

Biochemistry 521

Dr. Zucal Suo
740 Bio. Sci.
suo.3@osu.edu
688-3706 (o)

Teaching Assistants:

Mr. Wade Duym
726 Biological Sciences
duym.2@osu.edu

Ms. Soojin Lee
752 Biological Sciences
lee.1998@osu.edu

Lab Supervisor:
Ms. Cynthia Hatfield
868B Bio. Sci.
292-9482
hatfield.66@osu.edu

1

Biochemistry 521

Prerequisites: Biochemistry 511 or 613 or equiv and
past the placement test

Textbooks: Laboratory Manual
Biochemical Techniques by Robyt and White

Read the required text before coming to the lab!

Basis for Grading

Two Midterm exams	2x10%
Final exam	25%
Unannounced Quizzes	15%
Unknowns (~ 4)	10%
Practical exam	10%
Lab reports, techniques, etc.	20%

- No makeup quizzes

2

Laboratory Reports:

1. Work independently in the preparation of lab reports
2. Computer-generated lab reports
3. All lab reports are due one week after completion of the experiment.
4. Late reports lead to points deduction (1.5 points/day)
5. No report will be accepted if it is more than one week late.
6. Grading for each lab report (10 point total):
 - Introduction (1 point)
 - Experimental methods (1 point)
 - Raw data (1 point)
 - Calculations (1 point)
 - Results (3 points)
 - Discussion (3 points)

3

Lab Rules

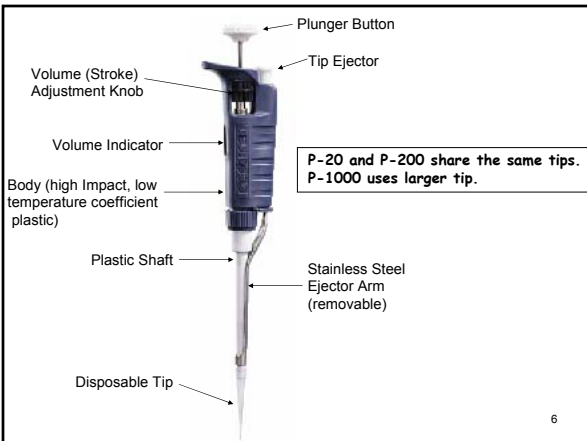
1. Take turns to use equipment
2. Be considerate and be a good citizen
3. Be Cooperative with your partner
4. Dispose trash in proper trash containers (do not throw away used beakers and flasks, leave them in a specific sink).
5. Be gentle to the equipment
6. Follow safety rules
7. Take detail notes
8. Return your pipet set to GTA at the end of each day

4

Session 1

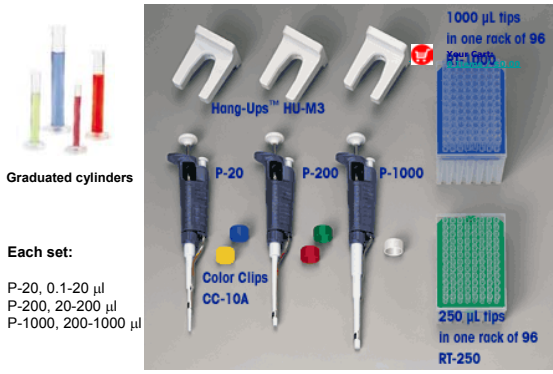
Pipetting and Weighing

5



6

Quantitative Liquid Transfer



Operation Procedures

1. Set the desired volume by dialing downward.
2. Attach a new pipet tip firmly
3. Depress the plunger to the first positive stop
4. Holding the pipet vertically, immerse the tip into the sample liquid to a depth of: 1-2 mm for P-20 and P-200 or 2-4 mm for P-1000.
5. Allow the plunger to return slowly to the Up position. *Never snap up plunger.*
6. Wait for 1-2 seconds to draw a full-volume of sample.
7. Depress the plunger to the Second Stop. This severs to *rinse* the tip.
8. Repeat Steps 5 and 6 to draw sample again into tip.

Operation Procedures

9. Withdrawn the tip from sample liquid. Wipe any fluid remaining on the outside of the tip using a Kimwipe. Do not touch the tip opening.
10. Dispense sample: place the tip end against the sidewall of a receiving vessel, depress Plunger slowly to the First Stop, wait for 1-3 seconds, depress Plunger to the Second Stop, then repeat several times to expel any liquid in the tip. **Suggestion:** withdraw the pipet with its tip sliding along the wall of the vessel.
11. Return plunger to Top Position, then discard the tip by depressing the tip ejector button.

Pipetting Guidelines

1. Pre-rinse tip.
2. Hold a pipet vertically;
3. No air bubbles;
4. No sample splash or jump;
5. Proper pipet tips;
6. No leakage;
7. Never grease any components of a pipet.
8. Temperature, density, acid/corrosives;
9. Regular calibration
10. Do not abuse pipets

10

Pipet Calibration

1. Weigh a weighing boat or a 1.7 ml Eppendorf tube.
2. Set a desired volume of a pipet: e.g. P-20, 10 μ l; P-200, 100 μ l; and P-1000, 500 μ l.
3. Draw distilled water carefully and dispense the sample into a pre-weighed receiving vessel.
4. Accurately weigh the receiving vessel and record the total weight.
5. Repeat the above procedures four more times.
6. Assume $D_{\text{water}} = 1 \text{ g/ml}$, determine the mean volumes, standard and average deviation. Then calculate percent error and reproducibility.
7. Check if your set of pipets is within the accuracy range (< 5% of the standard). If not, report to us.

11

Performance Specifications

Model	Accuracy	Reproducibility
P-20	< 0.1 μ l @ 1-10 μ l < 1% @ 10-20 μ l	< 0.04 μ l @ 2 μ l < 0.05 μ l @ 10 μ l < 0.06 μ l @ 20 μ l
P-200	< 0.5 μ l @ 20-60 μ l < 0.8% @ 60-200 μ l	< 0.15 μ l @ 25 μ l < 0.25 μ l @ 100 μ l < 0.30 μ l @ 200 μ l
P-1000	< 3 μ l @ 100-375 μ l < 0.8% @ 375-1000 μ l	< 0.6 μ l @ 250 μ l < 1.0 μ l @ 500 μ l < 1.3 μ l @ 1000 μ l

12

Weighing



Range: 0-160 gram
Accuracy: 0.01 mg



0-2100 gram
0.1 gram

Weighing Boat



Weighing paper

13

Error Analysis & Significant Figures

Average: $\frac{\sum x_i}{n}$

Mean deviation: $\frac{\sum |x_i - \bar{x}|}{n}$

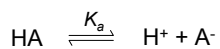
Standard deviation: $\sqrt{\frac{\sum (x_i - \bar{x})^2}{n - 1}}$

Example: 1.32 ± 0.04 g

14

pH and Buffers

$$\text{pH} = -\log_{10}[\text{H}^+]$$



$$\text{p}K_a = -\log_{10}K_a$$

$$\text{pH} = \text{p}K_a + \log_{10}\left\{\frac{[\text{A}^-]}{[\text{HA}]}\right\}$$

Buffer Effective Range: $\text{pH} = \text{p}K_a \pm 1$

15

pH and Buffers

$$\text{pH} = -\log_{10}[\text{H}^+]$$

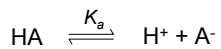
0.1 M HCl, pH = 1

0.1 M NaOH, pOH = 1 \Rightarrow pH = 13
since pH + pOH = 14

What is the pH of 0.1 M acetic acid? pH = 1?

16

pH and Buffers



$$\text{p}K_a = -\log_{10}K_a$$

$$\text{pH} = \text{p}K_a + \log_{10}\left\{\frac{[\text{A}^-]}{[\text{HA}]}\right\}$$

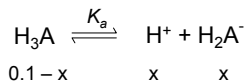
A^- is the conjugate base

1. When $[\text{A}^-] = [\text{HA}]$, pH = $\text{p}K_a$
2. When $[\text{A}^-] \neq [\text{HA}]$, pH \neq $\text{p}K_a$

17

5. What is the pH of each of the following?

e) 0.1 M H_3PO_4 (pKa's = 1.96, 6.8, 12.3)



$$\frac{[\text{H}^+][\text{H}_2\text{A}^-]}{[\text{H}_3\text{A}]} = K_{a1} \Rightarrow \frac{x^2}{0.1 - x} = 10^{-1.96}$$

$$X = 0.028 \text{ M}$$

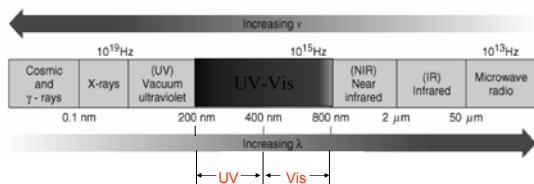
$$\text{pH} = -\log_{10}0.028 = 1.55$$

18

Session 2

Spectrophotometry

Electromagnetic Radiation and Spectra



$$\lambda = c/\nu$$

$$E = h\nu = hc/\lambda$$

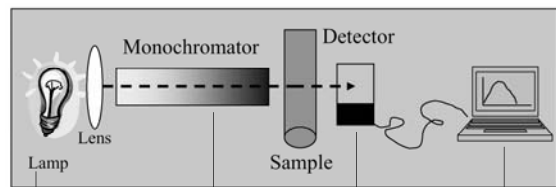
UV/Visible Spectroscopy

- Most UV/visible spectrophotometers cover from
 - 200 to 400 nm (the near ultraviolet) and
 - 400 nm (violet light) to 700 nm (red light)

Region of Spectrum	Wavelength (nm)	Energy (kcal/mol)
ultraviolet	200-400	71.5 - 143
visible	400-700	40.9 - 71.5

C-C bond Disassociation energy ~ 95Kcal/mol

Single Beam + Memory



Tungsten lamp: 400-700 nm
Deuterium lamp: 200-400 nm

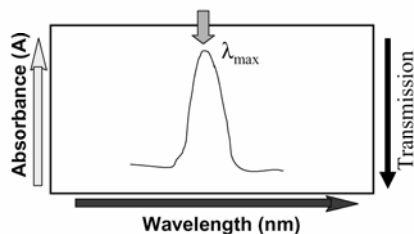
Phototube or
Photomultiplier

Recorder

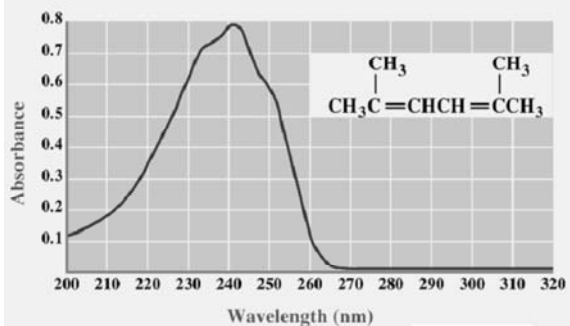
Selecting a narrow band of wavelengths

UV/Vis Spectroscopy

- UV-Vis spectral data are plotted as absorbance (A) versus wavelength (nm)

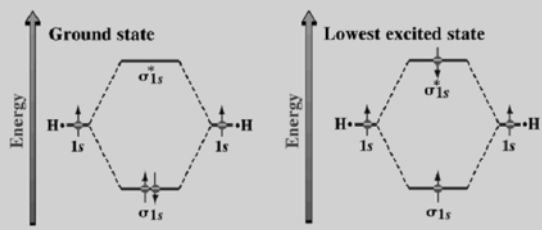


UV spectrum of 2,5-dimethyl-2,4-hexadiene



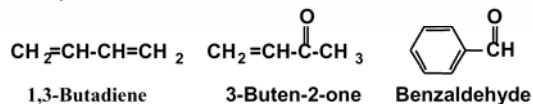
Origin of UV-Vis Absorbance MO Theory

MO energy diagram for the hydrogen molecule, H₂



Electronic Transitions

- Absorption of UV-vis radiation results in transition of electrons from a lower energy occupied MO to a higher energy unoccupied MO
- For example, π to π^* transitions in conjugated systems such as



UV/Vis Spectroscopy

- Absorbance: a quantitative measure of the extent to which a compound absorbs ultraviolet-visible radiation at a particular wavelength

$$\text{Absorbance (A)} = \log \frac{I_0}{I}$$

Where:

I_0 is the intensity of the incident radiation on the sample
 I is the intensity transmitted through the sample

UV/Vis Spectroscopy

- Transmission: a quantitative measure of the extent to which a compound absorbs ultraviolet-visible radiation at a particular wavelength

$$\% \text{Transmission (T)} = \left[\frac{I}{I_0} \right] \times 100$$

Where:

I_0 is the intensity of the incident radiation on the sample

I is the intensity transmitted through the sample

Beer-Lambert law

- Beer-Lambert law: the relationship between absorbance, concentration, and length of the sample tube

$$\text{Beer-Lambert Law: } A = \epsilon c l$$

A = absorbance

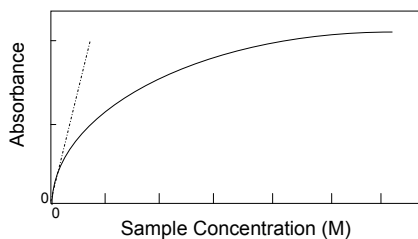
c = concentration ($\text{mol} \cdot \text{liter}^{-1}$)

l = length of the sample tube (cm)

ϵ = molar absorptivity ($\text{liter} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

Experimental values of ϵ range from 0 to 10^6

The ϵ values of DNA and protein can be calculated.
for large DNA plasmids: $\epsilon = 0.02 \text{ cm}^2/\text{ng}$



When A is out of the linear range, dilute your samples before measuring absorbance. When you calculate the sample concentration, do not forget the dilution factor.

$$A = \epsilon c l$$

Samples or Equipment	Suggested Linear Absorbance Range (A)
Proteins	0.05-0.3
DNA	0.05-0.3
Shimadzu	< 1.0

- If $A < 0.05$, the signal/noise is too low, leading to errors.
- UV or Vis light passes through your sample solution.
- Cuvettes must be transparent at the wavelengths you are measuring.

Bench Work

1. Absorption Spectrum of cytochrome C

- Zero the absorbance of a buffer from 355-600 nm
- Measure the absorbance spectrum of a cytochrome C solution from 355-600 nm,
- What is λ_{\max} ?

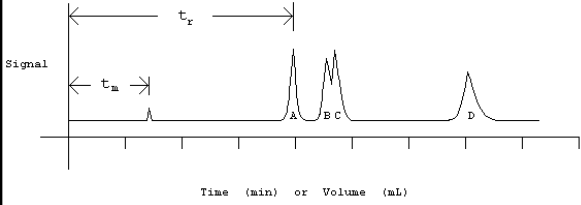
2. Measure ϵ_{415} of cytochrome C (MW = 12.4 kDa)

- Prepare four different samples of cytochrome C
- Measure A_{415} of each sample
- Calculate ϵ_{415} of cytochrome C

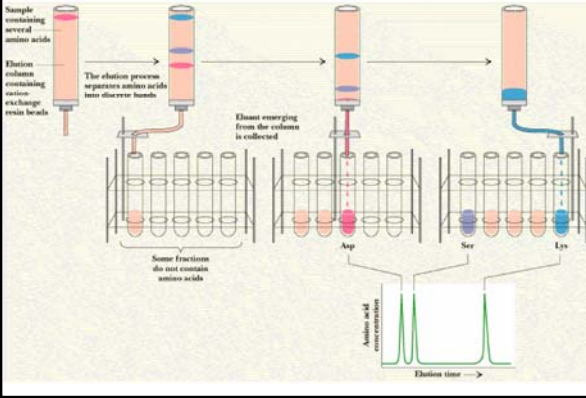
Attention: linear range, slope.

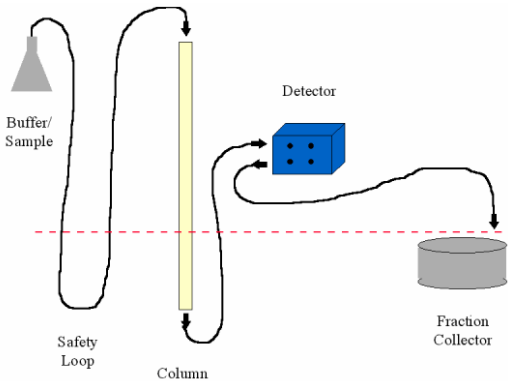
Session 3 CHROMATOGRAPHY

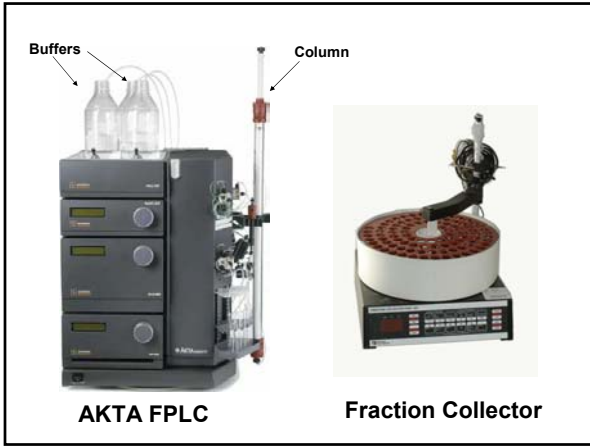
Chromatography is a separation process that involves partitioning a protein (or any other soluble analyte) between an insoluble **stationary phase** and a **mobile phase** that passes over its surface



Column Chromatography







Column Chromatography

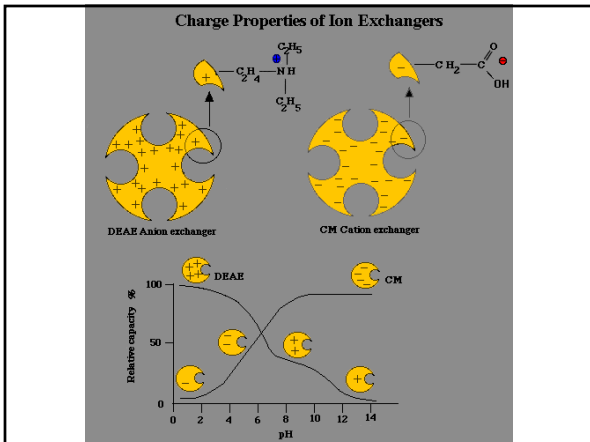
A) Ion Exchange Chromatography:

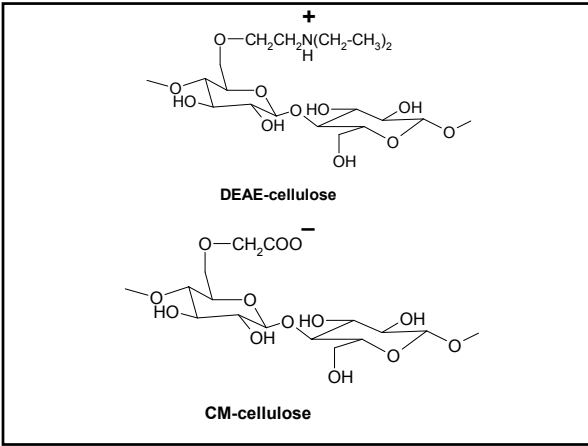
- cation
- anion

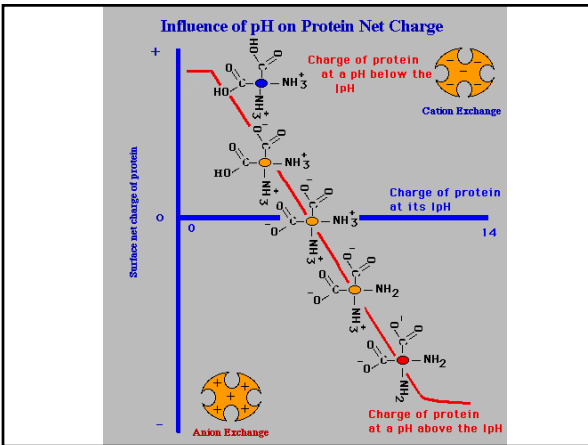
B) Gel Filtration Chromatography

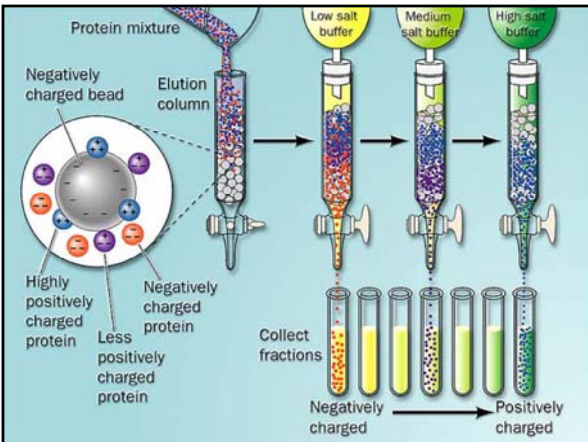
C) Hydrophobic Interaction Chromatography

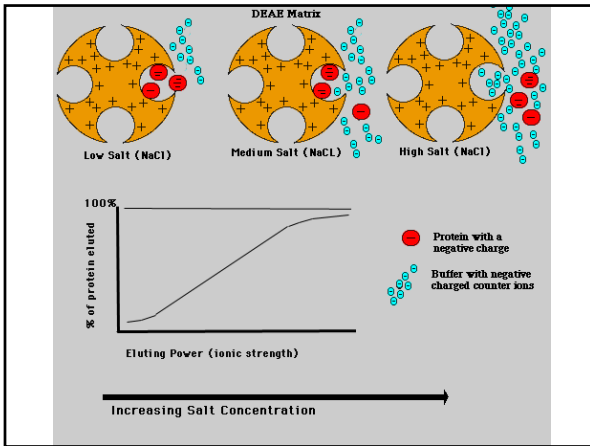
D) Affinity Chromatography







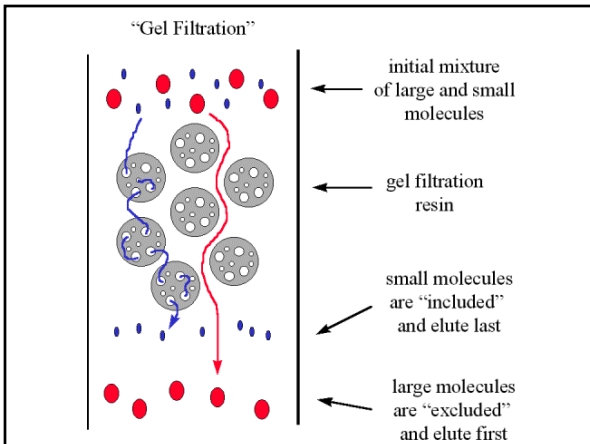


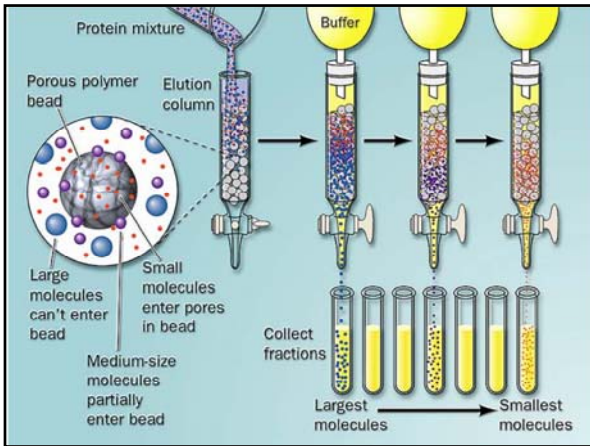


Gel filtration

Gel filtration does not rely on any chemical, affinity or charge interaction with the protein, rather it is based on a physical property of the protein – **size**.

- Large proteins which cannot enter these pores pass around the outside of the beads.
- Smaller proteins which can enter the pores of the beads have a longer, tortuous path before they exit the bead.



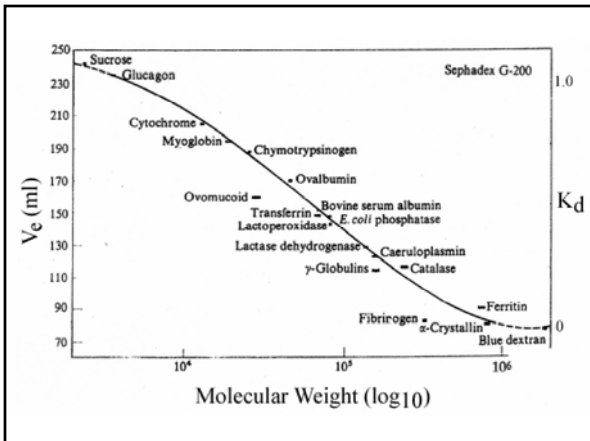


Void Volume

The void volume is the volume of mobile phase (V_m or V_0) in a column.

e.g. if the stationary phase occupies 40% of the total column volume, the void volume would be 60% of the total column volume.

Consider a column that is 25 cm long with an inner diameter of 1 cm. The total column volume is 19.6 ml ($V = \pi r^2 L = 3.14 * 0.5^2 * 25$ cm). If the mobile phase occupies 60% of the column volume, the void volume is 11.8 mL.



Gel filtration

1. Protein purification
2. Protein size estimation
3. Protein desalting

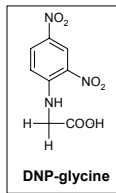
Bench Work

1. Separate BSA from red cytochrome C using cation exchange chromatography

- a. Low salt buffer
- b. High salt buffer

2a. Measure void volume of a gel-filtration column using Blue Dextran

2b. Separate BSA from yellow DNP-glycine using gel filtration chromatography



CM Sephadex: a weak cation exchanger, a carboxyl methyl group

Sephadex G50: size exclusion matrix

Sephadex: glucose-based polymers linked through α -1,6 hydroxyl groups

Component	Color	λ_{\max} (nm)	MW (Da)	pI
Blue dextran	blue	620	~2 million	<2
Cytochrome c	red	410	12,400	10
BSA	colorless	280	68,750	5.82
DNP-Glycine	yellow	360	241	3

Precaution:

1. Check columns for leakage before packing
2. Do not dry resin and pack the column evenly.
3. Get rid of air bobbles from the column
4. Do not disturb the top surface of the packed resins.
Make the surface even.
5. Pay attention to which protein elutes first.
6. Apply low salt buffer first, then high salt buffer to an ion exchange column.

Session 4

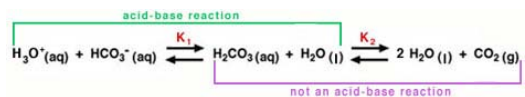
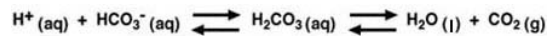
The pKa of TRIS & Heat of Ionization (ΔH)

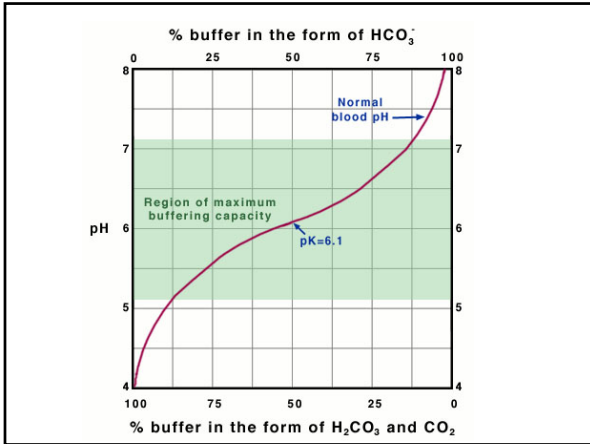
Biological Functions are Sensitive to pH

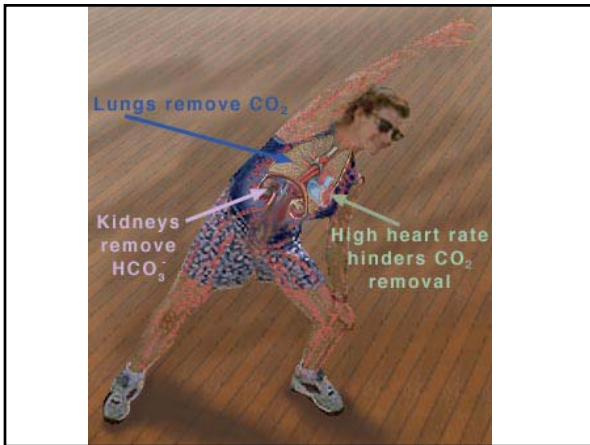
- Blood pH is 7.4
- Blood pH above 7.4 = alkalosis
- Blood pH below 7.4 = acidosis
- A change of pH of 0.2 units in either direction is considered serious
- Blood pH is mainly regulated by the lungs and kidneys in mammals

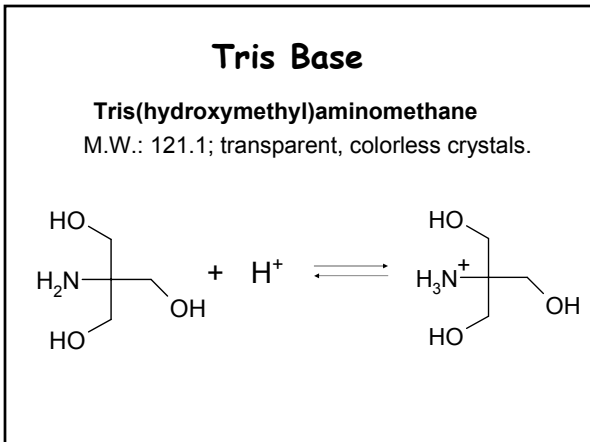
2. The Chief Mammalian Blood Buffer is a Mixture of Bicarbonate and Carbon Dioxide

There are also other buffers in blood, such as proteins and phosphate, but they are less important.







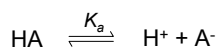




$$E = \text{constant} + \frac{2.303RT}{F} \text{pH}$$

Calibration using standard buffers

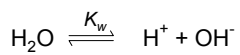
pH and Buffers



$$\text{p}K_a = -\log_{10} K_a$$

$$\text{pH} = \text{p}K_a + \log_{10} \left\{ \frac{[\text{A}^-]}{[\text{HA}]} \right\}$$

$$\text{p}K_a = \text{pH} - \log_{10} \left\{ \frac{[\text{A}^-]}{[\text{HA}]} \right\}$$



$$K_w = [\text{H}^+][\text{OH}^-] = 10^{-14} \text{ M}$$

$$\text{pH} + \text{pOH} = 14$$

$$\text{pH} = \text{pOH} = 7, \text{ neutral solution}$$

pH Buffers at Various Temperatures

Temperature (°C)	4.01 Buffer	6.86 Buffer	9.18 Buffer
	0.05M Potassium Acid Phthalate	0.025M KH_2PO_4 0.025M Na_2HPO_4	0.01M Borax
0°	4.00 pH	6.98 pH	9.46 pH
10°	4.00 pH	6.92 pH	9.33 pH
20°	4.00 pH	6.88 pH	9.22 pH
25°	4.01 pH	6.86 pH	9.18 pH
30°	4.02 pH	6.85 pH	9.14 pH
40°	4.04 pH	6.84 pH	9.07 pH
50°	4.06 pH	6.83 pH	9.01 pH
60°	4.09 pH	6.84 pH	8.96 pH

Van't Hoff Equation

$$\log \frac{K_2}{K_1} = \frac{-\Delta H}{4.576} \left[\frac{1}{T_2} - \frac{1}{T_1} \right]$$

$$pK = -\log_{10} K$$

$$\log \frac{K_2}{K_1} = pK_1 - pK_2$$

Units:

- ΔH : calories; T: °K

Bench Work

1. pKa at Room Temperature and 0 °C
2. pH meter
3. Calculate the heat of ionization of Tris
4. Statistical errors
