THE DEPARTMENT OF MEDICAL BIOCHEMSTRY

COLLEGE OF HEALTH SCIENCES, UNIVERSITY OF ILORIN, ILORIN.

PRACTICAL BIOCHEMISTRY

MANUAL

PRACTICAL BIOCHEMISTRY MANUAL

BY

THE DEPARTMENT OF MEDICAL BIOCHEMISTRY

COLLEGE OF HEALTH SCIENCES

UNIVERSITY OF ILORIN,

ILORIN.

CHAPTER ONE

STATISTICAL TREATMENT OF DATA

The results or data obtained are the most important ingredients in an experiment. Therefore, it is appropriate to begin a book on practical biochemistry with the analysis and treatment of data. It is often necessary, in carrying out biological experiments to ascertain the reliability of the data obtained. This is usually done by estimating the errors inherent in the experiment. The best method of estimating the error of any measurement is to repeat the measurement several times in order to see how closely the results obtained agree with one another.

Experimental errors are not mistakes (which can be avoided), rather, they are results of imperfections in the apparatus, difficulties of personal judgment, limitations of the scale, approximations in the theory used, unwanted fluctuation, etc. Since such errors are inevitable, every measurement or experimental determination should be accompanied by some indication of its reliability. This is usually done by stating the limits of error, e.g. $T = 20.1 \pm 0.1$ C or $pK = 6.02 \pm 0.02$. In normal scientific practice these limits are not limits of maximum possible errors, but estimates of the "standard error".

The standard error is a statistical index and supposes that in a large number of similar measurements the Magnitude and sign of the unknown error present would fluctuate in a random manner. It is also assumed that the average error over a large number of measurements would be zero. If this is not the case, then the measurement is said to have a bias; the error is then a systematic error.

The word 'error' is used in a scientific context to mean a deviation from the true value which cannot be avoided; it is not an error in the ordinary use of that word. If an athlete runs 100 meters in 10.8 seconds on one occasion, 10.6 seconds on another occasion and 11=.0 seconds on a third occasion, we do not regard these variations as error. They may be due to changes in weather conditions, personal fitness or some Physiological conditions. We could express this scientifically by stating that the athlete's mean time is 10.8 second with a maximum error of 0.2 seconds.

Systematic Error

Any error which affects the measurement in a regular and essentially predictable way is said to be a systematic error. Errors in the calibrating of a scale or due to leaking burettes are common examples of systematic errors. These errors usually introduce a definite bias one way or the other' i.e. they will tend to give a positive error or a negative error.

In principle, systematic errors can be corrected for and eliminate. However, in practice there are two main difficulties in achieving this: firstly, some systematic errors may remain undetected, and secondly, it may be impracticable to calculate the corrections. The best way to detect systematic errors is to use completely different methods to measure the same quantity. If two independent methods given different results, then a systematic error exists in at least one of them and possible sources should be looked for.

Random Errors

If it is possible to eliminate all systematic errors in an experiment, some errors, usually called random error, will undoubtedly persist. Unlike systematic errors, random errors cannot eliminated since they are irregular and errors may arise from a number of sources including reading errors (i.e. errors in reading a calibrated instrument or errors due to the limitations of the scales used), errors due to personal judgments in the making of adjustments, error due to fluctuation in temperature or other physical conditions during the period of the experiment. These errors can be eliminated by making repeated a whole experiment, or by letting different people make the same measurement. In normal volumetric titrations, burette readings are usually taken three or four times for the purpose of eliminating or minimizing random errors. These random errors show up in the 'scatter' of points when the results are plotted on a graph.

Sometime, variability in repeat readings is not due to errors but arises as a result of natural variations or fluctuations in the quantity being measured. For example, the diameter of a wire or glass tube varies along its length, the room temperature varies with the time of day. Such fluctuations will affect the reliability of the men value adopted in exactly the same way as experimental errors.

In scientific experiments the limits of errors are usually quoted, e.g. enzyme activity = 6.43 ± 0.46 moles/mg protein/min. The limits estimate the magnitude of random errors which may be present in the determination of enzyme activity. This provides a measure of the precision of the measurement. *Accuracy*, on the other hand, means closeness to the true value.

Accuracy and Precision

The meanings of the terms *"accuracy"* and *"Precision"* should be understood very early in their practical course by all students of Biochemistry. Accuracy refers to the correctness of a measurement or determination which precision refers to the reproducibility of the accurate it is. The closer several results is to the true value, the more accurate it is. The closer several results of a given measurement are to each other, the more precise they are, regardless of whether or not they are accurate.

For example, in a simple acid/base titration experiment in which HCI is titrated against NaOH, using methyl orange as an indicator, four students obtained the following results:

	1st Reading	2nd Reading	3rd Reading	4th Reading	Mean Titre
Student A	38.7	36.7	37.6	37.9	37.7
Student B	39.4	39.5	39.5	39.6	39.5
Student C	36.4	36.5	36.5	36.6	36.5
Student D	35.3	37.3	35.7	37.5	36.5

If the true titre value, supplied by the technologist who prepared the solutions, is 36.5, the results above show that student A had a poor precision and accuracy, B had a good precision but poor accuracy, C had a good precision and accuracy while D had poor precision but good accuracy, although through pure chance (magic, giraffe method, etc.) not as a result of any diligence on his p[art. Fig. 1 shows a graphical representation of the students 'titration results.

It is therefore, important to base the grading of students' experiments work on a mark scheme which takes cognizance of both precision and accuracy.

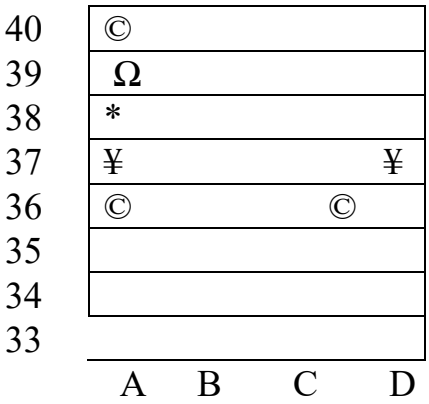

Fig. 1.1: Graphical representation of the students' titration results.

Standard Error

The limits of error quoted in scientific literature are usually not the outside limits of maximum possible errors; they represent the standard error of the result obtained. This is defined as the root-mean-square of the random errors that would occur in a large number of similar measurements. If in an experiment we make say ten determinations of some quantity Q and obtain the values 10.0, 10.1, 10.2, 10.3, 10.1, 20.2, 10.4, 10.3, 10.2 and 10.2, we assume that the best value, i.e. the value closest to the true value of Q, assuming the absence of systematic errors, the arithmetic mean of these readings, i.e. 10.2. However, if we quote this value in a scientific paper we need to give any reader who might wish to repeat the experiment some idea of the 'spread' of values that he is likely to obtain.

Such a reader might be interested in two things. First, he might wish to know how much any one determination is likely to differ from deviation of the measurements. If n determinations of quantity are made and the values x1, x2, x3 ……..xn are obtained the mean value is

$$\text{Standard deviation (s)} = \frac{d_1^2 + d_2^2 + \ldots\ldots. dn^2}{n}$$

Where $d_1 == x_1 - x$; $d_2 = x_2 - x$; $d_3 = x_3 - x$; etc.

Calculations using this formula can be tedious, and for $3 < n < 12$ a sufficiently accurate estimate of standard deviation may be made using the approximate formula

$$S=w / vn$$

Where w is the range of values. The range of a set of value is defined as the greatest minus the smallest value. In the example given above the range is 0.4 (i.e. 10.4 – 10.0). Thus, Q ==10.2 ± 0.15. s is so defined tat for most sets of measurement 70 5 of the individual values will lie within the spread of values indicated by the standard deviation. In the example above, these is a 70% chance that any single measurement will lie between 10.05 and 10.35.

In Biochemistry it is of far greater interest to estimate the standard error of the mean (S_m) of a set of values.

$$S_m = s/\sqrt{} n \text{ (n}$$

Where n is the number of determinations, and w is the range.

S_m gives n estimate of the precision of the mean of t of value in the example above, Q = 10.20 The standard deviation is the root ± 0.03. S_m is defined such that in a example above, Q = 10.20 ± 0.003. S_m is defined such that in a repetition of the experiment to determine Q there is an approximately70% + chance that the mean value of Q obtained will lie between 10.17 and 10.23.

The following general rules can be applied for the determination of standard deviation or standard error.

1. If n repeated readings have range w (i.e. the difference between the smallest and the largest values) then the standard deviation of the reading can be estimated by the formula.
2. It can be shown that the standard error of the mean (S_m) of a series of n values whose standard deviation is α is given by

$$(S_m) = \alpha / \triangle n$$

This is valid for all value of n. Therefore, $(S_m) = w / n$

The standard error assesses the precision of the mean or its liability to be incorrect because of random experimental errors. It gives no information about possible systematic errors.

Plotting Straight Line Graphs

In plotting graphs the scales should be such that they can be used directly and easily without auxiliary calculations. This condition is satisfied only by scales such as:

1" of 1 cm = 1,2,5 units

Or 10,20 50 units

Or 0.1, 0.2, 0.5 units.

Scales like 2" = 5 units; 1" = 3 or 4 units should be avoided.

If the points on a graph are supposed to lie on a straight line but in fact show considerable scatter, a best-fitting line should be drawn through the experimental points, taking all the points account. It is a mistake to select a group of point which seems collinear and reject others. It is also a mistake to force a line through the origin. A scattered graph is the inevitable result of experimental error and is normal. Careless readings, errors in mathematical calculations, will greatly increase the scatter and will also distort the line.

The following numerical method of fitting a straight line can be very useful:

Divide the points into three groups nearly equal as possible, putting the odd group in the middle as in the following examples

With 8 points 3,2,3

With 8 points 3,3,3

With 8 points 3,4,3

With 8 points 4,3,4

If there are n points altogether and r points in the two end groups, the gradient of the best fitting line may be estimated by the formula:

$$g = \frac{y_n + y_{n-1} + \ldots + \ldots y_{n-r=1} - y_r - y_{r-1} \ldots y_1}{X_n + x_{n-1} + \ldots + \ldots X_{n-r+t} + x_{r-1} - \ldots\ldots - x_i}$$

For example, if n = 8; or r = 3 and this becomes

$$g = \frac{y_8 + y_7 + y_3 + y_2 - y_1}{y_8 + y_7 + y_3 + y_2 - y_1}$$

This method is equivalent to taking the gradient of the line joining the centroid of the end groups, and is therefore called the 'controid methods'.

".

To get the equation of the best line we arrange for the line to have this gradient and also to pass throguh the centroid of all the points $(X_1 Y)$ where

$$x = (x_1 + x_2 + \ldots x_n) / n$$

$$y = (y_1 + y_2 + \ldots\ldots y_n) / n$$

Therefore,

$$(y - y) = g (x - x)$$

Errors of Scope and Intercept

The standard error of the gradient may be estimated from the scatter of the points by the formula:

$$S_g = 4w/nR \quad (3 \leq n \leq 12)$$

Where n is the number of point, w is the range of vertical scatter and R is the total range of the x coordinate between the first and the last point on the graph.

Fig 1.2: determination of the standard error of a slope.

Statistical analysis of Data: student 't' Test

The student 't' test, introduced by Gossett (who published his researches under the pseudonym 'student') is frequently used to demonstrate statistical of the difference between the mean value of two random sample drawn from normal populations.

$$t = /x - x$$

$$€=1/n_1 + 1/n_2$$

Where

$$€=n_1S_1 + n_2 S_1$$

X_1 and x_2 are the means of samples.

S_1 and S_2 are the standard deviations of samples,

N_1 and N_2 are the sizes of samples (i.e. the number of independent observations in each sample).

The probability corresponding to a particular 't' value can be checked in a table of 't' distribution.

Experiment 1: Exercise on Plotting of graphs and calculation of standard errors.

Many biochemical experiments involve the fitting of experimental data into graphs, and the calculation of expressions including the slope and intercept of

straight lines, together with the corresponding errors. This exercise on plotting of graphs is designed to enable the student gain experience in the use of graphs to obtain information. This will be particularly useful in experiments involving the use of spectrophotometers and plotting of standard curves as well as in studying enzymatic reactions.

The tables below gives values of y for various value of a variable x. Carry out the following steps to obtain valuable information from the graphs.

1. Plot a graph of y against x for each set of data.
2. Calculate the best slope, m (using the centroid method) and the intercept c on the y axis and draw the line corresponding to these.
3. Calculate the standard errors of the slope and intercept.
4. Calculate the ⌂- m/c and, using the rules for the combination of errors, calculate the standard error in each case.

Data Set 1

X	y
10.03	1.105
10.12	1.130
10.20	1.205
10.27	1.225
10.35	1.300
10.46	1.380
10.55	1.455
10.68	1.560

Date Set 2

x	y
12.00	7.24
10.00	6.25
8.00	5.26
6.00	4.24
5.00	3.74
4.00	3.25
3.00	2.75
2.50	2.50
2.00	2.25

Data Set 3

X	y

Date Set 4

x	y

6.25	1.54	0.16	0.65
12.5	5.88	0.08	0.17
25.0	20.0	0.04	0.05
50.0	50.0	0.02	0.02
100.0	0.80	0.01	0.0125
200.0	94.12	0.005	0.011
400.0	98.46	0.0025	0.010
800.0	99.61	0.0025	0.010

Experiment 2 : Calibration of volumetric apparatus.

The purpose of this experiment is to compare the accuracy and precision of a measuring cylinder, a pipette and a burette. You will be provided with some clean, dry volumetric apparatus a measuring cylinder, a pipette, a burette and five stoppered volumetric flasks carry out the following procedure in determining the accuracy and precision of the apparatus.

1. Weigh the five stoppered volumetric flasks provided. The flasks must be clean, dry and cold before weighing. Rinse the measuring cylinder provided and fill it to the 25 ml mark with distilled water at room temperature. (Record the temperature of the water, using a thermometer). Carefully pour the water into one of weighed volumetric flasks. Similarly measure 25 ml of water using the cylinder, into each of the other weighed flasks. Weighed each of the flasks and calculate the mean volume of the water, using the density of water at the temperature of the experiment, and the standard error of the mean.
2. Dry the five volumetric flasks and check the dry weights again.
3. Using the procedure given in (1) above, calibrate the 25 ml pipette. Check the temperature of the distilled water and calculate the mean and standard error of the mean as in (1) above.
4. Dry and reweigh the five volumetric flasks

5. Calibrate the burette by filing it with distilled water to a level above the zero mark. Run out water slowly until the bottom of the meniscus is on zero. Run out exactly 25 ml of water into one of the weighed volumetric flasks. If by mistake the 25 ml mark is overshot, read the exact volume of the water you have run out (to the nearest 0.01 ml) and compute the weight of 25 ml of water by simple proportion. Repeat for the other four volumetric flasks. Calculate the mean volume, and the standard error of the mean, of the burette.
6. Compare the measured volumes and their standard errors with the nominal volume for the measuring cylinder, pipette and burette. Comment on the differences observed in terms of the relative accuracy and precision of these measuring devices.

Chapter Two
Buffer Solutions

Many biochemical processes take place in aqueous solutions and their rates of reaction depend on the H^+ concentrations in the surrounding medium. The physical properties of solutions of macromolecules such as proteins, polysaccharides and nucleic acids may be drastically altered by manipulating the H^+ concentrations in such solutions.

The H^+ concentration in solutions is usually expressed in terms of pH. This is defined by the mathematical expression:

$$pH = - \log_{10}(H^+)$$

The square bracket denotes concentrations in moles per litre.

In living cells, the pH of many reactions involving enzymes are carefully regulated and maintained within narrow limits. This is achieved with the aid of biological buffers. A buffer is a system which tends to resist changes in pH when small amounts of H^+ or OH^- are added. In the laboratory, buffers are prepared by mixing a solution of a weak acid with a solution of a salt of the acid.

The pH of a buffer solution can be expressed by the Henderson –Hasselbech equation. This equation is important for the understanding of buffer action as well as the acid-base balance in cells. It is derived from the expression for the dissociation constant of an acid.

Consider the weak acid HA which dissociate thus:

$$HA \rightarrow H^+ + A^-$$

$$K = \frac{[H^+][A]}{[H]}$$

$$H^+ = \frac{K[HA]}{[A]}$$

Taking the logarithm of both sides

$$\text{Log}[H^+] = \log K + \frac{\log[H^{A]}}{[A]}$$

$$\text{and } - \log [H^+] = - \log K - \frac{\log[HA]}{[A]}$$

$$pH = pK - \frac{\log[HA]}{[A]}$$

$$pH = pK + \frac{\log[A]}{[HA]}$$

This is the Henderson – Hasselbach equation and is usually expressed as:

$$\mathbf{pH} = pK + \frac{\log[salt]}{[acid]}$$

The pH of a solution may be measured approximately with indicators or more accurately by a pH meter which uses a glass electrode and a calomel reference electrode. Acid/base indica are weak acids or bases. They are present in solution as mixtures of the ionized or unionized forms; the ratio of the two forms depends on the pH of the solution. The two forms also have different colours. Thus, it is possible to estimate the pH of a solution from the colours given by various indicators. The pH may then be determined more accurately by comparing the colour of a suitable indicator in the unknown solution with its colour in buffer of known values.

Experiment 3: Determinations of the pH of solutions

Two solution A and B of unknown pH are provided. To 5 ml of each solution add 2 drops of an indicator. Repeat this for each of the indicators listed below; observe the colour obtained and draw conclusions about the approximate pH of the solution. In order to determine the pH more accurately, use the procedure outlined below to prepare a series of buffer solutions where successive members differ by 0.2 pH units. The pH of the buffers should be in the neighbourhood of the approximate pH of the test solution. Put 5 ml of each of the prepared buffers into separate test tubes and add 4 drops of a suitable indicator to each test tube. Compare the colour of ‘5 ml of the unknown solution, to which 4 drops of the same indicator have been added, with that of the buffer solutions and hence determine the pH of the test solution as accurately as possible.

Preparation of the buffer solutions

A buffer solution of specified pH may be prepared as follows: pipette 10 ml Nach into a 100 ml measuring cylinder add (10 + x) ml of 1M solution of a weak acid from a burette, where x is calculated from the equation are:

(i) Ethanoic acid, pK_a + $\log \frac{10}{x}$ = pK_a + 1 - log x

(ii) Dihydrogen phosphate, pK_a = 7.1`0

$H_2PO_4 \rightarrow HPO_4^{2-} + H^+$

Indicator	pH Range	Acid Colour	Basic Colour
Thymol blue	1.2 – 2.8	red	yellow
Bromophenol blue	2.8 – 4.6	yellow	violet
Congo red	3.0 – 5.0	violet	orange
Bromocresol green	3.7 – 5.4	yellow	blue
Alizarin R. 3	4.0 – 6.0	yellow	purple
Methyl red	4.4 – 6.0	red	yellow
Chlorophenol red	4.8 – 6.4	yellow	red
Bromocresol purple	5.2 – 6.8	yellow	purple
Bromothmol blue	6.0 –7.6	yellow	blue
Phenol red	6.8 – 8.4	yellow	red
Cresol red	7.2 – 8.8	yellow	red
Thymol blue	8.0 – 9.6	yellow	blue
Phenolphthalein	8.2 – 100	colourless	red.

Experiment 4: Accurate determination of the pH of colourless solutions

Prepare a series of buffer solutions in test tubes by missing 0.1m KH_2PO_4 and 0.1m Na_2HPO_4 in various proportions as shown below. Add five drops of bromothymol blue indicator into each test tube and record the colours observed in each tube and record the colours observed in each tube. Add 5 drops of the bromothymol blue into each of the two solutions of unknown pH provided and estimate as accurately as possible the pH of the two solution.

Tube no	1	2	3	4	5	6	7	8	9	10	11
0.1m KH_2PO_4 (ml)	10	9	8	7	6	5	4	3	2	1	-
0.1M Na_2HPO_4(ml)	-	1	2	3	4	5	6	7	8	9	10
pH	4.6	5.6	6.3	6.5	6.6	6.8	6.9	7.1	7.4	7.7	9.2

Experiment 5: Determination of pK value

The pk value of an unknown solution can be determined from the titration of a weak acid with a strong alkali.

Reagent

0.1MNaOH solution; 0.1M phosphoric acid; methyl orange and phenolphthalein indicators.

Method

Pipette 10 ml of 0.1 phosphoric acid into a conical flask or any other suitable container. Add 5 drops of methyl orange indicator and titrate with 0.1M NaOH solution. Repeat the titration with 5 drops of phenolphthalein in place of methyl orange. In each case take the reading when the colour of the indicator begins to change. At this point the NaOH should be added drop by drop. Plot a graph of pH against the titre value and estimate the approximate pKJ and pK2 value for phosphoric acid. Calculate the 1st and 2nd dissociation constants for phosphoric acid.

Chapter three

Spectrophotometry

Spectrophotometry is the technique for the determination of the concentration of solutions by measuring the amount of absorption of light rays are passed through them. It is one of the most valuable analytical techniques in biochemistry. Unknown compounds may also be identified by their characteristic absorption spectra in the ultraviolet, visible or infrared. Enzyme-catalyzed reactions are usually followed by measuring the appearance of the product or disappearance of the substrate, using a spectrophotometer.

Fig. 3.1: The Electromagnetic spectrum

Spectrophotometry

A Spectrophotometry is an instrument used to measure the amount of light that is absorbed or transmitted by a solution. The essential components of a Spectrophotometry are shown in fig. 3.2. they include the following: (1) a light source, (2) a collimator or focusing device that transmits an intense straight beam of light, (c) a monochrometor or prism which divides the light beam into its component wavelengths, (d) a device for selecting the desired wavelength, (e) a compartment (test tube or cuvette) in which the sample is placed, (f) a photoelectric detector and (g) an electrical meter to record the output ofr the detector.

Fig. 3.2: Essential components of a Spectrophotometre

Beer – Lambert Law

When a ray of monochromatic light with an initial intensity I_o, passes through a solution of an absorbing solution in a colourless solvent, some of the light will be absorbed so that the intensity of the transmitted light, I, is less than I_o.. The fraction of the incident light absorbed is proportional to the number of solute molecules in the light path. The relationship between 1 and I_o is defined by the Beer-Lambert Law which state as follows:

The fraction of the incident light that is absorbed by

A solution depends on the thickness of the light path

(cuvette), the molar concentration of the absorbing compound in

the solution and the chemical nature of absorbing compound

The relationship between concentration, length or thickness of the light path, and the light absorbed by a particular substance can be expressed mathematically in the following equation.

$$A = \log_{10} I_0/I = etc$$

\where, I_0 = Incident light

I = transmitted light

e = molar extinction coefficient

t = thickness of light path (cuvette)

c = molar concentration of the absorbing substance in solution

Experiment 6: Measurement og the absorbance of coloured compounds

Principle

Coloured compounds have their own characteristic absorbance spectra, hence

A mixture of two or more coloured compounds can be resolved by measuring each of them at the wavelength of its maximum absorption.

Method

Determine the absorption spectra of the three dyes provided by measuring the absorbance of a 1% solution of each of them at various wavelengths. Plot a graph of absorbance against wavelength of maximum absorbance for each dye.

Mix the solutions of the three dyes in a test tube and determine the absorption spectrum of the mixture. How does the mixing affect the absorption spectrum?

Experiment 7: Demonstration of Beer – Lambert Law

Prepare a range of concentrations of one of the dyes used in the absorbance of each solution and plot it against the concentration of the dye in each tube (in mg/ ml or ug/ ml).

Tube no	1	2	3	4	5	6	7
Bromophenol Blue (10mg/I) (ml)	1	2	4	5	5	8	10
Distilled Water(ml)	9	8	7	6	5	2	0
Absorbance							

Repeat the whole experiment with the dyes methyl orange, eosin and tartrazine.

Experiment 8: Spectrophotometric determination of inorganic phosphate.

Principle

Inorganic phosphate react with ammonium molybdate in an acidic medium to form phosphomolybdic acid. The addition of a reducing agent reduces the molybdenum in phosphomolybdic acid to give a blue colour.

Materials

Reagent A – Ammonium molybdate solution.

Grind 2g of ammonium molybdate into powder and dissolve it in 9 ml of cone. Sulphuric acid pour the solution into 200 ml of distilled water in a measuring

cylinder and make the volume to 310 ml with more distilled water. Store the solution in a plastic bottle.

Reagent B – 9% ascorbic acid solution

Dissolve 18g of L-ascorbic acid in a little quantity of distilled water in a 200ml star volumetric flask and make up top the mark with the distilled water.

(Note: the cone acid is to be added to water, not vice versa!!)

Reagent C (to be prepared just before use)

Mix 2 ml of the 9% ascorbic acid solution (Reagent B) with 8 ml of the ammonium molybdate solution (Reagent A).

Standard curve for inorganic phosphate determination

Prepare a series of phosphate solutions in test tubes by mixing 1mM Na_2HPO_4 and distilled water in various proportions as shown below. Add 2 ml of reagent C to each test tube and allow to stand for 10 min for colour development. Read the absorbance of the solution at 820 nm content of tube 1 (which has no phosphate) should serve as the blank,

Plot a graph of absorbance against concentration of phosphate.

Tube No	1	2	3	4	5	6	7	8	9	10	11
1mM NaH_2PO_4 (µl)	10	9	8	7	6	5	4	3	2	1	-
0.1M Na_2HPO_4 (ml)	-	1	2	3	4	5	6	7	8	9	10
pH	4.6	5.6	6.3	6.5	6.6	6.8	6.9	7.1	7.4	7.7	9.2

Experimental Biochemistry

Experiment 9: Spectrophotometric estimation of haemoglobin.

There are several method for the estimation of haemoglobin in a given blood sample, value of any method depends on its accuracy , speed and

convenience, this also depends on colour of haemoglobin pigment produced, the haemoglobin standard used and the instrument employed.

Three haemoglobin varieties are found in human blood :

1. Foetal haemoglobin ;
2. Inactive haemoglobin (methaemoglobin , carboxyhaemoglobin and sulphaemoglobin).

The substance used to produce the pigment desired should be capable of converting the inactive haemoglobin pigment into the chosen derivative so that they do not interfere with subsequentcolour matching with the standard. The pigment should be easy to prepare and should give stable, optically clear solution .the usual pigments are: oxyhaemoglobin, carboxyhaemoglobin , alkaline haernatin. Acid haematin and cyanmethaemoglobin.

Principle of Cyanmethaemoglobin method

Blood is diluted in a solution contain potassium cyanide and Potassium ferricyanide (Drabkin's solution). Methaemoglobin and carboxyhaemoglobin are rapidly converted into cyanmethaemoglobin (haemoglobin cyanide). The absorbance of the solution is then measured in a spectrophotometer at 540 nm.

Reagent

1. Blood of known haemoglobin concentration.
2. Physiological saline (o.9%Nacl).
3. Drabkin's solution.

Procedure

1. Dilute the standard blood provide as follows:

Standard (Hb Conc.=15g/100 ml)	Physiological saline	Final concentration (g/100 ml)
0.2	0.8	3.0
0.4	0.6	6.0
0.6	0.4	9.0
0.8	0.2	12.0
1.0	-	15.0

2. Pipette 0.002 ml of each dilution into 4ml Drabkin's solution in a test tube and mix well.

   Read the absorbance at 540 nm using 4ml Drabkin's solution as blank. Plot a graph of final concentration against absorbance.

3. Estimate the haemoglobin concentration of the three blood sample A, B and C provided by pipetting 0.02 ml of each absorbance of the test sample into 4ml of Drabkin's solution and reading the absorbance at 540 nm. Read off the haemoglobin concentrations of the test samples from the stand curve.

Experiment 10: Spectrophotometric estimation of protein content of dilute solutions.

Protein concentration of dilute solution in the region of 10 to 200 µg/ml can be determined by measuring the *difference* in absorbance at 215 and 225 nm according to the procedure murphy and kies[2]. All protein absorb strongly below 230 nm due to the presence of peptide bonds. A standard curve can be prepared by plotting a graph of the difference A) against various concentration of a standard protein (e.g. bovine serum albumin). As an approximation,

The protein concentration can be calculated from the following formula:

$$\text{Concentration } (\mu g/ml) = 144(A215min - A224nm)$$

Method

Prepare a standard curve by devolving various amounts of a standard protein (bovine serum albumin) in 5m M NaOH as shown in the following table. Measure the concentration of the two protein sample And B provided from your graph.

Absorbance at 215 11m Absorbance at 225 run Difference (11A)

Tube No.	1	2	3	4	5	6	7	8
Bovine serum Albumin (1 mg/ml) (µl)	0	10	20	50	80	100	150	200
5mMNaOH (µl)	100	990	980	950	920	900	850	800
Absorbance at 215nm								
Absorbance at 225nm								
Difference (A)								

Chapter Four

Carbohydrates

Carbohydrates are polyhydroxy-aldehydes and ketones. They have the general formula $C_2 (H_2 O)y$ and are called carbohydrate (or hydrates of carbon) because they contain hydrogen and oxygen in the same proportion as in water . however, certain compounds, which could be classified as carbohydrates by virtue of their properties, do not conform to the general formula, e.g. 2 –deoyribose, $C5H_{10}O4$. There are other compounds which conform to the general formula, but which are

not necessarily carbohydrates, e.g. formaldehyde, CH_2O, ethanoic acid, $C_2H_4O_2$ etc.

Carbohydrates aremore abundant in nature than all other organic compounds of biological importance. The most abundant carbohydrate is cellulose which is found in woody structures and fibres of plants. Starches are also abundant in grains, tubers and roots where they serve as food reserves for plants.

Classification of Carbohydrates

Carbohydrates may be classified into three main groups:

1. Monosarccharides	(i)	Hexoses: glucose, fructose, galactose.
	(ii)	Pentoses: ribose, ribulose, xylose.
2. Disaccharides		Sucrose (glucose – fructose) Maltose (glucose – glucose) Lactose (glucose-glucose).
3. Polysaccharides		Starch (polyglucose) Cellulose (polyglucose).

Experiment 11: Tests on Carbohydrates

(Note: Use 1 solutions of carbohydrates for all tests unless otherwise stated)

1. Molish's test.

Concentrated sulphuric acid hydrolyzes glycosidic bonds to give the monosaccharides which are then dehydrated to furfural and its derivatives. These combine with a naphtholnto give a purple complex.

To 1ml of test solution, add 3 drops of 1% a-nephthol in ethanol. Mix and pour 1ml of cone. H_2SO_4 slowly down the side of the tube to give a separate lower layer. A violet colour at the junction of the two layers indicates the presence of a carbohydrate. All carbohydrate except amino sugars give a positive reaction. This reaction depends on the condensation of a-naphthol with the furfural formed by the action of H_2SO_4on the carbohydrate.

2.Benedict's Test

Add 5 drops of the test solution to 2 ml of Benedict's qualitative reagent. Heat in boiling water bath for 5 minutes. If the test solution contain a reducing sugar, a yellow brick-red precipitate of cupric oxide should be observed. If the blue colour of the Benedict's reagent persists, add more drops of the test solution and boil the mixture. Repeat this with fructose and glucose as positive controls.

3.Barfoed's Test

Add 1 ml of test solution to 2 ml of Bargoed's reagent. Boil for 1 minute and allow to stand. Barfoed's reagent is weakly acidic and can be reduced by monosaccharides to give brick-red precipitate of cuprous oxide. This precipitate is less dense that that produced with Benedict's solution.

4. Fehling's Test

Mix equal volumes of Fehling' solutions A and B, add 5 drops of the test solution to mixture and boil. The appearance of a brick-red precipitate indicates the presence of reducing sugar.

5. Fermentation test

Yeast ferments some carbohydrates to give alcohol and carbon dioxide which can be detected by the use of a suitable apparatus. For a hexose sugar, the reaction is as follows:

$$C_6 H_{12} O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Mix equal volumes of the test solution and the yeast suspension and place fermentation tube. Incubate the tube, in an upright position, at 37° C for 1 hour (or two hours at room temperature) and observe the evolution of carbon dioxide. Note the sugars which are fermented and to what extent. What controls would you set up at the same time?

6. Bial's Test for Pentoses

When pentoses are heated with cone. HC!, a furfural is formed which condenses orcinol in the presence of ferric ions to give a blue-green colour. Prolonged heating of some hexoses may yield hydroxymenthyl furfural which also reacts with orcinol to give colour complexes.

Add about 2 ml of the test solution to 5 ml of bial's reagent intest tube and boil. A green colour indicates the presence of a pentose sugar. cool the tube, then add 2 ml of an alcohol and shake.

7. Seliwanoff's Test for Ketoses e.g. fructose

Ketoses are dehydrated more rapidly than aldoses to give furfural derivatives.

Add 2 drops of the test solution to 2 ml of seliwanoff's reagent and warm in a boiling water bath for 1 minute. The appearance of a deep red colour indicates the presence of ketose sugar.

8. Formation of Osazones

Compounds containing the –CO-CHOH- group form crystallingosazones with phenylhydrazine. The osazones crystals have characteristic shapes and melting points which assist in the identification of the reducing sugar.

CHO CH=N=NH-Ph

CHOH + H_2N-NH-Ph CHOH =H_2O

R R

Aldose Phenylhdrazine Phenylhydrazone

CH=N-NH-Ph CH=N-NH-Ph

CHOH C=N-NH-Ph +H_2N-Ph

R R +NH_3

Osazone

$+H_2N$-NH-PH

Glucose, fructose ands mannose, which have the same configuration on the carbon atoms 3, 4, 5 and 6, form the same osazone.

Acidify 5 ml of the sugar solution 10 drops of glacial acetic acid. Add 3 drops of phynlhydrazine solution and pich of solid sodium acetate. Warm in a water bath for 5 min with occasional shaking. Filter and warm the filtrate in the water bath for a further 20 minutes. Cool very slowly and examine the osazone crystals under the microscope. Draw the crystals.

9. Test for starch

Add 2 drops of iodine solution to 5ml of the starch solution provided. Note the colour changes. Repeat the test on freshly pieces of yam, cassava, cocoyam and potato.

Experiment 12: Quantitative estimate of glucose (Benedict's Method)

Standardize the Benedict's quantitative reagent provided by running a 0.5% solution of glucose contained in a burette into s conical flask containing a boiling mixture of 10 ml of Benedict's reagent, 2g anhydrous Na_2CO_3 and a glass bead, until the blue colour is completely discharged. The glass bead is added to prevent violent splashing of the reactants in the conical flash. Estimate the amount of glucose required to discharge the blue colour.

Determine the concentration of glucose in the sample of diabetic urine provided. Express you results as milligram glucose per 100 ml of urine.

Chapter five

Lipids

Lipids belong to a class of biomolecules which are generally insoluble in water but are soluble in non-polar, organic solvents wuch as acetone, benzene, chloroform, diethylther, petroleum ether, e.t,c, the class includes a heterogeneous

group of various structural types which may be classified on the basis of their chemical composition.

Classification

Lipids may be classified into the following groups:

1. Fatty acids. All organic acids are usually classified as fatty acids. They may be saturated or unsaturated.
2. Neutral fat. This groupd includes the mono-, di- and triacylglycerols. They are the most abundant lipids in animals tissues.
3. Phospholipids. This group includes phosphatidic acid phosphatidylethanolamine (cephalin), phosphatidylcholine (lecithin), phosphatidylserine, phosphatidylinoline,phosphatidylglycerol and diphosphatidyiglycerol (cardiolipin).
4. Sphingolipids, including sphingomyelins, cerebrosides and gangliosides.
5. Steroids, including sterols and steroid hormones.
6. Prostaglandins.
7. Waxes.

Experiment 13: Qualitative Tests on Lipids

Carry out the following tests on the lipids provided olive oil, groundnut oil, palm oil, coconut oil, margarine, glycerol, palmitic acid, stearic acid, phosphatidylcholine and cholesterol.

1. Compare the solubility of the lipid in water, ethanol, chloroform and diethyl ether by adding two drops of the lipid to 5 ml of solvent. Describe what you observe and state the class to which each lipid belongs.
2. Put one drop of a chloroform (or ether) solution of the lipid on a clean sheet

of filter paper and allow it to dry. Describe the grease spot which is formed on the paper and measure it diameter (in cm).

3. Heat 2 drops of the lipid with a pinch of dry potassium hydrogen sulphate ($KHSO_4$) until theacid fumes of acrolein are produced. Write an equation for the reaction.
4. Dissolve a little quantity of the lipid in diethyl ether and add a dilute, slightly alkaline solution of phenophathalein drop by drop. The discharge of the pink colour indicates the presence of free fatty acids. Note how many drops of phenolphthalein are required to discharge the colour.
5. Dissolve 5 drops of the lipid in 5ml of chloroform. Add Dam's iodine solution (or bromine water) drop by drop into the mixture, shaking after each addition, until the iodine bromine) fails to be decolourized. Note the number of drops required to reach the point. This is a rough measure of the degree of unsaturation of the lipid, and is directly proportional to its iodine value.
6. Liebermann-Burchard Test for Cholesterol: Carefully add 1 ml of acetic anhydride to 1 ml of a chloroform solution of cholesterol (mg/ml), followed by 2 drops of conc, sulphuric acid. Note the change in colour. An anhydride reacts with cholesterol solution to produce a characteristic blue-green colour.
7. Salkowski Test for Cholesterol: When sulphuric acid is added to a chloroform solution of cholesterol, characteristic colour develop in the two layers. Carefully add 1 ml of conc. Sulpuric acid to 1 ml of a chloroform solution of cholesterol to form two layers. Describe the colours produced.

Experiment 14: Determination of plasma cholesterol

Cholesterol is widely distributed in animal tissues.Its concentration in the blood cell remain constant, even in disease. However, the plasma concentration of cholesterol is sensitive changes in the diet, state of health, etc. the determination of plasma cholesterol is based or Liebermann – Burchard reaction. Plasma proteins are first precipitated by a mixture of ether ethanol which also remove cholesterol and other lipids at the same time. The organic solvent removed by evaporation and the dry residue is dissolved in chloroform.

Pipette 0.2 ml of plasma into a centrifuge tube containing 2 ml of ether and 8 ml of absolute ethanol. Cork the test tube with a dry stopper and shake vigorously for two minutes. Centrifuge the test tube with its content on the bench centrifuge and carefully decent the clear supernatant into a hot residue in three separate washings using 1 ml chloroform each time. Combine these in a stopped, graduated glass cylinder and make it up to 5ml with chloroform.

In another set of graduated glass cylinder, set up a series of cholesterol standards and a b containing 5 ml of chloroform.

Add 2 ml of acetic anhydride to each tube, followed by 3 drops of cone. Sulphuric acid. Mix the contents by inverting the cylinder several times, then stand them in a dark cupboard for minutes. Compare the test and solution by reading the absorbance in spectrophotometer at 680nm.

(Note: It is essential to use dry glassware in carrying out all the tests).

Experiment 15: Iodine Value of Lipids

The iodine number of a lipid is defined as the number of grams of iodine which can absorbed by 100 grams of the lipid. It is a measure of the degree of unsaturation of the fatty acid or iodine monochloride is used for this test. The iodine equivalent of the bromine used in the reaction is then obtained by adding potassium iodine to the mixture and titrating with a standard solution of potassium thiosulphate.

Determine the iodine equivalent of the blank by pipetting 5ml of chloroform into a conic flask. Add 5 ml of Dam's iodine from a burette. Cork and shake thoroughly, then place the flask in a dark cupboard for 10 minutes. Add 5 ml of 10% potassium iodine solution followed by 20 ml of water. Mix thoroughly and titrate the liberated iodine with the standard thiosulphate provided. Towards the end of the titration, i.e. when the solution has a pale straw colour, add ml of a starch solution and continue the titration until the blue colour disappears. The conical flask must be shaken thoroughly throughout the titration to ensure that all the iodine is removed from the chloroform layer.

Repeat the entire procedure using, in place of chloroform, 5 ml of a 0.5% chloroform solution of (a) olive oil, (b) groundnut oil (c) coconut oil, (d) margarine and (e) glycerol.

Calculate the iodine values of the various lipids.

The difference between the blank and test reading (BI –T) gives the number of mls of 0.1 thiosulphate needed to react with the equivalent volume of iodine. The amount of lipid taken 0.025g and 1 litre of 0.1m iodine contains 12.7g of iodine. Therefore, the iodine number can be calculated as follows:

$$\text{Iodine number} = (BI - T) \times 12.7/1000 \times 100/0.025$$

$$= (bi - t) \times 50.8.$$

Calculations

-C = C – ICI → -CH – CH –

I CI

EXCESS ICI + KI → KCI + I_2

-CH = CH - + Br → -CH(Br) – CH (Br)

EXCESS Br_2 + 2KI – 2KBr + I_2

$2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2e$

$I_2 + 2e \rightarrow 2I$

$2S_2O_3^{2} + I_2 \rightarrow S_4O_6^{2-} + 2R$

Let (BI – T)= Y ml

2 moles of thiosuulphate ($2S_2O_3^{2}$) = I moles iodine (I_2)

1 litre of 0.1M $2S_2O_3^{2}$ will contain0.1y/1000 moles.

yml of 0.1m $2S_2O_3^{2}$ will contain 0.1y/1000 moles

2 moles of $2S_2O_3^{2}$) → 1 moles of I_2

1 moles of $2S_2O_3^{2}$) → ½ moles of I_2

.. 0.1y/1000 moles of $2S_2O_3^{2}$ → ½ x 0.1y/1000 1 moles of I_2

= ½ x0.1y/1000 x 254g of I_2

1 mole of lipid used =5ml of 0.5% solution

I.e.100ml of lipid contains 0.5g l

and 5 ml of lipid contains 0.5/100*5g=0.025g

0.025g of lipid absorbs1\2*0.1ly/1000*254*100/0.025

=(1\2*10*254y)\25

=50.8y

Experiment 16: Acid value of Lipids

Freshly prepared animal and vegetable oils contain very little free fatty acids (F.F.A.). However, during storage the oils may become rancid due to peroxide formation at the double bonds as a result of oxidation by atmospheric oxygen and hydrolysis by microorganisms. This results in the liberation of free fatty acids. With low grade vegetable oils, as much as 30% of the oil may be degraded in a very short time. The amount of free fatty acid present is, therefore, an indication of the age and quality of the oil.

The acid value is defined as the number of milligrams of potassium hydroxide required to neutralize the free acid present in 1 gram of the lipid.

Weigh out exactly 10g of the lipid add 50 ml of chloroform. Add 1 ml of phenolphthalcin solution, mix thoroughly and titrate with 0.1M KOH from a burette until the faint pink colour persists for 20 to 30 seconds. Notes the volume of alkali used and calculate the acid value of the lipid provided.

Experiment 17: Saponification Value of Lipids

Principle

When glyceryl esters are refluxed with alkali they give glycerol and the potassium salts of the fatty acids (soaps). The saponification value is the number of milligrammes of KOH required to neutralize the fatty acids resulting from the complete hydrolysis of 1 g of the lipid. The saponification value gives an indication of the nature of the fatty acids in the lipid since the longer the carbon chain the less acid is liberated per gram of lipid hydrolysed .

Procedure

Weigh 1 g of the lipid in a beaker and dissolve it in 3 ml of a mixture of ethanol ad ether (1:1 v/v). quantitatively transfer the contents of the beaker to a 250 ml conical flask by rinsing the beaker three times with more of the solvent then add 25 ml of 0.5m alcoholic KOH and attach to a reflux condenser. Set up another reflux

condenser as blank with everything present except the lipid and heat both flasks on a boiling water bath for 30 min. Leave to cool to room temperature and titrate 0.5 M HCI using phenolphthalein as indicator,

The differences between the blank and test reading gives the number of mls of 0.5M KOH required to saponify 1 g of the lipid.

CHAPTER SIX

AMION ACIDS AND PROTEIN

Experiment 18: Test on amino acid protein

1. Ninhydrin reaction:

(a) Spot the solutions of the amino acids provided on a filter paper, add a drop of ninhydrin reagent to each spot and hold the paper over a Bunsen flame to dry. Note and comment on, the different colours produced.

(b) Place 1 ml of a neutral solution of each amino acid in a test tube; add 5 drops of ninhydrin solution and boil for 1 min. note the colours produced. Repeat the test on serial dilutions of glycine to determine the limits of sensitivity of the reaction.

2. Xanthoproteic reaction

Add 1 ml of conc. Nitric acid to 1 ml of the amino acid solution. Warm the mixture in a boiling water bath and allow to cool. Note the formation of precipitates and colours. Add sufficient 40% NaOH to make the solution strongly alkaline. A yellow colour in acid solution which turns bright

orange in alkali indicates the presence of one or more of the aromatic amino acids tryptophan.

3. Millon's reaction

Add 5 drops of Millon's reagent to 1 ml of the test solution and warm the mixture in a boiling water bath for 10 min cool to room temperature and add 5 drop of sodium nitrate solution. The formation of a brick-red colour indicates the presence of a phenolic amino acid, i.e. tyrosine or its derivatives. Phenois and naphthols also give positive results.

4. Violet ring test

Add 2 ml of glacial acetic acid that has been exposed to light to 2 ml of the test solution. Pour 2 ml of conc. Sulphuric acid carefully down the sides of the test tube in a sloping position so as to form two layers. A violet ring at the interface indicates the present of tryptophan.

5. Ehrlich's test

Add 2 ml of Ehrlich's reagent to 0.5 ml of the test solution Note the colours formed.

6 Nitroprusside test

This group react with sodium nitroprusside ($Na_2Fe(CN)_5NO$) in the presence of excess ammonia to give a red colour . Mix 0.5 ml of a fresh solution of sodium nitroproside with 2 ml of the test solution .add 0.5 ml ammonium hydroxide.

7. Sagakuchi reaction:

The guanidine group of arginine reacts with ⌂–napphthol and an oxidizing agent such bromine water to give a red colour. Mix 1 ml of 40% sodium hydroxide with 3 ml arginine solution and add2 drops of ⌂–naphthol. Mix thoroughly and add about 4 drops of bromine water. Not the colour formed.

8. Biuret test

To 2 ml of the test solution, add 5 drops of biuret reagent. Mix thoroughly and note colours formed. This is a test for the presence of two or more peptide bonds in solution. The intensity of the colour produced in proportional to the number of peptide bonds present.

Experiment 19: Determination of protein: Biuret Method

The biuret reagent detects the presence of peptide bonds in solution and can therefore be used for the quantitative determination of proteins in solutions. When compounds contain peptide bonds are treated with alkaline copper sulphate, a purple coloured complex is obtained. The intensity of the colour is proportional to the number of peptide bonds in solution.

Procedure

You are provided with two protein sample A and B, e.g. serum (or plasma) and rat liver homogenate. Place the following reagent into various test tubes:

	Test (ml)	Blank (ml)
Test sample	0.1	-
3 Sodium deoxycholate	0.4	0.4
Distilled water	0.5	0.6
Biuret reagent	4.0	4.0

Mix the contents of the test tubes by gentle shaking and let stand for 20 minutes for colour development. Then read the absorbance of each test solution against the blank at 540 nm.

You are also provided with a standard solution of albumin (bovine serum albumin) with a concentration of 10 mg/ml. prepare a standard curve using various concentrations of the albumin. This can be done by replacing the test sample in the

table above with various amount, i.e. 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml, of the albumin. Adjust the amount of distilled water accordingly in order to keep the volume constant at 5ml. read the absorbance of the solution in each tube after 20 minutes and plot a graph of absorbance again concetrationof albumin. Estimate the concentrations of the protein samples provided from your graph.

Experiment 20: Determination of Protein: Lowry's Method

The amount of protein found in most biological membranes is usually very low and is best estimated using the procedure of Lowry et al[3]. The colour reagent used in this method is a phosphor-18- molybdictungstic complex (a mixture of several molecular forms such as $3H_2O.P_2O_5$, $9M_0O_3$ AND $3H_2OP_2O_5$, $8M_0O_3$) which can be reduced by phenol groups giving a blue colour at alkaline Ph, Tyrosine (and tryptophan) present in the protein are responsible for the reduction of the phosphomolybdictungstic complex. This complex also called phenol reagent, is very unstable and decomposes in alkaline solutions. Since it interacts with tyrosine only at alkaline pH, an excess of the reagent must be added in order to get complete reaction. However, these high concentrations of the phosphomolybdictungstic acid can give turbidity due to the formation of insoluble salts. Folin and Ciocalteu reagent, they also added some bromine water to maintain the phospomolybdictungestic reagent oxidized during storage.

Lowery et al. found that the pre-treatment of the protein sample with alkaline copper markedly increased the colour developed in the reduction reaction of the phospomolybdictungestic reagent. In their assay system they also added a mixture of Na_2CO_3 – NaOH to buffer the pH around 10 and to neutralize the phosphoric acid produced by the degradation of the phospomolybdictungestic complex at alkaline pH.

REAGENTS

(i) Reagent a (2% Na_2CO_3) and 4g of sodium hydroxide pellets a little quantity of distilled water in a 1 litre standard volumetric flask and made it up to the mark with distilled water.

(ii) Reagent B (2% Sodium Potassium Tartrate)

Dissolve 20g of sodium potassium tartrate, $NaKC_4O_6.4H_2O$, in distilled water in a 1 litre standard volumetric flask and make it up to the mark.

(iii) Reagent C (1% Copper Sulphate)

Dissolve 10g of copper sulphate, $CUSO_4.5H_2O$, in distilled water in litre standard volumetric flask and make it up to the mark.

(iv) Reagent D (Alkaline Copper Solution)

This should be prepared fresh, just before use, by maxing 50 ml reagent. A with 0.5 ml reagent B and 0.5 ml of reagent c. Add the tartrate (reagent B) first to prevent the solution from growing cloudy.

(v) Reagent E (Folin –Ciocalteu Reagent)

This reagent is commercially available in 2M solutions and contains phospomolybdictungestic complex, lithium sulphate and bromine water. It should be diluted to 1M concentration on the day of the assay. If the commercial preparation cannot be procured, the instructions for preparing the reagent from it active ingredients can be found in the Appendix.

- **Method**

Place the following reagents into various test tubes

	Test	Blank
Test sample	10µl	-
Distilled water	590µl	600µl
Reagent D	3.0ml	3.0ml

Mix and wait for 10 minutes

Reagent E	0.3ml	0.3ml

Mix rapidly!!

The rapid mixing after addition or reagent E is important since reagent E is unstable at alkaline pH and can be destroyed before it has reacted with the protein copper complex.

You are also provided with a standard solution of albumin (bovine serum albumin) with concentration of 10mg/ml. Prepare a standard curve using various

concentrations of albumin, this can be done by replacing the test sample with various amounts of the albumin shown below. Adjust the amount of distilled water accordingly in order to keep the total volume constant. Read the absorbance of the solution in each tube after 30 minutes and plot a graph absorbance against concentration of albumin. Estimate the concentrations of protein sample provided from your graph.

Tube No	1	2	3	4	5	6	7
Bovine, Serum, Albumin (10 mg/ml (µl)	0	25	50	75	100	150	200
Distilled Water (µl)	1000	975	950	925	900	850	800
Reagent D (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Mix and wait for 10 minutes

Reagent D (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0

Read absorbance at 750 run after 30 minutes.

Experiment 21: Formol titration of ammonia and amino acids.

Cationic groups in amino acids and ammonium salts react with excess alkali to produce free amino groups. These can combine with excess formaldehyde to produce substituted amino alcohols which are neutral to phenolphthalein.

$R.NH_3^+ \rightarrow R.NH_2 + H^+$ (i)

$R.NH_2 + HCHO \rightarrow R.NH.CH_2OH$ (ii)

$H+ + OH \rightarrow H_2O$ (iii)

(from alkali)

In formol titration, the pH of the amino acid solution and that of the formaldehyde are separately adjusted to 8.5. the solutions of formaldehyde and

amino acid are then mixed and the resulting solution Is titrated back to pH 8.5 the amount of alkali used is approximately equivalent to the quantity of NH_3 ion.

Procedure

1. Measure 40 ml of commercial formalin into a cylinder. Add 80 ml of distilled water and 8 drops of phenolphthalein. Titrate the mixture with 0.1M NaOH to faint pink en – point. This to neutralize the free acids present in the formalin.

2. Estimate the ammonia and amino acid nitrogen present inyour urine. Neutralize 20 ml of the urine as described in 1 above. Now add to the urine 20 ml of neutralized formaldehyde solution. Titrate the mixture with 0.1M NaOH back to the faint pink colour.

Chapter Seven
Nucleic Acids

Living organisms are unique in the sense that they reproduce exact copies of themselves and undergo mutation.

Deoxyribonucleic acid (DNA) is the primary genetic material. It is a long, threadlike, macromolecule made up of a large number of deoxyribonucieotides.

The purine and pyrimidine base of DNA carry genetic information, while the sugar and phosphate molecules serve as the backbone in maintaining the structure.

DNA contains four kinds of bases: two purine and two pyrimidine. The purines are adenine (a0 and guanine (G) while the pyrimidine are thymine (T) and cytosine (C).

Experiments which led to the discovery of the genetic role of DNA were started by Griffith in 1982. He worked with the bacterium Diplococcus pneumonia, usually referred to as pneumococcus, the causative organism of pneumonia. This bacterium usually exists in two forms: smooth (S) and rough ®, based on the type of colonies they form. S cells are surrounded by a smooth polysaccharide capsule and are pathogenic. R cells have no capsules and are non-pathogenic. There are also at least two types of S bacteria, namely IIS and IIIS, which can be distinguished on the basis of the chemical composition of their polysaccharide capsules. The ability to produce each of capsule can be inherited by subsequent generations of bacteria. However, a given S type of pneumococcus can undergo mutation to produce a genetic variant. About one of 10 million S bacteria can be transformed in this way to produce a colony of R cells and vice versa.

Griffith injected mice with a mixture of live R and heat killed S bacteria and found that the mice died, where either live R or heat-killed S bacteria injected alone were not lethal to the mice. The blood of the dead mice, on examination, was found to contain live S bacteria. It was concluded that the heat-killed S bacteria had somehow transformed the liver R bacteria into live S bacteria. The transforming principle was subsequently inditified in 1944 by Avery, MacLeod and McCarthy as DNA. The experimental basis for their conclusion can be summarized as follows:

(1) The purified, highly active transforming principle gave an elemental chemical analysis which was similar to that of DNA.
(2) The optical, ultracetrifugal, diffusion and electrohoretic properties of the purified material were also similar of those of DNA.
(3) Trypsin and chymotrypsin didi not affect the transforming activity of the material, thereby showing that the material is not protein in nature.

(4) Ribonuclease had no effect on the transforming principle where as its transforming activity was lost following the addition of deoxyribonuclease. This showed conclusively that the transforming material is DNA.

In 1953, Watson and Crick deduced the three- dimensional structure of DNA and immediately inferred its mechanism of replication. They analyzed x-ray diffraction photographs of DNA fibres taken by Franklin and Wilkins and deduced a structural model which eventually proved to be correct. The important features of their model are:

(1) The molecule consists of two helical polynucleotide chains which are coiled around a common axis. The chains run in opposite directions.
(2) The purine and pyrimidine based are on the inside of the helix while the phosphate and deoxyribose units are on the outside. The plans of the bases are perpendicular to the axis of the helix. The planes of the sugars are lsmost at right angles to those of the bases.
(3) The diameter of the helix is 20Ă. Adjacent bases are separated by 3.4Ă along the helix axis and related by a rotation of 36 degrees. The fore, the helical structure repeats after 10 residues on each chain, i.e. at interval of 34Ă.
(4) The two chains are held together by hydrogen bonds between pairs of bases. Adenine is always paired with thymine while guanine is always paired with cytosine.
(5) The sequence of bases along a polynucleotide chain is nt restricted inany way. However, this sequence of bases carried genetic information.

The entire DNA molecule is usually very long. For example, the E. coli chromosome consist of a double helical DNA with 4 million base pairs. It has a mass of 2.6×10^6 daltons.

Experiment 22: isolation of ribonucleic acid (RNA) from yeast cells.

Ribonucleic acid (RNA can be obtained from yeast cells by extracting a homogenate of yeast with phenol. The phenol solution disrupts hydrogen bonding in the RNA molecule and denatuyres the proteins present in the cell. The resulting

turbid suspension is then centrifuged to obtain two phases; the lower phenol phase and the upper aqueous phase which contains carbohydrates and RNA Denatured proteins, which are found in both phases, can be removed by centrifugation. The RNA can then be precipitated with absolute ethanol.

Materials: Dried yeast, Phenol solution, Potassium acetate (200 gil, pH 5.0), Absolute ethanol, Diethyl etthe

Methods

Suspend 30 g of dried yeast in 120 ml of distilled water that has been heated to 37° C . Leave the suspension at this temperature for 15 min then add 160 ml of concentrated phenol solution. Care should be taken in handling phenol because it is corrosive! Stir the suspension with a glass rod for 30 min at room temperature, then centrifuge at with a glass rod for 30 min at room temperature, then centrifuge at 3,000g for 15 min at 4C in order to break the emulsion. Carefully remove the upper aqueous phase pipette and centrifuge at 10,000g for 5 min at 4^{0}C to sediment the denatured protein. Add potassium acetate to the supernatant to a final concentration of 20 g/l and precipitate the RNA by adding 2 volumes of ethanol. Cool the solution in an ice bucket and leave to stand for 1 hour. Collect the precipitate by centrifuging at 2,000g for 5 min at 4^{0}C. wash the RNA with ethanol/water (3.1 v/v), then with absolute ethanol and , finally with diethyl either. Dry at room temperature and record the weight of the RNA obtained.

Experiment 23: Isolation of deoxyribonucleic acid (DNA) from spleen.

Deoxyribonucleic acid (DNA) is foud almost exlusively in the nucleus of most animal and plant cells. It has also been isolated in some organelles such as mitrochondria and chloroplasts. How eve, the amount present in some tissues is quite small hence isolation of DNA from these tissues can be very difficult. Some tissues also contain considerale amounts of deoxyribonuclease (DNA asc) activity hence the DNA in these tissues are rapidly degraded into smaller fragments. A good source of DNA should have sufficient quantity of the material as well as a low DNAasc activity. The best sources of DNA are the thymus and spleen.

DNA is associated with a nucleoprotein which is soluble in water and in solutions of high ionic strength. It is insoluble in solutions of low ionic strength (0.05 – 0.25mM) and use is made of this property in the isolation DNA.

The tissue from which DNA is to be extracted is homogenized in isotonic saline buffered with sodium citrate, pH 7.0. in this medium the deoxynucleoprotein is precipitated while other macromolecules are solution. The nucleoprotein can then be dissolved in 2M NaCL. The protein can then be removed by treatment with a mixture of chloroform alcohol and DNA precipitated with ethanol.

Materials: spleen (from cow, sheep or ckicken); 0.15 M NaCl (buffered with 0.015 M sodium citrate, pH 7.0). 2 M Nacl,Chloroform/Amyl alcohol (6:1 v/v), Absolute ethanol, Diethyl ether.

Method

Cut 50g of spleen with a pair of fine scissors in a beaker and homogenize with 200 ml buffered saline for 1 min in Potter Elvehjem glass homogenizer equipped with a Teflon pest Centrifuge the homogenate at 5,000g for 15 min and re-homogenise the precipitate in 200 ml buffered saline. Discard the supernatant and suspend the combined sediments in 2 M NaCI to final volume of 1 litre. Remove any sediments in 2 M NaCI to final volume of distilled water. Stir the thick precipitate with glass rod and leave it to stand in the beaker for 30 min. remove any liquid in the beaker with filter paper.

Dissolve the mixture of DNA and protein in 100 ml of 2 M NaCI, add an equal volume of the chloroform/amyl alcohol mixture (6:1v/v) and blend for 30 seconds. Centrifuge the emulsion at 5,000g for 15 min and collect the upper aqueous layer containing the DNA into a 500 ml beak Repeat this procedure twice more by adding the organic solvent and centrifuging. Each time collect the supernatant into the 500 ml beaker.

Precipitate the DNA by slowly stirring 2 volumes of ethanol at 4C with the supernatant fraction and collect the thick mass with a stirring rod.

Remove any liquid with filter and collect the thick mass with a stirring rod. Remove any liquid with filter paper and weigh the dry DNA.

Experiment 24: estimation of RNA by the orcino reaction.

The orcinol reaction is a general reaction for the detection of pentose sugars (see page 22). The reaction depends on the formation of furfural when the pentose is heated with concentrate hydrocholoric acid. Orcinol reacts with the furfural in the presence of ferric chloride to give green colour which can be used to estimate th concentration of the RNA in the medium.

Method

You are provided with two samples of ribonucleic acid labeled A and B. mix 2 ml of each of the samples with 3 ml of orcinol reagent. Heat the mixture on a boiling water bath for 20 minutes cool and determine the absorbance at 665 nm against an orcinol blank. Repeat the experiment with the standard solution of ribonucleic acid provided and estimate the concentration of RNA in the unknown samples.

Experiment 25: Isolation of Plasmids by the method of Birnboim and Doly.

Plasmids are self-replication, extra chromosomal, DNA molecules found in bacteria. They are double-stranded, closed circular molecules ranging in size from 1 kb to 200 kb. Plasmids are very useful in DNA cloning, the most widely used cloning vector is the plasmid pBR322 which contains ampicillin and tetracycline resistant genes. It is a small plasmid with 4.3 kilo bases and it has single site for many restriction enzymes (see Fig. 7.1).

Plasmids can eisolated by a ini-cal procedure describe by Birnboim and Doly[5]. This procedure is based on the differential behavior of closed circular, open circular and linear DNA molecules under alkaline conditions. High alkaline pH bring about the separation of complimentary strands of DNA in open circular and linear duplexes but not in closed circular DNA. There, exposing the crude Iysate to a pH of 12, followed by neutralization results in the formation of insoluble

aggregates of denatured DNA which can be removed by centrifugation. Plasmid DNA can then be removed from the supernatant by ethanol precipitation.

Fig. 7.1: A restriction map of bacterial plasmid pBR 322.

Reagents

Reagents A:	Lysozyme	10 mg
	Glucose (20, w/v)	0.23 ml
	NaEDTA (0.1M)	0.5 ml
	Tris-HCI buffer, pH 8.0(1M)	0.125 ml
	Distilled Water	4.1 ml
Reagent B:	Sodium chloride (2 M)	1 ml
	SDS (10, w/v)	1 ml
	Distilled Water	8 ml
Reagent C:	Sodium acetate, pH 4.8 (3M)	4.8 ml
Reagent D:	Sodium acetate (0.1M) in Tris-HCL(0.5 M), pH 8.0	
Reagent E:	Tris-HCI (10 mM) plus NaEDT A (mM)	

Method

Pipette 1.5 ml of an overnight bacterial culture into an Eppendorf tube and harvest the cells by a brief centrifugation. Re- suspend in 0.1 ml of Reagent A and leave onice for 30 min. Add 0.2 ml of reagent B, vortx gently and leave on ice for 5 min. add 0.15 ml of reagent C, mix by inversion and leave on ice for 45 to 60 min, then centrifugefor 5 min and transfer the supernatant as much as possible into another Eppendorf tube.

Add 1 ml of cold ethanol (95%), mix and leave at 20C for 30 min. collect the DNA by centrifuging in a microcentrifuge for 10 min and re-suspend the pellet in 0.1 ml of Reagent D. precipitate the DNA again using cold ethanol (95%).

Vacuum dry the sample to remove ethanol and dissolve the residue in 20 µl of Reagent E. the resulting solution contains about 20 µg DNA.

Experiment 26: Splicing of DNA with restriction enzymes.

DNA can be cut at various points in the molecule with restriction enzymes. Restriction enzymes are endonucleases which cleave phosphodiester bonds of double-stranded DNA at specific points. There are over 600 restriction enzymes which have been classified into three types (I, II and III). Type II enzymes, which Recognise specific palindromic sequences, are widely used in cutting DNA molecules for gene cloning. the enzymes have been isolated from various species of bacteria and are referred to by the first two letters of the name of the bacterial species. For example, the restriction endonuclease Eco RI was the first restriction enzyme isolated from Escherichia coli RY 13. The table below gives information on some restriction enzymes.

Method

Pipette the following into a sterile Eppendorf tube:

DNA (1 mg/ml)	10 µ
10x reaction buffer	2 µl
Restriction enzyme (10 units)	2 µl
Distilled Water	6 µ
Otal volume	20 µl

Mix the contents by tapping the tuybe and by centrifuging in a microcentrifuge tube for 30 seconds. Incubate the tube for 1 hour at 37C. Transfer the tube to a water bath kept at 70C and leave it for 10 min. Pipette 5 µl of the mixture into another Eppendorf tube and add 5 µ loading buffer to it. Run electrophoresis on agarose gel to determine whether or not the DNA has been cleaved by restriction enzymes.

CHAPTER EIGHT

Enzymes

Chemical reactions in biological systems are catalyzed by specific proteins called enzyme. Most reactions that take place within the body of an organism do not normally occur at a fast rate in the absence of enzymes. Enzymes accelerate the reactions by factors of at least a million. They are highly specific in their activity with respect to the reactions catalyzed and their choice of substrates. The can also be extracted from the tissues where they occur.

Most enzymes are simple or conjugated proteins. They make up the largest andmost specialized group of proteins within the living organism

A thorough knowledge of basic enzymology (enzyme kinetics, feedback inhibition, regulation of metabolic pathways, active sites, etc) is needed to fully understand the metabolic processes in biological systems and their regulation.

Enzymes are also useful for diagnostic purposes. There are some disease conditions in which body organs/tissues are damaged, with the result that some enzymes are released into the bloodstream, thereby giving rise to elevated levels of these enzymes in the serum. On analysis of the patient's serum for the various enzymes, their altered patterns or activities give information about the organs affected and the typ f disase, i.e. they aid in diagnosis. An example is myocardial infarction (death of heart tissue due to interruption of blood supply to it). Other examples include kidney diseases, muscular dystrophy. Enzymes have also proved to be useful tools in biochemical research. For example, in the elucidation of the amino acid sequence of proteins, use is made of the high degree of specificity or some enzymes. Trypsin, a proteolytic enzyme found in the intestinal juice, is highly specific in its action. It cuts polypeptide chains on the carbozyl (COOH) side of the basic amino acids arginine and lysine. Chymotrypsin, on the other hand, preferentially attacks the carboxyl side of aromatic and bulky polar residues. The action of both enzymes on polypeptide chain yield peptides which overlap, hence the primary sequence of the protein can be determined.

Importance of Enzymes

(i) For an organism to survive, it requiresa steady supply of energy in the form of ATP. This is required for many on-going reactions within the living syste, e.g. muscle contraction and biosynthetic processes. About 1/3 of the ATP produced at rest is used to maintain the Na/K+ gradient across the plasma membrane. This gradient is of vital importance

- For controlling cell volume
- For maintaining excitability of nerve and muscle, and
- For driving the active transport of sugarsan amino acids.

Some metabolic processes generate energy in the form of ATP, e.g. glycolysis, TCA cycle and –Oxidation of fatty acids. All the steps in these metabolic pathways are catalysed by enzymes. Enzymes are also required for the hydrolysis of food in the digestive system before absorption and ATP production.

(ii) Organisms need to synthesize new molecules to replace old ones. Pathways such as fatty acid synthesis, gluconeogenesis, protein synthesis, etc, are also catalyzed by enzymes.

(iii) Organisms need to get rid of by-products of metabolic activities, such as urea. The pathway by which this is achieved also involves enzymes. In getting rid of xenobiotics (food additives etc.),enzymes are needed to catalyze their conversion to less toxic compounds which can then be eliminated from the body.

(iv) Enzymes determine the survival rate of the organism. They are important in development, growth and remodeling. Controlled tissue degradation, e.g. metamorphosis (of the tadpole to the adult, and involution of the tail in the adult toad.

Classification of Enzymes

Enzymes are usually classified into six main groups.

1. Oxidoreductases.
2. Transferases.
3. Hydrolases.

4. Lyases.
5. Isomerases.
6. Ligases (Synthetases).

Oxidoreductases are responsible for oxidation and reduction processes (redox reactions). Transferases are responsible for the transfer of functional groups. Hydrolases participate in hydrolysis reactions. Lyases catalyse isomerization reactions, e.g. racemases and epimerizes. Ligaese are involved in the formation of bonds with ATP cleavage.

Experiment 28: Determination of the activity of urinary amylase,

A known quantity of soluble starch is digested with varying amounts of urine for 30 minutes at 37C. After cooling, iodine is added to determine the smallest quantity of urine that will completely digest starch to a point at which it no longer gives a blue colour.

Reagents:

Stock Solutions: 2% Starch solution (2g/10ml)

10 sodium chloride solution (10g/10 ml)

0.02M Iodine.

Working Solution: 0.1 % starch solution (5 ml of 2% starch made up to 100 ml).

Procedure

Prepare a series of tubes containing various concentration of freshly voided urine as shown blow. Add 2 ml of 1% starch solution to each tube, incubate ate 37 C for 30 minutes, then cool at room temperature, or by placing the tubes in cold tap water, then cool at room temperature, or by placing the tubes I cold tap water, then add a few drops of iodine to test for the presence of starch that starch that has not been hydrolysed by the action of the amylase calculate the diastolic inde. This is the number of mls of 0.1% starched digested by 1 ml of urine at 37 C in 30 minutes.

Repeat the experiment and add 2 drops of 10% sodium chloride solution to each tube before incubation. Comment on the difference in enzyme activity in this case

Tube No	1	2	3	4	5	6	7	8	9	10
Undiluted urine (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Distilled water	0. 0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0.1 Starch (ml)	2	2	2	2	2	2	2	2	2	2

Tube No	11	12	13	14	15	16	17	18	19	20
1/10 diluted urine (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Distilled water (ml)	0. 0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0.1 %Starch (ml)	2	2	2	2	2	2	2	2	2	2

Calculations

Xml of urine contains 2 mg of amaylase; then 1 ml of urine will contain 2/x mg of amylase. If the dilution of the urine is, say, 1:4, then amylase activity is 2/0.25 or 8 units/ ml.

Experiment 29 : Determination of the Adenosine Triphosphatase activities in rat brain.

Various types of adenosine triphosphatase (ATPase) activities are found in biological+ membranes. A Na^+, K^+ – activated, Mg^{2+} - dependent ATPase was first reported in crab nerve by Skou in 1957. Subsequent work has established the nearly ubiquitous occurrence of a similar Na^+, K^+ – ATPase in most of the biological tissues that have been studied, including the erythrocyte. Such studies have also established a striking correlation between the properties of the enzymatic system and feature of active transport of monovalent cations.

The existence of a Ca^{2+}stimulated, Mg dependent ATPase (C^{2+}, Mg^{2+}ATPase) in the erythrocyte member of workers who noted that the simultaneous presence of d $Mg^{2+}$and C^{2+} increases the total ATP hydrolysis in

isolated membranes from human erythrocytes, and at the same time inhibits the Na^+, K^+ - ATPase. The Ca^{2+}, Mg^{2+}-ATPase of the erythrocyte membrane is now generally accepted as the enzyme responsible for maintaining the concentration of Ca^2 + inside the cell at levels much lower than in the surrounding medium. The concentration of Ca^{2+} inside the erythrocytes is usually about 1 µM or less. On the other hand, the Ca^{2+} concentration in the surrounding plasma is about 1 –mM. Ca^{2+} penetrates across the membrane erythrocytes into the cytoplasm by passive diffusion. The efflux of Ca^{2+}, however, is mediated by n active process which Ca^{2+} out using ATPases is shown below:

Table 8.1: Constituents of the reaction media for the assay of Mg-ATPase, Ca - ATPases and Na , K –ATPase

	Mg^{2+}ATPase (mM)	Ca^{2+} -ATPases (mM)	Na^+ , K^+ –ATPase (mM)
NaCI	100	-	100
KCI	20	120	20
$MgCI_2$	5	5	5
$CaCI_2$	-	0.2	-
EDTA	0.5	-	0.5
Tris Buffer, pH 7.4	30	30	30
ATP	1	1	1
Ouabain	0.05	-	-

ATP, Adenosine triphosphate; Tris, Tris, Tris (hydroxymethyl) aminomethane; EDTA, Ethylene diamineteraacetic acid.

Tube (µI)	A	B	ENZYME BLANK	ATP Blank
Buffer(µI)	500	500	500	500
$CaCI_2$	20	120	20	
EDTA (µI)	5	5	5	
Distilled Water (µI)	-	0.2	-	
Rat brain homogenate	10	10	-	10

Mix and pre-incubate for 3 minutes at 37 C, then add ATP.

10mM ATP (μI)	100	100	100	-

Incubate at 37C for 30 minutes (with constant shaking), then stop the reaction with 0.2 mI of 5 sodium dodecyl sulphate (SDS). Estimate the amount of inorganic phosphate produced using the procedure in previous experiment.

Chapter

Vitamins

Vitamins are essential constituents of the diet which are required for the maintenance of the body's normal metabolism. Although they are present in minute amounts in foodstuffs, deficiency of any of the vitamins lead to corresponding disease.

There are two major classes of vitamins: the fat-soluble and the water-soluble vitamins. The former includes the vitamins A, d, E and K which are soluble in organic solvents such as ether and chloroform. The latter includes the B group of vitamin (thiamine, riboflavin, nicotinamide, folic acid, pyridoxine acid, p-amino benzoic acid, biotin, inositol, cyanocobalamin and choline) as well as vitamin c (ascorbic acid).

There are two methods for determining the amounts of vitamin occurring in thediet. Firstly, the bioassay methods which involve growth effects on bacterial cultures and, secondly, colorimetric methods which require the use of a variety of chromogenic reagents.

Experiment 30: Qualitative Test for Vitamin A

Principle

A solution of antimony trichloride in chloroform gives a transient blue colour in the presence of vitamin A.

Method

Place 2 ml of a saturated solution of antimony trichloride in chloroform in a test tube. With a clean dry, pipette add 0.2 ml of a 20% solution of cod liver oil in chloroform. Mix and observe the development of a blue colour.

(Note: Antimony trichloride is poisonous, and care should be taken in handling it!!).

Experiment 31: Quantitative determination of ascorbic acid content of orange juice

Principle

Ascorbic acid exhibits stron reducing properties in solution. It reduces the ble dye 2,6- dichiloropheno indophenols into its pinks form.

Method

To 1 ml of orange juice, add 25 ml of 5% acetic acid andtitrate with 2,6-dichlorophenol indophenols until the pink colour persists for about 15 seconds. Standardise the indophenols solution by titrating 5 ml of a solution containing 5 mg/100 ml of pure ascorbic acid. Perform duplicate titrations in each case. Calculate the ascorbic acid content of the organe juice in mg/100 ml

Experiment 32: Quantitative determination of ascorbic acid content of red pepper.

Principle

The principle is the same as for Experiment 31

Method

Weigh one red pepper and grind it up with little glacial acetic acid in a mortar. Transfer the extract quantitatively with distilled water into a 50 ml volumetric flask. Make up to the mark with more distilled water and filter rapidly. Run the filtrate from a burette into a test tube containing 1 ml of the dye 2,6-dichlorophenol indophenols and 1 drop of dilute acetic acid until a permanent pink colour is obtained. Repeat the titration, using a solution containing 5 mg/100 ml of pure ascorbic acid in place of the red pepper extract. Calculate the amount of ascorbic acid in 100g of red pepper.

Chapter Ten

Separation Methods

Dialysis

Dialysis is a method used to separation large and small molecuyles on the basis of their permeability through a semi permeable membrane. The mixture of small and large molecules is usually placed in a dialysis bag and immersed in a large volume of aqueous solvent. The small molecules pass through the membrane into the solvent until an equilibrium is reached. By dialyzing against running water or by repeatedly changing the solvent the small molecules can be completely removed from the mixture.

Chromatography

Chromatograph is an analytical technique for separating substances in solution. It works on the principle of redistribution of the substances between two or more phases. One of these phases is held stationary by adsorption on a suitable support, while the other liquid phase moves continuously over it. The various phases which could be used in chromatographic procedures include:

(i) Two immiscible liquids as in partition chromatography.
(ii) A liquid and the surface of a solid adsorbent such as chalk or aluminium oxide as in adsorption chromatography.
(iii) A gas and an adsorbent such as oil or grease, as in gas chromatography.

Two of the most Widely used chromatographic techniques are paper chromatograph and thin layer chromatograph. The separation of mixtures of amino acids, sugars and many other biological susstances by the process of paper chromotograph has been found to be very useful. When a strip of filter paper is suspended vertically with the lower end dipping into a mixture of water an organic solvent in-butanol, n-propanol, butanoic acid) the mixture move up the filter paper. The water in the mixture is strongly held by the filter paper and represent the stationary phase which the organic solvent for which the paper has little attraction represent the mobile phase.

In thin layer chromatography, an adsorbent in the form of a slurry is spread in thin layer on a glass plate. A wide range of adsorbents are sued for thin layer chromatography. These include silica gel, alumina, diatomaceous earth, cellulose powder, polyacrylamide,magnesiumphosphate andcalcium sulphate.

Experiment 33: Separation of gari from sucrose by dialysis.

Method

Soak 10g of gari in a beaker and add six cubes of sugar (sucrose). Pour distilled water into beaker until the garri is submerged. Stir the contents and allow the gari to absorb water, pour contents into a dialysis bag and immerse into a beaker of distilled water after tying dialysate and test for the present sucrose and starch, then immerse the dialysis bag in fresh distilled water. At the end of the experiments, examine the content of the dialysisi bag for the presence of starch and sucrose.

Fig. 10.1: principles of dialysis

Experiment 34: demonstration of Donnan membrane equilibrium

Materials

1. Dialysis tubing
2. Gelatine Solution (20 g/l).
3. Thymol blue indicator (2 f/l in 20% ethanol v/v)
4. Hydrochloric acid (0.1M)
5. Sodium hydroxide (0.1M).

Method

Put 20 ml of galatine into a boiling tube and add 2 drops of thymol blue indicator. Add 0.1M NaOH drop by drop until the colour turns green (pH about 9). In another boilding tube add drops of indicator and NaoH to 20 ml of distilled

water until the same shade of green colour I obtained. Put the gelatin in a dialysis bag of suitable size, suspend it in a beaker of distills. Water and leave it to stand. Observe the colour changes in the solution inside and outside the bag.

Repeat the experiment, but this time adjust the initial pH of the gelatin and water to about to give a yellow colour. Comment of the colour changes observed in both cases.

Experiment 35: Separation of sugars by paper chromatography.

Cellulose in the form of filter paper provides a convenient support for the aqueous phase in partition chromatography since it readily absorbs a thin layer of the aqueous phase. The mobile phase is usually an organic liquid in equilibrium with the stationary phase. The solute distributes itself between the stationary solvent phase an the components in a mixture. The extent of separation of the components depend on the difference in their R_f values. The R_f values is related to the partition coefficient of the componenet between the stationary phase and the mobile phase.

$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solute}}$

A mixture of sugars (e.g. glucose, fructose, xylose, arabinose andsucrose) can be separated by the use of paper chromatography. The mixture is spotted onto a sheet of filter paper, dried and the chromatogram is developed by allowing the solvent to flow along the sheet. The solvent front is marked and, after drying the paper, the positions of the individual compounds present in the mixture can be visualized by a suitable staining reaction. The ratio of the distance moved by a compound to that moved by the solvent (R_f values) is constant for a particular **compound, the solvent system and the paper used, if the temperature, pH and solute concentration are carefully controlled.**

Fig. 10.2: Measurement of R_f values.

Method

Draw a line about 5 cm from th end of a dry strip of whatman No.1 filter paper. Apply 10µ1 of a 1% solution of each of the following sugars to the filter paper at intervals of 4 cm along the line you have drawn: lactose, glucose, galactose, arabinose, xylose, sucrose. Dry the spots in a stream of hot air (or in the sun).

Sport 10µl of the mixture of sugars provided along the same line but at a distance of 4cm to the last sugar spot. Run the chromatogram in isopropanol/water solvent (4:1 v IV) and, paper at room temperature in a fume cupboard. Visualise the spots by spraying the filter with freshly prepared aniline-diphenylamine in the fume cupboard. Heat the paper briefly in the oven at 100 C to obtain good results. Identify the sugars in the mixture by comparing their movement with those of the standards and by measuring their R_f values.

Experiment 36: separation of amino acids by paper chromatography.

Method

Draw a line about 5cm from the end of a dry strip of Whatman No.1 filter paper. Apply drops of a 1% solution of each oor the following amino acids to the filter paper at intervals of 4 cm the line you have drawn; glycine, alanine, glutamic acid, tyrosine, phenylalaline. Dry the spots in a stream of hot air (or in the sun).

Sport 10µ1 of the mixture of amino acids provided along the same line but at a distance from the last amino acid spot. Run the chromatogram in butanol/ glacial acetic acid/water solvent (12:3:5 v /v/v) and dry the paper at room temperature. Visualize the spots by spraying the filter paper with freshyly prepared 0.2% ninhydrin reagent in butanol or acetone. Heat the paper briefly in an oven at 100C to obtain good results. Identify the amino acids in the mixture by comparing their rate of movement with those of the standards and measuring the R_f values.

Experiment 37: separation of serum proteins by electrophoresis on cellulose acetate.

Electrohoresis is a technique that is widely used in the separation of proteins and macromolecules in biological fluids such as serum, urine, cerebrospinal fluid, gastric juice. This can be very useful in the analysis of changes in serum proteins in disease condition Cellulose acetate electrophoresis is a very rapid and convenient method for this type of analysis.

Method:

Moisten a strip of cellulose acetate paper measuring 10 cm by 2.5 cm by placing it on the surface of a barbitone buffer, pH 8.6, in a flat dish. Allow the strip to soak in buffer, then immerse completely by gently roacking the dish. Remove the strip with forceps, blot it gently and place the electrophoretic apparatus. Connect it to the buffer compartments with filter paper with switch on the current and adjust to 0.4mA per centimeter of strip. Apply a spot of serum with microlitre pipette about one- third the length of the strip from the cathode.

Run the electrophoresis for 1 – 2 hours, remove the strip and stain with Ponceau S for four minutes. The Ponceau S stain contain trichloroacetic acid which fixes the protein hence application of heat before staining is not necessary. Remove the excess dye from the strip by washing repeatedly in 5% acetic acid. Describe the electrophoresis pattern you observe.

(Note: The electrophoresis can also be run for 20 minutes at a current of 1.0 mA).

Experiment 38: Seperation of leaf pigments by paper adsorption chromatography

Method:

Homogenize the leaves by grinding with pestle in a porcelain mortar. Extract the pigments by shaking the homogenate with a mixture of petroleum either, methanol and benzene (45:15:5 v/v/v). Remove the residue by filtration and wash the filterate four times with water to remove the methanol (Do not shake vigorously to prevent the formation of an emulsion). Remove the last traces of water by adding anhydrous sodium sulphate. Filter to remove the solid and concentrate extract by allowing some of the liquid to evaporate until you have 2 – 3ml of extract left.

Prepare a chromatography column (20 cm x 1 cm) and pack the column with slurries of the stationary phase in petroleum ether. Layer the stationary phase on top of each other in the following order; alumina (5cm), calcium carbonate (7cm) and sucrose (7cm), inserting a filter paper disc between each adsorbent. Wash the column with several volumes of the eluting solvent, benzene/petroleum ether (1:4 v/v).

When the top of the column in almost dry, add the extract elute with the solvent. Collect fractions of various coloured pigments and plot their absorption spectra.

Chapter Eleven

Urinalysis

The urine is the most easily obtained and most easily tested of all the body fluids. The testing of urine (or urinalysis) is capable of providing a great deal of useful information. Some unsuspected diseases may be unmasked by routine analysis of urine for its constituents and characteristics.

Experiment 39: test for physiological constituent of urine.

1. Tests of acidity and alkalinity.

Test the reaction of freshly voided urine to blue and red litmus paper. Record your results. Repeat the test with urine which has been allowed to stand for at least 10 hours.

2. Test for chlorides.

To 5 ml of fresh urine in a test tube, add 1 ml of conc. Nitric acid and 2 ml of 3% silver nitrate solution. Record your observations.

3. Tests for calcium and phosphate.

Add a little quantity of conc. Ammonia to 10 ml of urine in a test tube and record what you observe as the test tube cools. Filter the content and discard the filtrate. Wash the residue with a little quantity of distilled water and discard – the

supermatant. Dissolve the residue in hot dilute acetic acid and divide the resulting solution into two portions labeled A and B. to the portion labeled A, add a little quantity of conc. Nutric acid and 5 ml ammonium molybdate and boil. To the portion labeled B, add 5ml of 2% potassium oxalate solution. Record your observations.

4. Test for sulphate.

To 5 ml of fresh urine in a test tube, add 1ml of conc. Hydrochl;oric acid and 5 ml of 10% barium chloride solution. Record your observations.

5. Test for urea.

To 5 ml of fresh urine in a test tube, add 1 ml of alkaline sodium hyp obromite solution

Record your observations and write an equation for the reaction.

6. Test for creatimine

To 5 ml of fresh urine in a test tube, add 1 ml of saturated solution of picric acid and 10 drops of 10% NaOH. Repeat the test with 5 ml of water in another test tube and compare the colour changes in both test tubes.

7. Test for urine acid.

Put a few drops of fresh urine in a crucible and add 2-3 drops of conc. Nitric acid. Carefully evaporate to dryness on a water bath and allow to cool. Observe the colour changes on cooling and give an explanation for what you observe.

Experiment 40: tests for abnormal constituents of urine.

Abnormal constituent of urine can be detected by means of Ames reagent strips or tablets tests. These are similar to the strips for testing blood constituents (see diagram). To obtain accurate results the following precautions should be taken:

1. Store the bottles in a cool, dry place at temperatures below 30C, away from heat, moistur, direct sunlight, acid, alkali or volatile fumes. The bottles should also not be put in the refrigerator.

2. Only take out sufficient strips or tablets for immediate use. Do not remove the dessicant packet from the bottle. Replace the bottle cap immediately and tightly. Do not touch the test area of the strip.

3. Ensure that specimen containers are absolutely clean and free from contaminants.

4. Use freshly voided, well mixed, unrefrigerated urine. Do not acidify, centrifuge or filter specimen prior to testing.

5. Accurate timing is vital. All tests should be read at exactly the times stated on the bottles and instruction leaflets. Colours developing after thesetimes should be ignored.

6. Reagent strips or tablets should not be transferred from one bottle to another.

Reagents strips for testing abnormal constituents of urine include Albustix for protein Clinistix and Diastix for glucose, Urobilistix for urobilinogen, Ketostix for ketone bodiketodiastix for ketones and glucose. There are also some reagent strips with multiple functions. These include Labstix, Bili-Labstix, Multistix and N-Multistix. The use of the multi-reagent strips will beexplained during the laboratory session.

Some useful information

1. Test for glucose in urine.

   Normal urine does not contain glucose. The presence of glucose in urine is indicative glycosuria or diabetes mellitus. It is the usual practice to test the urine of pregnant women in antenatal clinics to detect cases of glycosuria.

2. Test for bilirubin

Normal urine does not contain bilirubin. If bilirubin is present in urine it is always in conjugated form and is generally indicative of hepatic or biliary disease. Infectious hepatitis, and the potentially more serious serum hepatitis, are of particular importance in hospitals and laboratories where close contact between patients and members of staff may be expected and where parenteral transmission of the causative virus may occur.

3. Test for urobilinogen.

Normal urine contains small quantities of urobilinoge, up to 4 mg/ day, which is formed in the large intestine by the action of bacterial enzymes on conjugated bilirubin. The ability of the liver to excrete the urobilinogen transported to it in the portal circulation may be reduced in disease. In this case more urobilinogen will enter systemic circulation and will be available for excretion in the urine. Therefore, urinary urobilinogen is a test of liver function.

Appendix

PREPARATION OF REAGENTS AND SOLUTIONS

02662X Barfoed's Reagent

Materials:

Copper acetate (66g)

Glacial acetic acid (10ml)

Procedure: Dissolve 66g of copper acetate and 10ml of glacial acetic acid ina about 800 ml of boiling distilled water. Make the solution up to 1 litre. Allow to stand overnight in a cylinder and decant off the supernatant for the precipitate which forms.

02711X Benedict' Reagent (Qualitative)

Materials:

Copper sulphate (17.5g)

Sodium citrate (175g)

Sodium carbonate, anhydrous (100g)

Procedure: Heat 100g of anhydrous sodium carbonate and 175g of sodium citrate in about 600ml of distilled water until they dissolve. Filter (if necessary) into a measuring cylinder. Dissolve 17.5g of copper sulphate in 100 ml of distilled water and add it slowly, with constant stirring, to the carbonate-citrate solution. Make the solution up to 1 litre with distilled water.

02712X Benedict' Reagent (Quantitative)

Materials:

Sodium carbonate, anhydrous (100g)

Sodium citrate (100g)

Potassium thiocyanate (125g)

Potassium ferrocyanide(5%)

Copper sulphate (18g)

Procedure: Dissolve 100g of anhydrous sodium carbonate in 200 ml of cold distilled water. Dissolve 100g of sodium citrate and 125g of potaiumthiocynate in 600 ml of boiling distilled water. Mix the two solutions. Dissolve 18g of copper sulphate in 100 ml of distilled water and add it slowly, with constant stirring,to the above solution. Then add 5 ml of 5% potassium ferrocyanide and make the solution up to 1 litre.

Procedure: Dissolve 20mg of potassium ferricyanide and 50 mg of potassium cyanide in of distilled water. The pH of the solution should be 9.6.

06227X Fehling's Solution A

Materials:

Copper sulphate (69.28g)

Procdure: Dissolve 69.28g of copper sulphate in distilled water and make up to 1 litre.

06228X Fehling's Solution B

Materials:

Potassium sodium tartrate (350g)

Sodium hydroxide (1.00g)

Procedure: Dissolve 3SOg of postassium sodium tartrate and 100g of soodiem hydroxide distilled water and make up to 1 litre.

06182X Folin & Coicaltau Pheno Reagent

Materials:

Sodium tungstate (100g)

Sodium molybdate (25g)

Phosphoric acid, 85 (50 ml)

Conc. HCI (100 ml)

Lithium sulphate (150g)

Procedure: Dissolve 100g of sodium tungstate and 25g of sodium molybdate in about 700 ml a distilled water. Add 50 ml of 85 phosphoric acid and 100 ml of cone HCI. Reflux for 10 hours using an all glass apparatus; then add 150g of lithium sulphate, 50 ml of distilled water and a few drops of bromine. Boil the mixture without a condenser for 15 minutes remove excess bromine. Allow to cool, then make the solution up to 1 litre with distilled water and filter (Whatman No. 1).

06402X Fouchet's reagent (for bilirubin)

Materials:

Trichloroacetic acid (25g)

Ferric chloride (10)

02796X Bial's Reagent (for pentoses)

Materials:

Orcinol (1g)

Conc. HCI (500 ml)

Ferric chloride (1%)

Procedure: Dissolve 19 of orcinol in 500 ml of conc. HCIand add 30 drops of ferric chloride.

02821X Biuret's Reagent

Materials:

Copper sulphate (1.5g)

Potassium sodium tartrate (6.0g)

Potassium iodide (1g)

Sodium hydroxide (30g)

Procedure: Dissolve 1.5g of copper sulphate and 6.0g of potassium sodium tartrate in 500 ml (distilled water in a 1 litre standard volumetric flask. With constant shaking, add 19 of potassium iodine and 300 ml of 10 sodium hydroxide. Make the solution up to 1 litre with distilled water.

04126X Dam's Iodine

Materials:

Pyridine (8.2 ml)

Conc. Sulphuric acid (6 ml)

Bromine (2.6 ml)

Glacial acetic acid (983 ml)

Brown bottles

Procedure: Dissolve 8.2 ml of pyridine and 6ml of conc. H2SO4 in 20 ml of cold glacial acetic acid. Add this to a solution of 2.6 ml bromine in 20 ml glacial acetic acid. Dilute the mixture to 1 litre with glacial acetic acid and keep in the dark.

04815X Drabkin's solution

Materials:

Potassium ferricyanide (20mg)

Piotassium cyanide (50g)

Procedure: Dissolve 25g of trichloroacetic acid in distilled water, add 0ml of hlroide and make the solution up to 100ml.

06413X Foulger's Reagent (for fructose)

Material:

Stannous chloride (10g)

Urea (10g)

Conc. Sulphuric acid

Procedure: Dissolve 10g of stannaous chloride and 10g of urea in 100ml of 40% H_2SO_4.

09001X Iodine Solution(0.1M)

Material:

Iodine(12.685g)

Potassium Iodine

Procedure: Dissolve 12.685g of iodine in 150 ml of 12% K solution in a 1 litre volumetric flask. Shake until the iodine dissolves completely, then make solution up with distilled water.

14748X Ninydrin Reagent

Material:

Ninhdrin (2g)

Ethanol, absolute.

Procedure: Dissolve 2g of ninhdrin in 100ml of absolute ethanol.

19736X Seliwanoff's Reagent (for fructose)

Material:

Reorcinol (0.5g)

Conc. HCI

Procedure: Dissolve 0.5g of resorcinol in 100 ml of 50% hydrochloric acid.

20001X Topfer's Reagent

Material:

Dimethyl Yellow (0.5g)

Ethanol, absolute.

Procedure: Dissolve 05g of dimethly yellow in 300 ml of ethanol and add 200 ml of distilled water.

(million's reagent is a mixture of mercuric nitrate and nitrite).