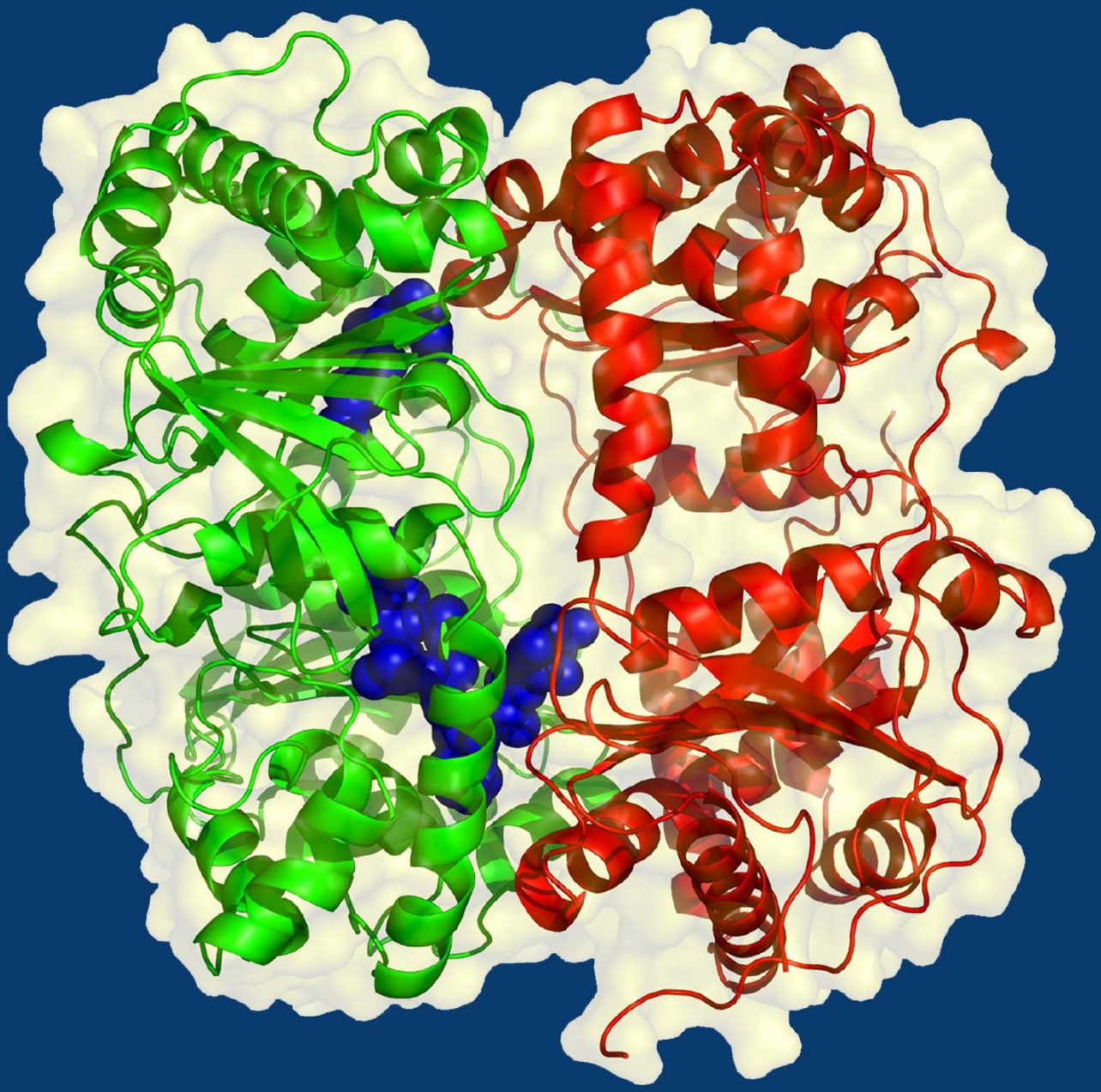
MARMARA UNIVERSITY
FACULTY OF ARTS AND SCIENCES
DEPARTMENT OF CHEMISTRY

BIOCHEMISTRY LABORATORY MANUAL



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Cover: Structure of the complex of beta-amyloid (blue) with human insulin degrading enzyme.

Laboratuvar Kuralları ve Güvenlik

Biyokimya laboratuvarı güz döneminde, biyokimyanın konusunu oluşturan biyomoleküllerin çeşitli özelliklerinin incelenmesi ve biyokimyada yaygın olarak kullanılan tekniklerle ilgili genel prosedürlerin tanıtılması hedeflenmektedir. Ayrıca biyokimyada sık olarak kullanılan bazı cihazların kullanımı öğretilecektir.

Her labarotuvardan önce o gün yapılacak olan deneyi mutlaka dikkatle okuyunuz. Her deneyden önce bir kısa sınav yapılacaktır. Bu sınav sonucunda başarısız olan öğrenci o deneye alınmaz.

Laboratuvarda çalışırken laboratuvar önlüğü, laboratuvar eldiveni ve koruyucu gözlük kullanılır. Önlük ve eldivenlerinizi laboratuvardan ayrılıncaya kadar çıkarmayınız. Bazı yumuşak lensler laboratuvar kimyasalları ile tepkimeye girerek rahatsızlık oluşturacağından, eğer mümkünse kontak lenslerinizi laboratuvara girmeden önce çıkarınız. Deney esnasında laboratuvar görevlilerinin talimatlarını dikkatle uygulayınız. Deney bitiminde tüm cihazları kapatıp, çalıştığınız yerin temizliğini iyi bir şekilde yapınız.

Bazı kimyasallar zehirli, mutajenik, karsinojenik veya teratojenikdir (doğum kusurlarına yol açan). Bu kimyasalların kullanımında ve atılmalarında laboratuvardaki görevlilerin yaptığı uyarıları dikkate alınız.

Tüm öğrencilerin bir laboratuvar not defteri tutmaları istenmektedir. Deney esnasında yaptığınız her bir ölçümü, deney aşamasını, dikkatle laboratuvar not defterinize not alınız. Her deneyin tamamlanmasından sonra en geç bir sonraki deneyden bir gün önce deney raporlarınızı teslim ediniz. Bu raporlar
http://kmy.fef.marmara.edu.tr/ogrencilere/biyokimya-anabilim-dali-duyurular/
web sayfasında bulunan Rapor Formatına göre hazırlanmalıdır.

Öğrenciler deneyleri gruplar halinde yapmaktadırlar ancak her bir öğrenci kendi yorumlarını, kendi hazırladığı deney raporu ile sunmalıdır. Birbirinin aynı olan ve/veya çalışma sorularına cevap verilmemiş deney raporları kabul edilmez.

Her dönemde bir ara sınav yapılır. Deney raporlarının %20'si ve ara sınavın %80'i alınarak öğrencinin vize notu saptanır. Ara sınavdan sonra yapılan deneyler için de deney raporlarının %20'si ve finalin %80'i alınarak öğrencinin final notu saptanır.

Her deneyden önce yapılan quiz sınavına girmeyen ya da sınavda başarısız olan öğrenci o deneye giremez ve girmediği deneyin raporunu veremez.

Biyokimya laboratuvarında devam zorunluluğu %80'dir. 3. kez laboratuvara gelmeyen bir öğrenci devamsızlıktan kalmış olur ve sınavlara alınmaz.

EXPERIMENT I

BUFFER SOLUTIONS AND pI OF AMINO ACIDS

Buffer Solutions

Buffers are aqueous systems that tend to resist changes in pH when small amounts of acid (H^+) or base (OH^-) are added. A buffer system consists of a weak acid (the proton donor) and its conjugate base (the proton acceptor).

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

Henderson-Hasselbalch Equation
(A^-: Salt / HA: Acid)

This equation could be used to calculate theoretical pH value of a buffer solution. As seen from the equation pH of the buffer solution is dependent on the concentration ratio of weak acid (or weak base) and its conjugated base (conjugated acid).

When you consider acetic acid/sodium acetate buffer system, when the concentration of the proton donor (acetic acid) exactly equals that of the proton acceptor (acetate), the buffering power of the system is maximal; that is, its pH changes least on addition of H^+ or OH^-. Since log1 is equal to zero the pH at this point for acetic acid is equal to its pKa (4,76) and known as **optimal pH**. One pH unit above or lower of optimal pH is called as **buffering range** (for this example pH= 3,76-5,76).

The intracellular and extracellular fluids of multicellular organisms have a characteristic and nearly constant pH (blood pH 7,4; stomach fluid pH 1,5; pancreatic fluid pH 8). The organism's first line of defense against changes in internal pH is provided by buffer systems. The cytoplasm of most cells contains high concentrations of proteins, and these proteins contain many amino acids with functional groups that are weak acids or weak bases. For example, the side chain of histidine has a pKa of 6.0 and thus can exist in either the protonated or unprotonated form near neutral pH. Proteins containing histidine residues therefore buffer effectively near neutral pH. Nucleotides such as ATP, as well as many metabolites of low molecular weight, contain ionizable groups that can contribute buffering power to the cytoplasm. Two especially important biological buffers are the phosphate and bicarbonate systems.

The normal pH value for the body fluids is between pH 7.35 and 7.45. When the pH value of body fluids is below 7.35, the condition is called acidosis, and when the pH is above 7.45, it is called alkalosis. Metabolism produces acidic products that lower the pH of the body fluids. For example, carbon dioxide is a by-product of metabolism, and carbon dioxide combines with water to form carbonic acid. Also, lactic acid is a product of anaerobic metabolism, protein metabolism produces phosphoric and sulfuric acids, and lipid metabolism produces fatty acids. These acidic substances must continuously be eliminated from the body to maintain pH homeostasis. Rapid elimination of acidic products of metabolism results in alkalosis, and the failure to eliminate acidic products of metabolism results in acidosis.

No.	Name	pH range	Temperature	pH change per °C
	General buffers			
1	KCl/HCl (CLARK and LUBS)[1]	1.0- 2.2	Room	0
2	Glycine/HCl (SØRENSEN)[2]	1.2- 3.4	Room	0
3	Na citrate/HCl (SØRENSEN)[2]	1.2- 5.0	Room	0
4	K biphthalate/HCl (CLARK and LUBS)[1]	2.4- 4.0	20° C	+0.001
5	K biphthalate/NaOH (Clark and LUBS)[1]	4.2- 6.2	20° C	
6	Na citrate/NaOH (SØRENSEN)[2]	5.2- 6.6	20° C	+0.004
7	Phosphate (SØRENSEN)[2]	5.0- 8.0	20° C	−0.003
8	Barbital-Na/HCl (MICHAELIS)[3]	7.0- 9.0	18° C	
9	Na borate/HCl (SØRENSEN)[2]	7.8- 9.2	20° C	−0.005
10	Glycine/NaOH (SØRENSEN)[2]	8.6-12.8	20° C	−0.025
11	Na borate/NaOH (SØRENSEN)[2]	9.4-10.6	20° C	−0.01
	Universal buffers			
12	Citric acid/phosphate (MCILVAINE)[4]	2.2- 7.8	21° C	
13	Citrate-phosphate-borate/HCl (TEORELL and STENHAGEN)[5]	2.0-12.0	20° C	
14	BRITTON-ROBINSON[6]	2.6-11.8	25° C	at low pH 0 at high pH −0.02
	Buffers for biological media			
15	Acetate (WALPOLE)[7,9]	3.8- 5.6	25° C	
16	Dimethylglutaric acid/NaOH[10]	3.2- 7.6	21° C	
17	Piperazine/HCl[11,12]	4.6- 6.4 8.8-10.6	20° C	
18	Tetraethylethylenediamine*[12]	5.0- 6.8 8.2-10.0	20° C	
19	Trismaleate[7,13]	5.2- 8.6	23° C	
20	Dimethylaminoethylamine*[12]	5.6- 7.4 8.6-10.4	20° C	
21	Imidazole/HCl[14]	6.2- 7.8	25° C	
22	Triethanolamine/HCl[15]	7.0- 8.8	25° C	
23	*N*-Dimethylaminoleucylglycine/NaOH[16]	7.0- 8.8	23° C	−0.015
24	Tris/HCl[7]	7.2- 9.0	23° C	−0.02
25	2-Amino-2-methylpropane-1,3-diol/HCl[7,13]	7.8-10.0	23° C	
26	Carbonate (DELORY and KING)[7,17]	9.2-10.8	20° C	

Buffer No.	Stock solutions		Composition of the buffer
	A	B	
1	KCl 0.2-N (14.91 g/l)	HCl 0.2-N	25 ml A + x ml B made up to 100 ml
2	Glycine 0.1-molar in NaCl 0.1-N (7.507 g glycine + 5.844 g NaCl/l)	HCl 0.1-N	x ml A + (100 − x) ml B
3	Disodium citrate 0.1-molar (21.01 g $C_6H_8O_7 \cdot 1H_2O$ + 200 ml NaOH 1-N per litre)	HCl 0.1-N	x ml A + (100 − x) ml B
4	Potassium biphthalate 0.1-molar (20.42 g $KHC_8H_4O_4$/l)	HCl 0.1-N	50 ml A + x ml B made up to 100 ml
5	As No. 4	NaOH 0.1-N	50 ml A + x ml B made up to 100 ml
6	As No. 3	NaOH 0.1-N	x ml A + (100 − x) ml B
7	Monopotassium phosphate 1/15-molar (9.073 g KH_2PO_4/l)	Disodium phosphate 1/15-molar (11.87 g $Na_2HPO_4 \cdot 2H_2O$/l)	x ml A + (100 − x) ml B
8	Barbital sodium 0.1-molar (20.62 g/l)	HCl 0.1-N	x ml A + (100 − x) ml B
9	Boric acid, half-neutralized, 0.2-molar (corr. to 0.05-molar borax: 12.37 g boric acid + 100 ml NaOH 1-N per litre)	HCl 0.1-N	x ml A + (100 − x) ml B
10	As No. 2	NaOH 0.1-N	x ml A + (100 − x) ml B
11	As No. 9	NaOH 0.1-N	x ml A + (100 − x) ml B
12	Citric acid 0.1-molar (21.01 g $C_6H_8O_7 \cdot 1H_2O$/l)	Disodium phosphate 0.2-molar (35.60 g $Na_2HPO_4 \cdot 2H_2O$/l)	x ml A + (100 − x) ml B
13	To citric acid and phosphoric acid solutions (ca. 100 ml), each equivalent to 100 ml NaOH 1-N, add 3.54 cryst. orthoboric acid and 343 ml NaOH 1-N, and make up the mixture to 1 litre	HCl 0.1-N	20 ml A + x ml B made up to 100 ml
14	Citric acid, monopotassium phosphate, barbital, boric acid, all 0.02857-molar (6.004 g $C_6H_8O_7 \cdot 1H_2O$, 3.888 g KH_2PO_4, 5.263 g barbital, 1.767 g H_3BO_3/l)	NaOH 0.2-N	100 ml A + x ml B
15	Sodium acetate 0.1-N (8.204 g $C_2H_3O_2Na$ or 13.61 g $C_2H_3O_2Na \cdot 3H_2O$/l)	Acetic acid 0.1-N (6.005 g/l)	x ml A + (100 − x) ml B
16	ββ-Dimethylglutaric acid 0.1-molar (16.02 g/l)	NaOH 0.2-N	(a) 100 ml A + x ml B made up to 1000 ml (b) 100 ml A + x ml B + 5.844 g NaCl made up to 1000 ml (NaCl ≙ 0.1-molar)
17	Piperazine 1-molar (86.14 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
18	Tetraethylethylenediamine 1-molar (172.32 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
19	Tris acid maleate 0.2-molar (24.23 g tris[hydroxymethyl]-aminomethane + 23.21 g maleic acid or 19.61 g maleic anhydride/l)	NaOH 0.2-N	25 ml A + x ml B made up to 100 ml
20	Dimethylaminoethylamine 1-molar (88 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
21	Imidazole 0.2-molar (13.62 g/l)	HCl 0.1-N	25 ml A + x ml B made up to 100 ml
22	Triethanolamine 0.5-molar (76.11 g/l) containing 20 g/l ethylenediaminetetraacetic acid disodium salt ($C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$)	HCl 0.05-N	10 ml A + x ml B made up to 100 ml
23	*N*-Dimethylaminoleucylglycine 0.1-molar (24.33 g $C_{10}H_{20}O_3N_2 \cdot \frac{1}{2}H_2O$/l) containing NaCl 0.2-N (11.69 g/l)	NaOH 1-N 100 ml made up to 1 litre with A	x ml A + (100 − x) ml B
24	Tris 0.2-molar (24.23 g tris[hydroxymethyl]aminomethane/l)	HCl 0.1-N	25 ml A + x ml B made up to 100 ml
25	2-Amino-2-methylpropane-1,3-diol 0.1-molar (10.51 g/l)	HCl 0.1-N	50 ml A + x ml B made up to 100 ml
26	Sodium carbonate anhydrous 0.1-molar (10.60 g/l)	Sodium bicarbonate 0.1-molar (8.401 g/l)	x ml A + (100 − x) ml B

pH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16a	16b	17	18	19	20	21	22	23	24	25	26	pH
1.0	54.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.0
1.2	36.0	11.1	9.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.2
1.4	23.2	26.4	17.9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.4
1.6	14.7	36.2	23.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.6
1.8	9.3	43.9	27.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.8
2.0	5.9	50.7	30.2	...	...	...	...	...	...	...	...	...	74.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.0
2.2	3.8	56.5	32.2	...	...	...	...	...	...	...	...	98.8	68.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.2
2.4	...	62.3	34.1	41.0	...	...	...	...	...	...	...	94.5	64.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.4
2.6	...	68.4	36.0	34.3	...	...	...	...	...	...	...	90.0	61.3	1.6	...	...	...	...	...	...	...	...	...	...	...	...	...	2.6
2.8	...	74.7	37.9	27.8	...	...	...	...	...	...	...	85.1	58.9	3.6	...	...	...	...	...	...	...	...	...	...	...	...	...	2.8
3.0	...	81.0	39.9	21.6	...	...	...	...	...	...	...	80.3	56.9	5.7	...	...	...	...	...	...	...	...	...	...	...	...	...	3.0
3.2	...	86.2	42.1	15.9	...	...	...	...	...	...	...	76.0	55.2	7.8	...	7.0	14.4	...	...	...	...	...	...	...	...	...	...	3.2
3.4	...	90.3	44.8	10.9	...	...	...	...	...	...	...	72.0	53.9	9.9	...	13.3	20.9	...	...	...	...	...	...	...	...	...	...	3.4
3.6	...	...	47.8	6.7	...	...	...	...	...	...	...	68.4	52.9	11.7	10.9	20.7	26.8	...	...	...	...	...	...	...	...	...	...	3.6
3.8	...	...	51.2	3.3	...	...	...	...	...	...	...	65.1	51.8	13.5	16.6	26.3	32.4	...	...	...	...	...	...	...	...	...	...	3.8
4.0	...	...	55.1	0.0	...	...	...	...	...	...	...	62.0	50.7	15.3	23.9	32.4	36.6	...	...	...	...	...	...	...	...	...	...	4.0
4.2	...	...	60.0	...	3.0	...	...	...	...	...	...	59.1	49.7	17.5	33.5	36.2	40.3	...	...	...	...	...	...	...	...	...	...	4.2
4.4	...	...	66.4	...	6.7	...	...	...	...	...	...	56.4	48.6	19.7	39.3	39.3	43.1	...	...	...	...	...	...	...	...	...	...	4.4
4.6	...	...	74.9	...	11.1	...	...	...	...	...	...	53.7	47.5	21.9	44.9	41.3	45.7	94.3	...	...	...	...	...	...	...	...	...	4.6
4.8	...	...	85.6	...	16.5	...	...	...	...	...	...	51.2	46.4	24.1	56.6	43.5	48.3	91.5	...	...	...	...	...	...	...	...	...	4.8
5.0	...	...	100.0	...	22.6	...	99.2	...	...	...	...	49.0	45.4	26.3	67.8	45.7	51.5	87.8	...	...	...	...	...	...	...	...	...	5.0
5.2	...	...	...	...	28.8	87.1	98.4	...	...	...	...	46.9	44.3	28.6	76.8	48.4	53.6	83.6	94.3	3.2	...	...	...	...	...	...	...	5.2
5.4	...	...	...	...	34.4	78.0	97.3	...	...	...	...	44.7	43.2	31.0	84.0	51.3	58.2	77.6	87.8	5.0	...	...	...	...	...	...	...	5.4
5.6	...	...	...	...	39.1	70.3	95.5	...	...	...	...	42.4	42.0	33.4	89.3	55.0	63.6	71.8	83.1	7.3	94.3	...	...	...	...	...	...	5.6
5.8	...	...	...	...	42.4	64.5	92.8	...	...	...	...	40.0	40.8	35.8	...	58.8	68.7	66.5	77.6	9.7	91.7	...	...	...	...	...	...	5.8
6.0	...	...	...	...	45.0	60.3	88.9	...	...	...	...	37.4	39.7	38.3	...	63.9	73.6	61.8	71.7	12.4	88.0	...	...	...	...	...	...	6.0
6.2	...	...	...	...	46.7	57.2	83.0	...	...	...	...	34.5	38.4	40.8	...	69.5	78.5	58.2	66.4	15.2	83.3	43.4	...	...	...	...	...	6.2
6.4	...	...	...	...	...	54.8	75.4	...	...	...	...	31.4	37.0	43.3	...	74.1	83.3	55.5	61.7	17.9	77.9	40.4	...	...	...	...	...	6.4
6.6	...	...	...	...	...	53.2	65.3	...	...	...	...	27.9	35.6	45.8	...	83.5	87.4	...	58.0	20.8	72.0	36.5	...	...	...	...	...	6.6
6.8	...	...	...	...	...	...	53.4	...	...	...	...	23.5	34.2	48.3	...	87.4	91.0	...	55.3	22.2	66.6	31.4	...	...	...	...	...	6.8
7.0	...	...	...	...	...	...	41.3	53.3	...	...	...	19.0	32.9	50.9	...	90.0	93.2	...	...	23.7	61.9	25.4	86.2	86.4	...	...	...	7.0
7.2	...	...	...	...	...	...	29.6	55.0	...	...	...	13.8	31.7	53.4	...	91.8	94.9	...	...	25.2	58.1	19.6	79.6	80.6	44.7	...	...	7.2
7.4	...	...	...	...	...	...	19.7	57.6	...	...	...	9.8	30.6	55.8	...	93.0	95.8	...	...	26.7	55.3	14.6	71.3	72.8	42.0	...	...	7.4
7.6	...	...	...	...	...	...	12.8	60.8	...	...	...	6.8	29.6	58.2	...	93.8	96.8	...	...	28.6	...	10.2	62.0	63.2	39.3	...	...	7.6
7.8	...	...	...	...	...	...	7.4	65.2	53.0	...	...	4.6	28.8	60.5	...	...	...	...	...	31.2	...	6.6	52.0	52.1	33.7	43.9	...	7.8
8.0	...	...	...	...	...	...	3.7	70.6	55.4	...	...	...	28.1	62.8	...	...	...	...	...	33.9	...	...	42.0	41.1	27.9	41.6	...	8.0
8.2	...	...	...	...	...	...	...	75.9	58.0	...	...	...	27.6	65.0	...	...	...	...	46.4	36.9	...	...	31.9	31.4	22.9	38.4	...	8.2
8.4	...	...	...	...	...	...	...	81.2	62.1	...	...	...	27.0	67.2	...	...	...	...	43.9	39.9	...	...	22.5	23.0	17.3	34.8	...	8.4
8.6	...	...	...	...	...	...	...	86.2	66.9	94.7	...	...	26.3	69.3	...	...	...	...	40.9	42.7	45.4	...	16.0	15.9	13.0	30.7	...	8.6
8.8	...	...	...	...	...	...	...	90.1	73.6	92.0	...	...	25.2	71.3	...	...	...	45.5	36.8	...	42.8	...	11.7	10.3	8.8	23.3	...	8.8
9.0	...	...	...	...	...	...	...	93.2	83.5	88.4	...	...	24.0	73.2	...	...	...	43.2	31.8	...	39.2	...	...	...	5.3	17.7	...	9.0
9.2	...	...	...	...	...	...	...	...	95.6	84.0	...	...	22.6	75.1	...	...	...	40.0	26.2	...	34.7	...	...	...	...	13.3	10.0	9.2
9.4	...	...	...	...	...	...	...	...	...	78.9	87.0	...	21.4	77.0	...	...	...	35.8	20.4	...	29.3	...	...	...	...	9.2	18.4	9.4
9.6	...	...	...	...	...	...	...	...	...	73.2	75.5	...	20.2	78.8	...	...	...	30.8	15.2	...	23.6	...	...	...	...	5.2	29.3	9.6
9.8	...	...	...	...	...	...	...	...	...	67.2	65.1	...	19.0	80.4	...	...	...	25.0	10.8	...	19.0	...	...	...	...	4.1	42.0	9.8
10.0	...	...	...	...	...	...	...	...	...	62.5	59.6	...	18.1	81.8	...	...	...	19.4	7.4	...	13.1	...	...	...	...	2.3	53.4	10.0
10.2	...	...	...	...	...	...	...	...	...	58.8	56.4	...	17.1	83.1	...	...	...	14.3	...	...	9.2	...	...	...	...	...	63.7	10.2
10.4	...	...	...	...	...	...	...	...	...	55.7	54.1	...	16.5	84.3	...	...	...	10.0	...	...	6.2	...	...	...	...	...	73.1	10.4
10.6	...	...	...	...	...	...	...	...	...	53.6	52.3	...	16.0	85.4	...	...	...	6.9	...	...	...	...	...	...	...	...	81.2	10.6
10.8	...	...	...	...	...	...	...	...	...	52.2	...	...	15.5	86.5	...	...	...	...	...	...	...	...	...	...	...	...	87.9	10.8
11.0	...	...	...	...	...	...	...	...	...	51.2	...	...	14.7	87.8	...	...	...	...	...	...	...	...	...	...	...	...	...	11.0
11.2	...	...	...	...	...	...	...	...	...	50.4	...	...	13.5	89.3	...	...	...	...	...	...	...	...	...	...	...	...	...	11.2
11.4	...	...	...	...	...	...	...	...	...	49.5	...	...	11.7	91.3	...	...	...	...	...	...	...	...	...	...	...	...	...	11.4
11.6	...	...	...	...	...	...	...	...	...	48.7	...	...	9.1	94.5	...	...	...	...	...	...	...	...	...	...	...	...	...	11.6
11.8	...	...	...	...	...	...	...	...	...	47.6	...	...	5.5	99.0	...	...	...	...	...	...	...	...	...	...	...	...	...	11.8
12.0	...	...	...	...	...	...	...	...	...	46.0	...	...	1.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.0
12.2	...	...	...	...	...	...	...	...	...	43.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.2
12.4	...	...	...	...	...	...	...	...	...	39.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.4
12.6	...	...	...	...	...	...	...	...	...	31.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.6
12.8	...	...	...	...	...	...	...	...	...	21.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.8

Isoelectric Points of Amino Acids

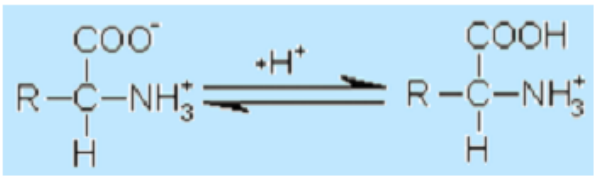
Amino acids are amphoteric substances and contain two functional groups; carboxylic acid (-COOH) and amino ($-NH_2$). When an amino acid is dissolved in water, it exists in solution as the dipolar ion, or **zwitterion**.

Neutral:

(Zwitterion)

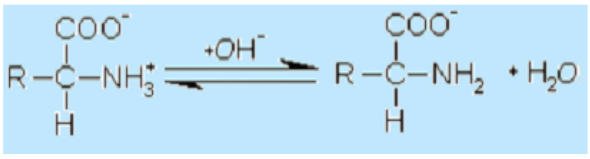
Acidic:

(Cation)



Basic:

(Anion)

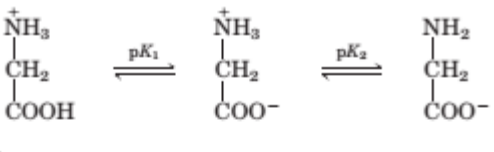


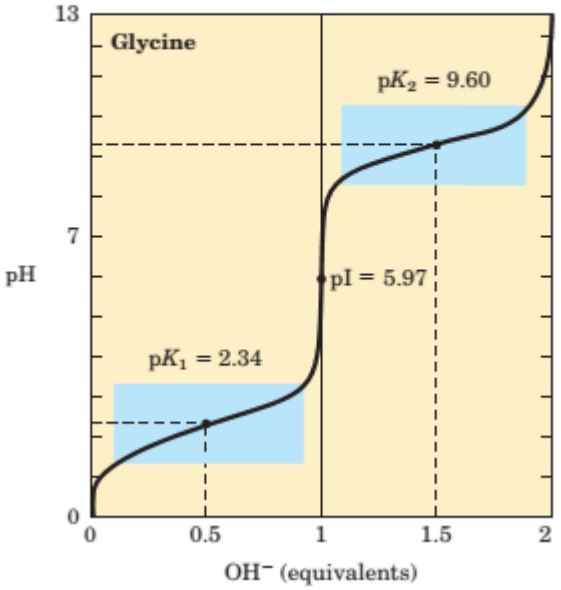
A zwitterion can act as either an acid (**proton donor**) or a base (**proton acceptor**). Substances having this dual nature are **amphoteric** and are often called ampholytes (from "amphoteric electrolytes").

Due to their amphoteric nature amino acids can be titrated with both acids and bases. Since amino acids bear at least two functional groups than can be titrated by they have two equilibrium constants (K) and two ionization constants (K_i). The characteristic pH at which the positive and negative electrical charges are equal and net electric charge is zero is called the **isoelectric point** or isoelectric pH, designated pI.

$$pI = pH = \tfrac{1}{2}\,(pK_1 + pK_2)$$

Acid-base titration involves the gradual addition or removal of protons. Figure shows the titration curve of the diprotic form of glycine. The plot has two distinct stages, corresponding to deprotonation of two different groups on glycine.



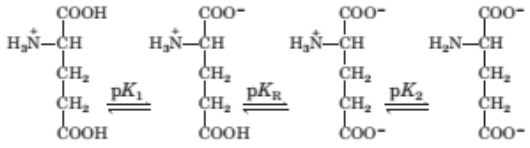


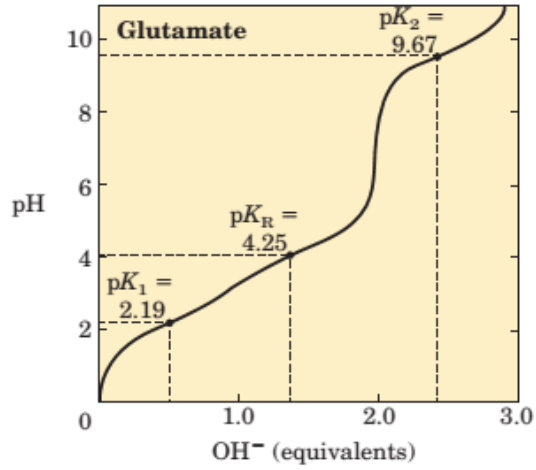
The shaded boxes, centered at about pK_1=2.34 and pK_2=9.60, indicate the regions of greatest buffering power. Glisin can be used as a buffer solution at +/- 1 pH range of these pK values. That means glycine has two regions of buffering power. At pH 5.97, the point of inflection between the two stages in its titration curve, glycine is present predominantly as its dipolar form, fully

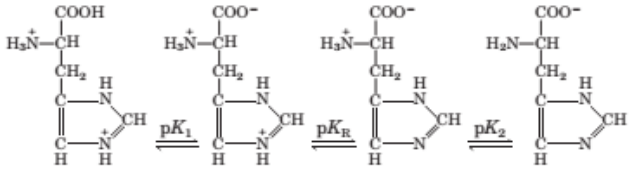
$$\text{pI} = \frac{1}{2}(pK_1 + pK_2) = \frac{1}{2}(2.34 + 9.60) = 5.97$$

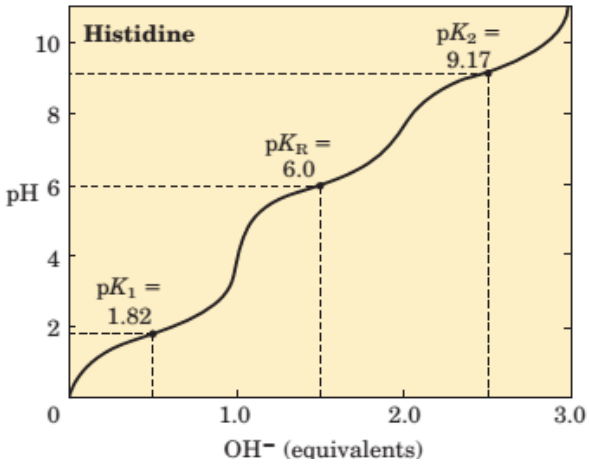
ionized but with no *net* electric charge, which is the pI of glycine.

Amino acids with an ionizable R group have more complex titration curves, with three stages corresponding to the three possible ionization steps









CHEMICALS

HCl, NaOH, Glycine

METHOD

Prepare 50 mL of 0.1 N Glycine. After calibrating the pH meter, pour 20 mL of glycine solution to a beaker and determine the pH of the solution. Titrate your solution with 0.1 N HCl. After each addition of 500 µL of acid mix the content of the beaker vigorously and determine pH. Continue this process until there is no discernible change of the pH of the solution.

Wash and dry the electrode of the pH meter. Pour 20 mL of glycine solution to another beaker and determine the pH of the solution. Titrate your solution with 0.1 N NaOH as described above. Write down the data you obtain in data sheet.

Acid-Base titration data sheet:

For acid titration:

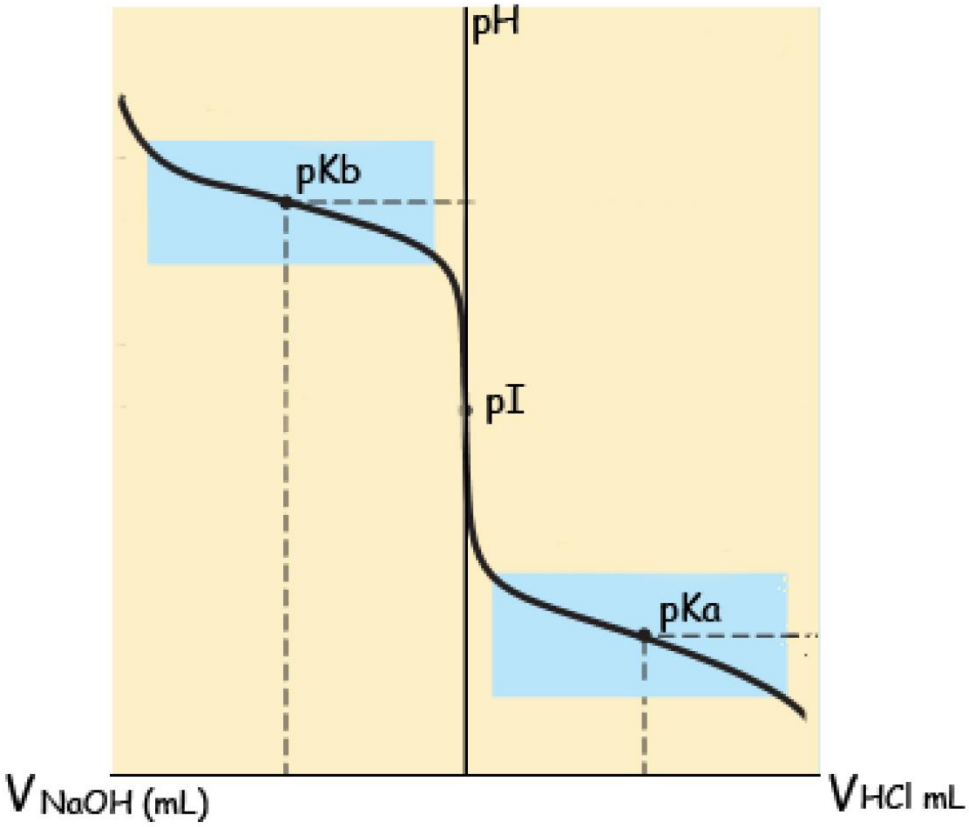
HCl (mL)	0	1															
pH																	
HCl (mL)																	
pH																	
HCl (mL)																	
pH																	
HCl (mL)																	
pH																	

For base titration:

NaOH (mL)	0	1															
pH																	
NaOH (mL)																	
pH																	
NaOH (mL)																	
pH																	
NaOH (mL)																	
pH																	

CALCULATION

Draw the graph between added acid or base volume (mL) to pH on a graph paper (milimetric paper). Using the graph determine pKa, pKb and pI values for glycine. Compare your findings with the theoretical values for glycine.



QUESTIONS

1- How do you calculate the pI value of glutamic acid and histidine? What is the importance of different pI values of amino acids?

2- A buffer contains 0.010 mol of lactic acid (pKa=3.86) and 0.050 mol of sodium lactate per liter. (a) Calculate the pH of the buffer. (b) Calculate the change in pH when 5 mL of 0.5 M HCl is added to 1 L of the buffer. (c) What pH change would you expect if you added the same quantity of HCl to 1 L of pure water?

EXPERIMENT II

SEPARATION OF AMINO ACIDS USING PAPER CHROMATOGRAPHY

Chromatography is a separation technique that employs the dispersion difference of the compounds in a mixture between two different phases to separate them from one another. The one of the phases is called as stationary phase and the other is called as mobile phase.

Stationary phase generally provides a support while mobile phase is responsible to carry the components of the mixture. Components of the mixture differently interacts with the mobile and stationary phase, thus move with different velocities. Separation techniques that uses this principle called as chromatographic techniques and can be classified in five group according to force that used for separation; adsorption, dispersion, ion exchange, gel filtration and affinity.

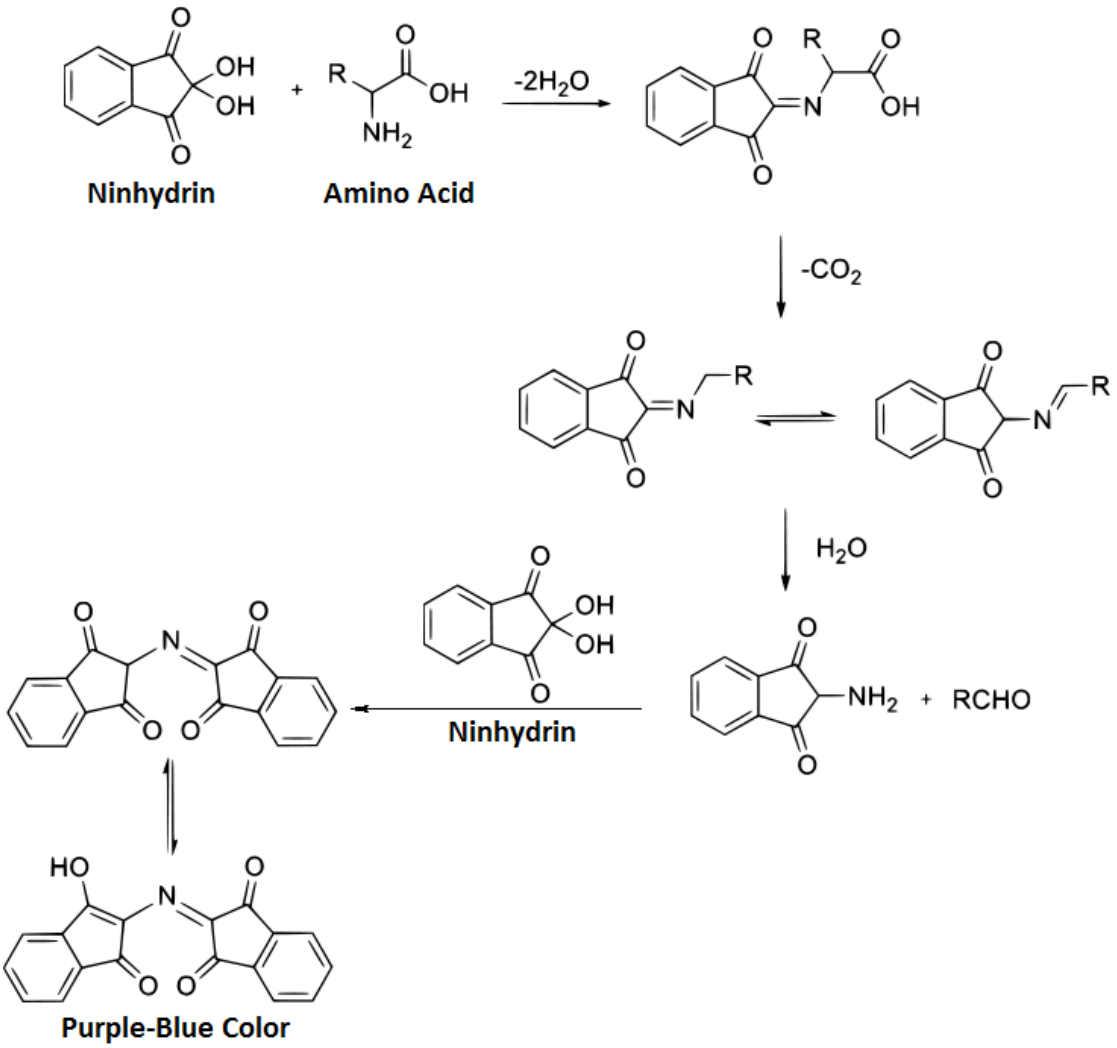
Chromatographic techniques used in the field of biochemistry to purification of a desired compound (such as proteins from plant or animal tissue), to concentrate a diluted solution, to analyze a compound's purity and to determine metabolites of an organism.

Paper chromatography, is a special form of dispersion chromatography. Whatman Chromatography Paper used in this experiment produced from pure cellulose, without using any chemical additives and has uniform pore sizes. Compounds that desired to be separated moves along the Whatman paper by the capillary effect of the mobile phase with different velocities according to their interaction with the mobile phase.

If the components of the mixture are colored, they can be seen on the Whatman paper as round spots. If they are colorless they can be visualized by spraying a special reagent or having them inspected on UV light. A quantitative or semi-quantitative analysis can be done using spot size and color intensity.

Retention factor (Rf) is defined as the ratio of the distance travelled by the center of a spot to the distance traveled by the solvent front. Rf value can be used to determine the components of a mixture. Each compound has a characteristic Rf value when analyzed at the equal conditions such as, temperature, type of mobile phase etc.

$$R_f = \frac{\text{migration distance of substance}}{\text{migration distance of solvent front}}$$



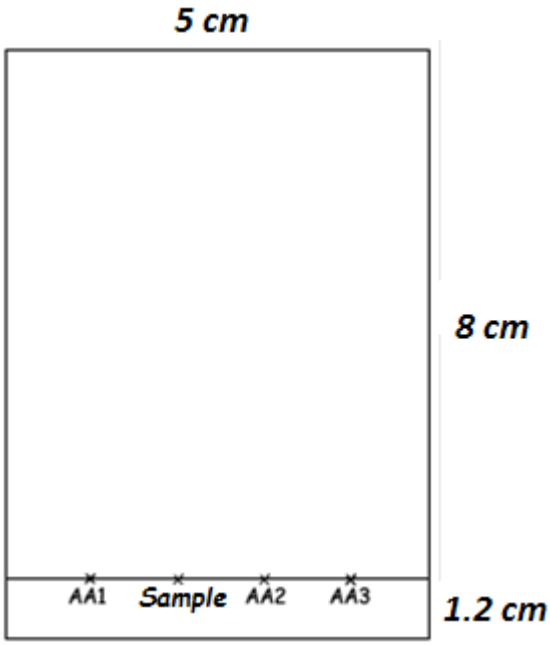
CHEMICALS

Asetic acid, n-butanol, ninhydrin, aspartic acid, leucine, valine, arginine, proline, phenylalanine, Hydrochloric acid, Whatman No.1 chromatography paper.

METHOD

Mobil phase prepared as n-butanol: acetic acid: water (65:15:25). Put 10 mL of mobile phase (it should be raised approximately 1 cm from the bottom) in chromatography tank and cover. In order to saturate the chromatography tank with mobile phase vapor do not remove the lid until you place the chromatography paper.

Using a pencil, prepare your chromatography paper according to the diagram given below.

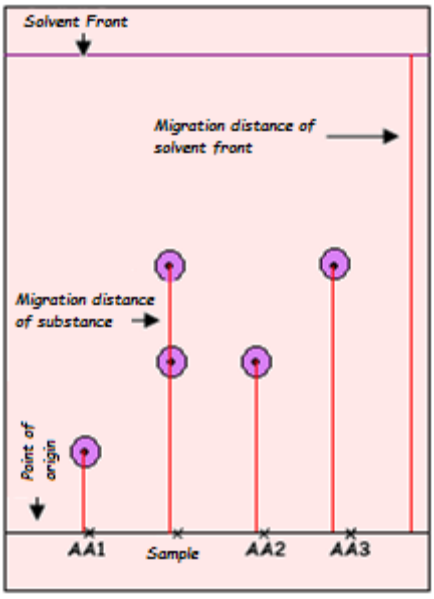


Apply 3 standard amino acid (dissolved in 1N HCl) and sample which are given to your experiment group to your chromatography paper using a capillary tube. Application spot radius should not be exceed to 3-4 mm. If the application spot is not big enough you may apply your solution for a second time after the first drop completely dried.

Group 1: Proline, arginine, valine, sample 1
Group 2: Aspartic acid, leucine, phenyl alanine, sample 2

When all the samples applied to the chromatography paper wait for the spots to completely dry and place your chromatography paper in to chromatography tank. Applications spots should not be directly in contact with the mobile phase. Allow the mobile phase flow through the chromatography paper until 0.5 cm left from the top. Remove the paper and mark the solvent front using a pencil. Dry your paper in the laboratory oven for about 3 minutes.

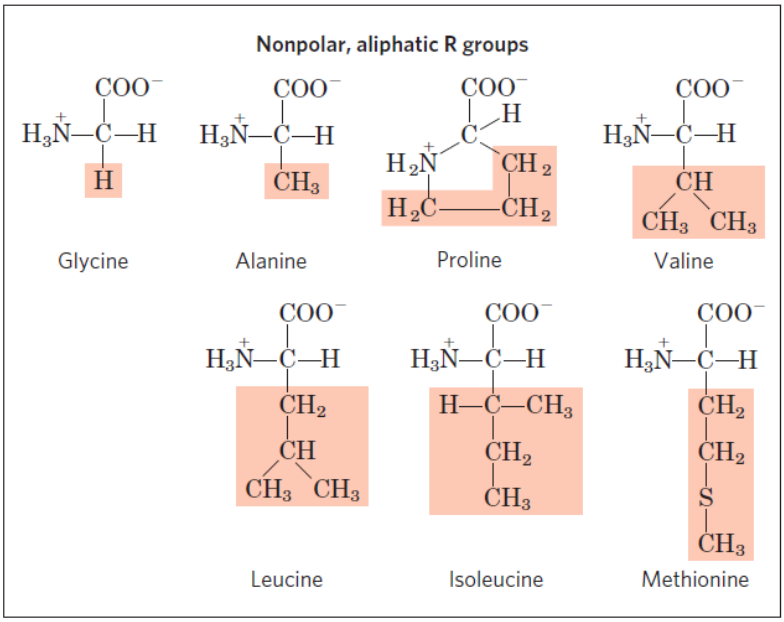
After removing your paper from the oven, thoroughly spray it with ninhydrine reagent in a fume hood and place it again in the oven and wait until all the spots appear. Calculate the Rf of the samples and standards using the following diagram and table.

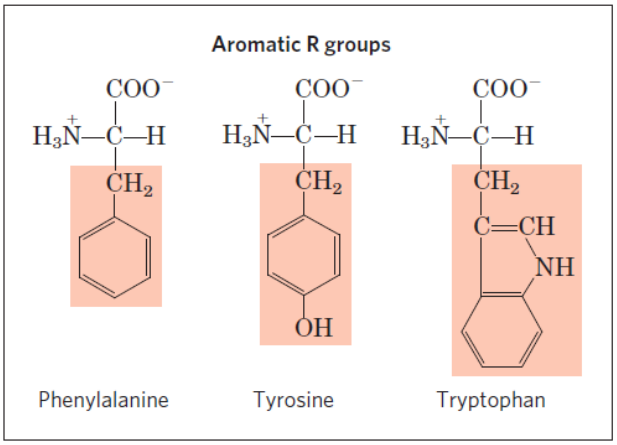


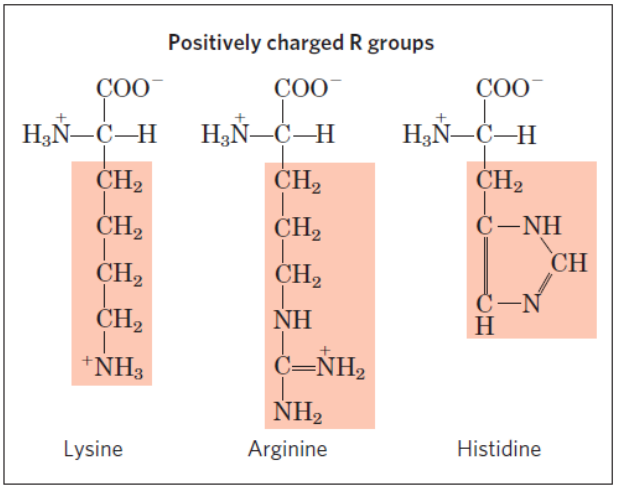
Amino Acid	Color of the Spot	Rf value
Aspartic acid		
Leucine		
Valine		
Arginine		
Proline		
Phenylalanine		
Sample 1		
Sample 2		

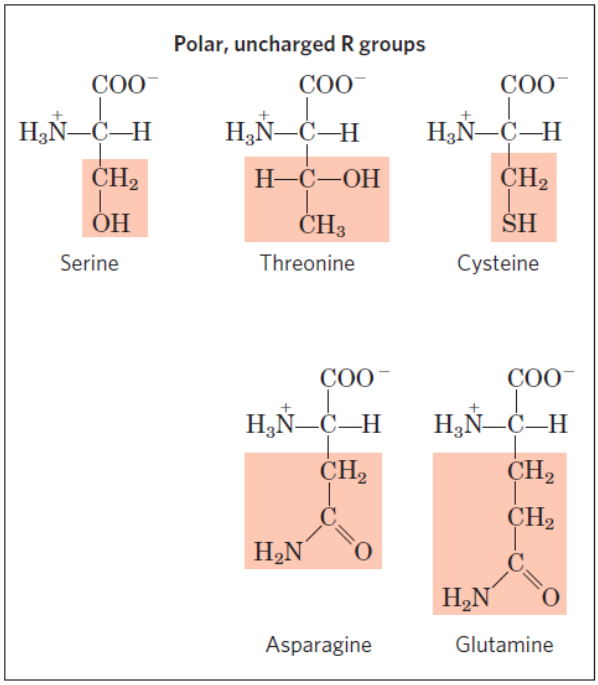
QUESTIONS

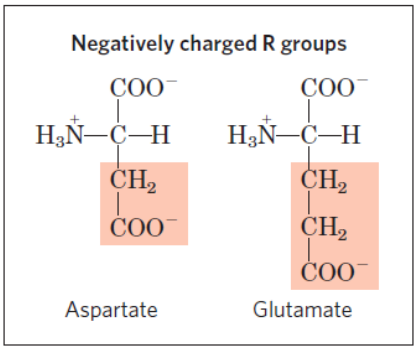
1- Why different amino acids have different Rf values? If you separate a mixture of amino acids consist of glutamic acid, histidine, glycine, tryptophan and isoleucine with paper chromatography using NH_3: Benzene (10:90) as a mobile phase what do you expect the Rf values of the amino acids will be?









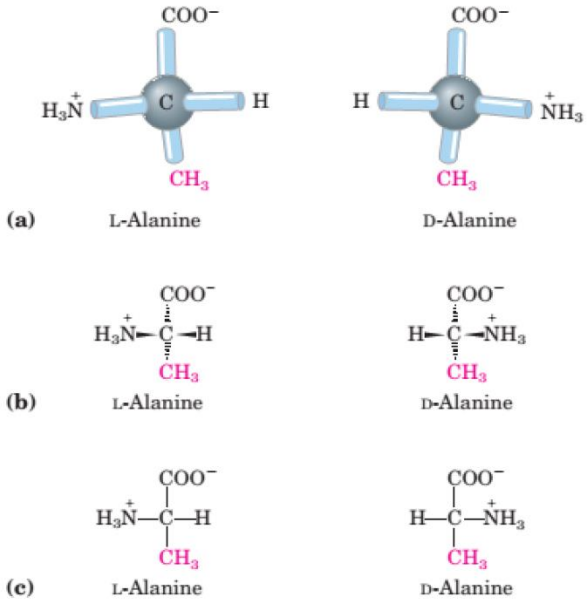


EXPERIMENT III

PROPERTIES OF AMINO ACIDS AND PROTEINS

I- AMINO ACIDS

Amino acids are organic compounds that contains at least one **amino** or one **carboxyl** functional groups. There are over 300 naturally occurring amino acids and 20 of these amino acids enter protein structure. The amino acid residues in protein molecules are exclusively L stereoisomers.



Amino acids are joined together by **peptide (amide)** bonds to form **polypeptides** and **proteins**. Amino acids are soluble in water but not soluble in non-polar organic solvents.

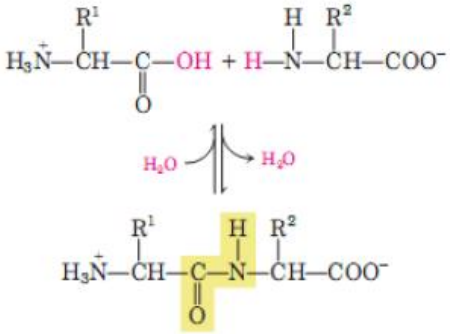
Solubility of amino acids increase in both acidic and basic solution, which means they are amphoteric in character. The melting points of amino acids are in the range of 200-300 °C.

The pH of a solution when a pure amino acid dissolved in pure water called a **isoionic point.** Solubility of amino acids and proteins in their isoionic point is maximum.

8 amino acids (valine, isoleucine, leucine, threonine, methionine, phenylalanine, tryptophan and lysine) cannot be synthesized by humans and these amino acids are called as essential amino acids.

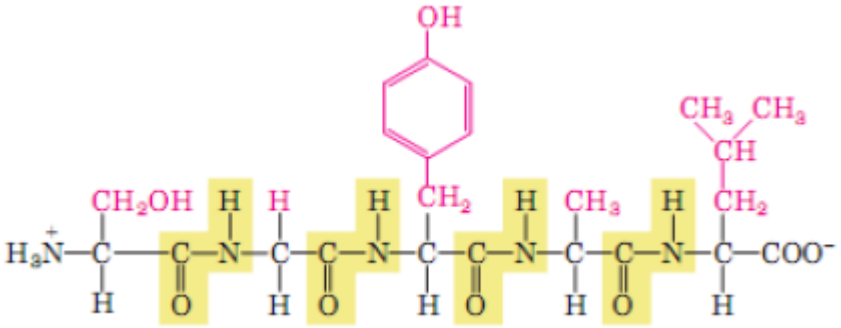
II- PEPTIDS

Two amino acid molecules can be covalently joined through a substituted amide linkage, termed a **peptide bond**, to yield a **dipeptide**. Due to its resonance forms this peptide bond is very stable. Three amino acids can be joined by two peptidebonds to form a **tripeptide**; similarly, amino acids can be linked to form **tetrapeptides**, **pentapeptides**, and soforth. When a few amino acids (10 or so) are joined in this fashion, the structure is called an **oligopeptide**. When many amino acids (50-100) are joined, the product is called a **polypeptide.**



Acidic or basic properties of peptides are depended to terminal amino **(N-terminal)** and carboxyl groups **(C-terminal)** and ionized functional groups in their side chains.

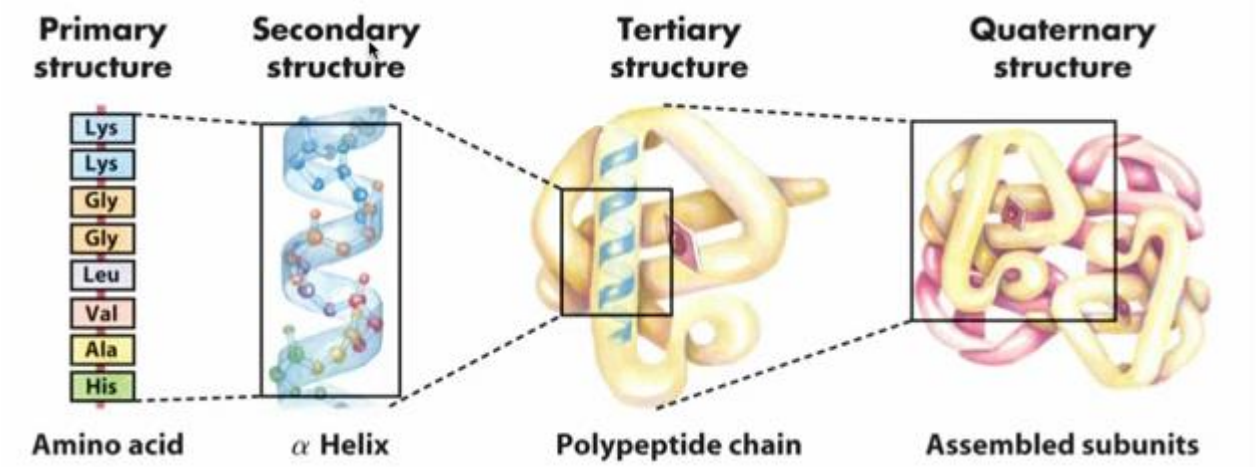
Peptides are written in a way that their N-terminal amino acid residue is on the left and C-terminal one is on the right. This pentapeptide can be named as serylglycyltyrocylleucine or Ser-Gly-Tyr-Ala-Leu.



III- PROTEINS

Molecules referred to as proteins generally consist of 100 or more amino acids. However, polypeptide chains which have lower than 100 amino acids can also be termed as proteins as long as they have definite three dimensional conformations and functions. Half of dry weight of a cell consists of proteins. They serve for many functions such as enzymes, hormones, receptors, immune system molecules and coagulation factors.

The primary structure consists of a sequence of amino acids linked together by peptide bonds and includes any disulfide bonds. The resulting polypeptide can be coiled into units of secondary structure, such as a helix. The helix is a part of the tertiary structure of the folded polypeptide, which is itself one of the subunits that make up the



A loss of three-dimensional structure sufficient to cause loss of function is called **denaturation**. Heat, strong acids (HCl, trichloric acid, perchloric acid etc.) organic solvents (ethanol, acetone etc.), cross linkers (formaldehyde, glutaraldehyde), chaotropic agents (urea 6-8 M, guanidine chloride 6M), reducing agents (2-mercaptoethanol, dithiyothreitol, iodoacetate), salts at high concentration (ammonium sulfate), heavy metal salts (mercury(II)chloride, copper(II)sulfate etc.) and certain detergents (sodium dodecilsulfate) cause denaturation of proteins.

Certain proteins denatured by heat, extremes of pH, or denaturing reagents will regain their native structure and their biological activity if denaturizing agents removed. This process is called **renaturation**. The quaternary, tertiary and secondary structures of a denaturated protein are partially destroyed. If primary structure is also affected this type of denaturation called as **degradation** and it is irreversible.

Salts increase the solubility of the proteins at lower concentrations. +2 charged salts such as $(NH_4)_2SO_4$ are more effective than +1 charged salts such as NaCl and NH_4Cl. At high concentration levels, salts precipitates proteins.

CHEMICALS

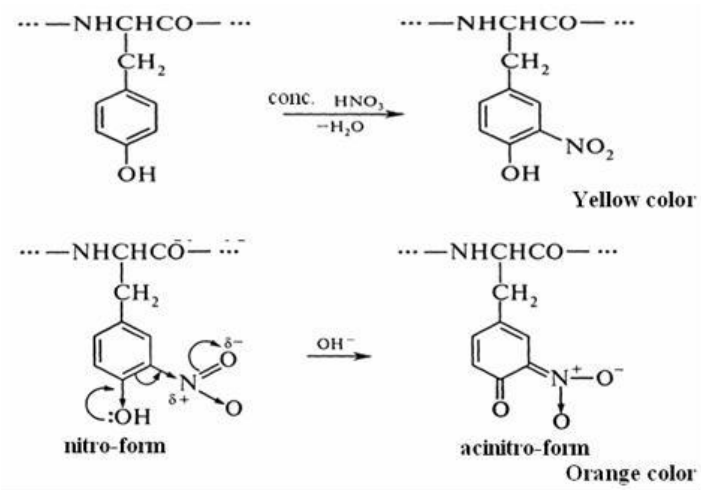
Glycine, arginine, glutamic acid, phenylalanine, tyrosine, tryptophan, histidine, cysteine, cystine, casein, egg albumin, sulfuric acid, nitric acid, hydrochloric acid, sodium hydroxide, mercury (II) chloride, sodium nitroprusside, copper(II)sulfate, copper(II)acetate, cadmium sulfate and bismuth (II) nitrate.

METHOD

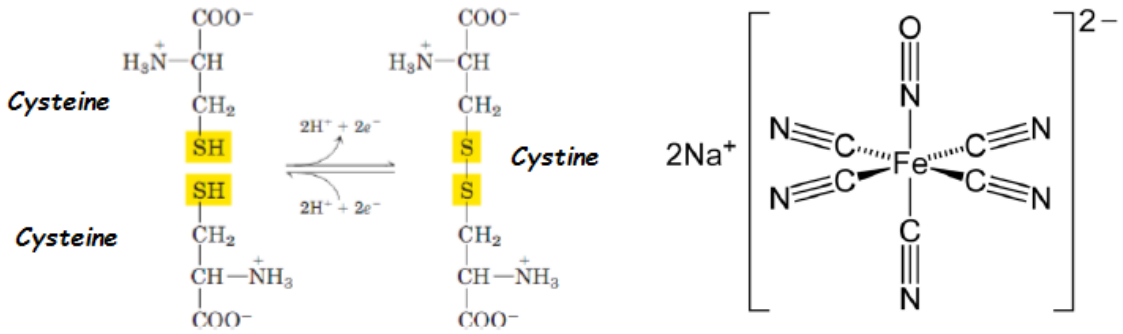
I- Reaction of Amino Acids

Solubility and pH of amino acids: Take small amounts of solid arginine, glycine, glutamic acid and phenylalanine in 4 test tubes. Add equal amounts of water in each tube and observe the solubility. Determine and record the pH of these solutions.

Xanthoproteic reaction: Aromatic amino acids in the presence of concentrated nitric acid turn to their yellow colored nitro derivatives. In separate test tubes add 5 drops of nitric acid onto tyrosine, tryptophan, phenylalanine, histidine and casein solutions. Add additional 5 drops of sulfuric acid to the phenylalanine test tube. Heat the solutions in water bath for 5 minutes and observe the color change. After tubes cool to the room temperature add 10% NaOH solution until the pH of your solution became basic. Record the color change.



Millon Reaction: (Millon Reagent: 15 g $HgCl_2$/ 100 mL %50 HNO_3). Phenol containing tyrosine amino acid is the only amino acid that gives this reaction. First phenol ring of the tyrosine became nitrated and this nitro tyrosine gives a red color with the mercury ions in the solution.



Nitroprusside reaction: Amino acids that contains -SH functionality gives this reaction. Only amio acid that contains -SH group is cysteine. Take cysteine, cysteine and egg albumin solutions in 3 test tubes and add a small amount of %10 NaOH and %2 sodium nitroprusside. Observe and record the color changes. Add a small amount of HCl in the casein tube and record your observations.

Tyrptophane reaction Take tryptophan and glycine in two test tubes and add same volume of acetic acid. Carefully add sulfuric acid to the test tube and observe the changes. This test is specific to tryptophan.

General qualitative reactions of amino acids:

Reaction	Reagent	Amino acid/ functional group	Color
Ninhydrine	Ninhydrine	Free NH_2	**Purple**
Biuret	Alkaline $CuSO_4$	Amide (peptide) bonds	**Blue**
Lowry	Phosphomolibdate	Tyrosine	**Blue**
Millon	HNO_3, HNO_2, $HgNO_3$	Tyrosine	**Red**
Xantoproteic	Boiling HNO_3	Tryptophan, tyrosine, phenylalanine	**Yellow**
Ehrlich	Der. HCl / p-dimethylamino benzaldehyde	Tryptophan	**Blue**
Sakaguchi	α- Naphtol, NaOCl or NaOBr	Arginine	**Red**
Nitroprusside	Sodium nitroprusside, NaOH	Cysteine	**Red**
Sullivan	Sodium-1,2-naphtoquinon-4-sulfonate and $NaHSO_4$	Cysteine	**Red**
Lead Sulfur	NaOH, Lead acetate	Cysteine	**Black**
Hopkins-Cole	Glyoxylic acid and H_2SO_4	Tryptophan	**Purple**
Pauly	Sulfanilic acid, HCl, Na_2CO_3, $NaNO_2$	Histidine, Tryptophan	His: **Yellow** Trp: **Purple**

II- Reactions of proteins

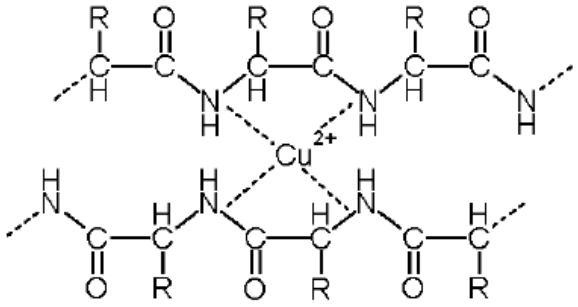
Denaturation of Proteins:

Effect of Heat: Heat a small amount of egg albumin solution and observe.

Effect of concentrated acids: Take small amount of nitric acid in a test tube and add egg albumin solution in a drop-wise manner.

Effect of heavy metals: Add small amount of cadmium sulfate to the egg albumin solution and record your observations.

Biuret reaction: Compounds that contain one or more peptide bonds form violet or purple colored coordination complexes with alkaline copper sulfate. Take glycine, casein and egg albumin in 3 test tubes and add small amount 10% NaOH and five drops of copper sulfate. Record your observations.



Question

1- Draw Glu-His-Trp-Ser-Gly-Leu-Arg-Pro-Gly peptide. What is the net charge of this peptide at pH 3, 8 and 11? What can you say about pI value of this peptide?

2- What kind of changes happens in the structure of proteins when they are separately incubated with concentrated salt solutions and organic solvents?

		pK_a			
	M_r	pK_1 (—COOH)	pK_2 ($—NH_3^+$)	pK_R R Grubu	pI
Gly G	75	2.34	9.60		5.97
Ala A	89	2.34	9.69		6.01
Pro P	115	1.99	10.96		6.48
Val V	117	2.32	9.62		5.97
Leu L	131	2.36	9.60		5.98
Ile I	131	2.36	9.68		6.02
Met M	149	2.28	9.21		5.74
Phe F	165	1.83	9.13		5.48
Tyr Y	181	2.20	9.11	10.07	5.66
Trp W	204	2.38	9.39		5.89
Ser S	105	2.21	9.15		5.68
Thr T	119	2.11	9.62		5.87
Cys C	121	1.96	10.28	8.18	5.07
Asn N	132	2.02	8.80		5.41
Gln Q	146	2.17	9.13		5.65
Lys K	146	2.18	8.95	10.53	9.74
His H	155	1.82	9.17	6.00	7.59
Arg R	174	2.17	9.04	12.48	10.76
Asp D	133	1.88	9.60	3.65	2.77
Glu E	147	2.19	9.67	4.25	3.22

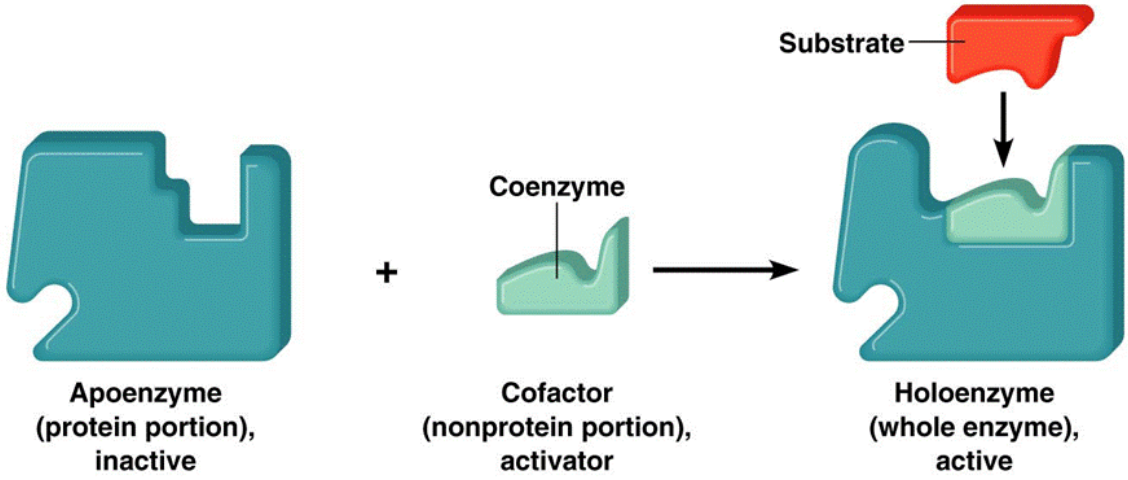
EXPERIMENT IV

EFFECT OF POLYPHENOL OXIDASE ENZYME ON OXIDATION OF CATECHOL

Enzymes catalyze chemical reaction of living organisms. They have a high degree of specificity for their substrates, they accelerate chemical reactions tremendously, and they function in aqueous solutions under very mild conditions of temperature and pH. Few nonbiological catalysts have all these properties.

With the exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost.

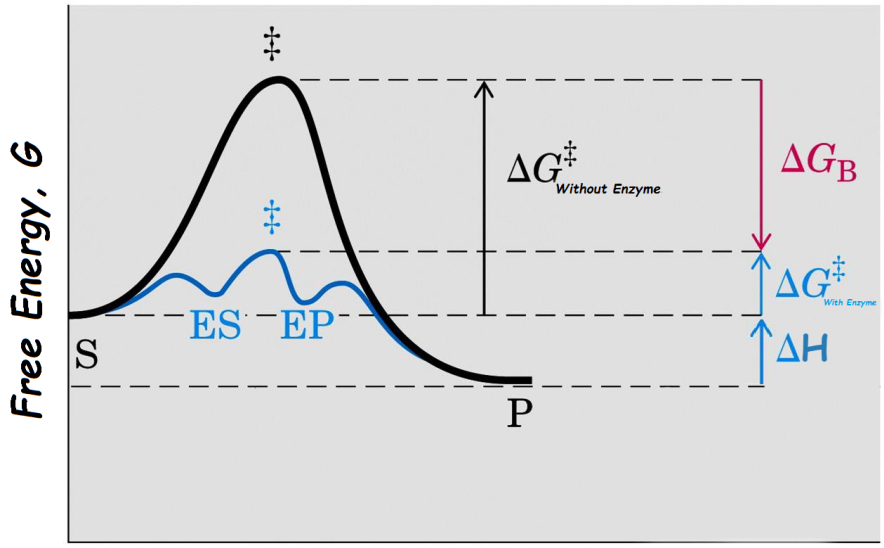
Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a **cofactor**—either one or more inorganic ions, or a complex organic or metalloorganic molecule called a **coenzyme**. A coenzyme or metal ion that is very tightly or even covalently bound to the enzyme protein is called a **prosthetic group**. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a **holoenzyme**. The protein part of such an enzyme is called the **apoenzyme** or apoprotein. Finally, some enzyme proteins are modified covalently by phosphorylation, glycosylation, and other processes. Many of these alterations are involved in the regulation of enzymeactivity.



The distinguishing feature of an enzyme-catalyzed reaction is that it takes place within the confines of a pocket on the enzyme called the **active site**. The molecule that is bound in the active site and acted upon by the enzyme is called the **substrate**.

A simple enzymatic reaction might be written where E, S, and P represent the enzyme, substrate, and product; ES and EP are transient complexes of the enzyme with the substrate and with the product.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (1)$$



At the beginning of the reaction substrate concentration is high and product concentration is low. Rate of ES formation will be equal to the rate of ES breakdown.

$$k_1 [E] [S] - k_2 [ES] = k_3 [ES] - k_4 [E] [P] \qquad (2)$$

Since [P] at the start of the reaction is negligible,

$$k_1 [E] [S] = k_2 [ES] + k_3 [ES] \; ; \; \frac{[E] [S]}{[ES]} = \frac{K_2 + K_3}{K_1} = K_m \qquad (3)$$

The term $(k_2+k_3)/k_1$ is defined as the **Michaelis constant**, Km. is the substrate concentration yielding a velocity of $V_{max}/2$

$[E_0]$, is the initial enzyme concentration and [E] is the enzyme concentration of any given time, $[E] = [E_0] - [ES]$.

When we apply it to equation 3;

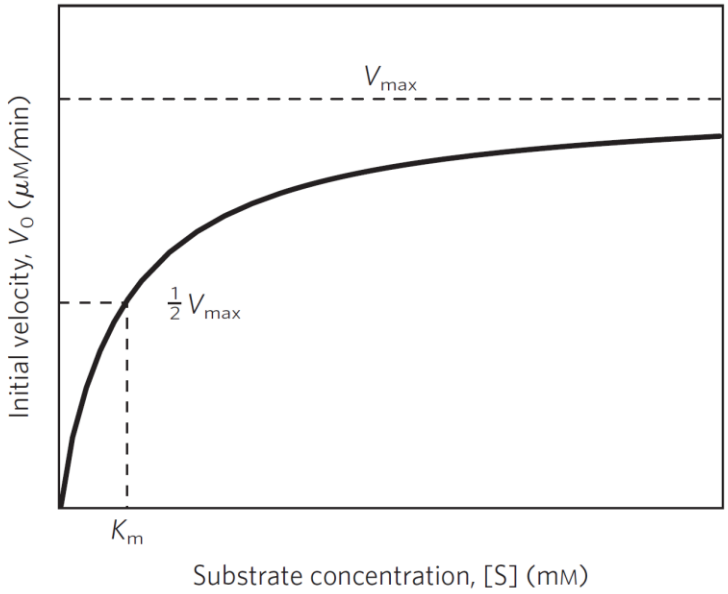
$$\frac{[E_0] - [ES]}{[ES]} = \frac{K_m}{[S]} \; ; \; \frac{[E_0]}{[ES]} = \frac{K_m}{[S]} + 1 \qquad (4)$$

Because the maximum velocity occurs when the enzyme is saturated (that is, with [ES]= [E_0]), Vmax can be defined as $k_2[E_0]$. When we apply it to equation 4;

$$\frac{[E_0]\,K_3}{[ES]\,K_3} = \frac{V_{Maks}}{V_0} = \frac{K_m}{[S]} + 1 \qquad (5)$$

We can now express V_0 in terms of [ES];

$$V_0 = \frac{V_{Maks}\,[S]}{K_m + [S]} \qquad (6)$$



This is the **Michaelis-Menten equation**, the rate equation for a one-substrate enzyme-catalyzed reaction. According to equation 6 relations between V_0 and [S] could be given with **Michealis Menten graph**.

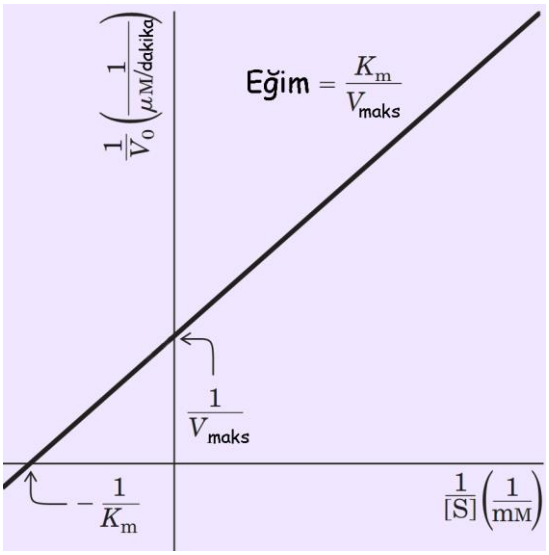
In low substrate concentrations since K_m >> [S], [S] is negligible and equation 6 turns into $V_0=V_{max}$ [S] / K_m and in high substrate concentrations since K_m << [S], [K_m] is negligible and equation 6 turns into $V_0=V_{max}$. The Michaelis-Menten equation can be algebraically transformed into equations that are more useful in plotting experimental data. One common transformation is derived simply by taking the reciprocal of both sides of the Michaelis-Menten equation:

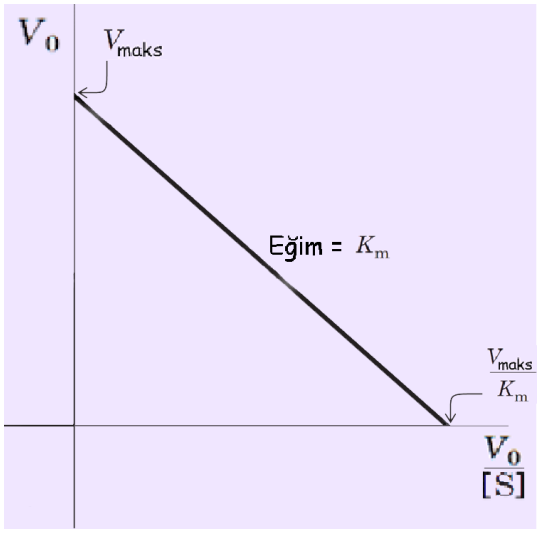
$$y = mx + b \qquad \frac{1}{V_0} = \frac{K_m}{V_{maks}}\frac{1}{[S]} + \frac{1}{V_{maks}}$$

This form of the Michaelis-Menten equation is called the **Lineweaver-Burk equation**.

Multiplying both sides of the Lineweaver-Burk equation by Vmax and rearranging gives the **Eadie-Hofstee equation**:

$$V_0 = (-K_m)\frac{V_0}{[S]} + V_{max}$$

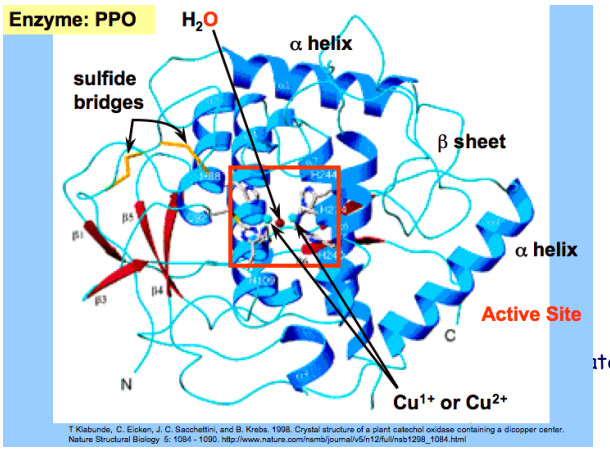


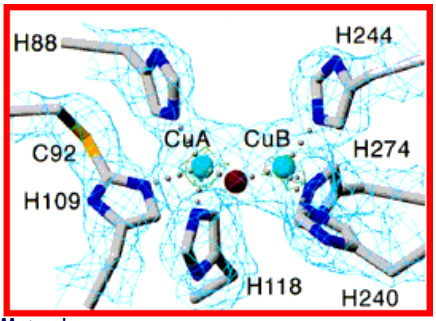


Units of activity (U) are typically used to describe enzyme catalytic activity, where a unit (U) refers to the amount of enzyme that catalyzes the conversion of 1 micromole (µmole) of substrate per minute. S**pecific activity** is defined in terms of enzyme units per mg enzyme protein. **Turnover number** (Kcat) is the number of substrate molecule each enzyme site converts to product per second. **Katal** is the amount of an enzyme needed to transform one mole of substrate per second.

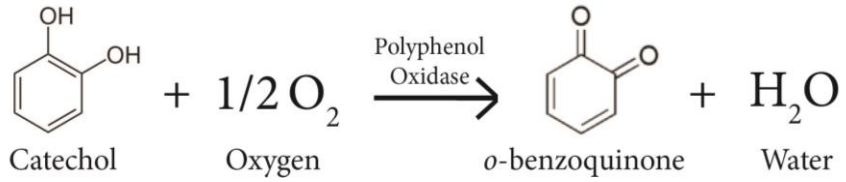
Enzyme inhibitors are molecular agents that interfere with catalysis, slowing or halting enzymatic reactions. This process is called as **enzyme inhibition**. There are two broad classes of enzyme inhibitors: reversible and irreversible.

Polyphenol oxidases (PPOs) are a group of copper enzymes that are able to catalyze the oxidation of aromatic compounds by oxygen. There are two main types of PPOs: laccases (EC 1.10.3.2) and tyrosinases (EC 1.14.18.1). Tyrosinases catalyze two kinds of reactions: ortho hydroxylation of monophenols, such as l-tyrosine; and the oxidation of this and other o-diphenols to o-quinones.





In this experiment we will investigate the enzymatic reaction of catechol to benzoquinone.



While catechol solution is colorless, benzoquinone solution is brown therefore rate of the reaction could be monitored colorimetrically. Also, 1,2-benzoquinone have a maximum absorption at 480 nm and therefore amount of product could be determined spectrophotometrically.

The aim of this experiment is determining Vmax and Km of polyphenol oxidase using catechol as substrate.

CHEMICALS

Citric acid- phosphate buffer pH 6, catechol, benzoquinone.

METHOD

Preparation of Crude Extract: Peel and slice the potato into small pieces. Homogenize your sample using 200 mL of Citric acid- phosphate buffer pH 6 in a laboratory blender. Since enzyme activity greatly affected by heat, all other steps of the purification have performed on ice. Filtrate the homogenate using glass wool and keep the enzyme solution on ice.

Effect of substrate concentration on reaction rate: Prepare 6 test tube as shown in the table (without enzyme).

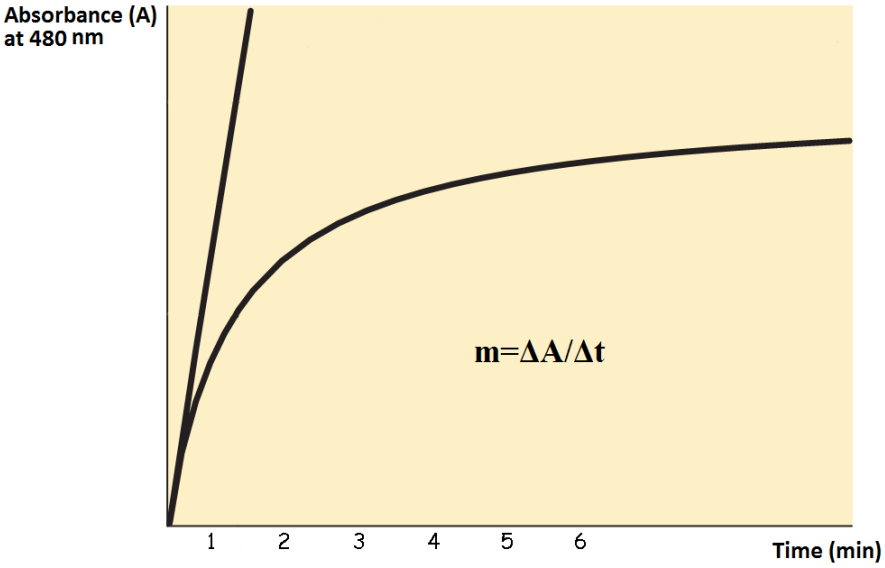
Tube no:	**Substrate Catechol 0.01 M (mL)**	**Water (mL)**	**Enzyme (µL)**	**Absorbance**						**Calculated reaction rate**
				1. min.	**2. min**	**3. min**	**4. min**	**5. min**	**6. min**	
1	0	10	-							
2	1	9	500							
3	2	8	500							
4	3.5	6.5	500							
5	5	5	500							
6	7	3	500							
7	9	1	500							

Determine the absorbance of the test tubes number 2-7 per minute at 480 nm.

Extinction constant: Prepare100 mL 0.01 M benzoquinone and determine its absorbance at 480 nm.

LAB REPORT

For tube number 2-7 draw the absorbance – time graph.



Draw tangent lines for each hyperbolic curve and calculate the slope of each line.

Since m= $\Delta A/\Delta t$, and $A=\varepsilon.c.\ell$ V_0 at each reaction could be calculated from the equation $V_0=m / \varepsilon . \ell$

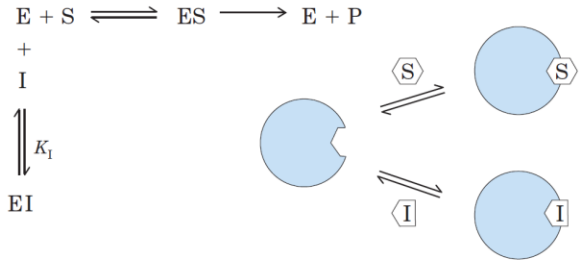
Draw Michealis Menten and Lineweaver-Burk graph and calculate Km and Vmax using Lineweaver-Burk plot.

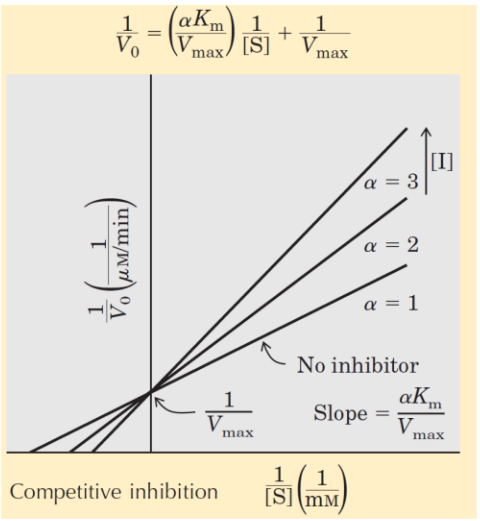
Marmara University
Faculty of Arts and Sciences
Department of Chemistry/ Biochemistry

QUESTION

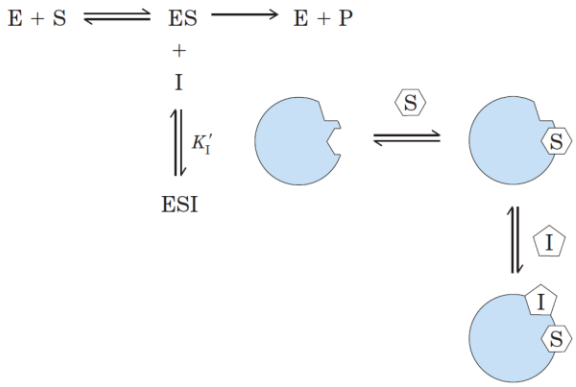
1- For the competitive, noncompetitive and uncompetitive inhibition type investigate following figures and determine relationship between inhibitor concentration and Vmax and Km for each mechanism.

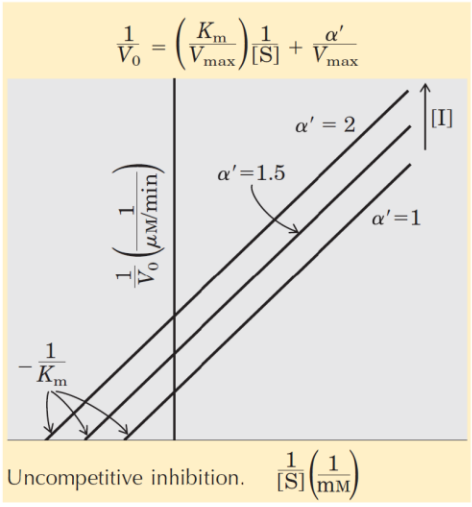
(a) Competitive inhibition



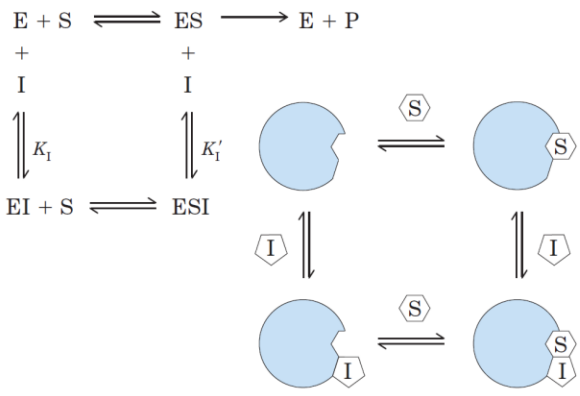


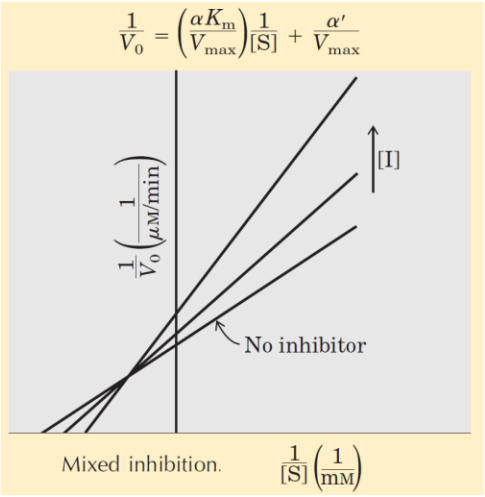
(b) Uncompetitive inhibition





(c) Mixed inhibition





EXPERIMENT V

PURIFICATION OF PROTEINS AND QUANTITATIVE PROTEIN DETERMINATION

I- Protein isolation and dialysis

In order to investigate its structure and properties a protein must be obtained in pure form. Since proteins are easily denatured and found as a complex mixture in biological materials (such as body fluids and tissue extracts), their purification is quite difficult.

Source for obtaining proteins are usually tissues and cells. First step in any purification process is destroying (or lysing) the cell and solubilize its protein content in **lysis buffer**. This solution is called as **crude extract** and it contains both targeted protein and all the other molecules in the cell that soluble in chosen lysis buffer.

Later, crude protein extract fractioned according their different properties such as solubility, size, electrical charge, and polarity. Various separation techniques could be use in that purpose; differential centrifuge, salting out, precipitation with cold acetone or alcohol, ultrafiltration, and various chromatographic methods.

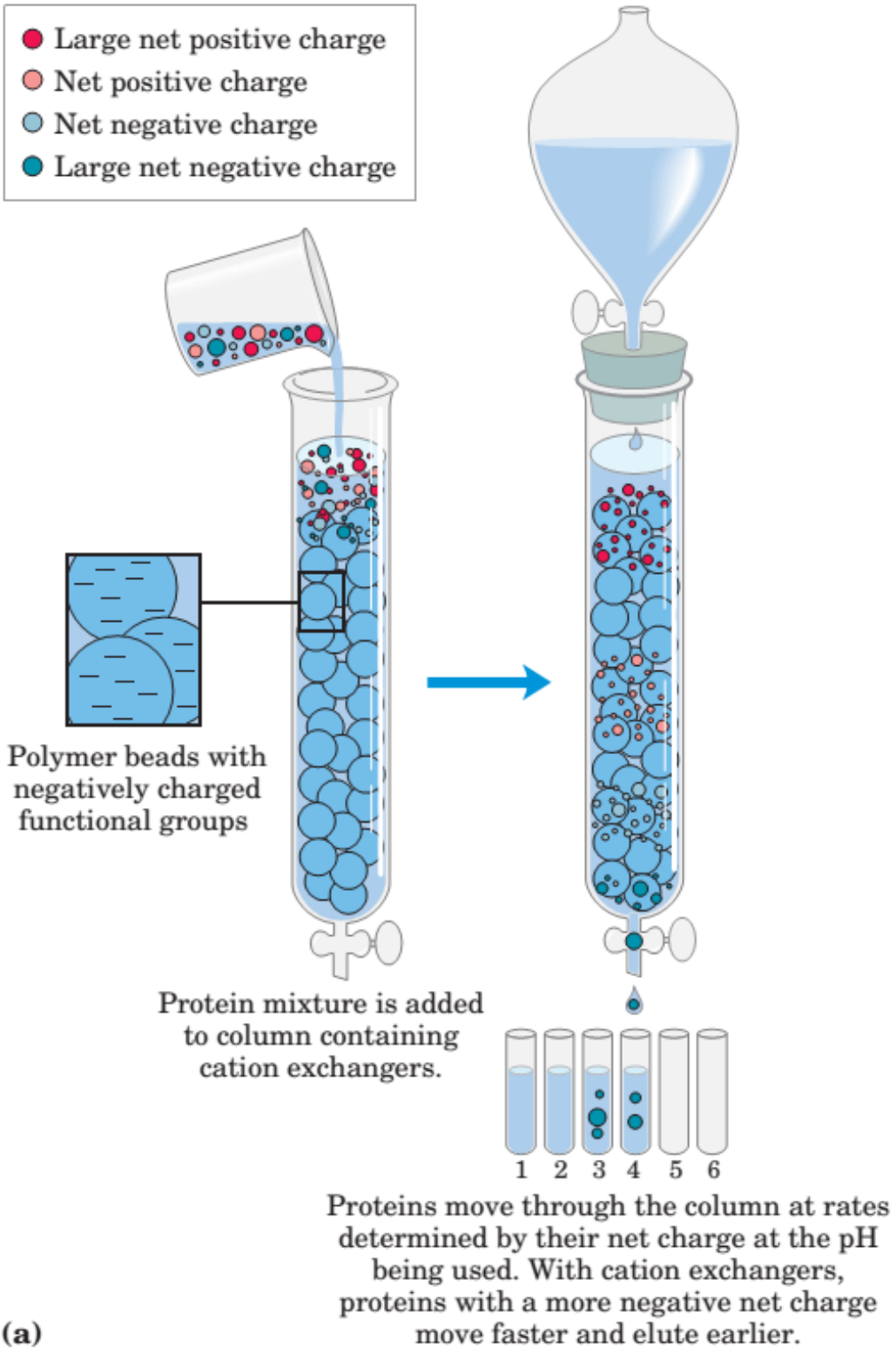
A sample methodology for purification of an enzyme from crude extract is given below:

a- Obtaining crude extract using a lysis buffer
b- Salting out using ammonium sulfate (solubility in salt solutions)
c- Ion exchange chromatography (electrical charge difference)
d- Size exclusion (molecular sieve) chromatography
e- Affinity chromatography (using a specifically binding ligand)

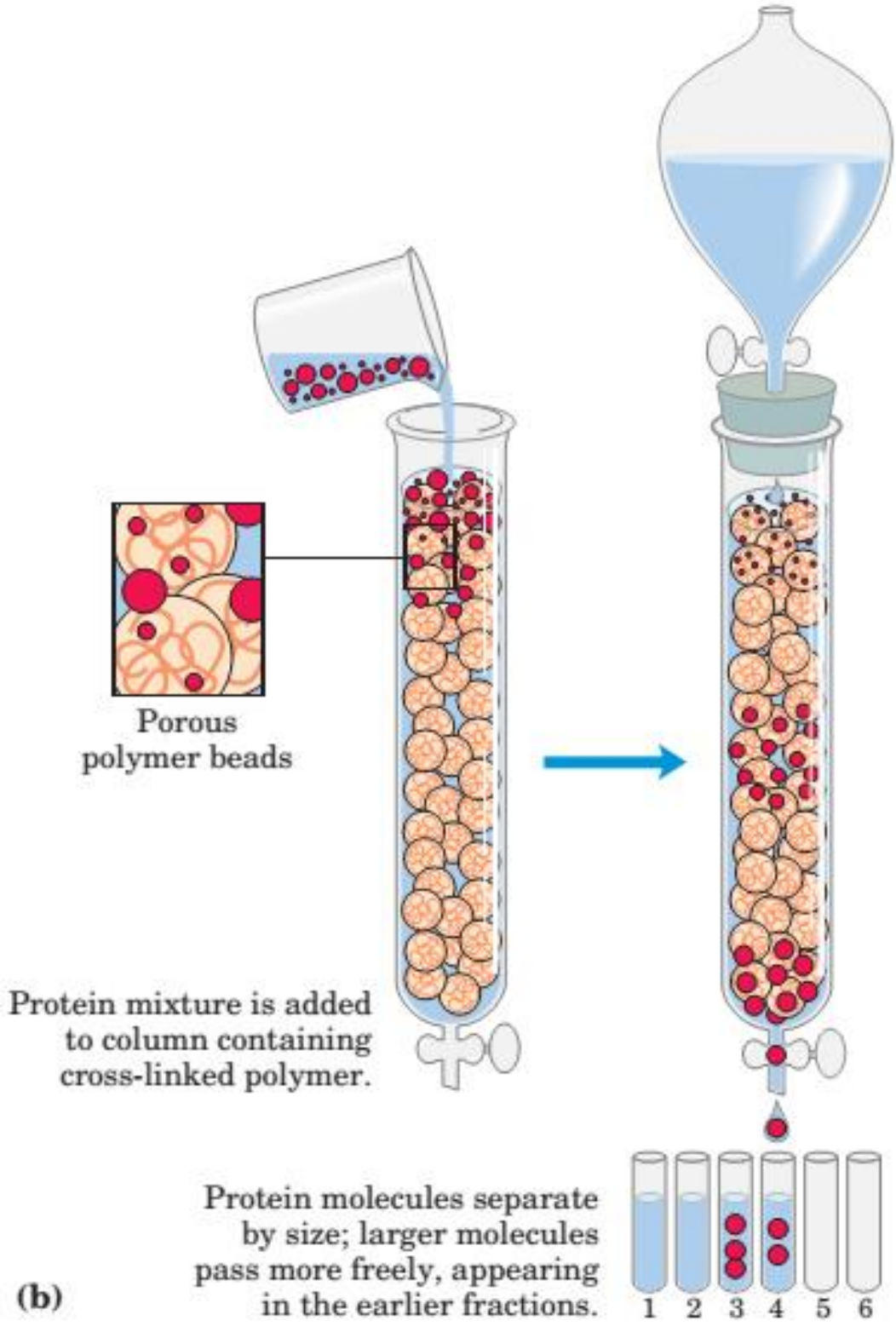
Proteins become more and more pure in each purification step although they lost activity. When working with enzyme pH and temperature of the solutions should be controlled carefully.

Process Step	Fraction volume (mL)	Total Protein (mg)	Activity (Unit)	Specific activity(Unit/mg)
Crude extract	1400	10000	100000	10
Salting out	280	3000	96000	32
Ion exchange	90	400	80000	200
Size exclusion	80	100	60000	600
Affinity	6	3	45000	15000

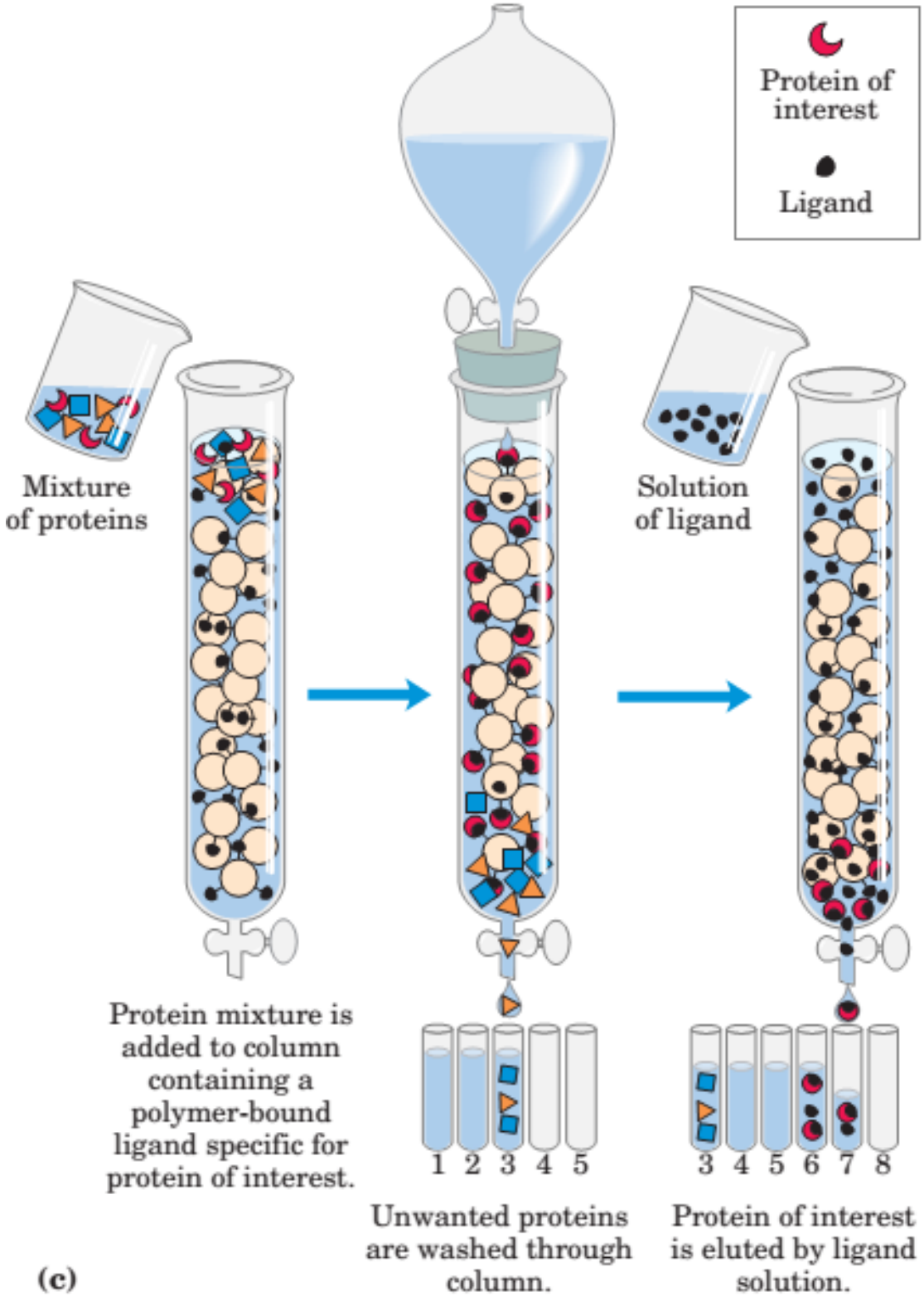
Ion Exchange Chormatography



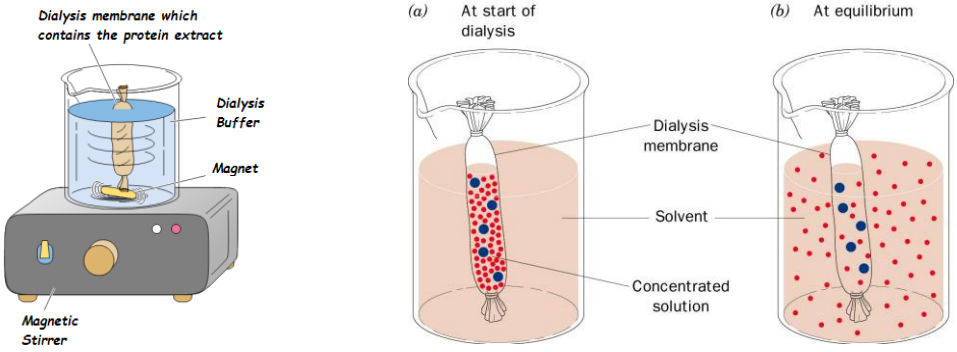
Size Exclusion (Molecular Sieve) Chromatography



Affinity Chromatography



Salt ions that remains in the solution after the salting out interferes with the following experiments and therefore should be removed. Gel filtration, ultracentrifuge and dialysis could be used for that purpose. Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane.



Membranes used in dialysis are made of cellulose and have definite pore size (usually 12000 Da). Salts at low concentration usually used as dialysis buffer. According to the rule of diffusion salts in the protein extract move to the outside of the membrane until concentration is equal both outside and inside of the dialysis tubing. However, since they are much bigger than salt molecules, proteins stay inside the dialysis sack. Equilibrium is reached approximately in 4-6 hours. At this point, dialysis buffer replaced with a fresh one. Dialysis usually complete in 24-48 hours.

Quantitative Determination of Proteins

In every step of the protein purification, it is important to know the quantity of the proteins. Generally, protein concentration of the sample dictates which methods could be used. There are numerous methods developed for determination of protein concentration. Most commonly used ones are simple absorbance, Lowry and Bradford methods. Each of these methods has their own advantages and disadvantages. In these methods, a protein with known concentrations used as a standard sample and a calibration plot prepared for that protein.

Simple Absorbance Method:
Principle: Tyrosine and tryptophan residues of the proteins have a maximum absorbance at 280 nm.

Advantages: Fast, cheap, low amount of sample required, no chemical modification done on the proteins.

Disadvantages: Low sensitivity (0.05–2 mg protein/mL), interference with nucleic acids and other aromatic compounds, high protein-to-protein differences.

Lowry (Folin-Ciocalteau) Method:
Principle: Biuret reaction (peptide bonds react with alkaline copper solutions to form Cu^{+}) coupled with Folin-Ciocalteau reaction (Phosphomolybdotungstic acid under Cu^{+} catalysis react with aromatic amino acids to form heteropolymolibden blue.)

Advantages: Medium protein-to-protein differences, higher sensitivity (0.01–1 mg/mL protein)

Disadvantages: High interferences with certain buffers, drugs, detergents, EDTA, ammonium sulfate, glycine, mercaptoethanol etc. pH should be 10-10.5 and mixing after the addition of reagent is important.

Bradford Method:
Principle: Coomassie Brilliant Blue G250 (CBB) dye reacts with basic amino acid residues in the protein.

Advantages: Faster, cheaper, easier and more sensitive (0.001 mg/mL protein) than Lowry Method. Also interferences with commonly used chemicals are lower than Lowry.

Disadvantages: Interference with ampholites and detergents. Relatively higher protein-to-protein differences.

CHEMICALS
NaCl, Bovine Serum Albumin (BSA), Bradford Reagent, Ovalbumin.

METHOD

Simple Absorbance Method

Serum physiologique solution: %0.897 NaCl (250 mL)
BSA stock solution: 100 mL %0.2 BSA solution (prepared in Serum physiologique solution)

Prepare protein standard solutions according the following table and measure the absorbance of standard solutions and samples at 280 nm. Draw the calibration plot (concentration (µg/mL) vs. absorbance) using the standard protein solutions and from the equation of the plot calculate the concentration of the samples.

Tube No	BSA Stock (mL)	Serum physiologique (mL)	Absorbance (280 nm)	Protein concentration (µg/mL)
1	0.0	3.0		
2	0,5	2,5		
3	1.0	2.0		
4	1,5	1,5		
5	2,0	1,0		
Sample 1	0,1	2,9		
Sample 2	0,1	2,9		
Sample 3	0,1	2,9		
Sample 4	0,1	2,9		

Bradford Method

Protein Stock solution: Ovalbumin stock solution (2 mg/mL) should be diluted 20 times to a final concentration of 0.1mg/mL. Bradford reagent should be at room temperature. Prepare protein standard solutions according the following table and measure the absorbance of standard solutions and samples at 595 nm. Draw the calibration plot (concentration (µg/mL) vs. absorbance) using the standard protein solutions and from the equation of the plot calculate the concentration of the samples.

Tube No	Ovalbumin Stock (µL)	Water (µL)	Bradford reagent (µL)		Absorbance (595 nm)	Protein concentration (µg/mL)
1	0	800	200	Mix and incubate for 10 minutes.		
2	20	780	200			
3	40	760	200			
4	60	740	200			
5	80	720	200			
Sample 1	2	798	200			
Sample 2	2	798	200			
Sample 3	2	798	200			
Sample 4	2	798	200			

QUESTIONS

1- How do you determine protein concentration using bicinchoninic acid assay?

EXPERIMENT VI

PROPERTIES OF CARBOHYDRATES

Carbohydrates are **polyhydroxy aldehydes or ketones**, or substances that yield such compounds on hydrolysis. Many, but not all, carbohydrates have the empirical formula $(CH_2O)_n$; some also contain nitrogen, phosphorus, or sulfur. There are three major size classes of carbohydrates: monosaccharides, oligosaccharides, and polysaccharides.

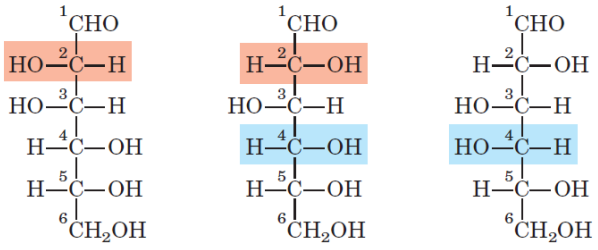
Monosaccharides, or simple sugars, consist of a single polyhydroxy aldehyde or ketone unit. Monosaccharides are colorless, crystalline solids that are freely soluble in water but insoluble in nonpolar solvents. The simplest monosaccharides are glyceraldehyde and dihydroxyacetone. All the monosaccharides except dihydroxyacetone contain one or more asymmetric (chiral) carbon atoms and thus occur in optically active isomeric forms.

Oligosaccharides consist of short chains of monosaccharide units, or residues, joined by characteristic linkages called glycosidic bonds. The most abundant are the disaccharides, with two monosaccharide units. The **polysaccharides** are sugar polymers containing more than 20 or so monosaccharide units

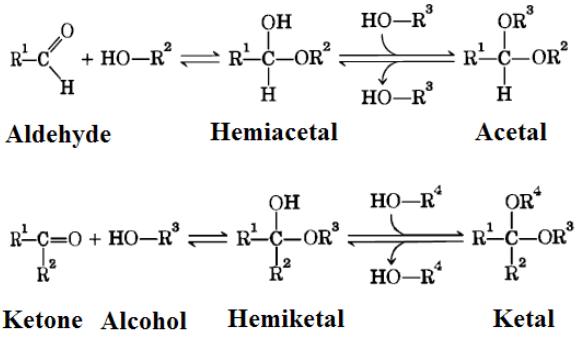
Some carbohydrates provide energy to metabolism (sugars) and also have the ability of short term energy storage (starch and glycogen). They are structural components of bacteria (proteoglycan) and plant (cellulose) cell walls and nucleic acid. Polysaccharides that located the outer surface of the cell are important for the cell signalization.

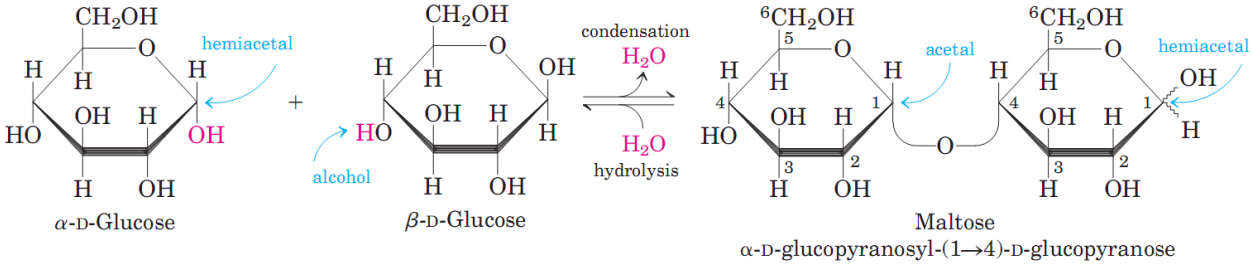
Carbon-containing compounds commonly exist as **stereoisomers**, molecules with the same chemical bonds and same chemical formula but different configuration, the fixed spatial arrangement of atoms. A carbon atom with four different substituents is said to be asymmetric, and asymmetric carbons are called **chiral centers**. Stereoisomers that are mirror images of each other are called **enantiomers**. Enantiomers have nearly identical chemical reactivities but differ in a characteristic physical property: their interaction with plane-polarized light; their **optical activity**. In separate solutions, two enantiomers rotate the plane of plane-polarized light in opposite directions, but an equimolar solution of the two enantiomers (a **racemic mixture**) shows no optical rotation. Compounds without chiral centers do not rotate the plane of plane-polarized light. Distinct from configuration is molecular **conformation**, the spatial arrangement of substituent groups that, without breaking any bonds, are free to assume different positions in space.

Two sugars that differ only in the configuration around one carbon atom are called **epimers**.

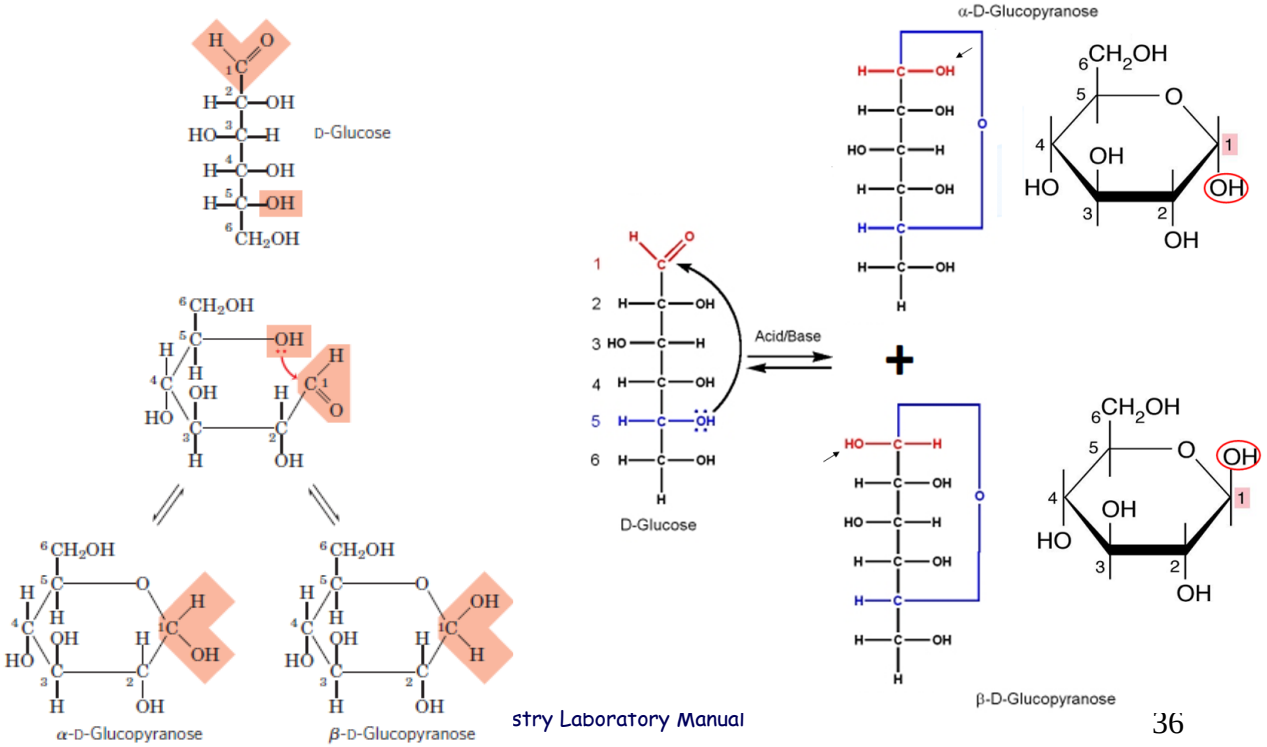


In aqueous solution, all monosaccharides with five or more carbon atoms in the backbone occur predominantly as cyclic (ring) structures in which the carbonyl group has formed a covalent bond with the oxygen of a hydroxyl group along the chain. The formation of these ring structures is the result of a general reaction between alcohols and aldehydes or ketones to form derivatives called **hemiacetals** or **hemiketals**. Addition of the second molecule of alcohol produces the full acetal or ketal, and the bond formed is a glycosidic linkage. When the two molecules that react are both monosaccharides, the acetal or ketal formed is a disaccharide.

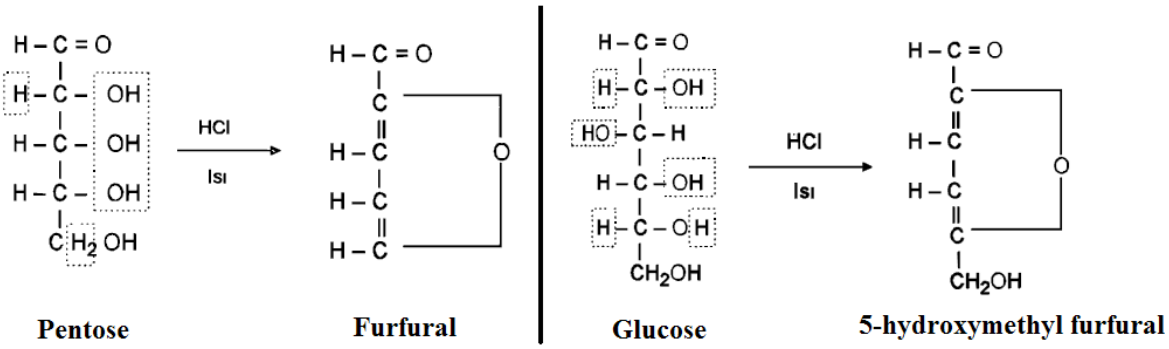




The reaction with the first molecule of alcohol creates an additional chiral center (the carbonyl carbon). Because the alcohol can add in either of two ways, attacking either the "front" or the "back" of the carbonyl carbon, the reaction can produce either of two stereoisomeric configurations, denoted **α** and **β**. Isomeric forms of monosaccharides that differ only in their configuration about the hemiacetal or hemiketal carbon atom are called **anomers**, and the carbonyl carbon atom is called the **anomeric carbon**.



In strong acidic conditions, glycosidic bonds of the carbohydrates hydrolyzed and resulting monosaccharides dehydrogenates. Thus, pentoses turn into furfurals and hexoses turn into 5-hydroxymethylfurfural. These molecules decompose and produce ketoaldehydes which combines with various phenols to produce colored compounds



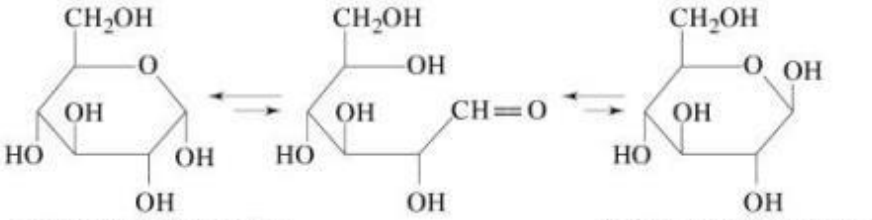
Pentose **Furfural** **Glucose** **5-hydroxymethyl furfural**

Specific rotation is the angle of rotation in degrees of the plane of polarization of a ray of monochromatic light that passes through a tube 1 decimeter long containing the optically active substance in solution at a concentration of 1 gram per millimeter (or 100%).

$$[\alpha]_{\lambda}^{T} = \frac{\alpha_{\lambda}^{T}}{c \cdot l}$$

$[\alpha]$ = specific rotation, l = optical pathlength in dm;
λ = wavelength, T = temperature, α = optical rotation,
c = concentration in g/100ml

The α and β anomers of D-glucose interconvert in aqueous solution by a process called **mutarotation**, in which one ring form (say, the α anomer) opens briefly into the linear form, then closes again to produce the β anomer. Thus, a solution of α-D-glucose and a solution of β-D-glucose eventually form identical equilibrium mixtures having identical optical properties. This mixture consists of about one-third α-D-glucose, two-thirds β-D-glucose, and very small amounts of the linear and five-membered ring (glucofuranose) forms. Rate of the mutarotation is generally slow but diluted alkaline and acidic solutions catalyze it.

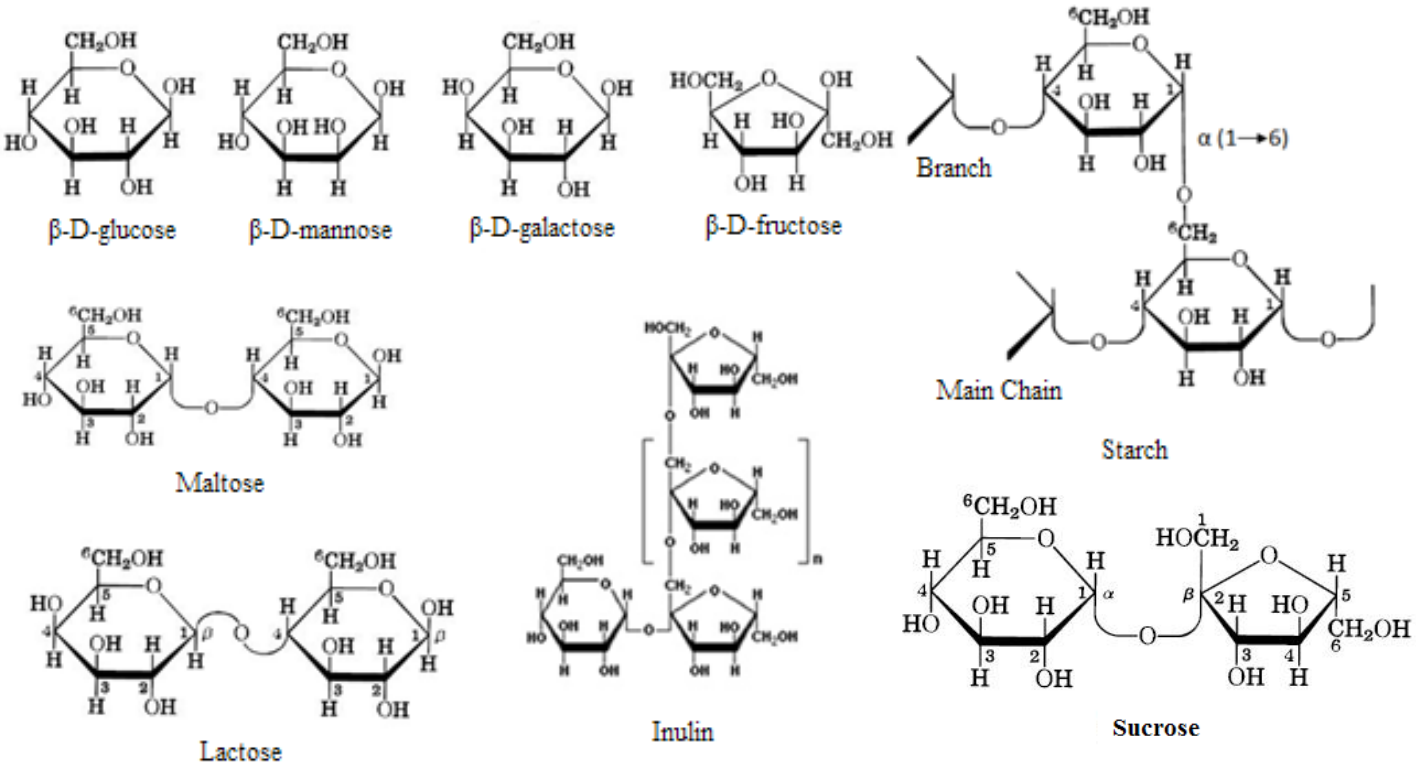


α-D-Glucophranose (36%)
m.p. 146 °C
Specific rotation: 112.2°

β-D-Glucopyranose (64%)
m.p. 148-155 °C
Specific rotation: 18.7°

When either α-form or β-form of glucose is dissolved in water, optical rotation changes and eventually a value of + 52.6° is obtained. Thus α-form changes to β-form (or vice versa) and eventually a solution containing 36% of α-form and 64% of β-form is obtained. This phenomenon is known as *mutarotation*.

CHEMICALS



1% glucose, sucrose (saccharose), starch, galactose, fructose, lactose, maltose and innulin solutions.

Saturated glucose and lactose solutions.

Sulfuric acid, phenyl hydrazine, acetic acid, sodium acetate.

Molisch's reagent: %10 α-naphthol in ethanol, conc. sulfuric acid.

Barfoed's Reagent: 6% Copper Acetate containing 1 mL of acetic acid

Fehling's Reagent: Reagent A: 6.98% $CuSO_4$ (Copper sulfate first dissolve in 1 mL of conc. sulfuric acid) Reagent B: Contains %35 sodium potassium tartrate and 12% sodium hydroxide.

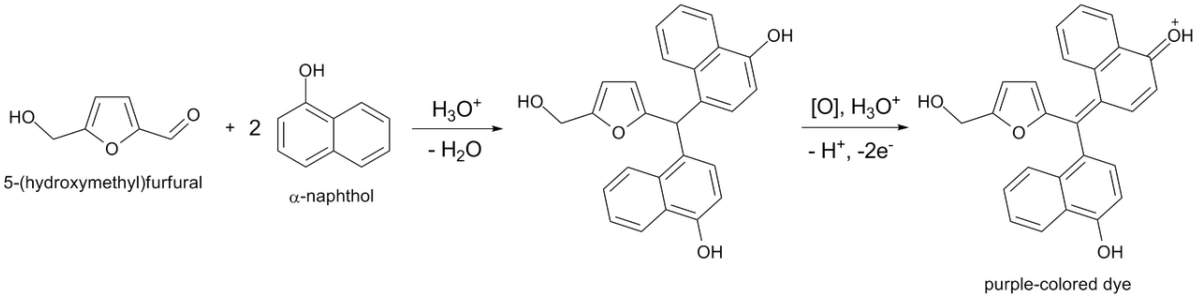
Seliwanoff's Reagent: 0,5% resorcinol dissolved in 25% v/v HCl

Rothenfusser's Reagent: 2g diphenylamine dissolved in 20 mL of ethanol, and 80 mL glacial acetic acid and 100 mL hydrochloric acid added to the solution

METHOD

Molisch's test

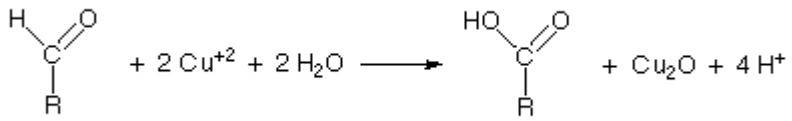
Take 3 test tube and add approximately 0.5 mL glucose, sucrose and starch solutions using a Pasteur's pipette. Add 4-5 drop of **Molisch's reagent** and mix vigorously using a whirlmaker. In a fume hood, incline the test tube and slowly add approximately 1 mL of concentrated sulfuric acid so that it forms a separate layer (this is a ring experiment). An appearance of reddish violet or purple ring at the junction of two liquids is observed in a positive Molisch test.



Carbohydrates when treated with concentrated sulfuric acid undergo dehydration to give furfural derivatives. These compounds condense with α-naphthol to form colored products. This test is given by **all carbohydrates.**

Barfoed's test

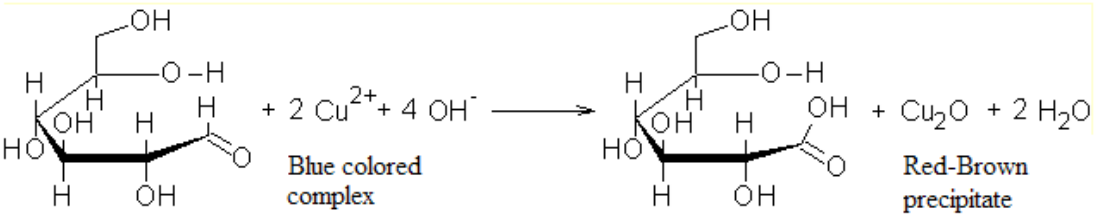
Take 7 test tube and add approximately 1 mL glucose, galactose, fructose, sucrose, lactose, maltose and starch solutions using a Pasteur's pipette. Add approx. 1 mL of **Barfoed's reagent** and place in the boiling water bath for exactly 3 minutes. When the waiting period is over cool the test tubes under running water and observe. A scanty brick red precipitate observed in a positive reaction.



This test is used to distinguish reducing monosaccharides from disaccharides by controlling pH and time of heating. On prolonged heating disaccharides can also give this test positive.

Fehling's Test:

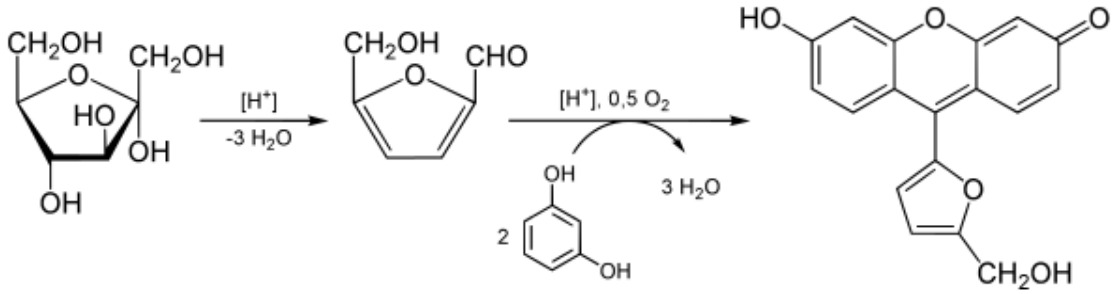
Take 5 test tube and add approximately 0,5 mL glucose, fructose, sucrose, lactose, and inulin solutions using a Pasteur's pipette. Add approx. 1 mL of **Fehling's reagent working solution** which is prepared by mixing Fehling Reagent A and Reagent B at equal volume. Place the test tubes in hot water bath and observe.



Carbohydrates which contains a free hydroxyl group at their anomeric carbon called as reducing sugars. Reducing sugars give positive Fehling's test.

Seliwanoff's Test

Take 5 test tube and add approximately 0,5 mL glucose, fructose, sucrose, lactose, and inulin solutions using a Pasteur's pipette. Add approx. 2 mL of **Seliwanoff's reagent** and place the test tubes in boiling hot water bath for 1 minute exactly. When the waiting period is over cool the test tubes under running water and observe. A cherry red color is observed in a positive reaction.



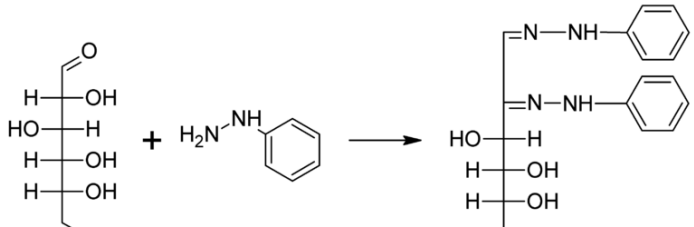
This test is given positive by ketohexoses which on treatment with hydrochloric acid form 5-hydroxymethyl furfural and reacts with resorcinol. Overheating of the solution should be avoided.

Rothenfusser's test:

Take 5 test tube and add approximately 1 mL glucose, fructose, sucrose, lactose, and inulin solutions using a Pasteur's pipette. Add approx. 1 mL of **Rothenfusser's reagent** and place the test tubes in boiling hot water bath for up to 5 minutes. Positive test is given by ketoses.

Osazone Test (This test will be done by the instructor)

Take 3 test tube and add 0,5 mL of phenyl hydrazine and 0,5 mL of acetic acid. Shake vigorously and add 0,5 g sodium acetate. Completlely dissolve the mixture by heating and add 3 mL of glucose, lactose and galactose solutions to each test tube, separately. Place in a boiling water bath for at least 30 minutes. Allow them to cool slowly and examine the crystals under the microscope.



A solution of reducing sugar when heated with phenyl hydrazine, characteristic yellow crystalline compound called Osazone are formed. These crystals have definite crystalline structure, precipitate time and melting point for different reducing sugars. Glucose, fructose and mannose produce the same Osazone because of the similarities in their molecular structure.

QUESTIONS

1- What is the invert sugar? Please give a brief information about its structur, chemical composition and usage.

EXPERIMENT VII

IODOMETRIC DETERMINATION OF GLUCOSE

Iodine oxidize sugars to aldonic acids in weal alkaline conditions. This reaction occurs easily for aldoses but ketoses give this reaction very slowly.

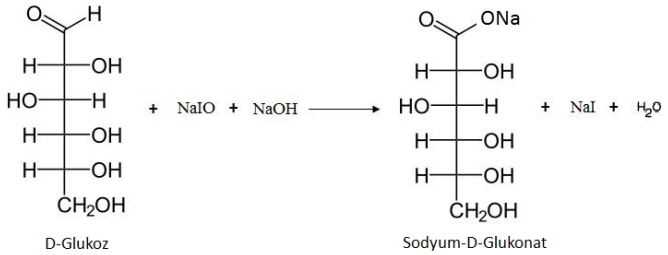
Since iodine is poorly soluble in water its solubility increased using potassium iodide.

$$I_2 + I^- \longleftrightarrow I_3^-$$

Like other halogens iodine gives redox reactions in alkaline conditions to produce IO^- and I^-.

$$2\ NaOH + I_2 \longrightarrow NaOI + NaI + 3H_2O$$

Hipoiodite that produce in this reaction reacts with aldoses in alkaline conditions to generate aldonic acids.



With the adition of acid, remaining hypoiodite ion gives a redox reaction with the excess iodide ion to produce iodine.

$$IO^- + I^- + 2HCl \longrightarrow I_2 + H_2O + 2Cl^-$$

Resulting iodine could be titrated by thiosulfate to determine the amount of hypoiodite that required to oxidize sugars. And using this value we can quantitatively analyze the glucose content of a sample.

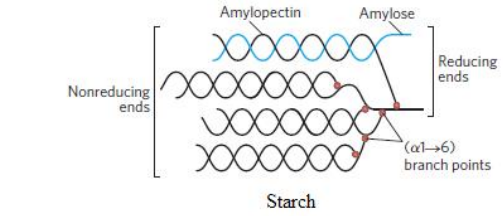
$$I_2^0 + 2S_2O_3^{2-}\ (\text{thiosulfate}) \longrightarrow S_4O_6^{2-}\ (\text{tetrathionate}) + 2I^-$$

Resulting iodide ion reacts with starch to give a blue color therefore starch can be used as a indicator for this titration.

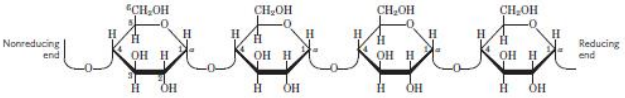
Starch contains two types of glucose polymer, amylose and amylopectin. Amylose consists of long, unbranched chains of D-glucose residues connected by (α1→4) linkages (as in maltose). Such chains vary in molecular weight from a few thousand to more than a million. Amylopectin also has a high molecular weight (up to 200 million) but unlike amylose is highly branched. The glycosidic linkages joining successive glucose residues in amylopectin chains are α1→4); the branch points (occurring every 24 to 30 residues)

are (α1→6) linkages. Amylose could be obtained in crystal form and due to its helix structure can react with iodide to give blue color.

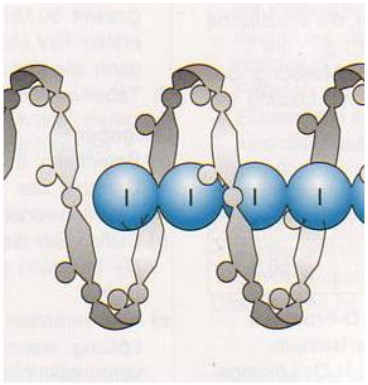
CHEMICALS



Starch



Amylose



0.5%

Glucose, 1% Honey (1 g honey dissolved in 100 mL water) 0.1 N Iodine (0.1N Iodine: Weigh 13 g iodine and 20 g potassium iodide together and dissolve in water and make up to 1 liter. Store in amber colored bottle), 15% Na_2CO_3, 0.05N Thiosulfate solution (12.4 g $Na_2S_2O_3$. $5H_20$ or 7.9g $Na_2S_2O_3$ dissolved in 1L), 10% HCl (27.4 mL conc. HCl dissolved in 100 mL) and 1% soluble starch (prepared in boiling water).

METHOD

Erlen Flask	**Water (ml)**	**0.5% Glucose (ml)**	**15% Na_2CO_3**	**0.1N İodine (ml)**		**10% HCl (ml)**		**Used $Na_2S_2O_3$ (mL)**	**Glucose concentration (mg/mL)**
1	5	-	1	7,5	**Incubate the flasks for 30 minutes in the dark**	1,5	**Titrate with 0.005 N thiosulfate until the color turn to yellow. Then add 1%starch and titrate until colorless**		
2	4	1	1	7,5		1,5			
3	2,5	2,5	1	7,5		1,5			
4	-	5	1	7,5		1,5			
Sample	4	1 mL from 1% honey solution	1	7,5		1,5			

Using the calibration plot between the amount of glucose versus used $Na_2S_2O_3$ volume, determine the glucose concentration of the honey sample.

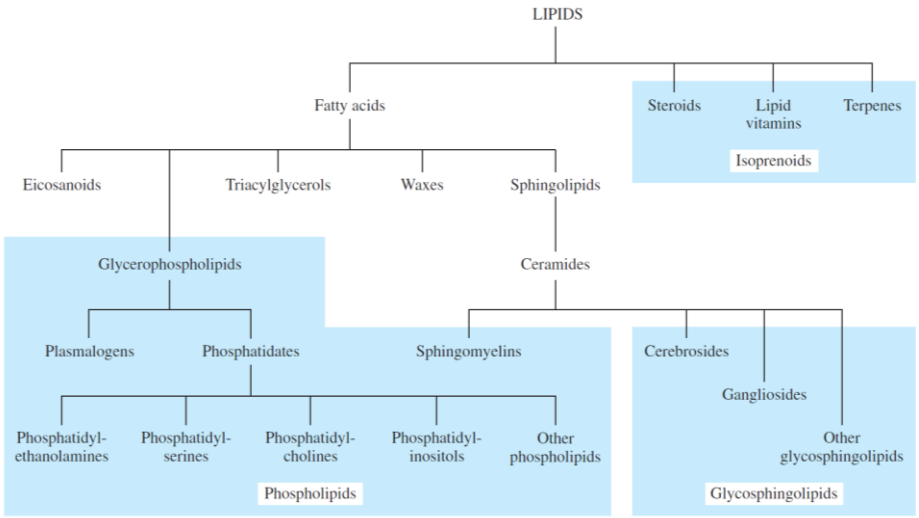
QUESTION

1- What is a polysaccharide? What are the functions of the polysaccharides?

EXPERIMENT VIII

PROPERTIES OF LIPIDS

Biological lipids are a chemically diverse group of compounds, the common and defining feature of which is their insolubility in water. Lipids can be classified as follows.

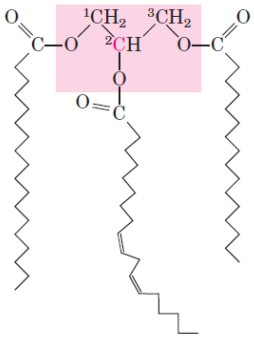


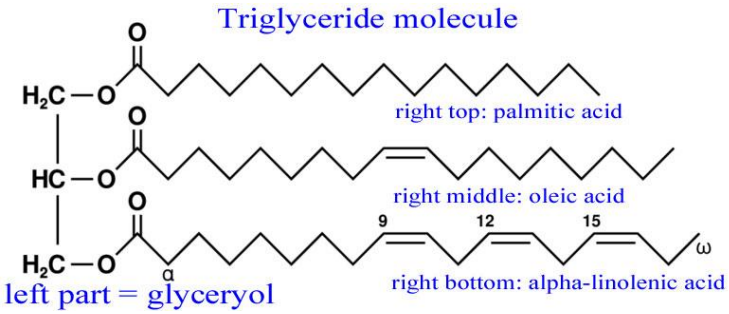
The biological functions of the lipids are as diverse as their chemistry. Fats and oils are the principal stored forms of energy in many organisms. Phospholipids and sterols are major structural elements of biological membranes. Other lipids, although present in relatively small quantities, play crucial roles as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, "chaperones" to help membrane proteins fold, emulsifying agents in the digestive tract, hormones, and intracellular messengers.

Fatty acids are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long (C4 to C36). In some fatty acids, this chain is unbranched and fully saturated (contains no double bonds); in others the chain contains one or more double bonds

Carbon skeleton	Structure*	Systematic name†	Common name (derivation)	Melting point (°C)	Solubility at 30 °C (mg/g solvent)	
					Water	Benzene
12:0	$CH_3(CH_2)_{10}COOH$	*n*-Dodecanoic acid	Lauric acid	44.2	0.063	2,600
14:0	$CH_3(CH_2)_{12}COOH$	*n*-Tetradecanoic acid	Myristic acid	53.9	0.024	874
16:0	$CH_3(CH_2)_{14}COOH$	*n*-Hexadecanoic acid	Palmitic acid	63.1	0.0083	348
18:0	$CH_3(CH_2)_{16}COOH$	*n*-Octadecanoic acid	Stearic acid	69.6	0.0034	124
20:0	$CH_3(CH_2)_{18}COOH$	*n*-Eicosanoic acid	Arachidic acid	76.5		
16:1(Δ^9)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	*cis*-9-Hexadecenoic acid	Palmitoleic acid	1-0.5		
18:1(Δ^9)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	*cis*-9-Octadecenoic acid	Oleic acid	13.4		
18:2($\Delta^{9,12}$)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	*cis*-,*cis*-9,12-Octadecadienoic acid	Linoleic acid	1-5		
18:3($\Delta^{9,12,15}$)	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	*cis*-,*cis*-,*cis*-9,12,15-Octadecatrienoic acid	α-Linolenic acid	−11		
20:4($\Delta^{5,8,11,14}$)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$	*cis*-,*cis*-,*cis*-,*cis*-5,8,11,14-Icosatetraenoic acid	Arachidonic acid	−49.5		

The simplest lipids constructed from fatty acids are the triacylglycerols, also referred to as triglycerides, fats, or neutral fats. Triacylglycerols are composed of three fatty acids each in ester linkage with a single glycerol. Those containing the same kind of fatty acid in all three positions are called simple triacylglycerols and are named after the fatty acid they contain. Most naturally occurring triacylglycerols are mixed; they contain two or more different fatty acids.





CHEMICALS

Ethyl alcohol, petroleum ether, chloroform, benzene, carbon tetrachloride, Wijs Reagent (ICl in acetic acid), 10% KI, 0.1 N $Na_2S_2O_3$ solution (24.8 g $Na_2S_2O_3$. $5H_20$ or 15.8g $Na_2S_2O_3$ and 1g Na_2CO_3 dissolved in 1L), 1% Starch (Used in boiling water)

METHOD

Solubility of triglycerids

(WARNING! This experiment must be done in the fume hood and the wastes will be discard inside the fume hood !!!)

Take 5 test tubes and add 5 drops of sunflower oil. To each test tube add 1.5 mL of one of the following solvents; ethanol, petroleum ether, chloroform, benzene and water. Mix the test tubes' content using a whirlmaker and observe.

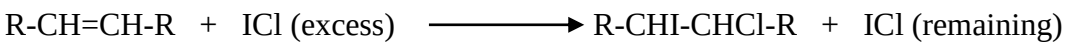
Heat the test tube with ethanol in 70°C water bath, and observe if there are any changes to the solubility.

pH of the triglycerids

Take 3 test tube and using a Pasteur's pipette add approx. 0,5 mL of olive oil, 0,5 mL of sunflower oil and a small amount of butter using a glass rod (carefully place the butter at the bottom of the test tube) a small amount of butter. Then add approx. 3 mL of ethanol to the test tubes, shake vigorously using a whirlmaker and heat for 2 minutes in 70°C water bath. Mix them again thoroughly using a whirlmaker and determine their pH values using a pH strip.

Determination of the saturation degree of triacylglycerids

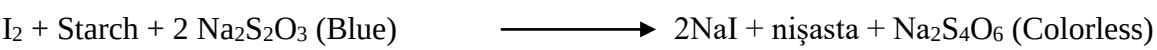
The **iodine value** (or **iodine index**) is the mass of iodine in grams that is consumed by 100 grams of a triglycerid. Iodine values are used to determine the amount of unsaturation in fatty acids. This unsaturation is in the form of double bonds, which react with iodine compounds. Wijs method is commonly used to determine the degree of saturation (or iodine value) of the fats and oils. The higher the iodine number, the more C=C bonds are present in the fat.



Iodine that used in the reaction calculated as follows;

$$ICl\ (used) = ICl\ (excess) - ICl\ (remaining)$$

The amount of the remaining ICl could be determined by the addition of excess KI to the solution and consequent titration of the resulting I_2 with thiosulfate.



Erlen Flask	**Oil or fat (g)**	**CCl_4 (mL)**		**Wijs reaktifi (mL)**		**%10 KI (mL)**		**Used $Na_2S_2O_3$**
1	Sunflower oil 0.2 g	15	**Thoroughly mix to solve oils and fats**	25	Cover the Erlenmeyer Flasks and incubate for 30 minutes in the dark	20	**Titrate with 0.1 N thiosulfate until the color turn to yellow. Then add 1% starch and titrate until colorless**	
2	Olive Oil 0.2 g	15		25		20		
3	Butter 0.4 g	15		25		20		
4	0	15		25		20		

QUESTION

1- What are the iodine values of the commonly consumed oils and fats? Which type of oils are better?

$$Iodine\,Value = \frac{(B-S)\times M \times 12.96}{wt.\,of\ oil\,(g)}$$

Where:

B = Titration of blank (mL)

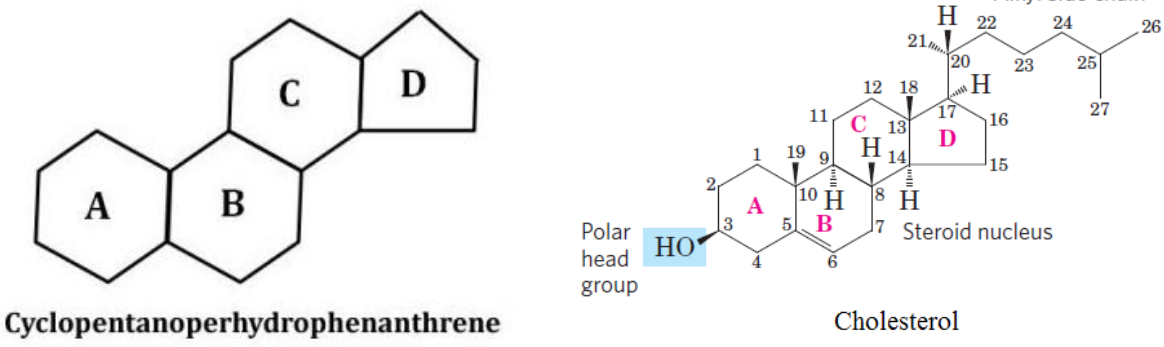
S = Titration of test solution (mL)

M = Molarity of $Na_2S_2O_3$

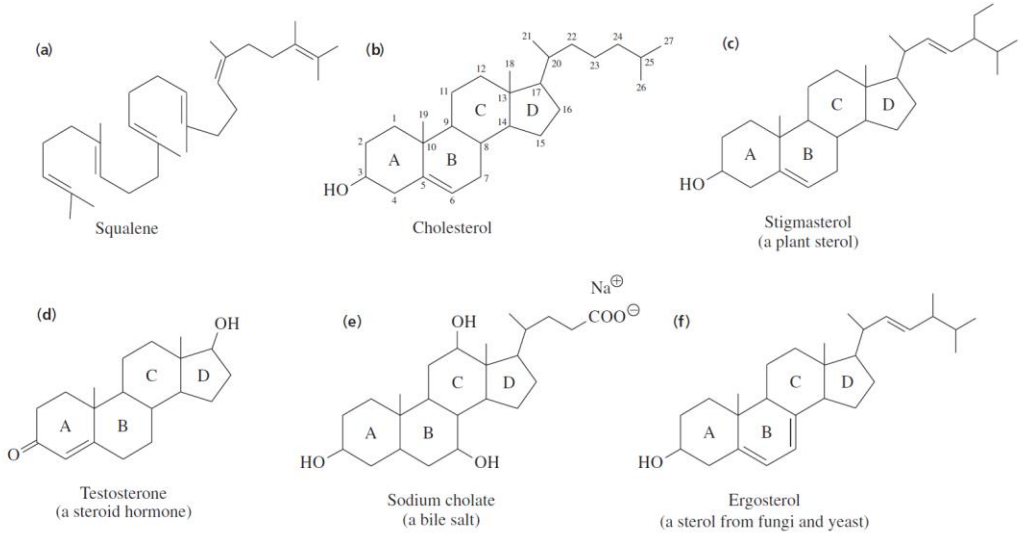
EXPERIMENT IX

LIPID EXTRACTION AND STEROL DETERMINATION

Sterols are secondary alcohols which contains cyclopentanoperhydrophenanthrene ring system. Cholesterol, the major sterol in animal tissues, is amphipathic, with a polar head group (the hydroxyl group at C-3) and a nonpolar hydrocarbon body (the steroid nucleus and the hydrocarbon side chain at C-17), about as long as a 16-carbon fatty acid in its extended form. Similar sterols are found in other eukaryotes: stigmasterol in plants and ergosterol in fungi, for example. Bacteria cannot synthesize sterols; a few bacterial species, however, can incorporate exogenous sterols into their membranes



In addition to their roles as membrane constituents, the sterols serve as precursors for variety of products with specific biological activities. Steroid hormones, for example, are potent biological signals that regulate gene expression. Bile acids are polar derivatives of cholesterol that act as detergents in the intestine, emulsifying dietary fats to make them more readily accessible to digestive lipases.



CHEMICALS

Lieberman's reagent (60 mL acetic anhydride, 10 mL sulfuric acid, 30 mL acetic acid and 0.6 g sodium sulfate)

Preparation of the Lieberman's reagent: In a round reaction flask which is on an ice bath add 60 ml of cold acetic acid. While mixing continuously, slowly add 10 mL of concentrated sulfuric acid. Finally add 30 mL of acetic acid and 0.6 g of anhydrous sodium sulfate. Reagent could be stored for about a week.

10 mM cholesterol standard: 0,191 g cholesterol/ 50 mL chloroform: methanol 1:1
5 mM cholesterol standard: Take 10 ml from 10 mM cholesterol standard and add 10 mL of chloroform: methanol 1:1
2.5 mM cholesterol standard: Take 10 ml from 5 mM cholesterol standard and add 10 mL of chloroform: methanol 1:1

METHOD

Lipid extraction

In a plastic centrifuge tube take 1g of egg yolk or butter add 10 mL of chloroform: methanol 2:1 and mix thoroughly with a whirlmaker. Centrifuge for 10 minutes at the highest rpm available and transfer the supernatant in a clean test tube.

Add 3 mL of 0,02% $CaCl_2$ mix thoroughly with a whirlmaker, wait for phases to be completely separated and then discard the upper phase.

Add same amount of chloroform:methanol:water 2:50:50, mix thoroughly with a whirlmaker, wait for phases to be completely separated and then discard the upper phase.

Measure the volume of the extract.

Qualitative determination of cholesterol

Salkowski's Test

Take 2 test tube and add 0,5 mL of lipid extract and a small amount of cholesterol in each of the test tubes and solve in 2 mL of chloroform. In a fume hood, incline the test tube and slowly add approximately 2 mL of concentrated sulfuric acid so that it forms a separate layer (this is a ring experiment). An appearance of reddish ring at the upper phase is observed in a positive test.

Quantitative determination of cholesterol

Lieberman's Test

Tube No		Liebermann Reagent		Absorbance at 550 nm
1	0,5 mL chloroform: methanol 2:1	5 mL	Cover the test tubes and incubate in 35 °C water bath for 10 minutes	
2	0,5 mL 2,5 mM Cholesterol standard	5 mL		
3	0,5 mL 5 mM Cholesterol standard	5 mL		
4	0,5 mL 10 mM Cholesterol standard	5 mL		
5	0,5 mL Lipid extract	5 mL		

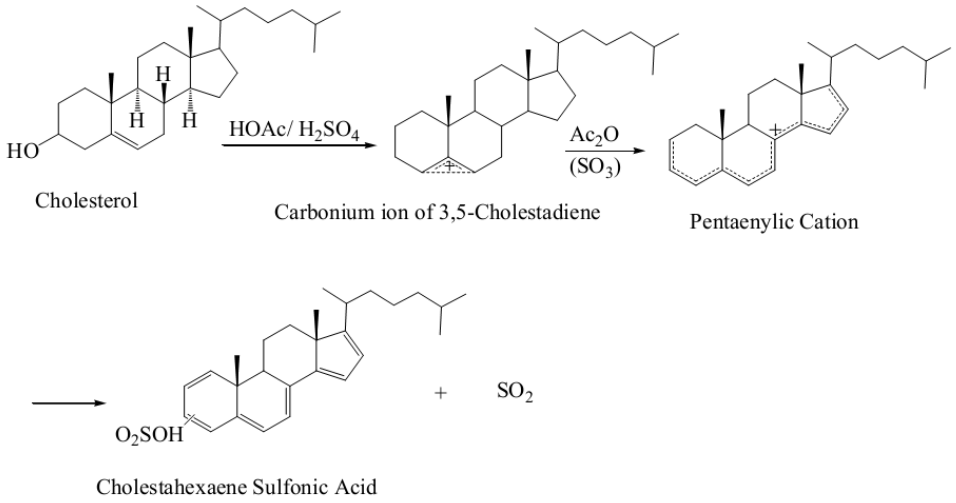


Figure 1. Mechanism for Liebermann-Burchard reaction.

Using the calibration plot between the amount of cholesterol versus absorbance, determine the cholesterol concentration of the butter and yolk.

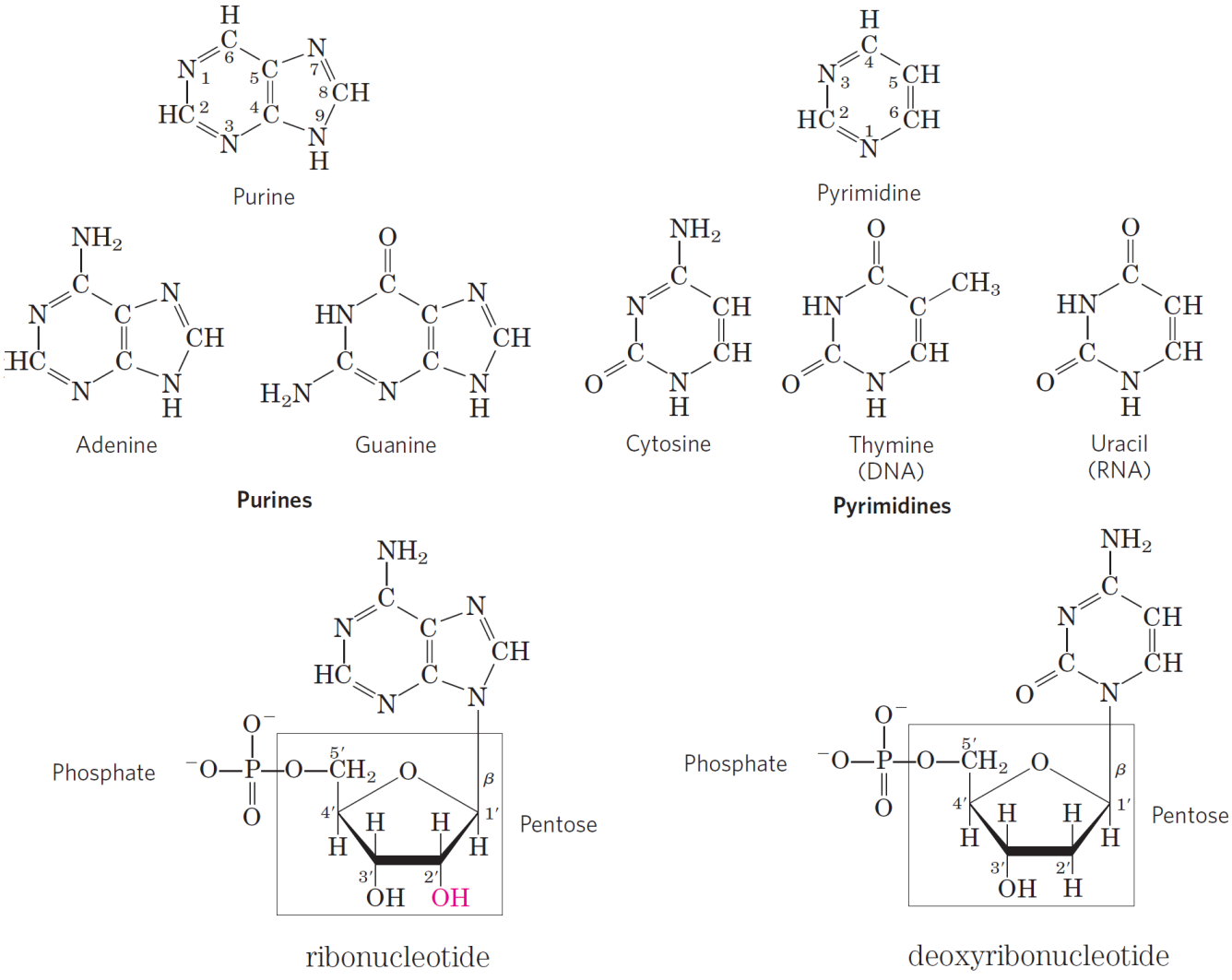
QUESTION

1- What are the functions of the sterols in human body?

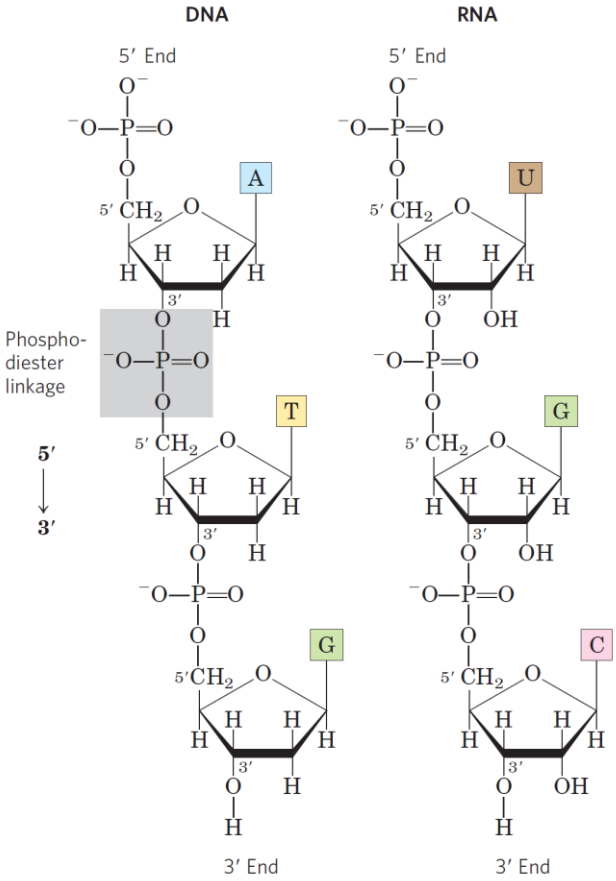
EXPERIMENT X

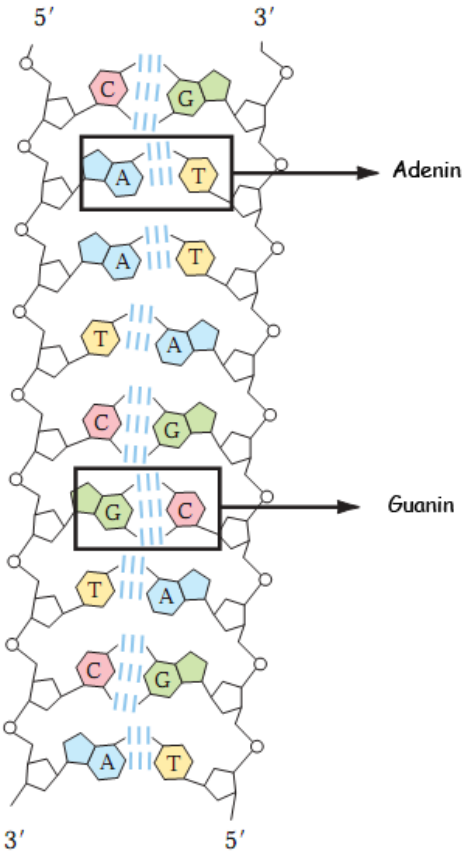
ISOLATION AND PROPERTIES OF DNA

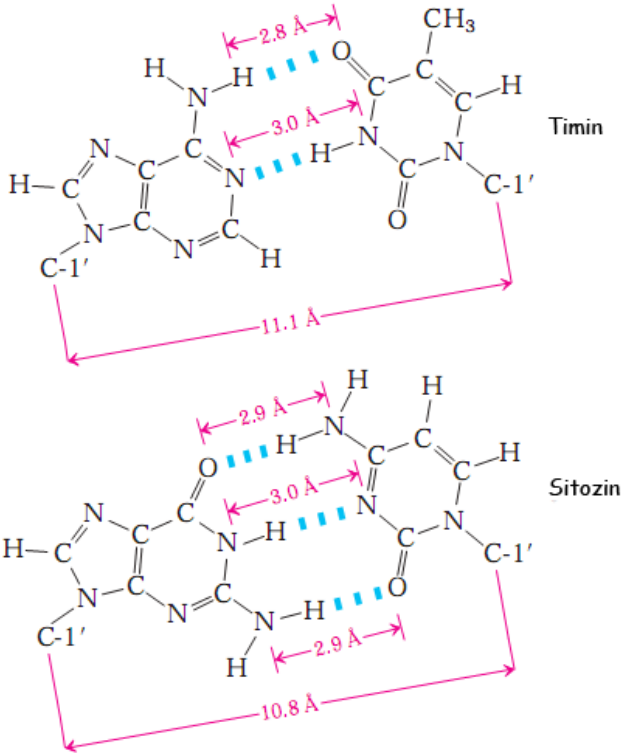
Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are the molecular repositories of genetic information. **Nucleotides** are the building blocks of these macromolecules. Each nucleotide consists of **a sugar**, **a phosphate** and **a nitrogenous base**.



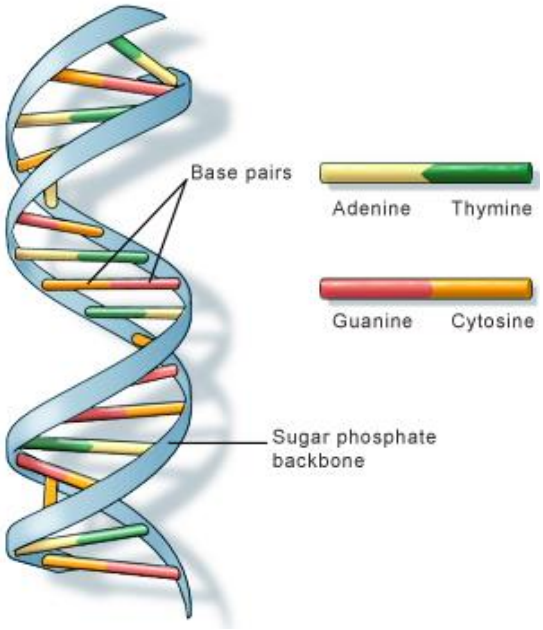
In nucleic acids, nucleotides are connected by 3′,5′-phosphodiester bridges. Phosphodiester bridges between sugar molecules make up the backbone of nucleic acids.


DNA
RNA
5′ End
5′ End
Phospho-
diester
linkage
5′
3′
3′ End
3′ End


5′
3′
Adenin
Guanin
3′
5′


2.8 Å
3.0 Å
Timin
11.1 Å
2.9 Å
3.0 Å
Sitozin
2.9 Å
10.8 Å

In DNA double helix adenine-timine (A=T) and guanine-cytosine (G≡C) pairs with each other. Hydrogen bonds between A-T and G-C pairs (**horizontal interactions**) and interactions between bases (**vertical interactions**) support the double helix structure of DNA.

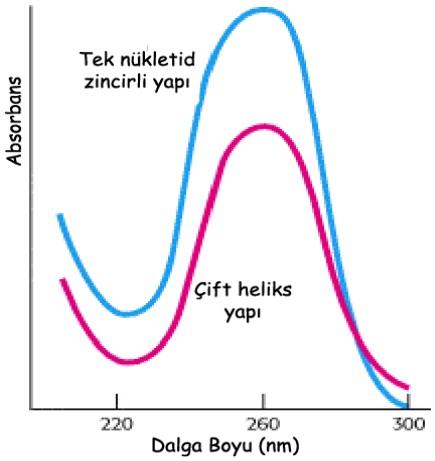


Just as heat and extremes of pH denature globular proteins, they also cause **denaturation**, or **melting**, of double-helical DNA. Disruption of the hydrogen bonds between paired bases and of base stacking causes unwinding of the double helix to form two single strands, completely separate from each other along the entire length or part of the length (partial denaturation) of the molecule. No covalent bonds in the DNA are broken.

The close interaction between stacked bases in a nucleic acid has the effect of decreasing its absorption of UV light relative to that of a solution with the same concentration of free nucleotides, and the absorption is decreased further when two complementary nucleic acid strands are paired. This is called the **hypochromic effect**. Denaturation of a double-stranded nucleic acid produces the opposite result: an increase in absorption called the **hyperchromic effect**. The transition from double-stranded DNA to the single-stranded, denatured form can thus be detected by monitoring UV absorption at 260 nm.

If DNA chain shortens as a result of covalent bond breakage of one of both chain, this event called as **DNA degradation**.

Each species of DNA has a characteristic denaturation temperature, or melting point (t_m; formally, the temperature at which half the DNA is present as separated single strands): the higher its content of G≡C base pairs, the higher the melting point of the DNA. This is because G≡C base pairs, with three hydrogen bonds, require more heat energy to dissociate than A=T base pairs. Thus the melting point of a DNA molecule, determined under fixed conditions of pH and ionic strength, can yield an estimate of its base composition.



The ability of two complementary DNA strands to pair with one another can be used to detect similar DNA sequences in two different species or within the genome of a single species. If duplex DNAs isolated from human cells and from mouse cells are completely denatured by heating, then mixed and kept at about 25 °C below their t_m for many hours, much of the DNA will anneal (renaturate). This process known as **hybridization**.

Double stranded DNA at 50µg/mL concentration has an absorbance of 1.000 at 260 nm (l=1cm). For single stranded DNA this value is 40 µg/mL.

Purity of nucleic acids can be determined by the ratio of $A_{260\ nm}$/ $A_{280\ nm}$. For DNA this ratio is 1.8, and for DNA this ratio is 2.0. If ratio is lower than these values, that means DNA (or RNA) sample **contaminated** with proteins.

An **isotonic solution** refers to two solutions having the same osmotic pressure across a semipermeable membrane. This state allows for the free movement of water across the membrane without changing the concentration of solutes on either side. A common example of an isotonic solution is saline solution (%0.876 NaCl, serum physiologique).

CHEMICALS

NaCl, EDTA, SDS, ethanol.

Method

*Extraction solution***:** 0.15 M NaCl and 0.1 M EDTA. 0,925 g EDTA and 2,19 g NaCl dissolved in 250 mL distilled water. Stored in refrigerator and used cold.

2M NaC solution: 11.7 g NaCl dissolved in 100 mL distilled water.

DNA isolation:

Take 10g tissue and homogenize it with 100 mL cold extraction solution. Then add 1 mL of 10% SDS to that solution and after mixing centrifuge it at 4000 rpm for 10 minutes. Transfer 4 ml of supernatant into a new centrifuge tube and add 8 mL cold 2M NaCl solution. Once more mix and centrifuge it at 4000 rpm for 5 minutes and transfer 4 ml of supernatant into a new glass test tube. Add 8 mL of cold ethanol to the test tube and observe changes. Centrifuge the test tube for 1-2 minutes at 4000 rpm and decant the supernatant. Add 1-2 mL 0,1M EDTA solution to the test tube and wash the precipitate to remove ethanol remnants. Finally solve the pellet into low amount of extraction solution.

DNA Melting:

Check the concentration and purity of DNA sample by the ratio of $A_{260\ nm}$/ $A_{280\ nm}$

Prepare and do the experiment according to following table.

Tube	DNA isolate (mL)	Process	Process	Process	Absorbance (260 nm)	Absorbance (280 nm)
1	5	Keep in room temperature	Keep in room temperature	Keep in room temperature		
2	5	Heat to 80°C (10 minutes)	Keep in room temperature	Keep in room temperature		**Absorbance not measured**
3	5	Heat to 80°C (10 minutes)	Keep in ice bath (5 minutes)	Keep in room temperature		

Question

1- What is Genomics? Why genomic studies are important?