UNIVERSITY OF NAIROBI

COLLEGE OF BIOLOGICAL AND PHYSICAL SCIENCES

DEPARTMENT OF BIOCHEMISTRY

Practical Course for First Year students of B.Sc. Agriculture, B.Sc. Food Science and Nutrition and B.Sc. Range Management

Revised and re-edited by Prof. J.K. Munyua and C. Karuri Kamweti - November 2000

TABLE OF CONTENTS

AIMS AND OBJECTIVES OF "PRACTICAL BIOCHEMISTRY"

The experiments described in this manual have been compiled for the students of first year B.Sc. Agriculture, B.Sc. Food Science and Nutrition and B.Sc. Range Management, Biochemistry, University of Nairobi. The experiments will supplement the lectures in biochemistry that are given during the same period. Each practical ends with questions that should be answered and submitted for marking. By answering the questions, you will obtain a better and longer-lasting understanding of biochemical techniques and the intricacies of biochemistry. When you have finished the course you should be able to:-

1. Follow biochemical procedures to obtain relevant data
2. Interpret data from a biochemical experiment
3. Write a concise logical report based on laboratory findings
4. Assess the application and limitation of common biochemical tests in quality control
5. Correctly measure the volume of liquids and operate simple equipment
6. Relate the practical work with the theoretical aspects covered in class
7. Carry out simple biochemical calculations involving SI units
8. Appreciate the role of control experiments in acquisition of relevant laboratory data.
9. Predict which biochemical technique might be useful in solving a particular problem later in your profession.

DIRECTIONS FOR PRACTICAL LABORATRY 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 case of qualitative tests, the quantities recommended are only approximate, but it should be appreciated that gross deviations from the recommended proportions of reagents and test materials may invalidate the test. In 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 either by 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 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 any 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 20° or 25°C.

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 for 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 tip 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:

50 ml burettes are graduated to 0.1 ml but the levels of liquid can be estimated to 0.01ml. A burette must be absolutely clean to deliver accurately and must first be tested with water to make certain it drains without leaving droplets behind. Be sure the burettes 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. 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 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 of the 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 0.002 ml. They are used in the same manner as microburettes, 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 magnitude in a semi-micro titration is of course very serious. Semi-microburettes are 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 are required to obtain chemically clean glassware.

Cleaning of burettes and pipettes requires special attention since improper drainage of these due to greasy surface introduce 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 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 much solution 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.

THEORETICAL INTRODUCTION TO METHODS OF EXPRESSING CONCENTRATIONS IN VOLUMETRIC ANALYSIS AND IN 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. Therefore 1 molar of NaOH solution contains 40.01 gms of NaOH in 1000 mls of water (H_20).

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 the acid-base which yields or combines with exactly 1.0080 grams of H^+ (the weight of one gram of H), this process of quantitative combination, leading to neutralisation of solution, is called titration.

Examples: The molecular weight of NaOH is 40.01. One molecule of NaOH can combine with one of H^+

($H^+ + OH^- \longrightarrow H_2O$), 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 36.47g. A normal solution of HC1 contains 36.47 HCl per litre of the solution since one molecule of HCl yields one H^+. However, sulfuric acid contains two ionizable hydrogen atoms. The equivalent weight of H_2SO_4 is therefore the gram-molecular weight which is 98.02 divided by 2 = 49.042 g and therefore a Normal solution for H_2SO_4 contains 49.042 g. H_2SO_4 per litre.

Other Methods of expressing concentration

A Molar solution - is one containing 1.00 grams molecular weight of a solute dissolved in 1000 g. of solvent (note the distinction between a molal and a molar solution). Molality is properly used in calculations involving the colligative properties, such as osmotic pressure, freezing and boiling point depression and elevation, and certain other thermodynamic properties of water and other solvents.

Percent by weight indicates grams of solute in 100 g. of solution (w/w). This is the expression used for commercially available solution.

Percent volume indicates grams of solute in 100 ml. of solution. This is by far the more commonly used expression involving per cent (w/v).

Volume percent. This expression is commonly used to indicate concentration of a solution in a liquid. 50% alcohol means that 100 ml. of solution contains 50 ml of alcohol.

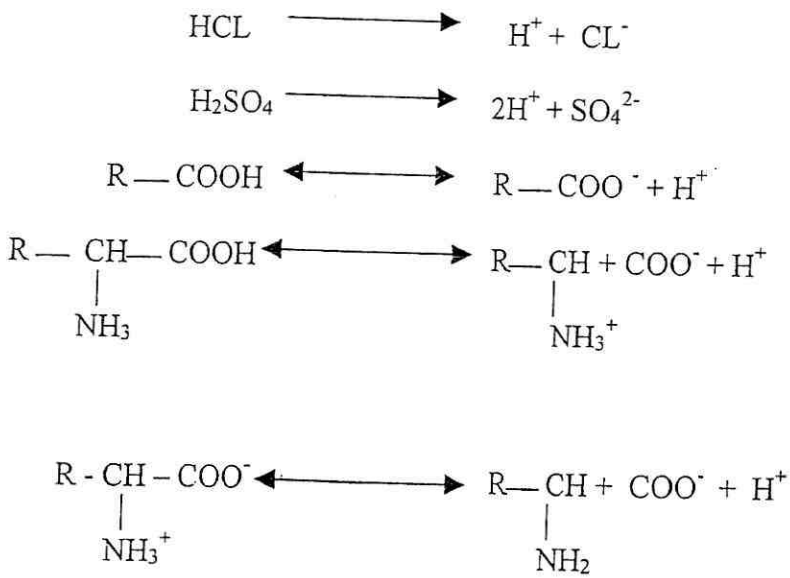
Milligrams per cent (mg. 1%) is a term often used in expressing concentration of materials in blood and refers to milligrams of solute per 100 ml. of solution.

PRACTICAL 1

ACID-BASE TITRATIONS/ pH MEASUREMENTS

pH - is a measure of hydrogen ion [H^+] concentration. This is usually done in solutions containing mineral acids like HCl, amino acids, fatty acids, proteins, peptides or nucleic acids. This is because these molecules contain hydrogen ions that dissociate when dissolved in water and contribute to the increase in hydrogen ion concentration.

Example



The pH is defined as the - log $_{10}[H^+]$. That is the log of one over the concentration of hydrogen ion. Then using this equation we can calculate hydrogen ion concentration if we know the pH. Solutions usually have pH which range from 0 - 14. The instrument which is used for pH-measurement is a pH- meter which utilizes a glass electrode.

Reagents and Materials

pH meter and magnetic stirrer
Straight pipettes
Burette, 10 ml. or 25 ml.
Phenolphthalein (indicator)
.01 M Hydrochloric acid
.01 M Acetic acid
.01 M Sodium hydroxide
Teat Pipettes

Procedure

Calibrate the pH meter using buffers, one on the acid and one on the alkaline side.
Place 25 mls. of 0.01 M HC1 in a 100 ml. beaker.
Add 2 - 3 drops of phenolphthalein
Adjust the pH meter electrodes so that the pH can be measured i.e the electrodes are well immersed in the solution.
From a burette add successive 2.5 ml. aliquots of 0.01 M NaOH and record the pH after each addition.
Continue the titration until about 25 mls of 0.01 M NaOH have been added or the phenolphthalein changes colour to pink.
Repeat the procedure using 10 ml of 0.01 N acetic acid.
Combine the data and on a graph paper plot the results from the **two titrations into a single graph** using the ml of NaOH on the **Ordinate** and the pH observed on the **Abscissa**.

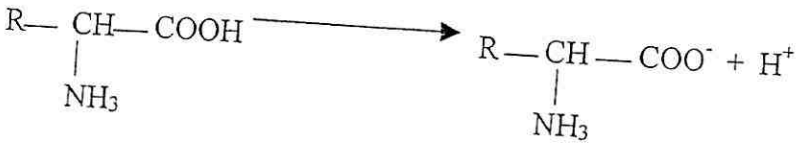
Questions:

1. Write the law of Mass Action and Henderson-Hasselbalch equation.
2. What is the pH of; 10 ml HCl , 10 ml NaOH

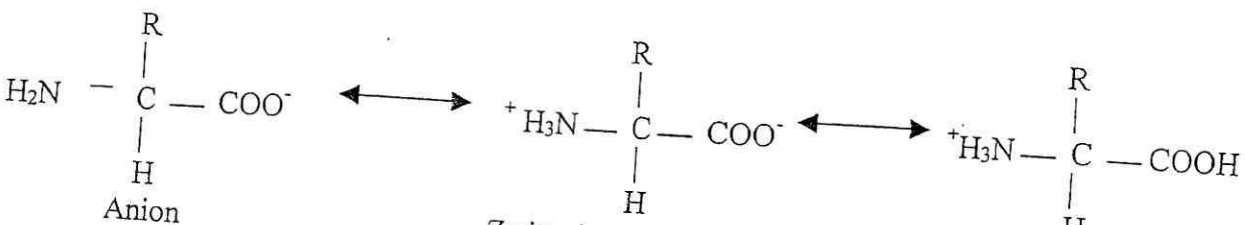
PRACTICAL 2

FORMOL TITRATION OF AMINO ACIDS

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

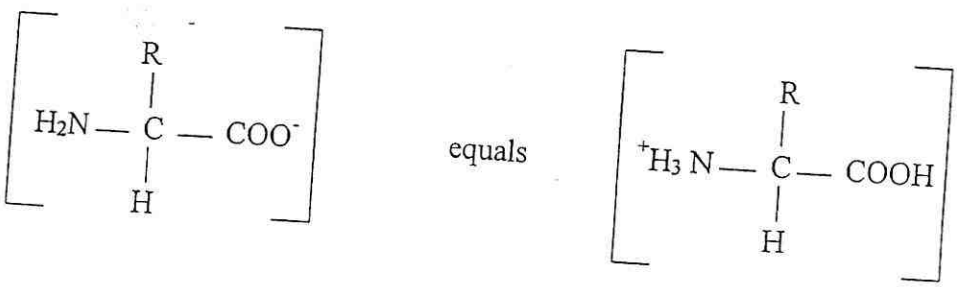


When an amino acid is dissolved in water a **zwitterion** or **dipole** is formed which then shifts to an anion at a high pH or a cation at a low pH as follows:



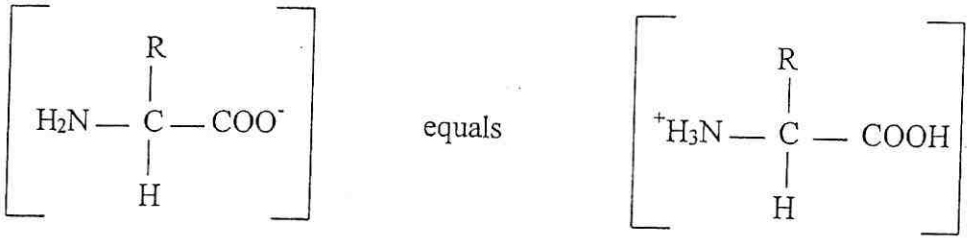
Anion — Zwitterion or Dipole — Cation

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



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

has a pK_2 where;



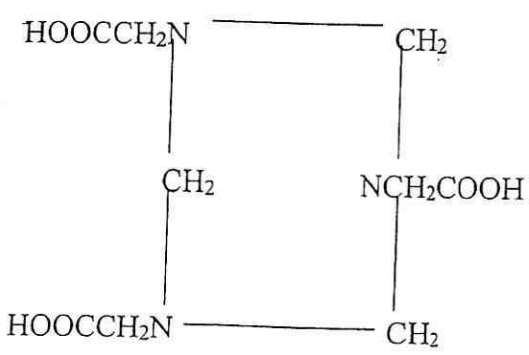
Both pK_1 and pK_2 involve the shift of a portion of H^+ and can be calculated by the **Henderson-Heselbalch** equation. For glycine $pK_1 = 2.35$ and $pK_2 = 9.78$. Between the two points there is a pH at which the molecule carries a net charge of zero called the **isoelectric point** or **pI** . For glycine pI = 6.06. That is

$$pI = \frac{pK_1 + pK_2}{2} = \frac{2.35 + 9.78}{2} = 6.06$$

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

Amino - acids having -SH, phenolic, $-NH_2$ or COOH groups as the R group also dissociate giving pK_3 and pK_4.

Formaldehyde and other aldehydes react with the amino groups of amino acids to give METHYLOL derivatives : $HN_2 + HCHO \longleftrightarrow NHCH_2OH$. Reaction of glycine with formaldehyde leads to nitromolecular cycleation to result in formation of the cyclic tricarboxymethyl trimethylene triamine:



$HOOCCH_2N$ ——————— CH_2

Formaldehyde does not react with NH_3^+ groups. Because of this, reaction of formaldehyde with $NH_2CH_2COO^-$ pulls the equilibrium

$^+NH_3CH_2COO^-$ ⟷ $NH_2CH_2COO^- + H^+$

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

Reagents and Materials
0.1 M Hcl
0.1 M Glycine solution
0.1 M NaOH solution
Neutral Formalin solution
pH Meters
100ml Beakers

Procedure

Place 20 ml of the glycine solution in a 100 ml beaker.

Group 1 : Titrate the amino acid solution by adding 2 ml aliquots of the standard acid, mix and measure the pH of each successive addition until volume of 20 ml is reached.

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

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

Group 4 : Add formalin as in Group 2, do titration with alkali as in group 3.

Calculation:

Each group of 4 students combines the data into a single graph as follows: Plot equivalents of HC1 and NaOH on the ordinate vs pH on the abscissa. Calculate the various pK's of the amino and carboxyl groups **with** and **without** the presence of formalin.

PRACTICAL 3

SEPARATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

THEORY

The resolution of a mixture of chemical compounds can frequently be accomplished by the technique of partition chromatography on paper. This technique is based on the principle that when a substance is soluble in two mutually immiscible solvents, it will ditribute itself between the two phases in a definite proportion which can be expressed by the distribution of partition constant $K = C_1/C_2$ where C_1 and C_2 represent the equilibrium concentrations in each of the solvents. Thus amino acids or simple carbohydrates distribute themselves between water and some organic solvents such as butanol or phenol in characteristic ratios.

Filter paper may be looked upon as matrix of cellulose fibres containing a small amount of bound water that is able to act as a solvent for carbohydrates, amino acids, or for other substances. If the mixture of substances to be resolved is placed at one end of a sheet of paper and the organic solvent is allowed to flow through the paper, the components of the mixture will tend to distribute themselves between the flowing solvents and the bound water on the paper. The rate of movement of the mobile solvent depends upon its relative affinity or partition between the solvent and the water. The greater the affinity of the substance to the mobile phase, the more it will be carried with the flow. The sheet of paper makes possible an enormous number of partitions operations between the two phases and is thus able to separate compounds with quite similar structures. In some cases, the rate of movement of the components of the mixture is only determined by partition between the solvent and bound water but also by adsorption to the paper and by the relative differences in desorption by the mobile solvent. There are in fact instances where this is the only relevant factor. These extremes may be referred to as partition chromatography and adsorption chromatography, respectively.

Solvents, Paper and Equipment - Hundreds of solvents systems have been described and specified references should be consulted for a discussion of these.

Specific methods generally indicate the type of paper to be used. Sheets or strips of Whatman No.1 paper are probably used more than any other. There are also other types of paper for specific purposes. These include less dense paper for more rapid flow rates (Whatman No. 54 or 4), and thick paper with high capacity for preparative purpose (Whatman 3MM). Glass fibre "paper" is also used for chromatography involving solvents incompatible with cellulose.

The equipment required generally depends on the kind of chromatography to be employed. The simplest system consists of a closed container which provides a means of suspending the paper and for immersing one of the paper in the mobile solvent.

The container should be closed to avoid drafts that would cause localized drying of the paper, and the entire tank should be located in an area with little temperature variation. These conditions are critical for obtaining constant and reproducible migration of the solvent front along the paper.

In general, approximately 10-30 microlitre of the solution to be analysed is applied 3 or 4 inches from one end of the paper strip or sheet and allowed to dry. This position should be indicated with a pencil mark. The paper is then suspended in the chromatographic tank in one of two possible

a pencil mark. The paper is then suspended in the chromatographic tank in one of two possible ways. In the first, the solvent reservoir is located at the top of the tank. This is referred to as **descending** chromatography, whereas **ascending** chromatography refers to the case where the solvent reservour is located at the bottom of the tank and the solvent runs up into the paper. A small part (approximately 1 inch) of the paper at the end at which the sample was applied is allowed to extend into the solvent vessel. The tank is sealed.

The solvent will then move through the paper past the sample spot and into the paper. The time required for the solvent to move almost the entire distance of the paper will vary with the type of paper used and the particular solvent system. It should be examined periodically and removed from the tank just before the solvent front reaches the other end. The solvent front should be marked and the paper dried. The position on the paper of the components of the mixture is directed by specific staining methods. The ratio of the **distance of movement of a given component** and **the distance of movement of the solvent front** is called **Rf value** which can vary from 0 to 1. In given solvent system, each substance has a characteristic Rf value which is useful in its characterization. However, an authentic sample (called a "marker" is ordinarily run with the unknown.

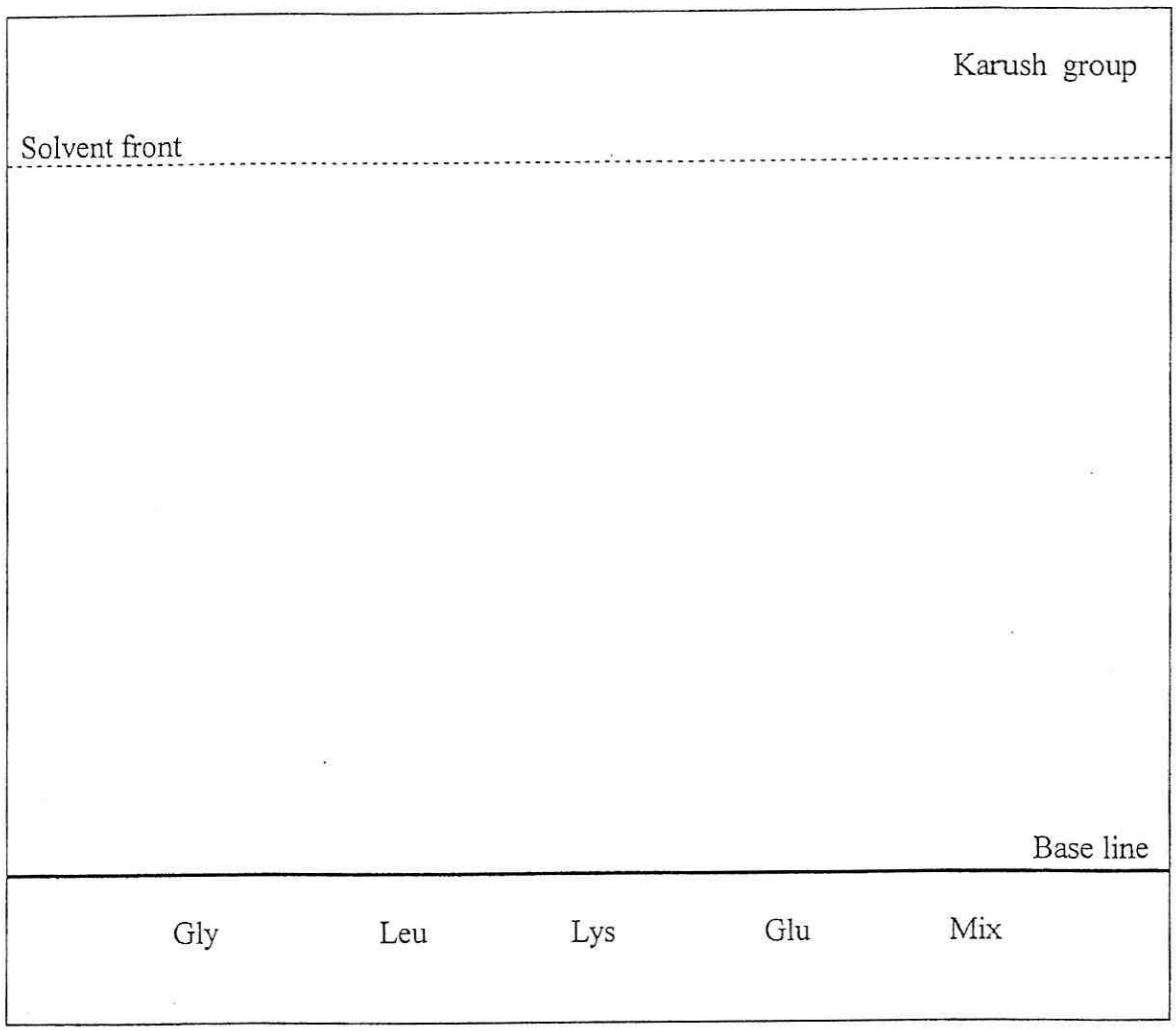
In some cases the rate of movement of component is too slow relative to the flow of the solvent through the paper. In this case resolution is enhanced by employing the descending method and allowing the solvent to run for a longer period of time so that the front moves off the end of the paper. In this case the Rf concept has no meaning and chromatography of the unknown mixture must be accomplished by simultaneous studies of known compounds which present in the mixture is suspected. Chromatography of an unknown component with a substance of unknown structure in two or more solvent systems is usually sufficient to characterize the unknown compound.

Reagents and Materials

Chromatography Tank (or sweet jar with lid)
Filter paper (20 x 18 cm.) Whatman Type No. 1
Wire Loops or capillary tubes
Ninhydrin solution - 0.2% in acetone
DL-aspartic acid
DL-leucine
DL-lysine
DL-tryptophan
Any three amino acid mixture or protein hydrolysate
Paper clips
Solvent system: Ethanol: Water: Ammonia (8 : 1 : 1)

Procedure

You are provided with a jar containing above solvent system. A 20 x 18 cm. sheet of paper is used for chromatography. Place it on a clean surface and avoid touching the paper as much as possible with bare hands. Draw a pencil line across the paper, 2.5 cm. from the bottom. Beginning 3 cm. from one side place pencil dots or cross at 3 cms. intervals along the line and mark these according to your different amino acids, and the mixture (see drawing).



Mix = Mixture of amino acid (or Protein hydrolysate)

To apply the test solution to the paper use the wire loop or capillary tube. Clean the loop by rinsing with water and heating with Bunsen burner to remove substances. Use separate capillary tube or loop for each amino acid.

Place your test solution on the pre-marked spots and see that the spots do not overlap and that the spots are not too close to the joint. Place the cylinder with the spotted end down in the tank, taking care not to let the paper touch the glass walls nor the spots touch the solvent in the bottom. Close

the tank with the lid.

No observation can be made while the chromagoram is running because the compounds used are colourless.
Run for 1 - 2 hours or until the solvent front has advanced to within 2 cm from the top of the paper. Mark the solvent front with a pencil, open the paper and let it dry in the air or in the oven.
When dry spray the chromatogram with ninhydrin solution, allow the solvent to dry, then heat the sheet in the oven for about 5 minutes. The position of the amino acid spots will be revealed by development of a **purple colour**. The colour is stable for some weeks if kept in the dark and free of acid vapours. For permanent record, outline the spots in pencil. **Measure the distance between the centre of each spot and the starting pencil line (base line).** The ratio of this distance between the original and the solvent front is the **Rf value** for a particular amino acid. Make a sketch of your results on paper provided to be handed in prior to your leaving the laboratory.

Questions

1. What is Rf of aspartic acid?
2. What is the Rf of leucine?
3. What is the Rf of tryphtophan?
4. Explain in your own opinion why the Rf of aspartic acid differs from that of leucine.
5. Why would lysine not elute together with aspartic acid?
6. Why must you not touch the filter paper with your fingers?
7. Why do you just apply only one spot of the mixture?
8. What is the chemical reaction between ninhydrin and the amino acid?
9. What does formaldehyde do to the amino acid so that it alters the titration curve?

PRACTICAL 5

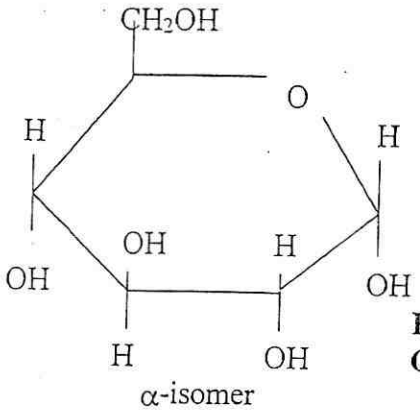
CARBOHYDRATES

THEORY

The principle carbohydrates in the living organism are hexoses composed of six carbon atoms such as glucose and fructose and the pentoses of 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 molecules. The simple sugars are also known as monosaccharides and occur in many forms in nature of varying chain length from 3 to 7 carbon atoms. As the study of carbohydrate metabolism progresses, example of all these will be found in the animal cell.

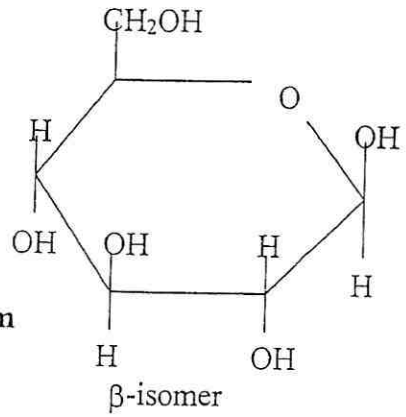
Carbohydrates can also be classified as polyhyroxyl alcohols having either an aldehyde group (aldoses) or a ketone group (ketoses). These groups will reduce certain metal ions, the sugar undergoind oxidation in the reaction forming a number of low molecular weight compounds. This reaction is the basis 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 isometric forms or anomeric forms.



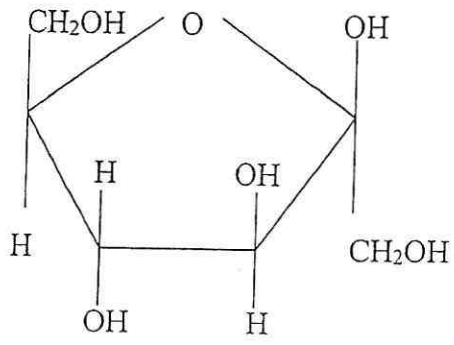
α-isomer

Pyran Ring Form Glucopyranose



β-isomer

Furan Ring Form of β-D-Fructofuranose



Monosaccharides polymerize into larger molecules as follows:-

Disaccharides, 2 units, example cane sugar
Oligosaccharides, up to 10 units.] - Dialyzable

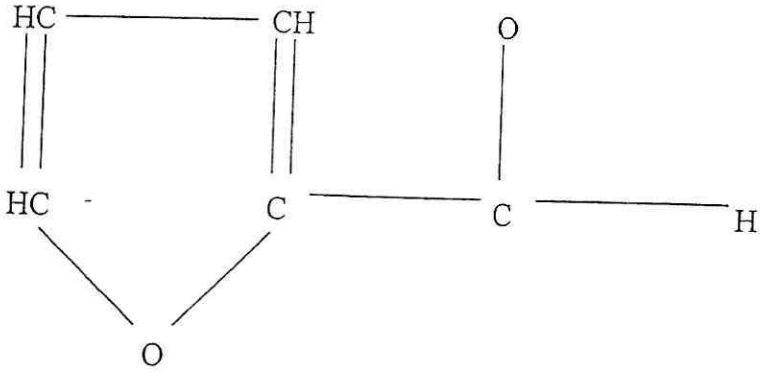
Polysaccharides, many units, examples starch and glycogen. These are classed as macromolecules and are non dialyzable.
Another characteristic property of simple sugars is the optical activity due to the presence of assymetric carbon atoms in the structure. This is manifested by the rotation of polarized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

Reagents and Materials

Carbohydrate test solutions: 1% solutions of glucose, fructose, pentose, sucrose and maltose.
Conc. H_2SO_4, conc. HCl
Molisch Reagent
Benedict's Reagent
Barfoed's Reagent
Seliwanoff's Reagent
Bial's Reagent.
Boiling water bath

A) Molisch Test:

The Molisch Test is a test for carbohydrates in general, concentrated sulfuric 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 then dehydrated to furfural.
Pentoses yeild furfural of the strudture;



Hexoses yield 5-hydroxy-methylfurfural, the ketohexoses reacting more rapidly than the aldohexoses. This is the basis of Seliwanoff's test for distinguishing between fructose and glucose. In the presence of Conc. H_2SO_4, phenolic compounds such as thymol and α-naphthol condense with the furfural to form purple coloured derivatives. This coloured complex is the basis of Molisch test.

Procedure:

Pipette 2 ml of sugar solutions into separate test tube. Add 2 drops of the Molisch reagent. Mix thoroughly, incline the tube, and pour 1 to 2 ml of conc. H_2SO_4 down the side, slowly and carefully, 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 contain a potential aldehyde or ketone group. The group is designated as potential for in the pyran or furan ring structure the group has the hemiacetal structure rather than an aldehyde or ketone structure. As already described this group will reduce certain metals forming oxidized low molecular weight compounds of the sugar.

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

Benedict's test is carried out in an alkaline solution of sodium carbonate. The precipitation of Cu $(CO_3)_2$ is prevented by adding citrate a molecule having free hydroxyl groups which form a complex with the Cu^{++}

Under carefully controlled conditions, this reaction serves as an excellent quantitative procedure for sugars.

Procedure

Pipette 1 ml of each test solution into different test tubes. Add 5 ml of Benedict's Reagent to each of the test tubes. Place all the tubes simultaneously in a boiling water bath and heat for 3 minutes. Cool and observe.

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

C. Barfoed' Test

Barfoed's test is also a copper reduction test but is carried out in acid rather than alkaline solution, conditions in which sugars are weaker reducing agents $Cu^{++} \longrightarrow Cu^{+}$ is only reduced by monosaccharides. It can be used therefore to distinguish the monosaccharides from disaccharides. However, on prolonged boiling the acid may hydrolize some of the disaccharide and a slight positive result will be obtained.
Barfoed's Reagent cannot be used to test solutions containing chlorides such as urine, for a white precipitate of $CuC1_2$ forms.

Procedure

Pipette 1 ml of each test solution into different test tubes. Add 5 ml of Barfoed's Reagent to each test tube. Mix and place all the tubes in the boiling water bath. 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.
After 15 minutes remove any remaining tubes and allow them to stand and observe for any precipitate that may settle out. Note the time. 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 from furfurals more rapidly than aldoses so that comparison of the rate of colour formation can be used to distinguish between these two types of sugars.

Procedure

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

E. Bial's Test

Bial's test is for pentose sugars and depends on the rapid formation of the furfural from pentose under the specific conditions of the test. In this test orcinol reacts with the furfural yielding a blue-green colour.
This is the last of the specific tests that will be used in the study of carbohydrates. It should be noted that two tests, Benedict's and Barfoed's, depend on the reduction of copper and differences in the composition of the solution and the rate of reaction are used to obtain specific information. The other three tests depend on differential rates of dehydration to form furfural derivatives which have in turn reacted with phenolic compounds forming coloured complexes.

Procedure

Pipette 2 ml of each of the test solutions into different test tubes
Add 3 ml of Bial's reagent
Mix, and boil for one minute
A positive reaction is indicated by the **appearance of a green colour**
Hexoses, which form hydroxy-methylfurfural yield a yellow to brown colour.

Questions

1. Which of these disaccharides give reducing sugar on hydrolysis?
   a. Sucrose
   b. Maltose
   c. Lactose

2. What monosaccharides will form furan and which will form pyran ring? glucose, ribose, deoxyribose, galactose or mannose.

3. Oxidation of sugars by acids will give certain oxidative products, name them.

4. How is iodine used to identify different polysaccharides.

5. How do you differentiate between a fructose and glucose.

6. Differentiate between Benedict's Test and Barfoed's Test.

PRACTICAL 9

HALOGEN ABSORPTION BY THE DOUBLE BOND OF UNSATURATED FATTY ACIDS

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

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

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

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

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

Reagents and Materials

Chloroform
Oleic acid
0.5 M Stearic acid: 143 gm/L of chloroform
Cottonseed Oil
Hubl's Iodine Solution (Freshly prepared)

Procedure

This experiment will be carried out by students working in pairs under supervision of an instructor so that each pair will have access to the colorimeter when needed since the time at which readings are made are essential to the success of the study.

To each of four clean, dry test tubes:
Add 10 ml of chloroform
Add 5 ml of Hubl's iodine solution
At 2 minute intervals add to:
Tube 1: 0.1 cottonseed oil and mix
Tube 2: 0.1 ml oleic acid and mix
Tube 3: 0.1 ml stearic acid and mix
Tube 4: This is the control and is used to set the instrument at a scale setting of 100% with a filter which transmits at 420 mu (filter No. 600 or 620).
Read absorbance of each tube **exactly** 5 minutes and 20 minutes after adding the oil or fatty acid.

Calculations

Tube 4 has absrobance A_1 , of the original iodine concentration. The absorbance A_2, of the other tubes is the amount of iodine remaining unreacted; the difference, $A_1 - A_2$ is the iodine which has reacted.
Plot the value of difference for each time for each fat as a bar graph.

PRACTICAL 11

COLORIMETRY AND PHOTOMETRY

THEORY

When a ray of monochromatic light of initial intensity (I_o) passes through a solution in a transparent vessel some of the incident light is absorbed so that the intensity of the transmitted light (**I**) is less than that of (I_o). Some loss of intensity of light occurs due to scattering and absorption by particles or molecules in the solution and reflection on the interfaces. The relationship between I_o and I depends mainly on the path length of the absorbing medium and the concentration (**C**) of the absorbing solution. These factors are related in the laws of Lambert and Beer.

Lambert's Law:

It states that when a ray of monochromatic light pases through an absorbing medium, its intensity decreases exponentially as the length of the the absorbing medium increases.

Beer's Law

States that when a ray of monochromatic light passes through an absrobing medium, its intensity decreases exponentially as the concentration of the absorbing medium increases.

These two laws are combined together in the **Beer-Lambert** Law as follows;

$$I = I\,o_e^{-k3cl}$$

Transmittance

The ratio of intensitites is known as transmittance (T) and is usually expressed as a percentage.

$$\text{i.e. } \%T = I/Io \times 100 = e^{-k3cl}$$

This is not commonly used since a plot of percent transmittance (T) against concentration (C) gives a negative curve, instead extinction is used.

Extinction:

This is the negative logarithim on both sides of the above equation (or if logarithims are taken on both sides the above equation instead of ratio then:

$\%T = I/Io \times 100 = e^{-k3cl}$

$\log_e I_o/I = k_3cl$

$Log_{10} I_o/I = k_3cl / 2.303$

$Log_{10} I_o/I = kcl$

The expression $Log_{10} I_o/I$ is known as the absorbance (A). The absorbance is some times referred to as the optical density (O.D.) or extinction (ϵ), but these latter two names are no longer recommended. Absorbance (A) is more fequently and currently used.

Therefore,
$A = kcl$ or ϵcl

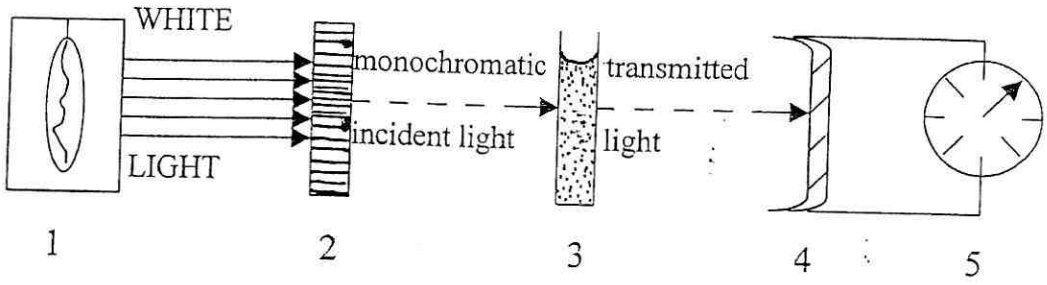
Where A = Absorbance
k = Molar extinction coefficient
c = Concentration of absorbing medium
l = Pathlength travelled by light = 1cm

It is frequently necessary in the laboratory to determine the total amount of some substances in solution quantitatively. If the substance is coloured, or can be altered to form a coloured substance, the amount present can be measured by colorimetry. The principle on which this is based is the Beers-Lambert Law which states that the amount of light absorbed as it passes through a coloured solution is proportional to length of the light path, Lambert Law, and to the concentration of the solute, Beer's Law. This principle is used for most of the quantitative work in Biochemistry, and simply means that given the same depth of solution, that having the most intense colour contains the largest amount of solute or the substance being determined.

It is the inherent nature of an atom or molecule to exist in discrete energy states. Each atom or molecule can exist only at certain energy levels and not at other. We find that for each of these particles there are characteristic energies which may be absorbed lifting the molecule from one permitted energy level to another possible. It is the particular absorption of given wavelength (monochromatic light) impinges upon a solution containing molecules capable of absorbing this wavelength, a reduction in the light intensity will occur because some of its energy is absorbed. There will be no or little interference by molecules of different substances as their possible molecular energy changes do not correspond to particular wavelength used hence monochromatic light increases the accuracy of the determination.

The simplest way of doing colorimetry is the preparation of a series of known solutions of graded concentrations and with which the unknown is compared until an appropriate matching of colour is obtained. Elaborate devices have been developed to assist in colour comparison, but all depend on the judgement of the individual observed and are nearly worthless **if a person is colour blind**.

It is now customary to use a photoelectric device to measure the intensity of transmitted light which eliminates the subjective error. These vary greatly in complexity from the simple filter photometer to the most elaborate spectrophotometers, but irrespective of the complexity of the instrumentation, the basic principle is the same.
The mechanism of the photometer is illustrated in the diagram.



1	2	3	4	5
Light Source	Coloured Filter	Known or Unknown Solution	Photo Cell	Meter Printer out Device, Digital, Graphical e.t.c.

NOTE:

1. This is normally any electric bulb, operated by a battery or on a regulated mains to control the fluctuation.
2. A filter is used to isolate the wavelength of light that is maximally absorbed by the solute increasing the sensitivity.
3. The known or unknown solutions are placed in a cuvette or glass tube.
4. The photo cell converts the energy of the transmitted light to electrical current.
5. The amount of current is measured by a galvanometer.

The reading is most commonly in terms of absorbance **(A)** or optical density **(O.D.)** which are essentially the same. The optical density increases logarithmically as the amount of absorbing solute increases.
In all determinations a "control" solution composed of the solvent and reagents is used to set the instrument at zero optical density. The unknown is then placed in the light path and its optical density is measured. The amount of solute present can be determined by comparing this reading with the reading of known solutions. An instrument is calibrated for a specific determination by preparing series of known, measuring their optical densities and plotting these against the concentrations on graph paper. This is called a **"standard curve"** and can then be used for all subsequent determinations of this particular substance.

5. The photocell converts the energy of light to electrical current.
6. The meter measures the amount of current.

The reading is most commonly in terms of absorbance (**A**) or optical density (**O.D**), which are essentially the same. The optical density increases logarithmically as the amount of absorbing solution increases.

In all determinations a control solution of the solvent and the reagents is used to set the instrument at zero optical density. The unknown is then placed in the light path and its optical density measured. The amount of solute present can be determined by comparing this reading with the readings of the known solutions. An instrument is calibrated for a specific determination by preparing series of known, measuring their optical densities and plotting these against the concentrations on graph paper. This is called a standard curve and can be used for all subsequent determinations of this particular substance.

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

Theory

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 lengths at which the absorbances of bromophenol blue and methyl orange are maximum. You will then proceed to investigate the relationship between the absorbance concentrations of these two dyes at the wavelengths you will have determined.

Materials and reagents

1. Colorimeter/ digital spectrophotometer
2. Bromophenol blue (10 mg/litre) dye
3. Methyl orange (10 mg/litre) dye
4. Unknown mixture of the two dyes

Method

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

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Bromophenol (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	2.0	1.5	1.0	0.5

1. Set the wavelength
2. Zero the spectrophotometer with distilled water (Blank)
3. Record the absorbance
4. Alter the absorbance and repeat step 2 and 3
5. Plot values of absorbance against the concentrations of the dye in each tube in µg/ 5ml
6. Repeat the above experiment using methyl orange.
7. Finally use the information gained in this experiment to determine the concentration of each dye present with the dye methyl mixture.

8. How do the curves of the extinction against the concentration conform to Beer's law?

THE ABSORBANCE CURVES OF BROMOPHENOL AND METHYL ORANGE

Materials and reagents
These are as in the previous experiment.

Method

Determine the absorbance of each dye in each turn against the range of wavelengths with the spectrophotometer. NB; The instrument must be reset on zero absorbance with distilled water in the cuvette for each wavelength.

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

Questions

1. What is the wavelength that gives maximum absorbance for each dye?
2. How does mixing the dyes affect the absorption spectrum?
3. 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 and reagents
1. p-Nitrophenol (10 rnM)
2. HCl (10 mM)
3. NaOH (10 mM)
4. Spectrophotometer
5. Volumetric flask (100ml)
6. Volumetric flasks (100 mls)

Method
Dilute the p-nitrophenol solution 0.2-50 mI 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 and 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.