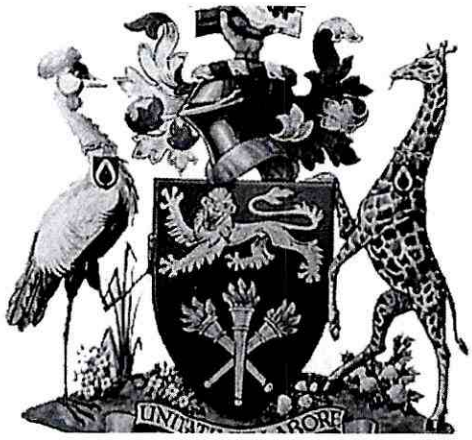

UNIVERSITY OF NAIROBI

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL

FOR

BACHELOR OF VETERINARY MEDICINE (BVM I)

2019

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General instructions for laboratory practicals in Biochemistry

A) Conduct of experiments

The student should read and become thoroughly familiar with the following rules and requirements. Each student is held responsible for all the materials in these instructions.

1. It is absolutely essential to study systematically the laboratory experiment before class. It is impossible to finish the experiments required unless each student definitely plans his/her work. More so, satisfactory observations, better understanding of the processes involved, and definite saving of time will be accomplished by carefully determining the objective of the experiment, the principles involved in the procedure, and the value of the evidence obtained.

2. Neatness and cleanliness are as important in the laboratory as a proper systemization of the procedure. All the procedure, especially in working with the delicate biological products. All precautions used in qualitative and quantitative analyses must be observed. The instructor in determining the course grade will appraise the techniques with which the student conducts his/her experiments.

3. The student must bear in mind that in working with biological materials he/she is usually dealing with organic compounds of a particular labile character. As little time as possible should be allowed to elapse before completing a preparation or determination using animal or plant tissues or fluids. Due to the complexity of compounds, the presence of catalytic agents which hasten processes of deterioration, and to the peculiar colloidal condition of many of the constituents in biochemistry, it is necessary to adhere carefully to the details in order to obtain the best yield and purity of products. In many cases these instructions are based upon empirically devised experiments partly because the systems are so complex in nature and partly because of the limited state of our knowledge. That is, the biochemist combines chemically precise quantitative methods with the comparative modes of study of the biologist.

4. When the experiment is in the nature of an unknown determination, or when the results of an experiment are to be submitted to the instructor, reports must be in your laboratory book. The report should contain sufficient experimental data and all calculations to permit verification of the results.

5. The instructors are available for the purpose of supervision. Questions are welcomed, provided the student is certain that he/she cannot answer the questions with some additional thought before monopolizing the instructor's time.

PRACTICAL ONE

Titration of acid and base

Introduction

Mole Chemistry
Methods of expressing the concentration of solutions

Molarity
The molarity is the number of moles of solute in 1000 mls of solution. The usual symbol for molarity is M. Equal volumes of the same molarity contain the same number of solute molecules. Remember Avogadro's number.

Molality
The molality of a solution is the number of moles of solute in 1,000 g of solvent. The usual symbol for molality is m. All solutions of the same molality contain the same number of solute molecules in equal weights of solution. Rarely used.

Equivalent weight or combining weight
Equivalent weight or combining weight of an element or ion is its atomic or formula weight divided by its valence. Elements entering to combination always do so in quantities proportional to their equivalent weights.

Normality
A normal solution contains one gram molecular weight of the dissolved substance divided by the hydrogen equivalent or valence of the substance per 1000 mls of solution. The symbol for normality is N.
In analytical chemistry involving chemicals between solutions, it is the practice to express concentrations in terms of Molarities or Normalities.

Reagents and Materials
1. Standard: Sodium carbonate (Na_2CO_3; M.Wt. 106) solution containing 0.08gm/10- ml)
2. Unknown Acid: 5.5 ml of concentrated Hydrochloric acid (HCl) diluted with 1000-ml of distilled water.
3. Unknown Alkali: Prepare a saturated solution of Sodium hydroxide (NaOH, M.Wt. 40.00) and allow it to stand for several days.
4. Dilute 5.0 ml of the clear supernatant to 1000 mls with distilled water.
5. Indicator solutions: Methyl red solution; Phenolphthalein solution.

Procedure
A. Into a clean 50-ml beaker, pipette exactly 10 ml of the Sodium carbonate solution (0.08 g/10 ml). Add 2 drops of Methyl red indicator.
Titrate the base with the HCl solution from a 10-ml pipette at a rate of 1 ml while mixing. Just before the end point is reached, boil the solution to expel the CO_2,

cool and complete the titration. Accurately read and record the volume of the acid used. Run this determination in triplicate.

B. Using the HCl filled pipette, titrate 10-ml portion of the NaOH using Phenolphthalein solution as the indicator. Accurately read and record the volume of the acid used. Run this determination also in triplicate.

Results

Calculate

1. The Normality of the standard solution of Na_2CO_3.
2. The Molarity of this solution.
3. The Normality of the acid and the alkali used in the titration.

Questions

1. What is the molarity and normality of:
   a. acetic acid (60.032 g/litre)
   b. sulfuric acid (98.016 g/litre)
2. A student needs 200 ml of 0.1 M NaOH solution. What weight of solid is used?
3. How many grams of glucose are required to prepare 200 ml of 0.5-M solution?

PRACTICAL TWO

Determination of pH without use of standard buffer solution

Introduction

Buffer solutions and pH

The animal body is composed of about 70 % water and 30 % dry matter and there are of course a great many dissolved salts or ions in the fluid, among the most important being "Hydrogen ion" H^+. First we need to be clear about what this term means. The common designation of H^+ suggesting a hydrogen atom which has lost its electron or more exactly a proton which carries a unit positive charge. Such a particle does not exist free in solution, but occurs as the strong acid of water, the hydronium ion, H_3O^+. So what we really mean when we speak of the hydrogen ion concentration is the hydronuim ion concentration.

$$H^+ + H2O \longleftrightarrow H_3O^+$$

The range of $[H^+]$ compatible with life is from 10^{-7} to 10^{-8} M and under normal conditions this is very closely controlled.

This experiment is designed to give experience in measuring hydrogen ion concentration and knowledge about the mechanism of control of $[H^+]$, which exists in tissues. However, we will not study them "in vivo" in the animal, but rather in the test tube or "in vitro".

First the Bronsted's definitions:

An acid is a substance that liberates or donates protons and a base is a substance that accepts protons.

Thus $HCl \longrightarrow H^+ + Cl^-$ (in water solution)
(acid) (base)

Or $H_2O \longrightarrow H^+ + OH^-$

NaOH is the sodium salt of the base OH^- or hydroxyl ion and becomes an alkali. This concept of acids and bases is important for it will be expanded later in an explanation of the mechanism of all chemical reactions that occur in the animal body. The concentration of ions in solutions is usually expressed as normality or molarity such as 1N or 0.1 M. The system becomes cumbersome when expressing the (H^+) of water or biological fluids such as 0.0000001 N or $1x10^{-7}$ N. This has led to the use of the term pH in which "p" signifies that the negative logarithm is used.

Thus:
pH = -log of the $[H^+]$

Or
pK = -log of K, the equilibrium constant.

Buffers

Water has the properties of both an acid and a base. Applying the Law of Mass Action to the equation:

$H^+ + OH^- = H_2O$, the equilibrium constant, K, may be expressed:

$$K = \frac{[H^+][OH^-]}{[H_2O]}$$

Since water is negligibly dissociated, the concentration of water is essentially constant and can be combined with K- Kwater.
Then

$$Kwater = [H^+][OH^-] = 10^{-14}$$

This is the ionic product at 22^0C. The value of 10^{-14} is derived from water conductivity measurements. When this expression is converted to the negative logarithm (p = -log).

$$pKwater = 14 = pH + pOH$$

This may be similarly applied to the dissociation of an acid in the terms of Bronsted's definition:

$$\underset{\text{(Acid)}}{Hac} = \underset{\text{(Base)}}{H^+ + Ac^-}$$

The equilibrium constant for the dissociation of the acid becomes:

$$Ka = \frac{[H^+][Ac^-]}{[HAc]}$$

or in terms of $[H^+]$ concentration:

$$[H^+] = \frac{Ka\,[HAc]}{[Ac]}$$

Expressing this in negative logarithms (p):

$$pH = pKa + \log \frac{[Ac^-]}{[HAc]}$$

This expression is known as the Henderson-Hasselbalch equation, and can be used in several ways. If the pKa is known for an acid, the ratio of the concentration of the salt to the acid can be used to prepare solutions of known pH as in the first part of the experimental work. An unknown pKa can be determined by measuring the pH of an acid as it is neutralized by a base, this you will do in the second part of this experiment.

As living cells are sensitive to changes in reactions of pH it is important that they be protected against such changes by the buffering action of the constituents of the tissue fluids, which provide the immediate environment.

Buffer solutions resist the effect of changes that might be expected by addition of a strong acid and a salt of that acid or alkali. They are solution of either a weak acid and a salt of that acid or a weak base and a salt of that base. Examples are:

Acetic acid + Sodium acetate
Ammonium hydroxide + Ammonium chloride

The reaction on the addition of a strong acid

$$\text{Acid} + \text{Buffer} \longleftrightarrow \text{Buffer} + \text{Salt}$$

$$H^+Cl^- + HAc + Na^+Ac \longleftrightarrow HAc + Na^+Ac^- + Na^+Cl^-$$

The H^+ combines with a strong base, the acetate, to form a weak or undissociated acid.
The reaction of the addition of an alkali:

$$\text{Alkali} + \text{Buffer} \longleftrightarrow \text{Buffer} + \text{Water}$$

$$Na^+OH^- + HAc + Na^+Ac^- \longleftrightarrow HAc + Na^+Ac^- + H_2O$$

The hydroxyl combines with hydrogen from the acetic acid to form the weak acid water.
The pH of a buffer system is described by the Henderson-Hasselbalch equation and the "capacity" is determined by the concentrations.
Indicators are organic acids, which in solution exhibit one colour as the undissociated acid and a different colour as the base.

$$H^+IN^- \longleftrightarrow Na^+IN^-$$
Red Yellow

The color change occurs at the point which the pH of the solution equals the pK and they are most commonly used to determine the end point of acid-base titrations. Since the colour change occurs gradually over a short range of pH, they can be used to measure the pH of an unknown solution by comparing colors.

Table of Indicators

Indicator (dye)	PK	pH range	Color change
Topfer's reagent	3.3	2.9 – 4.2	Red – Yellow
Bromophenol Blue	4.7	4.0 – 5.6	Yellow – Blue
Methyl red	5.1	4.2 – 6.3	Red – Yellow
Bromocresol purple	6.3	5.2 – 6.8	Yellow – Purple
Bromothymol blue	7.1	6.0- 7.6	Yellow – Blue
Phenol Red	7.9	6.8 – 8.4	Yellow – Red
Cresol Red	8.1	7.2 – 9.6	Yellow – Red
Thymol Blue (2^{nd} change)	8.9	8.0 – 9.6	Yellow – Blue
Phenolphthalein	8.7	8.3 – 10.0	Colorless-Pink
Thymol blue (1^{st} change)	1.65	1.2 - 2.8	Red – yellow
Bromocresol green	4.7	4.0 - 5.6	Yellow – blue

Reagents and Materials

1. Two solutions X and Y of unknown pH
2. 0.1 M NaOH solution
3. 0.1 M HCl solution
4. Alkaline indicator (dye)
5. Acid indicator (dye)
6. Test tube rack with 9 spaces in a row

Procedure

1. The first step in this procedure is to determine the approximate pH of the unknown solution so that a suitable indicator may be selected from the different indicators with which you are provided.To find out approximate pH range, first use wide range pH paper.

2. Treat a small portion of the unknown solution with 1m drop of indicator solution and compare the colour obtained with 0.1 N HCl and 0.1 M NaOH with the same indicator handled in the same way.

3. The same indicator is used for determining the pH of the unknown, if the colour obtained with the unknown is intermediate between the acid and alkali colours of the chosen indicator.

4. If on the other hand the unknown shows either the full acid or the full alkaline colour with the indicator selected, it is unsuitable and another indicator must be tried in the same way, until the right indicator with the effective range has been found.

5. Set up 18 test tubes of approximately equal bore in 2 parallel rows of 9 in a rack, and add into the back row from left to right, 1, 2, 3, 4, 5, 6, 7, 8, and 9 drops of indicator respectively. Proceed in the same manner with the front row, but from right to left.

6. Add 1 drop of 0.1 M NaOH to each tube in the back row, and 1 drop of 0.1 M HCl to each tube in the front row.

7. Make each tube up to 10 mls with distilled water.
   Each tube contains the same amount of indicator but in different ratios of alkaline: acid from as shown in the table below

Tube pair	1	2	3	4	5	6	7	8	9
Drops in alk. Series	1	2	3	4	5	6	7	8	9
Drops in acid series	9	8	7	6	5	4	3	2	1
Ratio of alk:acid form	1/9	2/8	3/7	4/6	5/5	6/4	7/3	8/2	9/1
Log. Ratios	-.95	-.6	-.37	-.18	0	+.18	+.37	+.6	+.95

8. To determine the pH of an unknown, place 10 drops of indicator in a tube of the same bore as used for the standards and make up to 10 mls with the unknown solution. Match this tube with the standard pairs, to obtain the degree of ionization of the indicator, and then apply the equation,

$$pH = pK + \log \frac{\text{alkaline}}{\text{acid}} \text{ ratio}$$

   If the match is between 2 tubes the pH for each should be worked out and the average taken. The indicator chosen should have a pK near the pH of the solution under test. If the pH of a solution like urine is required compensation must be made for the colour by placing a tube of the solution behind the standard pair being examined and a tube of water behind the test.

PRACTICAL THREE

Titratable and actual acidity

Introduction

The pH Meter and the Glass Electrode Theory
In modern laboratory practice pH is usually measured by electrochemical methods. The apparatus used is called the pH meter, and allows much greater accuracy than can be attained using indicators. These methods depend on the fact that when a metal electrode is placed in a solution of one of its salts the metal may gain or lose electrons:

$$M \longleftrightarrow M^{+} + e$$

And thus acquires an electrical potential (electrode potential, E) which depends on the nature of the metal and the concentration of the metal ion solution:

$$E = k + \frac{RT}{n^{F}} \log_e [M^{+}]$$

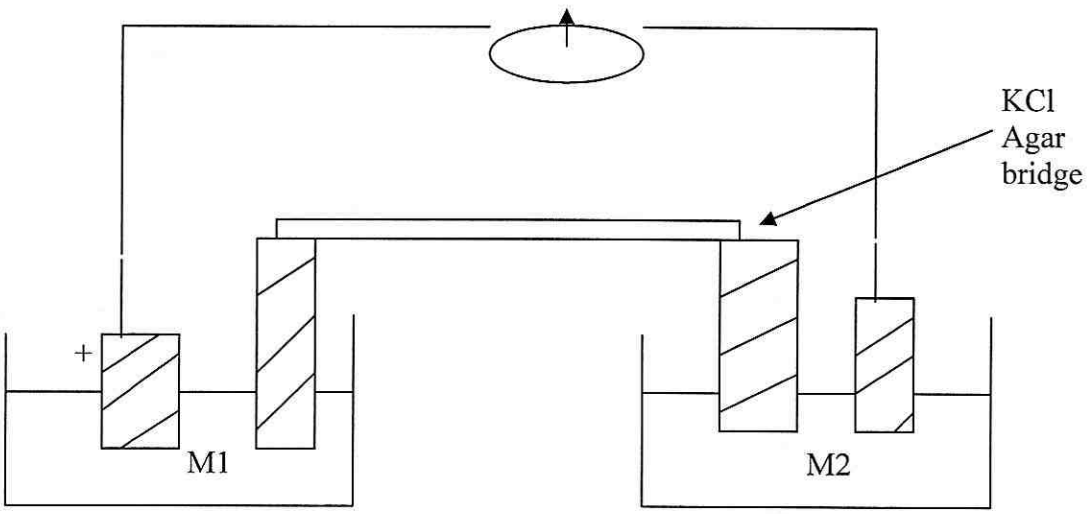
where K is a constant for the system, R is the gas constant, T the absolute temperature and F the Faraday constant, 96,500 coulombs and [M] the concentration of the metal.

This is also true of hydrogen in contact with a solution of hydrogen ions; i.e. the potential of a hydrogen electrode depends on the $[H^{+}]$ or pH of the solution in which it is immersed. A single electrode potential cannot be measured, but when two such electrodes are joined by a conducting "bridge" they form a galvanic cell and the potential difference between the electrodes can be measured.
In electrometric pH measurement the hydrogen electrode is usually coupled with a standard calomel electrode.

As the hydrogen electrode is a cumbersome affair it is replaced in standard practice by the glass electrode immersed in 1 N HCl. The bulb is of special glass which is permeable to hydrogen ions. When this electrode is immersed in a solution containing hydrogen ions it assumes a potential which is governed by the expression:

$$Eg = kg + \log_e [H^{+}]$$

Coupled with the standard calomel electrode the glass electrode forms a cell of which the electromotive force is at constant temperature, determined solely by the hydrogen ion concentration of the solution surrounding the glass electrode. The enclosed portion of the pH meter is essentially a voltmeter for measuring the e.m.f.



The acidity of a solution can be estimated in two ways which actually have two slightly different meanings. In a 0.01 M solution of a weak acid such as acetic acid there are equal moles of hydrogen and this can be verified if they are titrated with a 0.01 M NaOH to a neutral pH or with indicator phenolphthalein. If we measure the pH of the original solutions they will be quite different and the changes in the pH that will occur during titration will be different. HCl readily donates its hydrogen ion or proton to water forming the hydronium ion and the concentration or "H^+ activity" can be measured with a pH meter. In a solution of acetic acid of equal molarity only about 1 % of the hydrogen on the molecule is released into the solution to form the hydronium ions and the pH or $[H^+]$ is appreciably less. If we add hydroxyl ions to the HCl solution they react with the hydronium to from water which is a weak acid:

$$Cl^- + H_2O + NaOH \longleftrightarrow 2H_2O + Na^+Cl^-$$

When hydroxyl ions are added to acetic acid most of the hydroxyl reacts with the hydrogen which is part of the acetic acid molecule and there is only a small change in the $[H_30^+]$ or pH.

$$CH_3COOH \longleftrightarrow H_2O + CH_3OO^- + Na^+$$

This is an example of buffering, which is most easily defined as a reaction, which converts a strong acid, or base which is highly dissociated to a weak acid or base which is only slightly dissociated. A buffer solution is a mixture of a weak acid or base with the salt of that acid or base. From your study of the Henderson-Hassalbalch equation you can see that an appropriate mixture of the acid and the anion can prepare a standard solution of a standard solution of a known pH.

Reagents and Materials

1. pH meters and stirring rods
2. 10-ml and 25-ml burettes or straight pipettes
3. Phenolphthalein indicator solution
4. 0.01 M Hydrochloric acid
5. 0.01 M Acetic acid
6. 0.01 M Sodium hydroxide

Procedure

1. Calibrate the pH meter using two standard buffers of pH values 4.0 and 9.2 respectively.
2. To a beaker containing 25 mls of 0.01 M HCl, add 2 – 3 drops of phenolphthalein solution and measure the pH taking care to ensure that the electrode is well immersed in the solution.
3. From the burette or straight pipette add successive 2.5-ml aliquots of 0.01 M NaOH, stir and record the pH after each addition. CARE: Do not break the electrode!
4. Continue the titration until 30 mls of the 0.01 M NaOH have been added and the phenolphthalein changes colour permanently.
5. Repeat the procedure using 10 mls of 0.01 M Acetic acid.

Results

Plot the results from the two titrations on a graph paper using the ml of NaOH on the X-axis and the pH observed on the Y-axis.
Plot also for the Acetic acid.
From the graph, determine the pH of the solution containing 17 mls of 0.01 M NaOH.

Questions

1. Write the Law of Mass Action and Henderson-Hasselbalch equation.
2. What is the pH of
   (a) 10^{-3} N HCl
   (b) 10^{-2} N NaOH
3. Calculate the pH of a solution prepared by mixing 15 ml of 0.1 N CH_3COOH and 10 ml of 0.1 N NaOH.

PRACTICAL FOUR

Formal titration of amino acids

Introduction

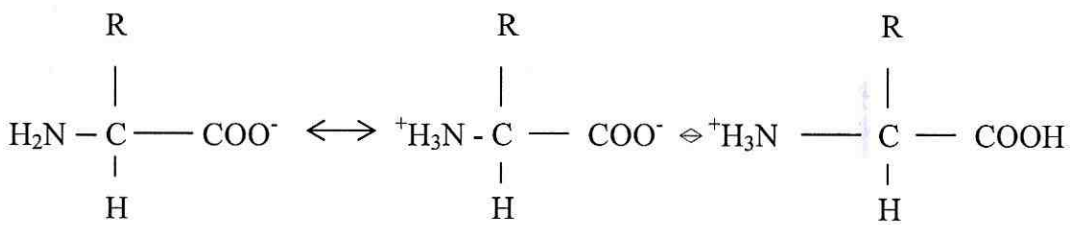
Amino Acids Theory

An amino acid essentially is a single carbon atom, to which are attached three groups: a hydrogen, an amine group, a carboxyl group and the functional group which distinguishes each of the amino acids and is designated as the R group. The first two groups act as an acid and a base as follows:

$$-COOH \longleftrightarrow COO^- + H^+$$

$$-NH_2 + H^+ \longleftrightarrow -NH_2^+$$

When an amino acid is dissolved in water a zwitterion or dipole is formed which then shifts to an anion at a high pH or a cation at a low pH as follows:



Anion Zwitterion or Dipole Cation

In reaction 1, ^-OH is added to remove H^+ or to increase the pH and an anion is formed. This reaction has a pK_1 at which:

In reaction 2, H^+ is added moving to a more acid pH and the amino acid forms a cation.

This also has a pK_2 where:

Both pK_1 and pK_2 involve the shift of a portion of H^+ and can be calculated by the Henderson-Hasselbalch equation. For glycine pK_1 +2.35 and pK_2 +9.78 Between the two points there is a pH at which the molecule carries a net charge of zero called the isolelectric point or pI.

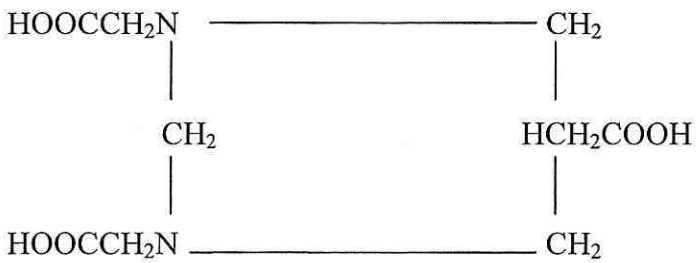
For glycine pI = 6.06

Also $pI = \frac{1}{2} (pK_1 + pK_2)$

Amino acids having –SH, phenolic, $-NH_2$ or COOH groups on the R group also dissociating giving pK_3 and pK_4.
Formaldehyde and other aldehydes react with the amino groups of the amino acids to give methylol derivatives:

$$NH_2 + HCHO \longleftrightarrow NHCH_2OH$$

Reactions of glycine with formaldehyde lead to nitromolecular cyclelation to result in formation of the cyclic tricarboxymethyl trimethylene triamine:



Formaldehyde does not react with NH^{3+} groups. Because of this, reaction of formaldehyde with $NH_2CH_2COO^-$ pulls equilibrium:

$$^+NH_3CH_2COO^- \longleftrightarrow NH_2CH_2COO^- + H^+$$

to the right which lowers the apparent value of pK.
The class will calculate the values of pK_1 and pK_2 of glycine in the presence of formalin.

Reagents and Materials

1. 0.1 M HCL
2. 0.1 M Glycine solution
2. 0.1 M NaOH solution
3. Neutral Formalin solution

Procedure

To a 50-ml beaker add 20 mls of the glycine solution.

Group 1: Titrate the amino acid solution by adding 2.0 ml aliquot of the standard acid, mix and measure the pH of each successive addition of 2.0 ml until 25 mls is reached.

Group 2: Add 10 mls of neutral formalin to the glycine solution. Mix and measure the pH with a pH meter. Titrate with standard acid as in group 1 above.

Group 3: Titrate as in group 1 but add successive aliquots of the standard alkali solution instead of the acid solution.

Group 4: Add formalin as in group 2, to titrate with alkali as in group 3.

Calculation

Each group of 4 students combines the data into a single graph as follows:
Plot equivalents of HCl and NaOH on the x-axis versus pH on the y-axis.
Calculate the various pK's of the amino and carboxyl groups **with** and **without** the presence of formalin.

PRACTICAL FIVE

Separation of amino acids by paper chromatography

Introduction

Amino acids analysis is invaluable for the study of metabolism, the study of protein structure and for the determination of abnormal composition of urine and blood in diseases. In this experiment, the stationary phase is supported by the paper. Other suitable supports are silica gel or cellulose powder. These are finely divided solids and must be on a thin sheet of glass ("glass plate").

A mixture of low Molecular Weight Compounds may often be separated by means of partition chromatography. This method is based on the relative distribution of the compound between two solvents in which it has different solubilities. This partitioning effect may be carried out in solution (as in counter current distribution) or, more commonly, on inert supporting medium.

The medium in paper chromatography consists of a network of hydrophilic cellulose fibres. The solvent system usually contains an organic alcohol, water and either a strong acid or a strong base. The cellulose binds a layer of water, which represents the stationary phase. The mobile phase is the remaining solvent, which migrates up the paper by capillary action, the organic component having the greatest mobility. A compound applied as a sample will distribute itself between the stationary water phase and the mobile organic phase depending on its relative affinity (solubility) for the two solvents. This partitioning effect is repeated many times and since the relative affinity is characteristic for a given compound, a separation is achieved. The more hydrophobic (non-polar) components migrate the furthest, the more hydrophilic (polar) remain near the origin.

The separated amino acids on the chromatogram are located by treating the sheet with ninhydrin, which reacts with amino acids forming purple to red spot where the amino acid is located. The reaction depends upon the presence of a free amino group, the nitrogen of the amine reacting and forming a link between two monomers to form a coloured dimer. The complete reaction will be found in any good text book on biochemistry.

Reagents and Materials

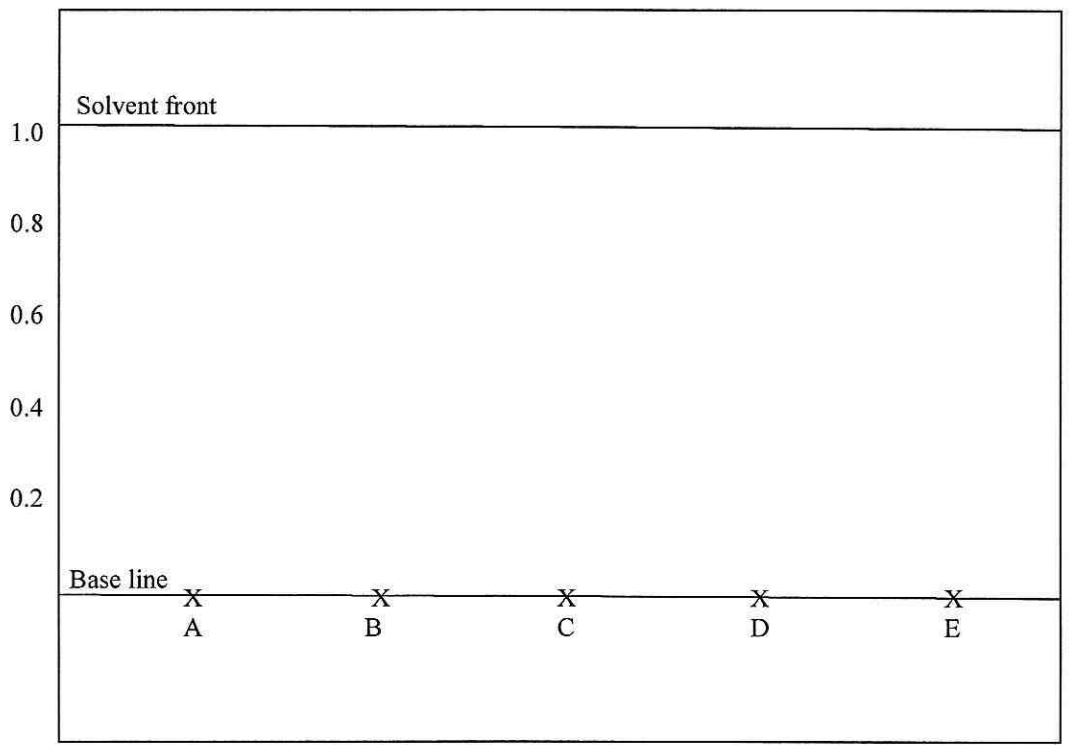
1. Chromatography sweet jars with lid
2. Whatman No. 1 Filter papers size 25 x 25 cm.
3. Capillary tubes
4. Dip tray for Ninhydrin (under hood)
5. Paper Clips
6. Solution (1 mg/ml in water) of glycine, alanine, leucine, tryptophan, glutamic acid and lysine
7. Amino acid mixture of three of the above (labeled G)
8. Solvent: Ethanol: Water: Ammonia (specific gravity 0.880) 80:10:10
9. Ninhydrin solution (dissolves 200 mg in 100 ml Acetone; keeps indefinitely in a refrigerator).

Procedure

First pour the solvent provided into the jar to a depth not over 1 cm (about 50 ml), replace the lid to allow the tank to become saturated with the solvent vapour.

N. B. Avoid the fumes and spillage.

1. Draw a pencil line across the paper, 2.0 cm from the bottom. Beginning 4 cm from one side place pencil dots or crosses at 3 cm intervals a long the line and mark these according to your different amino acids (see drawing below, not to scale!).



2. Apply the test solution to the pre-marked spots using a clean capillary tube for each solution. Touch the end of the capillary tube containing the solution to the paper lightly in order to transfer as small a volume as possible. (Clearly label the spots in pencil).

3. Dry the spots before clamping the two edges of the paper with the clips to form a cylinder, taking care to ensure that the spots do not overlap and are not too close to the joint. Place the cylinder with the spotted end down in the tank while ensuring that the paper does not touch the glass walls nor the spots touch the fluid in the bottom and close the tank with the lid.

4. Run the chromatogram for a minimum of 1 hour 30 minutes or until the solvent front has advanced to within 2 cm at the top of the paper. Mark the solvent front with a pencil, open the paper and allow to dry.

5. Dip the dry chromatogram in the tray containing Ninhydrin, allowing the solvent to dry before heating the sheet in an oven for about 5 minutes. The position of the amino acid spot will be revealed by development of a purple colour. The colour is stable for some weeks if kept in the dark and free of acid vapours. For permanent record, outline the spots in pencil.

Results

Measure the distance between the centre of each spot and the starting pencil line. The ratio of this distance, between the origin and the solvent front is the R_f value for the amino acid. Determine the R_f values of the test samples. Can you relate the observed R_f to the structural differences between the amino acid?

PRACTICAL SIX

Qualitative test for proteins and amino acids

(Colour tests)
Reagents and Materials

1. Protein: (a) Powdered egg albumin
   (b) 5 % solutions of casein and gelatin in NaOH
2. Amino acid solutions: 1 % solutions of Cystine, Tyrosine, Tryptophan, phenylalanine and arginine.

3. 2M NaOH solution
4. 10M NaOH solution
5. 1% $CuSO_4$ solution
6. Millon-Nasse Reagent
7. 1% $NaNO_2$
8. 2M H_2SO_4
9. Pyridine solution
10. 20 % sodium sulphite solution
12. 0.02 % α-Naphthol in 95 % Ethanol
13. Alkaline Hypobromite solution
14. Conc HNO_3
15. Lead acetate solution
16. Glyoxylic acid
17. Conc. H_2SO_4
18. Buffered ninhydrin
19. Ice
20. Water-bath

A) General composition test for proteins

Since the proteins always contain nitrogen in addition to carbon, hydrogen and oxygen a positive test for this element indicates the possibility that the material under examination is a protein. A negative test for nitrogen definitely eliminates the possibility of protein.

Procedure

1. Transfer a small amount of powdered egg albumin into a dry test tube.
2. Suspend a strip of moistened litmus paper in the tube.
3. Place across the mouth of the test tube a piece of filter paper moistened with lead acetate solution.
4. Heat the powder and observe.

Result

The powder char, indicating the presence of carbon

The fumes of ammonia evolved turn the colour of the indicator paper towards the alkaline side indicating the presence of nitrogen. The deposition of moisture on the side of the test tube indicates the presence of hydrogen and oxygen.

B) Biuret test (Proteins)

The Biuret reaction is given by all substances whose molecules contain two carbamyl (-$CONH_2$) groups joined either directly together or through a single atom of nitrogen or carbon. Proteins respond positively since there are pairs of -CO-NH- groups (two or more peptide bonds). The test takes the name from the compound Biuret, which also gives the same reaction. The colour is due to a co-ordination complex formed by Cu^{++} with the -NH groups of the peptide linkage in alkaline solution. It can also be used for quantitative determination of protein.

Procedure

1. To 2.0 ml of 2 N NaOH add two drops of 1 % $CuSO_4$ solution. Divide the solution into 2 parts.
2. To one add a few drops of protein solution, and to the other a few drops of water as a control. A pink to violet colour is given by the protein solution, depending upon the nature of the protein.
3. Repeat the test for the amino acid solution.
4. Observe any color changes.

3. Ninhydrin test for amino acids

When amino acids are heated with Ninhydrin they are quantitatively deaminated and an intense blue to purple colour appears. This reagent reacts with all the amino acids, peptides and proteins or derivatives that contain a free amino acid and a free carboxyl group.

Reagents and Materials

Buffered ninhydrin

a. Citrate buffer pH 5.0 - Dissolve 21.008 gm. Citric acid in 200mls of distilled water, add 200mls of 1 M NaOH solution and dilute to 500mls with distilled water.
b. Ninhydrin - Dissolve 0.5 gm of ninhydrin in 100mls of the above buffer.

Procedure

1. Place 1.0 ml of amino acid solution in a clean test tube.
   Neutralize if necessary, using phenolphthalein as indicator, and then add 1 ml of buffered ninhydrin.
2. Place test tube in boiling water and observe colour change.

Result

A purple colour will be formed if the test is positive.
Repeat the test using a protein solution and record your observations.

C) Millon-Nasse test (tyrosine)

This is an adoption of the Millon Test and can be used in the presence of considerable quantities of inorganic salts especially NaCl as in the detection of protein material in urine. The reaction is due to the presence of hydroxyphenyl group $-C_6H_4$-OH in the protein molecule; but any phenolic compound which is unsubstituted in 3, 5 position such as tyrosine, carbolic acid or thymol will give the reaction. Proteins respond positive with Millon-Nasse when tyrosine is present.

Procedure

1. Transfer 5 mls of protein solution or amino acid solution respectively into different test tubes.
2. Add 1 ml of Millon-Nasse reagent to each.
3. Place the tubes in a boiling water bath for 10 minutes.
4. Cool and add 1 ml of 1 % $NaNO_2$ solution.

Result

A deep-red colour indicates presence of tyrosine.

D) Sakaguchi test (arginine)

This test indicates the presence of Arginine and depends upon the presence of the guanidino groups of the Arginine molecule.

The reaction of the guanidino group with C-α--Naphthol is the basis of the test. Since the developed colour decomposes rapidly, urea is used to stabilise the reaction.

Procedure

1. Prepare in a 250 ml beaker as an ice bath.
   Place two test tubes into the ice-bath, one containing 1 ml of the protein solution, the other 1 ml of amino acid solution.
2. Chill thoroughly for 5 minutes then add 0.2 ml of 2 M NaOH to each, and 0.2 ml of α-C-Naphthol solution to both test tubes.
3. Add 3 drops of Hypobromite solution. Mix well.

Result

A red colour denotes the presence of guanidino group. Add 0.2 ml of urea to stabilize the colour.

E) Xanthoproteic test (tyrosine and tryptophan)

This reaction depends on nitration of the phenyl group $-C_6H_5$ or aromatic ring, it is givenby all molecules containing this structure such as tyrosine, tryptophan or phenol, but phenylalanine does not respond to this test as it is ordinarily performed.

Procedure

1. To 2.0 ml protein solution or amino acid solution, add 1.0 ml conc. HNO_3. A yellowish precipitate forms.
2. With gentle boiling it will change to deep yellow, and slowly dissolve.

3. After cooling slowly add 2.0 ml 10 N NaOH. An orange-red ring will form at the interface in a positive reaction.

F) Sulphur Test (For Cysteine, Cystine and Methionine)

This test indicates the presence of cysteine and cystine as they both contain sulphur in their molecule.

Cysteine is recognized as being present as such in the intact protein molecule but it is not ordinarily obtained as one of the products of protein hydrolysis unless precaution is taken against oxidation. It is readily converted by oxidation of the –SH group into various sulphinic acids and into cystine.

Procedure

1. To test tube. Add 1.0 ml test solution, 2.0 ml of 10N NaOH and 2 drops of lead acetate solution.
2. Boil for 1-2 minutes. The alkali liberates –S from cystine and cysteine and forms sodium sulphide, which forms a black or brown colouration of PbS with the lead acetate.

G) Glyoxylic Acid Test (Hopkins-Cole Test, Tryptophan)

This is a test for tryptophan free or combined with protein.When a solution of tryptophan or protein containing this compound is mixed with glyoxylic acid and the mixture is layered over conc. H_2SO_4 a violet ring appears at the interface of the liquids. It is a characteristic of tryptophan, which is the only amino acid in protein, which contains the indole group.

Procedure

1. To 2.0 ml test solution add 2 or 3 drops of glyoxilic acid solution and mix well.
2. Pour conc. H_2SO_4 carefully down the side of the tube to form 2 layers. Allow to stand for 10 minutes.

A positive test is indicated by the formation of a purple or violet ring at the interface.

Table of Summary of Colour Tests

compound →	Tyrosine	Tryptophan	Arginine	Phenyalanine	Cysteine	Casein	Gelatin
General test ↓							
Biuret							
Ninhydrin							
Millon's							
Sakaguchi							
Xantho-proteic							
Sulphur							
Glyoxylic							

PRACTICAL SEVEN

Hydrolysis of proteins and peptides

Introduction

This procedure is used to obtain the free amino acids residues of a protein. The hydrolysis can be accomplished by an acid, an alkali or enzymes; with each of these the peptide bond is broken at certain points along the chain, yielding peptides. Neither acids nor alkalis are structurally specific catalysts, so that the longer the hydrolysis is continued, the greater number of fragments and the smaller the size.

Complete hydrolysis with an acid requires 24 hours and results in destruction of some of amino acid and the formation of humine, a dark coloured sludge which is the result of condensation of tryptophane with carbohydrate. Alkaline hydrolysis results in destruction of cystine and arginine is converted to ornithine and urea. Complete hydrolysis with the enzymes is difficult and the solutions will contain hydrolytic products of the enzymes as well as the protein.

If hydrolysis is not carried to completion the peptides can be separated and the amino acid residues of each peptide determined. This technique is used in the determination of the amino acid sequences of the primary structure. In this experiment the protein gelatine and the tripeptide, glutathione will be hydrolyzed by Hydrochloric acid, HCl.

Reagents and Materials

1. 0.5 % Solution of Gelatine
2. 0.2 % Solution of Glutathione
3. 0.2 % Solution of Cystine, Glutamic acid and Glycine
4. Conc. HCl; 6 M HCl
5. 30 % Trichloroacetic acid
6. Solvent: N-Butanol: Glacial acetic acid: Water (4:1:1)
7. 0.5% Ninhydrin solution (dissolved in acetone or ethanol)

A) Hydrolysis of Protein

Procedure

1. Put 12 ml of gelatin solution in a large test tube, add 3 ml of conc. HCl from a burette and mix well.
2. Immediately pipette 2 ml of the mixture into a tube containing 0.5ml of 30% TCA. This is the control of zero time.
3. Stopper with digest mixture and place it in a beaker of boiling water.
4. Withdraw 2mls samples at 15, 30, 45 and 60 minutes pipetting each into a different test tube containing 0.5mls of 30 % TCA
5. After all the samples have been collected, filter and apply a spot of each to marked positions on a sheet of chromatographic paper. Staple into a cylindrical form and place into the chromatographic jar, previously prepared with solvent.

6. Chromatograph for at least two hours.
7. Dry, mark the solvent front, and stain with the Ninhydrin solution, and then sketch the results.

B) Hydrolysis of a peptide

Procedure

1. This should be carried out at the same time as the hydrolysis of the protein.
2. Place 1 ml of 0.1 % Glutathione solution in a test tube and add 0.5 ml of 6 M HCl then heat for 60 minutes in a beaker of boiling water.
3. Apply a sample of the hydrolysate to a sheet of chromatographic paper. To assist in identification of the products of hydrolysis apply spots of the solutions of Cystine, Glutamic acid and Glycine.
4. Chromatograph as in part A of this experiment.
5. Sketch the results.

C) Test for identification of Sulphur in proteins (for Cysteine and Methionine)

Procedure

This test indicates the presence of Cysteine and Cystine as they contain Sulphur in their molecule.

Cysteine is recognized as being present as such in the intact protein molecule but it is not ordinarily obtained as one of the products of protein hydrolysis unless precautions are taken against oxidation. It is readily converted by oxidation of -SH group into various sulphuric and sulphonic acids and into Cystine.

1. To a test tube add 1 ml test solution, 2 ml of 10 M NaOH and 2 drops Lead acetate solution.
2. Boil for 2 minutes.
3. Observe the alkali colouration of PbS with the lead acetate.

D) Test for Identification of Phosphorous in Proteins

The conjugated proteins differ from the simple proteins in that they are combined with some non-protein substances such as nucleic acids, carbohydrates or phosphates in a manner, which confers new and characteristic properties on the complex formed.

They are classified according to the nature of the group attached and amongst them you find the phospho-proteins which contain phosphate bound in ester linkage to the hydroxyl amino acids serine and threonine of the peptide chain.

Sodium hydroxide splits off the bound phosphate yielding inorganic phosphate. Examples for phospho-proteins include casein from milk, ovavitelin from egg yolk, and proteins associated with the feeding of the young.

Procedure

1. Place 2 mls of casein solution in a test tube and add 5 drops of 2 M NaOH. Boil for 5 minutes.
2. Cool and adjust pH to about 4.5 with 2 M H_2SO_4 using indicator paper.
3. Centrifuge off the precipitate and transfer the supernatant into a clean test tube.
4. Add in sequence: 1 ml of molybdic acid reagent, 1 ml of sodium sulphite solution and 1 ml of Hydroquinone solution.

Result

A blue colour develops due to formation of a phosphomolybdate complex indicating the presence of inorganic phosphate.

PRACTICAL EIGHT

Determination of total protein by Biuret and Folin-phenol method

Introduction

In a previous experiment the Biuret reaction was used as a qualitative test for protein. The test will be used in this experiment for the determination of protein using a spectrophotometer. The first part is the preparation of a standard curve which will then be used to measure the protein concentration in the unknown solutions. This curve will also be used later in other studies on proteins.

Since the accuracy of all your work depends on the accuracy of the standard curve, care must be taken in this work and all determinations should be done in triplicate. Each run should also include a reagent blank or control containing only the reagents and no protein, which is used to adjust the instrument to zero absorbance.

A) Total Protein by Biuret Method

Reagents and Materials

1. Biuret reagent
2. 0.85 % NaCl
3. Standard Protein containing 0.2 g/100 ml
4. Unknown protein solutions of 5-7 g/100 ml

Procedure

I) Preparation of the standard curve

1. Prepare standard curve by setting up 3 sets of 5 tubes as follows:

Tube No.	1	2	3	4	5	6 (blank)
Std. Protein soln. (ml)	5.0	4.0	3.0	2.0	1.0	0.0
0.85% saline soln.(ml)	0.0	1.0	2.0	3.0	4.0	5.0
Biuret reagent (ml)	5.0	5.0	5.0	5.0	5.0	5.0
Total volume (ml)	10.0	10.0	10.0	10.0	10.0	10.0
Protein conc. (g/10 ml)	0.01	0.008	0.006	0.004	0.002	0.0

2. Allow 30 minutes for full colour development.
3. Read in the O.D. at 538 nm.
4. Plot the standard curve on the graph paper.

II) Determination of an unknown Protein Concentration

The unknown has been prepared at concentration equivalent to that of blood, or about 5gm to 7gm per 100 ml so that this procedure can be used later from the determination of serum or plasma proteins. Since this concentration is greater than that of the standard curve, suitable dilutions will have to be made to obtain a concentration, which can be read on the curve.

Procedure

1. Dilute 1 ml of unknown to 10 ml with 0.85 % saline.
   The concentration now is 0.5 - 0.7 gm/100 ml.
2. Take 3 test tubes and pipette 1 ml of the diluted unknown into each.
3. Add 5mls of Biuret reagent to all the five tubes. Allow 30 minutes for full colour development.
4. Read the absorbance with a spectrophotometer at 538 nm.

Calculations

The quantity of protein in the unit volume of 10 ml used in the determination is obtained from the standard curve. This is the amount of protein that was contained in 0.1 ml of the original unknown solution. Therefore the concentration in the original per 100 mls of the solution is obtained as follows:

$$\text{Unknown conc. gm/100ml} = \frac{\text{Amount in sample}}{0.1} \times 100$$

B) Total Protein by Folin-Phenol Method

This reagent is used most frequently for the development of phenol, and can be used to determine protein quantitatively by a reaction with the amino acid tyrosine with phenolic side chain. The sensitivity can be greatly increased by treating the protein solution first with copper ions, which form a complex similar to Biuret, followed by reaction with the Folin-Phenol reagent. The reduction of phosphotungstate and phosphomolybdate by the tyrosine to the blue dyestuffs constitutes the reaction. This reaction depends on the tyrosine content of the protein, therefore varies with the amount of this amino acid in the protein.

Reagents and Materials

1. Alkaline copper solution
2. Folin-Phenol reagent, dilute 1:2 before use.
3. Standard protein casein 0.3 gm/liter (300 mg/ml).
4. Unknown 150 mg/ml.

Procedure

I) Preparation of the standard curve

1. Prepare the following sets of tubes in triplicate,

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**
Protein soln (ml)	0.1	0.2	0.4	0.6	0.8	0.0
Distilled water (ml)	0.9	0.8	0.6	0.4	0.2	1.0
Alk. Cu^{2+} soln (ml)	8.0	8.0	8.0	8.0	8.0	8.0

Mix well and allow to stand for 15 minutes, then add

Folin – phenol reagent	1.0	1.0	1.0	1.0	1.0	1.0

Add rapidly and mix well, allow 30 minutes for full color development
Read absorbance with spectrophotometer at 600 nm.
Plot the standard curve.

II) Determination of Protein Content of an Unknown Solution

Use an aliquot in tube 1 of this procedure containing 60 μg to 240 μg of protein. This may require testing several different sized aliquots to obtain the proper dilution.

Lipids

Theory

Lipids are a class of compounds that are distinguished by their relative insolubility in water and solubility in organic solvents. The structure of lipids is composed to a great extent of carbon and hydrogen of about equal electronegativity and little tendency to form polar molecules. Fatty acids are long chains of these two atoms and make up most of the compounds recognized as fats or triglycerides. Since these acids are non-polar, they will dissolve in non-polar fluids such as diethyl ether or chloroform. All lipid molecules have polar areas as part of the structure such as the glycerol of triglycerides and these change the solubility behaviour to a certain extent. Various examples of this will be found in later experiments, but this series is devoted to neutral fats or triglycerides, the energy storage compound of animals.

Neutral fats and oils are esters of the trivalent alcohol glycerol (CH_2OH-$CHOH$-CH_2OH) and different types of fatty acids. These may be straight or branched chained, saturated or unsaturated, aliphatic or aromatic hydrocarbons with one or two carboxyl groups. The chain length and the degree of unsaturation of the fatty acid residues determine the physical character of the triglycride. Usually, the harder the fat or the higher its melting point, the greater the saturation with long chained fatty acids. Oils owe their physical properties to unsaturated fatty acid residues, very short-chain saturated fatty acid residues, or both.

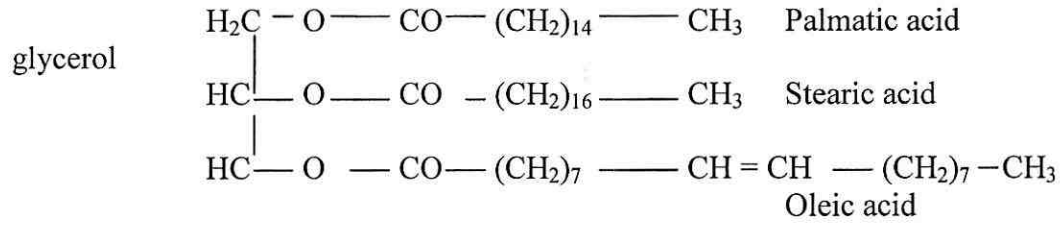
Further, physical features of triglycerides governed by the character of the fatty acid side chains are solubility, susceptibility to rancidity and the so called "fatty characterizing number" (acid number, iodine number, e.t.c.)

Solubility

Substances which form true solutions in water dissolve due to polarity of the solvent and solutes dissociate into individual molecules or ions. If the interactions between solvents and solute are less the inter-molecular forces of the solute or the solvent emulsions or suspensions will form.The nature and extent of interactions is determined by the nature of the molecular groupings present in that particular compound. In fat molecules the hydrophobic fatty acid residues predominate over the hydrophilic glycerol component, hence fats form emulsions with water but true solutions with organic solvents.

Naturally occurring animal fats consist largely of mixed glycerides of Oleic acid $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$, Palmitic acid $CH_3(CH_2)_{14}COOH$ and Stearic acid $CH_3(CH_2)_{16}COOH$. Simple triglycerides, such as tristearins, are rarely met in nature; hence a natural fat is a mixture of different compounds rather than a pure one. Therefore it is more convenient to take a certain weight such as a gram of fat as reference for analysis rather than a mole in the definitions of acid number, iodine number, e.t.c.

The following triglyceride is an example of an individual fat molecule:



There are many possible distributions of the individual fatty acid residues in triglycerides. In contrast to animal fats, the vegetable oils olive, rape-seed, palm, coconut, linseed or castor oils, contain both the triglycerides of palmitic, stearic and oleic acids and the glycerides of polyunsaturated acids. These should not be confused with mineral oils, which are hydrocarbons not esters.

The higher unsaturated fatty acids as linoleic acid ($C_{18\ 9,12}$), linolenic acid ($C_{\ 18,12,15}$) and arachidonic acid ($C_{20\ \ 5,8,11,14}$) are called "essential fatty acids" or "Vitamins" i.e. the mammalian organism is unable to synthesize them and therefore depends upon dietary sources. They are particularly important in nutrition of the young.

Triglycerides are energy storage compounds whereas the phospholipids, sphingomyelins and cerebrosides, sterols and waxes are functional molecules which play a role in metabolism and structure of cells.

PRACTICAL NINE

Determination of saponification number of triglycerides

Introduction

The saponification number is the number or milligrams of KOH required to saponify 1.0 gram of the triglyceride. The fat from a lamb will require less KOH to saponify it than butter fat since it is composed mainly of C_{16} and C_{18} fatty acids whereas butter is composed of much shorter acids too (down to C_{14}).

A known amount of the fat is hydrolyzed with an excess of a standard alcoholic KOH for 30 minutes. The excess KOH is titrated with HCl and the amount of KOH needed to saponify the fat is calculated.

The importance of this determination lies in the relationship between saponification number and the chain length of the fatty acid residues. Three molecules of KOH are required to neutralize three fatty acid molecules regardless of their size. Thus, the saponification value of a given weight of fat varies inversely with the average molecular weight of the fatty acid residues.

It is thought that hydrolysis of triglycerides during saponification occurs stepwise rather than as a simultaneous release of the three fatty acid at once.

Reagents and Materials

1. Fat samples 4.0 % w/v solution in Ethanol i.e., I: Beef fat and II: Butter fat.
2. 0.3 M KOH dissolved in 90 % Ethanol.
3. 0.3 M HCl.
4. Phenolphthalein solution.
5. Reflux air condenser: 18 inches length of glass tubing in rubber stopper fitted with a layer of aluminum foil.

Procedure

Each determination should be run in duplicate.

1. Add 1 gm of fat, 25 ml of 0.3 M alcoholic KOH, and a sample of glass beads to a 250 ml flask and boil gently for 30 minutes under a reflux condenser.
2. Cool and add 3 drops of Phenolphthalein.
3. Titrate with 0.3 M HCl. (This titrates excess KOH)
4. Carry out a blank titration of 25 mls of the alcoholic KOH with 0.3 M HCl.

Reactions

i. Triglyceride $\xrightarrow{\text{Hydrolysis}}$ Glycerol + 3 fatty acids

ii. Fatty acid + KOH $\xrightarrow{\text{neutralization}}$ Salt-Fatty acid + H_2O

Calculations

i. Standardization (KOH): (ml-HCL)(M) = mEq - KOH
ii. Excess (KOH) : (ml-HCL)(M) = mEq – KOH

iii. Difference between 1 and 2 = mEq - KOH used in saponification

iv. Saponification Number = $\frac{\text{(meq - KOH) (mg Equiv. wt.-KOH)}}{\text{sample wt.}}$

PRACTICAL TEN

Halogen absorption by the double bond of unsaturated fatty acids

Introduction

An unsaturated fatty acid is one which contains one or more doublebond(denotedby) in the reaction chain. These bonds can be hydrogenated or saturated with H_2, process for "hardening of fats" as it increases the melting point. Unsaturated fatty acids, free or esterified, will also react with halogens by addition of two halogen atoms at one double bond.

$$R_1 - CH = CH - R_2 \; + \; I_2 \longrightarrow R_1 - \underset{\displaystyle I}{\underset{|}{CH}} - \underset{\displaystyle I}{\underset{|}{CH}} - R_2$$

The amount of halogen which reacts is directly proportional to the number of -C=C- bonds present. A qualitative method of indicating unsaturation consists of estimating the decolourization of a bromine or iodine solution by various lipids.

The "iodine number" is a quantitative value which reflects the degree of unsaturation of a fat. By definition it is the amount of iodine in grams absorbed by 100 g of fat. Bromine is usually used since it is more reactive than iodine, but the amount of halogen absorbed is finally expressed as grams of iodine. The value is influenced by the fat proportion of each unsaturated fatty acid present, the degree of unsaturation of each acid and the mean molecualr weight of the fat.

The velocity of halogen addition to unsaturated fatty acids depends upon the structure of the acid, the kind of halogen and the solvents and catalysts used. Addition of iodine takes place slowly but this reagent has the advantage of specificity for addition whereas chloride or bromine is also capable of substitution reactions. Iodine will be used in the form of Hubl's reagent and photometric measurements used to measure the rate of halogen addition, and uses the concept of comparing the rate of reaction as a means of comparing the structure of two similar compounds.

Reagents and materials

1. Chloroform
2. Oleic acid
3. Stearic acid
4. Cotton seed oil
5. Hubl's iodine solution (use when freshly prepared only).

Procedure

Students in pairs under close supervision will carry out this experiment so that both students have access to the spectrophotometer at the time for reading the absorbances.

To each of the four clean test tubes:
Add 10mls of Chloroform
Add 5mls of Hubl's iodine solution

Mix well.

At 2 minute intervals add to:

Tube 1: 0.1 ml cottonseed oil and mix
Tube 2: 0.1 ml oleic acid and mix
Tube 3: 0.1 ml Stearic acid and mix
Tube 4: Nil (This is the control tube and is used to set the spectrophotometer to read zero absorbance at 420 nm).

Read absorbance of each tube exactly 5 minutes and 20 minutes after adding the oil and fatty acid.

Results and Calculations

Tube 4 has an absorbance A_1 of the original iodine concentration. The absorbance A_2 of the other tubes is the amount of iodine remaining unreacted. The difference, $A_1 - A_2$ is the iodine which has reacted.

Graph

Plot the value of the difference for each time for each sample of fat as a bar graph.

PRACTICAL ELEVEN

Rancidity of fats by acid number

Introduction

Rancidity is the deterioration of fats that occur due to the effect of

a) Hydrolysis of fat by microbial lipases to yield free fatty acids
b) Oxidation of atmospheric oxygen at double bond.

Rancidity imparts a characteristic unpleasant odour to the fat, which arises from free fatty acids and form short chain aldehydes, which form, by further reactions of the peroxides. Increasingly degree of unsaturation increases the susceptibility to rancidity as the –C=C- bonds facilitate peroxide formation. A further type of rancidity is the so called "ketonic rancidity" following the action of fungi or moulds on saturated free fatty acids, which is part in process of cheese production.

Acid Number

During the hydrolytic process of rancidity, free fatty acids arise which can be used as a measure of the extent to which rancidity has occurred. The amount is given by the "acid number" which is the number of milligrams of KOH required to neutralize the free fatty acids in 1 gram of a triglyceride.

Reagents and Materials

1. 4 % w/v samples of cottonseed oil
   - Sample I: Stored at 5^0C (refrigerated)
   - Sample II: Stored at room temperature, in sunlight and air bubbled through it
   - Sample III: Sample II with Vitamin E added as an anti-oxidant.
2. Absolute Ethanol
3. Phenolphthalein solution
4. 0.3 M KOH

Procedure

Determinations to be done in duplicates

1. Transfer 1.0ml of the fat solution to a 250ml conical flask.
2. Add 24mls of absolute ethanol.
3. Add a few drops of phenolphthalein and titrate at once with 0.3 M KOH till pink colour persists for five seconds.

Results
Determine the volume of KOH used.

Calculations
If the test solution contains 4 % fat, the acid number of the fat can be calculated similar to the calculation of the saponification number using the following formula:

$$\text{Acid No.} = \frac{(\text{ml KOH}) \times (\text{milli Eq. Wt. of KOH}) \times (\text{M})}{\text{Sample weight}}$$

Calculate the acid number for the test samples using the above formula.

PRACTICAL TWELVE

Separation of lipid extract from liver by thin layer chromatography (TLC)

Introduction

The modern basis of thin layer chromatography (TLC) was, however, laid by the extensive studies of Stahl, 1956, who first developed a practical laboratory technique for producing uniform thin layers of various powdered materials on glass. The term thin layer was introduced to indicate that a layer of slurried materials had been applied to a sheet of glass and that this layer was about 25 μm (=0.25 mm = 0.010 inches) thick, thus differentiating the techniques from paper chromatography. A synonym sometimes used is open column chromatography, since the chromatography results are frequently comparable to those obtained with a conventional column. In fact, the experiences from one procedure can often be applied to designing a chromatographic separation by the other.

Basically TLC is the technique of separation and identification of chemical substances by a moving solvent on a thin layer of suitable adsorbent. This adsorbent, generally with a binder, is deposited on a sheet of glass or other material, which serves as an inert support for the layer. The layer is prepared by making a slurry of finely-divided layer material with a suitable liquid such as water, pouring it on glass plate spreading it in a thin and even layer, and drying it. The dried adsorbent adheres to the plate.

The adsorbent that you will use is SILICA GEL, a form of silicon dioxide (SiO_2) that has an affinity for lipid molecules, binding the highly polar phospholipids more tightly than the less polar compounds. An organic solvent moving up the plate will separate a mixture of lipids on the basis of relative affinities of the lipids for the silica gel and for the solvent or **mobile phase**. A non-polar solvent (e.g. chloroform) wills separate the neutral lipids leaving the phospholipids at the origin. More polar solvent mixtures (e.g. chloroform, methanol, and acetic acid) will separate a mixture of phospholipids while the neutral lipids will move with the solvent front.

Reagents and Materials

1. Capillary tubes
2. TLC plates (activated)
3. Chromatography Jars with lid
4. Chromatography Solvent: Methanol: Chloroform: Water (30:70:5)
5. Iodine vapour chamber (under hood)

Procedure

You are provided with a liver extract, which has been prepared in the following manner: Fresh liver cut into small pieces was ground with sand in a mortar, with continual addition of methanol-chloroform (1:2). After overnight standing the filtrate was washed with a small amount of saline in a separatory funnel. The saline washing is discarded and TLC provides you in the crude methanol-chloroform extract for separation into constituent lipids.

1. Apply a drop of the extract with a capillary tube 2.5 cm from the narrow edge of the glass plate and 1.5 cm from each side. You should apply continually very small amounts since the smaller the size of the spot the better are the results.
2. When the plates with the applied sample extract is completely dry, place it into a jar containing methanol: chloroform: water in the ratio 30:70:5
3. Let the solvent ascend up the plate one half to two thirds of the 20 cm.
   This will take approximately 30 minutes. Do not run this for more than 45 minutes.
4. Remove the plate, dry in the air and place into a vapour atmosphere of iodine in a jar for 10 minutes.

Results

Make a sketch of the results and calculate the R_f of the main components separated.

PRACTICAL THIRTEEN

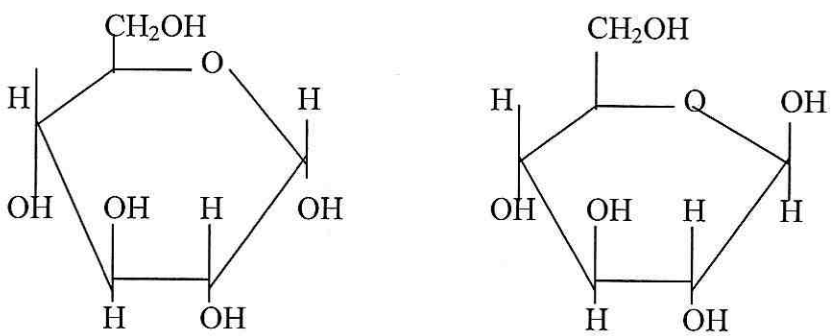
Qualitative test for carbohydrates

Introduction

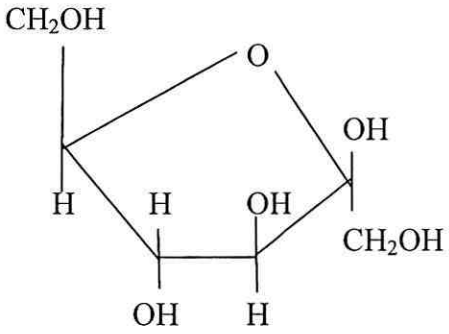
The principal carbohydrates in the animal organism are hexoses composed of six carbon atoms such as glucose and fructose and the pentoses or five-carbon atom sugars. The hexoses supply a large part of the energy required by the cell and can be metabolizedto pentoses which are an important part of the nuclei acid molecules. The simple sugarsare also known as monosaccharides and occur in many forms in nature of varying chain length of 3 to 7 carbon atoms. As the study of carbohydrate metabolism progresses examples of all of these will be found in the animal cell.

Carbohydrates can also be classified as polyhydroxyl alcohols having either an aldehyde These groups will reduce certain metal ions, the sugar undergoing oxidation in the reaction forming a number of low molecular weight compounds. This reaction is the basis of the Benedict's test for reducing sugars and for the quantitative determination of glucose in body fluids.

Both hexoses and pentoses form pyran and furan ring compounds in solution. Glucose, the most common hexoses, forms a pyran ring with two isometric forms.



α-isomer Pyran Ring Form β-isomer
Glucopyranose



Furan Ring Form of β-D-Fructofuranose

Monosaccharides polymerize into larger molecules as follows:
Disaccharides, 2 units, example cane sugar
Oligosaccharides, up to 10 units
...Dialyzable

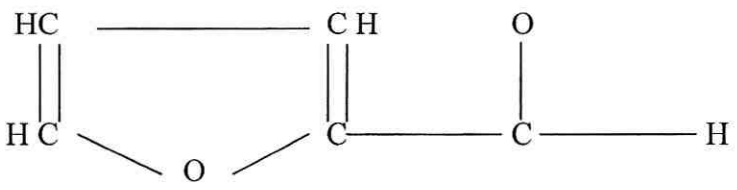
Polysaccharides, many units, example starch and glycogen. These are classed as macromolecules and are non-dialyzable.
Another characteristic property of simple sugars is the optical activity due to the presence of asymmetric carbon atoms in the structure. This is manifested by the rotation of polarized light, which will be demonstrated later in the course.

Reagents and Materials

1. 1 % solutions of Glucose, Fructose, Pentose, Sucrose and Maltose
2. Conc. H_2SO_4
3. Conc. HCl
4. Molisch's reagent
5. Barfoed's reagent
6. Seliwanoff's reagent
7. Bial's reagent

A) Molisch Test

The Molisch test is a general test for carbohydrates. Concentrated Sulphuric acid causes a dehydration of the monosacharides forming a molecule of furfural. If a polymeric saccharide is present it will be hydrolyzed by the acid to the monomer and then dehydrated to furfural.
The pentose yield furfural of the structure:



Hexoses yield 5-Hydroxy-methyl-furfural, the ketohexoses reacting more rapidly than the aldohexoses. This is the basis of Seliwanoff's test for distinguishing between fructose and glucose.

In the presence of conc. H_2SO_4, phenolic compounds such as thymol and alpha-naphthol condense with the furfural to form purple coloured derivatives. This colour complex is the basis of Molisch test.

Procedure

1. Pipette 2 ml of the sugar solutions into separate test tubes.
2. Add 2 drops of the Molisch reagent and mix thoroughly.
   Incline the test tube and pour 1-2mls of conc. H_2SO_4 down the side, slowly and carefully, to form two layers.

Result

The formation of a purple ring at the interface indicates the presence of a carbohydrate.
A green colour is not a positive reaction.
In case of doubt, repeat the test using a more dilute sugar solution.

B) Benedict's Test for Reducing Sugars

Alkaline copper reagents have been used for a long time as a sensitive test for reducing sugars which contain a potential aldehyde or ketone group. The group is designated as potential for the pyran or furan ring structure, the group has the hemiacetal structure rather than an aldehyde or ketone structure.
As already described this group will reduce certain metals forming oxidized low molecular weight compounds of the sugar.

$$Cu^{++} + \text{Glucose} \longrightarrow Cu^{+}\text{ (insoluble)} + \text{oxidized products}$$

Benedict's test is carried out in an alkaline solution of sodium carbonate.
The precipitation of Cu $(CO_3)_2$ is prevented by adding citrate, a molecule having free hydroxyl groups which form a complex with the Cu^{++}.
Under carefully controlled conditions, this reaction serves as an excellent quantitative procedure for reducing sugars.

Procedure

1. Pipette 1.0 ml of each of the test solutions into different test tubes.
2. Add 5.0 mls of Benedict's reagent to each of the test tubes.
3. Place all the tubes simultaneously in a boiling water bath and heat for 3 minutes. Cool and observe.

Result

The presence of reducing sugar is indicated by the formation of a red to yellow precipitate.

The sensitivity of this test should be demonstrated by diluting the glucose test solution 1 to 4 and 1 to 10 and repeating the test.

C) Seliwanoff's Test

This is used to distinguish fructose, a keto sugar, from glucose, an aldose. Ketoses form furfurals more rapidly than aldoses so that a comparison of the rate of colour formation can be used to distinguish between these two types of sugars.

Procedure

1. Pipette 1.0 ml of each of the test solutions into different test tubes. Also prepare a blank tube with 1.0 ml of distilled water.
2. Add 5.0 ml of Seliwanoff's reagent to each tube.
3. Mix well and heat for exactly 60 seconds in a boiling water bath.

Results

Observe and record the results after exactly 60 seconds.
Continue the heating for five minutes recording any changes that occur in a table summarizing your observations.

D) Bial's Test

This is a test for pentose sugars. The test depends on the rapid formation of the furfural from pentose under the specific conditions of the test. In this test Orcinol reacts with the furfural yielding a blue-green colour.

This is the last of the specific tests that will be used in the study of carbohydrates. It should be noted that two tests, Benedict's and Barfoeds, depend on the reduction of copper and differences in the composition of the solution and the rate of reaction are used to obtain specific information. The other three tests depend on differential rates of dehydration to form compounds forming coloured complexes.

Procedure

1. Pipette 2.0 ml of each of the test solution into different test tubes.
2. Add 3.0 ml of Bial's reagent.
3. Mix and boil for one minute.

Results

A positive reaction is indicated by the appearance of a green colour.
Hexoses, which form hydroxy-methyfurfural, yield a yellow colour.

E) Barfoed's Test

Barfoed's test is also a copper reduction test but is carried out in the acid rather than alkaline solution, conditions in which sugars are a weaker reducing agents and

$$Cu^{++} \longrightarrow Cu^{+}$$ is only reduced by monosaccharides.

It can be used therefore to distinguish the monosaccharides from disaccharides. However, on prolonged boiling the acid may hydrolyze some of the disaccharides and a slight positive result will be obtained.

Barfoed's reagent cannot be used to test solutions containing chlorides such as urine, for a white precipitate of $CuCl_2$ forms.

Procedure

1. Pipette 1 ml of each test solutions into different test tubes.
2. Add 5 ml of Barfoed's reagent to each tube.
3. Mix and place all the tubes in the boiling water bath.
4. Observe them carefully for the appearance of the red precipitate of CuO_2 and record the number of minutes required, remove the positive and allow it to stand.
5. After 15 minutes, remove any remaining tubes and allow them to stand and observe for any precipitate that may settle out. Note the time.
6. Record the observations in a table.

PRACTICAL FOURTEEN

Nucleic acids

Extraction and hydrolysis of RNA

Introduction

Nucleic acids are macromolecules of great importance which can be extracted in sufficient quantity for study from yeast or, if possible, from cells of the thymus gland. The nucleic acids are partially split from the protein of the nucleoprotein in yeast by dilute alkali and purified by precipitation with ethanol.

Acid hydrolysis and composition determine degraded RNA further by the appropriate tests. Three major components make up the structure of the molecule: purine or pyrimidine base, carbohydrate and phosphoric acid.

A) Extraction of RNA

Reagents and Materials

1. Dried yeast
2. 0.04 M NaOH
3. 3M H_2SO_4
4. 3M Acetic acid
5. Litmus paper
6. 95 % ethanol containing 10 ml of con. HCl/L
7. Biuret reagent

This part should be done by groups of 5 students.

Procedure

1. Place 20g of dried yeast in a 250 ml flask.
2. Add 200mls of 0.04 M NaOH stir and suspend the yeast.
3. Heat to boiling for 30 minutes then cool.
4. Centrifuge and test the precipitate for protein using the Biuret test that you have already done.
5. Decant the supernatant into a clean beaker and acidify with 0.8 ml of 16 M Acetic acid (use litmus paper to test the acidity).
6. Add 150mls of acidified ethanol, stirring thoroughly but gently to ensure complete precipitation of nucleic acids.
7. Decant the supernatant.
8. Wash the precipitate with 25 ml acetone, stirring up the precipitate and transferring it to a filter paper on a funnel. Several washings with the acetone will be needed to complete the transfer.
9. Spread the paper out and allow the precipitate to dry on a hot plate (Do not burn the paper!).
8. Test a small amount of the sample for protein (use the Biuret test).

B) Hydrolysis of RNA

Procedure

1. With the aid of the instructor, determine the weight (W_1) of the precipitate that you have prepared with the filter paper.
2. Transfer the precipitate into a 100-ml flask and add exactly 15mls of H_2SO_4.
3. Weigh the filter paper (W_2) and calculate the weight of the precipitate from the difference between W_1 and W_2.
4. Hydrolyze by heating for 3 minutes in boiling water. Cool.
5. Dilute to 75mls with distilled water and use 10ml portion for each of the following tests.

C) Determination of Composition of RNA

Carry out the Molisch test and Bial's test.

D) Quantitative Determination of Ribose

Reagents and Materials

Orcinol Reagent: 10 % Orcinol in 35 % Ethanol
Ribose standard: 10mg of ribose dissolved in 100 ml of distilled water.

Procedure

Filter the hydrolysate if it still contains precipitate
Place the following solutions in the appropriate test tube.

Tube No.	1	2	3	4	5
Orcinol Reagent (ml)	3.0	3.0	3.0	3.0	3.0
Ribose standard (ml)	0.0	1.0	2.0	0.0	0.0
Hydrolysate (ml)	0.0	0.0	0.0	3.0	3.0
Water (ml)	3.0	2.0	1.0	0.0	0.0

Mix and heat for 45 minutes in boiling water
Read the absorbance at 540 nm.

Results

Calculations

$$\text{mg Ribose/mg RNA} = \frac{\text{Reading of Unknown}}{\text{Reading of std}} \times \frac{\text{mg Ribose in std.} \times \text{Vol. Used}}{\text{conc. RNA (mg/ml)} \times \text{Vol. Used}}$$

E) Test for Purines and Pyrimidines

Reagents and Materials

1. 15 % Ammonium hydroxide (NH_4OH)
2. 0.1 M Silver nitrate

Procedure

1. Place 1.0 ml of hydrolysate in two test tubes labelled A and B.
2. Add 2 drops of NH_4OH to make the solution alkaline into tubes A, B and C.
3. Add 3 drops of Silver nitrate to tubes A and C.
4. Add 1.0 ml of distilled water to tube C, and 3 drops of distilled water to tube B.

Results

Observe carefully and record your results.
A precipitate of silver salts of the bases constitutes a positive test.
Which are the controls (tubes) in this experiment?

PRACTICAL FIFTEEN

Determination of serum bilirubin

Introduction

Bilirubin is formed from hemoglobin in the reticuloendothelial system and then circulates bound to plasma albumin in low concentrations in the blood. Increased concentrations of bilirubin in the blood cause jaundice. Jaundice is mostly connected with liver and bilary tract diseases.

Bilirubin is obtained from evaporated bile or powder gallstones when these are successively extracted with ether hot water, 10% acetic acid, ethanol and hot glacial acetic acid. This extraction composition removes sterols, bile salts, biliverdin and inorganic salts. This residue is then dried and extracted with hot chloroform from which bilirubin can be extracted. Bilirubin is insoluble in water, dilute acids and the common fat solvents, but dissolves in hot choloform and alkalis.

Bilirubin is determined or estimated using a diazo reagent which reacts with bilirubin to form a purple compound azobilirubin. In normal persons, bilirubin is in the range 0.2 – 0.6mg bilirubin/100ml serum. Since jaundice is due to increase in the concentration of bilirubin in the blood, its concentration therefore gives a measure of the intensity of jaundice. Pernicious anemia usually gives values of less than 3 mg/100 ml.

High levels of bilirubin of greater than 4 mg/100ml are associated with obstructive and hemolytic jaundice. However higher values 20 -30mg/100ml is as a result of obstructive jaundice, which as a rule gives higher value than hemolytic. Obstruction of the head of the pancreas when complete as in case of carcinoma may reach values as high as 20 mg/100ml. Higher values (20-30mg/100ml) may be reached during acute infective and toxic hepatitis.

Treatment of bilirubin in the serum of plasma with diazotized sulphanilic acid (diazo reagent) gives a rose mauve colour with bilirubin. The colour intensity, which gives the bilirubin concentration, is read in a spectrophotometer.

Reagents

1. Solution A =2.0g of sulphanilic acid dissolved in 15 ml of conic. HCL and diluted to 1 litter with water.
2. Solution B =0.5% sodium nitrite.
3. Diazo reagent (prepared freshly by adding 0.3ml of solution B to 10ml of solution A).
4. Diazo blank reagent – 15ml conc. HCL in 1 litre water.
5. Saturated ammonium sulphate.
6. 85% ethanol or absolute methanol.
7. Artificial methyl red standarad (equivalent to 0.8mg/100ml) dissolved in chloroform.

Procedure

1. **Indirect reaction (total bilirubin)**

Set two centrifuge tubes as follows;

Tube 1: Indirect Blank
- 1.0ml serum
- 0.5ml diazo blank reagent
- 0.5ml saturated ammonium sulfate
- 8.0 ml 85% ethanol or absolute methanol
- Mix by inversion to allow air bubbles to escape.

Tube 2: Indirect Sample
- 1.0ml serum
- 0.5ml diazo reagent
- 0.5ml saturated ammonium sulfate
- 8.0ml 85% ethanol or absolute methanol
- Mix by inversion to allow air bubbles to escape
- Stopper the contents.

Leave the solution lying horizontally with an even distribution of the precipitate for 30 minutes.
Centrifuge and read the absorption of the supernatant at 540nm.

2. **Direct reaction**

Set two tubes as follows;

Tube 1: Direct Blank
- 0.5ml diazo reagent
- 8.5ml water
- Mix by inversion to allow the air bubbles to escape

Tube 2: Direct Sample
- 0.5ml diazo reagent
- 8.5ml water
- Mix by inversion to allow the air bubbles to escape

To each tube add 1ml of serum
Mix the contents and read the absorption at 540nm exactly 15 minutes after the addition of the serum

Standard

10ml of the methyl red is read against water. This corresponds to a serum bilirubin of 0.8mg/100ml analyzed exactly as for total bilirubin

Calculate the amount of bilirubin as mg/100ml serum

PRACTICAL SIXTEEN

Extraction of serum lipids and analysis by thin layer chromatography

Introduction

Lipids are compounds that are insoluble in water and soluble in a variety of organic solvents such as ether, chloroform and hexane. The major classes of lipids are triglycerides, non-esterified (free) fatty acids and sterols (principally cholesterol)

In medicine, elevated serum lipid levels (hyperlipaemia) occur in such disease states as diabetes mellitus and hypothyroidism. In addition a correlation between serum lipid levels and the incidence of coronary heart disease and atherosclerosis has been noted.

Most lipids are not found in free form in the blood or other tissues. They are associated with proteins that form lipoproteins. To analyze blood or tissue lipid content it is usually necessary to separate the lipids from the proteins and other water-soluble substances by extraction with organic solvents.

The relative ease with which lipids may be extracted may be extracted depends on the degree of their association with tissue proteins. The loosely – bound "neutral" lipids e.g. triglycerides and cholesteryl esters are easily extracted with non-polar solvents (such as chloroform). Extraction of the polar phospholipids-protein complex. In the extraction process the hydrophobic lipid molecules "dissolve" into the organic phase, leaving most of the water-soluble proteins, carbohydrates, amino-acids and salts in the aqueous phase. The two phases are separated and the organic phase is analyzed for lipid content.

TLC is somewhat similar in principle to the paper chromatography techniques. The main difference is that in place of the hydrated cellulose fibres of the paper, the adsorbent or stationary phase in TLC is in the form of a very thin film of powdered material fixed on an inert rigid support such as a glass plate. Techniques of sample application, "running", detection, and calculation of Rf are all similar to those of paper chromatography.

Reagents and materials

1. Fresh serum
2. Extraction solvent; chloroform: methanol (2:1)
3. Chromatographic solvent (in chromatographic jars)
   Polar solvent – chloroform: methanol: acetic acid: water (25: 15: 4: 2)
   Non-polar solvent – chloroform: acetic acid (99:5:0.5)
4. Methanol
5. Chloroform
6. Two TLC silica gel glass plates, 5x20cm

7. Extraction tube
8. Centrifuge tubes
9. Capillary pipettes
10. Iodine vapor chamber (under hood)

Procedure

Extraction of serum lipids

1. Place 1.0ml of serum in an extraction tube and add 7.0ml of chloroform-methanol (2:1) mixture.
2. Shake vigorously for 3 minutes (a white precipitate will form)
3. Transfer the mixture to a centrifuge tube for 10 minutes. The aqueous and organic phase will separate into two layers with a dense white precipitate of protein at the interface.
4. Take care not to mix the two phases, remove all the upper aqueous phase and the white precipitate. If necessary discard a small portion of the organic layer to ensure complete removal of all water and protein.
5. Transfer the organic phase to a clean, dry 50ml beaker and carefully evaporate to near dryness on a hot plate.
6. When nearly dry, add 1.0ml of methanol to aid in moving traces of water. Repeat this process.
7. When dry, add 0.5ml of chloroform to dissolve lipids. The solution should be yellow, and there may be a small amount of insoluble residues.

TLC of lipid extract

The adsorbent that you will use is silica gel, a form of silicon dioxide that has an affinity for lipid molecules, binding the highly polar phospholipids more tightly than the less polar compounds. An organic solvent moving up the plate will separate a mixture of lipids from the silica gel and the solvent or mobile phase. A non-polar solvent such as chloroform which separates the neutral lipids will separate a mixture of lipids from the silica gel and the solvent or mobile phase. A non-polar solvent such as chloroform which separates the neutral lipids solvent will move with the solvent front. In today's practical you will use both types of solvent system. Using the non-polar solvent, cholesteryl esters, free triglycerides, acid cholesterol and the phospholipids will move with the solvent front. A polar solvent will separate neutral lipids, free fatty acids, phosphatidyl ethanolamine, phosphatidyl, serine, phosphatidyl choline and sphingomyelin.

Procedure

1. Place two TLC plates on a clean dry work surface with coated side up
   Handle plates only by the edges and take care not to disturb the coating.
2. On each plate, make a single, faint pencil mark approximately 2cm from one end and 2.5cm from either side. This dot will be the origin where you will sport the sample.

3. Carefully apply the lipid extract to each plate at this spot. To do this, dip a capillary pipette into the extract until the tip fills to a depth of about 1cm. This volume is about 10 micro-litres.
4. Briefly and repeatedly touch the tip of the pipette to the pencil dot and allow a small amount of the extract to flow on to the plate. Let the spot dry between each application and take care to keep the spot small, about 0.5cm diameter.
5. Repeat until a volume of about 20 micro-litres has been applied to each plate.

PRACTICAL SEVENTEEN

Isolation of phospholipids

Introduction

Lipids are an important class of biochemical compounds that fulfil several several functions, including energy storage (e.g. triglyceride) and membrane structure (e.g. cholesterol and phosphatidyl choline, also called lecithin). The basic structure of the major class of lipids, the triglycrides, is a glycerol molecule to which are attached fatty acids at carbons 1 and 2. The hydroxyl group on carbon 3 may be free (e.g. diglyceride) or esterified with fatty acids (e.g. triglyceride) or another organic group (e.g. phosphoryl choline group of lecithin). Depending on nature of the substituent at the carbon 3, a lipid may either be polar (e.g. lecithin) or non-polar (e.g. triglyceride). This difference is exploited in the resolution of lipid mixture by TLC. Spots of lipids are revealed on the TLC plate by incubation by iodine vapor. The iodine molecules bind reversibly to the double bond of unsaturated fatty acids. Such fatty acids are often substituted at carbon 2.

You will extract lecithin from egg using an organic solvent and purify it by precipitation with acetone. Egg your also contains triglycerides, sphingomyelin and cholesterol, however, sphingomyelin is insoluble in either, and cholesterol is not precipitated from either by acetone. The degree of purity of the cholesterol is not precipitated from either by acetone. The degree of purity of the lecithin can be estimated by measuring its phosphate content, since one molecule of phosphate would be derived from one mole of pure lecithin. Alternatively, contaminating lipids may be resolved and identified by TLC. In the following practical you will use your purified lecithin to study the action of the enzyme phospholipase A.

Reagents and materials

1. Egg yolk (previously weighed)
2. Ether
3. Ethanol-ether mixture (2:1, v/v)
4. Acetone
5. 5M H_2SO_4
6. Ammonium molybdate reagent
7. Standard phosphate solution (1.0 micromol/ml)
8. 0.5M Trichloroacetic acid (TCA)

Procedure

1. Crush the egg yolk with a glass rod and mix with ethanol-ether (75ml). Allow to stand for about 10 minutes, stirring occasionally.

2. Filter through a fluted filter paper moistened with ethanol-ether. Remove the residue from the paper, mix well with another portion of ethanol-ether (20ml) and filter. Combine the two filtrates and discard the residue.

3. Take a sample (1ml) of the combined filtrates and save it for analysis in the next practical. Evaporate the remainder to dryness on a hot plate.

4. While waiting, prepare in duplicate a range of phosphate concentrations at suitable intervals between 0 and 1.0ml of standard phosphate solutions (0-2.0 micromoles). Make each volume up to 1.0ml with water. To each add 0.5M TCA (2.0ml).

5. Dissolve the residue from step 4 in ether (10ml). With continual stirring until the precipitated lecithin stick together. Collect the precipitate by filtration and dry it between two filter papers. Weigh the lecithin in a tarred sample bottle and record the yield.

6. Weigh 10mg of your lecithin into a sample bottle. Add 5M sulfuric acid (0.5ml) and incubate the bottle at about 200^0 C for about 30 minutes to digest the lecithin.

7. During this time finish doing the standard assay procedure as follows. To the tubes containing phosphate solution and acid, add ammonium molybdate reagent (1.0ml) to each and after mixing well, read the absorbance at 600nm.

8. After the digestion of lecithin is complete, allow the bottle to cool before adding slowly 3ml of water (CARE). Heat this diluted solution for about 10minutes in boiling water-bath to hydrolyze and pyrophosphate formed during the digestion. Filter the solution through a moist filter paper. Make the volume up to 25ml with water.

9. Determine the total phosphate content of the solution by taking a sample (1.0ml) and apply the same procedure as for the standard assay method.

10. Draw the standard curve for phosphate, plotting micromoles of phosphate on the X-axis. Calculate the %age content of lecithin in the egg yolk and the %age purity of your lecithin sample. Keep your lecithin preparation and the egg yolk solution for the next practical.

Discussion

Comment on your results and the purity of your lecithin sample. Incorporate the results of the next practical in your discussion.

PRACTICAL EIGHTEEN

Characterization of a phospholipids

Introduction

Higher animals breakdown ingested lipids in the small intestines by the action of hydrolytic enzymes (lipases) present in the secretion of the pancreas.
One such enzyme, phospholipase A, catalyses the conversion of lecithin (phosphatidyl lysophosphatides. It does this by selectively removing a fatty acid residue from this position are generally unsaturated. The source of the phospholipase in this experiment is in the (pancreatin).

Since lecithin is almost insoluble in water and therefore unavailable to the enzyme, its effective concentration is increased by the preparation of a lecithin emulsion using an organic solvent. The emulsion also contains calcium ions (to activate the enzyme and to precipitate the fatty acid as it is formed) and buffer of pH 8.0 (to maintain optimal activity of phospholipase A). The products of the enzymatic reaction are analyzed by TLC in two different solvents systems.

Solvent A – separates only polar lipids, whereas
Solvent B – separates only non-polar lipids.

Reagents and materials

1. Lecithin (from previous practical)
2. Egg yolk solution (from previous practical)
3. Lysolecithin (marker lipid, 10mg/ml in methanol)
4. Oleic acid (marker lipid, 5mg/ml in chloroform)
5. Chloroform-methanol solution (3:1, v/v)
6. Pancreatin solution (50mg/ml in 0.2M borate buffer, pH 8.0 (containing 20mM calcium chloride)
7. TLC plates coated with silica gel G.
8. Solvents (mobile phase)
   Solvent A – Chloroform: methanol: acetic acid: water (65:8:4)
   Solvent B – Hexane: diethyl ether: acetic acid (80:20: 1)

Procedure

1. Weigh out some of your lecithin preparation (about 30mg) into a sample bottle. Dissolve the lecithin in chloroform-methanol solution (1.0ml).

2. Using the capillary tubing, apply 2 or 3 spots of the lecithin solution to each of the two TLC plates, allowing each spot to dry before applying the next. Apply the same at a point about 2 cm from the bottom of the plate and about 2cm in from the bottom of the plate and about 2cm in from the right hand side.

3. To the bulk of the lecithin solution in the sample bottle, add pancreatin solution (1.0ml). Close the bottle with its stopper and shake the bottle well to form the creamy emulsion. Enclose the bottle in your first and continue shaking.

4. After 15 minutes remove a sample from the incubation mixture and apply it as a spot at a point about 2cm in from the left-hand side of each plate. Try to apply the same size sample as that of lecithin solution.

5. Repeat step 4 after 30 minutes, placing the sample about 3cm away from the 15 minutes sample.

6. During the incubation period apply samples of egg yolk solution, lysolecithin solution and oleic acid solution to each TLC plate. The spots should be about 3 cm apart. Try to apply the same size sample of each and keep the sample spot as small as possible.

7. Separate the non-polar lipids by chromatography of one of the plates in solvent B, and the polar lipids in solvent A. this should take between 30 minutes and one hour.

8. When solvent B has risen to within a few centimeters of the top, remove the plate and mark the solvent front. Allow the solvent to evaporate from the plate. Stain the lipids by placing the plate for about 20 minutes in the tank containing iodine vapor.

9. Mark the position of each spot as quickly as possible after removing the plate from the iodine vapor, as the stain gradually fades. Calculate the Rf value for each component and try to identify them from the following Rf values:

    0.8 – 0.9 cholesterol ester
    0.4 – 0.6 triglycerides
    0.1 – 0.3 fatty acids
    0.05 cholesterol
    0.0 Phospholipid

10. Repeat steps 8 – 9 for the polar lipids on the plate A.

Discussion

Comment on your results, indicating whether they agree with the mode of action of phospholipase A proposed in the introduction. Include the TLC results for your samples of egg yolk and lecithin in the discussion of the previous experiments.

Questions

1. Why is lysolecithin only weakly stained with iodine vapor, whereas oleic acid of similar concentration is heavily stained?

2. Why is it important to have the incubation mixture well buffered?
3. Lysolecithin is a powerful detergent and it is a product of the reaction. Would you expect it to stimulate or inhibit the enzymatic reaction, and why?
4. What is the natural detergent which emulsifies the lipid in the human intestine and where it is produced?

PRACTICAL NINETEEN

Hemoglobin determination

Introduction

The determination of hemoglobin is one of the most frequently used and often the most poorly conducted clinical determination. There are a wide variety of methods used of varying degrees of simplicity, but ultimately all depend on one of two methods for standardization, either the oxygen binding power or the iron content of the hemoglobin. The former is a gasometric procedure and measures the functionally active hemoglobin. The second depends on the colour of blood, either diluted or undiluted, or on the colour produced by various reagents.

A. Determination of hemoglobin by Wong method

Theory

The colorimetric determination of iron in hemoglobin is easily done and serves as a good base for standardization of other procedures. Values are expressed in grams of hemoglobin per 100ml of blood. In the method of Wong, the iron is detached from the hemoglobin with concentrated sulfuric acid and potassium persulfate. The proteins are then precipitated with tungstic acid and the iron determined colorimetrically.

Reagents and materials

1. Saturated potassium persulfate
2. 10% sodium tungstate solution
3. Standard Iron solution (0.1 mg ferric iron/ml)
4. 3.0N potassium thiocyanate solution (POISONOUS)
5. Concentrated sulfuric acid

Procedure

1. **(unknown)**
   Transfer 0.5ml of well mixed oxalated whole blood to a 50ml volumetric flask.
   Add 2ml of iron free concentrated sulfuric acid. Mix by whirling for one or two minutes.
   Add 2ml of saturated potassium persulfate solution.
   Mix and dilute to about 25ml with water.
   Add ml of 10% sodium tungstate solution.
   Mix, then cool to room temperature under the tap, and dilute to volume with water.
   Stopper and mix by inversion.
   Filter through a dry paper, collecting the filtrate in a dry flask.

2. **(standard)**
   Prepare a standard in a second 50ml volumetric flask by adding about 25ml of water in the flask.
   And the following:

2.0ml of concentrated sulfuric acid, and
2.0ml of saturated potassium persulfate solution, and
2.5ml of standard iron solution.
Cool to room temperature, dilute with water to the mark, and mix.

3. **(blank)**
   Prepare blank similar to the standard but omitting the standard iron solution.

4. Measure in separate test tubes 10ml of;
   a. Unknown
   b. Standard, and
   c. Blank

   To each add
   - 0.5ml of saturated potassium persulfate solution, and then,
   - 2.0ml 3N potassium thiocyanate solution.

Mix by inversion and read with the next 30 minutes, at a wavelength of 480nm on the spectrophotometer.

Calculation

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times \frac{0.25}{1} \times \frac{100}{0.5} \times \frac{1}{3.4} = \text{grams haemoglobin/100ml blood.}$$

B. Determination of hemoglobin by cyanomethemoglobin method

Theory

This procedure depends on the conversion of hemoglobin to the very stable cyanomethemoglobin which can be kept in the refrigerator once standardized.
It must, however, be standardized by a procedure such as the iron method of Wong.

Reagents and materials

Cyanomethemoglobin Reagent. (POISONOUS)

Procedure

From a burette acid 5ml of cyanomethemoglobin reagent into a clean test tube. Pipette 0.02ml of the standardized blood by the iron method into the test tube and rinse out the pipette in to the solution.
Mix and allow to stand for 10 minutes.
Prepare a similar tube with the unknown blood.
Compare the standardized blood with that of the unknown using a spectrophotometer, using the cyanomethemoglobin reagent as a blank, at a wavelength of 520 – 550nm.

Calculations

$$\text{Hemoglobin, gm/100ml} = \frac{\text{O.D. unknown}}{\text{O. D. known}} \times \text{gm/100 of standard}$$

C. Test of the occurrence of occult blood

Introduction

It is necessary to test the feces or urine for the presence of blood, where it is not easily detected unless in high concentrations. Tests have been devised which will detect even minute traces of blood. In this experiment you will carry out some tests and make an estimate of the sensitivity.

The ortho-tolidine or benzidine test

This test is similar in principal to the guaiacum test except it uses a derivative of the aniline dyes, ortho-tolidine which is oxidized by hydrogen peroxide to a blue color in the presence of a catalyst such as the iron in the haem. In both of these tests the iron catalyses the decomposition of the hydrogen peroxide to nascent oxygen, which oxidizes the dye. For this reason Fe (III) and Cu (II) can interfere with the test.

Reagents and materials

1. Urine, with and without blood
2. Blood dilutions: 1:25, 1:50, 1:100, and 1:1000.
3. 0.001M $CuSO_4$
4. Ortho-tolidine solution
5. 3% Hydrogen peroxide.

Procedure

In series of seven test tubes place 2ml of each test solution, and . 001M CuSO4 in the seventh.

To each of the above tubes add,

4 drops of the ortho-tolidine solution, and then
4 drops of hydrogen peroxide

Record your results.

D. Pyridine-haemochromogen test

In this experiment sodium hydroxide is used to dissolve the blood forming alkaline haematin. This is reduced by glucose to reduced alkaline haematin, which then combines with pyridine to form pyridien-haemochromogen.

Reagents and Materials

1. Takayama's solution
2. Microscope slides
3. Cover slips

Procedure

Place one drop of blood on a microscope slide and spread into a thin film. Add two drops of takayama's solution, cover with a cover slip.

Allow to stand for 5 to 10 minutes.

Salmon pink crystals of pyridine-haemochromogen form.

Sketch the shape of the crystals.

PRACTICAL TWENTY

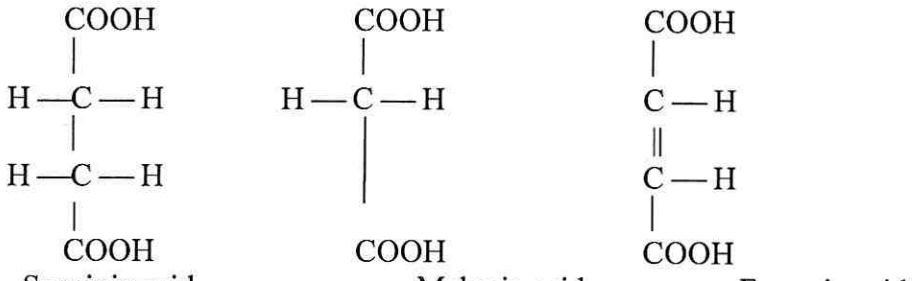
Oxidative enzymes in tissues

Introduction

Heart muscle is an excellent tissue from which to prepare a homogenate containing the enzymes, which carry out the oxidative reactions. The particular system to be studied in this experiment is the succinic acid dehydrogenase, which oxidizes succinic acid to fumaric acid. This reaction will also demonstrate the effect of competitive inhibition in the presence of malonic acid.

In the cell the hydrogen removed from a metabolite is normally transferred by the coenzyme to the respiratory chain. In vitro methylene blue can perform this function as a hydrogen acceptor and as a result of the reaction is converted to the colorless reduced form of the dye. The rate of the reaction can be measured by timing the disappearance of the blue color from the solution.
Methylene blue is easily re-oxidized by atmospheric oxygen and therefore is unstable for more exact measurements using a spectrophotometer. The dye neotetrazolium is more stable in the reduced form and can be used for more precise studies by extracting it from the reaction mixture and measuring its absorption in the spectrophotometer.

Competitive inhibition is based on a close structural similarity between a compound and the substrate for an enzyme. The inhibitor will combine with the binding site of the enzyme but the reaction cannot be completed because of the differences in the spacial arrangement of the elements of the compound.



Succinic acid Malonic acid Fumaric acid

Preparation of cardiac tissue homogenate

The tissue homogenate will be prepared cooperatively by the students of each aisle. The subsequent study of the enzyme will be carried out individually using this homogenate. The preparation should be kept cold.

Reagents and Materials

1. Waring homogenizer or potter-Elvehjem homogenizer
2. Buchner funnel, suction pump
3. Knife and cutting board
4. Ice bath
5. Frozen bovine heart muscle
6. 1 L flask
7. Ice cold distilled water
8. 0.001% methylene blue
9. 0.1M sodium succinate
10. 0.1M sodium malonate
11. 0.1M phosphate buffer, pH 7.4

A. Preparation of tissue homogenate

Procedure

Finely slice the frozen heart muscle and place it in the beaker.
Extract 3 times with 200ml portions of ice cold distilled water.
Decant and discard the water each time.
Filter the hash on the buchner funnel with suction and transfer the residue to the blender jar.
Add 250ml of cold phosphate buffer.
Homogenize the mixture for 2 minutes.
Keep the homogenate in the ice bath.

B. Measurement of succinic acid dehydrogenase

Reagents and Materials

1. 0.001% Methylene blue
2. 0.1M phosphate buffer, pH 7.4
3. 0.1M sodium succinate
4. Rubber stoppers.

Procedure

Prepare the following series of 6 test tubes, adding the homogenate last and rapidly as possible.
Stopper and mix by inverting the tube.
Begin timing the rate of the disappearance of the color at this point, recording the time it takes each tube to be decolorized.

TUBE	1	2	3	4	5	6	7
0.1%Methylene blue	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water	5.0	5.0	4.5	4.0	3.5	3.0	4.0
Buffer pH 7.4	2.0	2.0	2.0	2.0	2.0	2.0	2.0
0.1M succinate	2.0	0.0	2.0	2.0	2.0	2.0	2.0
Homogenate	0.0	2.0	0.5	1.0	1.5	2.0	2.0

Plot the elapsed time for decoration against the amount of homogenate added as the abscissa.

C. The effect of competitive inhibition

Prepare 6 test tubes as follows,

Tube	1	2	3	4	5	6	7
0.1%Mehtylene blue	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Buffer, pH 7.4	2.0	2.0	2.0	2.0	2.0	2.0	2.0
0.1M Succinate	2.0	2.0	2.0	2.0	2.0	2.0	1.0
0.1M Malonate	0.0	0.5	1.0	2.0	3.0	4.0	1.0
Water	3.0	2.5	2.0	1.0	0.0	0.0	4.0
Homogenate	0.0	2.0	2.0	2.0	2.0	2.0	2.0

Plot the elapsed time for decolourization vs mal./suc. Ratio.

D. Oxidative enzymes of liver

Procedure
Repeat part A and B substituting liver for heart muscle.

E. Oxidative enzymes in tendons

Repeat part A and B substituting tendon for heart muscle.

PRACTICAL TWENTY ONE

Enzyme kinetics

Introduction

The sequence of an enzyme-catalyzed reaction can be described by the following generalized reactions;

ENZYME + SUBSTRATE ⇔ ENZYME - SUBSTRATE + COMPLEX

ENZYME – SUBSTRATE – COMPLEX ⇔ ENZYME + PRODUCTS.

Evidence for the formation of a complex between the substrate and the enzyme has been found by spectrophotometric experiments, so that the validity of this statement will be assumed. The formation of a complex emphasizes the importance of the tertiary structure of the enzyme, which orients the functional groups of the amino acids forming the binding site.

In vivo, the first reaction is generally the rate limiting step, for in the cell the second reaction will go essentially to completion by removal of the product by the next step of the sequence. The reactions can be studied in vitro using a purified enzyme system, by varying the concentrations of the reactions (enzyme, substrate and products). The role of the environment can be measured by varying the pH and the temperature.

The enzyme used is a purified preparation of alkaline phosphatase, a phosphoric monoester hydrolyze. The substrate is disodium phenylphosphate, which yields phenol on hydrolysis, which can be measured with the folin-phenol reagent in a spectrophotometer. The hydrogen ion is controlled by a glycine buffer and the pH modified by changing the ratio of glycinate to glycine according to the Henderson-Hassaelbalch equation. The temperature is easily controlled, but it must be remembered that enzymes are proteins and are easily denatured by temperatures above 40°C centigrade.

Reagents and Materials

1. Glycine-Na glycinate buffers of pH 9.0, 9.5, 10.0, 10.5, 11.0
2. 0.01M disodium phenyl phosphate (DPP)
3. 0.02M disodium phenyl phosphate (DPP)
4. 1M NaOH
5. 0.05M Na_2HPO_4
6. Alkaline phosphatase solution, 5mg/100ml, 10mg/100ml, 20mg/100ml.
7. Folin-phnol reagent
8. Alkaline copper reagent.

A. Determination of effect of pH

Procedure

Enzyme conc. 10mg/100ml (dilute 1:20)
Substrate: 0.02M DPP
NB: T = Test & C = Control

Set up 10 test tubes, 2 at each pH, as follows,

Tube No.	1		2		3		4		5	
PH of Buffer	9.0		9.5		10.0		10.5		11.	
	T	C	T	C	T	C	T	C	T	C
Buffer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.02M DPP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Enzyme	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-
Water	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2

Place in a water bath at 37° C for 15 minutes, for enzyme reaction.
Remove the tubes from the water bath and add,

Alkaline copper reagent	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Mix well and allow to stand for 15 minutes, and then add

Folin - Phenol reagent	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Allow 30 minutes for color development.

Make up a blank tube as follows,

(This will help you to zero the spectrophotometer for all the tests).
1ml water
8ml alkaline copper reagent and allow 15 minutes color development.
Add 1ml of folin-phenol and allow 30 minutes color development.

Determine the amount of phenol liberated using the Folin-phenol methods and the standard curve, which was prepared in the earlier experiments.
Measure the absorbance with the spectrophotometer at a wavelength of 600nm.
Plot the μ-moles of phenol liberated versus pH on the Y-axis.

B. The effect of temperature

Procedure

Enzyme conc. 10mg/100ml
Substrate: 0.02M DPP

Prepare 5 pairs of test tubes as follows,

Tube	1		2		3		4		5	
	T	C	T	C	T	C	T	C	T	C
Buffer, pH 10	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Substrate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Mix thoroughly and then add,

Enzyme	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-
Water	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2

Incubate for 15 minutes, at the following temperatures

Temperature °C	0	20	40	60	80

Remove from water-bath and add,

Alk. Cu^{2+} reag.	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Allow 15 minutes color development, then add

Folin-phenol reag	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Allow 30 minute color development.

Determine the amount of phenol liberated as in part A.
Plot moles of phenol versus temperature on the Y-axis

C. The effect of substrate concentration

Procedure

Enzyme conc. 10mg/100ml
Substrate: 0.01M, 0.02M and 0.04M DPP

Prepare the following set of tubes,

Tube	1	2	3	4	5	6	7	8	9	10
Buffer, pH 10	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Water	0.4	0.3	0.2	0.1	0.2	0.1	0.0	0.5	0.2	0.1
0.01M DPP	0.1	0.2	0.3	0.4	-	-	-	-	-	-
0.02M DPP	-	-	-	-	0.3	0.4	0.5	-	-	-
00.04M DPP	-	-	-	-	-	-	-	-	0.3	0.4
Enzyme	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Incubate all tubes for 15 minutes at 37°C,
Remove from water-bath, then add

Alk. Cu^{2+} reag.	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Allow 15 minutes for color development, and then add

Folin-phenol reag.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Allow 30 minutes color development.

Determine phenol liberated as before.
Plot u-moles of phenol liberated versus moles of substrate on the Y-axis.

D. The effect of enzyme concentration

Procedure

Enzyme conc. 5mg/100ml, 10mg/100ml, 20mg/100ml.
Substrate: 0.02M DPP

Prepare 8 tubes as follows,

Tube No.	1	2	3	4	5	6	7	8
Buffer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Substrate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Water	0.15	0.05	0.1	0.05	-	0.1	0.05	0.25
Enzyme, 5mg/100ml	0.1	0.2	-	-	-	-	-	-
Enzyme, 10mg/100ml	-	-	0.15	0.2	0.25	-	-	-
Enzyme, 20mg/100ml	-	-	-	-	-	0.15	0.2	-

Incubate for 15 minutes at 37°C
Remove from the water-bath, then add

Alk. Cu^{2+} reag.	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0

Allow 15 minutes color development, the add

Folin-phenol reag.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Allow 30 minutes for color development.

Determine phenol liberated versus μ-grams of enzyme on the Y-axis.

E. The effect of end product concentration

Procedure

Enzyme conc. 10mg/100ml.
Substrate: 0.01M DPP
End product: 0.05M Na_2HPO_4

Prepare the following sets of tubes,

Tube No.	1	2	3	4	5	6
Buffer, pH 10	0.5	0.5	0.5	0.5	0.5	0.5
0.05M Na_2HPO_4	0.5	0.5	0.5	0.5	0.5	0.5
0.01M DPP	-	0.1	0.2	0.3	0.4	0.3
Water	0.4	0.3	0.2	0.1	-	0.3
Enzyme	0.2	0.2	0.2	0.2	0.2	-

Incubate for 15 minutes at 37°C.
Remove from the water-bath, and then add

Alk. Cu^{2+} reag.	8.0	8.0	8.0	8.0	8.0	8.0

Allow 15-minute color development, and then add

Folin-phenol reag.	1.0	1.0	1.0	1.0	1.0	1.0

Allow 30-minute color development.

Determine phenol liberated as before.
Plot u-mole of phenol liberated versus μ-moles of PO_4 Y- axis.

Standard curve for phenol

Reagents and Materials

1. Phenol std. 90 μ-gm/ml (0.09gm/l of L-Tyrosine in dilute HCL)
   Dilution of HCL = 1ml conc HCL + 2ml H_2O
2. Alkaline copper reagent
3. Folin-phenol reagent

Prepare the following sets of tubes as follows,

Tube No.	1	2	3	4	5	6
Phenol solution	0.1	0.2	0.4	0.6	0.8	0.0
Water	0.9	0.8	0.6	0.4	0.2	1.0
Alk. Cu^{2+} reag.	8.0	8.0	8.0	8.0	8.0	8.0

Mix well and allow to stand for 15 minutes.

Folin- phenol reag.	1.0	1.0	1.0	1.0	1.0	1.0

Mix and allow to stand for 30 minutes.
Read the absorbance at 660nm using a spectrophotometer.
Plot phenol conc. (u-moles) versus O.D. on the Y-axis.

NB:
M.wgt. phenol = 94g.
L-tyrosine + HCL = phenol.

PRACTICAL TWENTY TWO

Determination of liver glycogen and blood glucose and the effect of fasting

Introduction

Fasted rats have been deprived of food for 72 hours before been killed. Fed rats have been fasted for 72 hours then fed for 72 hours with either a high carbohydrate diet containing 35% sucrose or high protein diet (lean meat).

Reagents and Materials

1. 60% KOH
2. 1N H_2SO_4
3. 3% Perchloric acid
4. 90% Ethanol
5. 70% Ethanol
6. 1N NaOH
7. Standard glucose 180mg/L (180 u-gm/ml)
8. Glucose reagent;

   Solution A: 40 u-gm peroxidase and 2.5 mg glucose oxidase dissolved in 0.2M phosphate buffer of pH 7.0

   Solution B: 5mg O-dianisidine hydrochloride /ml (H_2O).

   Working solution: Take 100ml of solution A and mix with 1ml of solution B, just before the experiment.

Principal

i. Glucose oxidase catalyses the reaction,
   β-D- glucose + H_2O + O_2 → D-gluconic acid + H_2O_2
   D- gluconolactone is first formed and then hydrolyzed to D-gluconic acid.

ii. Peroxidase catalyses the reaction, the decomposition of hydrogen peroxide is the indicator reaction.
   H_2O_2 → H_2O + O_2

iii. The oxygen liberated oxidizes a hydrogen donor DH_2, in this case O-dianisidine to a colored compound D.
   O_2 + DH2 → $2H_2O$ + D

   The amount of dye formed DH_2 is a measure of the glucose oxidized.

Procedure

A. Preparation of liver extracts

1. Weigh 0.2gm liver samples and place in centrifuge tubes immediately after killing the animals provided to you.
2. Add 0.2ml of 60% KOH to the liver sample and place it in boiling water-bath for at least 30 minutes.
3. Add 2ml of water during this period to counter evaporation losses.
4. After digestion add another 0.4ml of water, followed by 90% ethanol.
5. Just bring the ethanol to the boiling point and then remove from the water-bath.
6. Wash the pellet twice, first by resuspending in 2ml of 70% ethanol then resuspend in 2ml of 1N H_2SO_4.
7. Place in boiling water-bath again for 1 hour.
8. Neutralize the acid by adding 2ml of 1N NaOH.

B. Preparation of blood extract

To 1ml of fresh blood add 9ml of 9% perchloric acid.
Centrifuge the extract and retain the supernatant for the glucose assay.

C. Dilution of the liver and liver extracts

Dilute the extracts from the rats as follows,

i. Rat fed on normal diet (control) - 1:50
ii. Rat that has been fasted and then refed (refed) - 1:50
iii. Rat that has been fasted (fasted) - 1:5

D. Determination of glucose in the liver extract

Tube No.	1	2	3	4	5	6	7	8	9
Fasted rat	0.2	0.2	-	-	-	-	-	-	-
Refed rat	-	-	0.2	0.2	-	-	-	-	-
Control rat	-	-	-	-	0.2	0.2	-	-	-
Std. Glucose	-	-	-	-	-	-	0.2	0.2	-
Water	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.0
Glucose reag.	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Mix well
Leave in dark for 30 minutes.
Read O.D. at 435nm.

E. Determination of glucose in the blood extract

Repeat as in step D using blood extract.