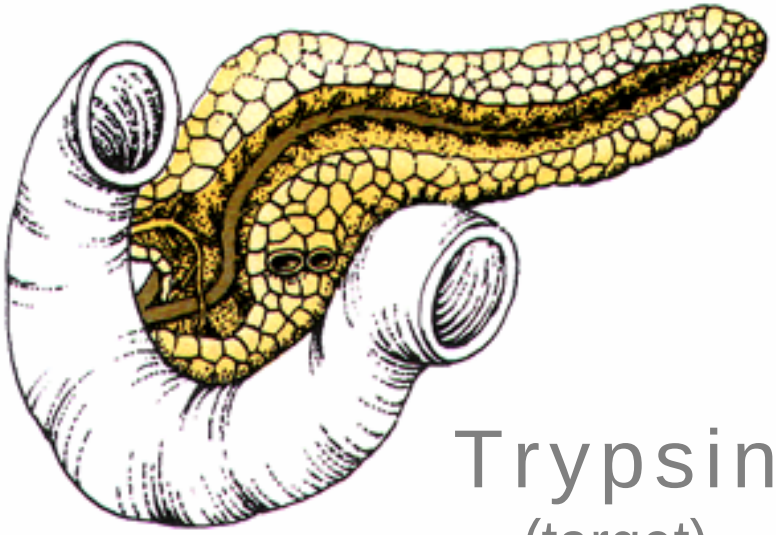


Metal **C**helate **A**ffinity **C**hromatography

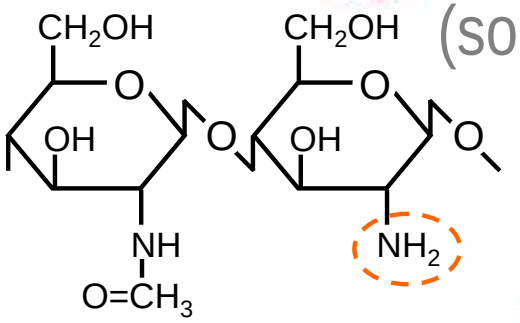
Juang RH (2005) EPA

親和層析法實例 Materials used in an example

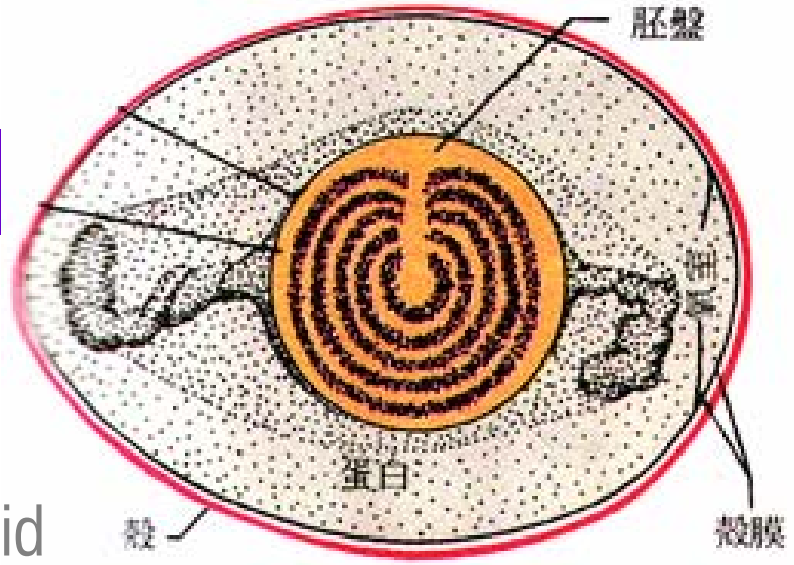
Chitin
(solid support)



Trypsin
(target)



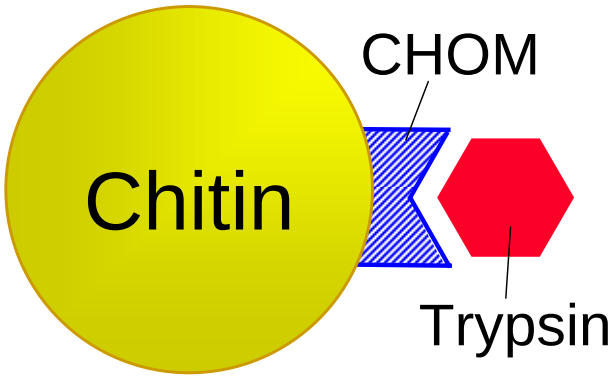
glutaraldehyde

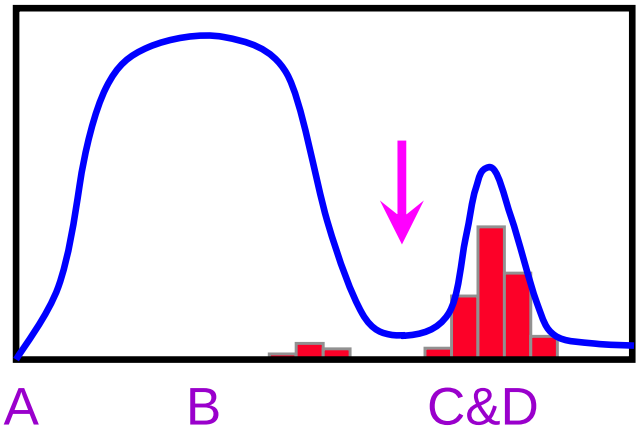


CHOM
(ligand, bait)
雞蛋粘多糖蛋白
Chicken ovamucoid
Trypsin inhibitor

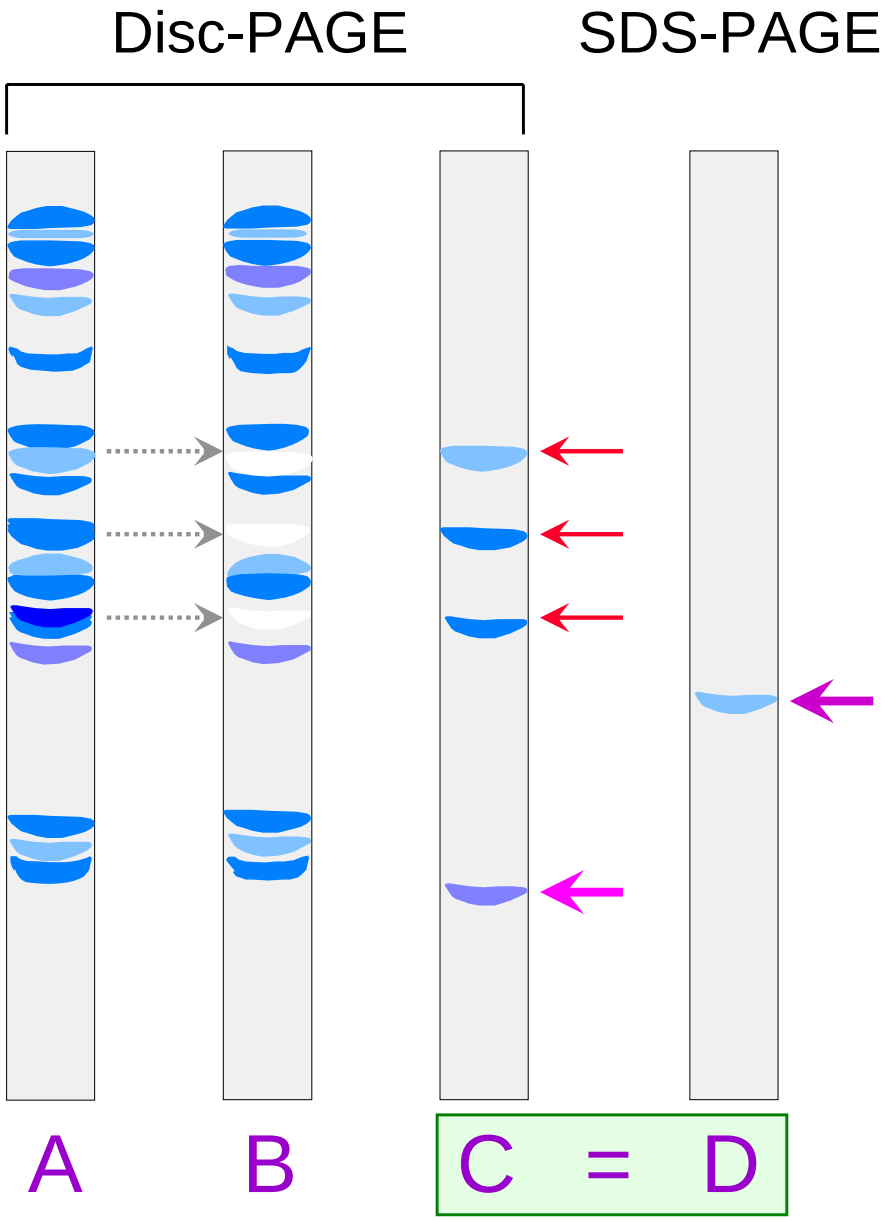
Juang RH (2005) ECX

以親和層析法純化 Trypsin purification





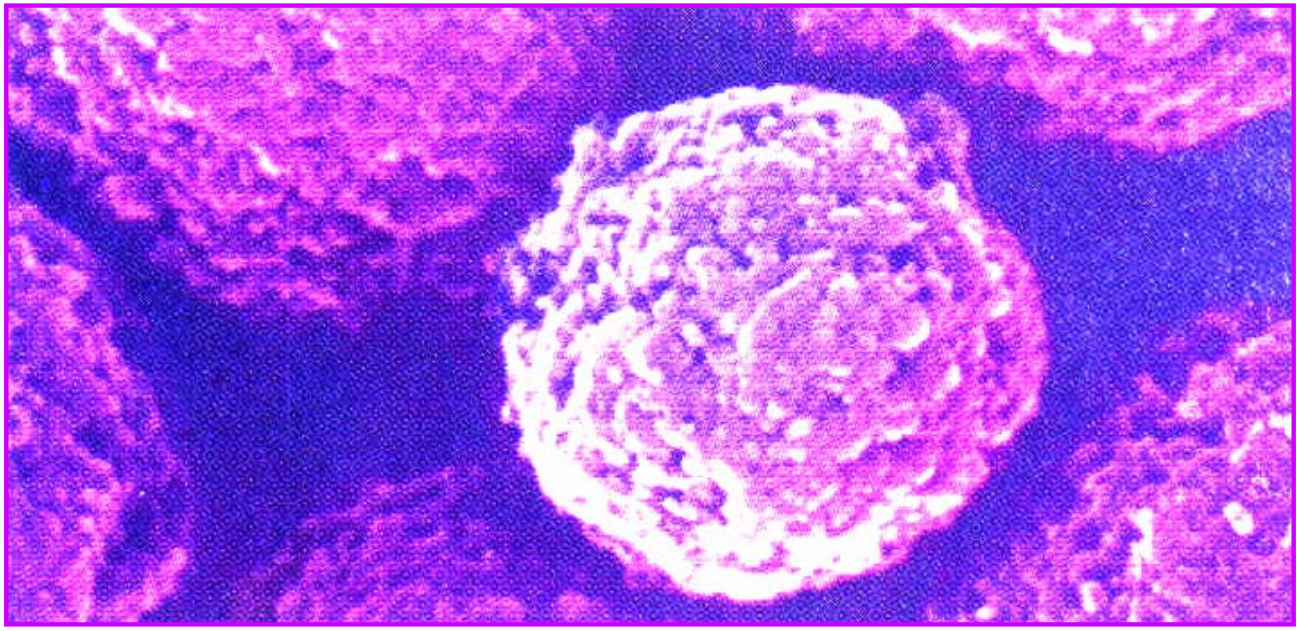
以膠體電泳檢定親和層析
操作過程各步驟的樣本
Check by PAGE →



Juang RH (2005) EPA

Hydroxyapatite 吸著劑

磷酸鈣陶土 $(Ca_5(PO_4)_3OH)_2$

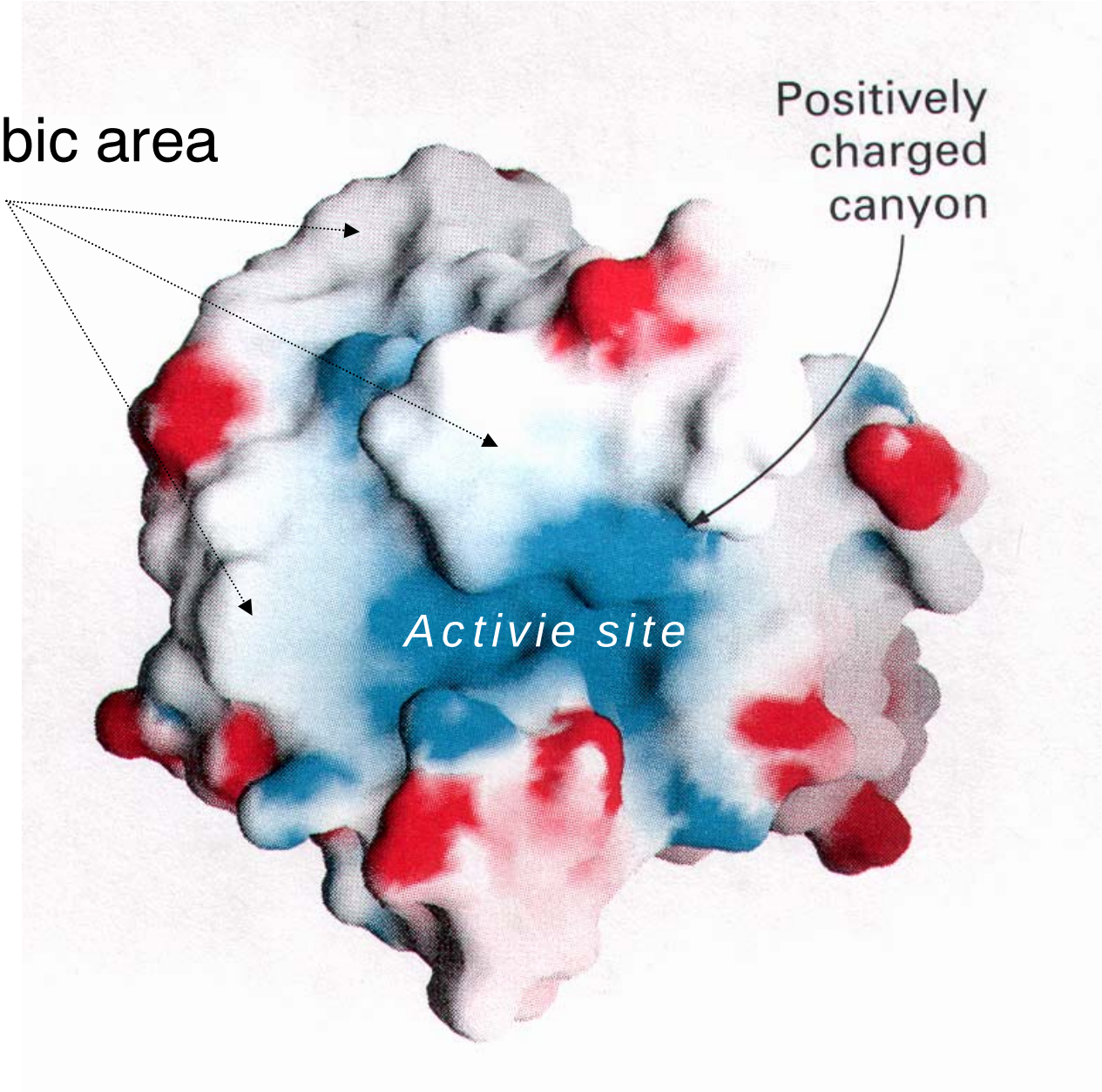


可選擇 NaCl 或 磷酸 等不同溶離條件

Elution by NaCl or phosphate gradient leads to different results

Bio-Rad CHT

蛋白質表面的極性或非極性分布



Superxoide dismutase (SOD)

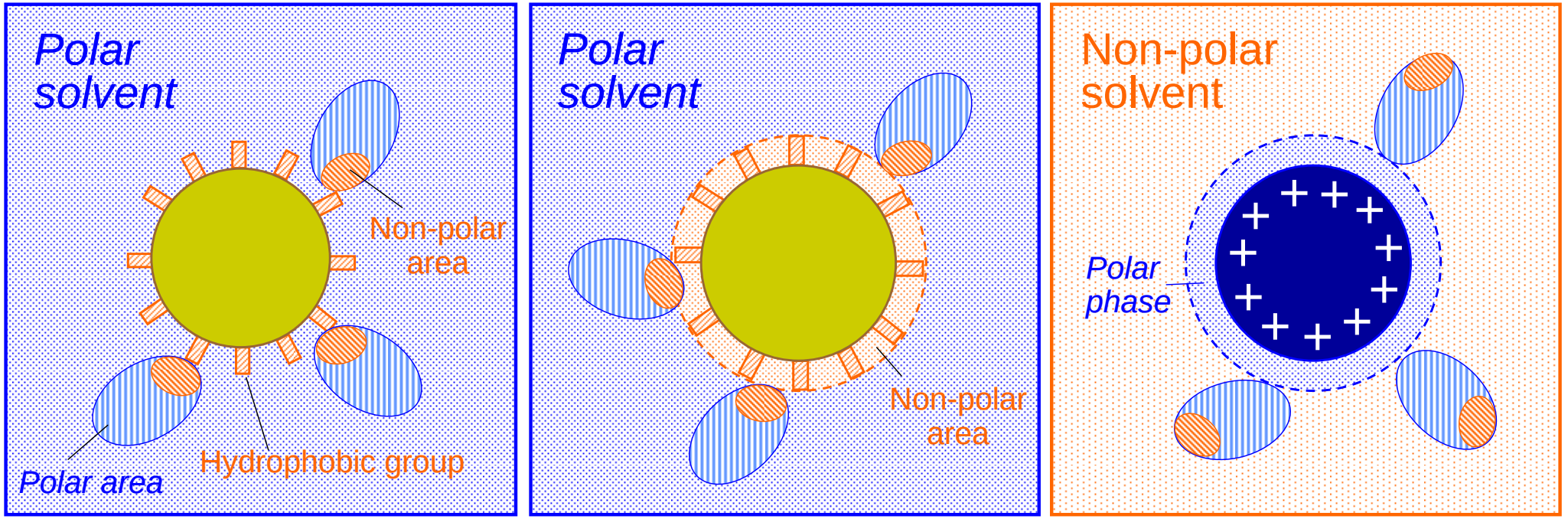
Stryer L (1995) Biochemistry 4/e Fig. 21-36

疏水性及反相層析法 Hydrophobic interaction

C_{18} = $-C_{18}H_{37}$

HIC (liquid-solid)

Reverse Phase Chromatography (liquid-liquid)



Using non-polar groups as a stationary phase

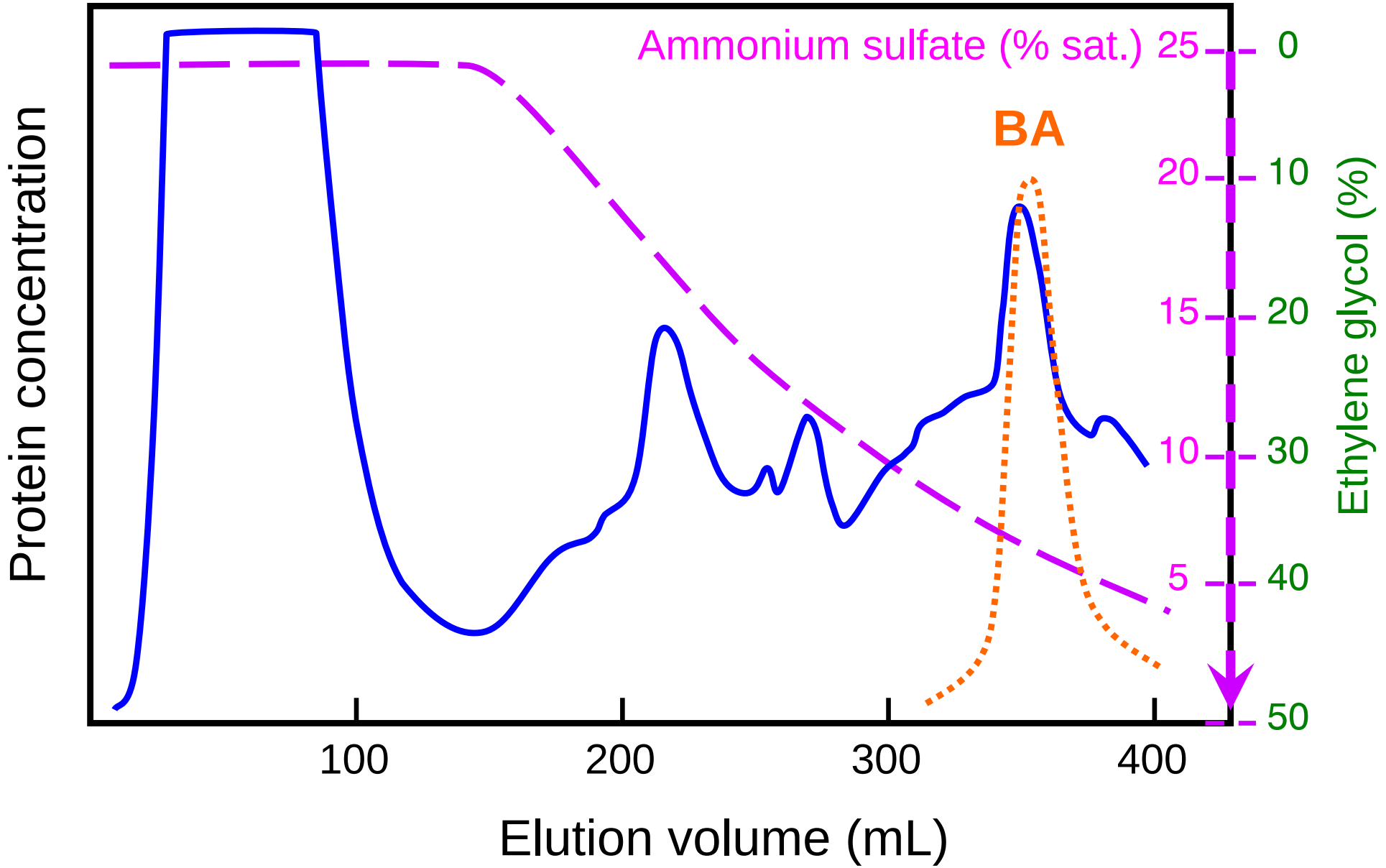
Using ion-exchanger

C6 = $-C_6H_{13}$

Phenyl = $-C_6H_6$

Juang RH (2005) EPA

Hydrophobic interaction chromatography (HIC)



Pharmacia HIC

4 其它純化或分離方法 Other purification methods

- 4.1 製備式電泳 Preparative electrophoresis

  蛋白質色帶由原態電泳中直接切除出來

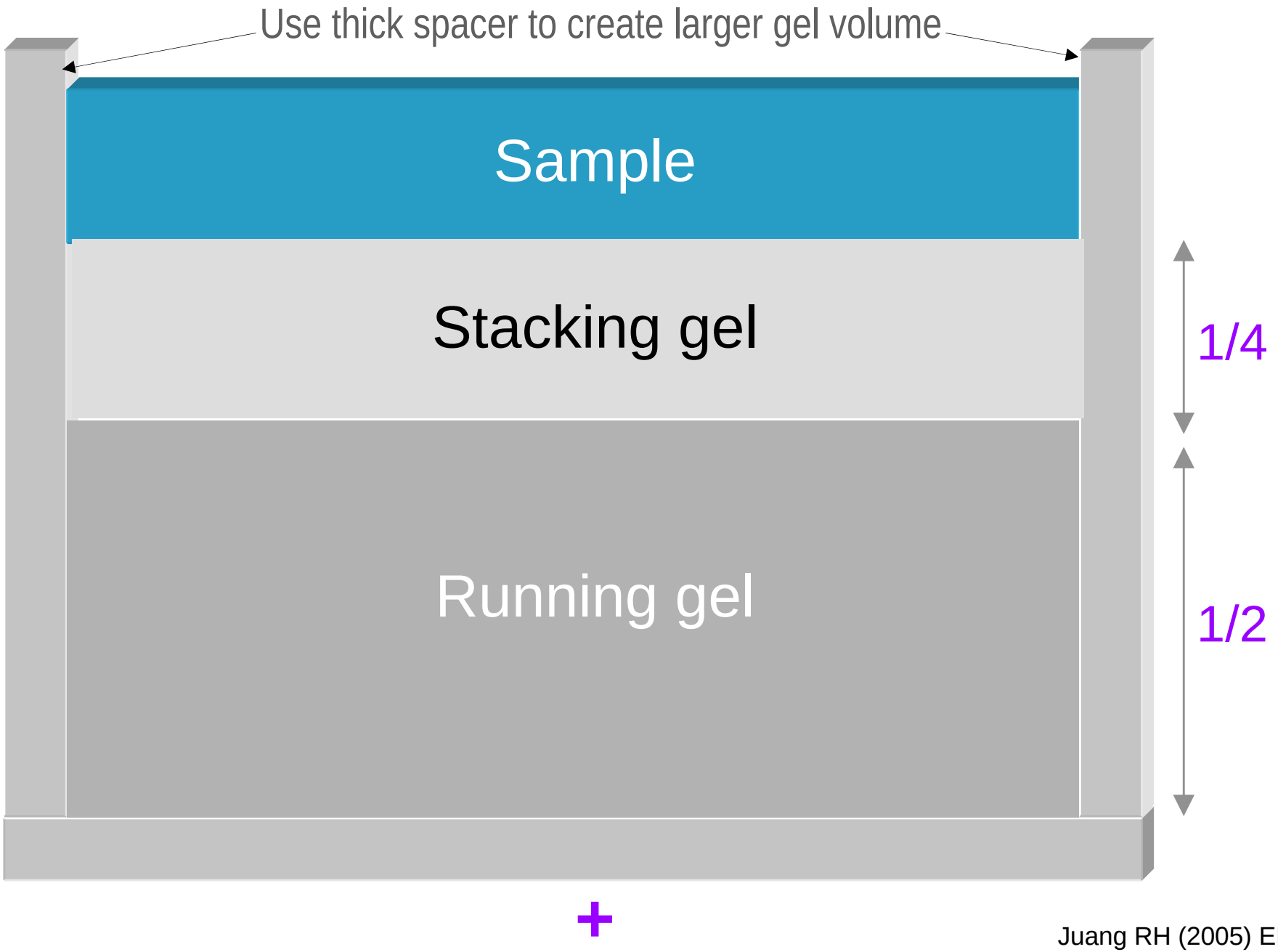
- 4.2 超高速離心法 Ultracentrifugation

  各種分子的沉降係數不同來進行分離

- 4.3 超微薄膜過濾法 Ultrafiltration

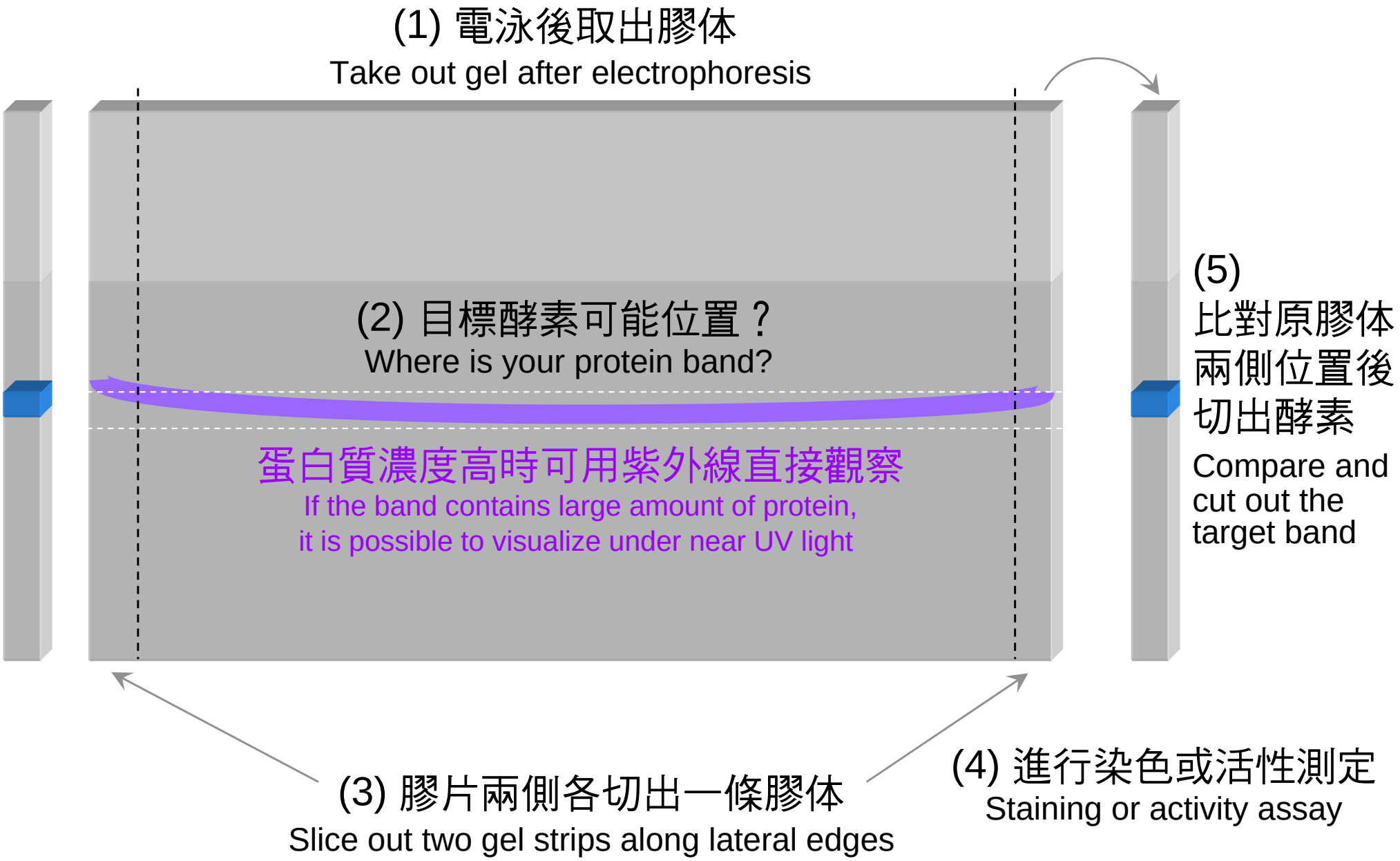
  超微薄膜可以用來脫鹽及濃縮蛋白質

製備式電泳膠片 Preparative gel format



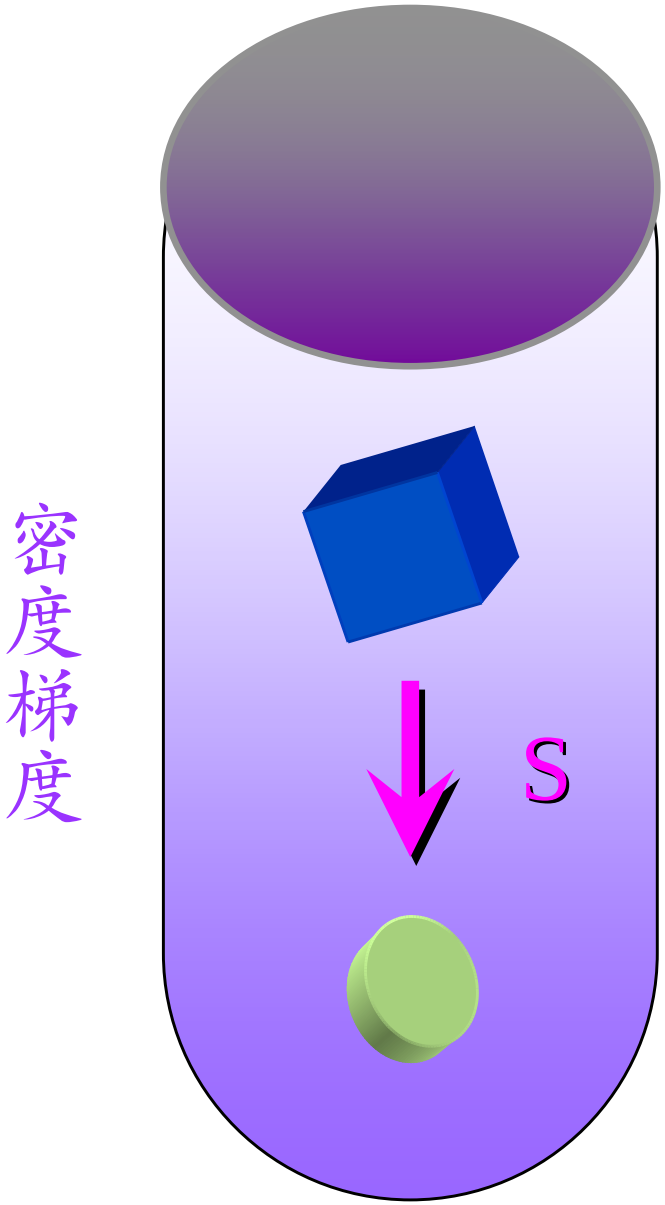
Juang RH (2005) EPA

製備式電泳操作 Detect protein band on the gel



Juang RH (2005) EPA

超高速離心 Sedimentation coefficient



Svedberg unit

粒子在密度梯度離心時的沈降速率

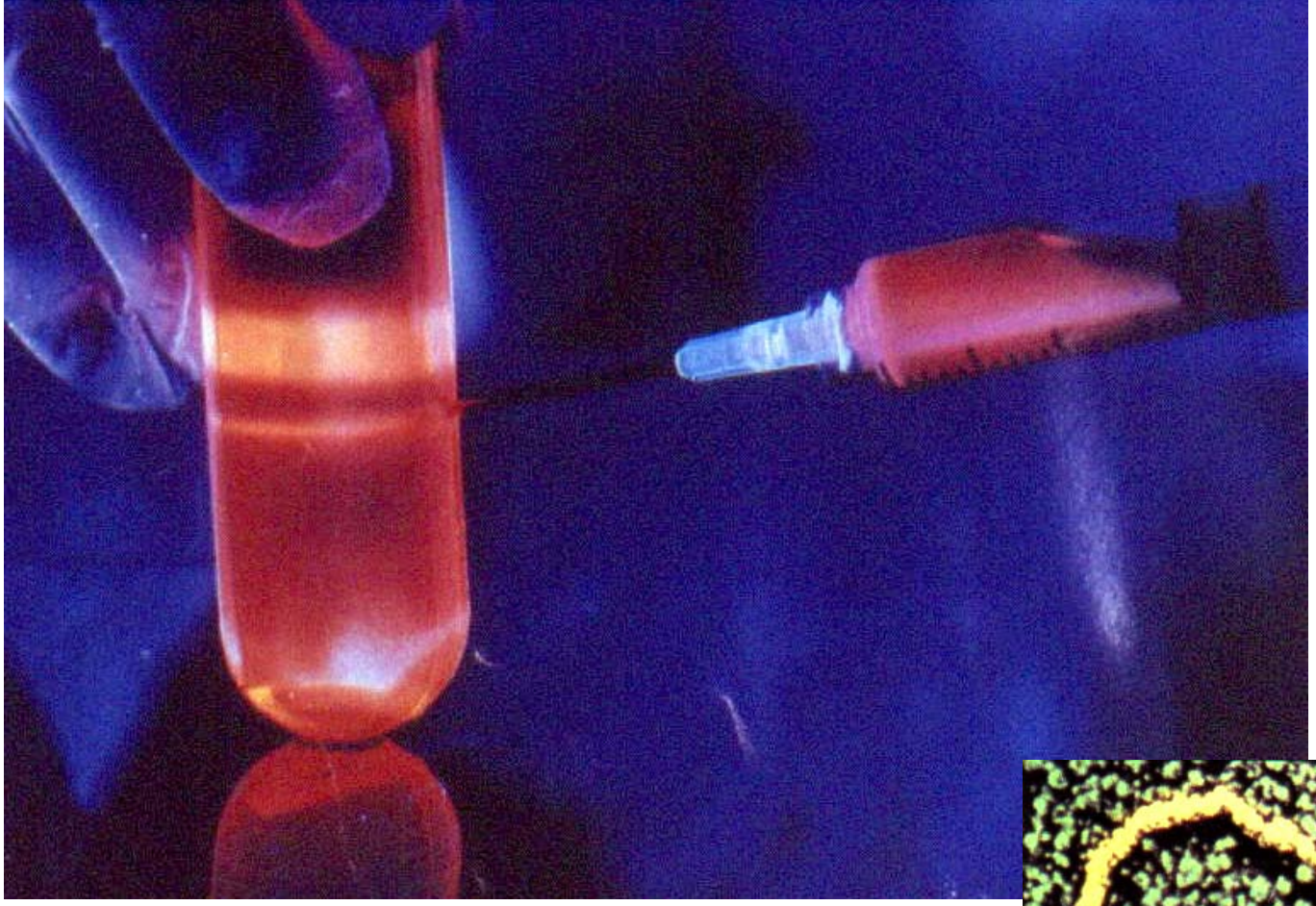
The sedimentation velocity of a particle when it is centrifuged in a density gradient

分 子 量 molecular weight

分子密度 molecular density

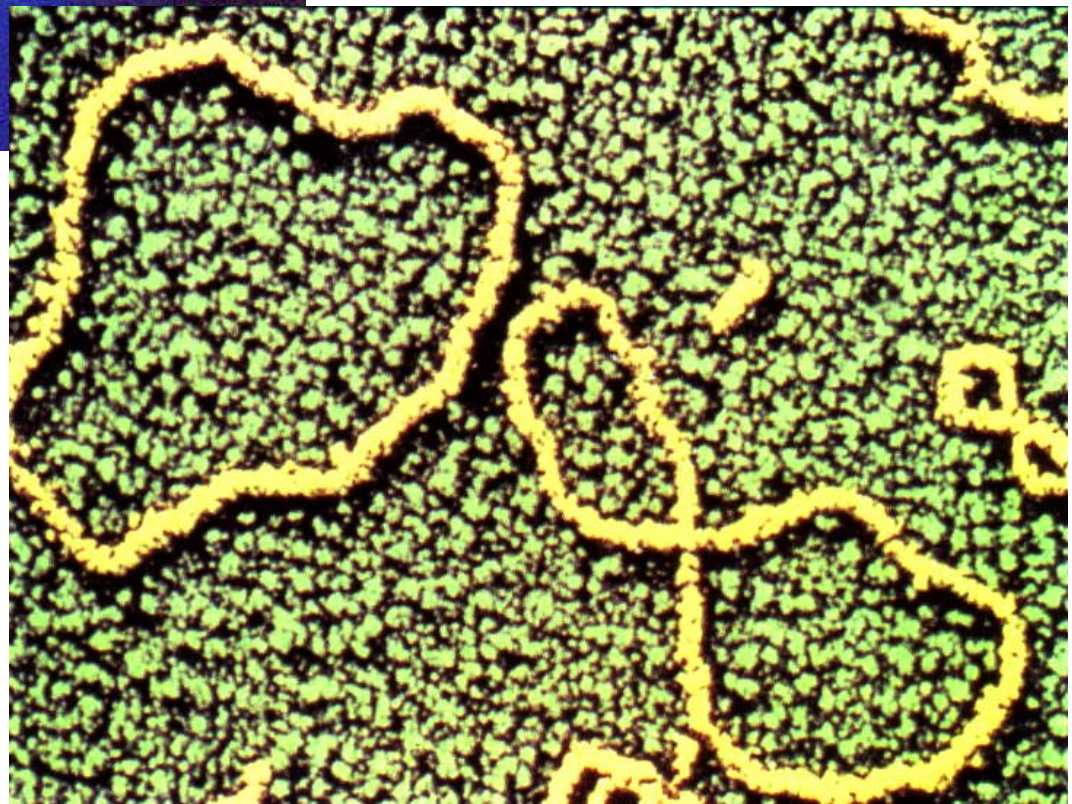
分子組成 molecular composition

分子形狀 molecular shape



以超高速離心大量製備質體

Ultracentrifugation is used to prepare plasmid in large scale



兩種超高速離心比較 Two ultracentrifuge types

Centrifuge	Sedimentation Velocity	Sedimentation Equilibrium
also called →	Zone Centrifugation	Isopycnic Equilibration
Gradient formation	Precast (sucrose, glycerol)	During centrifugation (CsCl)
	Shallow gradient, lower density	Steep gradient, higher density
Suitable samples	Similar density, different MW	Similar MW, different density
	Protein	Nucleic acid / cell organelle
Centrifugation conditions	Lower speed, not complete sedimented, stop at proper time	Completely sediment to where the density is equilibrated, high speed, long running time
	區帶離心法	等密度平衡離心法

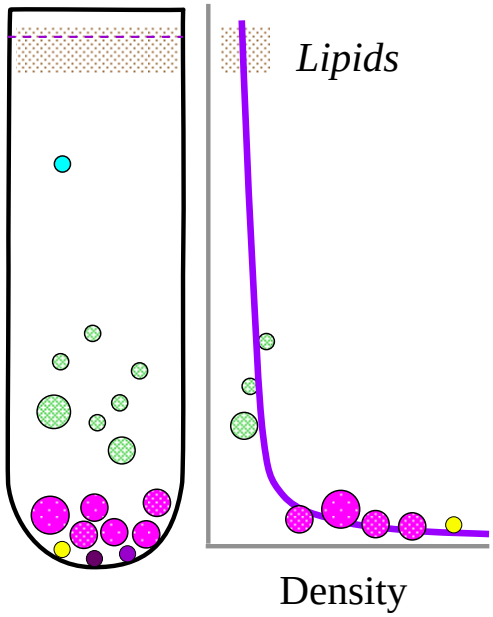
Juang RH (2005) EPA

各種高速離心法比較 Comparison of centrifuges

High speed

Ultracentrifugation

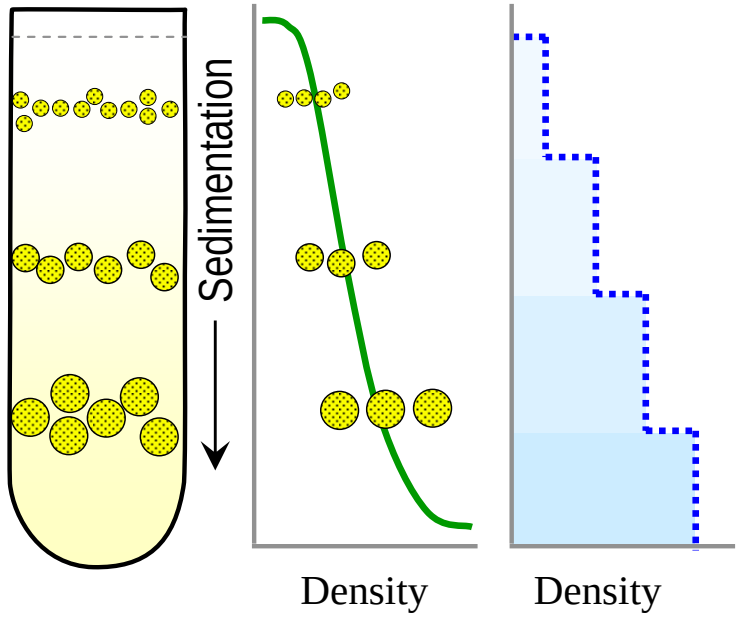
Gravity Centrifugation
(No density)



一般的重力離心僅把顆粒與溶液分離開來

Utilize gravity force to separate particles from the solution

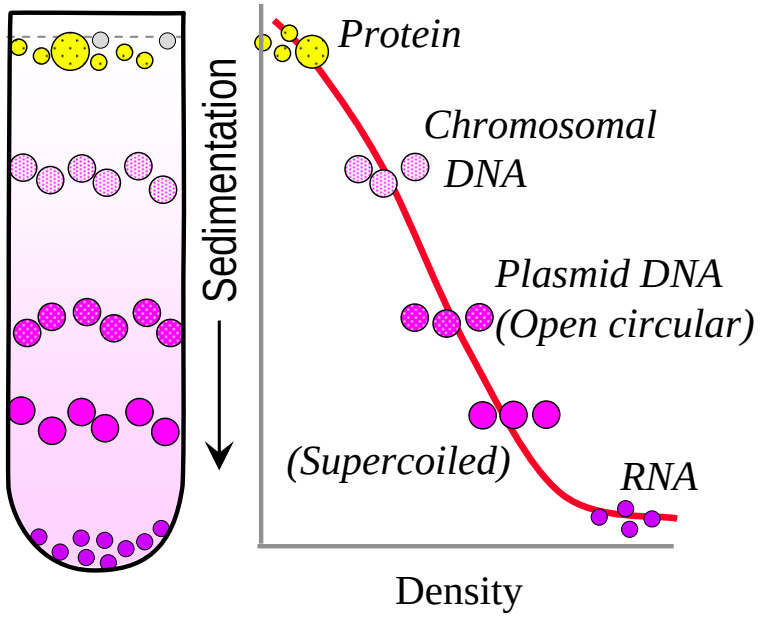
Zone Centrifugation
(Precast) → (step-wise)



樣本：多為蛋白質
密度相似、分子量不同者

Sample: protein (similar density, but different in MW)

Isopycnic Equilibration
(CsCl gradient forming)



樣本：多為核酸
密度不同、分子量相似者

Sample: nucleic acid (similar MW, but different in density)

Juang RH (2005) EPA

4 其它純化或分離方法 Other purification methods

- 4.1 製備式電泳 Preparative electrophoresis

  蛋白質色帶由原態電泳中直接切除出來

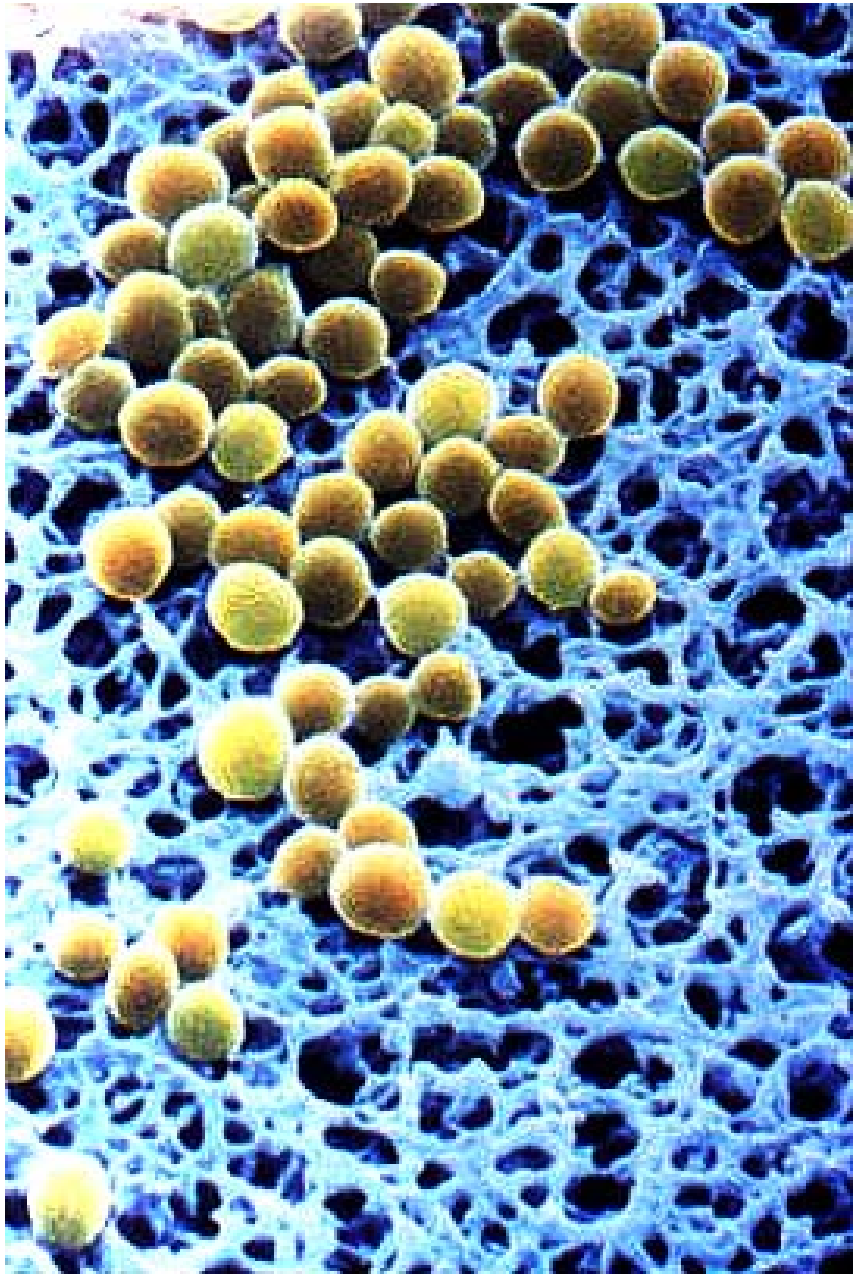
- 4.2 超高速離心法 Ultracentrifugation

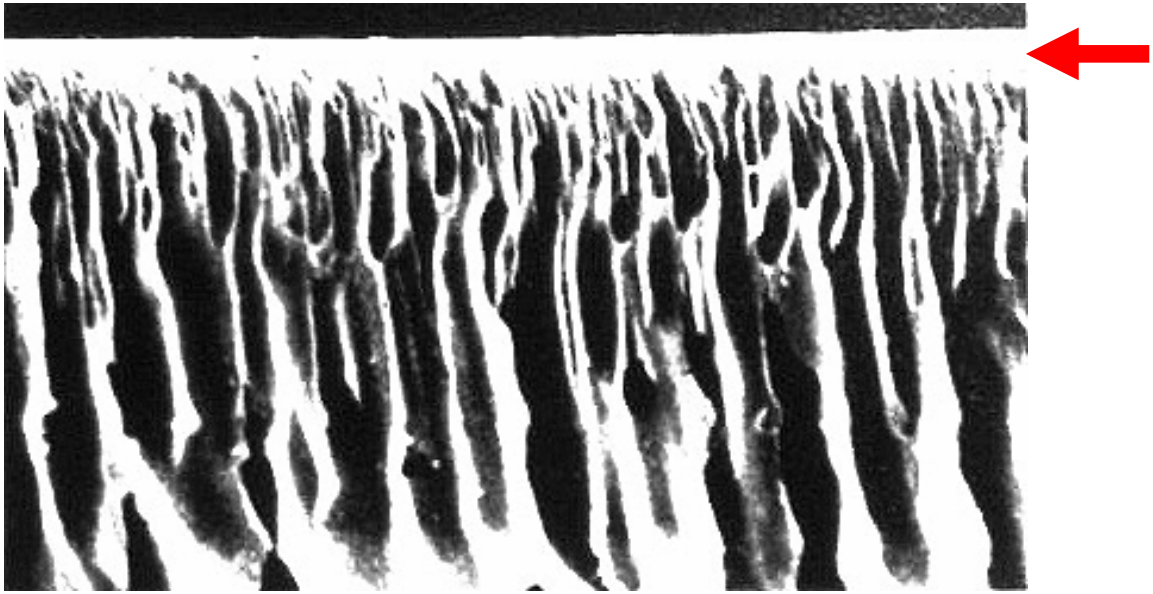
  各種分子的沉降係數不同來進行分離

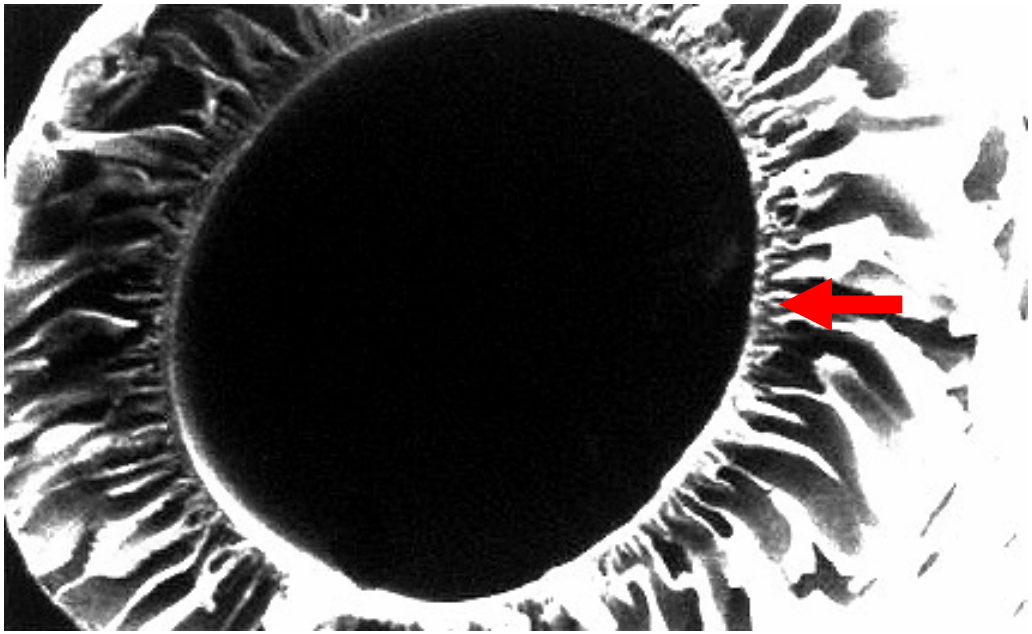
- 4.3 超微薄膜過濾法 Ultrafiltration

  超微薄膜可以用來脫鹽及濃縮蛋白質

超微薄膜技術 Ultrafiltration technology

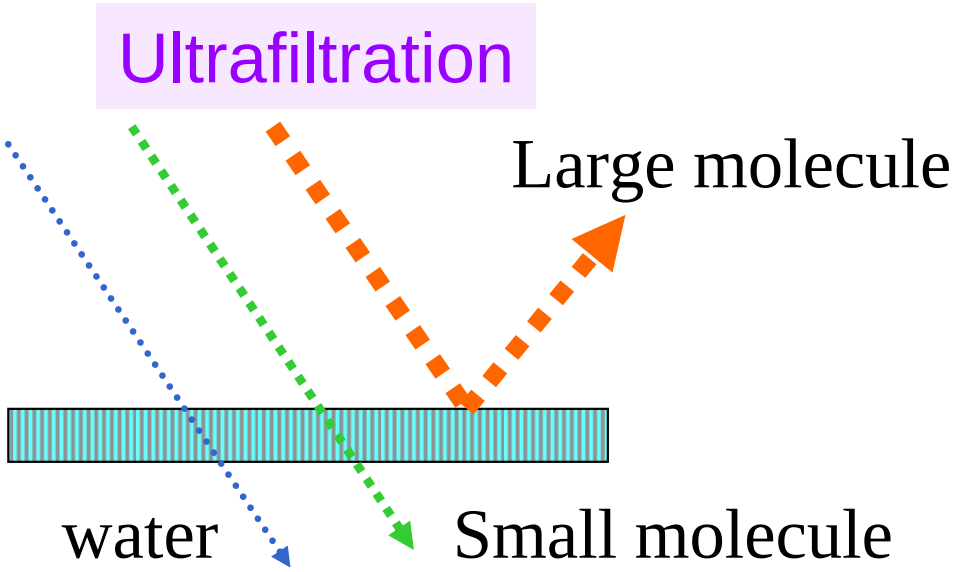


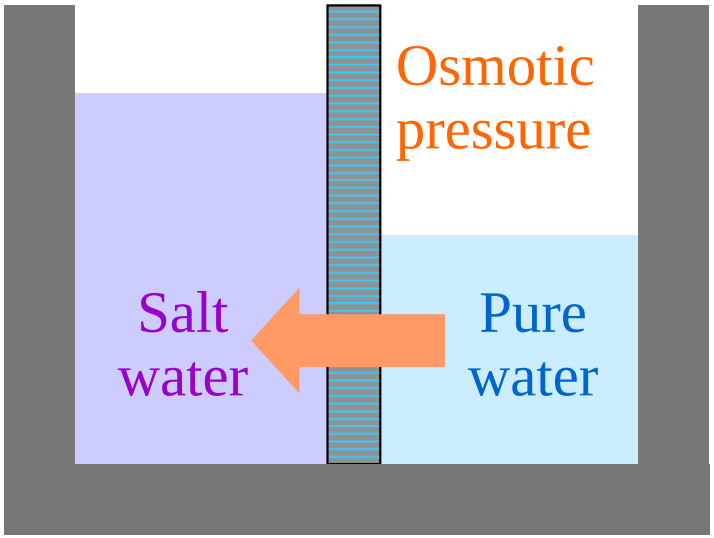


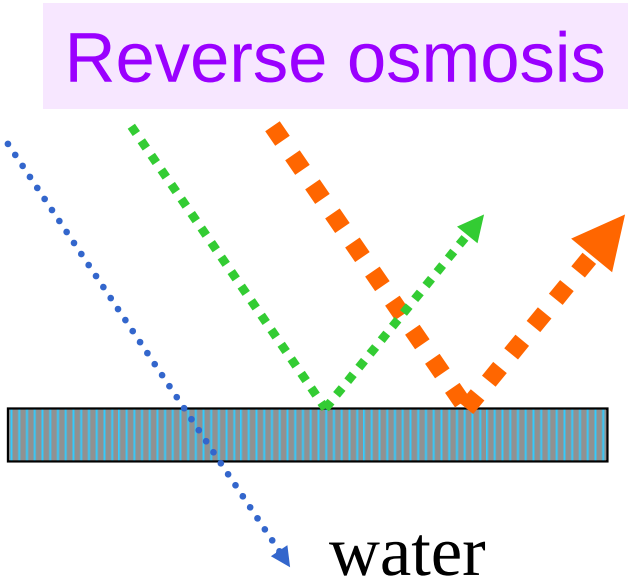


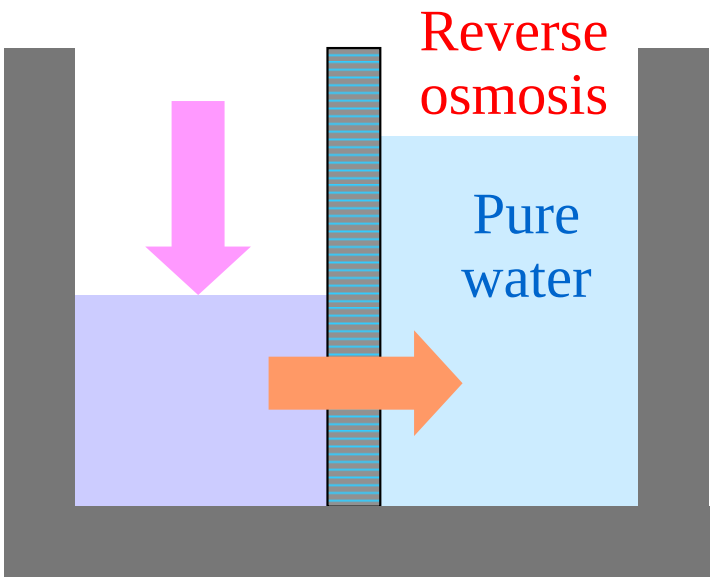
Amicon catalogue

超微薄膜及逆滲透 Ultrafiltration and RO



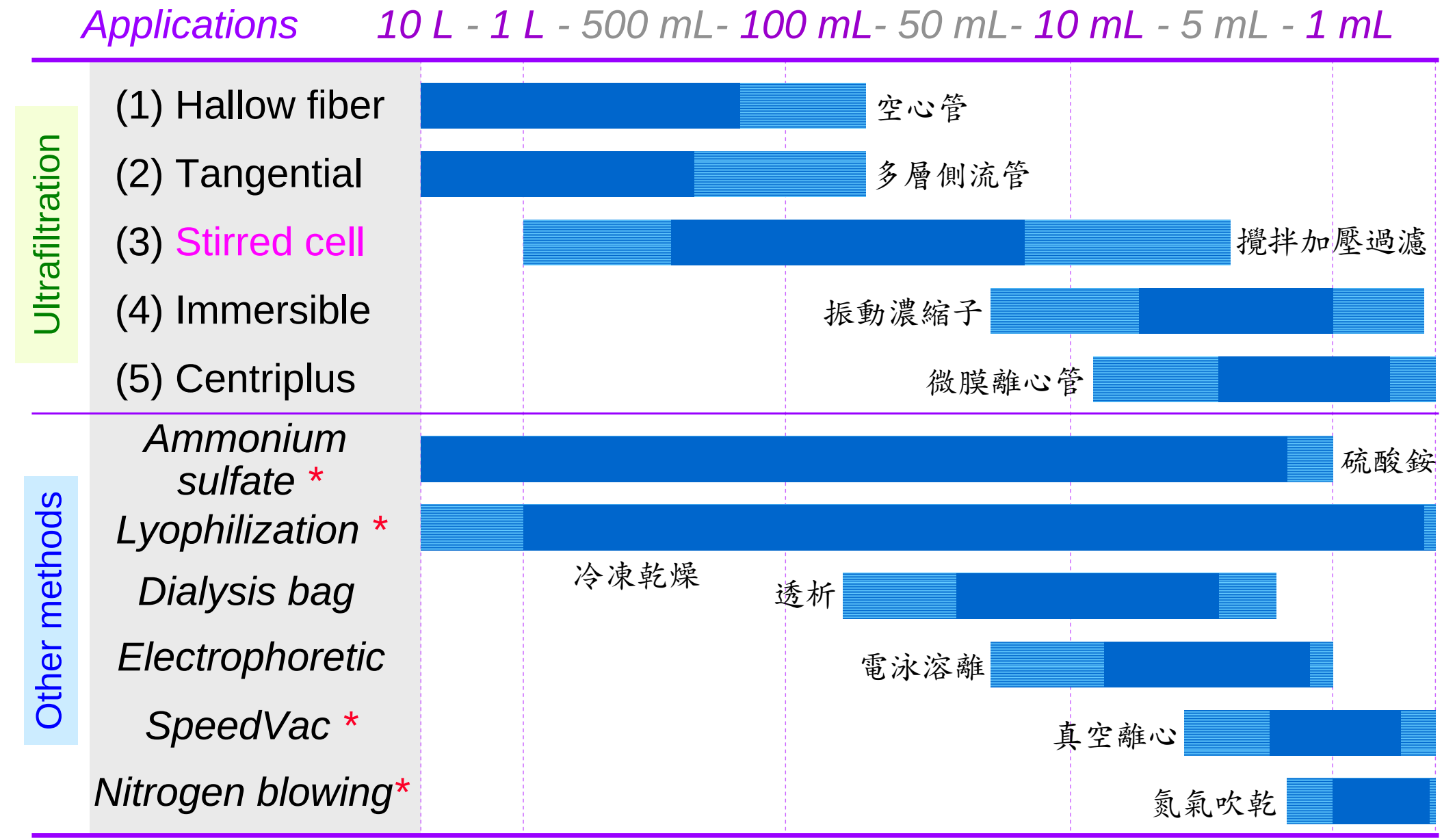






Juang RH (2005) EPA

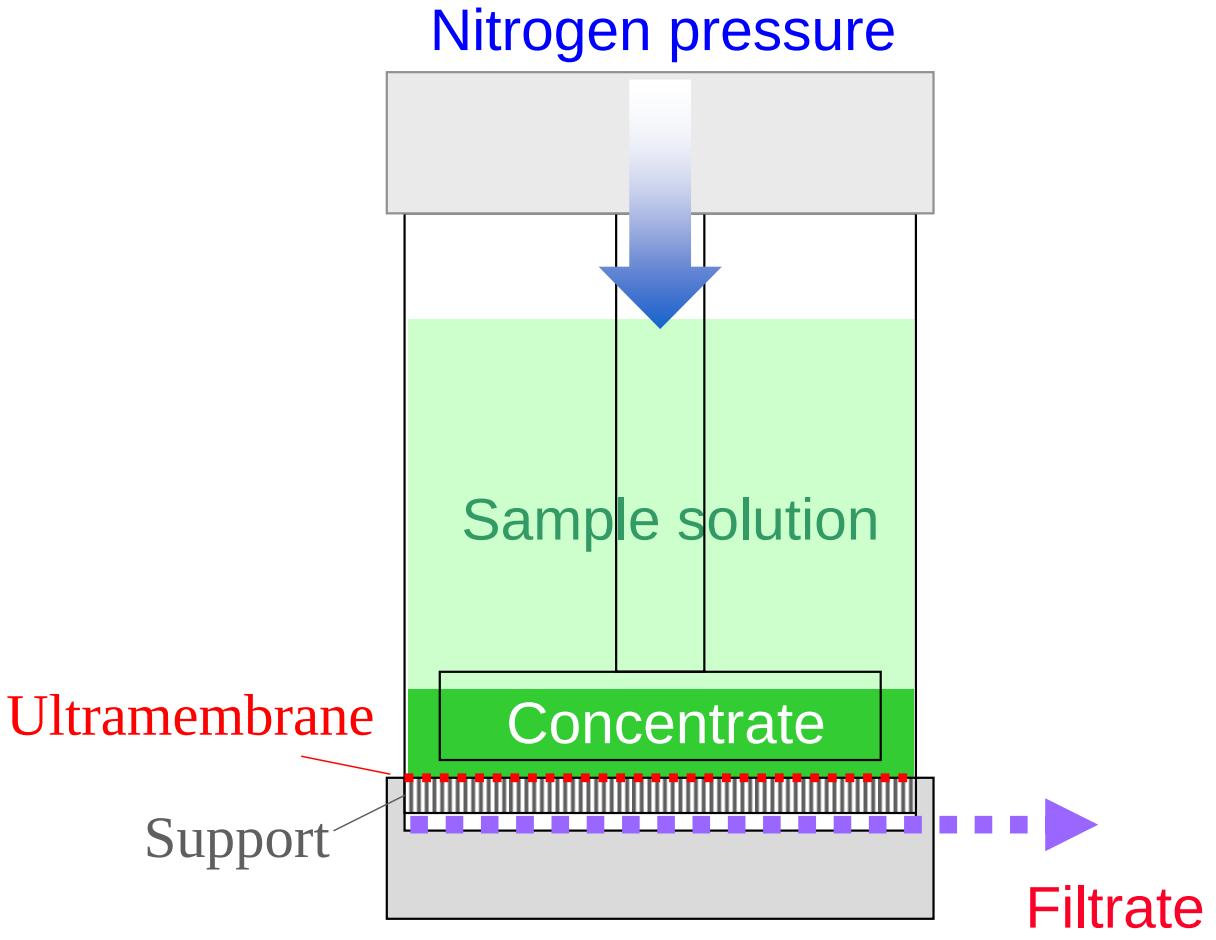
各種濃縮方法的使用範圍 Useful ranges



* The salt concentration increases in the sample

Juang RH (2005) EPA

超微薄膜濃縮裝置 - Stirred cell



Amicon Stirred Cells

Juang RH (2005) EPA

5 純化策略 Purification strategy

5.1 純化步驟設計 Design a purification protocol

一邊進行純化工作，一邊改進純化方法或步驟

摸著石頭過河 (Trial and error)

5.1.1 影響純化的因素 Critical factors in purification

要求高活性、高回收率、高純度、方便快速、經濟

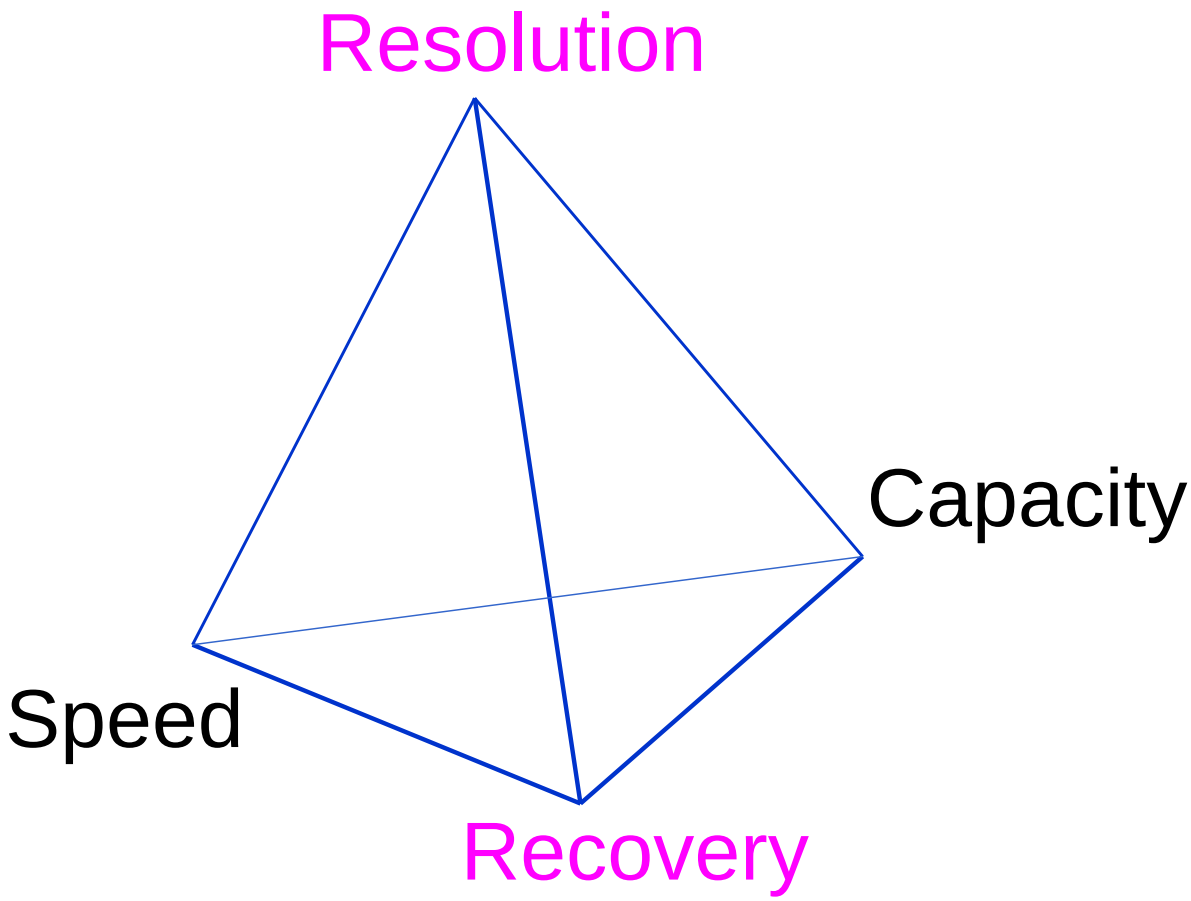
5.1.2 組合純化步驟 Set up purification steps

組合各種方法以達純化效率

5.2 純化結果 Purification table

以純化表來檢討純化效果

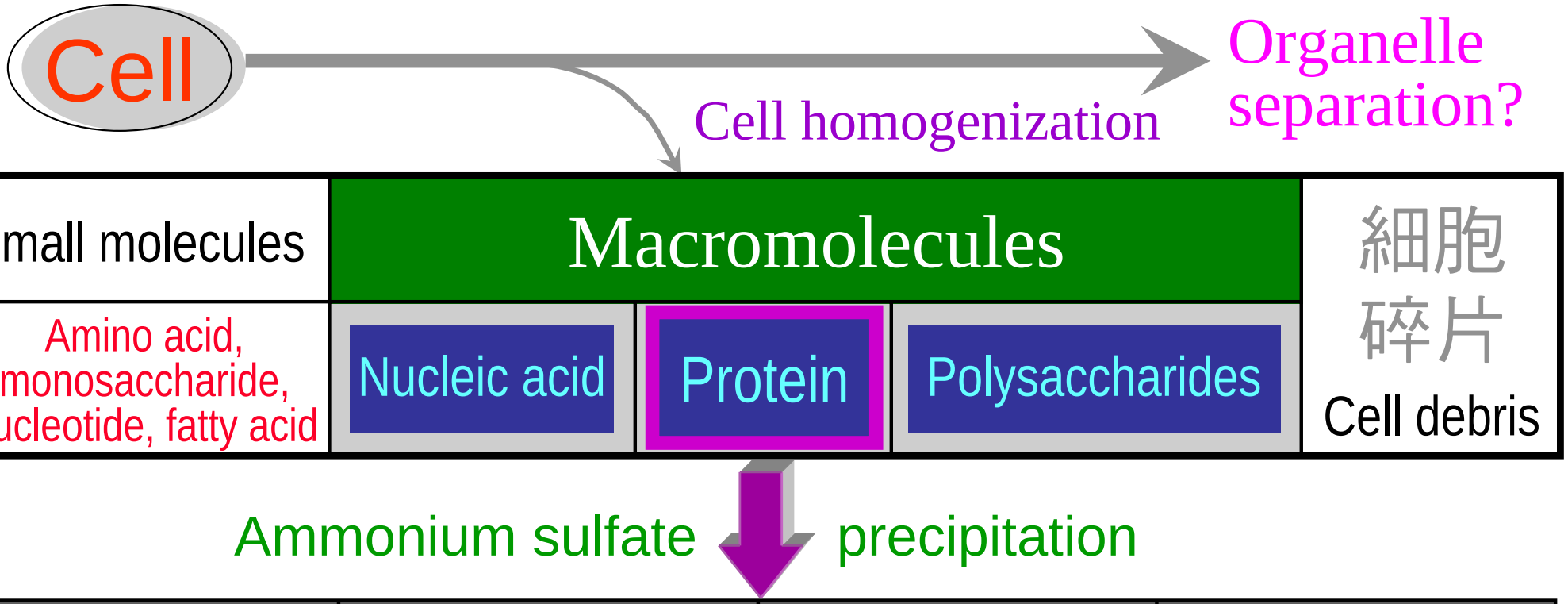
檢討純化效果 Critical factors



- 高活性
  High activity
- 高回收率
  High recovery
- 高純度
  High purity
- 方便與快速
  Rapid
- 經濟
  Economy

Juang RH (2005) EPA

各種純化或分析方法的原理



Molecular size	Molecular charge	Molecular polarity	Affinity
Gel filtration, SDS-PAGE, Ultrafiltration	Ion exchange, Chromatofocusing, Disc-PAGE, Isoelectric focusing	Reverse phase chromatography, HIC, Salting-out	Affinity chromatography, Hydroxyapatite

Basic protein properties useful for planning separation steps

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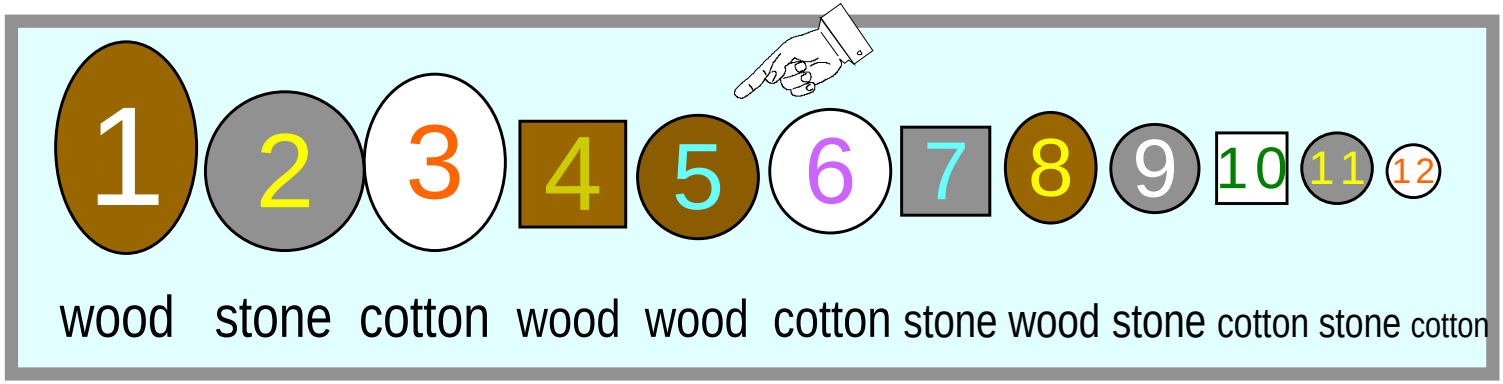
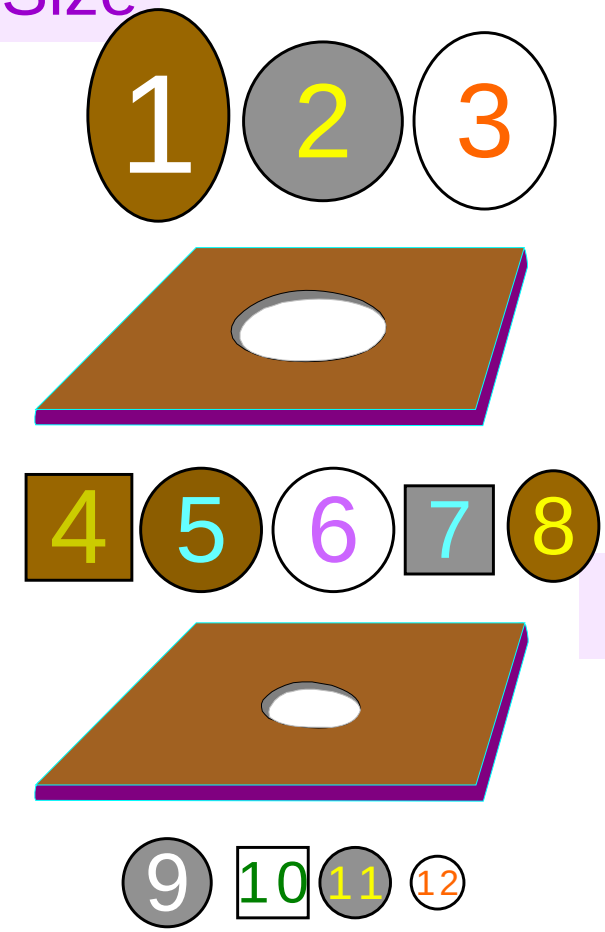
如何分離這些大小物件

shape
size
density

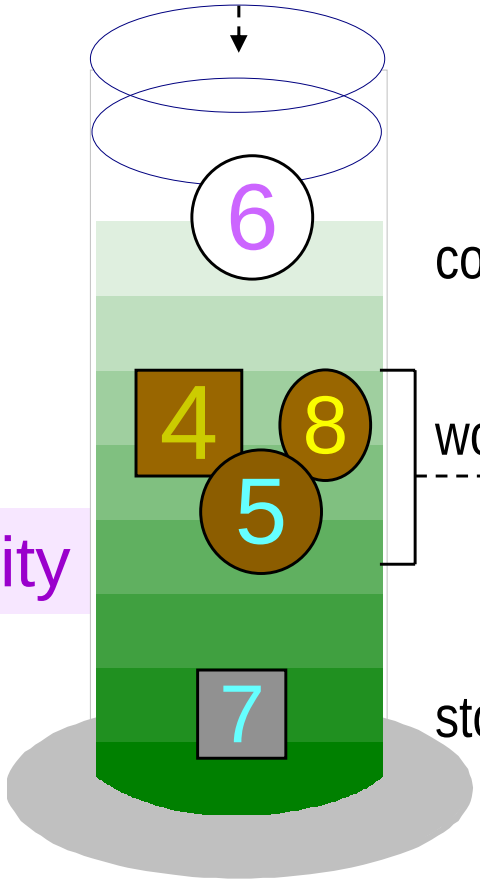


Size



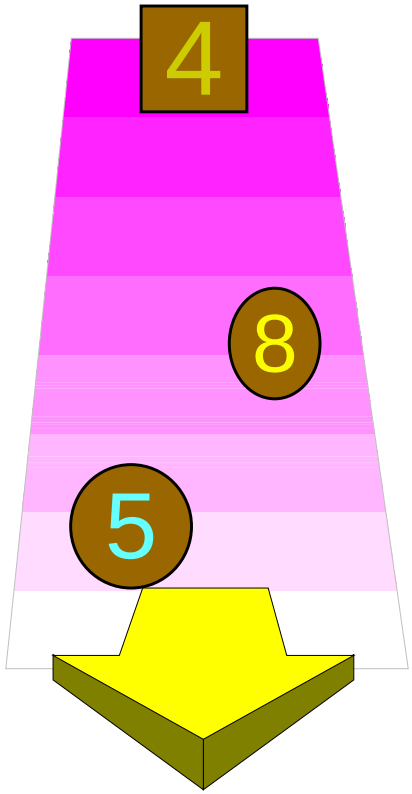
Sieving different sizes

Density



Sedimentation in different speed

Shape



Rolling down in Different speed

How to separate these 12 objects?

Juang RH (2005) EPA

組合純化步驟 Set up your purification protocol

純化流程基本骨幹

Basic backbone for purification

硫酸銨分劃
Ammonium sulfate fractionation

分子表面極性不同
The polarity of the protein

離子交換法
Ion exchange

分子淨電荷不同
The charge of the protein

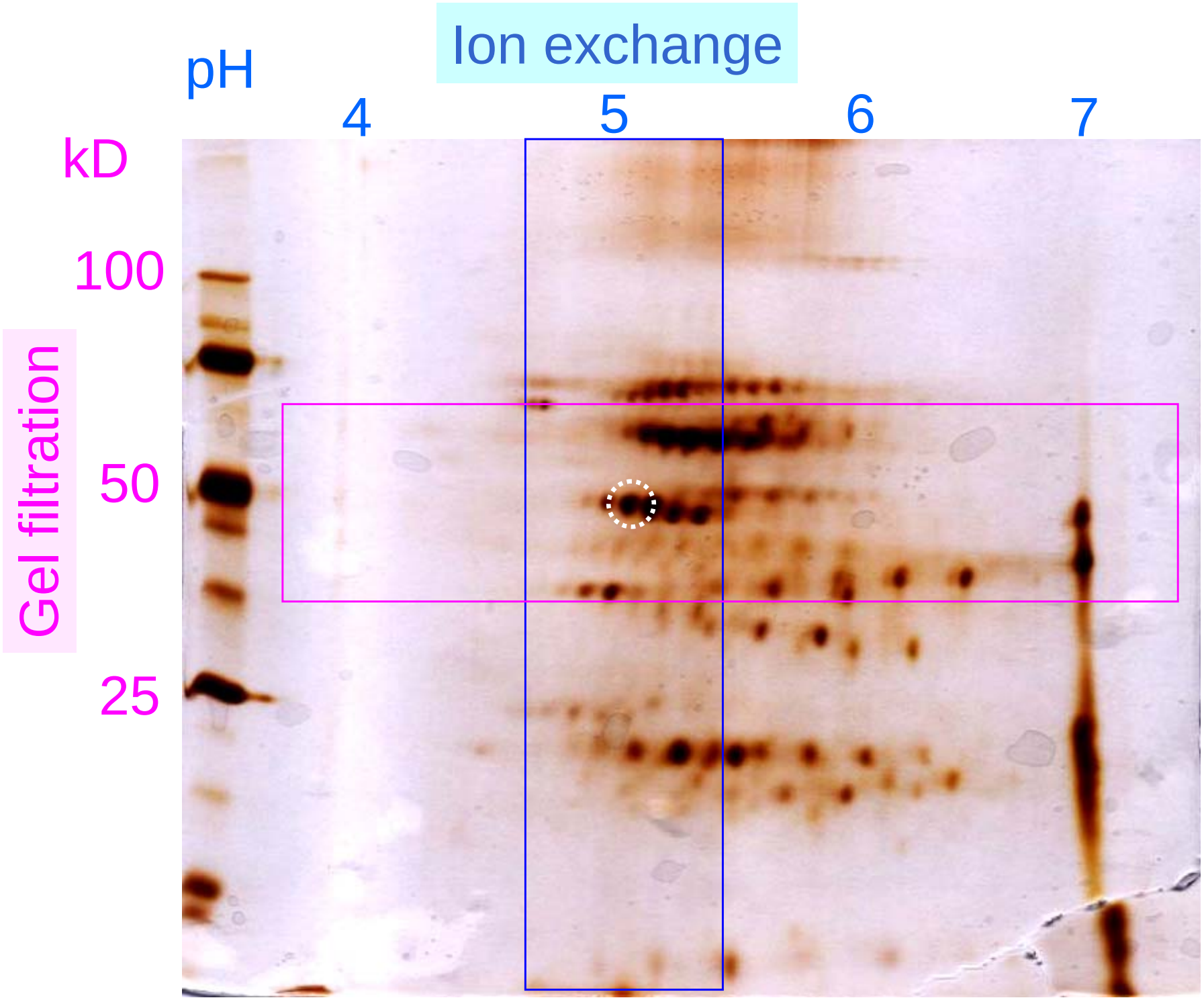
膠體過濾法
Gel filtration

分子量大小不同
The molecular size of the protein

HIC? Hydroxyapatite? Affinity chromatography? Another ion exchange?

Juang RH (2005) EPA

純化過程的鳥瞰

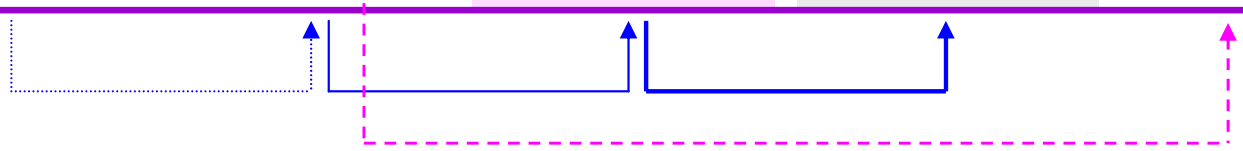


The result of purification after two chromatographic steps

Juang RH (2005) EPA

蔗糖合成酵素之純化表 Purification table

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold (fold)	Recovery (%)
Crude extraction	1,070	9,672	9.0	1.0	100
Protamine sulfate precipitation	800	12,555	15.7	1.7	130
Ammonium sulfate (35-55% sat)	250	6,610	26.4	2.9	68
Sepharose CL-6B gel filtration	53	5,789	111.3	12.4	60
DEAE Sepharose ion exchange	8.6	2,960	344.2	38.2	31



From 100 g rice grain at its milky stage (乳熟期)

Juang RH (2005) EPA