NATIONAL OPEN UNIVERSITY OF NIGERIA

SCHOOL OF SCIENCE AND TECHNOLOGY

COURSE CODE: General Biochemistry Laboratory 11

COURSE TITLE: BIO 218

National Open University of Nigeria (NOUN)

Course guide

BIO 218 General Biochemistry Laboratory 11

CONTENTS PG

1.0 INTRODUCTIONS

BIO 218: GENERAL BIOCHEMISTRY LABORATORY II, a one credit unit course is a 200 level second semester course of the National Open University of Nigeria (NOUN). The course will be available to all students who require basic knowledge in General Biochemistry Laboratory conduct and practices

Biology as a subject in the natural sciences and applied sciences deals with the study of the living world (both plants and animals) and their interaction with one another and with their immediate environment. I. e. it studies the biotic and abiotic factors of the natural world. Biology is an intellectual discipline that deals mostly with organisms found in the field and those kept under laboratory investigation to find out the effect of nature on their development and how they perpetuate from generation to generation.

The course guide tells you briefly what the course is all about, what course material you will be using and how you can effectively work your way through this material.

2. 0 WHAT YOU WILL LEARN IN THIS COURSE

The entire aim of Bio 218 (General Biochemistry Laboratory 11) is to introduce you to general texts in concentration, Reaction of carbohydrate, thin layer of chromatographic separation of sugar. Estimation of glucose in biological fluid (blood and urine). Analysis of lipids for double bond and free fatty acids. Separation by thin layer chromatography. Separation and purification of nucleic acids. Estimation of DNA and RNA. Estimation of phosphate and titratable acidity.

3.0 COURSE AIMS AND OBJECTIVES

This course aimed at given you the fundamental base line on the concept of concentrates and carbohydrate reactions, chromatographic separation of sugar and the estimation of glucose in fluids like blood and urine, analysis of lipids and nucleic acids. The course is generally aimed at:

- Knowing what concentrate is all about
- Identifying different carbohydrates
- Describing the different reactions of carbohydrates, and how to use chromatographic seperation
- Knowing the procedures for the estimation of blood and urine glucose and the importance of testing blood glucose level for diabetics
- Knowing the principle of determination of Iodine number of a fat/lipid
- Understanding the importance of DNA and RNA as components of nucleic acid
- Knowing how to get rid of excess acid in the body

- Knowing how to determine phosphate and titrable acidity in urine

4.0 WORKING THROUGH THIS COURSE

To complete this course, you are required to read the study units and read other reference text listed if you can lay hands on them. The course should take you through out the duration of the semester (about 15weeks) to complete. Under section 5.0 you find listed all the components of the course, what you have to do and how you should allocate your time to each unit in order to complete the course successfully on time

5.0 Course Materials

The main components of the requirements for this course are:

1. The study guide
2. Study units
3. Other reference materials
4. Assignment file
5. Presentation schedule
6. Materials for practical work.

6.0 STUDY UNITS

The units in this course are listed here below:

- **GENERAL TEXTS IN CONCENTRATION**
- **REACTIONS OF CARBOHYDRATE, THIN LAYER OF CHROMATOGRAPHIC SEPARATION OF SUGAR**
- **ESTIMATION OF GLUCOSE IN BIOLOGICAL FLUID (BLOOD AND URINE)**

- **ANALYSIS OF LIPIDS FOR DOUBLE BOND AND FREE FATTY ACIDS**
- **SEPARATION OF LIPIDS BY THIN LAYER CHROMATOGRAPHY**
- **SEPARATION AND PURIFICATION OF NUCLEIC ACIDS**
- **ESTIMATION OF PHOSPHATE AND TITRATABLE ACIDITY CONTENT**

7.0 REFERENCES AND OTHER RELATED MATERIALS

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Smith, I. Chromatographic and Electrophoretic Techniques, 4th edn. Vol. 1. Chromatography. Heineman, London, 1976.

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Presentation Schedule

You will be at an advantage if you follow the schedule of submission of your assignments as well as attend all tutorials

9.0 Assessment

There are two phases of assessment in the course. The tutor - marked assignments and the written examinations you are expected to use information, knowledge and techniques in the course to tackle your assignments. You are also to submit your assignment to your course tutor for formal assessment as stipulated in your presentation schedule and assignment file.

The work you submit to your tutor for assessment will count for 30% of your total course. You will be expected to sit for an examination for two or three hours. The exams will contribute 70% of your total course work.

10.0Tutor-Marked Assignments (TMA's)

The assignment questions for the units in this course can be found in the assignment file. You will be able to complete your assignment from the information and materials contained in your study units, reference materials, books and practical exercises.

You will however be at an advantage if you can read more widely as a degree student. The references you are given will give you these additional readings.

11.0 Final Examination and Grading

The final examination for Bio 215 will be a two or three hour practical examination which has a value of 70% of the total course grade. The examination will consist of questions which reflect the types of self - testing, practice exercises and tutor marked problems you have previously encountered. All areas of this course will be assessed.

You will succeed if you use the time between finishing the last unit and sitting the examination to revise the entire course.

12.0 How To Get The Best From This Course

In NOUN distance learning programme the, study units replace the lecturer. That to say there is no physical contact between student and lecturer in the class room.

This is one of the great advantages of distance learning (the course material with study guide and self assessment questions). You can read and work through specially designed study materials at your own pace and at a time and place that suits you best. Handle the reading of the course material as if you are listening to a lecture. The study unit will equally guide you when to read other materials, just as the lecturer might tell you in a class exercise; your study unit provides exercises for you to do at appropriate points.

Each of the study unit follows a common format. The first item is an introduction to the subject matter of the unit and how a particular unit is integrated with units and the course as a whole. Next is the set of learning aims/objectives. This let you know what you should do by the time you have completed the unit. You should use these aims/objectives to guide your study. When you have finished the unit you must go back and check whether you have achieved the aims/objectives highlighted in the unit. If you make a habit of doing this you will significantly improve your chances of passing the course.

The main body of the unit guides you through the content of the course. Some units require time more than the other to master. Self-test are interspersed throughout the units and answers are given at the end of the units. Working through these tests will help you to achieve the objectives of the unit and prepare you for the assignments and the examination. You should do each self test as you meet it in the study unit. There will also be numerous examples given in the study units; work through these when you come across them too.

13.0 SUMMARY

The following is practical strategy for working through the course.

Read this course guide thoroughly

Organize a study schedule.

Refer to the study units for more details.

Note the time you are expected to spend on each unit and how the assignments relate to the units and details of your tutorials.

You need to gather all this information in one place, such as your diary or a wall calendar. Whatever method you choose to use, you should decide on and write in your own dates on how to work on each unit.

The major reason that students fail is that they lag behind with their course work. If you get into difficulties with your schedule please let your tutor know before it is too late.

Turn to unit 1 and read the introduction and the aims/objectives for the unit

Assemble the study materials if you are required to do so.

Work through the unit. The content of the unit itself has been arranged to provide a sequence for you to follow. As you work through the unit you will be instructed to read from other articles. Use the unit to guide your reading.

Review the aims/objectives for each study unit to confirm that you have achieved them.

If you feel uncertain about any of the objectives, review the study materials or consult your tutor.

When you are confident that you have achieved a unit's objectives, you can then start the next unit.

Always ensure that you precede nit by unit as you follow the study material and try to pace your study so as to keep yourself on schedule.

Your tutor will mark and comment on your assignments.

Keep a close watch on your progress and on any difficulties you might encounter and provide assistance to you during the course. You must mail your tutor-marked assignments to your tutor well before the due date (at least two working days are required). They will be marked by your tutor and returned to you as soon as possible.

Do not hesitate to contact your tutor if you need help. The following might be circumstances in which you would find help necessary. Contact your tutor if

i. You do not understand any part of the study units or the assignment.
ii. You have difficulty with the self-tests or exercises
iii. You have a question or problem with an assignment or with the grading of an assignment.

You should try your best to attend the tutorials. This is the only chance have to face to face to face contact with your tutor and ask questions which are answered instantly. You can raise any problem encountered in the course of your study. To gain the maximum benefit from tutorials, prepare a question list before attending them. You will learn a lot from participating in the discussions actively.

1.

BIO 218 GENERAL BIOCHEMISTRY LABORATORY II

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UNIT 1 GENERAL TEXTS IN CONCENTRATION

CONTENTS

1.1 Introduction

Concentration is important in the preparation of reagents in the laboratory for any biochemical reaction and it is related to amount of substance in a solution. Amount is a physical quantity just as mass, volume and area; its unit is the mole. It gives the number of molecules or atoms or ions or electrons in a given volume of weight of substance.

1.2 Objectives

Upon completion of studying this unit, you should be able to know:

1. What is meant by a concentration of a sample?
2. How to calculate the concentration of a solution
3. How to prepare a known concentration of a reagent

1.3 MAIN BODY

1.3.1 What is a Concentration?

Concentration can be defined as amount per unit volume. If a mixture contains components A_1, A_2, A_3, etc, then the concentration of A_1 in the mixture is:

$$= \frac{\text{Concentration of } A_1 \text{ in the sample}}{\text{Quantity of sample}}$$

A similar equation may be written for any other component. The term concentration is used to denote the amount of solute dissolved in a given quantity of solvent or solution. The concentration of a solution can be expressed either qualitatively or quantitatively. The terms concentrated and dilute are used to quantitatively describe a solution. A solution with a relatively small concentration of solute is said to be dilute, while one with a large concentration is said to be concentrated. These expressions suggest that concentration may be given in more than one unit, depending on the units in which the quantity of component or quality of sample is expressed.

1.3.2 Expression of Concentration

Molarity: The concentration of a given solution is given in terms of the amount (number of moles) of the substance it contains in a known volume. Concentrations of substances are hence measured in terms of molarity 'M'. For a given solution,

$$\frac{\text{Concentration in gdm}^{-3}}{\text{Mass of one mole of the substance}}$$

In terms of amount of number of moles

$$= \frac{\text{number of moles (amount)}}{\text{Volume in dm}^3}$$

i.e amount = molarity x volume.

Molarity was some years back used for a given amount of a substance per unit volume. The correct terminology for molarity today is concentration. Its unit is gdm^{-3} when defined as the number of grams of solute dissolved in 1 dm^3 of solution, and mol dm^3, when defined as the amount of the substance per dm^3.

$$\text{Concentration (C)} = \frac{\text{amount (n)}}{\text{Volume (v)}}$$

Hence, n = CV

$$\text{Mass concentration (Cm)} = \frac{\text{mass (m) in g}}{\text{Volume (V) in } dm^3}$$

And the unit is g dm^{-3} (g/dm^3)

$$\text{Avogadro's constant (L)} = \frac{\text{no. of particles or ions or atoms (N)}}{\text{Amount of the species (n)}}$$

Therefore, N = nL

Molality: The molality is the number of moles of solute in one kilogramme of solvent. Such a solution is said to have a molality of one with respect to the solute.

$$\text{Molality} = \frac{\text{number of moles of solute}}{\text{Mass of solvent (kg)}}$$

Normality: this is the number of gram-equivalent weighs of solute per dm^3 of solution. This term is no longer in use, but will be found in some old text bookds

$$\text{Nomality} = \frac{\text{equivalents solute}}{\text{Volume of solution in } dm^3}$$

The gram-equivalent weight of an acid is that weight of the acid in grams which produces 1.008g of hydrogen ion, H^+ from the following ionizations.

$$HCl \rightarrow H^+ + Cl^-$$

36.5g 1.00g

$H_2SO_4 \rightarrow 2H^+ + SO_4^{2-}$

It is obvious that the gram-equivalent weight of hydrogen chloride is 36.5g and that of sulphuric acid is 98 ÷ 2 or 49g. That is, a normal solution of hydrochloric acid contains 36.5g, and of sulphuric acid 49g of acid in 1 litre. The gram-equivalent of alkalis (or carbonates) is that weight in grams of the alkali (or carbonate) that reacts with 1.008g of hydrogen ion. From the following reactions:

$NaOH\ (or\ KOH) + H^+ \rightarrow Na^+\ (or\ K^+) + H_2O$

40g 56g 1.008g

$Na_2CO_3 + 2H^+ \rightarrow 2Na^+ + H_2O + CO_2$

106g 2 x 1.008g

Hence, the gram-equivalent weight of sodium hydroxide is 40g, of potassium hydroxide is 56g and that of Na_2CO_3 is 106 ÷ 2g or 53g. That is, a normal solution of NaOH contains 40g of the alkali in one litre; for KOH, the corresponding weight is 56g and for Na_2CO_3 is 53g.

Weight Percentage (%)

Weight percentage is defined as the ratio of the weight of solute to the total weight of solute plus solvent multiplied by 100.

$$\text{Wt/wt \%} = \frac{S_1}{S_1 + S_2} \times 100$$

Example:

Volume Percentage (%)

Volume percentage is defined as the ratio of the volume of solute to the total volume of solute plus solvent, multiplied by 100.

$$\text{Vol/vol \%} = \frac{V_2}{V_2 + V_1} \times 100$$

Weight/volume percentage (%w/v)

Weight/volume percentage is defined as the number of grams of solute per one hundred cm^3 of solution multiplied by 100.

$$\text{Wt/vol \%} = \frac{\text{grams of solute}}{\text{Volume of solution in } cm^3} \times 100$$

1.3.3 Standard solution: A standard solution is a solution of known concentration. It is one that contains a known mass of the substance in a known volume of solution. If A is a known standard solution of HCl and B contains 2.50g anhydrous Na_2CO_3 in 500 cm^3 of aqueous solution, and if 23.15 of A neutralized 25.00 cm^3 of B, then

a). Mass concentration of B is calculated thus:

500 cm^3 solution contains 2.50g Na_2CO_3

□· 1000 cm^3 (1 dm^3) solution will contain $\frac{2.50 \times 1000}{500}$ g/dm^3

= 5.00 gdm^{-3}

Since molar mass of B = 106 g dm^{-3}, then,

b). Concentration of B = $\frac{\text{mass concentration of B}}{\text{molar mass of B}}$

$$= \frac{5.0 \text{ g dm}^{-3}}{106 \text{ g mol}^{-1}}$$

= 0.047 mol dm^{-3}

For the reaction $Na_2CO_3(aq) + 2HCl(aq) \rightarrow 2NaCl(aq) + H2O(l) + CO_2(g)$

$n = \frac{\text{amount of acid}}{\text{amount of base}} = \frac{2}{1}$ or 2

So,

$\frac{CaVa}{CbVb} = \frac{2}{1}$ and therefore

$$\text{Concentration of acid} = \frac{2 \times 0.047 \text{ mol dm}^{-3} \times 25.00 \text{ cm}^3}{23.15 \text{ cm}^3}$$

$= 0.102 \text{ mol dm}^{-3}$

c). If it is required to calculate the concentration of a solution of H_2SO_4 which contains 9.8g of pure acid in 125 cm^3 of solution, then,

$$\text{amount of acid} = \frac{\text{mass of acid}}{\text{molar mass of acid}}$$

$$= \frac{9.8 \text{ g}}{98 \text{gmol}^{-1}}$$

$= 0.10$ mol.

$$\text{Concentration of the acid} = \frac{\text{amount}}{\text{Volume}}$$

But volume of acid = 125 cm^3 or dm^3 or 0.125 dm^3

Therefore

$$\text{Concentration of acid} = \frac{0.10 \text{ mol}}{0.125 \text{ dm}^{-3}}$$

$= 0.80 \text{ mol dm}^{-3}$.

1.3.4 Preparation of various concentrations of reagents

i). **H_2SO_4**

The concentration of concentrated laboratory H_2SO_4 is about 18 M. 1000 cm^3 conc. H_2SO_4 contains 1.84 kg or 1840 g and so, 1 cm^3 contains 1.84 g of the acid. Therefore the density H_2SO_4 of it is 1.84 g cm^{-3}. If the % assay of the acid is 97%, then pure acid

per cm^3 = $\frac{97}{100}$ x 1.84 g

= 1.7848 g

But molar mass of 1 M H_2SO_4 or 1 mole of H_2SO_4 = 98 g mol^{-1}. Therefore:

If 1.7848g H_2SO_4 is contained in 1 cm^3 of pure acid then, 98.0g will be contained in

$\frac{1 \times 98}{1.7848}$ cm^3 or 54.9 cm^3. This is approximately 55 cm^3. Therefore, for 1 mole H_2SO_4 (1.0 M) to be prepared, 55 cm^3 of the concentrated acid (Density of technical grade H_2SO_4 is 1.84 g cm^{-3}) will be added to 945 cm^3 (1000-55 cm^3) of distilled water in a 1 mm^3 volumetric flask. The technique of doing this is as follows:

Measure out about 55 cm^3 of the concentrated acid into a measuring cylinder, and add it slowly to 945 ml (cm^3) of distilled water in a volumetric flask or a beaker with constant stirring. The solution is roughly 1 M (1 mol) H_2SO_4.

Different concentrations of the acid can be prepared by taking the advantage of the proportion of volume of concentrated acid. Since 1 mol H_2SO_4 = 98g of the acid in 1000 cm^3 or 49g H_2SO_4 in 500 or 9.8g H_2SO_4 in 100 cm^3, it then follows that,

2 M H_2SO_4 = 110 cm^3 (55 x 2 cm^3) conc. H_2SO_4 in 890 cm^3 (1000-110 cm^3) distilled water

0.2 M H_2SO_4 = 11 cm^3 (55 x 0.2 cm^3) conc. H_2SO_4 in 989 cm^3 (1000-11 cm^3) distilled water or 1.10 cm^3 conc. in 98.9 cm^3 water.

2). NaOH

The molar mass of NaOH is 40 g mol^{-1}

1.0 mole (1.0 M) NaOH = 40g NaOH in dm^3 of solution or 4g NaOH in 100 cm^3 of solution or 10g NaOH in 250 cm^3 of solution. By proportion therefore:

2.0 M NaOH = 40 x 2g NaOH per dm^3 or 80g NaOH per dm^3.

0.1 M NaOH = 40 x 0.1 or 4.0g NaOH in 1000 dm^3.

1.4 Conclusion

Concentration is a physical quantity which we measured in quantitative chemical analysis of mixtures. This term deals with relative amount, as oppose to the absolute amount or quantity of a component of a mixture. The concentration of standard solution is usually expressed in molarity.

1.5 Summary

In this unit, you have learnt:

1. The meaning of concentration
2. The different expression of concentrations and how to calculate concentration
3. How you can prepare a standard solution.

1.6 Tutor-marked Assignments

1. List different expression of concentration
2. Explain how to prepare 1.0 M Na_2CO_3 and 0.25 M NaOH.

3. If X is a known standard solution of H2SO4 and Y contain 4.0 g anhydrous NaOH in 250 cm3 aqueous solution, and if 20.25 cm3 of X neutralized 25.00 cm3 of B, then

a) Calculate the mass concentration of Y

b) Concentration of Y

c) Concentration of the acid (A)

1.7 References/Further reading

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UNIT 2 REACTIONS OF CARBOHYDRATE, THIN LAYER OF CHROMATOGRAPHIC SEPARATION OF SUGAR

CONTENTS

2.1 Introduction

These set of practicals are designed to introduce the students to some of the common procedures in carbohydrate chemistry and biochemistry. Carbohydrates are one of the most important components in many foods. Carbohydrates may be present as isolated molecules or they may be physically associated or chemically bound to other molecules.

2.2 Objectives

Upon completion of studying this unit, you should be able to:

1. Identify different carbohydrates
2. Describe the different reactions of carbohydrates

3. Describe the principle of thin layer chromatography and how to use it to separate sugars.

2.3 Main body

2.3.1 What is a Carbohydrate?

Carbohydrates are very, important biological macromolecules that predominantly occur in almost all living things. They can be aldehydes or ketones with polyhydroxyl groups that contain carbon, oxygen and hydrogen in the ratio of 1:2:1 thus conforming to a general molecular formula of $(CH_2O)_n$. However, some carbohydrates contain elements like sulphur, nitrogen, and phosphorous, thus deviating from the general molecular formula. The basic units of carbohydrates are monosaccharides which cannot be split further by hydrolysis. Carbohydrates are also important components of some of the structural materials of living organisms. Some examples of this are the cell walls in plants, polysaccharides in the capsules of bacteria, and mucopolysaccharides of skin and connective tissue in animals. In addition, monosaccharides are an important part of biochemical compounds such as nucleic acids, coenzymes and flavoproteins. Carbohydrates are also involved in cell recognition, contact inhibition and the antigenic properties of blood group substances.

Carbohydrates have characteristics tests that can distinguish them from other molecules.

2.3.2 Qualitative Tests for Carbohydrates

2.3.2.1 Test for Simple Sugars

Principle: There are reducing sugars possessing between 3 to 7 carbon atoms in their structure and conform to identification reaction for aldehydes and ketones. Notable examples are glucose, ribose, erythrulose and glyceraldehydes.

1). **Molisch Reaction**

Principle: The solution is treated with concentrated sulphuric acid which hydrolysis glycosides to monosaccharides. The reaction takes advantage of the acid catalysed dehydration of simple sugar to produce either furfural or 5-OH methyl furfural which combines with α–naphthol to produce a purple complex.

Materials: Ethanol, α–naphthol, conc. H_2SO_4, glucose, test tubes.

Procedure: Add 9-12 drops of alcohol into a test tube containing few crystal of α–naphthol and add 3 drops of glucose. Mix well and pour a few drops of conc. H_2SO_4 down the side of the tube, which would form a layer at the bottom of the test tube, in the alcohol solution. A violet colouration (purple colour) at the junction of the two layers would be observed. The experiment should be repeated for fructose and a complex sugar.

2). Reaction with Fehling's Solution

Principle: This is based on the ability of sugars containing a free aldehyde or ketone group (or containing a ring which readily opens to give a free aldehyde or ketone) to reduce Cu^{++}. Other compounds can reduce Cu^{+} so Fehling's is not specific for sugar.

Materials: Fehling's reagent contains $CuSO_4$, potassium sodium tartrate, and NaOH; the tartrate prevents the precipitation of $Cu(OH)_2$, NaOH promotes the readily reducing of Cu^{++}; test tubes.

Procedure: Add 5 ml of Fehling's solution to a few crystals of glucose and boil. A red precipitate of copper (I) oxide is observed. A positive reaction is change of colour from blue to green or orange.

3). Reaction with Barfoed's Reagent

Principle: Barfoed's reagent is specifically used to distinguish simple sugar from complex sugars. The principle of this test is similar to Fehling's but the reagent is, instead of being strongly alkaline, is weakly acidic, (Cupric acetate dissolved in dilute acetic acid) and is reduced by monosaccharides but not by disaccharides. This reagent also reduces copper (II) oxide to copper (I) oxide giving a red precipitate on heating.

Procedure: Add few crystals of glucose into 5 ml of the reagent and boil. The experiment can be repeated using fructose and a complex sugar. A reddish brown precipitate indicates the presence of a reducing monosaccharide.

4). Benedict's test for Reducing Sugars

Principle: When Benedict's Solution reacts with a reducing sugar, the copper II ion will be reduced to copper I ion to give a reddish brown precipitation. This confirms the presence of a reducing sugar.

Benedicts modified the original Fehling's test to produce a single solution which is more convenient for tests, as well as more stable, than Fehling's reagent.

Procedure: Add five drops of the test solution to 2 ml of Benedict's solution and place in a boiling water bath for 5 min. examine the sensitivity of Benedict's test using increasing dilutions of glucose.

5). Iodine test for Polysaccharides

Principle: Iodine forms coloured adsorption complexes with polysaccharides; starch gives a blue colour with iodine while glycogen and partially hydrolysed starch react to form red-brown colours.

Procedure: Acidify the test solution with dilute HCl, then add two drops of iodine, and compare the colours obtained with that of water and iodine.

2.3.3 Quantitative determination of carbohydrates

1). Estimation of carbohydrates by the Anthrone method

Principle: The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses. Aldopentoses and hexuronic acids, either free or present in polysaccharides. The blue-green solution shows an absorption maximum at 620 nm, although some carbohydrates may give other colour. The absorbance depends on the compound investigated, but is constant for a particular molecule.

Procedure: Add 4 ml of the anthrone reagents to 1 ml of a protein-free carbohydrate solution and rapidly mix. Place the tubes in a boiling water bath for 10 min with a marble on top to prevent loss of water by evaporation, cool and read the absorbance at 620 nm against a reagent blank. Prepare standard curves for the glucose and glycogen solutions and compare them. Remember that glucose exists as the glycoside form ($C_6H_{10}O_5$) in glycogen of mol wt. 162, not 180.

2.) The determination of Glucose Concentration using Glucose Oxidase Method

Principle: Glucose oxidase is an enzyme found in the growth medium of *Penicillin notatum* and catalyses the oxidation of β-D-glucopyranose to D-glucono-1,5-lactone with the formation of hydrogen peroxide; the lactone is then slowly hydrolysed to D-gluconic acid. The enzyme is specific for β-D-glucopyranose, but most enzyme preparations contain mutarotase, which catalyses the interconversion of the α and β forms since the exists in a solution of glucose. Peroxidase is incorporated into the reaction mixture and catalyses the reaction of hydrogen

peroxide which produced oxygen. The oxygen produced reacts with a chromogen that produces a colour where intensity is directly proportional to the glucose concentration in the test sample. The absorbance of the mixture is measured 625 nm using Ortho-tolidine as the colour reagent (chromogen). Only α –isomer of glucose gives this reaction thus confirming its specificity.

Procedure: Pipette into ten test tubes 1ml each of a serial dilution of standard glucose solution in the range of 0.1mg/ml to 1.0mg/ml. Add also 1ml of glucose sample solution and 5ml of combined O-tolidine reagent into each of the ten test tubes. Allow the mixture to develop colour by standing for 8-10 min and read the absorbance of each at 625 nm against distilled water blank. The combined O-tolidine reagent contains 1 ml of 1mg/ml peroxidase enzyme, 1ml of 190 IU/ml oxidase, 150ml Acetate buffer and 1ml O-tolidine.

3). Quantitative Estimation of Reducing Sugars by the Dinitrosalicylate Method

Principle: Dinitrosalicylate [3,5-dinitrosalicylic acid (DNS)] is reduced at alkaline pH at 100^{o}C to 3-amino-5-nitrosalicylate by sugars containing aldehyde or ketone groups; the reduced DNS is reddish brown and can be estimated colorimetrically.

The chemistry of the reaction is complicated since standard curves do not always go through the origin and different sugars give different colour yields. The method is therefore not suitable for the determination of a complex mixture of reducing sugars.

Procedure: Prepare the DNS reagent just before use by mixing the stock solutions (1g/l solution of glucose, fructose and maltose in saturated benzoic acid) and add 1 ml of the reagents to 3 ml of the sugar solution in a test tube. Prepare a blank by adding 1 ml of the reagent to 3 ml of distilled water. Cover each tube with a marble and place in a boiling water bath for 5 min, cool to room temperature and read the absorbance at 540 nm against the blank. Note that all the tubes

must be cooled to room temperature before reading since the absorbance is sensitive to temperature. Prepare standard curves of the sugars provided and use them to estimate the concentration of the 'unknowns' provided. Comment on the result.

2.3.4 Thin Layer of Chromatographic Separation of Sugar

Chromatographic techniques employ mild conditions and separate molecules on the bases of differences of size, shape, mass, charge, solubility and adsorption properties. There are many different types of chromatography but they all involve interactions between three components: the mixture to be separated, a solid phase and a solvent. Sugars can be separated by thin layer chromatography (TLC) on silica gel G. the TLC plates are activated by heating at 105^{o}C for 30 min just before use.

Principle: Separation of compounds on a thin layer is similar to that of paper chromatography only that Silica gel G is the ideal support medium instead of cellulose that is used in paper chromatography, but has added advantage that a variety of supporting media can be used so that separation can be by adsorption, ion exchange, partition chromatography, or gel filtration depending on the nature of the medium employed. The method is very rapid and many separations can be completed in under an hour.

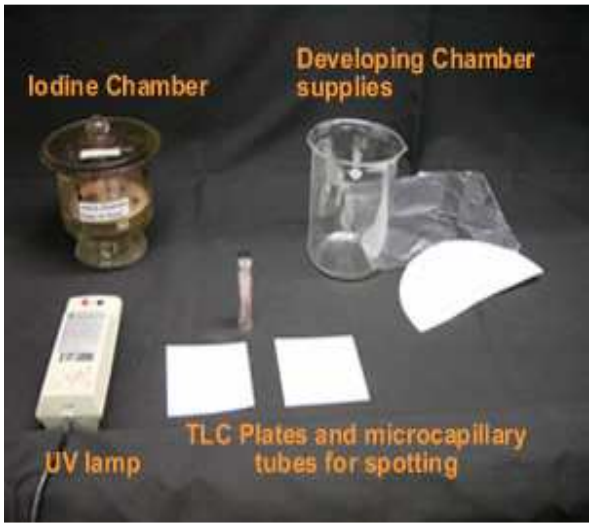


Plate 2.1: Apparatus required for Thin Layer Chromatography Technique

Adapted:http://www.wellesley.edu/Chemistry/chem211lab/Orgo_Lab_Manual/Appendix/Techniques/TLC/thin_layer_chrom.html#

Procedure: Add 3 ml of ethanol to 1 ml of fruit juice and centrifuge to remove denatured protein. Carefully spot the supernatant on to a thin layer plate together with some standard sugar solutions. Place the plate in a chamber saturated with solvent and develop the chromatogram until the solvent front is close to the top of the plate. Draw a line across the plate at this point and remove the chromatogram when the solvent reaches the mark. Dry the plate in a stream of cold air and locate the sugars by spraying the plates with freshly prepared aniline-diphenylamine in a fume chamber and heating briefly at 100^{o}C. Note the colour of each sugar and use this and the R_f value to identify the sugars present in the carbohydrate fruit juice.

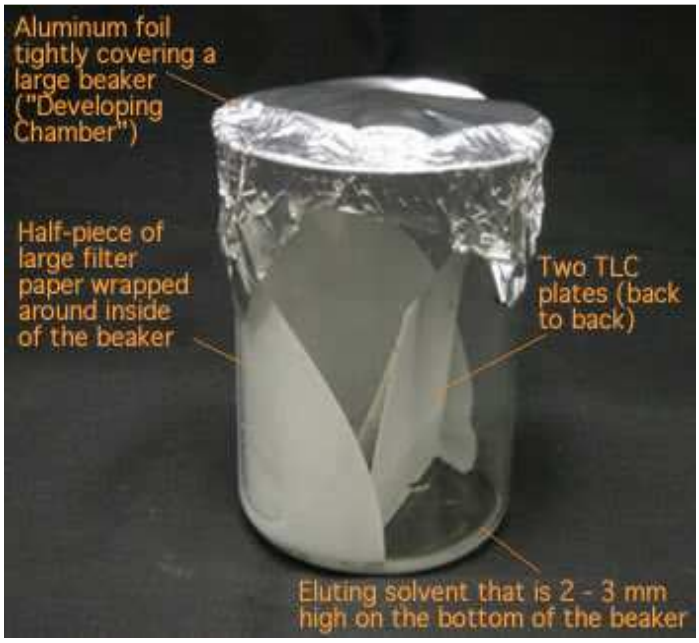


Plate 2.2: Plate Showing TLC Developing Tank

Adapted:http://www.wellesley.edu/Chemistry/chem211lab/Orgo_Lab_Manual/Appendix/Techniques/TLC/thin_layer_chrom.html#

After the solvent has risen to near the top of the plate (about 1 cm from the top), remove the plate and mark the solvent front with a pencil. Keep the plates in the hood until the majority of the eluting solvent has evaporated from the plates. Examine the plate under UV light to see the components as dark spots against a bright green-blue background.

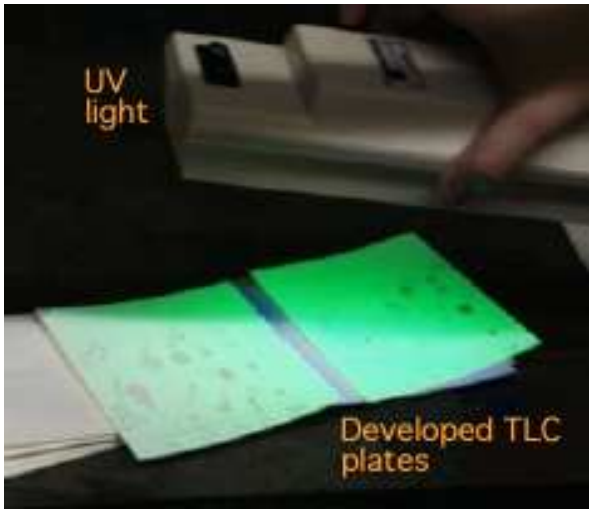
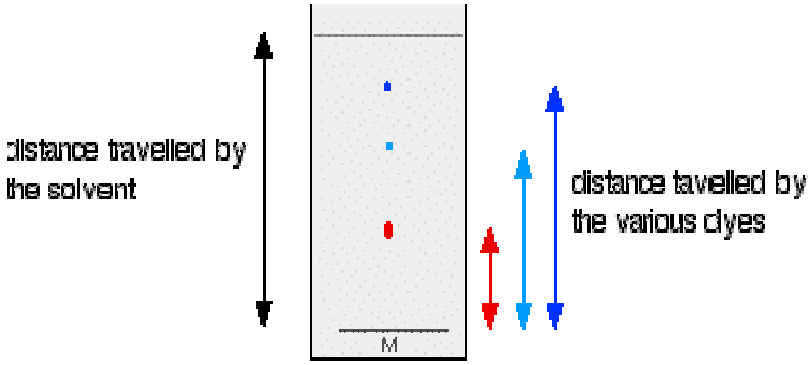


Plate 2.3: Developed Plate under UV light to see the components as dark spots against a bright green-blue background.

Adapted:http://www.wellesley.edu/Chemistry/chem211lab/Orgo_Lab_Manual/Appendix/Techniques/TLC/thin_layer_chrom.html#

Measuring R_f values

If all you wanted to know is how many different sugars made up the mixture, you could just stop there. However, measurements are often taken from the plate in order to help identify the compounds present. These measurements are the distance travelled by the solvent, and the distance travelled by individual spots. When the solvent front gets close to the top of the plate, the plate is removed from the beaker and the position of the solvent is marked with another line before it has a chance to evaporate. These measurements are then taken:



Source: www.chemguide.co.uk/.../thinlayer.html

2.4 Conclusion

Carbohydrates are one of the most important components in many foods and they have characteristics test that can distinguish them from other macromolecules and to separate them from one another.

2.5 Summary

In this unit, we have discussed:

1. Various reactions of carbohydrates for their identification and quantification
2. How thin layer chromatography can be used for separation of sugars

2.6 Tutor-marked Assignments

1). List the common uses of Thin-Layer Chromatography

2). What are the different classes of carbohydrates?

3.) What are the differences between simple and complex sugars?

2.7 References/Further Readings

Plummer, T.D. (1987). An Introduction to Practical Biochemistry. 3rd edition. McGraw-Hill, England. Pp 166-188.

Amadi, B.A., Agomuo, E.N., and Ibegbulem, C.O. (2004). Research Methods in Biochemistry. Supreme, Owerri.

Adapted from Mohrig, 1st ed., pp. 151-162.): Thin Layer Chromatography. Retrieved: http://www.wellesley.edu/Chemistry/chem211lab/Orgo_Lab_Manual/Appendix/Techniques/TLC/thin_layer_chrom.html#

Jim Clark (2007). Thin Layer Chromatography. Retrieved: http:// www.chemguide.co.uk/analysis/chromatography/thinlayer.html

UNIT 3 ESTIMATION OF GLUCOSE IN BIOLOGICAL FLUID (BLOOD AND URINE)

CONTENTS

3.1 Introduction

Diabetics cannot regulate their blood sugar levels because of failure of their pancreas to produce sufficient insulin. The blood sugar level is the amount of glucose (sugar) in the blood expressed as millimoles per litre (mmol/l).blood glucose should have a range of 4-8mmol/l. Therefore testing for blood glucose levels is an important daily routine for diabetics.

3.2 Objective

Upon completion of studying this unit, you should be able to understand:

1. The procedures for the estimation of blood and urine glucose
2. The importance of testing blood glucose level for diabetics

3.3 Glucose in the blood

Glucose passes into the blood from the gut and liver, and is taken up by the tissues, including the liver. Levels of blood glucose are sensitively regulated by hormones and in many diseases level of glucose alters. Estimation of blood glucose is a very important technique in clinical biochemistry, and in research in biochemistry of hormones.

3.3.1 Estimation of Glucose by Folin-Wu Method

Principle: the glucose is oxidized by alkaline Cu^{++}, (as in Benedict's test), and Cu_2O is formed. Cuprous oxide is re-oxidized by phosphomolybdic acid which is reduced to the dark blue phosphomolybdous acid. The concentration of phosphomolybdous acid, which is proportional to concentration of glucose (provided alkaline Cu^{++} and phosphomolybdic acid are present in excess), is measured colorimetrically.

Materials:

Procedure: Take three Folin-Wu tubes and a boiling tube.

a). To each of 2 F-W tubes, add 2 ml of protein free filtrate. To the third, add 2 ml of standard glucose. To a boiling tube, add 2 ml of water (for water blank).

b). To each tube add 2 ml of alkaline copper swirl and place tubes in a boiling water bath for exactly 8 min. transfer to a cold water bath or beaker. Leave for 5 min.

c). To each tube add 2 ml of phosphomolybdic acid and mix well. Leave for 3 min and dilute to 25 ml with distilled water. Mix well. For blank, dilute to 25 ml in a volumetric flask.

3.3.2 Estimation of Sugar in Urine by Benedict's Titration

It is sometimes necessary to estimate sugar in urine; but urine contains traces of phenols and uric acid which can interfere with sensitive colorimetric or iodometric methods.

Principle: Benedict's quantitative reagent contains cupric ions, citrate, potassium thiocyanate and potassium ferrocyanide. On heating with reducing sugars, Cu^{++} (blue) is reduced to Cu^{+} which precipitates as white cuprous thiocyanate, the ferrocyanide helps prevent deposition of Cu_2O. The extent of reduction depends on the sugar, and the conditions under which the titration is carried out.

Procedure:

1. Check the approximate concentration of glucose in the urine sample with Clinistix. This also identifies the sugar as glucose, since the test is highly specific.

2. If the sugar content is 0.5% or below from the Clinistix test, carryout the titration with undiluted urine. If it is between 1 and 2% dilute the urine 25 ml to 100 ml final volume if above 2%, dilute the urine 10 ml to 100 ml final volume.

3. The urine diluted as necessary is placed in a 5o ml burette and the volume adjusted to the zero mark. 25 ml of the Benedict's reagent is measured with a pipette into a porcelain evaporation dish, 20 g of crystallized sodium carbonate (or one-half the weight of anhydrous salt) is added, together with a small quantity of powdered pumice stone or anti-bumping granules, and the mixture heated to boiling over a free flame and stirred with a glass rod to aid in dissolving the bulk of the carbonate. The diluted urine is now run in from the burette rather rapidly, until a chalk-white precipitate forms in noticeable amount and the blue colour of the mixture begins to lessen perceptibly; after which the solution from the burette must be run in a few drops at a time, until the disappearance of the last trace of blue colour, which marks the end point. The final colour at the end point may be yellow or brown, owing to urinary pigments, but there should be no trace of blue (or green) colour. The solution must be kept vigorously boiling and stirred continuously throughout the entire titration. If the mixture becomes too concentrated

during the process, water may be added from time to time to replace the volume lost by evaporation.

Calculation:

The 25 ml of copper solution is reduced by exactly 50 mg of glucose. Therefore, the volume of urine run out of the burette to effect the reduction contained 50 mg of glucose. The formula for calculating the percentage of the sugar is as follows:

$$\frac{0.050}{y} \times D \times 100 = \text{Per cent glucose in original sample}$$

Where y is the number of ml of the diluted urine required to reduce 25 ml of the copper solution, and D is the dilution of the urine (D equals 1 for undiluted urine, 10 for urine diluted 1:10, etc.).

Carry out two more titrations, first adjusting the dilution of the urine sample, if necessary, to give a titre of order of 10-15 ml, e.g if you used urine diluted 25 ml to 100 ml , and got a titre of 7.5 ml urine, repeat the titrations using urine diluted 12.5 ml to 100 ml.

3.4 Conclusion

Levels of blood glucose are sensitively regulated by hormones and in many diseases, level of glucose alters. Estimation of blood glucose is a very important technique in clinical biochemistry and in research in biochemistry of hormones for diagnostic purposes.

3.5 Summary

In this unit you have learnt:

1. The procedures in the estimation of blood and urine glucose
2. The importance of these analyses.

3.6 Tutor-marked Assignments

1. What is blood sugar level and list its importance?

2. What is (are) the disease(s) associated with changes in the blood sugar level from the normal level?

3.7 References/Further Readings

Plummer, T.D. (1987). An Introduction to Practical Biochemistry. 3rd edition. McGraw-Hill, England.

UNIT 4 ANALYSIS OF LIPIDS FOR DOUBLE BOND AND FREE FATTY ACIDS

CONTENTS

4.1 Introduction

Lipids are naturally occurring compounds that are esters of long chain fatty acids. The iodine value of a lipid sample gives information on the degree of unsaturation of the lipid.

4.2 Objectives

Upon completion of studying this unit, you should be able to:

1). The definition of Iodine number

2) The principle of determination of Iodine number of a fat/lipid

3) The procedure for determining Iodine value

4.3 Iodine Number

The iodine number of a lipid or fat sample is defined as the number of grams of iodine absorbed by 100 grams of lipid. As a molecule of iodine (I_2) adds across each of the double bonds of an unsaturated fatty acid, the iodine number gives a measure of the degree of unsaturation of the fatty acids of a lipid.

Although the basis of the determination of iodine number is the addition of iodine across double bonds of unsaturated fatty acids, other halogens can be used to measure the degree of unsaturation. In the Dam method, bromine is used in preference to iodine because bromine reacts more readily with the unsaturated bonds than does iodine.

Principle: This test is carried out to calculate the relative unsaturation of fats since halogens add across the double bonds of unsaturated fatty acids to form addition compounds. Iodine monochloride (ICl) reacts with the fat in the dark and the iodine released (after adding potassium iodide (KI)) is titrated with standard thiosulphate to determine the amount of iodine consumed which will be compared with blank (without fat).

$CH_2{=}CH_2 + ICl \rightarrow CH_2I\ CH_2Cl$

$ICl + KI \rightarrow HCl + I_2$

Procedure: Pipette 10ml of the fat solution into a stoppered bottle; add 25ml of the ICl solution, stopper the bottle, and leave to stand in the dark for 1 hour, after shaking thoroughly. At the same time, set up a blank in which the fat solution is replaced by 10 ml of chloroform. Rinse the stoppers and necks of the bottles with about 50 ml of water, add 10 ml of KI solution, and titrate the liberated iodine with the standard thiosulphate. When the solution is a pale straw colour, add about 1 ml starch solution and continue titrating until the blue colour disappears. The bottles must be shaken thoroughly throughout the titration to ensure that all the iodine is removed from the chloroform layer.

The difference between the blank and the test readings (BL - T) gives the number of ml of thiosulphate needed to react with the equivalent volume of iodine. This is (BL - T) / 2 ml of 0.1 mol/litre iodine since 2 molecules of thiosulphate are needed for each iodine.

Molecular weight of I_2 = 2 x 127

Therefore, the weight of iodine in (BL - T) / 2ml of 0.1 mol/litre of iodine is:

(BL - T) / 2 x 0.1 x 2 x 127/ 1000g

The amount of fat taken was 0.2g so the iodine number is

(BL - T) x 127/ 1000 x 100 / 0.2

Iodine number = (BL - T) x 6.35g per 100g of fat.

4.4 Conclusion

The iodine value of a lipid sample gives information on the degree of unsaturation of the lipid. The iodine number of a lipid or fat sample is the number of grams of iodine absorbed by 100 grams of lipid.

4.5 Summary

In this unit you have learnt:

1. The meaning of iodine number
2. The principle of the method for the determination of iodine number
3. The procedure for determining iodine number

4.6 Tutor-marked Assignments

What is the significant of iodine number in the shelf-life of oils and their use in soap making?

4.7 References/Further Readings

Plummer, T.D. (1987). An Introduction to Practical Biochemistry. 3rd edition. McGraw-Hill, England

Amadi, B.A., Agomuo, E.N., and Ibegbulem, C.O. (2004). Research Methods in Biochemistry. Supreme, Owerri.

UNIT 5 SEPARATION OF LIPIDS BY THIN LAYER CHROMATOGRAPHY

CONTENTS

5.1 Introduction

Thin layer chromatography (TLC) is the most used method, it is applied to separate the components of a sample mixture by using the whole range of fundamental processes in chromatography: adsorption, distribution, exclusion − diffusion and ion exchange.

5.2 Objective

Upon completion of studying this unit, you should be able to:

1). Know the technique of separating lipids using TLC

5.3 Main Body

TLC is used mainly to separate and determine the concentration of different types of lipid groups in foods, *e.g.* triacylglycerols, diacylglycerols, monoacylglycerols, cholesterol, cholesterol oxides and phospholipids. A TLC plate is coated with a suitable absorbing material and placed into an appropriate solvent. A small amount of the lipid sample to be analyzed is *spotted* onto the TLC plate. With time the solvent moves up the plate due to capillary forces and separates different lipid fractions on the basis of their affinity for the absorbing material. At the end of the

separation the plate is sprayed with a dye so as to make the spots visible. By comparing the distance that the spots move with standards of known composition it is possible to identify the lipids present. Spots can be scraped off and analyzed further using techniques, such as GC, NMR or mass spectrometry. This procedure is inexpensive and allows rapid analysis of lipids in fatty foods.

Thin layer chromatography (TLC) is one type of chromatography; the stationary phase is a thin layer of adsorbent particles attached to a solid plate. A small amount of sample is applied (spotted) near the bottom of the plate and the plate is placed in the mobile phase. This solvent is drawn up by capillary action. Separation occurs as each component, being different in chemical and physical composition, interacts with the stationary and mobile phases to a different degree creating the individual bands on the plate. TLC can be utilized to identify the different lipids.

Principle: Lipids in biological material are present as a complex mixture and are first fractionated into a number of groups by solvent extraction. Resolution of the compounds within each group can then be carried out by thin layer chromatography. The solvent is selected according to the lipids under investigation, but, generally, neutral lipids are separated with non-polar solvents and charge lipids with polar solvents. A number of supporting media can be used for lipid fractionation and the actual choice of material depends on the group of lipids and solvent employed. Silica gel has been widely used and separation with this material is on the basis of adsorption.

In the following experiments, the lipids are separated into groups according to their polarity and examples from each group are included as representative standards. Some naturally occurring oils and fats are examined for the presence of the classes of lipid considered

Procedure: Clean the glass plates with ethanol then pour aqueous slurry of the gel on to their surface 250 µm thick. Activate the plates by heating at 110^{o}C for 1 h and allow to cool. Spot out 20-50 µl of an approximately 1 per cent w/v solution of each lipid in the solvent (petroleum ether, b.p.60-70^{0}C: diethylether: glacial acetic acid; 80:20:1) and develop the chromatogram. Locate the lipids by spraying with the dichlorofluorescein solution and view the plates in ultraviolet light (270 nm). The lipids show up as green fluorescent spots against a dark background. Alternatively, the spots can be visualized by spraying the plates with 50 per cent v/v sulphuric acid followed by heating in the oven at 110^{0}C for 10 min.

5.4 Conclusion

Lipids in biological material are present as a complex mixture and are first fractionated into a number of groups by solvent extraction. Resolution of the compounds within each group can then be carried out by thin layer chromatography. The lipids are separated into groups according to their polarity

5.5 Summary

In this unit you have learnt:

- The principle of TLC and how to separate lipids using TLC

5.6 Tutor Marked Assignment

- List the different types of lipids
- What is the difference between oils and fats

5.7 References/Further Readings

LAB CHROM 7 TLC Separation of Amino Acids Adapted from *Laboratory Experiments for Organic and Biochemistry*. Bettelheim & Landesberg (PA Standards for Sci & Tech 3.1.12.D; 3.4.10.A; 3.7.12.B) Retrieved January, 2011.

Hossu, A., Mihaela − Flory, M., Radulescu, C., Mihaela I., Magearu, V., TLC 'Applications on separation and quantification of fat-soluble vitamins' Rom. Biotechnol. Lett., Vol. 14, No. 5, 4615-4619 (2009)

Smith, I. Chromatographic and Electrophoretic Techniques, 4th edn. Vol. 1. Chromatography. Heineman, London, 1976.

Wilson, K., 'Chromatographic Techniques' in A Biologists Guide to Principles and Techniques of Practical Biochemistry, K.H. Goulding (eds). 3rd edn. Arnold, London.

Plummer, D.T., An Introduction to Practical Biochemistry. 3rd edn. McGraw-Hill, London, 1987.

UNIT 6 SEPARATION AND PURIFICATION OF NUCLEIC ACIDS

CONTENTS

6.1 Introduction

Nucleic acids are nitrogen-containing compounds of high molecular weight found in association with proteins in the cell. The nucleic acid-protein complexes are known as nucleoproteins, and these can be separated into the component proteins and nucleic acids by treatment with acid or high salt concentration. The proteins are basic in character and the nucleic acids are acidic. Two main groups of nucleic acids are known, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

DNA is intimately associated with the genetic material of the cell. The main function of DNA is to act as a store of genetic information while both DNA and RNA act as the template for the

synthesis of proteins in the cell with a triplet of three bases providing the genetic code for each amino acid.

6.2 Objectives

After going through this unit and performing the experiments sets in it, you should be able to:

- Isolate DNA and RNA from different tissues
- To understand the importance of DNA and RNA as components of nucleic acid
- The functions of both DNA and RNA.

6.3 Main body

6.3.1 The Isolation of RNA from Yeast

Principle: Total yeast RNA is obtained by extracting a whole cell homogenate with phenol. The concentrated solution of phenol disrupts hydrogen bonding in the macromolecules, causing denaturation of the protein. The turbid suspension is centrifuged and two phases appear, the lower phenol phase contains protein, and the upper aqueous phase contains carbohydrate and RNA. Denatured protein, which is present in both phases, is removed by centrifugation. The RNA is then precipitated with alcohol. The product obtained is free of DNA but usually contaminated with polysaccharide. Further purification can be made by treating the preparation with amylase.

Procedure: Suspend 30 g of dried yeast in 120 ml of water previously heated to 37^{o}C. Leave for 15 min at this temperature and add 160 ml of concentrated phenol solution (Care: corrosive). Stir the suspension mechanically for 30 min at room temperature, then centrifuge at 3000 g for 15 min in the cold to break the emulsion. Carefully remove the upper aqueous layer with Pasteur pipette and centrifuge at 10 000 g for 5 min in a refrigerated centrifuge to sediment denatured

protein. Add potassium acetate to the supernatant to a final concentration of 20 g/litre and precipitate the RNA by adding 2 volumes of ethanol. Cool the solution in ice and leave to stand for 1 h. collect the precipitate by centrifuging at 2000 g for 5 min in the cold. Wash the RNA with ethanol-water (3:1), ethanol, and finally, ether,; air dry and weigh. (Note: Yeast contains about 4 per cent RNA by dry weight).

6.3.2 The Isolation of DNA from pig spleen

Principle: Almost all cells contain DNA, but the amount present in some tissues is quite small so that they are not a particularly convenient source. In addition, some tissues contain high deoxyribonuclease (DNase) activity so that the DNA is broken down to smaller fragments. A convenient source for the isolation of DNA should therefore contain a high quantity of the material and have low deoxyribonuclease activity. Lymphoid tissue is very good in these respects and thymus is the best source, with spleen as a good alternative.

DNA is readily denatured and extreme care must be taken in order to obtain a product that is structurally related to that found in the cell. Mechanical stress and extreme physical and chemical conditions must be avoided and nucleases must be inhibited. Sodium citrate is therefore present in the solution to bind Ca^{2+} and Mg^{2+} which are cofactors for DNase and the stages until the removal of protein are carried out as rapidly as possible in the cold.

The nucleoprotein is soluble in water and solutions of high ionic strength, but is insoluble solutions of low ionic strength (0.05-0.25 mol/litre) and use is made of this property in the initial extraction. The tissues is first homogenized in isotonic saline buffered with sodium citrate, pH 7, when most other macromolecules pass into solution, leaving the insoluble deoxyribonucleoprotein which is then dissolved in 2 mol/litre saline.

The protein is removed by treatment with a chloroform/amyl alcohol mixture and the DNA precipitated with ethanol. The product is the dissolved in dilute buffered saline stored frozen. It is stable in this form for several months.

Procedure

Chop 50g of pig spleen into a small fragment and homogenize with 200ml of buffered saline for 1 min. centrifuge the suspension at 500g for 15 min and rehomogenized precipitate in a further 200ml of buffered saline discharge supernatant and suspend the combined sediment uniformly in 2 mol/liter NaCl to a final volume of 1 liter, when most of the material should dissovlve. Remove and sediment by centrifugation and stir the solution continuously with a glass rod while adding an equal volume of distill water. Spool the fibrous precipitate on to a glass rod and leave it to stand in a beaker for 30 min. during the time the clot will shrink and the liquid expressed should be remove with filter paper deoxyribonucleoprotien in about 100 ml of 2 mol/liter NaCl, add and equal volume of the chloroform/amyl alcohol mixture (6:1) and blend for 30 s. centrifuge the emotion at 5000g for 10-15 min and collect the upper (opalescent) aqueous layer containing the DNA. This is best carried out by gentle suction into a suitable container so that the denatured protein at the interface of the two liquid is not disturbed. Repeat the treatment with organic solvent twice more and collect the supernatant into a 500 ml beaker.

Precipitate the DNA by slowly stirring 2 volume of ice-cold ethanol with the supernatant and collect the mass of fibres on the glass stirring rod. Carefully remove the rod and gently press the fibrous DNA against the side of the beaker to expel the solvent. Finally, press the precipitate by dipping the rod into a series of solvent and expelling the solvent as described. Four solvent are used: 70% v/v ethanol, 80%v/v ethanol, absolute ethanol and ether. Remove the last traces of either by standing the DNA in a fume cupboard for about 10 min. Weigh the dry DNA and

dissolve by continuously stirring in buffered saline diluted one in ten with distilled water (2 mg/ml), store frozen until required.

6.3.3 The estimation of DNA by the diphenylamine reaction

When the DNA is treated with diphenylamine under acid conditions, a blue compound is formed with a sharp absorption maximum at 595 nm. This reaction is giving by 2-deoxypentoses in general and is not specific for DNA. In acid solution the straight form of deoxypentose is converted to the highly reactive β-hydroxylevulinaldehyde which reacts with diphenylamine to give a blue complex. In DNA, only the deoxyribose of the purine nucleotides reacts, so that the value of obtain represents half of the total deoxyribose present.

Procedure

Dissolve 10 mg of the nucleic acid in 50 ml of buffered saline, remove 2 ml and add 4 ml of diphenylamine reagent heat on a boiling water bath for 10 min cool and read the extinction at 595 nm. Read the test and standards against water blank. Assay the isolated nucleic acids and the commercial samples for DNA.

6.3.4 The estimation RNA by means of the orcinol reaction

This is a general reaction for pentoses and depends on the formation of furfural when the pentose is heated with concentrated hydrochloric acid. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour. Only the purine nucleotide gives any significant reaction.

Procedure: Mix 2 ml of the nucleic acid solution with 3 ml of orcinol reagent. Heat on a boiling water bath for 20 min, cool and determine the extinction at 665 nm against an orcinol blank.

6.3.5 Estimation of DNA Concentrations

The determination of the concentration of DNA or RNA in solution is a fundamental task in molecular biology. DNA is usually the limiting reagent in most experiments; therefore, the knowledge of its concentration is critical. Determination of the DNA concentration can be estimated either by qualitatively comparing the fluorescence of DNA bands in an agarose gel to a standard or by spectrophotometric means.

6.3.5.1 Qualitative Estimation

DNA fluorescence in the presence of ethidium bromide, and the intensity of the fluorescence is proportional to its concentration. As such, comparison of relative fluorescence of unknown DNA to known standards can be used as a rough estimation of DNA concentration. This comparison is usually made following the electrophoresis of the standard (for example, 1 microgram of Lambda DNA cleaved with the restriction endonuclease HindIII) and the unknown. The mass of 1 microgram of a Lambda-HindIII standard provides convenient references for comparison. An alternative method for estimating DNA concentration relies on the lower limit for visually detecting DNA in a gel. It has been estimated that a band of DNA below 5 nanograms is not detectable by the human eye. Serially diluting DNA to extinction, followed by electrophoresis, can also be used to measure DNA concentration. This technique is particularly if there is more than one species or fragment of DNA in a sample. In this manner, the concentration of individual bands can be estimated.

6.3.5.2 Quantitative Estimation of DNA Concentrations

DNA, RNA, and protein strongly absorb ultraviolet light in the 260 to 280 nm range. UV spectroscopy can be used as a quantitative technique to measure nucleic acid concentration and protein contamination. Nucleic acids strongly absorb at 260 nm and less strongly at 280 nm

while proteins do the opposite. The general rules for determining the concentrations of nucleic acids at 260 nm are:

1. 1 Optical Density (OD) unit of double-stranded DNA is 50 micrograms/ml.
2. 1 OD unit of single-stranded DNA is 33 micrograms/ml.
3. 1 OD unit of single-stranded RNA is 40 micrograms/ml.

Proteins absorb strongly at 280 nm where 1 OD unit is 1 mg/ml. When using UV spectroscopy for estimating DNA concentrations, it is very important to remove all protein and RNA from the DNA solution. Good estimations can only be made on clean preparations. An estimate of the purity of a DNA preparation can be made by measuring the absorbance at both 260 nm and 280 nm. Pure solutions of nucleic acid will absorb approximately twice as much at 260 nm as at 280 nm. Experimentally, the ratio of 260 nm/280 nm of a pure DNA solution is between 1.8 and 2.0. As protein contamination increases, the ratio decreases. Additionally, the presence of contaminating organic solvents, such as phenol, can affect estimations of concentration and purity.

Materials:

UV Spectrophotometer,

Quartz or UV compatible cuvettes,

TE buffer, DNA template

Procedure:

1. Fill the cuvette with water or TE buffer. Zero the spectrophotometer at 260 nm with this blank.
2. DNA from plasmid and genomic preparations is typically at a concentration exceeding 1 micrograms/microliter. Consequently, DNA is usually diluted before measuring its

absorbance. An unfortunate result of this measurement is that the DNA is expended as a result of the dilution. Be sure this is adequate DNA to waste. Start by diluting the DNA sample 1 microliter : 999 microliters of TE buffer (the dilution can be done directly in the cuvette). Mix the dilution thoroughly.

3. Measure the optical density (OD). Multiply the resulting OD by 50 micrograms/ml. For a 1:1000 dilution, the mass of DNA is equal to micrograms/microliter.
4. Similarly, the same sample can be measured at 280 nm. A ratio of the OD-260nm/OD-280nm is an indicator of DNA purity. A ratio of 1.8 or higher indicates minimal protein contamination.

6.4 Conclusion

Nucleic acids are nitrogen-containing compounds of high molecular weight found in association with proteins in the cell. The main function of DNA is to act as a store of genetic information while both DNA and RNA act as the template for the synthesis of proteins in the cell with a triplet of three bases providing the genetic code for each amino acid.

6.5 Summary

In this unit you have learnt:

- The importance of DNA and RNA as components of nucleic acid
- How to isolate DNA and RNA from different tissues
- How to estimate DNA and RNA both qualitatively and quantitatively
- The functions of both DNA and RNA.

6.6 Tutor-marked Assignments

- List the functions of DNA and RNA

- List some disease associated with nucleic acid

6.7 References/Further Readings

Plummer, D.T. (1987). An Introduction to Practical Biochemistry.(3rd ed)McGraw-Hill, London.

Methods in biotechnology (2009): http://biotechmethods.blogspot.com

UNIT SEVEN ESTIMATION OF PHOSPHATE AND TITRATABLE ACIDITY CONTENT

7.1 Introduction

Unlike dyes, most biochemical compound are colourless and can only be analyzed colorimetrically after reacting them with a specific chemical reagent to give a colour product. This point is illustrated in the measurement of inorganic phosphate, which is probably one of the commonest determinations carried out in a biochemical laboratory.

Urine contains wide variety of compound, and analysis of urine is an important method for investigating metabolism for both health and disease. This experiment focuses on how the body gets rid of excess acid. It show how careful analysis, and application of the principle of pH and buffer, can be used to obtain information on this.

7.2 Objective

After studying this unit, you should be able to:

1. How the body gets rid of excess acid

2. How to determine phosphate and titrable acidity in urine

7.3 Phosphate and titrable acidity in urine

The body normally needs to excrete acid because some metabolic products (carbon dioxide and ketone body) and also some food are acidic. The kidney normally allow excretion of urine of a higher concentration of free hydrogen ions (H^+) than that found in blood, and the pH of urine can be low as 4.5 although the pH of the blood is around 7.4. At pH of 4.5, urine contains H^+ at a concentration of only 10 - 4.5M, or 3x 10 – 5M, so H^+ will not be removed very satisfactorily from the body as free H^+ ions. On lowering the pH from 7.4 to 4.5 H^+ can combine any weak acids present, which have pka within this range, e.g

$HPO_4 = H_2PO_4^-$ pk 6.8

At pH 7.4, almost 75% of phosphate is in the form HPO_4 and 25% is $H_2PO_4^-$ but at pH 4.5 virtually all the phosphate is as $H_2PO_4^-$ urine and titrating it until the pH is again blood pH 7.4, the amount of titrable acidity in urine is determined.

Not all the titrable acidity is due to phosphate: other compound (e.g B-hydroxy butyric acid) can combine with significantly more H^+ at pH 4.5 than at 7.4; but for ketone bodies it is rather small because both acetacetatic and B-hydroxy butyric acid have pk below 4.5.

Not all excreted acidity is in fact “titrable acidity”. The kidney excretes some NH_4^+ ions, the amount of which rises in acidosis and each of these carries an H+ ion, which cannot be dissociated by the pH to 7.4, since the pk ammonia is above 9.

Titratable acidity is a total amount of acid in the solution as determined by the titratable using a standard solution of sodium hydroxide (titrant). The reaction’s completion is determined by a chemical indicator that changes its colour at this point.

Titratable acid is a term to describe acid such as phosphoric acid, sulfuric which are involve in renal physiology. It is used to explicitly excluded ammonium (NH_4^+) as a source of acid, and is part of the calculation for net acid excretion.

Method:

i). Measure the pH of sample accurately (using pH-meter). This will free acidity.

ii). Estimate phosphate content

iii). Measure titratable acidity.

1. **Measurement of pH:** Measure the pH of the sample accurately using pH-meter. For rough indication of pH, indicator-papers (pH-papers) can be used. These usually come with a colour-chart, so that after moistening the paper with the test solution you may match its colour against the chart to find out the pH.

2. **Measurement of Phosphate:**

Principle: Phosphate will be estimated by treating with molybdic acid which is then reduced by ascorbate to give a blue compound. The intensity of the blue colour is measured by the use of a colorimeter. Inorganic phosphate reacts with ammonia molybdate in an acid solution to form phosphomolybdic acid. Addition of a reducing agent reduces the molybdenum in the phosphomolybdate to give a blue colour, but does not affect the uncombined molybdic acid. In this method the reducing agent used is p-methyl aminophenol sulphate. The presence of copper in the buffer solution increases the rate at which the colour develops.

The principle of a calibration-curve is that known amounts of pure substance being assayed are treated, and then the readings taken. The known quantity of the substance is then plotted along

X-axis (abscissa), while the readings are plotted on the Y-axis (ordinate). The points obtained are then joined by straight line of smooth curve, as appropriate.

If the solution to be assayed contain phosphate, but of unknown concentration is put through exactly the same procedure as the standard solutions, then the reading obtained for it can be converted into quantity by reading across and then down from the graph directly.

Method: Dilute 1ml of urine to 100 ml with distilled water, and mix. Pipette 1ml of the diluted urine into a clean test tube, and 2ml into a second test tube. Pipette 1ml of standard phosphate (containing 5 µg of phosphorous per ml) into two other clean test tubes. Add water to each to bring volume to 4.2ml, and mix; then add 4.2ml of distilled water to a fifth tube (for use as a blank). From a burette, add 0.8ml of molybdic + ascorbic acid to each tube. Mix again; allow standing for 30 mins, and then reading the optical density (absorbance) of the first 4 tubes against the fifth as blank, at wavelength of 700 nm.

iii). **Titratable Acidity:** As explained earlier, that in order to obtain the excreted "titratable acidity", the amount of alkali needed to be added to an aliquot of urine to bring the pH to 7.4 should be determined, using a pH meter. However, in practice, it is reasonably satisfactory to use phenolphthalein as indicator, which gives an end point (faint pink) at pH 8.5-9. It is most important to stop the titration as soon as the end point is reached, otherwise ammonia ions will be titrated as well. However, this means that the phosphate present is titrated until it is 100 % HPO_4 instead of just 75% of it.

Methods: Place 20ml urine in a clinical flask and add 5 drops of phenolphthalein. Fill a burette with standardized NaOH (appropriately 100 nM). Titrate until the phenolphthalein just begins to produce a persistent pink colour. (Note: the actual colour-change may be difficult to detect,

because of the colour of the urine itself. Pipette 20ml of urine into another conical flask of the same size and use this for comparison, viewing both against a clean white background). Repeat until you are confident of the titratable acidity to ± 0.1ml.

7.4 Conclusion

This unit has described how the body gets rid of excess acid and we learnt that, urine contains wide variety of compound, and analysis of urine is an important method for investigating metabolism for both health and disease.

7.5 Summary

In this unit you have learnt:

1. The importance of phosphate and titrable acidity in urine
2. How to determine phosphate and titrable acidity in urine.
3. How much of titratable acidity is due to phosphate ($H_2PO_4^-$)

7.6 Tutor marked assignment

1. If the pH of urine is 5.2, the pka of phosphate (6.8); calculate from the Henderson-Hasselbalch equation the ratio of HPO_4 to H_2PO_4 in the urine.

$$pH \text{ (of urine)} = \log \frac{[HPO_4]}{[H_2PO_4]}$$

2. From the ratio of HPO_4/ H_2PO_4 in total concentration of HPO_4 = H_2PO_4 in urine' calculate concentration in the urine of HPO_4 and of H_2PO_4 in millimolar units.

7.7 Reference/Further Reading

Plummer, D.T. (1987). An Introduction to Practical Biochemistry.(3rd ed)McGraw-Hill, London.

White, Handler and Smith. Principles of Biochemistry (4th Ed.). chap. 33