2 酵素活性測定法 Enzyme activity assay methods

- 2.1 催化反應 Catalytic reaction

  活性測定時要注意一些基本原則

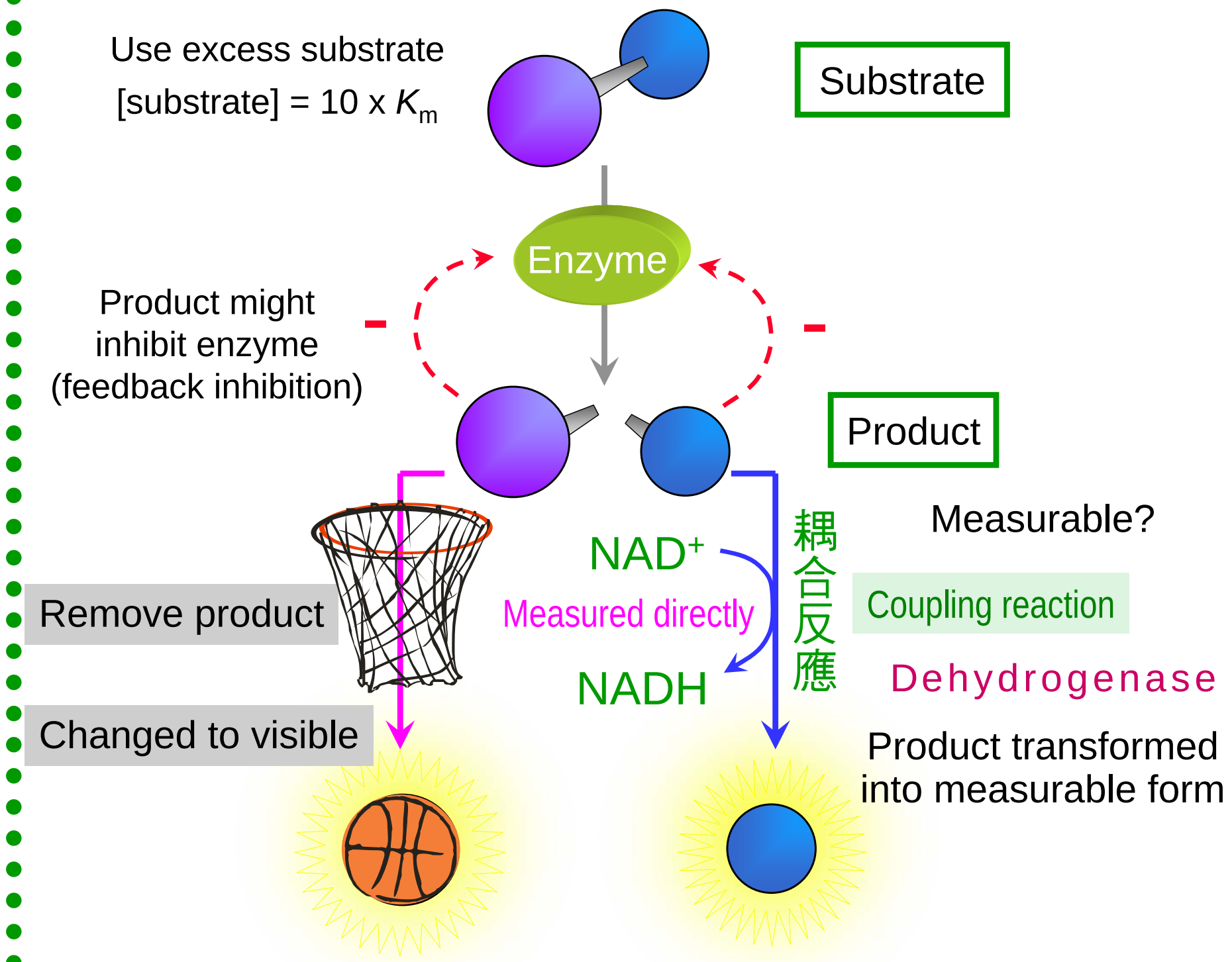
- 2.2 酵素活性分析 Enzyme assay methods

  反應速率 = 生成物 (P) / 時間 (t)

- 2.3 維持酵素活性 Maintain activity

  很多酵素在細胞外容易失去活性

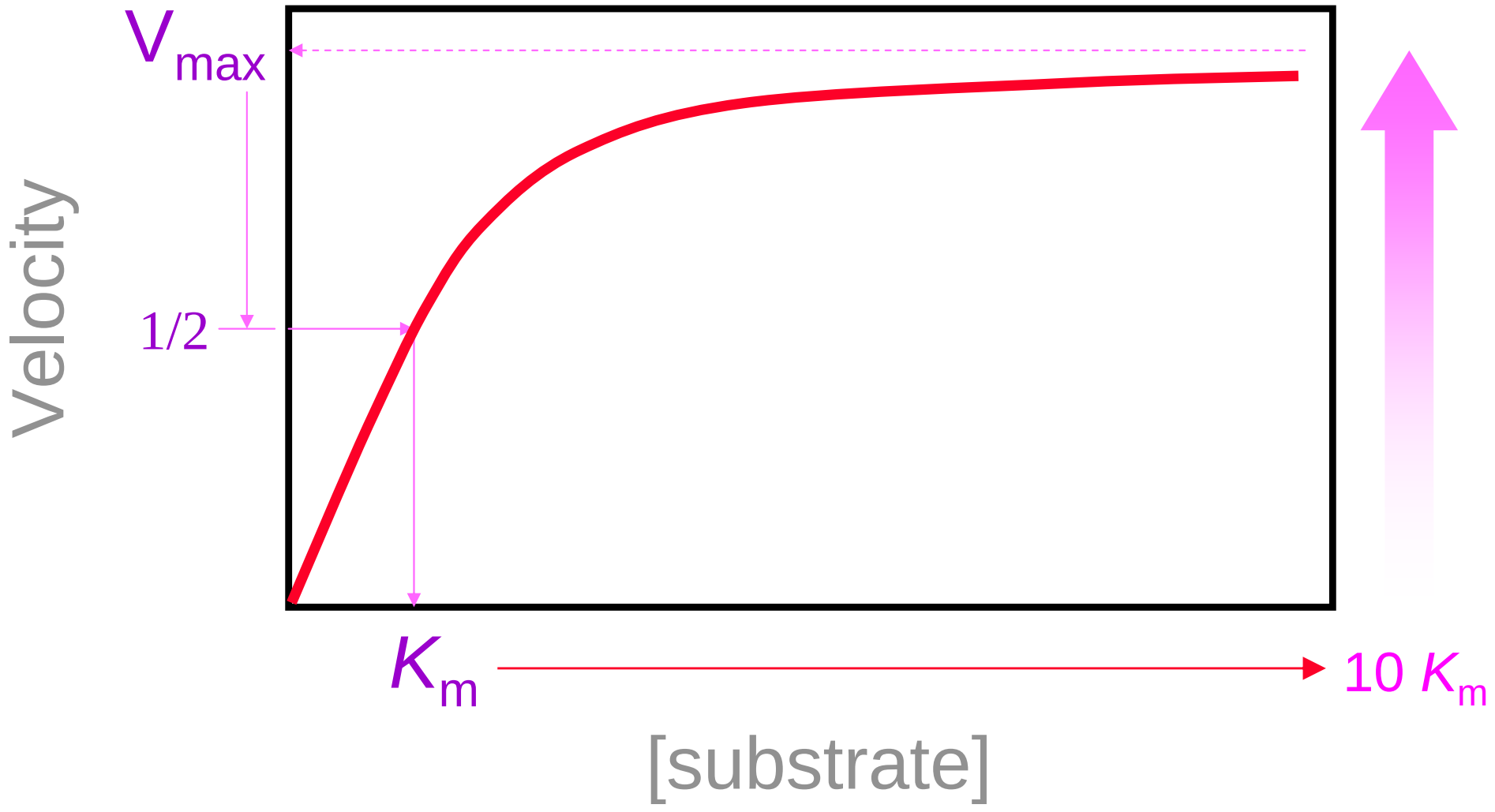
酵素反應及偵測方法



Enzyme catalysis and detection methods

Juang RH (2005) EPA

基質量使用十倍 K_m　[Substrate] = 10 K_m

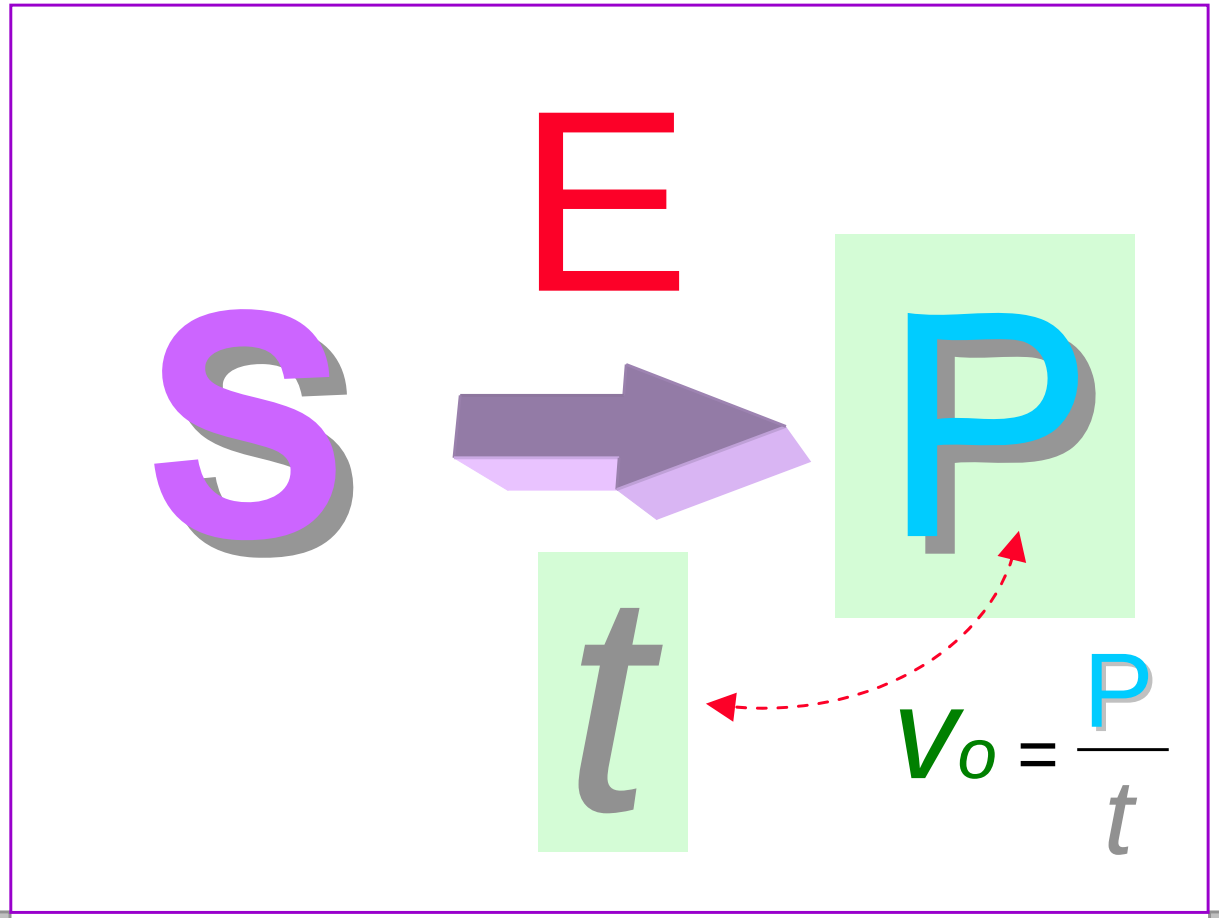


酵素活性測定 Determine enzyme activity (SEPt)

Optimized enzyme concentration

Substrate excess

10 x K_m



Product measurable

pH

酸鹼度

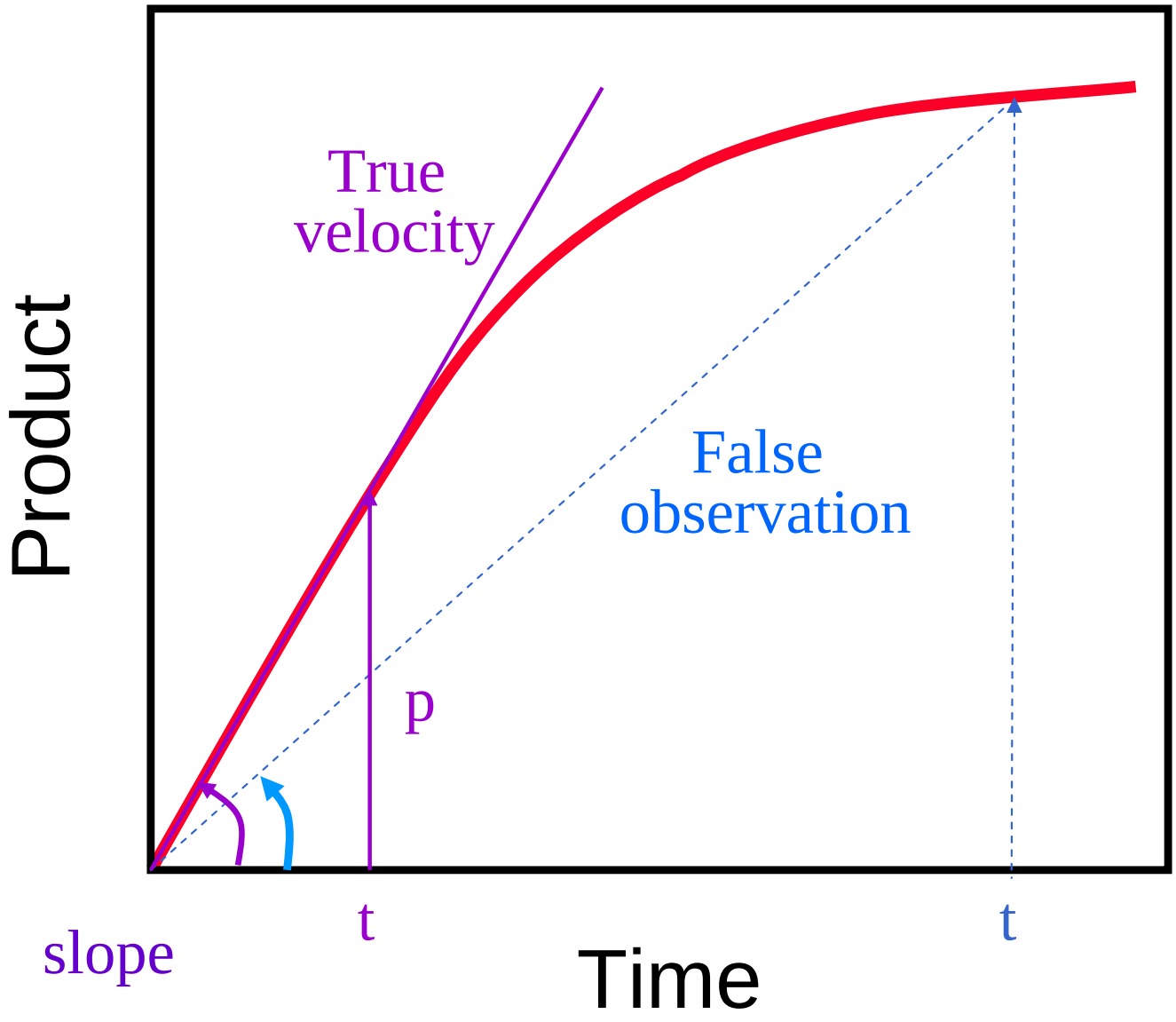
Proper reaction time

Temperature

溫 度

Juang RH (2005) EPA

反應速率需成線性 True velocity of the reaction



$$v_o = \frac{p}{t}$$

Juang RH (2005) EPA

2.2 酵素活性分析 Enzyme assay methods

- 2.2.1 酵素活性測定方法 Assay methods
  在一定時間內測得生成物的產量
- 2.2.2 中止酵素反應方法 Stop the reaction
  中止酵素的方法不得破壞生成物或干擾測定
- 2.2.3 連續測定法 Continuous measuring
  連續測定可不用刻意中止酵素反應
- 2.2.4 澱粉磷解脢活性分析 Assay for L-SP
  以生化方法可以偵測到澱粉磷解脢的活性

2.2.1 酵素活性測定方法 Assay methods

- a. 直接測定生成物 Measuring product directly

  酒精去氫酶 (alcohol dehydrogenase, deHase)

  $Alcohol + NAD^+ \rightarrow Acetaldehyde + NADH + H^+$

- b. 耦合反應法 Coupling reaction

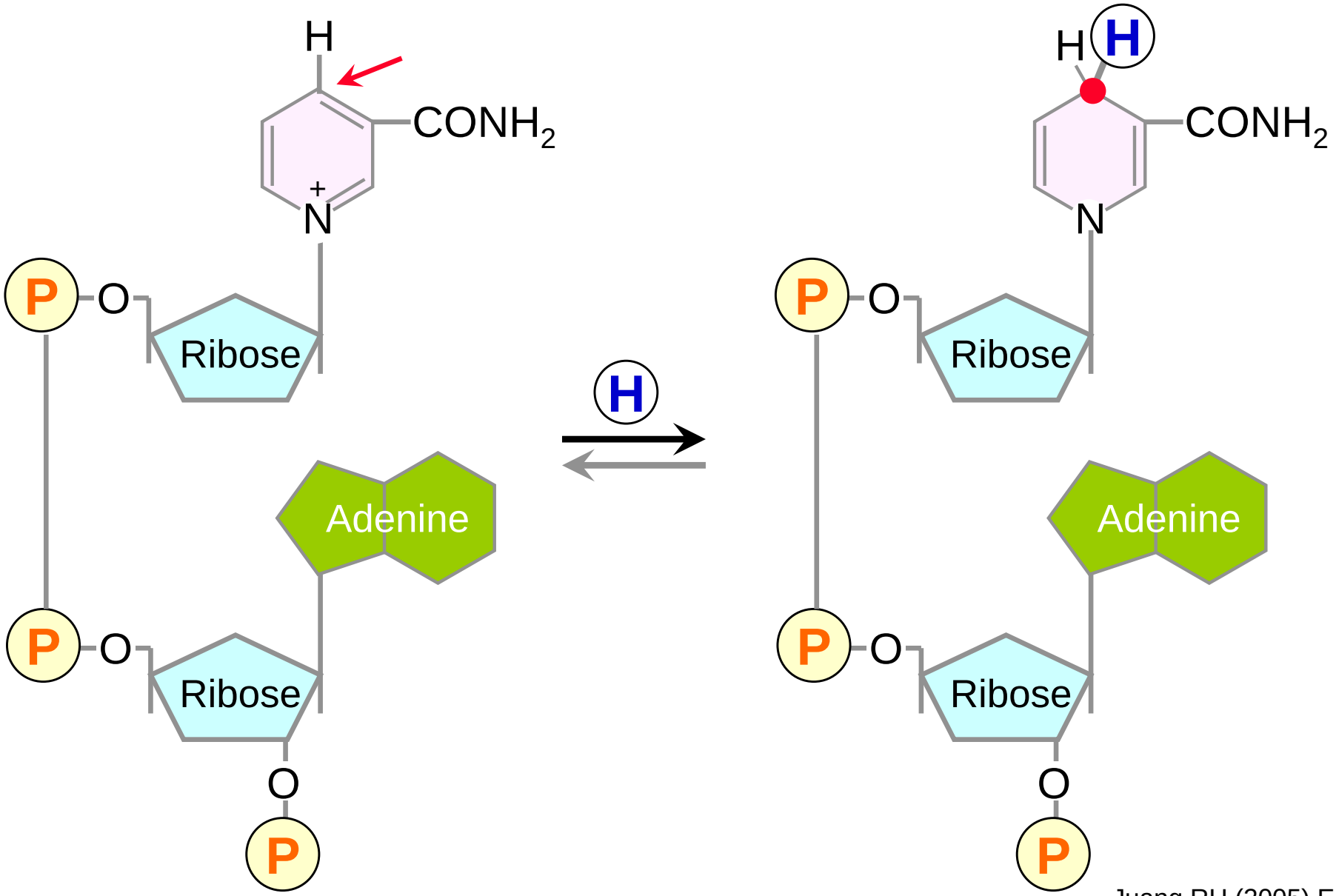
  S → P → Q 可耦合到去氫酶反應 (coupled to deHase)

- c. 化學測定法 Chemical method
- d. 放射線測定法 Tracer method
- e. 測壓法 Manometry (for gaseous product)
- f. 電極 Electrode (for pH or O_2 change)
- g. HPLC 檢定法 Your last choice

輔脢 NADH 作用機制 Action of coenzyme NADH

NADP+ Oxidized form

NADPH Reduced form



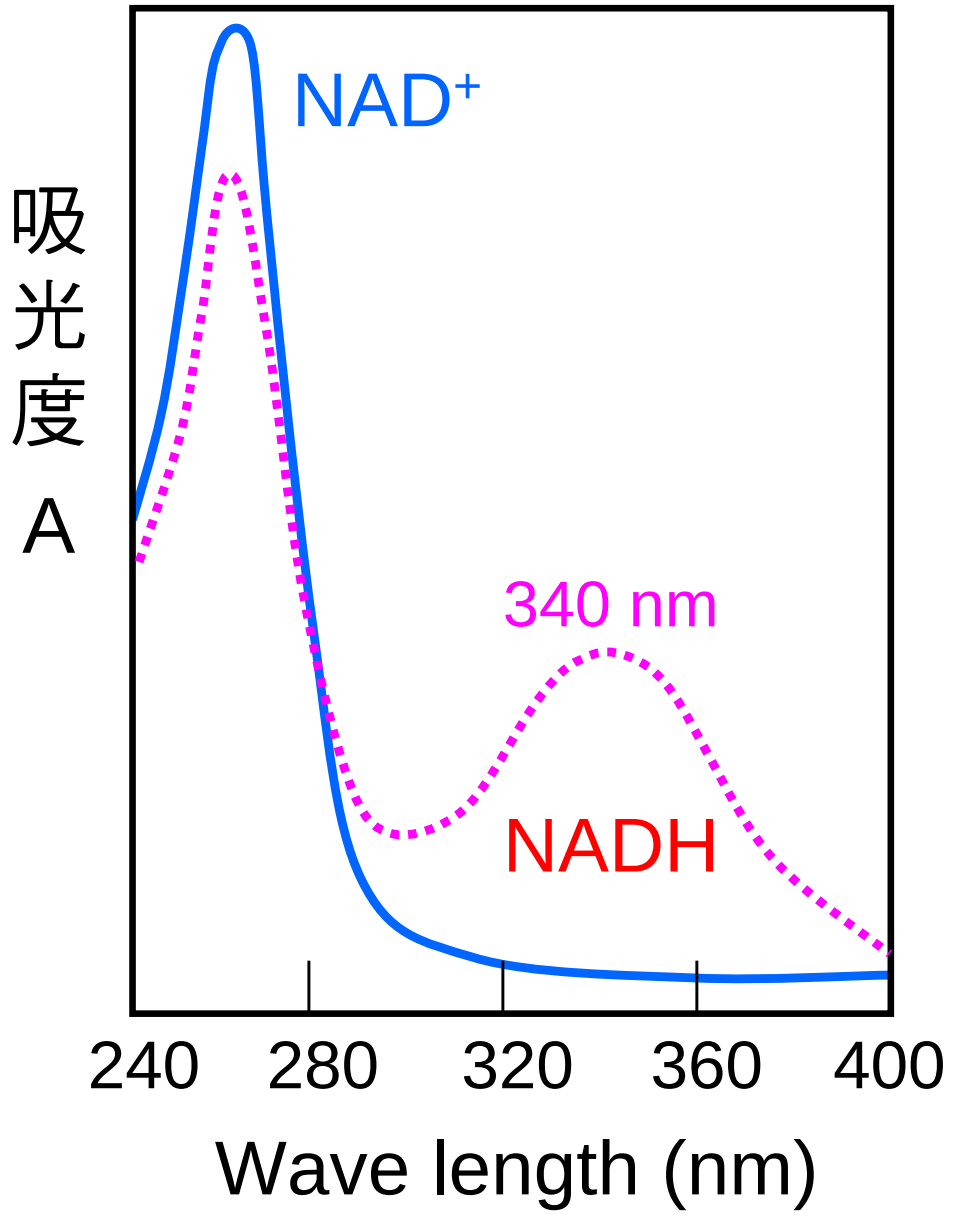
Adapted from Alberts et al (2002) Molecular Biology of the Cell (4e) p.86

Juang RH (2005) EPA

Coenzyme NADH

NAD$^+$/NADH 的轉換可以
耦合 340 nm 吸光度變化

$NAD^+ \rightleftharpoons NADH$ 340 nm



Dehydrogenase (去氫酶)

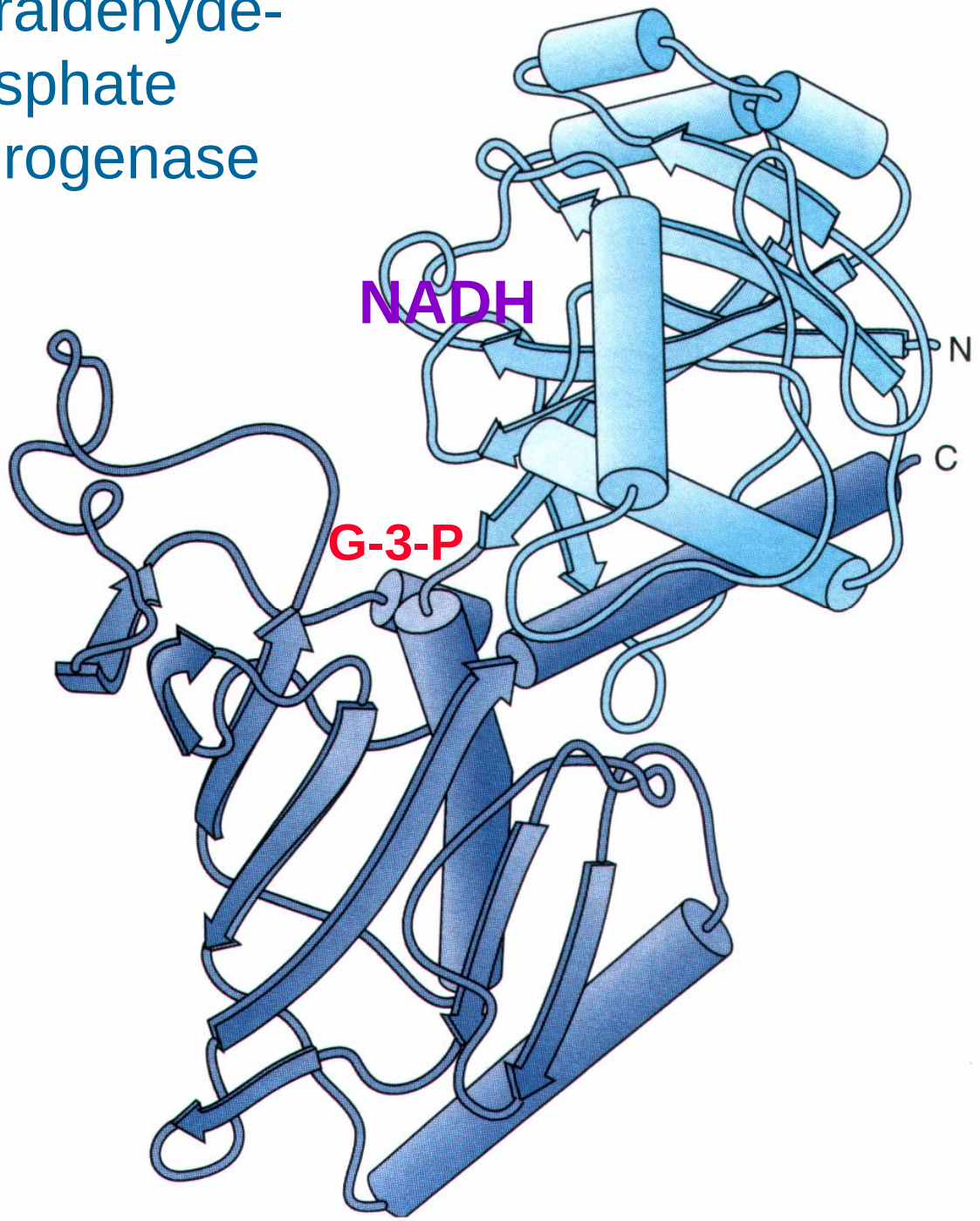
Glyceraldehyde-3-P deHase

- Dehydrogenases use NADH or NADPH as coenzyme
- Have similar NAD$^+$ Binding domain (Convergent evolution)

Juang RH (2005) EPA

一個典型的去氫酶

Glyceraldehyde-3-phosphate dehydrogenase



NADH Binding Domain (conserved)

substrate Binding Domain (variable)

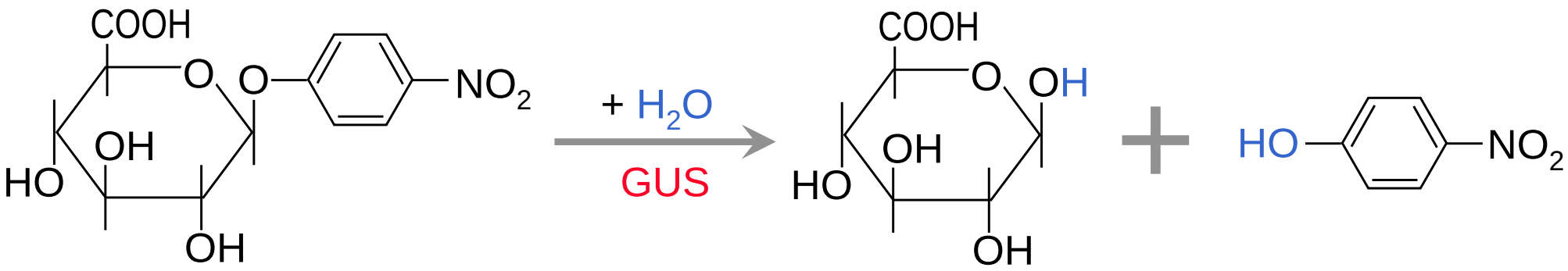
A typical dehydrogenase molecule contains two domains

Kleinsmith & Kish (1995) Principles of Cell and Molecular Biology (2e) p.25

GUS activity assay - using synthetic substrate

Substrate (colorless)

Products (yellow)



p-Nitrophenyl β-D-glucuronide (*p*NPG)

β-D-Glucuronic acid

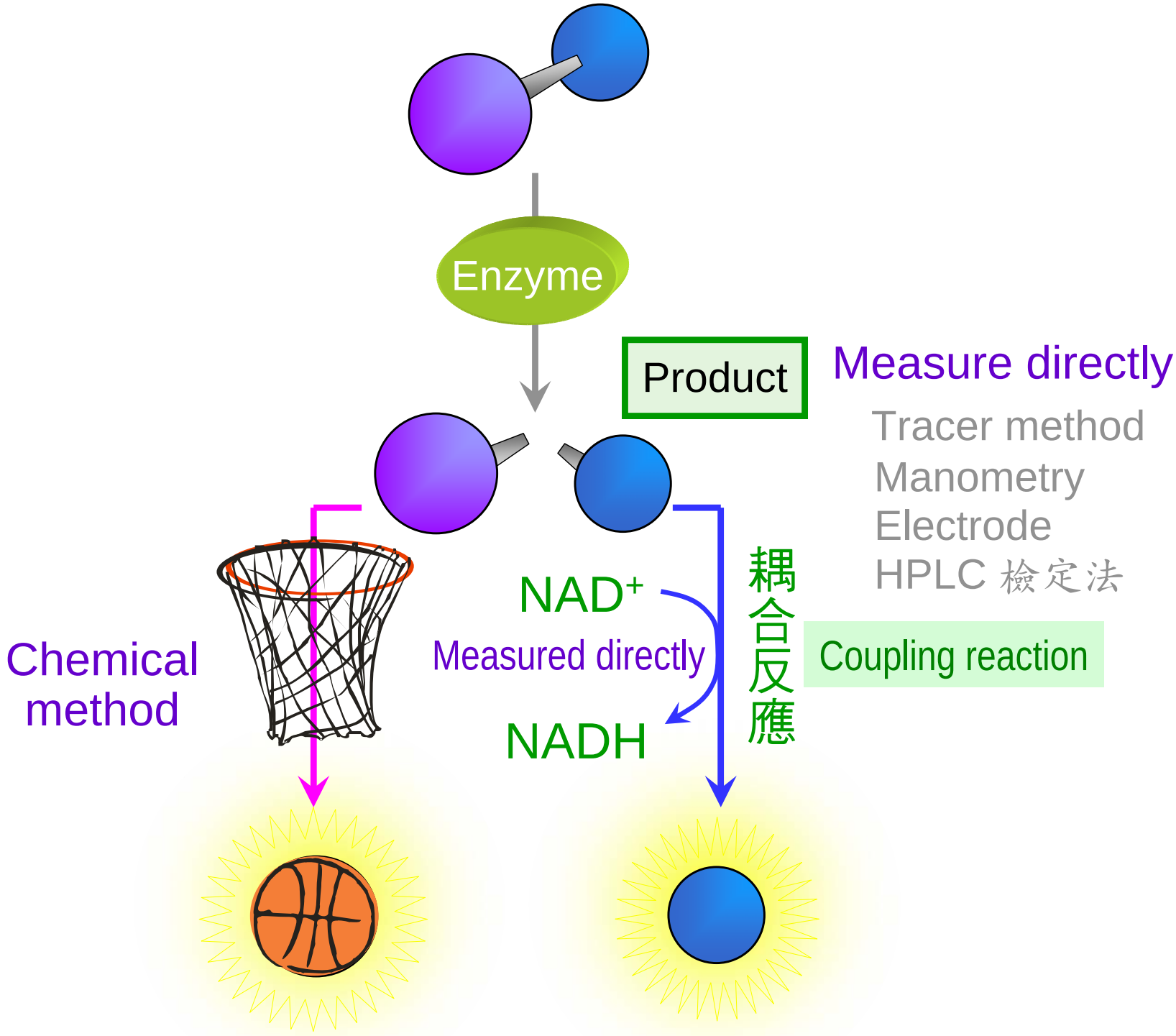
p-Nitrophenol (yellow)

415 nm

GUS (glucuronidase) is extensively used for a reporter in cloning.

Juang RH (2005) EPA

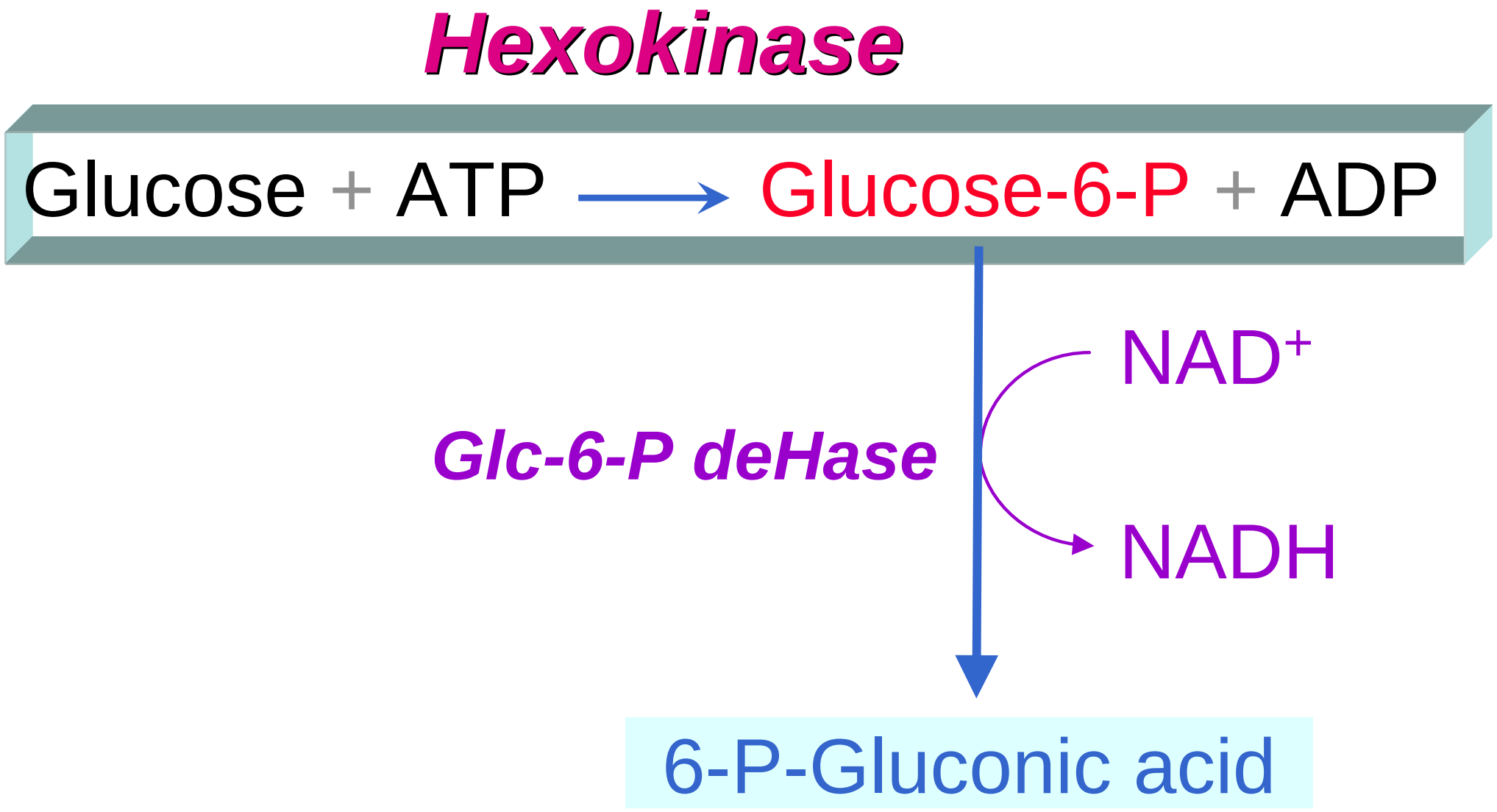
酵素偵測方法



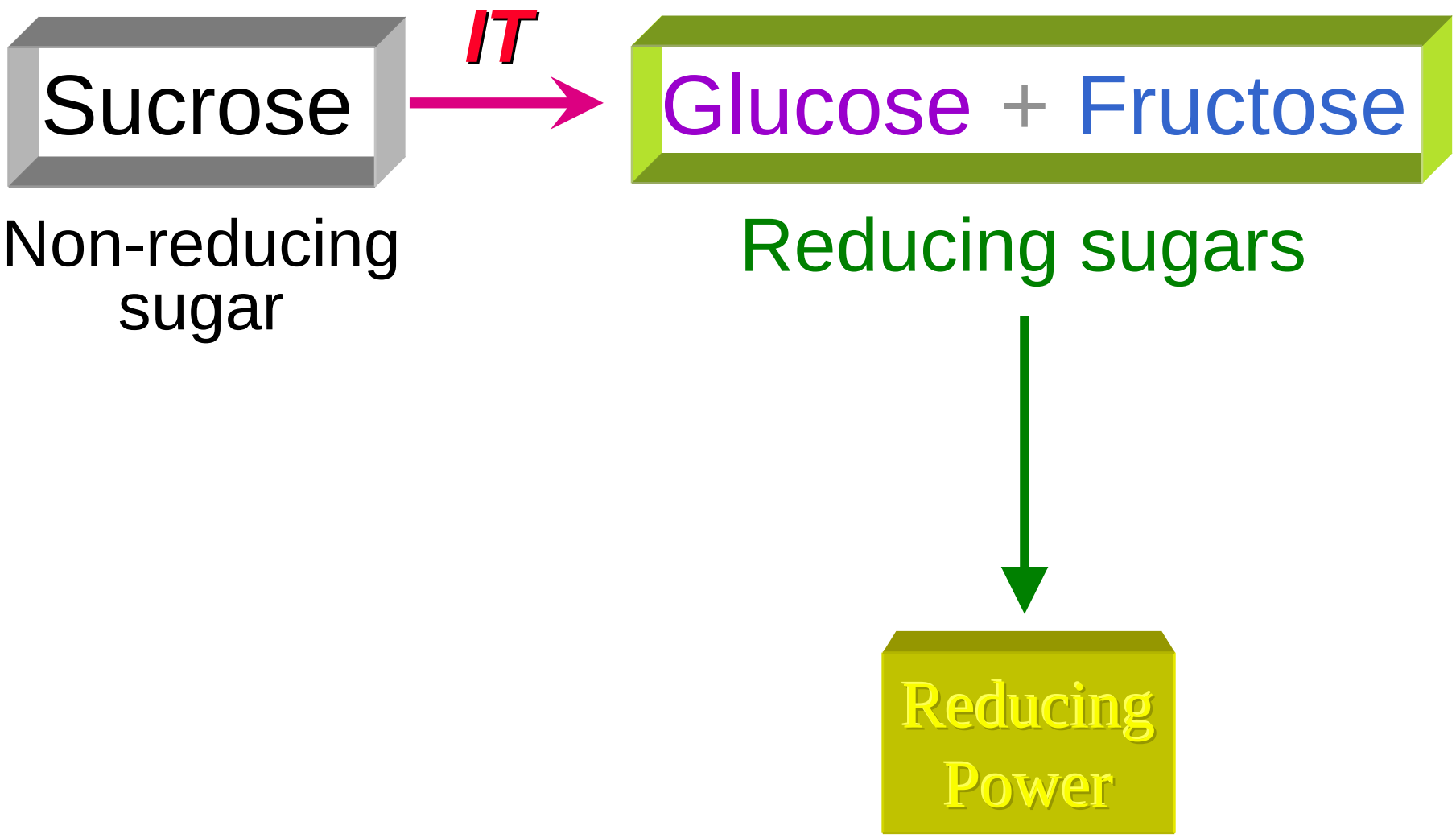
Enzyme detection methods

Juang RH (2005) EPA

Coupled to *dehydrogenase* (NAD^+-NADH)

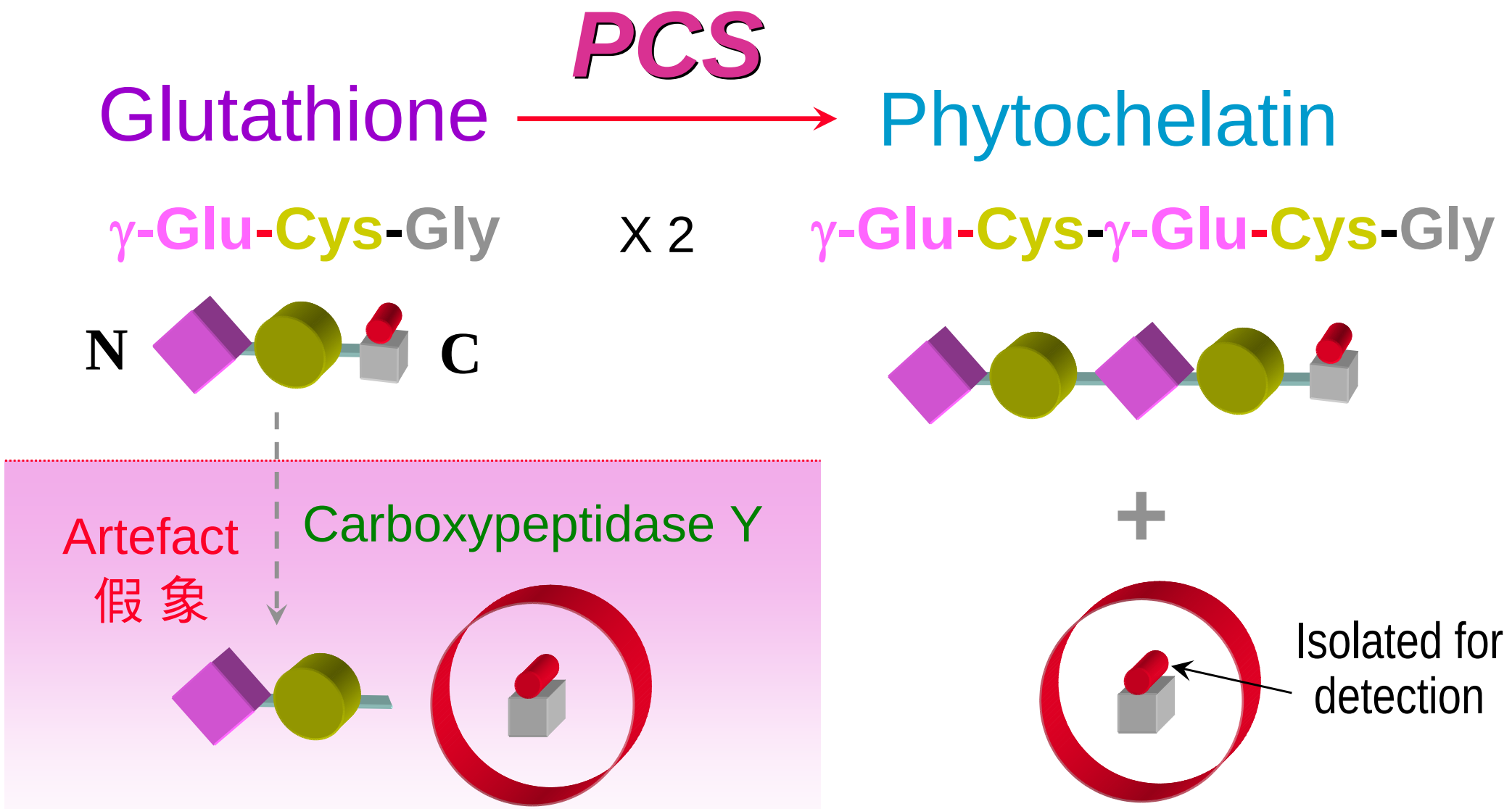


轉化腌 *Invertase (IT)*



Juang RH (2005) EPA

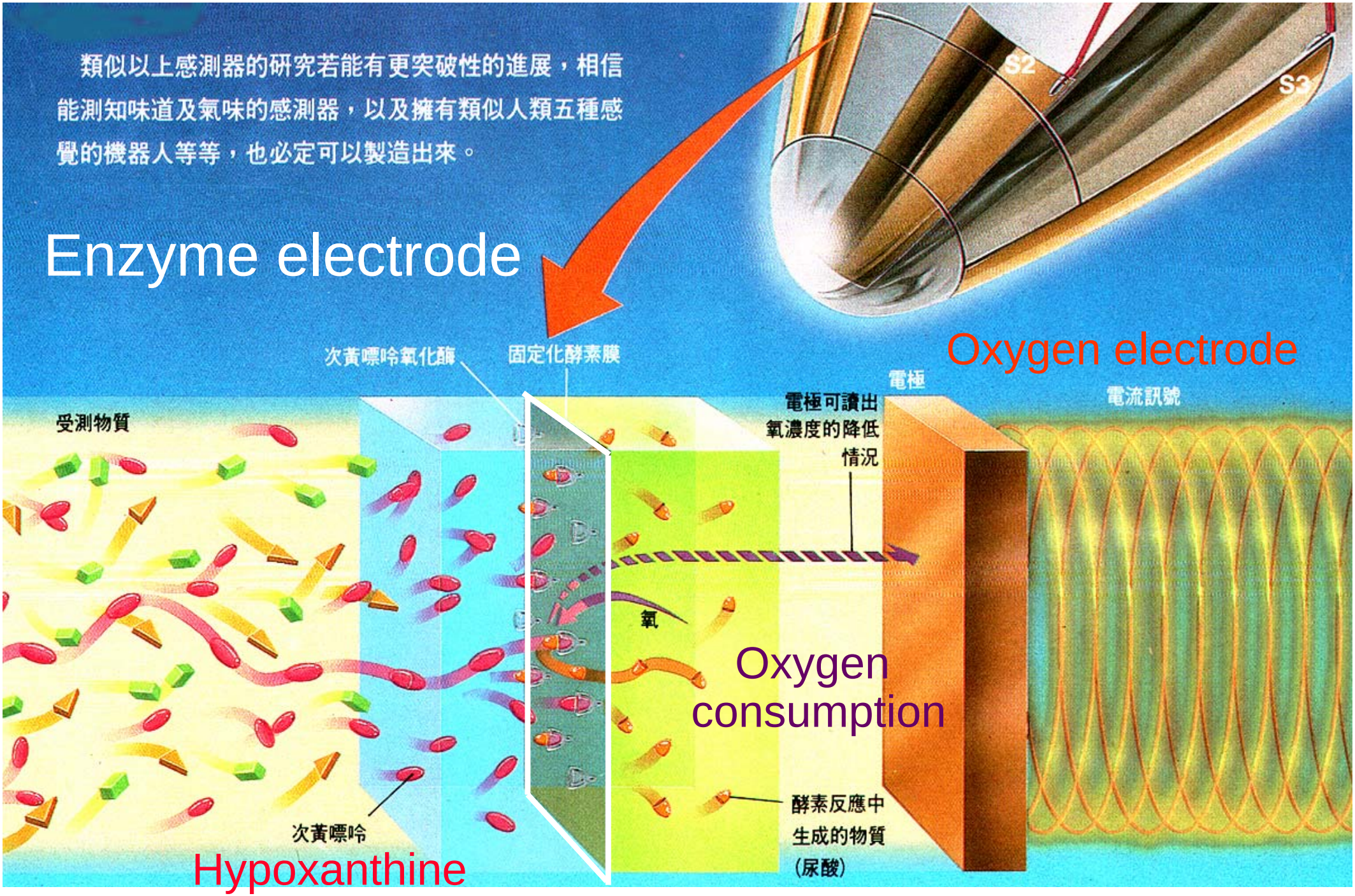
放射線測定法 Using radioactive tracer



It is safer to measure phytochelatin directly by HPLC

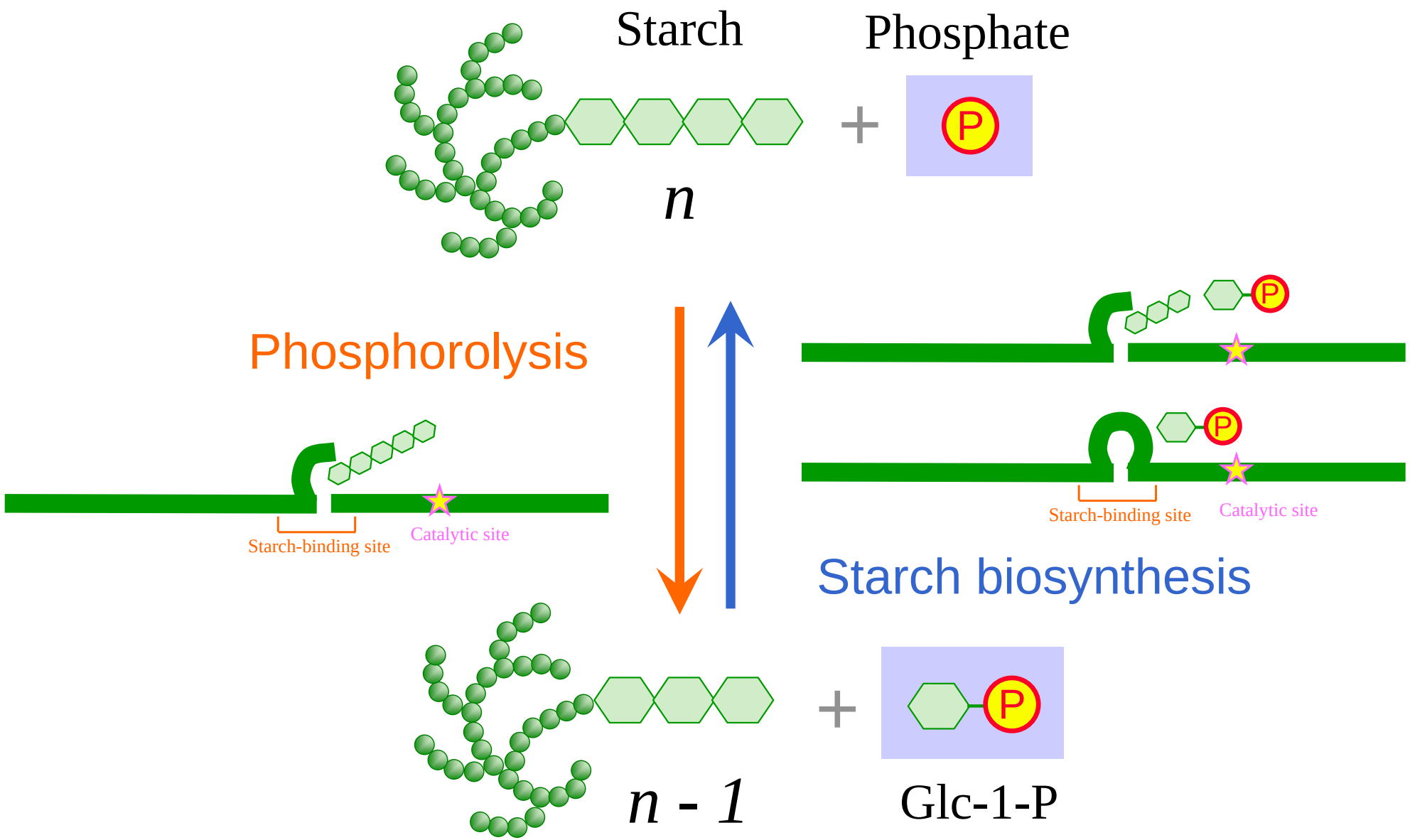
Juang RH (2005) EPA

A sensor to monitor the quality of meat



牛頓 (1985) no. 23 p.108

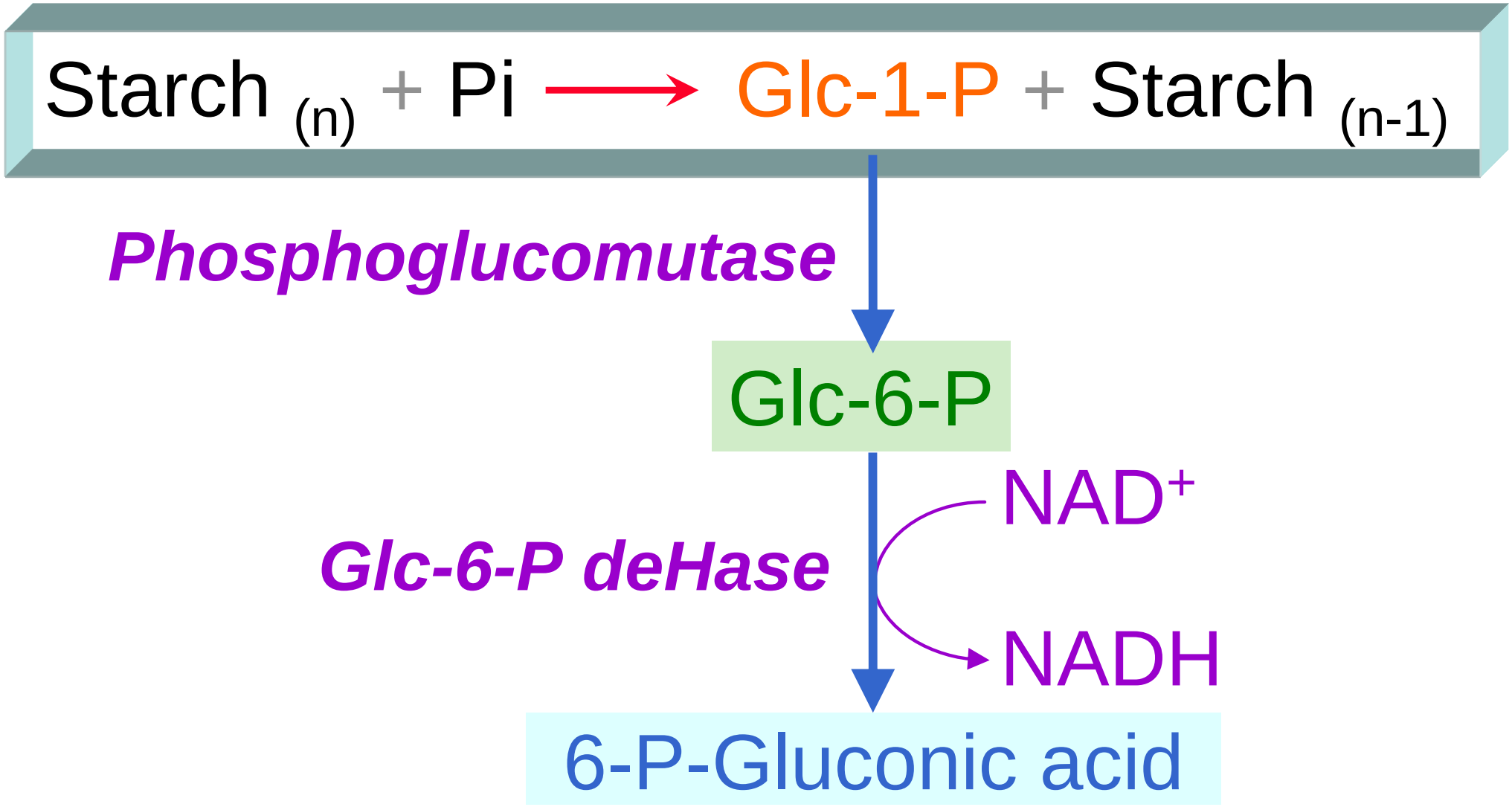
2.2.4 澱粉磷解脢活性分析 Assay for L-SP



Juang RH (2005) EPA

Coupled to *dehydrogenase*

Starch phosphorylase (phosphorolysis)

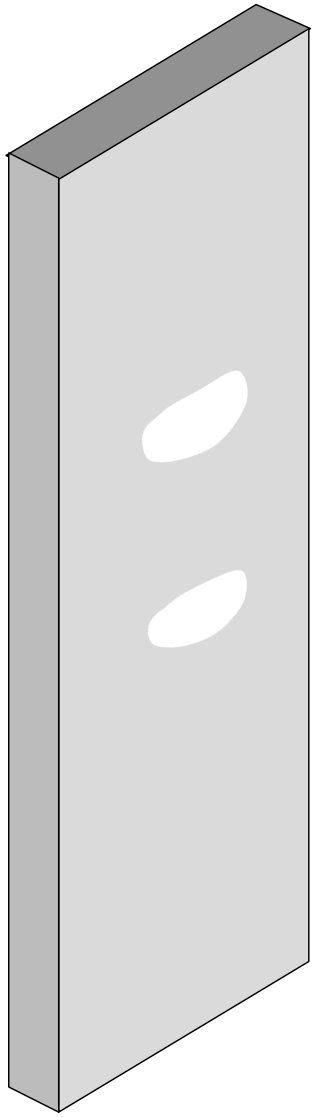


Juang RH (2005) EPA

澱粉磷解酶活性分析及干擾

A

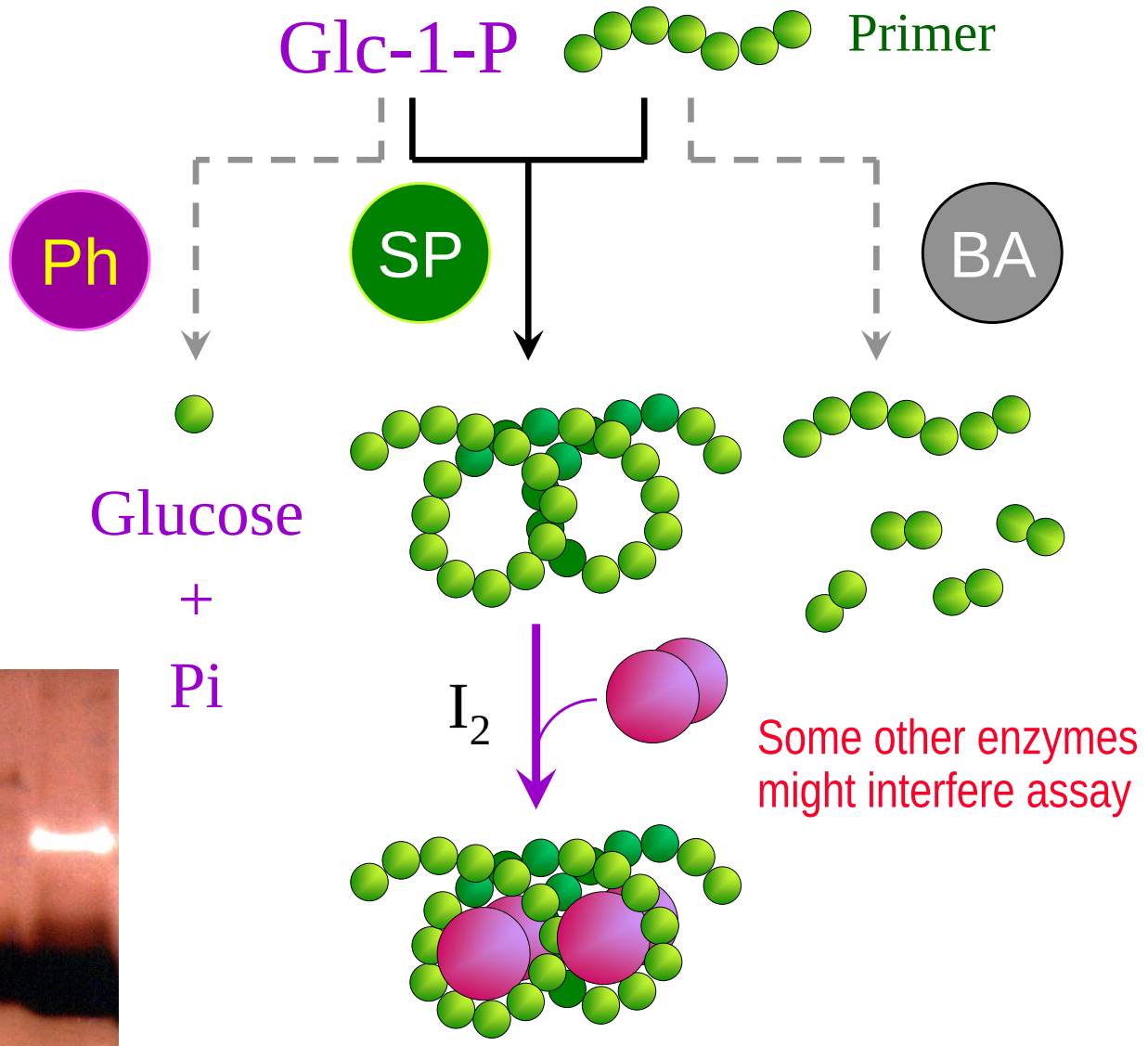
Activity staining



Native PAGE

B

Activity assay and interference



Direct observation of the color product

Some other enzymes might lead to false (+) or (-) results

Juang RH (2005) EPA

2.2.2 中止酵素反應方法 Stop enzyme reaction

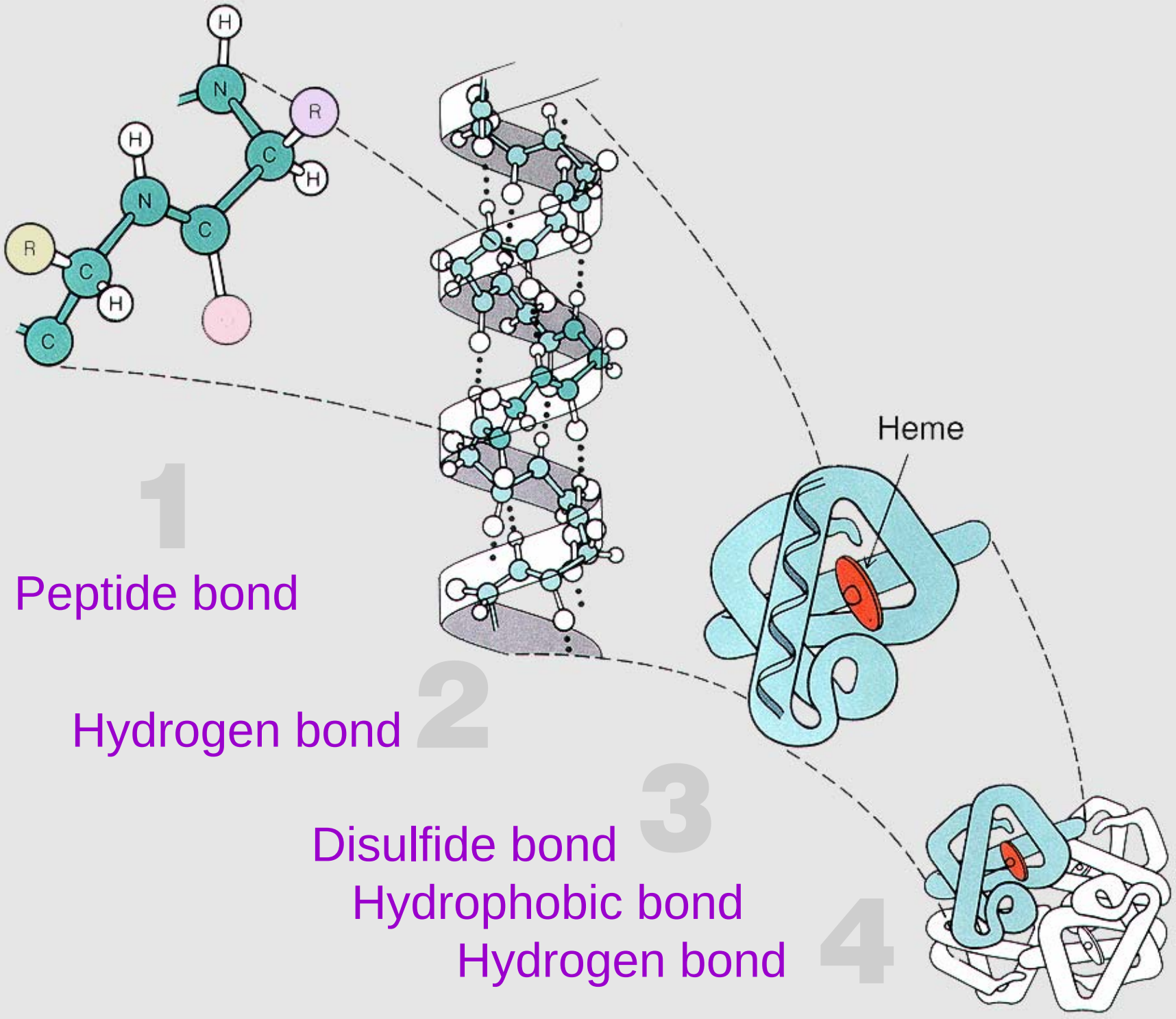
- How to denature the enzyme effectively ?

Change *p*H	TCA	Chemical
Rapidly heating	Boiling	Physical
Add denaturant	SDS	Chemical
Add metal chelator	EDTA	Chemical
Add enzyme inhibitor	PMSF	Chemical
Add non-radioactive substrate (pulse-chase)		Physical

- The product should not be destroyed
- No interference to the detection method

Juang RH (2005) EPA

蛋白質的四級構造

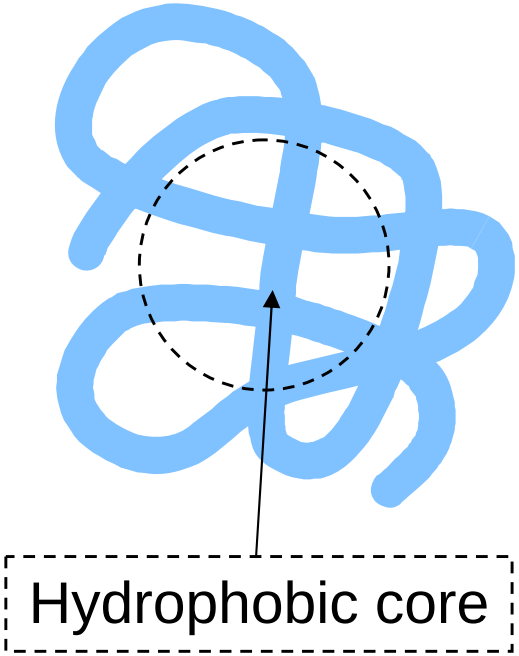


Mathews & van Holde (1990) Biochemistry (1e) p.200, Fig. 6.25

Four levers of protein structure

SDS 在蛋白質表面均勻敷上一層負電

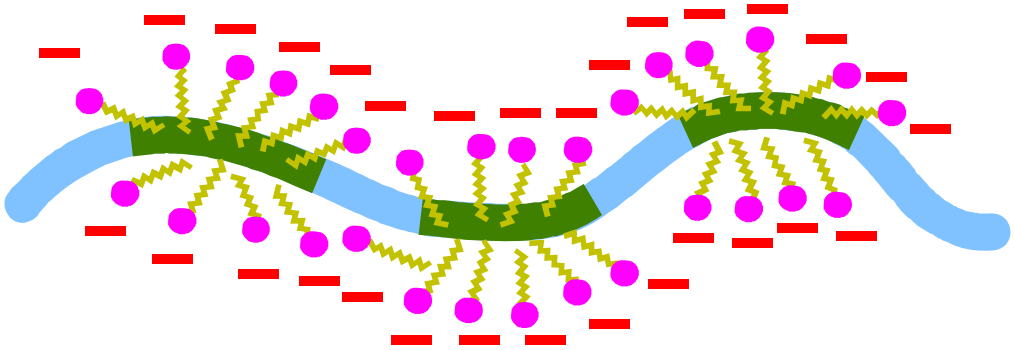
Native protein



SDS

boiling

Protein is denatured to linear form



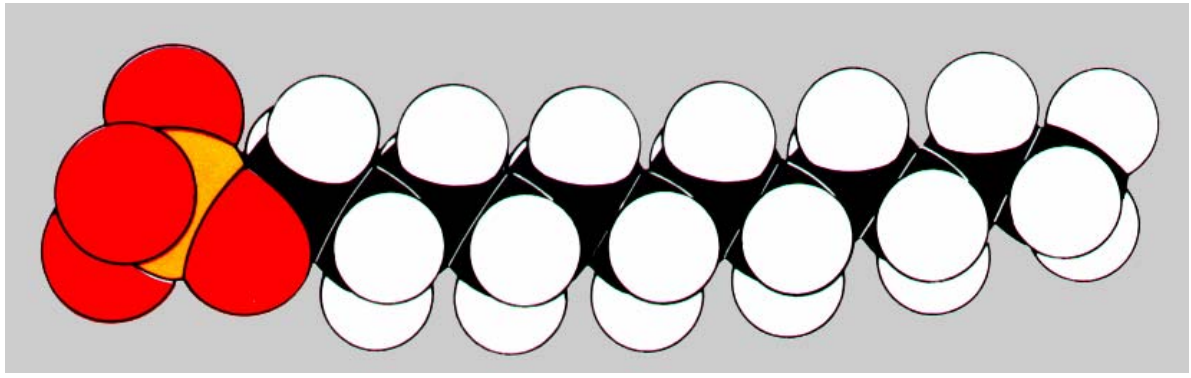
Its surface covered with negatively charged SDS uniformly

+ Mercaptoethanol: to break the disulfide bonds

Juang RH (2005) EPA

常見的變性劑

Urea	$NH_2\text{-}C(=O)\text{-}NH_2$
Guanidine HCl	$NH_2(=NH_2^+)\text{-}NH_2$
Mercaptoethanol	$HOCH_2CH_2SH$
SDS	$SO_4^-\text{-}(CH_2)_{11}CH_3$



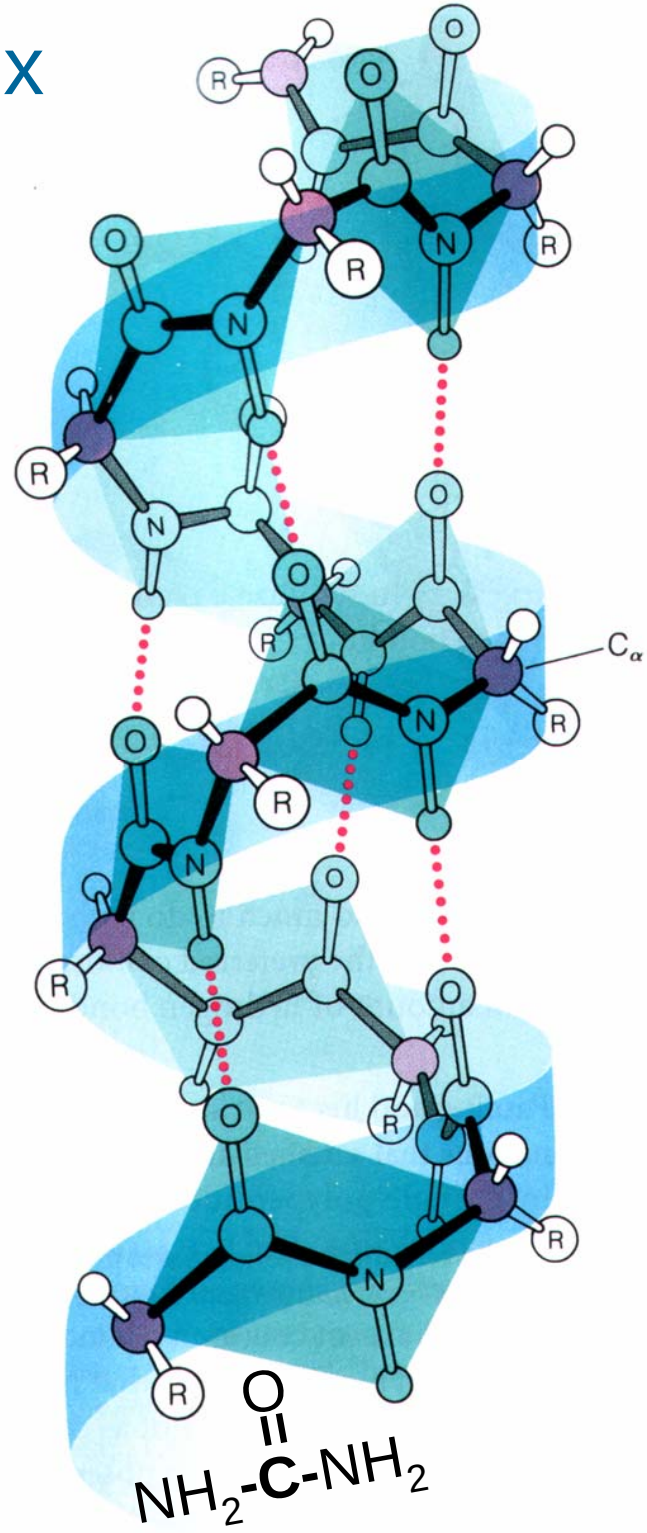
Common denaturants for proteins

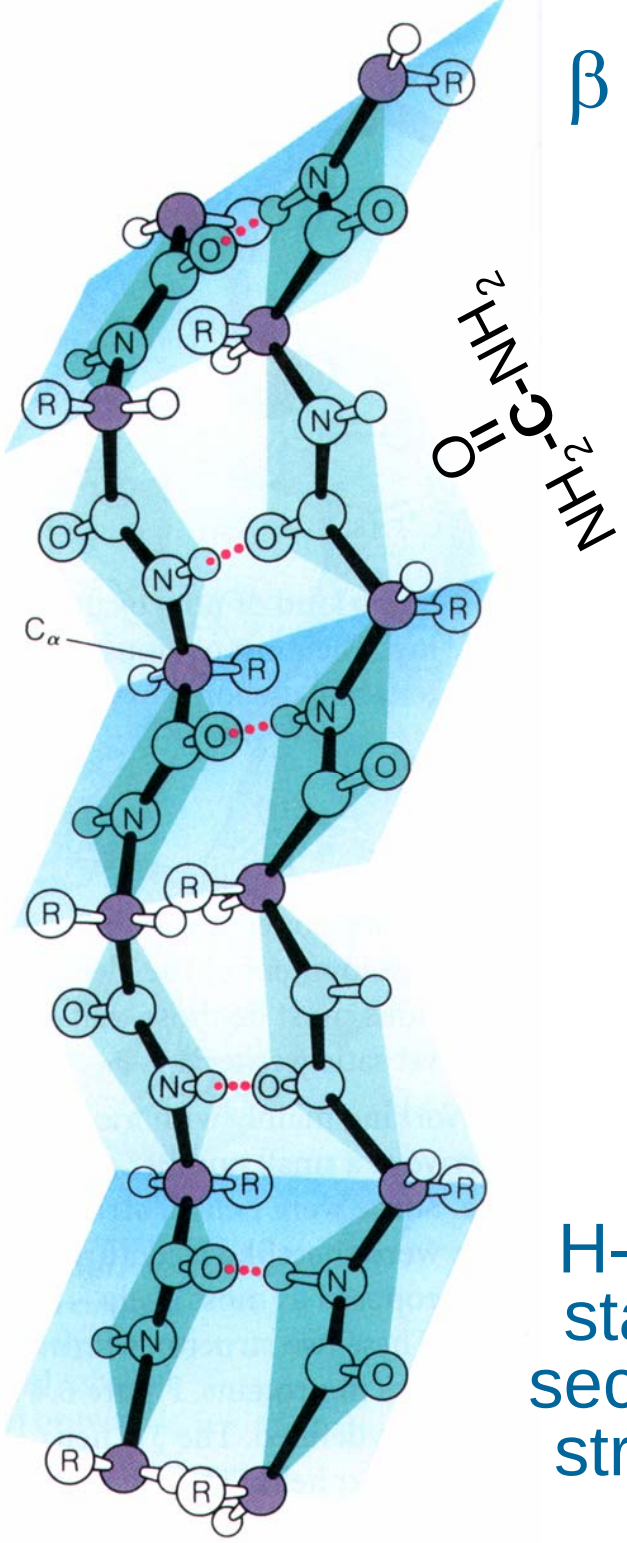
Juang RH (2005) EPA

蛋白質二級構造

α helix

β sheet

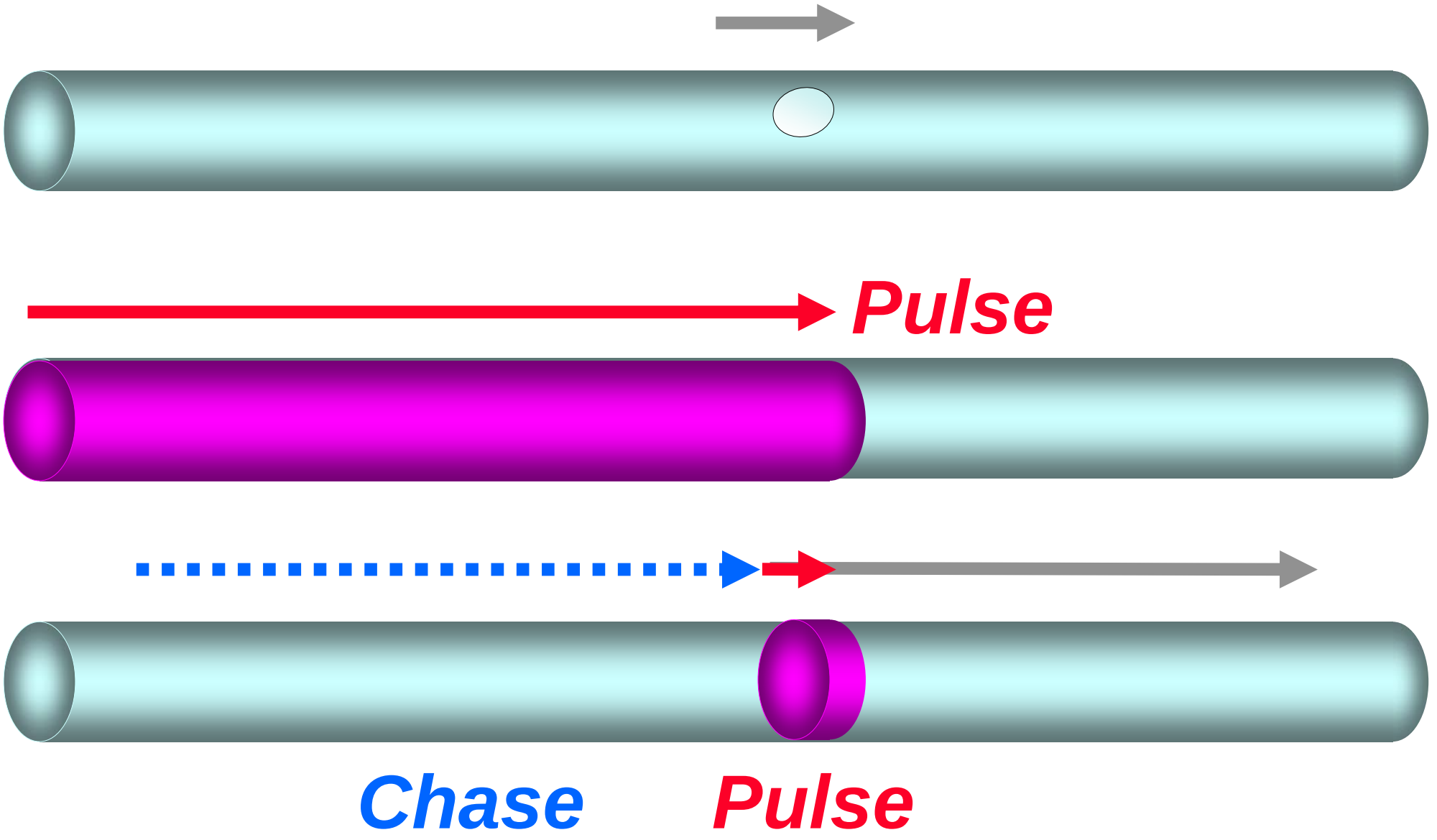




Mathews et al (2000) Biochemistry (3e) p.164

H-bonds stabilize secondary structure

Pulse-chase

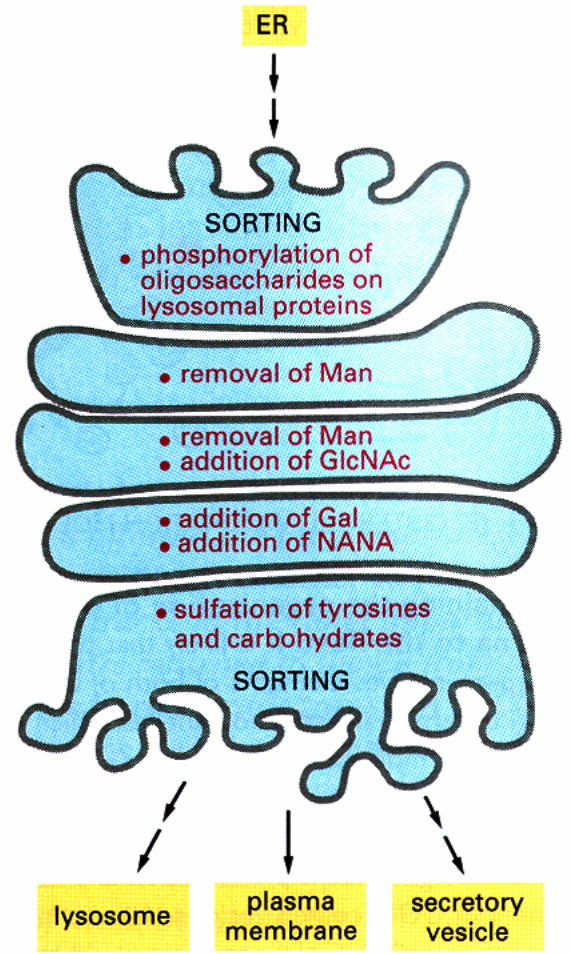


To measure the flow speed of water in a glass tube

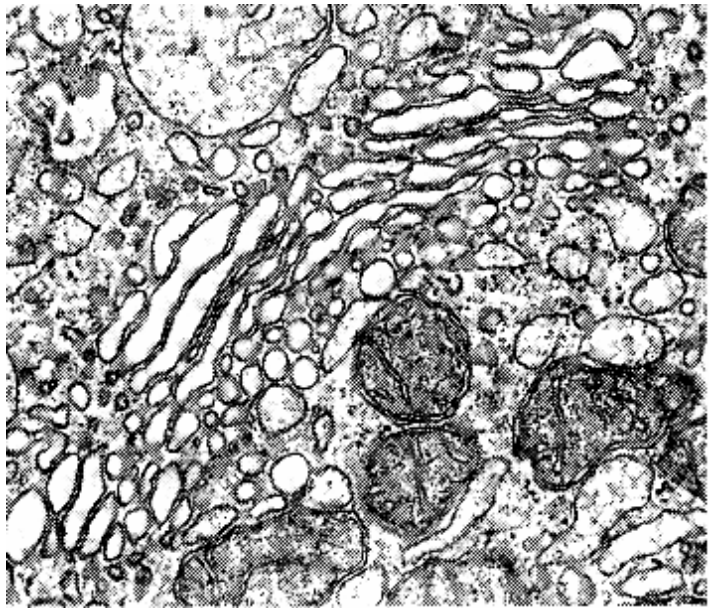
Juang RH (2005) EPA

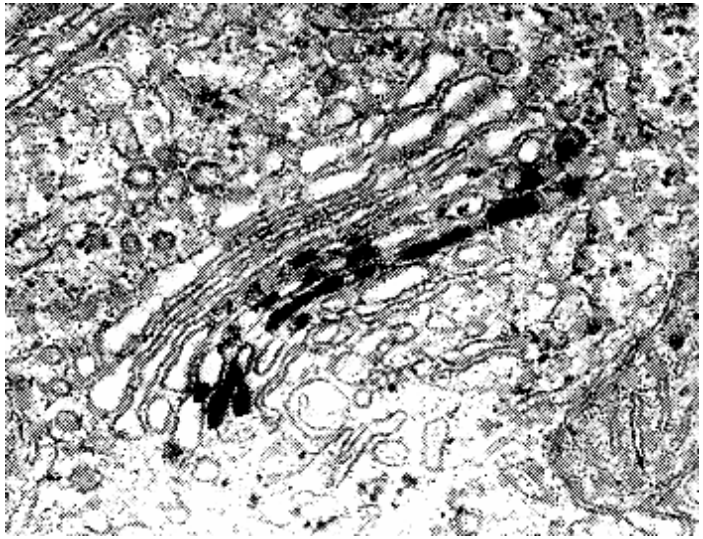
高爾基氏體的蛋白質運送 Golgi transportation

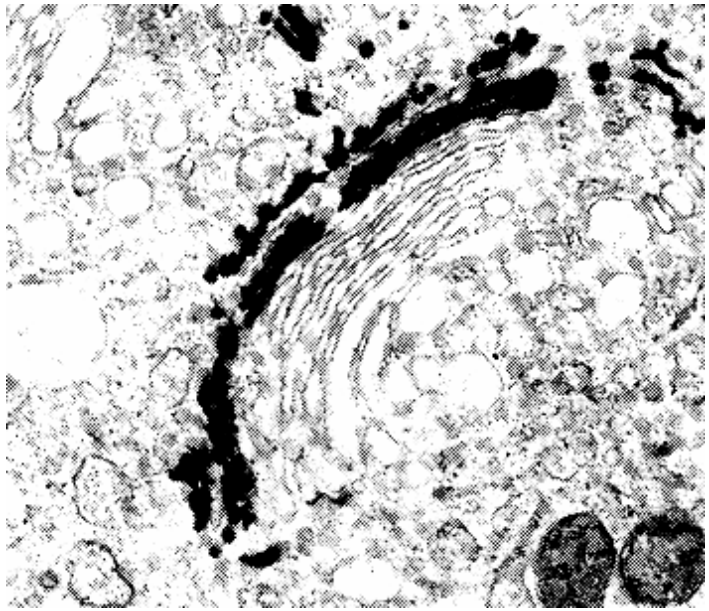
Inner side

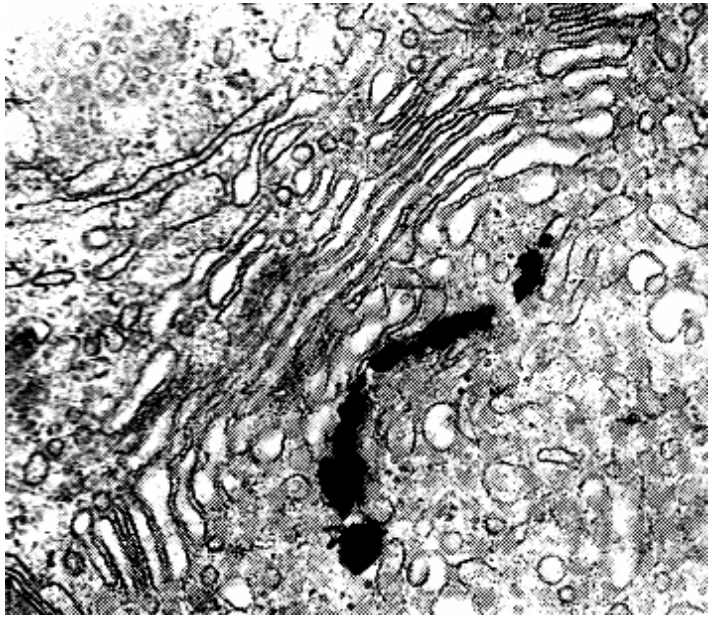


Outer side









Alberts et al (2002) Molecular Biology of the Cell (4e) p.736-737

2.3 維持酵素活性 Maintain enzyme activity

- 2.3.1 緩衝液 Buffer

  可維持穩定的酸鹼度及離子濃度以保酵素活性

- 2.3.2 試劑的保存 Reagents

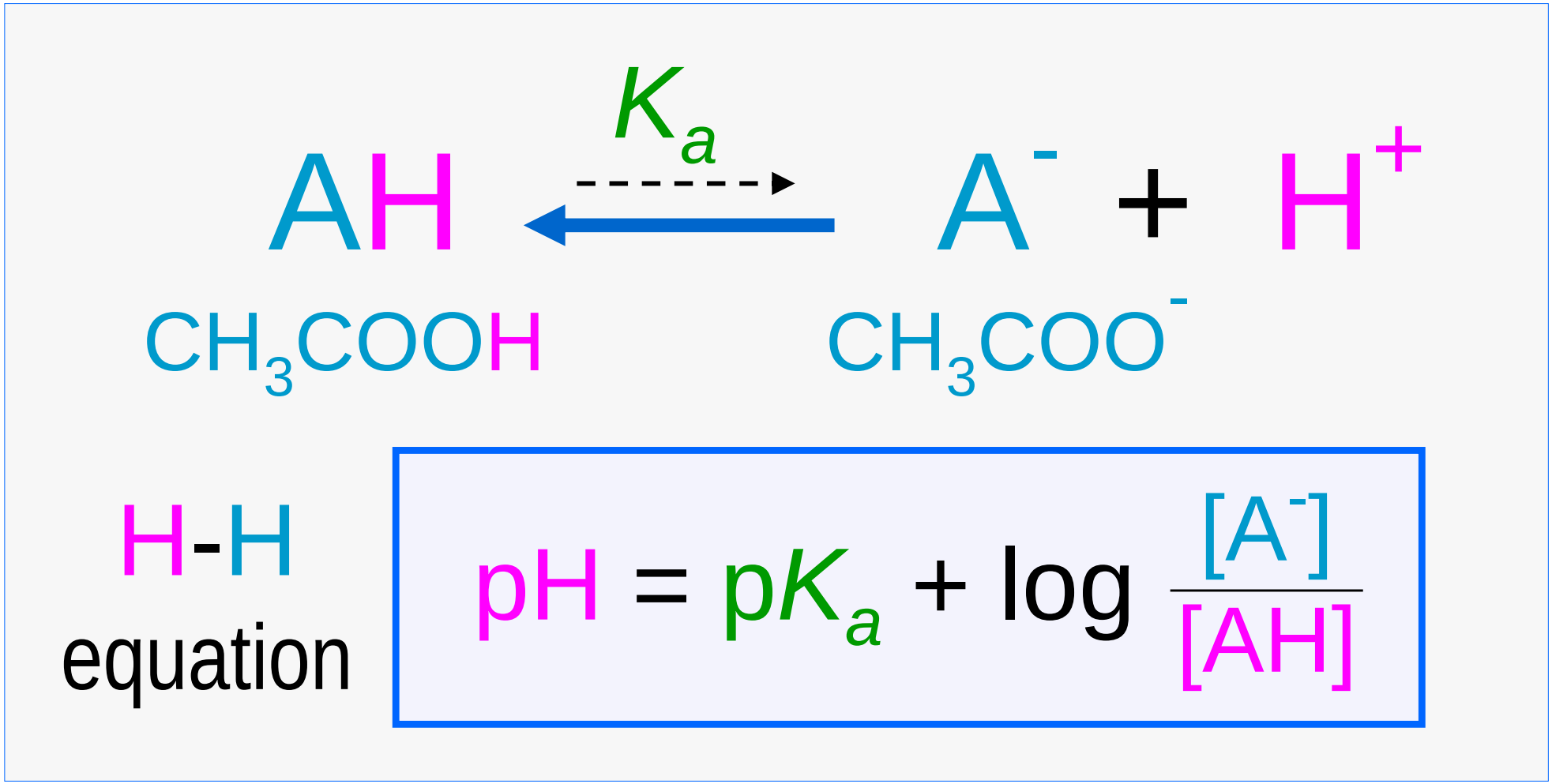
  試劑要依指示保存在適當的地方

- 2.3.3 酵素活性之維持 Maintain the activity

  注意酵素失活的原因有助保持其最高活性

- 2.3.4 酵素活性單位 Enzyme activity unit

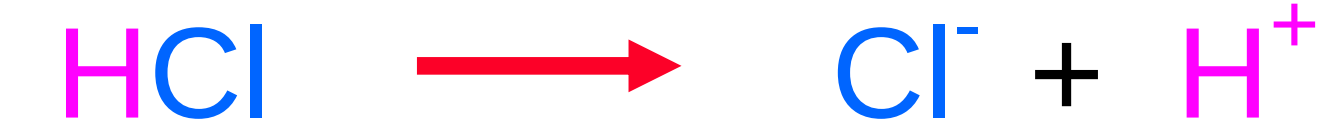
弱酸鹼可作為緩衝分子 Buffer is weak acid/base



H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

Strong acid



Juang RH (2005) EPA

如何推導公式

$$K_a = \frac{[A^-]\,[H^+]}{[AH]} \qquad \frac{\text{dissociated}}{\text{associated}}$$

(1) K_a 的定義

$$\log K_a = \log [H^+] \frac{[A^-]}{[AH]}$$

(2) 兩邊取 log

$$\log K_a = \log [H^+] + \log \frac{[A^-]}{[AH]}$$

分解右邊 log

$$-\log [H^+] = -\log K_a + \log \frac{[A^-]}{[AH]}$$

(3) 移項

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[A^-]}{[AH]}$$

(4) 定義 -log 為 p

Henderson-Hasselbalch equation

How the pK_a of a buffer contribute to its buffering effect

How to derive H-H equation from K_a

Juang RH (2005) EPA

弱酸如何作為緩衝液分子

$AH \rightleftharpoons A^- + H^+$

example Acetic acid

$CH_3COOH = CH_3COO^- + H^+$

◎ K_a is the dissociation constant

$$K_a = \frac{[A^-][H^+]}{[AH]} = \frac{1}{10^5}$$

pK_a of acetic acid ($pK_a = 5$)

◎ K_a is derived to get H-H equation

一、兩邊取 log

二、移項取出 $[H^+]$

三、定義 p 為 -log (pH = -log$[H^+]$)

H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

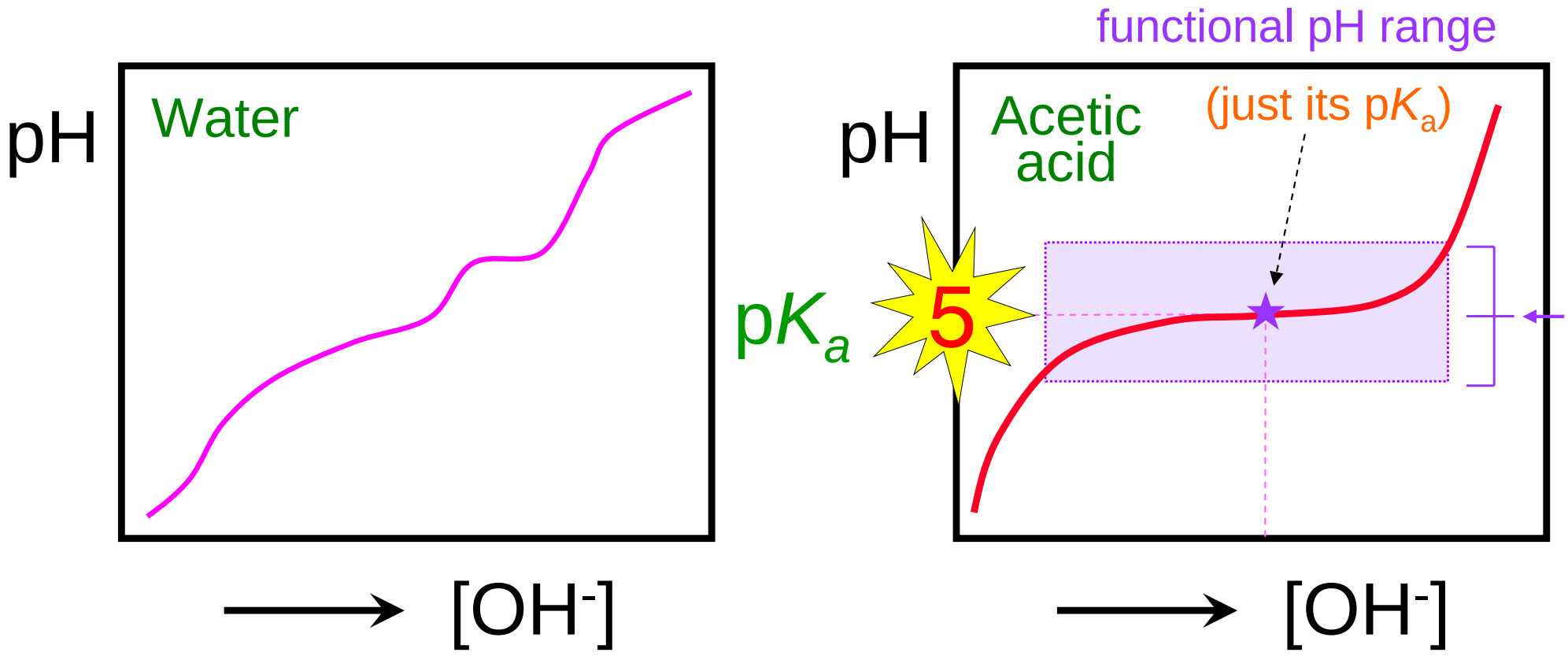
5 5

dissociated / associated

pH = constant pK_a ? ….. when $[A^-] = [AH]$, log 1 = 0

Why weak acid could serve as a buffer ?

弱酸在其 pK_a 上下有緩衝作用



H-H equation

$$pH = pK_a + \log \frac{[A^-]}{[AH]}$$

6 5

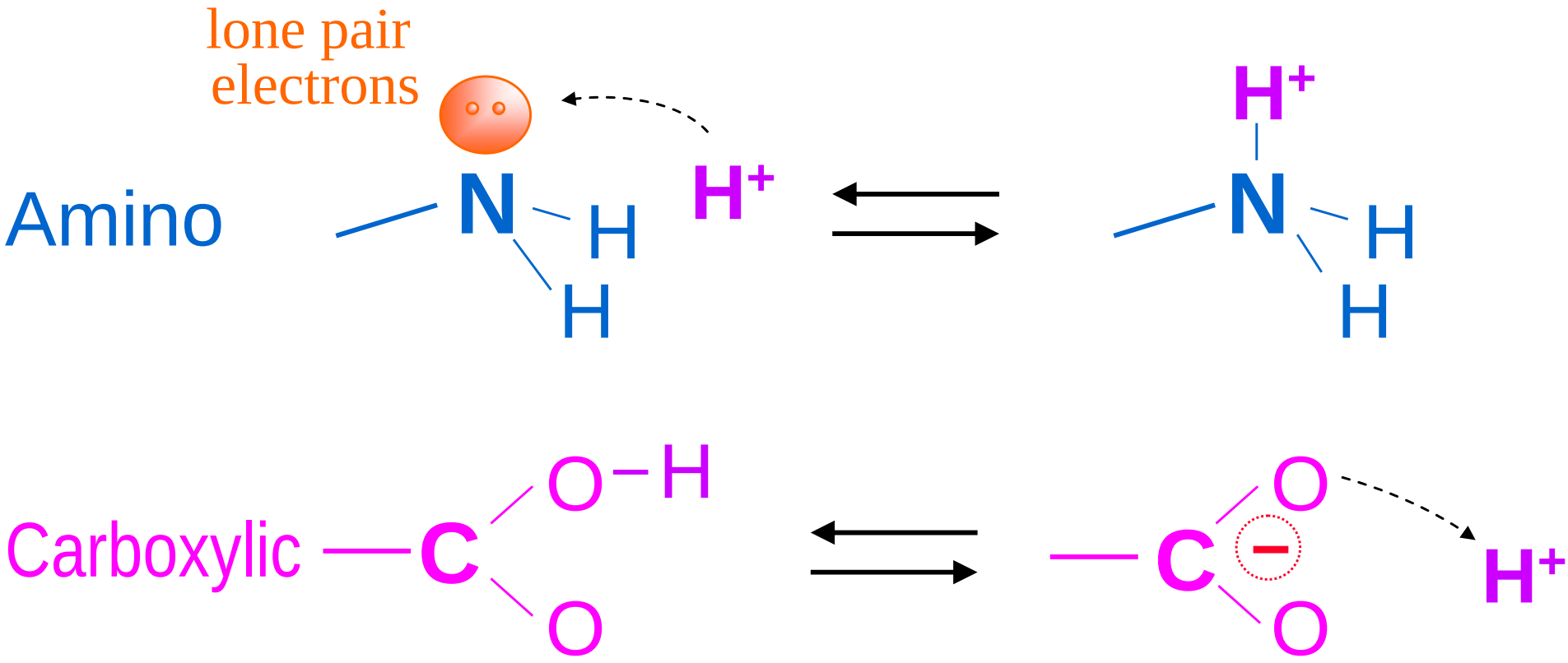
dissociated / associated

Acetic acid has highest buffer effect at its pK_a (pH 5)

Juang RH (2005) EPA

質子可以吸著或脫離一基團

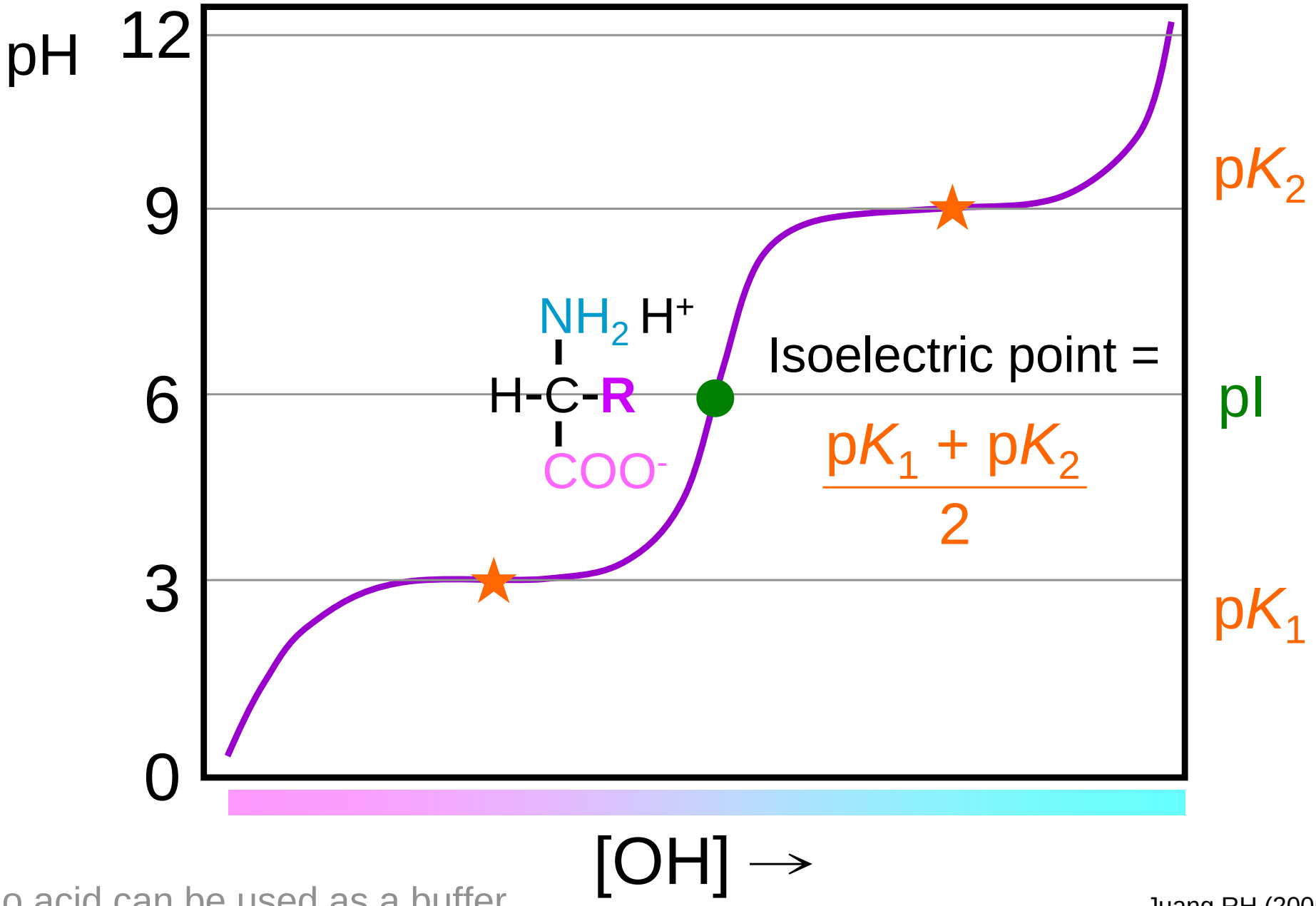
Proton： The smallest and most abundant particle in the living cell controlling the pH and the charge property of a molecule



Ampholyte: A molecule contains both positively and negatively charged groups

Proton can enter or leave a functional group relatively freely

胺基酸的緩衝作用範圍 Amino acid as a buffer



Amino acid can be used as a buffer

Juang RH (2005) EPA

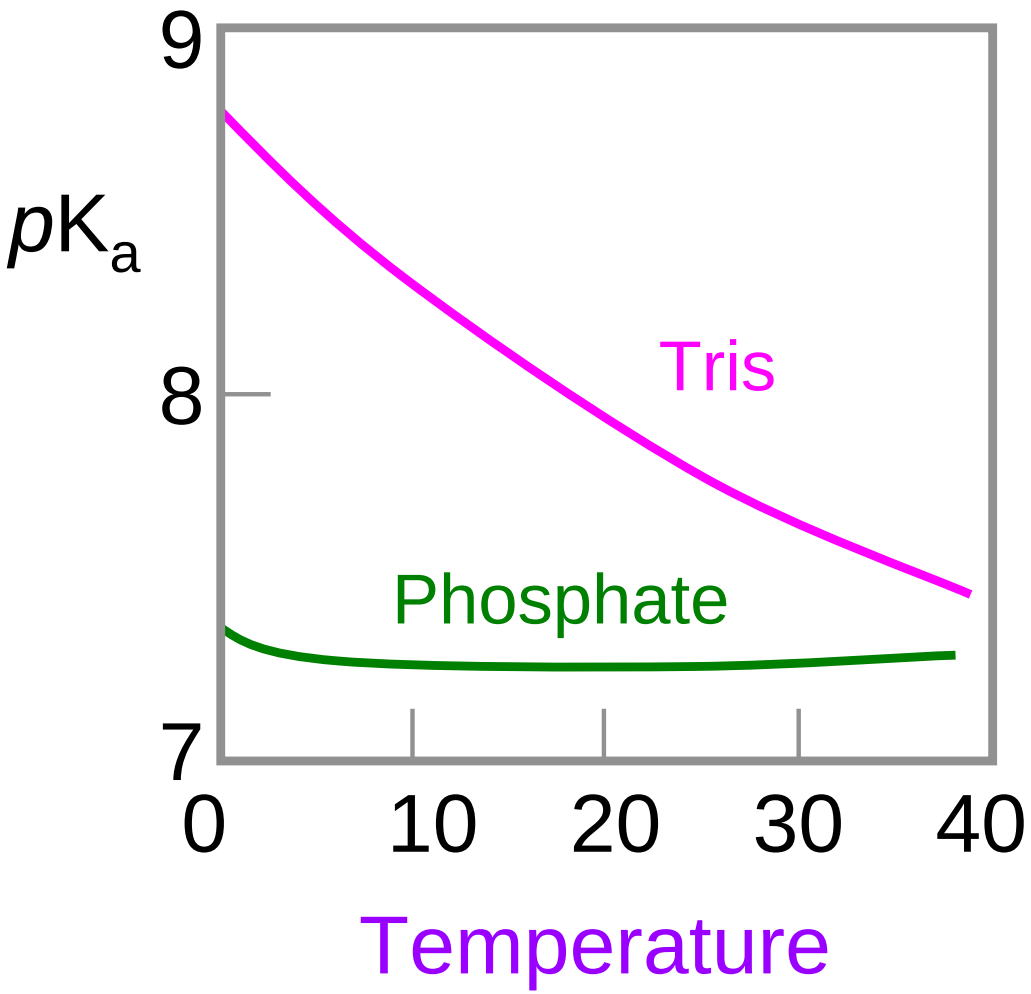
各種常用緩衝液及其使用範圍 Common buffers

Buffer	pH	Remarks
Formate	3.0 - 4.5	Volatile, could be removed by lyophilization
Citrate	3.0 - 6.2	Bind with divalent metal ions
Acetate	3.7 - 5.5	Volatile, could be removed by lyophilization
Phosphate	5.8 - 8.0	Precipitated with Ca; crystallized at low temperature
HEPES	6.5 - 8.5	Low toxicity, used in cell culture
Tris	7.1 - 8.9	pH effected by temperature; special pH electrode required
Borate	9.1 - 9.0	
Carbonate	9.7 - 10.7	Bind with divalent metal ions
Universal	2 - 12	Contains several buffers at various pH ranges

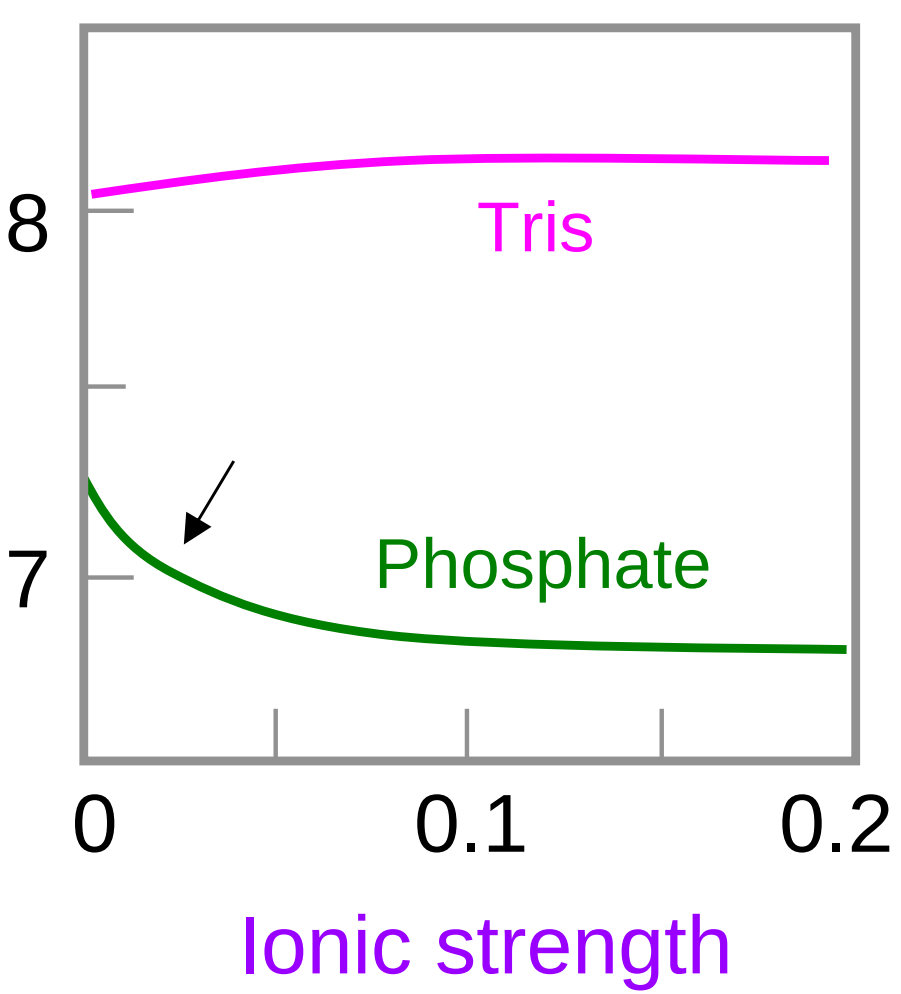
Juang RH (2005) EPA

緩衝液使用注意 Notice for two common buffers

Tris is affected by temperature



Diluted phosphate buffer raises its pH



Juang RH (2005) EPA

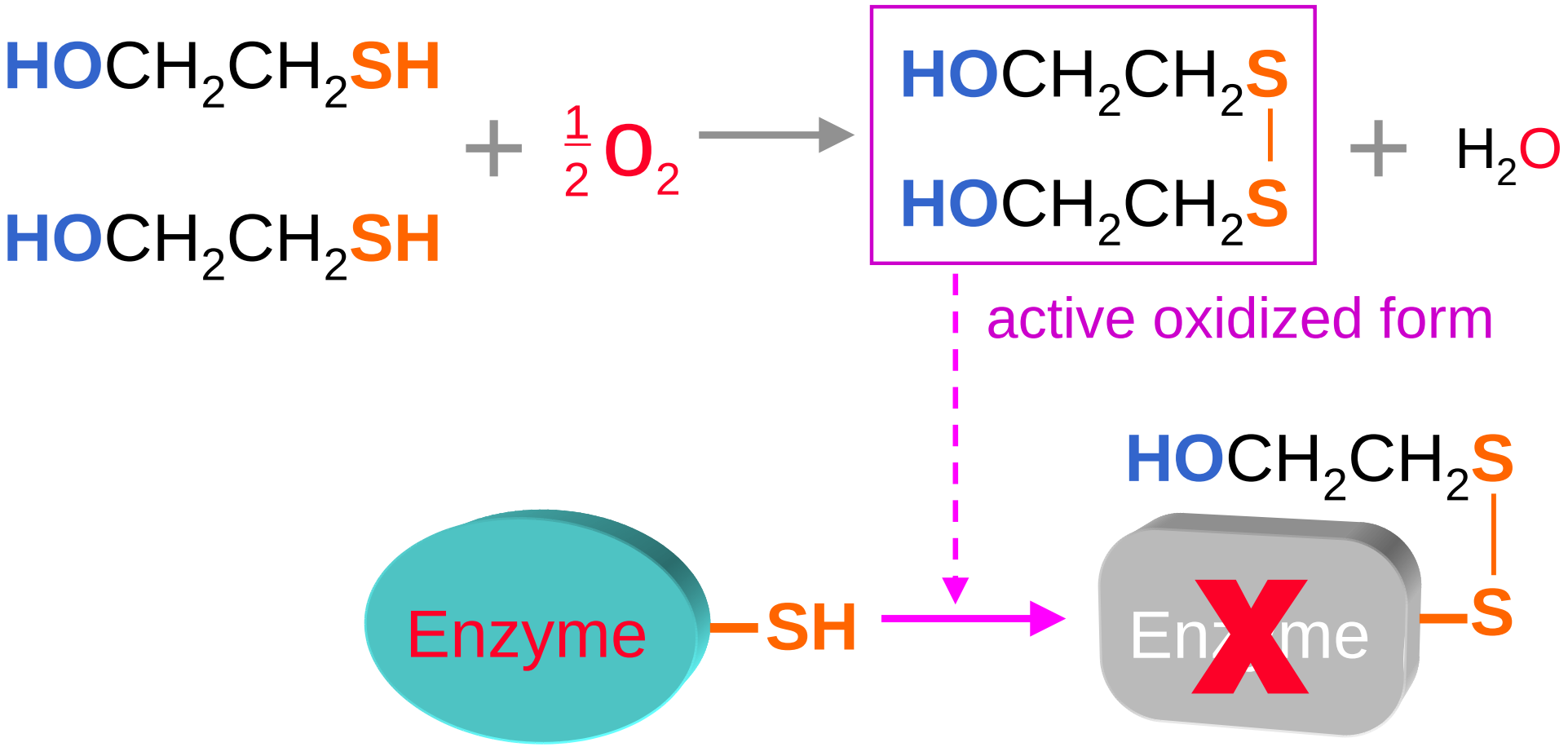
緩衝液常用的添加物 Some common additives

Additives	Action	Concentration
NaN_3 (sodium azide)	Antimicrobials	0.01%
EDTA	Remove metal ions	0.1 - 1 mM
β-Mercaptoethanol	Antioxidant	1 - 10 mM
Dithiothreitol (DTT or DTE)	Antioxidant	1 - 5 mM
BSA (bovine serum albumin)	Stabilizer	0.1 - 10 mg/mL
Tween-20, Triton X-100	Surfactant	0.5 - 0.05%
Glycerol, glucose	Antifreeze	50%
PMSF, TPCK, TLCK, benzamidine etc.	Proteinase inhibitor	Trace amount

Beta-*mercaptoethanol*

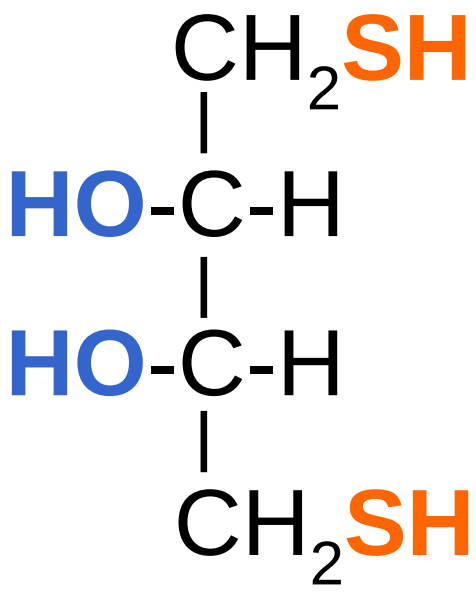
α β

HOCH_2CH_2**SH** **HO**CH_2CH_3

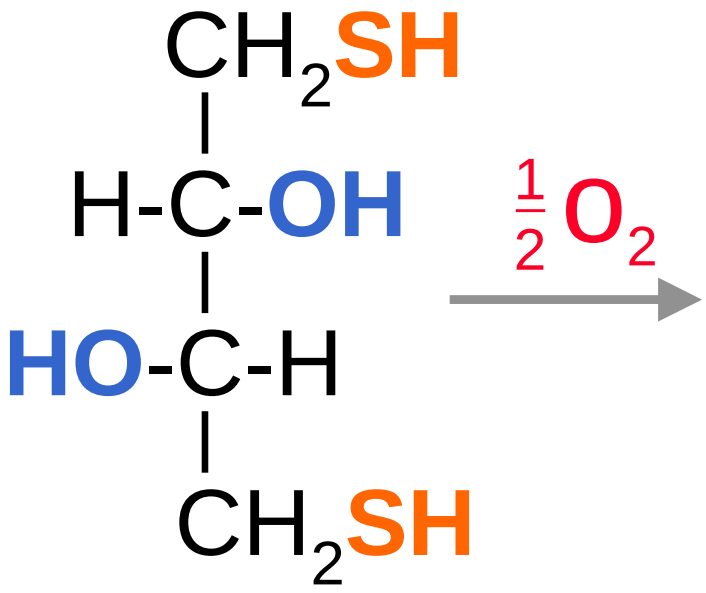


Juang RH (2005) EPA

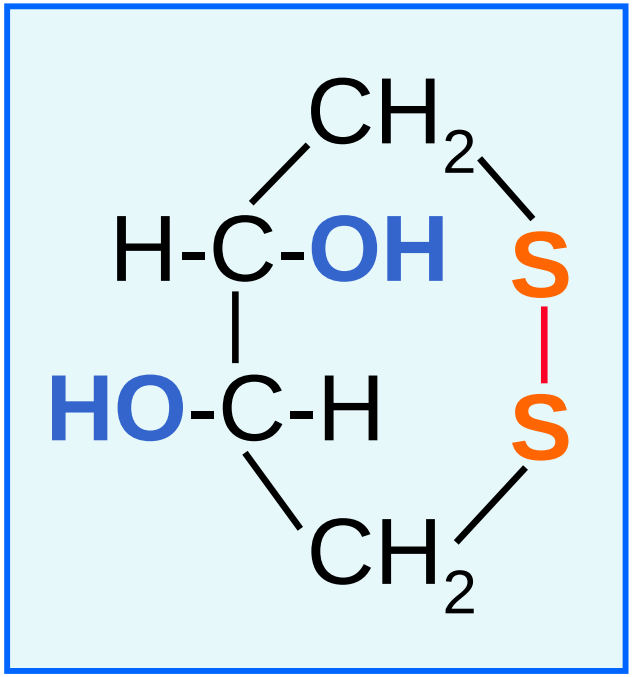
Dithiothreitol (DTT)



Dithioerythritol
(DTE)



Dithiothreitol
(DTT)



Stable cyclic
oxidized form

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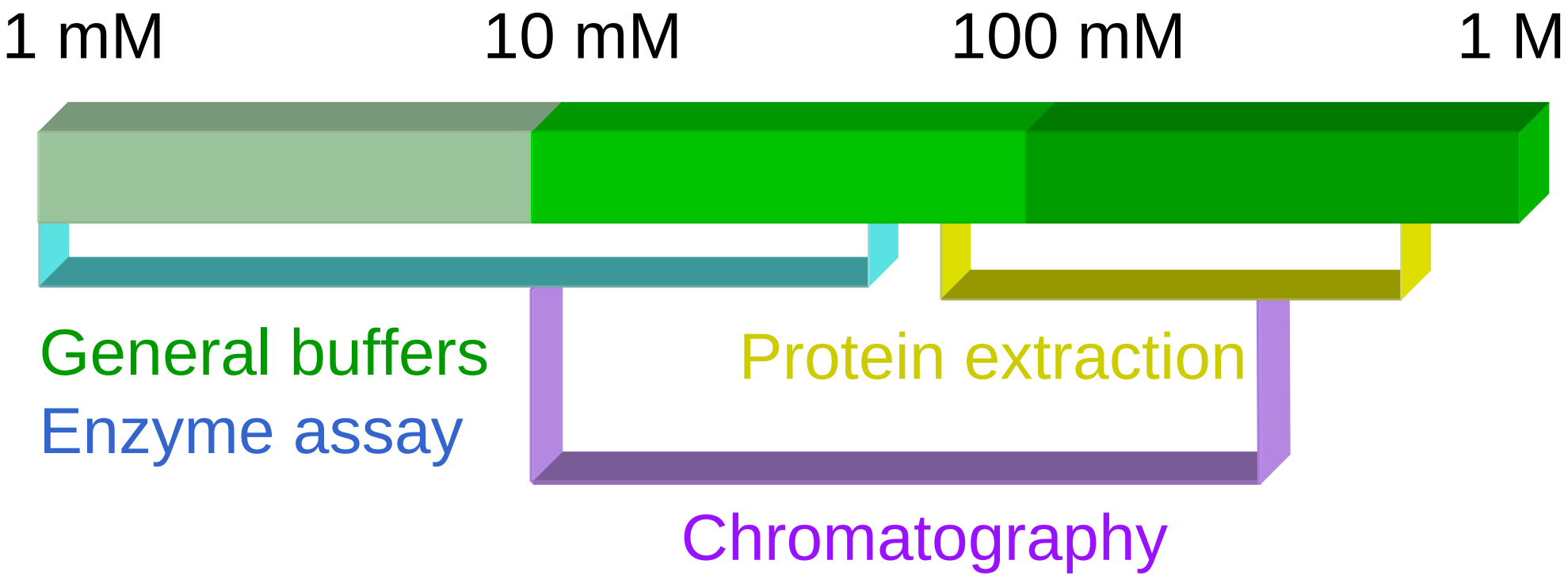
緩衝液使用濃度範圍 Concentration ranges



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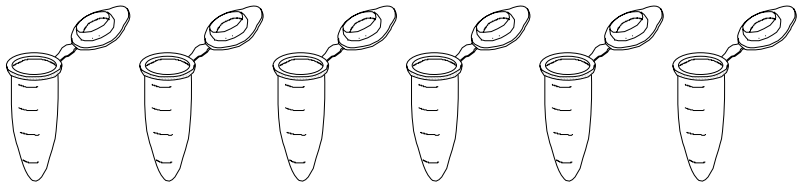
2.3 維持酵素活性 Maintain enzyme activity

- 2.3.1 緩衝液 Buffer
  可維持穩定的酸鹼度及離子濃度以保酵素活性
- 2.3.2 試劑的保存 Reagents
  試劑要依指示保存在適當的地方
- 2.3.3 酵素活性之維持 Maintain the activity
  注意酵素失活的原因有助保持其最高活性
- 2.3.4 酵素活性單位 Enzyme activity unit

試劑的保存注意事項



a. Avoid humidity

Open the bottle of a frozen reagent only when its temperature has been brought back to the room temperature

b. Stored in frozen state

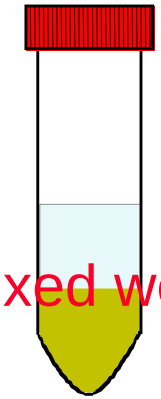
(1) Frequently used reagents should be frozen in aliquot

(2) Avoid repeatedly freezing-thawing

(3) Certain enzymes are very sensitive to freezing L-SP

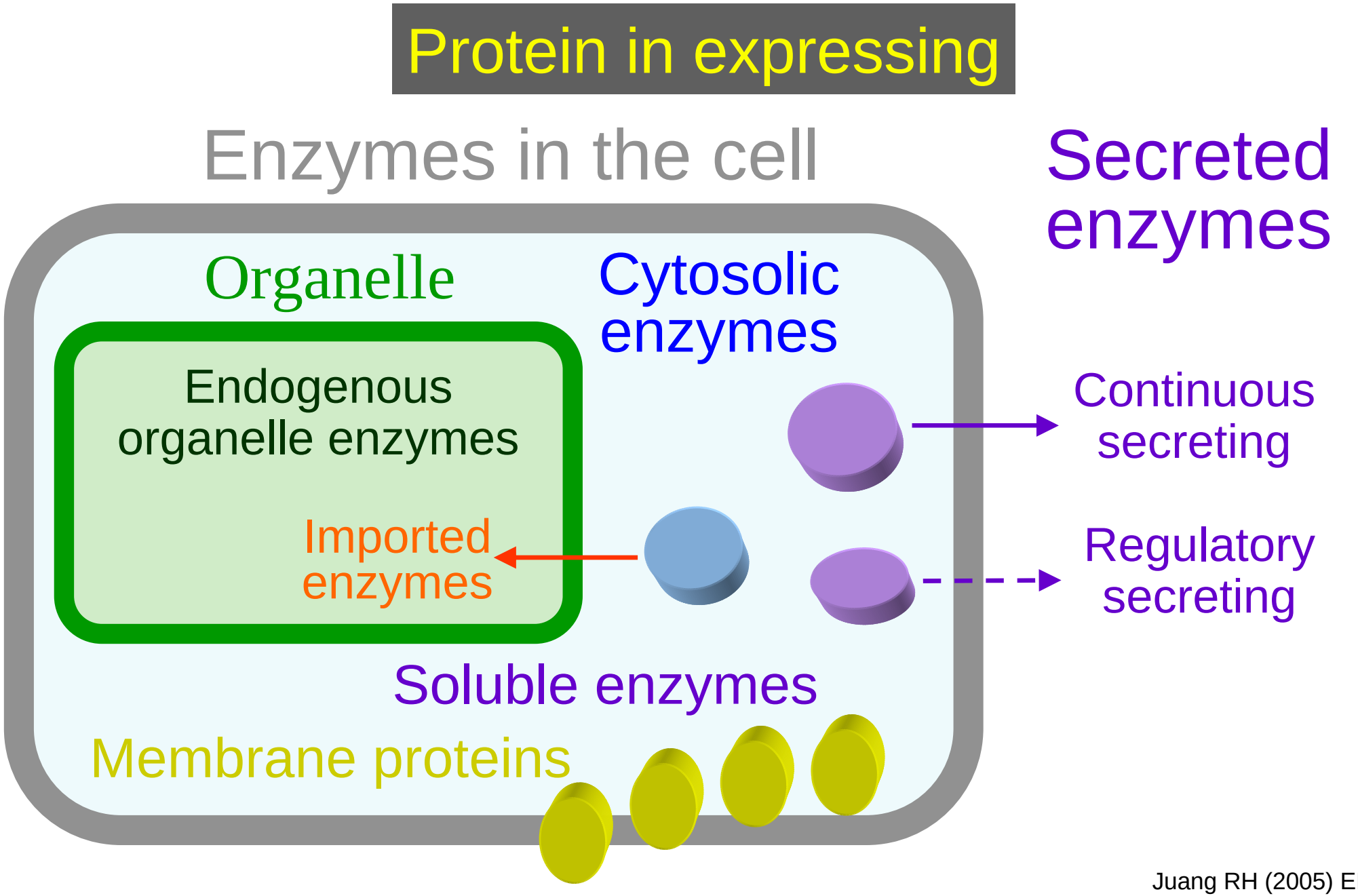
c. Frozen in glycerol

Protein stored in -20℃ in 50% glycerol



d. Avoid light and microbe

細胞內外酵素分佈 Cellular distribution of enzymes



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各種酵素的安定性都不同 Enzyme stability

Reasons enzyme lost its activity:

Protein *Denatured*	Physical/chemical denaturation
Active site destroyed	Chemical reaction
Protease proteolysis	Inactivated by degradation
Enzyme *Inhibitor*	Natural or synthetic inhibitors

Physical denaturation	Heating, freezing, foaming, adsorbing
Chemical denaturation	Extreme pH, oxidation, heavy metals

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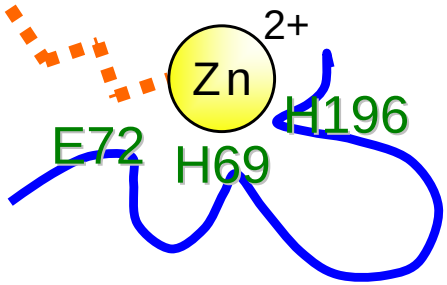
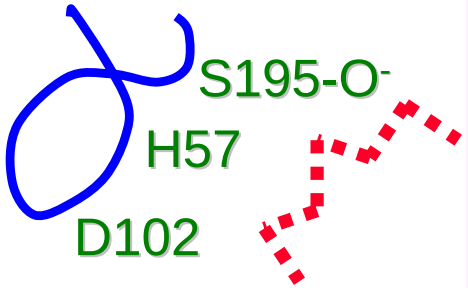
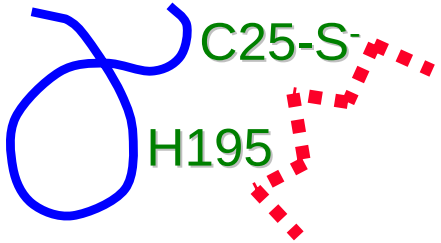
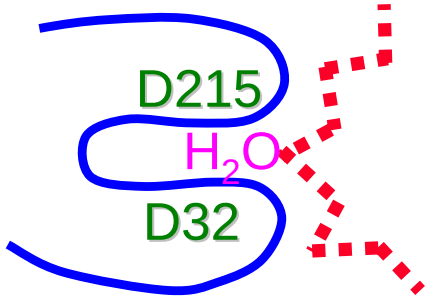
蛋白胨的專一性及其抑制劑 Protease families

Family	Example	Mechanism	Specificity	Inhibitor
Metal Protease	Carboxy-peptidase A		Non-polar	EDTA EGTA
Serine Protease	Chymotrypsin Trypsin		Aromatic Basic	DFP TLCK TPCK
Cysteine Protease	Papain		Non-specific	PCMB Leupeptin
Aspartyl Protease	Pepsin Renin		Non-specific	Pepstatin

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