UNIVERSITY OF NAIROBI

SCHOOL OF MEDICINE

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL FOR FIRST YEAR STUDENTS OF B.SC. NURSING

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DIRECTIONS FOR PRACTICAL LABORATORY CLASSES AND GENERAL INSTRUCTIONS

Before starting any experiment the student should read the instructions carefully, paying attention to all details, and should be quite certain about what he is trying to do. In the case of the qualitative tests, the quantities recommended proportions of reagents and test materials may invalidate the test. In the quantitative experiments, the routine must be rigidly followed and quantities measured accurately. For this purpose a measuring cylinder is usually quite inadequate because the graduations near the base may be very inaccurate. Accurate measurements of volume may be made by either burette or pipette. These are most accurate when measurements are made between two calibration marks.

The results of an experiment and the conclusions to be drawn from it should be written in ink, in your practical notebook, as soon as possible after the conclusion of the experiment. The section on results should include a full account of all observations. If you are in doubt about the significance of your findings, you should discuss them with your demonstrator.

LABORATORY RULES

1. Do not replace any solution in a reagent bottle, take only the minimum amount required.
2. Replace the stopper immediately a solution has been taken from a bottle and take care not to mix stoppers.
3. Do not put pipettes into bottles of quantitative reagents
4. Do not remove a bottle of special reagents from side shelves to your own benches
5. Never pipette a corrosive fluid, always use a measuring cylinder or burette
6. Good results are only obtained with clean apparatus: many erroneous results are due to dirty apparatus.
7. When you have finished work, leave your bench clean and dry, just as you should expect to find it. See that all waste materials is put in the waste boxes provided and not into sinks.

USE OF LAB EQUIPMENT FOR VOLUMETRIC AND GRAVIMETRIC METHODS

Volumetric Flasks: These are calibrated to contain a given volume at a specified temperature usually 20º or 25ºC.

Pipettes: There are three basic types of pipettes used in biochemical work. These are

Transfer Pipettes: (This type has a bulb in the middle). This is the most accurate type and is calibrated to deliver a certain volume. Such a pipette must be clean to deliver accurately. A test of cleanliness is to allow water to run out of it. If it drains smoothly, leaving no adhering drops within, it is satisfactory. If drops remain behind the pipette obviously it will not deliver the indicated volume.
There is a convention with regard to the use of the transfer pipette which must be observed for accurate delivery. The pipette is filled to about an inch above the calibration mark, the upper end

being held closed with the forefinger. The solution is allowed to escape, until the bottom of the meniscus is level with the mark, which should be at eye level. The tip is then touched for a second or two with inner side of a clean beaker or test tube (not receiving vessel). The solution is then allowed to flow into the receiving vessel by gravity until flow ceases. The transfer is then complete, although you will note that a small column of liquid still remains in the trip of the pipette. Do not blow this out into receiving vessel. The pipette is calibrated to deliver the specified volume if the above convention is observed in completing and delivery.

BURETTES

Macroburettes

Fifty ml burettes are graduated to 0.1 ml but the levels of liquid can be estimated to 0.01 ml. A burette must be absolutely clean to deliver accurately and must first be tested with water to make certain it drains without leaving droplets behing. Be sure the burrettes do not leak when the stopcock is closed. Before performing titration ensure that there are no air bubbles trapped, especially near the stopcock or the drawn-out tip. Bubbles usually can be removed by allowing one or two ml to flow out. In reading a burette be sure your eye is on the same level as the meniscus to avoid parallax errors. If the burette is allowed to drain with stopcock open the film of solution remaining on the inner surface will not drain as fast as the level of the solution and a large error can ensure unless one waits for a few minutes after closing the stopcock before making a reading, to allow this film to come into equilibrium with the column through surface tension effects. This is very important as many burettes are marked with a minimum emptying time which must be observed to get accurate readings. It is therefore advisable to allow drainage during a titration to occur at a rate considerably less than “wide open” to minimize such drainage errors.

For your guidance one drop of an aqueous solution delivered from a typical burette is equivalent to about 0.05 ml. Fractions of a drop are easily delivered by touching tip of burette to inner side receiving vessel.

Semi-microburettes

These are frequently used in biochemical analysis. Microburettes, have a total volume of 1.0, 2.0 or 5.0 ml and are graduated usually to 0.02 ml. They are used in the same manner as macroburettes, but drainage errors become much more serious. Remember that a single drop of an aqueous reagent may have a volume of about 0.05 ml. This usually loaded from a permanently connected reservoir of reagent by means of pressure from a hand-bulb.

Cleaning Glassware

Cleanliness of glassware is essential in all chemical manipulations, particularly in analytical work. Each student will clean his own equipment, and whenever possible this should be done immediately after use. If precipitates and solutions are allowed to dry on the surface of glassware, the latter is much more difficult to clean. This is particularly true of dried deposits of fats, proteins, tissues etc.

The best general cleanser for laboratory glassware is soap and warm water applied with still brush. Caution should be taken to avoid scratching the glassware with the metal handles of brushes. There are a wide variety of brushes available for different kinds of apparatus. Thorough rinsing with distilled water is required to obtain chemically clean glassware. Cleaning of burettes and pipettes requires special attention since improper drainage of these due to greasy surface introduces serious errors in quantitative work. A general test for cleanliness of burettes and pipettes is to fill them with H_2O and allow them to drain at a normal rate. If any visible drops or rivulets of H_2O are left behind on the surface of the glass then the surface is not clean enough to drain reproducibly. If pipettes are clean to begin with, they may require only copious rinsing with tap water and then distilled water immediately after their use to preserve cleanliness. After pipetting biological materials such as blood, immediately rinse the pipette with water. Further cleansing with soap may be necessary.

For stubborn cases, a detergent will be available. However, do not soak calibrated glassware in strong detergent solution for long periods since such solutions are alkaline and will etch the glass. Thorough rinsing is particularly important with glassware that is to be used in enzyme experiments, since many enzymes are strongly inhibited by detergents. Pipettes and burettes that cannot be cleaned satisfactorily with soap and water can be exchanged at the storeroom.

VOLUMETRIC ANALYSIS AND STOICHIOMETRIC CALCULATIONS

The concentrations of solutions are usually expressed in terms of molarity or normality. A molar solution contains one gram-equivalent weight of the substance in one litre of the solution. Example: The molecular weight of NaOH is 40.01. A molar solution of NaOH contains NaOH ntains 40.01g of NaOH per litre.

A normal solution contains one-gram equivalent weight of the substance in one litre of solution. The equivalent weight of the substance depends on the nature of the reaction for which the solution is used. In acid-base reaction which yields or combines with exactly 1.0080 grams H^+ (the weight of one gram ion of H). This process of quantitative combination, leading to neutralization of solution is called titration. Examples: The molecular weight of NaOH is 40.01. One molecule of NaOH can combine with one of H^+(H^+ + OH^-), hence the equivalent weight of NaOH is 40.01 g and a normal solution of NaOH has the same concentration as a molar solution. HCl has a molecular weight of 36.47. A normal solution of HCl contains 36.47 g HCl per litre of the solution since one molecule of HCl yields one H^+. However, sulphuric acid contains two ionizable hydrogen atoms. The equivalent weight of H_2SO_4 is therefore the gram-molecular weight divided by 2 or 98.08 = 49.08g and a normal solution for H_2SO_4 therefore contains 49.04g /litre of H_2SO_4.

Other methods of expressing concentration

A molal solution is one containing 1.00 grams molecular weight of a solute dissolved in 100 gm of solvent (note the distinction between a molar and molal solution). Molality is properly used in calculation involving the colligative properties, such as osmotic pressure, freezing and boiling point depression and evaluation, and certain other thermodynamic properties of water and other solvents. Percent weight indicated grams of solute in 100 g of solution (w/w). This is the

expression used for commercial available solutions. Percent volume indicates grams of solute in 100 mls of solution. This is by far the more commonly used expression involving per cent (w/w). Volume per cent. This expression is commonly used to indicate concentration of a solution of the solute in a liquid. 50% alcohol for example means we have 50 mls in 100 ml solution.

PRACTICAL ONE

THE BEER-LAMBERT'S LAW

Many biochemical experiments involve the measurement of a compound or group of compounds present in a complex mixture. Probably the most widely used method for determining the concentration of biochemical compounds is colorimetry, which makes use of the property that when white light passes through a coloured solution, some wavelengths are absorbed more than others. Many compounds are not themselves coloured, but can be made to absorb light in the visible region by reaction with suitable reagents. These reactions are often fairly specific and in most cases very sensitive, so that quantitities of material in the region of millimole per litre concentrations can be measured. The big advantage is that complete isolation of the compound is not necessary and the constituents of a complex mixture such as blood can be determined after little treatment. As discussed below, the depth of colour is proportional to the concentration of the compound being measured, while the amount of light absorbed is proportional to the intensity of the colour and hence the concentration.

The Beer-Lambert Law

When a ray of monochromatic light of initial intensity Io passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light I is less than Io. There is some loss of light intensity from scattering by particles in the solution and reflection at the interfaces, but mainly from absorption by the solution. The relationship between I and Io depends on the path length of the absorbing medium, I and the concentration of the absorbing solution, c. These factors are related in the laws of Lambert and Beer.

Lambert's Law: It states that when a ray of monochromatic light passes through an absorbing medium its intensity decreases exponentially as the length of the absorbing medium.

Beer's Law. It states that when a ray of monochromatic light passes through an absorbing medium its intensity decreases exponentially as the concentration of the absorbing medium increases. These two laws are combined together in the Beer-Lambert law:

$$I = I_o e^{-k3cl}$$

Transmittance

The ratio of intensities is known as the Transmittance (T) and this is usually expressed as a percentage.

$$\text{Per cent T} = \frac{I}{I_o} x100 = e^{-k3cl}$$

This is not very convenient since a plot of per cent transmittance against concentration gives a negative curve

Extinction: If negative logarithms are taken on both sides of the equation instead of a ratio then:

$$\log_e {I_o}/{I} = k_3 cl$$

$$\log_{10} {I_o}/{I} = {k_3 cl}/{2.303}$$

$$\log_{10} {I_o}/{I} = kcl$$

The expression $\log_{10} {I_o}/{I}$ is known as the Absorbance (A). The absorbance is sometimes referred to as the optical density or extinction, but this name is no longer recommended.

Therefore,

$A = k_3 cl$

If the Beer-Lambert law is obeyed and is kept constant, then a plot of extinction against concentration gives a straight line passing through the origin which is far more convenient than the curve for transmittance.

DEMONSTRATIONS OF BEER'S LAW USING BROMOPHENOL BLUE AND METHYL ORANGE

Principle

Coloured compounds have their own characteristic absorption spectra and careful selection of the wavelengths where maximum absorption is found enables a mixture of two coloured substances to be analysed. In this experiment you will first determine the wavelengths at which the absorbances of bromophenol blue and methyl orange are maximum. You will then proceed to investigate the relationship between the absorbance and concentration of these two dyes at the wavelengths you will have determined.

Materials

1 Colorimeter/digital spectrophotometer
2 Bromophenol blue (10 mg/litre)
3 Methyl orange (10 mg/litre)
4 An "unknown" mixture of the two dyes

Method

Prepare a range of concentrations of one of the dyes by setting up a series of tubes as below.

Tube	1	2	3	4	5	6	7	8	9	10	11
Bromophenol (10 mg/litre) (ml)	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Distilled water (ml)	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0

Place the filter which gave maximum extinction in the light path and zero the calorimeter with distilled water. Next record the absorbance of each solution and plot this against the concentration of dye in each tube in $\mu g / 5ml$.

1. Repeat the above experiment using the filter which gave maximum absorption with the methyl orange, one other filter, and, if possible, white light with no filter. How do the curves of extinction against concentration conform to Beer's Law?
2. Repeat the whole of the experiment with the dye methyl orange.
3. Finally, use the information gained in these experiments to determine the concentration of each dye present with the dye mixture.

THE ABSORBANCE CURVES OF BROMOPHENOL AND METHYL ORANGE

Materials

These are as in the previous experiment.

Method

Determine the absorbance of each dye in turn against the range of filters supplied with the colorimeter. If you use the spectrophotometer, you will not need to change any filters. Remember, the instrument must be reset on zero absorbance with distilled water in the cuvette for each filter.

Carefully note the wavelength of maximum transmission (minimum absorbance) of each filter and plot a graph of the absorbance recorded against this wavelength.

Questions

What is the wavelength that gives maximum absorbance for each dye? How does mixing the dyes effect the absorption spectrum.

1. Draw the structures of bromophenol blue and methyl orange. Comment on their key functional groups and their common laboratory uses.

THE ABSORPTION SPECTRUM OF P-NITROPHENOL

Materials

1. p-Nitrophenol (10 mM)
2. HCl (10 mM)
3. NaOH (10 mM)
4. Spectrophotometer
5. Volumetric flasks (100 mls)

Method

Dilute the p-nitrophenol solution 0.2-50 ml with (a) 10 mmol/litre HCl and (b) 10 mmol/litre NaOH. Determine the absorption spectra of each solution from 250 to 500 nm. Comment on the differences between the two spectra and calculate the molar extinction coefficient at the wavelength for the maximum absorption.

QUESTIONS

1. Show the dissociation reactions of p-nitrophenol that explain the colour changes in acid and basic media.
2. Comment on the acid/base properties of p-nitrophenol
3. Suppose the nitro group in p-nitrophenol was replaced by a carboxyl group, predict the effect of adding (a) HCl (b) NaOH to an aqueous solution as in the experiment above.
4. Explain the importance of p-nitrophenol in a medical biochemistry laboratory
5. A solution of 10^{-5} moles/litre of substance X shows a transmittance of 70.2 per cent at 260nm in a 1 cm cuvette. Calculate: (a) the absorbance (b) the transmittance in a 3cm cuvette (c) the absorbance of 50μ mole/litre of substance X in 1 cm cuvette.
6. A solution Y of 29.3 mg/litre has an absorbance of 0.25 at 260 nm. If the light path is 1cm and the molecular weight of Y is 586, calculate (a) the molar extinction coefficient (b) the transmittance of 10 μmol/litre of solution.
7. Calculate the molar absorption coefficient, ε, at 351 nm for aquocobalamin in 0.1 M phosphate buffer, pH 7.0, from the following data, which were obtained in a 1-cm cell.

Solution	**Concentration**	**I_o**	**I**
A	2.23×10^5 M	93.1	27.4
B	1.90×10^5 M	94.2	32.8

PRACTICAL TWO

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. 10 M NaOH solution
5. 1% $CuSo_4$ solution
6. Millon-Nasse Reagent
7. 1% $NaNO_2$
8. 2 M H_2SO_4
9. Pyridine solution
10. 20% sodium sulphite solution
11. 0.02 % α-Naphthol in 95% Ethanol
12. Alkaline Hypobromite solution
13. Conc HNO_3
14. Lead acetate solution
15. Glyoxylic acid
16. Conc. H_2SO_4
17. Buffered ninhydrin
18. Ice
19. 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 name from the compound Biuret, which also gives th 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% CuSo4 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 colour changes.

C. Ninhydrin test for amino acids.

When amino acids are heated with Ninhydrin they are quantitatively deaminated and an intense blue to purple colour appear. 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

(i) Citrate buffer pH 5.0 – Dissolve 21.008 gm. Citric acid in 200 mls of distilled water, add 200 mls of 1M NaOH solution and dilute to 500 mls with distilled water.
(ii) 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.

D. Million-Nasse test (tyrosine)

This an adoption of the Million 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 positively with Million-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 Million-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 tryrosine.

E. Sakaguchi test (arginine)

This test indicates the presence of Arginine and depends upon the presence of the guanidine groups of the Arginine molecule.
The reaction of the guanidine group with C-α—Naphthol is the basis of the test. Since the developed colour decomposes rapidly, urea is used to stabilize 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 guanidine group. Add 0.2 ml of urea to stabilize the colour.

F. Xanthoproteic test (tyrosine and tryptophan)

This reaction depends on nitration of the phenyl group $–C_6H_5$ or aromatic ring, it is given by 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 10M NaOH. An orange-red ring will form at the interface in a positive reaction.

G. 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 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 sulphuric acids and into cystine.

Procedure

1. To a 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.

H. Glyoxylic Acid Test (Hopkins-Cole Test, Tryptophan)

This is a test for tryptophan free or combined with protein. When 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

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

PRACTICAL THREE

EXTRACTION AND THIN-LAYER CHROMATOGRAPHY OF SERUM LIPIDS

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, phospholipids, non-esterified ("free") fatty acids and sterols (principally cholesterol).

In medicine, elevated serum lipid levels (***hyperlipidemia)*** occur in such disease states as diabetes mellitus and hypothyroidism. In addition a correlation between serum atherosclerosis and hyperlipidaemia has been noted.

Most lipids are not found in free form in the blood or other tissues. They are associated with proteins to form lipoproteins. To analyse 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.

In today's practical you will extract the lipids from serum and then separate the different lipid classes by thin layer chromatography.

Principle of lipid extraction

The relative ease with which lipids may be extracted depends on the degree of their association with tissue proteins. The loosely-bound "neutral" lipids (e.g triglycerides and cholesterol esters) are easily extracted with non-polar phospholipids, which are more tightly protein-bound, requires the addition of a polar compound (e.g methanol) to the extraction solvent to dissociate the 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 analysed for lipid content.

Principle of thin layer chromatography (TLC)

TLC is somewhat similar in principle to the paper chromatography technique. The main difference is that in place of the hydrated cellulose fibres of paper, the adsorbent or stationery 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 RF calculation are all similar to those of paper chromatography.

The absorbent that you will use is silica gel, a form of a 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 the relative affinities of the lipids for the silica gel and for the solvent or mobile phase. A non-polar solvent (e.g chloroform) will separate the neutral lipids leaving the phospholipids at the origin. More polar solvent mixtures (e.g chloroform; methanol; acetic acid) will separate a mixture

of phospholipids while the neutral lipids will move with the solvent front. In today's practical you will use both types of solvent system.

SOLVENT FRONT

Neutral Lipids		**Polar Lipids**
Free fatty acids		
Cholesterol ester		Phosphatidyl ethanolamine
Triglycerides		Phosphatidyl serine
Free fatty acids		Phosphatidyl choline
Cholesterol		Spingomyelin
ORIGIN		
(Nonpolar solvent)		

Reagents and Materials

1. Fresh Serum
2. Extraction solvent; chloroform: methanol (2:1)
3. Chlomatography: solvents (in chromatography jars);
   Chloroform: methanol: acetic acid water (25:15:4.2)
4. Methanol
5. Chloroform
6. TLC (2); silica gel on glass, 5 x 20 cm
7. Extraction tube
8. Centrifuge tube
9. Capillary pipette
10. Chromatography jars (2)

Iodine vapour chamber (under hood)

Procedures

Extraction of serum lipids

1. Place *1.0 ml of serum* in an extraction tube and add 7.0 ml of *chloroform: methanol (2.1)* mixture
2. Shake vigorously for 3 min (a white precipitate will form).
3. Transfer the mixture to a centrifuge tube and centrifuge for 10 min. The aqueous and organic phase will separate into two layers with a dense white precipitate of protein at the interface.
4. *Taking 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 50 ml beaker *and carefully* evaporate to near dryness on a hotplate.

6. When nearly dry, add 1.0 ml methanol to aid in moving traces of water. (Repeat) A technician will assist you in drying the samples.
7. When dry, add 0.5 ml of chloroform to beaker to dissolve lipids. The solution should be yellow, and there may be a small amount of insoluble residue.

TLC of Lipid Extract

1. Place two TLC plates on a clean dry work surface with coated side up. Handle plates only by edges and take care not to disturb the coating.
2. On each plate, make a single, faint pencil mark approximately 2 cm from one end and 2.5 cm from either side. This dot will be the origin where you will spot 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 1 cm. This volume is about *10µl* (microlitres). Briefly and repeatedly touch the tip of the pipette to the pencil dot and allow a small amount of the extract to flow onto the plate. Let the spot dry between each application and take care to keep the spot small, about *0.5 cm diameter*. Repeat until a volume of about 20µl has been applied to each plate.

Place one plate in the chromatography jar containing chloroform and acetic acid and the other in chloroform, methanol, acetic acid and water mixture. Ascend about two thirds the length of plate. This will require 30 to 45 min.

QUESTIONS

1. Make a diagram of the lipid chromatography from each solvent system, tentatively identifying each spot from the information in figures 1 and 2.
2. From each spot, calculate the Rf values i.e

$$RF = \frac{Distance\ from\ origin\ to\ center\ of\ spot}{Distance\ from\ origin\ to\ solvent\ front}$$

Present your measurements in tabular form.

3. Use chemical structures to illustrate and explain why phosphatidyl choline is more polar than a triglyceride; why a free fatty acid is more polar than a triglyceride and why cholesterol esters are less polar than free cholesterol.

PRACTICAL FOUR

TESTS FOR VARIOUS CARBOHYDRATES

Theory

The principle 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 metabolized to pentoses which are an important part of nucleic acid molecule's. The simple sugars are also known as monosacchrides (of varying chain length of 3 to 7 carbon atoms) and occur in many forms in nature. 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 polyhydroxy compounds having either an aldehyde group (aldoses) or a ketone group (ketoses). These groups will reduce certain metal ions with 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 hexose, forms a pyran ring with two isomeric forms.

Monosacchrides polymerize into large molecules as follows:

Disaccharides, 2 units, example cane sugar
Oligosaccharides, upto 10 units
Polysaccharides have many monosaccharide units. They include starch and glycogen.
These are classed as macromolecules and are non-dialysable.

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 discussed during your lecture sessions.

EXPERIMENT 1

REAGENT AND MATERIALS

Carbohydrate test solution: 1% solutions of glucose, fructose, pentose, sucrose and maltose.

1. Concentrated H_2SO_4
2. Molisch's reagent
3. Benedict's reagent
4. Barfoed's reagent
5. Seliwanoff's reagent
6. Bial's reagent

A. Molisch Test

The molisch test is a test for carbohydrates in general. Concentrated sulphuric acid causes a dehydration of the monosaccharide forming a molecule of furfural. If a polymeric saccharide is present, it will be hydrolysed by the acid to the monomer and the hydrated furfural.

Experiment

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

In the presence of concentrated H_2SO_4, phenolic compounds such as thymol and α-naphthol condense with furfural to form purple coloured derivatives. The coloured complex is the basis of Molisch's test.

Procedure

1. Pipette 2 ml of the sugar solution into separate test tubes
2. Add 2 drops of the Molisch's reagent
3. Mix thoroughly, incline the test tube, and put 1 to 2 ml of concentrated H_2SO_4 down the side, slowly to form two layers.

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, the test should be repeated on a more dilute 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 contains a potential aldehyde or ketone group. The group is designated as potential for the pyran of 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^{++} + Glucose → Cu^{+} (insoluble) + oxidized products

Benedict's a 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 ml of each test solutions into different test tubes
2. Add 5ml of Benedict's reagent to each of test tubes
3. Place all the tubes simultaneously in a boiling water bath and heat for 3 minutes
4. Cool and observe

The presence of reducing sugars is indicated by the formation of a red yellow precipitate. The sensitivity of this test should be demonstrated by diluting glucose test solution 1 to 5 and 1 to 10 and repeating the test.

C. Barfoed's Test

Barfoed's test is also a copper reduction test but is carried out in acid rather than alkaline solution. Where sugars are weaker reducing agents and Cu^{++} Cu^{+} is only reduced by monosaccharides.

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

Procedure

1. Pipette1 ml of each solutions into different test tubes
2. Add 5 ml of Barfoed's reagent to each test 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 Cu_2O and record the number of minutes required; remove the positive tube 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.

D. Seliwanoff's Test

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

Procedure

1. Pipette 1 ml of each of the test solutions into different test tubes
2. Also prepare a blank tube with 1 ml of water
3. Add 5 ml of Seliwanoff's reagent to each tube
4. Mix and heat for exactly 60 seconds in the boiling water bath
5. Observe and record the results at this time
6. Continue the heating for five minutes recording any changes that occur in a table summarizing your observation.

E. Bial's Test

Bial's test is for pentose sugars and it depends on the rapid formation of the furfural from pentose under 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, Benedict's and Burfoed's depend on the reduction of copper and differences in the consumption 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 furfural derivatives which have in turn reacted with phenolic compounds forming coloured complexes.

Procedure

1. Pipette 2ml of each of the solutions into different test tubes,
2. Add 3 ml of Bial's reagents
3. Mix and boil for one minute. A positive reaction is indicated by the appearance of a green colour. Hexose which from hydroxyl-methyfurfural yield a yellow to brown colour.

IODINE TEST FOR POLYSACCHARIDES

Theory

Iodine gives an intensive blue reaction when mixed with certain polysaccharide, but not with others. The 1-4 glycosidic linkage of polysaccharide form a helix, six pyranose groups forming one complete turn and the inner dimensions are such that an iodine molecule is suspended within a turn of the helix. The iodine behaves as an oscillator and absorbs all the energy of light except that of the short wave-length which gives the blue colour of the reaction. Other polysaccharides with α(-4) glycosidic linkage do not form a helix, and therefore, cannot form a red-violet colour with iodine. Polysaccharides can be hydrolysed by acids at about 100^0C, with hydrogen ions acting as the catalyst.

Reagents and Materials

1% solution of starch
1% solution of glycogen
1% solution of cellulose
1 M HCl
0.1 N. Iodine solution
Cotton Wool

Procedure

1. Prepare a test plate by putting a drop 0.1 N iodine solution in each depression.

2. Place 2 mls of each test solution in different test tubes
3. Add 1 M HCl to each tube, and stopper with cotton wool,
4. Heat the solutions in boiling water bath for 30 minutes
5. Test each polysaccharides at the start and at 3 min intervals during hydrolysis by adding a drop of the solution to the iodine on the test plate.

NB: Use a different row for each polysaccharide. Make a table on which you will record your results on polysaccharide-hydrolysis. As the hydrolysis is progressing test the nature of the colour formed by each polysaccharide.

1. Pipette 2 mls of the 1% starch solutions into a test tube and add one drop of the iodine solution.
2. Observe and record the result
3. Heat that solution, record the result and then cool it and again record the result on the colour change.
4. Add a few drops of 2 N NaOH and then acidify again with a few drops of acid
5. Record these observations.

Quantitative determination of glucose in urine using Benedict's method

Benedict's reagent for the estimation of reducing sugars contains potassium thiocynate as well as copper sulphate. In the presence of the former white precipitate of cuprous oxide. The small amount of potassium ferrocynide also aids in keeping cuprous oxide in solution. As the precipitate formed is white, the loss of all blue tint in the solution, indicates complete reduction of the copper. The alkali used is sodium carbonate, which has the advantage over hydroxides as it is less likely to cause destruction of small amounts of sugar. The solution also has the great advantage of being stable for an indefinite length of time. The method is recommended for its simplicity and accuracy.

Procedure

1. Place 10 ml of the quantitative BENEDICT'S REAGENT with a pipette into the 100 ml flask (flat bottomed flask) which is in your glass equipment.
2. Add the four or five glass beads provided. Place the flask on the asbestos gauze on your tripod and with a very small flame gently bring to the boil
3. On boiling, add drop wise to the solution (with 2.0 ml graduated pipette), your unknown glucose solution (urine). Start with your pipette filled to the 0.0 ml mark. Add drop wise till a precipitate forms, then continue to boil 1 or 2 minutes and add very slowly some further drops until the colour has entirely disappeared.
4. Make a note of the amount of sample used.
5. Repeat at least once or twice. Some students will have to continue adding their glucose solution patiently.

Calculate gm% glucose in your solution from the fact that 20 ml your quantitative Benedict's reagent are reduced by 50 ml of glucose standard convert gm % into molarity.

Questions

1. Differentiate between a reducing sugar and non-reducing sugar
2. Explain in details the effects of acid, alkaline and heating on starch.

PRACTICAL FIVE

SEPARATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

A mixture of low Mwt. 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 solubility. This partioning effect may be carried out in solution (as in counter current distribution) or, more commonly, on an 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 base. The cellulose binds to 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 partioning effect is repeated many times and since the relative affinity is characteristic for a given compound, a separation is achieved. The more hydrophobic (non-pollar) components migrate the furthest, the more hydrophilic (polar) remain near the origin.

In a given solvent system each amino acid migrates with a characteristic Rf value. Usually the migration of an unknown amino acid run as standards. Following the chromatography the amino acids are revealed as purple spots on the paper by spraying with a solution of ninhydrin and heating at 100^{o}c. Paper chromatography is often combined with paper electrophoresis in a two-dimensional form for the resolution of complex mixtures of amino acids and peptides, such as results from the hydrolysis of a protein. Electrophoresis in one dimension is followed by chromatography in the second dimension.

REAGENTS AND MATERIALS

1. Solution (1mg/ml in water) of glycine, alanine, leucine, tryptophan, glutamic acid, lysine.
2. A mixture of three of the above (labeled X)
3. Casein hydrolyse (20 mg/ml) casein protein hydrolysed in 5M H_2SO_4 at 100^{o} for 4h, then neutralized and deionized with Ba $(OH)_2$
4. Whitman No. 1 paper (20 cm x 18 cm)
5. Chromatography solvent (n-propane-0.88 ammonia, 7:3 v/v)
6. Ninhydrin reagent (1% in acetone)

Procedure

1. Pour the solvent (about 50 ml) into the chromatography bottle and replace the lid. Care: avoid the fumes and spillage.

2. Using a pencil draw a line across each of two papers about 2 cm from the lower edge. Mark on the line 4 points at roughly 4 cm intervals, taking care to start well in form the

left hand edge. Avoid touching the paper with your fingers except by the extreme edges.

3. Apply a sample of glycine solution to the first spot on one paper. Touch the end of the capillary tube containing glycine solution to the paper lightly in order to transfer as small a volume as possible. Label the spot in pencil.

4. Repeat application of the other amino acids, the unknown mixture and the casein hydrolysate. Use the individual capillary tubes with each solution so as to avoid contamination. Allow the spots to dry completely.

5. Fold the paper into a cylinder and staple the edges together so that they do not overlap. Place the cylinder, sample side down, in the chromatography bottle, taking care not to let the paper touch the glass walls. Replace the lid of the tank firmly.

6. Allow to run for about one and half hours. Develop the chromatogram by incubation at 100^{o}C for a few minutes.

7. Spray the paper lightly with ninhydrin solution inside the fume cupboard. Do not allow the paper to become completely soaked. Develop the chromatogram by incubation at 100^{o}C for a few minutes.

8. Outline each spot in pencil and mark the centre of the spot. Calculate the Rf value of each amino acid. Include the chromatogram (or a copy in your report).

Discussion

Comment on the separation of amino acids by single dimension ascending paper chromatography as evidenced by your own results. Can you identify any of the constituent amino acids of casein? Which amino acids were presented in the unknown mixture.

Questions

1. In preparing the casein hydrolysate, what is the precipitate formed by adding barium hydroxide to the hydrolysed mixture? Why is this step necessary.

2. Why is it necessary to add the solvent to the chromatography bottle well before starting the experiment?

3. Why should you not mark the sample sports on the paper with ink and why should you avoid touching the paper with your fingers?

4. Account for the Rf value of each amino acid in terms of its structure.

5. Is single dimension ascending paper chromatography an effective method for separating the amino acids in a protein hydrolysate? How could you improve the separation?