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LABORATORY SAFETY

The exposure to danger cannot completely be avoided when working in the laboratory. The fundamental rule of accident prevention must be upper most in the mind of scientist working in the laboratory. This rule is never to be careless or negligent and always to be attentive when on duty. The source of danger in the Biochemistry laboratory includes contact with poisonous substances flammable or explosive substances and exposure to electricity.

Working in the laboratory becomes less dangerous when the following basic rules are followed

1. All bottles containing chemicals and reagents should be clearly labelled and hazard noted
2. Never carry large bottles by the neck but hold the bottles with both hands
3. Keep bottles in use on shelves, not higher that eye level
4. Corrosive chemicals include strong acids or alkalis e.g. nitric, sulphuric and hydrochloric acids, sodium and potassium hydroxide. Take great care while opening these bottles, or when pouring such bottles. Always add contents slowly to water, preferably while cooling and stirring
5. Clothing spilled with corrosive substances must be discarded immediately
6. For acid splashes on the skin, wash thoroughly and repeatedly with water and then bath the affected skin with cotton soaked in 5% aqueous sodium carbonate
7. In case of acid splashes on the eye, wash the eyes immediately with copious amount of water, sprayed from a wash bottle or rubber bulb or hold the eye under a running tap. After washing, put 4 drops of 2% aqueous sodium bicarbonate into the eye, continue to apply bicarbonate solution in the eye until checked by a doctor.
8. In case of accidental swallowing of acids while using pipette, call a physician. Alternatively, give the victim two whites of egg mixed with 500ml of water or milk. If neither is available, give ordinary water to drink. Make the victim gargle with soap solution. If the lips and tongues are burned by acid first rinse thoroughly with water and then with 2% aqueous sodium bicarbonate.
9. In case of alkali splashes on the skin, wash thoroughly and repeatedly with water and then bathe the affected skin with cotton soaked in 5% acetic acid (or undiluted vinegar)

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10. For alkali splashes on the eye, wash immediately with water from a wash bottle; squirt the water into the corner of the eye. After washing with water repeatedly, further wash the eye with a saturated solution of boric acid.
11. In case of minor burns caused by heat, plunge the affected parts into cold water or ice to soothe the pain. Apply mercurochrome acriflavine ointment to the burns. Apply guaze dressing loosely. Never tear off the blisters that form over the burns.
12. In case of inhaling toxic vapour or gases (e.g. chloroform) or accidental swallowing while pipetting a poisonous solution, place the victim in open air while waiting for the physician.

13. In case of fire accident, the laboratory has been designed to enhance escape. The doors are escape routes, fire alarms have been installed, sand buckets and portable carbon dioxide extinguishing equipment (always painted red) are available to put out the fire.

REVIEW OF ANALYTICAL CHEMISTRY

Concentration

Concentration of a solution may be defined as the amount of the solute present in the given quantity of the solution. The common expressions of concentrations are:

1. **Percent**

There are three ways of expressing percentage composition of a solution.

a. Weight per unit weight (W/W). A 10% W/W solution contains 10g of solute in 90g of solvent
b. Weight per unit volume (W/V). A 10% W/V solutions 10g of solute dissolved in a final volume of 100ml solution (not the solvent)
c. Volume per unit volume (V/V). A 10% V/V solution contains 10ml of concentrate per 100ml of solution (not the solvent)

2. **Molarity**
   It is the number of moles of the solute dissolved per litre of the solution. A molar solution (1M) contains 1g mole of the solute dissolved per litre of the solution (the final volume of the solution s made 1 litre). A millimole is 1/1000 of a mole, i.e 1 millimolar (mM) solution per litre of solution.
3. **Normality**
   It is the number of gram equivalent of the solute dissolved per litre of the solution (1N)
   Since equivalent weight= $\frac{\text{molecular weight}}{\text{Valency}}$

   i. In case of monovalent compounds or elements N=M
   ii. When the valency is other than one
   N = M x Valency

In case of a normal solution, concentrations are expressed in terms of their combining weights.

A gram equivalent contains the same number of chemically active particles.

Other milliequivalent (mEq) is 1/1000 of an equivalent

$$\text{Meq} = \frac{\text{mg}}{\text{GmEqwt}}$$

to convert mg per 100ml to mEq/ Litre
Mg per 100ml X 10 = mg/L

So $\frac{\text{mg Per 100 L X 10}}{\text{Eqwt}} = \text{mEq/L}$

4. Molality

It is the number of moles of the solute dissolved per 1000gm of the solvent.

DILUTION PROBLEMS

Dilution is usually expressed as one unit of the original solution per total units of the final solution. For a 1:10 dilution, it is required that one unit of concentrated solution should be diluted to a total volume of 10 units. If several dilutions are made, multiply them together to arrive at the final concentration e.g.

If 20% solution is diluted 1:10 and then further diluted to 1:10

Final concentration = 20% x 1/10 x 1/10
= 0.2%

It is advisable to use diluted samples first and later on in calculation make use of dilution factor. This avoids erroneous results even due to slightest pippeting error.

Solve the following problem

1. What is the concentration in % W/V terms of the following solutions
   a. 7µg/µl b. 15mg/0.2ml c. 5000mg/2L d. 36mg/6ml
2. How many µg of x are there in 1ml of a 6% w/v solution of x
3. How many m-moles of glucose are there in 5ml of a 0.5M solution?
4. How many ml of a 10% W/V solution of A contains
   a) 0.1g b) 50mg C) 250 µg ?
5. You have a solution of penicillin containing 1 million international units per 0.5ml. How many ml would you inject to administer

a) 500,000 international units b) 5million international units c) 250,000 international units d) 3millin international units?

Since Eqwt = $\frac{\text{molecular weight}}{\text{Valency}}$

$\frac{\text{mg / 100ml X 10}}{\text{Molecular}} = \text{mEq/L}$
Valency

Or

$$\frac{\text{mg/100ml X Valency}}{\text{Molecular weight}} = \text{mEq/L---(1)}$$

Example

Conversion of normal range of Urea, i.e 21-53/dl to mEq/L

Using the above formula

Molecular weight of Urea = 60

Valency = 1

$$21\text{mg} = \frac{21 \times 10 \times 1}{60} = 3.5\text{mEq/L}$$

$$53\text{mg} = \frac{53 \times 10 \times 1}{60} = 9.0\text{mEq/L}$$

So, the range in mEq/L is 3.5-9.0mWq/L when valency is equal to l, mEq/L is equal to mmol/L because,

mmol = mg

Gram molecular weight

To convert mg per 100ml to mmol/L

mg per 100ml x 10 = mg/L

$$\text{So,} \quad \frac{\text{mg per 100ml x 10}}{\text{Molwt}} = \text{mmol/L ---------- (2)}$$

So, formula (1) is equal to formula (2) when valency is equal to one, i.emmol/L = mEq/L

EXPERIMENT 1: QUANTITATIVE ASSAYS IN BIOCHEMISTRY

The Use of Standard Curve

Majority of the assays in this course are trimetric, spectrophotometric or colorimetric.

The titrimetric procedure has already been dealt with in BCH 210P. Spectrophotometric and colorimetric assays depend on the absorption of radiation usually in the UV or visible region of the spectrum by the compound itself or by the compound itself or by a product to which it may be converted by a suitable chemical reaction. The concentration of the compound is related to the amount of light absorbed by the Beer – Lambert's law.

$\text{Log } I_0/I_t = Ecl$

Where I_o is the incident light,

It is the intensity of the transmitted light, c is concentration and I is the path length (in CM) of the solution through which the light has travelled. This is fixed at 1cm in most cases. E is a constant for a particular substance and is called the extinction coefficient. If c is measured in moles/litre, then E is called molar extinction coefficient. Obviously, the coefficient expressed on a mole basis is 1000-fold larger than the molar extinction coefficient. The fraction Io is called extinction (E) or commonly, but incorrectly optical density (OD) and it's measured directly by the spectrophotometer

To measure the concentration of a solution, the following steps are important

i. The value of E at the wavelength of interest must be known (usually where there is strongest absorption by the test substance)
ii. The spectrophotometer is set at this wavelength
iii. The solution is diluted to a concentration for which the extinction value of 0.8 is regarded as the upper limit for accurate measurement (solution in which this value is exceeded should be diluted appropriately and qualitatively

In colorimetric assays, a series of solutions containing the test substance at known concentrations ('standard') together with solution to be assayed are treated with one or more reagents to produce a coloured compound (chromophore) i.e. a compound that absorbs light at visible wavelengths. From the extinction values of the standards at this wavelength, a standard curve is plotted that relates the extinction to the amount (usually in micrograms) of the substances present. The extinction value of the unknown allows the calculation of its concentration by reference to the standard curve. This is called extrapolation. To obtain reliable results, the following points must be adhered to:

i. The total volume of all tests and standards after the addition of the colour reagents must be the same. Also, the volume of the colour reagents added must be constant throughout

ii. Since many of the reagents are themselves coloured, or may give rise to small amounts of the chromophore, a reagent blank must be included. This entails substitution of the standard or test solution with distilled water while all other reagents are present. It is common practise to set the reading of the spectrophotometer with the reagent blank

iii. Test solutions should be assayed in duplicate. If the two readings differ widely they should be repeated, not averaged.

A description of a typical colorimetric procedure may help you to understand this technique better. The concentration of a substance X in a solution is to be determined by the addition of 1ml of each of reagents A and B to 1ml of a solution containing X. A coloured complex is formed that absorbs maximally at 540nm (a solution of X in water at a concentration of 10mmol/100ml is available as the standard solution).

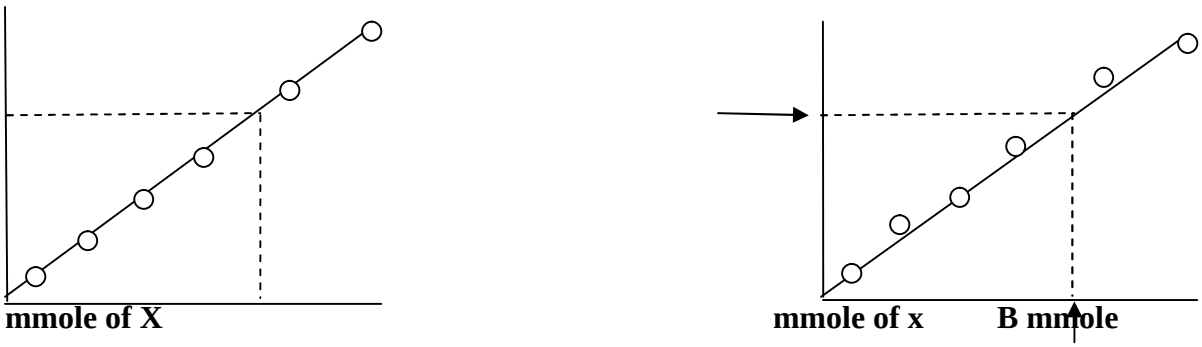
A series of standards is prepared by making serial dilutions of the stock solutions. For example, 0.1, 0.2, 0.3 and 0.4mmols/100ml of the standard may be made up to 1ml each to yield solutions containing 0.01, 0.02, 0.3 and 0.04mmols respectively.

Since the concentration of X is 10mmole/100ml i.e. 0.10mmole/ml, the highest standard (containing 1ml) has 0.1 mmoles of X present. A reagent blank consisting of 1ml of water is also prepared. To all tubes, 1ml of each of A and B are added and the colour allowed to develop. Note that the total volume in each tube is 3ml.

A table is drawn in the laboratory notebook into which readings can be entered as they are obtained. The table columns are headed as follows:

ml of standard (10mmole/100ml)	**mmole X**	**Extinction at 540nm**
0	0	0 (reagent blank)
0.2	0.02	
0.4	0.04	
0.6	0.06	
0.8	0.08	
1.0	0.10	

Extinction reading at 540nm is then entered into the appropriate rows/column in the table and a standard curve of extinction against the amount of X present is plotted with the readings. Suppose the 0.2ml of the unknown solution contain no more than 0.10mmole of X. To duplicate 0.2ml portions of the solution are added 0.8ml water (to bring to 1ml), plus 1ml of both A and B. The total volume is now 3ml which is identical in this respect to the volumes of the standard and reagent blank. The mean of extinction values obtained with the tests is used to extrapolate the amount of X in the unknown solution as shown below:



Suppose the extrapolated value is B mmoles. Therefore, 0.2ml of the test solution contains B mmoles of X. The concentration of the solution can be worked out viz:

0.2ml containing B mmoles

1ml contains B/0.2

100ml contain B/0.2 x 100 = 500 B mmoles

If the original guess that 0.2ml would be satisfactory turns out to be wrong resulting in an extinction that exceeds that of the highest standard, the assay is repeated using a smaller volume of the test solution. On no account should an attempt be made to bring the extinction value within the range of the standard curve by diluting the final coloured by diluting the final coloured mixture obtained after the addition of A and B.

Exercise

Suppose that the following extinction readings at 540nm were obtained for the amounts of X indicated. Assuming that the extinction obtained for 0.01ml of test solution was 0.075, plot a standard calibration curve and hence calculate the concentration of X in the unknown.

(Express your answer in µg/ml)

ml of standard (0.2 microgram/ml)	Extinction at 540nm
0.0	0.0
0.1	0.02
0.2	0.05
0.3	0.07
0.4	0.085
0.5	0.12
0.6	0.14
0.7	0.15

EXPERIMENT 2: DETERMINATION OF THE pH OF COLOURLESS SOLUTIONS BY MEANS OF INDICATORS

Introduction

The pH is the most convenient method of expressing acidity or alkalinity; an approximate idea of the pH of a solution can be obtained using indicators. There are organic dyes whose colour is dependent on upon the pH of the solution. Indicators are usually weak acids which dissociates in the solution

$$\text{Indicator} \longrightarrow \text{indicator}^- + H^+$$

Applying the Henderson-Hasselbach equation

$$pH = pKin + \text{Log} \frac{(\text{Indicator})}{(\text{Indicator})}$$

The two forms of the indicator have different colours and the actual colour of the solution will depend upon the pKin and the pH. The colour change which occurs over a wide range of pH 8.0 indicators cannot be used to determine the pH of a solution accurately. Only a small quantity of indicator being examined is utilised, otherwise the acid-base equilibrium will be displaced. Indicators are of more value in determining the end point of a titration. The indicator is so selected, that the colour changes at the equivalent titration carried out.

Indicator	**pH Range**	**Acid Colour**	**Basic Colour**
Thymol	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
Bromothymol blue	6.0-7.6	yellow	blue
Phenol	6.8-8.4	yellow	red
Cresol red	7.2-8.8	yellow	red

Thymol blue	8.0-9.6	yellow	blue
Phenolphthalein	8.2-10.0	colourless	red

Experimental procedure

1. Two solutions of unknown pH are provided.
2. To 5ml of each solution, add 2 drops of indicator. Repeat this for the indicators provided. Observe the colours and draw conclusions about the approximate pH of the solution.
3. To determine the pH of the test solution more accurately, use the buffer solutions provided with the known pH to compare the test results.
    i. Put 5ml of each buffered solution into separate tubes and add 4 drops of a suitable indicator to each tube. Compare the colour of 5ml of the unknown solution to which 4 drops of the same indicator have been added.
    ii. Determine the pH of the test solution as accurately as possible.

RESULTS

Solution	Colour	pH

DISCUSSION

Question

1. Explain the term pH
2. Why are the buffers necessary in biological systems?

EXPERIMENT 3: ACCURATE DETERMINATION OF THE pH OF COLOURLESS

Introduction

The pH of an unknown solution can be estimated using the pH meter. A pH meter consists of two electrodes

a. An indicator electrode (glass)
b. The reference electrode (calomel)

When these electrodes are placed into the solution of interest, the ions in the solution diffuses through the membrane to the electrodes, both reference and indicator electrodes are connected to a graduated meter. The difference in potential of the two electrodes is an indication of the pH of a solution.

Experimental procedures

1. You have been given two buffer solutions A and B.
   Prepare a series of buffer solutions in test tubes by mixing A and B in different proportions as shown in the table 1below and determine the pH of each tube. Add five drops of bromothymol blue indicator into each tube and record the colours observed in each tube. Add 5 drops of the bromothymol blue into each of the two solutions X and Y of unknown pH provided and estimate as accurately as possible the pH of the two solutions
2. You have been provided 3 solutions A_{10}, B_{12} and C_{14} of unknown pH using the pH meter accurately determine the pH of the solutions.

Results

Table 1

1 Tube No	1	2	3	4	5	6	7	8	9	10	11	Unknown X	Unknown Y
Buffer A													
Buffer B													
pH													
Colours Observed													

Table 2

Solution	pH
A_{10}	
B_{12}	
C_{14}	

Questions

1. Explain briefly why pH studies are important in medicine.
2. What is a buffer? What buffering mechanism in the body helps to maintain homeostasis?

EXPERIMENRT 4: TEST ON CARBOHYDRATES

Introduction

Carbohydrates are polyhydroxy- aldehydes and ketoses. They have the general formula $C_n(H_2O)_n$ and being hydrates of carbon contain hydrogen and oxygen in the same proportion as in water. Some compounds are carbohydrates by virtue of their properties but do not conform to the general formula e.g 2-deoxyribose $C_5H_{10}O_4$ while other have the formula e.g. formaldehyde CH_2O and ethanoic acid $C_2H_4O_2$ but are not necessarily carbohydrates.

Carbohydrates are more abundant in nature than all other organic compounds of biological importance e.g. cellulose, starch etc

Carbohydrates may be classified into three main groups:

I.	Monosaccharide	Hexose : glucose, fructose	
		Pentose: ribose, Xylose	
II.	Disaccharides	Sucrose	(glucose – fructose)
		Maltose	(glucose – glucose)
		Lactose	(glucose – galactose)
III.	Polysaccharides	starch	(Polyglucose)
		Cellulose	(Polyglucose)

Experimental procedures

This experiment is designed to illustrate some qualitative reactions of carbohydrates. Carry out the following tests on the carbohydrate solutions provided. Tabulate your results as **test/observation/inference.**

1. **Molisch's test**

i. Concentrated sulphuric acid hydrolyzes glucosidic bonds to give the monosaccharide which are then dehydrated to furfural and its derivates. These combine with α-naphtol to give a purple complex.

ii. To 1ml of test solution, add 3 drops of 1% α-naphtol in ethanol. Mix and pour 1ml of conc. 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 carbohydrates. All carbohydrates except amino sugar give a positive reaction. This reaction depends on

the condensation of α -naphtol and furfural formed by the action of sulphuric acid on the carbohydrate.

2. Benedict's Test

Add 5 drops of the test solution to 2ml of Benedict's quantitative reagent. Heat in a boiling water bath for 5 minutes. If the test solution contains a yellow or brick red precipitate of cupric oxide is observed, carbohydrate is present. But, if the blue colour of the Benedict's reagent persists, add more drops of the test solution and boil the mixture to observe the colour change.

3. Barfoed's test

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

4. Fehling's Test

Mix 1:1 (V/V) of Fehling's solution A and B. Add 5 drops of test solution to this mixture and boil. The appearance of a brick red precipitate indicates the presence of a reducing sugar.

5. Bial's test for pentoses

When pentoses are heated with conc. HCl, a furfural is formed which condenses with orcinol in the presence of ferric ions to give a blue-green colour while prolonged heating of some hexoses may yield hydroxymethyl furfural which also reacts with orcinol to give coloured complexes. Add about 2ml of the test solution to 5ml of Bial's reagent in a test tube and boil. A blue-green colour indicates the presence of a pentose sugar. Cool the tube and then add 2ml of ethanol and shake.

6. Seliwanoff's test for ketoses e.g. fructose

Ketoses are dehydrated more rapidly than aldoses to give furfural derivative which then condense with resorcinol to form a red colouration. Add 2 drops of the test solution to 2ml of Seliwanoff's reagent and warm in a boiling bath for 1 minute. The appearance of a deep red colour indicates the presence of a ketose sugar.

7. Test for starch

Add 2 drops of iodine to 5ml of the starch solution provided. Note the colour.

8. Formation of Osazone

Compounds containing the – CO – CHOH- group form crystalline osazones with phenyl hydrazine. The osazone crystals have characteristic shapes and melting points which assist in the identification of the reducing sugar. Phenylhydrazine reacts with the carbonyl group of a sugar to give the phenylhydrazone which then reacts further with two molecules of phenylhydrazine to form the osazone.

Question: Draw the osazone. Draw the osazone structure after observing them with the aid of a light microscope.

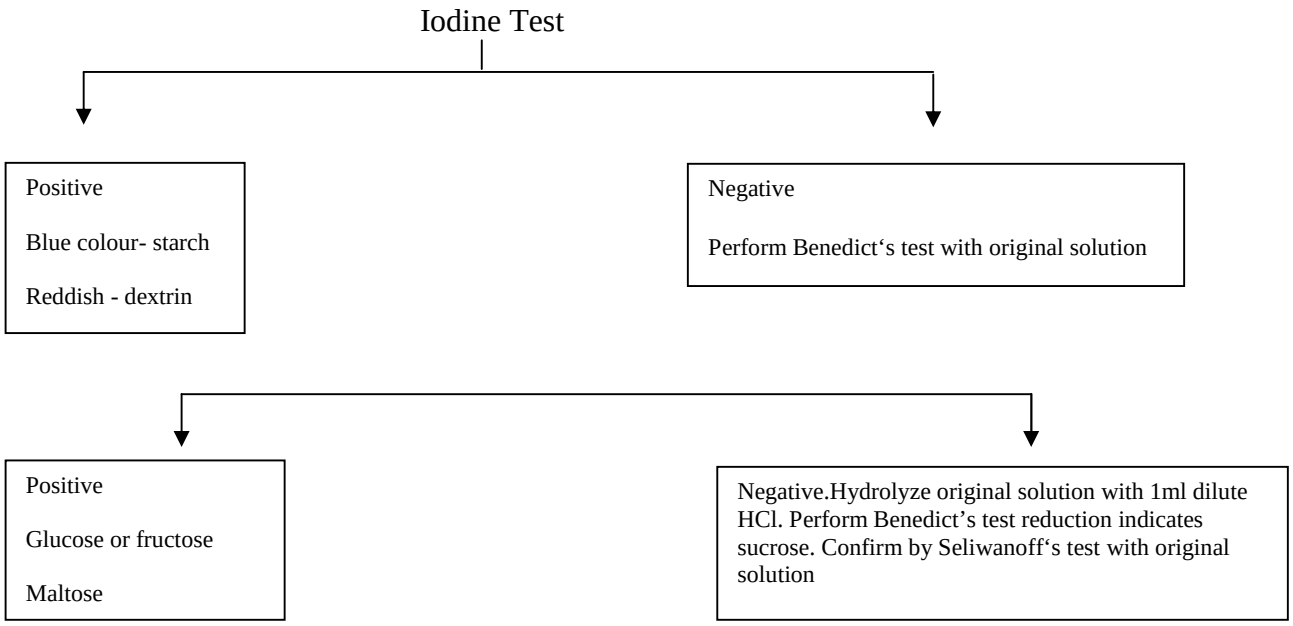
Test	Observation	Inference

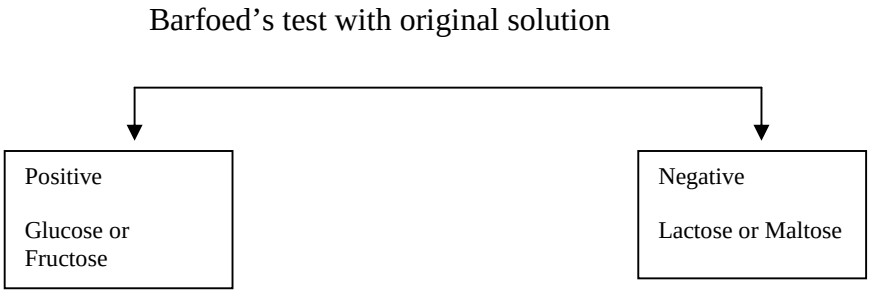
Test	Observation	Inference

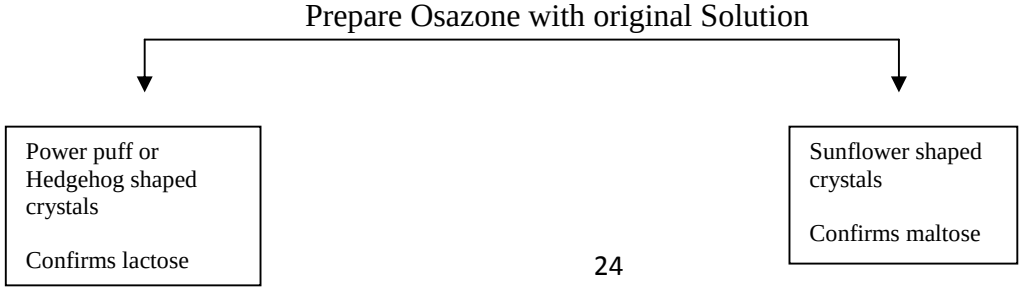
EXPERIMENT 5: DETERMINATION OF CARBOHYDRATE IN UNKNOWN SOLUTIONS

You have been provided with solutions of unknown carbohydrate. With the reagents provided, identify the unknown samples. Record your results in the tabular form **Test/Observation/ Inference**

<u>Identification of Carbohydrate in a given Solution</u>







Test	Observation	Inference

EXPERIMENT 6: QUANTITATIVE ESTIMATION OF GLUCOSE (BENEDICT'S METHOD)

Experimental procedure

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

Estimate the amount of glucose required to discharge the blue colour.

Carry out three determinations.

RESULTS

Urine Sample	Titre Value (cm^3)
i	
ii	
iii	

Question.

1. Calculate the concentration of glucose in the sample as:
   a. mg%
   b. mmol/Litre
   c. What is glycosuria? Why is it noticed in a patient's urine sample
   d. Propose a management regimen for glycosuria
   e. Which hormones help to maintain blood sugar levels in man?

EXPERIMENT 7: SOLUBILITY AND QUANTITATIVE TESTS ON LIPIDS

Carry out the following tests on the lipids provided:

Olive Oil

Groundnut Oil

Palm Oil

Coconut Oil Margarine

Glycerol

Palmitic acid

Cholesterol

Stearic acid

Phosphatidylcholine

1. Compare the solubility of the lipid in
   a. Water
   b. Ethanol
   c. Chloroform
   d. Diethyl ether

Perform the tests by adding 2 drops of the lipid to 5ml of solvent.

Describe what you observe and state the class to which each lipid belongs.

2. Put one drop of 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 its diameter (in cm)

Acrolein test

Heat 2 drops of the lipid with a pinch of dry potassium hydrogen sulphide ($KHSO_4$) until the acid fumes of acrolein are produced. Write an equation for the reaction.

Test for free fatty acid

Dissolve a little quantity of the lipid in diethyl ether and add a dilute alkaline solution of phenolphthalein in dropwise manner. The discharge of the pink colour indicates the presence of free fatty acids. Note how many drops of phenolphthalein required to discharge the colour. Compare the quantity of the oils.

Iodine value

Add 5 drops of the lipid to 5ml chloroform. Shake and add Dam's iodine solution or bromine water, drop by drop into the mixture, shaking after each addition until the iodine (or bromine) fails to be decolorized. Note the number of drops required to reach the end point. This is a rough measure of the degree of unsaturation of the lipid and it is directly proportional to its iodine value. Compare the quality of the oils.

Liebermann – Burchard test for cholesterol

Carefully add 1ml acidic anhydride to 1ml of cholesterol. Add 2 drops of conc. sulphuric acid. Note the change in colour. Acidic anhydride reacts with the cholesterol solution to produce a characteristic blue-green colour.

Salkowski test for cholesterol

Carefully add 1ml conc. Sulphuric acid to 1ml of a chloroform solution of cholesterol. Two layers form. The acid layer develops a yellow colour with a green fluorescence and the chloroform layer will give a range of colours first, from bluish red gradually to violet red.

Emulsification test

To 5ml water in a test tube, add one to two drops of oil and shake. The oil is dispersed into a virtual emulsion but on keeping, it rises to the top. Add a few drops of soap and solution then shake. The oil is now really emulsified.

Report your experiments under each test by tabulating **Test/Observation/Inference**

Question

1. Name the essential fatty acids
2. Draw the structure of cholesterol.

Test	Observation	Inference

EXPERIMENT 8: ESTIMATION OF GLUCOSE BY IODINE OXIDATION IN ALKALINE CONDITIONS

Principle

The principle underlying this experiment is that glucose is oxidised to gluconic acid by alkalinic iodine solution.

$C_5H_{11}O_5.CHO + I_2 + 3NaOH = C_5H_{11}0_5.CO_2Na + 2NaI + 2H_2O$

Excess iodine and alkali are added to the glucose solution and the excess iodine is then back-titrated with standardised sodium thiosulphate. Care must be taken not to add too large an excess of the iodine as this will cause further oxidation of the sodium gluconate formed (about 2ml of 0.05M I_2 is a suitable amount).

Reagents needed

1. Standardised sodium thiosulphate solution (0.102M)
2. Iodine solution (approx. 0.05M)
3. 2M HCl
4. Methyl red
5. Unknown glucose solution (4-10g/I)
6. 1% w/v soluble starch
7. Sodium Hydroxide (0.025M)

Procedure

Standardise the iodine solution with the standard sodium thiosulphate solution. Pipette 10ml of the unknown-strength glucose solution into a conical flask. Add 10ml of 0.05M iodine and 20ml of 0.025M NaOH. Shake well until the mixture becomes light – yellow (straw colour). Add 2 drops of 2M HCl to the mixture to bring its pH to about 5.0. Then add 1% starch solution as an indicator, in drops until the mixture turns blue to blue black.

Put the standardized 0.10M sodium thiosulphate solution in a burette and titrate against the blue-black colour disappears. Note and record the titre.

Calculations

Let M_S be the molarity $Na_2S_2O_3$ and let M_i be the molarity of the iodine solution.

From the equation reaction,

$$2Na_2S_2O_3 + I_2 \longrightarrow 2NaI + Na_2S_4O_6$$

It is clear that 1 mole of iodine is equivalent to 2 moles of $Na_2S_2O_3$ i.e. 1 mole of I_2 = 2 moles of $Na_2S_2O_3$

$$\frac{M_iV_i}{M_sV_s} = \frac{1}{2}$$ (where Vi and Vs are the volumes of iodine and $Na_2S_2O_3$ respectively)

$$\text{Hence } V_i = 1 \frac{M_sV_s}{2\,M_i} \text{ i.e } \frac{M_sV_s}{2M_i}$$

This is the volume of excess I_2. Therefore the volume of I_2 needed to oxidise 10ml of unknown glucose solution is

{(total volume of I_2) – Vi}ml

To convert this volume to mmoles of I_2, multiply by M_i i.e. mmoles of I_2 =

[(total vol. of I_2) – V_i] x M_i = mmoles of glucose in 10ml

Mg of glucose in 10ml solution = {(total vol. of I_2) – V_i] x M_i x 180

In mg/100ml, it becomes $\frac{[(\text{total vol. of } I_2) - V_i] \times M_i \times 180}{10} \times \frac{100}{1}$

(Note that 180 is the molar mass of glucose)

Exercise

The glucose content of a urine sample from a patient was determined by the method of iodine oxidation at alkaline pH I_2 (8ml of 0.05M) was added to 12.5ml of the urine sample and the mixture was titrated against standardised 0.025M $Na_2S_2O_3$ with starch as an indicator. If the titre obtained was 13ml, calculate the glucose the glucose content of the urine, and then comment on your results

EXPERIMENT 9: SUGAR CONTENT OF FRUITS

The sweetness of fruits is mainly due your 'invert sugar' an equimolar mixture of fructose and glucose. Some fruits also contain traces of sucrose.

In this experiment, the sugar present in oranges is extracted with water and the glucose content of 'invert sugar' is determined titrimetrically by oxidation with iodine in alkaline conditions. An aliquot of the sugar extract is then hydrolysed with acid and the glucose content of the hydrolysed sucrose is estimated using the iodine oxidation method described in the previous experiment. The protein content of oranges is very small and therefore it is unnecessary to prepare protein – free samples before the estimation of the sugar)

Note: this method can used to estimate all sugars with an aldehydes or potential aldehydes groups but since glucose is the only aldoses present in any substantial amount in oranges, the interference from other sugars is very minimal.

Reagents and apparatus

1. Ripe oranges
2. Standardised sodium thiosulphate solution (approx. 0.1M)
3. Iodine solution (0.05M)
4. 1% w/v soluble starch
5. 2M HCl
6. 0.5M NaOH
7. Methyl red
8. Homogenizer/blender

Procedure

Peel some oranges and removes and remove the white skin and seeds from the sections. Weigh a 50g – portion and homogenise in 50ml of water in a blender. Transfer the homogenate quantitatively to two 50ml-centrifuge tubes. Leave the tubes for about 10min with occasional stirring using a glass rod. Then centrifuge at 3000 rpm for 3 minutes. Decant the supernatant quantitatively into a 100ml volumetric flask and make up the mark.

a. Glucose content of unhydrolyzed extract

Pipette 5ml of orange extract into a conical flask. Add 10ml of 0.05M iodine and 20ml 0.025M NaOH. Shake the mixture thoroughly. Then add 1% starch solution as an indicator, in drops until the mixture turns blue or blue-black. Put the standardized 0.1M sodium thiosulphate solution in a burette and titrate. Note the colour change and record the titre.

b. Glucose content of hydrolysed extract

Pipette 25ml of orange extract into a 250ml conical flask, add 5ml of 2M HCl and heat in boiling water-bath for 20 min. Cool, adjust pH to about 6 with 2M NaOH using universal pH indicator paper. Transfer the hydrolysate to a 50ml volumetric flask and make up the mark. Pipette, in triplicate 5ml of hydrolysed orange extract into a conical flask. Add 10ml of 0.05M iodine and 20ml 0.025M NaOH. Shake the mixture thoroughly. Then add 1% starch solution as an indicator, in drops until the mixture turns blue or blue-black. Put the standardized 0.1M sodium thiosulphate solution in a burette and titrate. Note the colour change and record the titre.

Calculation

Let M_s be the molarity of $Na_2S_2O_3$, and let M_i be the molarity of the iodine solution.

Equation: $2Na_2S_2O_3 + I_2 \longrightarrow 2NaI + Na_2S_4O_6$

1 mole of iodine ≡ 2 moles of $Na_2S_2O_3$

$\frac{M_iV_i}{M_sV_s} = \frac{1}{2}$ (where V_i and V_s are the volumes of iodine and $Na_2S_2O_3$ used respectively).

Hence, $V_i = \frac{M_sV_s}{2M_i}$

a. Glucose content of unhydrolysed extract

The glucose content of 5ml of the orange extract (equivalent to about 2.5g of orange) can be calculated viz:

Volume of I_2 that oxidised 5ml of juice

= (total vol. of I_2) – (vol. of excess I_2)

Mmoles of I_2 = [(total vol. of I_2) – V_i] x M_i = mmoles of glucose in 5ml of juice

Mmoles of glucose in 100g fruit

$$= \frac{[(\text{total vol. of } I_2) - V_i] \times M_i \times 180 \times 100}{2.5g \qquad\qquad 1}$$

To get the invert sugar concentration, multiply the answer by 2

b. Glucose content of hydrolysed extract

The same procedure is used as in (a) above. However, note that 5ml of the hydrolysed extract will be equivalent to 1.25g of orange because of dilution i.e. 25ml of original juice was hydrolysed and made up to 50ml. Thus the dilution factor is:

$\frac{50ml}{25ml} = 2$

Hence if 5ml of original juice is equivalent to 2.5g of orange, 5ml of the hydrolysate will be equivalent to 2.5/2 = 1.25g of orange.

Exercise

1. Calculate the glucose content of the unhydrolyzed and hydrolyze orange extract.
2. Determine the invert sugar concentration for both unhydrolyzed and hydrolyzed orange extract.

CALCULATION AND DISCUSSION

EXPERIMENT 10: ISOLATION AND THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS OF LIPIDS

The purpose of this experiment is to teach you something about the methods involved in the isolation of lipids from a natural source and how to use thin layer chromatography in qualitative analysis of lipids.

The major components of the lipid class are the triglycerides, phospholipids and sterols.

Procedure

Source of Lipids: Hard boiled egg yoke

Extraction of lipids

1. Weight 0.3g – 0.5g of egg yolk into a beaker and add 2 – 3ml of chloroform/ methanol (2:1) mixture and stir with a clean glass rod. When it has become a fine suspension, add 8 – 10ml of the solvent system.
2. Swirl and allow to settle briefly (5 minutes) filter using a filter paper to get a clear yellow solution.
3. Transfer the filtrate to a separating funnel rinsing the beaker with 2-3ml of chloroform/methanol solvent system. Put all in a separating funnel and add 5ml of distilled water. Mix the two solutions by swirling the funnel gently. Rock the funnel to a horizontal position and then back to a vertical position four or five times.
4. Allow the funnel to stand vertically to enable the phases to separate. After 10- 15 minutes collect the lower lipid layer into a small conical flask.
5. Evaporate the lipid extract to almost dryness in a water bath.
6. Spot on thin layer chromatography plates, using capillary tubes, references standards are provided also.
7. Place the plates in a developing chamber which contains a solvent system (ether/hexane 1:1). When the solvent has almost risen to the top of the gel layer, remove the plate.
8. Mark the solvent front, air dry briefly and put the plates in the iodine chamber.
9. Many spots become clear. Take it out and mark the outline using a fine needle or pin
10. Compare it with the reference lipids.

Questions

1. Make a drawing of your plate. Comment on the observations
2. Calculate the Rf values of the lipid and identify the presence in egg yolk
3. Comment on the component of the egg yolk

DRAWING AND CALCULATION

DISCUSSION

EXPERIMENT 11: TESTS ON AMINO ACIDS AND PROTEINS

This experiment is designed to illustrate some qualitative reactions of proteins and their derivative. You have been provided with solutions of some proteins and amino acids. Perform the following colour tests on each and water blank. Tabulate your results in the format: **Tests / Observation / Inference**

1. **A. Ninhydrin reaction**
   Spot the solution of the different amino acid on a filter paper and a drop of ninhydrin reagent. Hold the paper over Bunsen flame to dry. Note and comment on the different colours produced.

   B. Place 1ml of the solution of each amino acid solution in a test. Add 5 drops of ninhydrin solutions and boil for 2 minutes in a water bath. Note the colours produced. Repeat the tests on serial dilutions of glycine to determine the limits of sensitivity of the reaction.

2. **Biuret test**
   To 2ml of the test solution, add 5 drops of biuret reagent. Mix thoroughly, note the colour formed. It is a test for the presence of two or more peptide bonds in a solution. The intensity of the colour produced is proportional to the number of peptide bonds present.
3. **Xanthoproteic reaction**
   Add 1ml of conc. nitric acid to 1ml 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 strong alkaline. A yellow colour in an acidic solution which turns bright orange in alkaline indicates the presence of one or more of the aromatic acid tryptophan, phenylananine or tyrosine.
4. **Millions' reaction**
   Add 5 drops of million's reagent to 1ml of the test solution and warm the mixture in a boiling water bath for 10 minutes. Cool to room temperature and add 5 drops 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 phenols and napthols also give positive results.
5. **Sagakuchi reaction**
   The guanidine group of arginine reacts with α-naphthol and an oxidising agent such as bromine water to give a red colour. Mix 1ml of 40% NaOH with 3ml of arginine solution. Add 2 drops of α-naphthol. Mix thoroughly and add 4 drops of bromine water (sodium hypobromine). Note the colour formed.
6. **Violet ring test (Formaldehyde)**
   Add 2ml of conc. H_2SO_4 down the sides of a test tube containing 0.5ml commercial formalin + 1ml test solutions. A bluish purple colour developing at the interface is positive. The test is specific for some indole derivatives e.g tryptophan

7. Cystine sulphur test

Boil 1ml test solution and 3ml of 40% sodium hydroxide for 3 minutes and cool. Add 3ml of 5% lead acetate solution. A dark gray black precipitate shows the presence of sulphur in the protein.

EXPERIMENT 12: DETERMINATION OF PROTEINS AND AMINO ACIDS IN UNKNOWN SOLUTIONS

Proteins give characteristic colours on treatment with certain reagents due to an amino acid or a class of amino acid having a characteristic group or certain groupings in the protein molecule. These reactions are useful in the identification of these amino acids and protein containing them

Experimental procedure

You have been provided with some unknown solutions of acids and proteins with some reagents. Identify the samples.

Test	Observation	Inference

EXPERIMENT 13: DETERMINATION OF PROTEIN: BIURET METHOD.

The biuret reagent detects the presence of peptide bonds in solution and can be used for quantitative determinations of protein in solutions. When compounds containing peptide bonds are used, a purple coloured complex is obtained. The intensity of the colour is proportional to the number of peptide bonds in the solution.

Experimental procedure

You are provided with two solutions of protein sample of unknown concentrations. Pipette the following into various test tubes.

1. Standard solution of albumin (bovine serum albumin 10mg/ml) using varying amounts of 0.1 ml-0.6ml.
2. Pipette 0.1ml of unknown sample in duplicate into tube 8 and 9
3. Adjust volume to same with distilled water
4. Add 0.4ml of 3% sodium deoxycholate to all tubes
5. Add 4.0ml of biuret reagent to all tubes
6. Read the tubes after 20 minutes at 540nm using the blank to zero the instrument

Results

TUBE NUMBER	1	2	3	4	5	6	7	8	9
Standard Protein (ml)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	--	--
Standard Protein (mg)									
Test Solution (ml)								0.1	0.1
Water (ml)	0.6	0.5	0.4	0.3	0.2	0.1	0.0	0.5	0.5
3% sodium deoxycholate (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Biuret reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
O. D. At 540nm									

Question

1. Plot a graph of absorbance against concentration of albumin
2. Estimate the concentration of the protein in the samples provided from your graph
3. What single factor affects the quality of a protein needed for growth?

GRAPH

DISCUSSION

EXPERIMENT 14: THE ESTIMATION OF RNA BY MEANS OF THE ORCINOL

Nucleic acids are macromolecules which consist of nucleotides as their monomeric units. They are of two main types namely: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both are involved in the transcription and translation of hereditary material. Each monomer or nucleotide is RNA, is made up of ribose sugar, a nitrogen base (purine or nucleotide) and phosphoric acids which is esterified to the carbon 3 – hydroxyl group of the ribose sugar.

The estimation of RNA by the above method is based on the formation of furfural from the pentose sugar, when heated with concentrated hydrochloric acid (note that, this is general reaction for pentose). Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour. Only the purine nucleotides give any significant reaction.

Reagents:

i. Standard RNA solution
ii. Sample of unknown concentration
iii. Orcinol reagent (Dissolve 1.0g of ferric chloride ($FeCl_3 6H_2O$) in 1 litre of concentrated HCl. Add 35ml of 6% w/v Orcinol in alcohol.
iv. Procedure
v. Mix 2ml of RNA solution (test sample) with 3ml of Orcinol reagent (in duplicate). Heat in a boiling water bath for 20minutes. Cool and read the extinction at 665nm using Orcinol reagent as blank.

Results

TUBE NUMBER	1	2	3	4	5	Unknown	
Standard RNA Solution (ml)	,,	0.5	1.0	1.5	2.0	2	2
Water (ml)	-	1.5	1.0	0.5	-	-	--
RNA concentration (mg)							
Orcinol reagent (ml)	3	3	3	3	3	3	3
O. D. At 665nm							

Questions

i. Prepare a calibration curve by plotting a graph of O. D against concentration (mg RNA)
ii. Determine the concentration of RNA in the sample provided
iii. Name the different types of RNA molecules present in cells that are actively involved in protein synthesis
iv. Briefly enumerate the role of each type of RNA

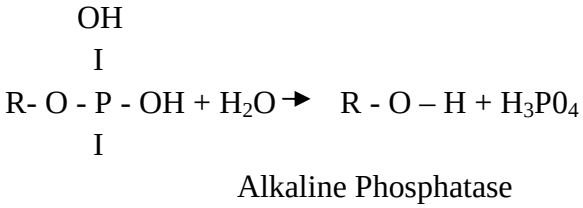
GRAPH

DISCUSSION

DISCUSSION

EXPERIMENT 15: THE EFFECT OF ENZYME CONCENTRATION ON THE REACTION RATE

In an enzyme catalyzed reaction, the rate of the enzymatic reaction is directly proportional to the enzyme concentration. When the substrate is in excess alkaline phosphates at alkaline pH, hydrolysis of a wide variety of phosphates monoesters occurs. Nitrophenylphosphate is the substrate while p-nitrophenol is the product. The yellow colour in alkaline solution can be measured colorimetrically after alkaline has been added to inactivate the enzyme



Procedure

1. Set up the following tubes in the water bath at 30°C for approximately three minutes and pipette the enzyme extract from 0.0ml – 0.60ml
2. Make the volume up to 2ml with the buffer solution provided
3. Start the reaction by adding 2ml of the substrate. At the end of the time interval (10 minutes), stop the reaction by adding 2.0ml of 0.4M NaOH solution
4. Mix and read the absorbance at 405nm immediately. use the blank tube to zero the instrument

Results

TUBE NO	1	2	3	4	5	6	7	8
Enzyme extract (ml)	0.0	0.05	0.10	0.2	0.3	0.4	0.5	0.6
Buffer Solution (ml)	2.0	1.95	1.90	1.8	1.7	1.6	1.5	1.4
Substrate (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
AFTER 10 MINUTES ADD 2.0ml 0.4MNaOH TO ALL TEST TUBES								
O.Dat405nm								

Questions

1. Plot a graph of activity [V] versus enzyme concentration [E] in ml

Clue

Unit of Enzyme of activity

Activity is also defined as number of µmoles of products formed per minute

Calculations

Suppose after 10 minutes incubation, reading of test (T) = 0.8 and blank (A) = A per minute as a result of enzyme action = $\frac{0.8 - 0.06}{10}$

Molarity of p- nitrophenol formed per minute

Molar extinction coefficient of p-nitrophenol = 18litres mol^{-1} cm^{-1}

$= \frac{0.80 - 0.06}{10 \text{ X } 18.18 \text{ X } 10^3}$ unit = x mole / L/ min

Converting moles to µmoles /L/ min

$= \frac{0.74 \text{ x } 10^6}{10 \text{ x } 18.7 \text{ x } 10^3}$ = µmoles formed in 6ml of reaction mixture

$= \frac{0.74 \text{ x } 10^6}{10 \text{ x } 18.7 \text{ x } 10^3} \text{ x } \frac{6}{1000}$ µmoles/min

This formed from 0.20ml of enzyme

1ml of enzyme will be

$= \frac{0.74 \text{ x } 10^6}{10 \text{ x } 18.8 \text{ x } 10^3} \text{ x } \frac{6}{1000} \text{ x } \frac{1}{0.20}$ µmoles/min/ml of enzyme

$= \frac{0.74 \text{ x } 3}{188}$ µmoles / min / ml

2. Explain how the different enzyme concentrations affect the same concentration of substrate in each tube.

CALCULATION

DISCUSSION

EXPERIMENT 16: DETERMINATION OF Km

Km is the affinity of an enzyme for the substrate. It is expressed in the same units as substrate concentration.

The Km determines:

a. How easily the enzyme can bind to the substrate
b. How fast a reaction can be

The smaller the Km value, the higher the affinity of the enzyme for the substrate and vice versa.

The value of Km for an enzyme can be determined by plotting I/V versus I/S. There are other types of plots to determine Km.

Procedure

1. Pipette into a series of test tube immersed in a 30°C water bath a range of substrate concentrations from 0.1 – 2.0 ml
2. Add the buffer solutions to make the volume the same in all tubes
3. Start the reaction by adding 0.2ml of the enzyme mix. At the end of 10 minutes interval, stop the reaction by adding 2.0ml of 0.4M NaOH. Mix and read the absorbance at 405nm as soon as possible. Use the blank to zero the instrument

Results

TUBE NUMBER	1	2	3	4	5	6
Substrate 5moles / I (ml)	0.1	0.4	0.8	1.2	1.6	1.8
Buffer Solution (ml)	1.7	1.4	1.0	0.6	0.2	-
Enzyme (ml)	0.2	0.2	0.2	0.2	0.2	0.2
AFTER 10 MINUTES ADD 2ml 0.4M NaOH TO ALL TEST TUBES						
0.D at 405 nm						

QUESTIONS

1. Calculate the activity of the enzyme in each tube
2. Plot a graph of [V] against [S]
3. Plots a graph of [I/V] against [I/S]
4. Determine the Km and Vmax of the enzyme

GRAPH

GRAPH

EXPERIMENT 17: DEMONSTRATION OF SOME ASPECTS OF ENZYME INHIBITION

An enzyme inhibitor is a compound that causes a decrease in the rate of catalytic reaction either by reacting with the enzyme to form an enzyme inhibitor complex or form an enzyme substrate inhibitor complex.

$E + I \longrightarrow EI$

$ES + I \longrightarrow ESI$

The formation of EI or ESI stops their action and products are not formed.

Procedure

1. Prepare a set of tube by pippeting 1 – 1.8ml of buffered inhibitor solution {inorganic phosphate (pi) 20mMoles/L}
2. Add 2ml of the substrate {5mMoles/L of p– nitrophenol phosphate}. Make up the volume to 3.8ml in each tube by adding sufficient buffer solution.
3. Pipette 0.2ml of enzyme solution into each tube to start the reaction mix
4. After 10 minutes, stop the reaction by adding 2.0ml of 0.4MNaOH to all test tubes. Mix and read immediately the absorbance at 405nm using the blank to zero the instrument

Results

TUBE NUMBER	1	2	3	4	5	6	7	8
	--	0.5	0.8	1.0	1.2	1.4	1.6	1.8
	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	1.8	1.3	1.0	0.8	0.6	0.4	0.2	0.0
	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
After 10 mins, add 2.0ml of 0.4M NaOH to all test tubes. Mix								
O. D at 405 nm								

Questions

1. Calculate the velocity of the enzyme in the different tubes.
2. Plot graph of I/V against I.
3. Calculate the Ki value of the inhibitor.
4. Why do we study inhibitor of enzymes in medicine?

GRAPH

DISCUSSION

EXPERIMENT 18: QUANTITATIVE ESTIMATION OF BLOOD SUGAR

Glucose is the sugar of blood fluids. Lactose and maltose completely hydrolyse into their corresponding monosacharides before absorption, they do not enter the blood stream. When present in blood, due to intravenous injection, disaccharides are treated as foreign substances and excreted in the urine.

Blood sugar estimation is clinically useful in assessing glucose tolerance. Hyperglycaemia is a common feature of decreased tolerance to glucose. Hyperactivity of thyroid, pituitary and adrenal glands can also elevate blood sugar which can also occur in a number of infectious diseases.

Hypoglycaemia may result from an overdose of insulin treatment of diabetes mellitus, hypothyroidism, hypopituitary and hypoadrenalism can cause a decrease in blood sugar levels.

The most common blood sugar level in the fasting state is in the region of 80mg% in normal persons. The level reaches nearly 165mg% one hour after oral ingestion of 50g glucose and return to the fasting level 1 of 120mg% after two hours in normal individuals. 120mg% and anything above is considered high.

Many quantitative methods are used in estimating blood glucose concentration in the laboratory. These methods are not entirely accurate since other substances present in blood as ascorbate, glutathione and acids esters may augment blood sugar concentration. The use of clinistix (analytical paper strip) rely on the activity of glucose oxidase. The glucose oxidase method is specific of glucose and provided a rapid but approximate indication of the amount of glucose in urine blood receptively.

In recent times, blood sugar levels are determined automatically by highly specific instrument.

Experimental procedure

1. Blood samples are provided with unknown glucose concentration.
2. Prepare a glucose standard curve by pippeting varying amount of working standard glucose solution (100mg/ml) into a series of test tubes.
3. Use 2ml of blood sample provided. Duplicate the tubes.
4. Make all the volume equal by adding distilled water.
5. Add Nelson-Somogyi's reagent 4 (prepared fresh).
6. Boil for 20minutes at 100°C in a water bath and cool down before adding Nelson-Somogyi's reagent 3.
7. Shake vigorously and immediately to remove CO_2 and allow to stand for 10minutes before adding water.
8. Read absorbance at 600nm using the blank to zero the spectrophotometer.

Results

TUBE NUMBER	1	2	3	4	5	6	7	8
Glucose standard (ml)	0.0	0.2	0.4	0.6	0.8	1.0	-	--
Glucose (mg)								
Unknown sample (ml)	--	--	--	--	--	--	1.0	1.0
Water (ml)	1.0	0.8	0.6	0.4	0.2	--	--	--
Nelson-Somogyi's reagent 4 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Boil for 20minutes at 100 degrees centigrade in a Boiling water bath and cool								
Nelson-Somogyi's reagent 3 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Water (ml)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
O. Dat600nm								

Questions

1. Plot optical density (O.D) against concentration (mg glucose), to get a calibration/ standard glucose curve. From the graph, extrapolate the concentration of sugar in unknown sample.
2. Express your result in mmole/L. Discuss the significance of your results.
3. Why is it necessary to add an anticoagulant or glycolytic agent to the blood sample?
4. Briefly discuss the role of insulin and glucagon in the maintenance of sugar level in man.

GRAPH

DISCUSSION

EXPERIMENT 19: ESTIMATION OF SERUM UREA

Amino acids and other nitrogenous compound present in the body give out NH_3 on oxidation and since NH_3 is toxic, it is readily converted to urea through the urea cycle. Urea gets excreted in the urine.

The normal concentration of urea in human blood serum is 3 – 7 mmole/litre. The urea concentration in blood serum is liable to a sizeable variation depending on the dietary protein intake.

Principle

This method is based on the property of urea to form a red coloured compound when reacted with diacetylmonxine in the presence of thiosemicarbazide and iron salt in a strong acidic medium. The intensity of the red colouration is presumed to be proportional to the concentration of urea in the serum.

Procedure

The urea concentration of blood serum is calculated by comparison with urea standard curve. To obtain the standard curve, dispense different concentration of urea varying from zero to 10 µmole into test tubes. Make the volumes up to 1ml by distilled water. Add 3ml of colour reagent to each test tube. Mix and boil for 20minutes, cool and read the absorbance at 520nm within 15minutes using 1ml water and 3ml colour reagent as blank.

Test Tubes

	1	2	3	4	5	6
Urea (ml)	0.0	0.2	0.4	0.6	0.8	1.00
Distilled H_2O (ml)	1.00	0.8	0.6	0.4	0.2	0.0
Colour Reagent	3.0	3.0	3.0	3.0	3.0	3.0
Absorbance L= 520nm						

Take 0.2ml of the serum sample into a test tube, make it up to 1ml by adding 0.8ml distilled water and add 3ml of colour reagent, mix and boil for 20minutes in a H_2O. Collect under the tap within 15 minutes using 1ml of H_2O and 3ml of colour reagent as blank. Read the absorbance at 520nm wavelength. Use the value to plot a graph of absorbance against urea concentration in µmole per litre.

Question

1. From the urea standard calibration curve, estimate the serum concentration.
2. Define dilution factor.
3. a. What is the normal concentration of urea in human blood serum?
   b. Is your serum urea concentration normal or abnormal? What do you think is responsible?
4. a. What specific factor could be responsible for variation of the normal serum urea concentration?
   bi. What is azotemia?
   ii. What are the various conditions that could bring about azotemia?
   c. Draw the structure of urea and the urea cycle.

GRAPH

DISCUSSION

EXPERIMENT 20: ESTIMATION OF SERUM PROTEINS

The normal serum pattern by electrophoretic analysis gives the following values on average

Albumin	55%
α_1Globulin	5%
α_2Globulin	8%
βGlobulin	14%
γGlobulin	18%

Most serum are synthesised in the liver. They are glycoproteins except albumin. Serum proteins are normally in the range of 6-8g/dl. Normal serum albumin is within the range of 3.5-5.5g/dl.

Variation in the normal pattern of these proteins occurs in disease states. In disease state, as a result of haemoglobin concentration total serum protein increase. Increase is observed in chronic infection due to augumented antibody production in condition such as cancers where they play a role in response to inflammation. Low levels of serum proteins are observed in liver disease, kwashiorkor and marasmus.

Experimental Method

The Lowry method is used for the clinical analysis of serum proteins.

You have been provided with the following

1. Serum (unknown concentration).
2. Folin-Ciocalteau reagent.
3. Standard albumin (0.5% Bovine albumin in 9% NaCl).

Procedure

1. Setup a series of test tubes as shown in the table below.
2. Make all the volume in the individual test tubes the same by adding distilled water.
3. Add 5ml of alkaline $CuSO_4$ solution to all tubes and mix.
4. Add 0.5ml of diluted Folin-Ciocalteau reagent, mix and leave on the bench for 30 minutes.
5. Read the absorbance of the test sample at 660nm.

Results

TUBE NUMBER	1	2	3	4	5	6	7	8
Standard protein µg/ml (ml)	0.0	0.1	0.2	0.4	0.6	0.8	-	-
Water (ml)	0.1	0.9	0.8	0.6	0.4	0.2	-	-
Test solution (ml) Concentration µg/ml	-	-	-	-		-	1.0	1.0
$CuSO_4$ solution (ml) Folin-Ciocalteau reagent (ml)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
O.D at 660nm								

Question

1. Calculate the concentration of albumin in 100ml sample.
2. Calculate the concentration of albumin in g/dl.
3. What can you deduce from this result?
4. In what condition is hypoalbuminemia observed in man/children?

CALCULATION AND DISCUSSION

EXPERIMENT 21: NORMAL URINE

Normal urine contains both inorganic and organic constituents. The inorganic constituents include Na^+, K^+, Ca^{++}, Mg^{++}, NH_4^+, Cl^-, H_2PO_4, SO_4 and traces of HCO_3^-. The normal nitrogenous constituents are urea, acid and creatinine. The total non-protein nitrogen varies from 10 – 15g/day depending mainly on the protein intake. In addition to these major organic constituents, detoxified products like indican and ethereal sulphates are found in urine.

Inorganic Constituents

Test for chloride

Chloride is precipitated as its silver with $AgNO_3$ in the presence of HNO_3.To 2ml of urine add 0.5 ml of concentrated HNO_3 and 1ml of $AgNO_3$. Note the white precipitate of silver chloride.

Test for sulphate

This is derived from the sulphate containing amino acids. About 85-95% of sulphur is excreted as inorganic sulphate (Na_2SO_3) and the rest as ethereal sulphates (cysteine, taurine, etc).

Barium chloride: To 2ml of urine add 2ml of 10% $BaCl_2$. Observe the white precipitation. Filter it and preserve the filtrate. This is test for inorganic sulphate.

To the above filtrate, add 1ml of conc. HCl and about 3ml of 10% $BaCl_2$ solution. Boil the mixture for 1 to 2 minutes. Note the appearance of turbidity.

This is test for ethereal sulphate.

Test for calcium

With potassium oxalate in acidic condition, calcium is precipitated as oxalate. To 2ml of urine add 5 drops of 1% acetic acid and 5ml potassium oxalate, white precipitation of calcium oxalate is formed.

Test for inorganic phosphorus

Upon warming with ammonium molybdate is precipitated as canary yellow ammonium phosphomolybdate.

To 5ml of urine, add a few drops of concentrated HNO_3 and a pinch of ammonium molybdate. Warm it and note the yellow precipitate.

Test for ammonia

Urinary ammonia is derived from glutamine and other amino acids in kidney. Also there is increase in ammonia production when acid forming foods are taken in; ammonia is evaporated when urine is alkaline.

To 5ml urine, add 2ml of 2% Na_2CO_3 till the solution is alkaline to litmus. Boil the solution.

Organic constituents

Test for urea

Urea is formed in the liver as the end product of protein metabolism and so its excretion depends on the protein intake. Among the organic nitrogenous constituents, the main one urea. About 80-90% of nitrogen in the urine is in form of urea. When urea is treated with sodium hypobromite, it decomposes to give nitrogen.

This reaction is the basis for the estimation of urea in urine

$$H_2NCONH_2 + 3NaOBr + 2NaOH \longrightarrow N_2 + Na_2CO_3 + 3NaBr + 3H_2O$$

Urea Sodium Hypobromite

To 2ml urine in a test-tube add 4-5 drops of sodium hypobromite. Note the effervescence of nitrogen gas.

Test of Uric acid

Uric Acid is the end product of purine metabolism uric acid is sparingly soluble in water. It is soluble in alkaline solution.

a. Phosphotungstic acid reduction test. Uric acid is a reducing agent in alkaline condition; it reduces phosphotungstic acid to tungsten blue. To 2ml of uric acid, add a few drops of phosphotungstic acid reagent followed by a few drops of 20% Na_2CO_3. Note the blue colour.

b. Shiff's test, uric acid reduces ammoniacal$AgNO_3$ to metallic silver.

Wet a piece of filter paper with a few drops of ammoniacal $AgNO_3$ solution. Add one or two drops of uric acid to the same paper. Note the formation of black colour due to the precipitation of silver.

EXPERIMENT 22: TRITRABLE ACIDITY AND AMMONIA IN URINE

The normal daily excretion of ammonia is about 0.7g on an average diet. The ammonia nitrogen forms 2.5-4.5% of the total urinary non-protein nitrogen (NPN). This percentage is referred to as ammonia coefficient. Acid forming foods increase this figure while those foods forming alkali will decrease. In metabolic acidosis, ammonia excretion is increased; a mechanism by the renal tubules to conserve Na^+.

The sum of titrable acidity and ammonia excretion in urine gives a measure of the extent to which the body is able to conserve base.

Titrable acidity is the term given to the volume of 0.1M NaOH required to make the urine alkaline. To phenolphthalein, potassium oxalate is added to precipitate calcium as calcium phosphate precipitate formed towards the end point.

Ammonia estimation is based on titration. After neutralising the titrable acid, neutral formalin is added to the sample of urine. The ammonium salts are decomposed, liberating the equal quantity of acids as per the following reaction.

$$4NH_4Cl + 6HCHO \longrightarrow \underset{\text{Hexamethylene}}{N_4(CH_2)_6} + 6H_2O + 4HCl$$

Tetra-amine

Formalin reacts with ammonium salts to give hexamethylene tetra-amine and an equivalent quantity of acid. The liberated acid is titrated against 0.1M NaOH with phenolphthalein indicator. The test tube value is proportional to the ammonia content of urine.

Note: The pink colour at the end of the formal titration should not be discharged on further addition of neutral formalin, showing the ammonium salts have completely been decomposed. Ammonia estimation by formal titration includes amino acids also as they form dimethylol denotative with formalin releasing H^+ ions.

The titrable acidity normally is in the range of 200-500ml of 0.1M NaOH every 24hours. The acidity is increased in metabolic acidosis and decreased in metabolic alkalosis.

Experimental procedure

Materials

0.1M sodium hydroxide

Potassium oxalate crystals

0.1% phenolphthalein in 50% alcohol

Neutral formalin

Titrable acidity

1. Pipette 20ml urine into a conical flask and add potassium oxalate and mix the content.
2. Add 2 drops of phenolphthalein indicator and titrate against 0.1M NaOH to a light pink end point. Carry out duplicated titration. Preserve the contents of the flask for formol titration.
3. Formol titration of ammonia.

Add 10ml of neutral formalin to the neutralised urine of the above experiment.

The pink colour disappears due to the liberation of acid. Titrate the contents to a light pink against 0.1M NaOH. Carry out duplicated titrations.

Calculations

1ml of 0.1M NaOH	=	3.675mg HCl
	=	1.7mg NH_3
		Since 20ml urine is taken
Titrable acidity		= (titre value x 5ml) per 100ml urine
	=	(titre value x 5 x 3.65) mg of HCl per 100ml urine
Ammonia	=	(Titre value x 5 x 1.7) mg per 100ml urine

Question

1. Explain what acidosis and alkalosis can mean to the normal function of the body's metabolism processes.
2. Explain briefly the role of glutamate in the removal of ammonia from the body.

DISCUSSION

EXPERIMENT 23: ESTIMATION OF URINARY CREATININE

Creatinine is a waste product derived from creatine. The quantity of creatinine and creatine nitrogen excreted per kg body weight is referred to as creatinine coefficient. It also represents the functional muscle mass in the body. Normal excretion is in the range of 1.0 – 1.2g/day. Very little creatine is normally found in adult urine.

Urinary creatinine is largely endogenous and is little influenced by diet. The diet excretion is therefore remarkably constant. In acute and chronic renal failure, serum creatinine is elevated. It is therefore useful in the diagnosis, prognosis, management and monitoring of renal failure.

Creatinine clearance is determined by measurement of serum and urine creatinine over a specific period. It is the best method to determine renal glomerular function. Low level suggest primary renal disease.

Principle

Creatinine in urine reacts with alkaline picrate to give orange or yellow – red colour (Jaffe's reaction). The colour is stable only for a short time after full development. Temperature has an appreciable effect on the rate and intensity of colour development.

Experimental procedure

1. Prepare a set of standard by pippeting exactly 0-2.5ml of the standard creatinine solution (0.003g/ml) into a series of clean dry test tubes.
2. Add sufficient distilled water to bring the volume in each test tube to exactly 3ml.
3. Pipette 2ml of the urine sample into two test tubes (duplicate samples), adjust the volume to exactly 3ml.
4. Freshly prepare the alkaline picrate reagent to each test tube. Mix the contents of the tube without delay and leave the 15 minutes
5. Measure the O.D of the colour at 510nm using the blank to zero the instrument. Note the dilution of the urine sample provided.

Questions

1. Prepare a table of your results.
2. Draw a graph showing O.D Vs mg creatinine for the standards. Use the graph to determine the mg creatinine in the unknown urine or serum sample.

TABLE

GRAPH

EXPERIMENT 24: TEST ON ABNORMAL URINE

Physiological characteristics of normal urine

Appearance: Normal urine is clear. Turbidity may develop on standing as the pH increases and phosphates precipitate.

Colour: Amber yellow is the colour of normal fresh urine. The colour may be light or dark depending on the volume. The colouring pigment is urochrome or urobilinogen.

Reaction: Fresh urine is usually acidic with a pH of 6.0 (range 4.7 to 8.0). The urine becomes more acidic on high protein diets as more phosphates are eliminated from protein catabolism. Urine on standing becomes alkaline by the bacterial action on urea and the formation of ammonia.

Specific Gravity: The specific gravity of physiological urine is in the range of 1.016 to 1.022. The variation is according to the concentration of solutes in the urine.

Volume: A normal adult excretes 800-200ml urine daily, according to the quantity of fluids ingestedand environmental temperature.

Chemical Constituents: The principal chemical components of urine are urea, uric acid, creatinine, amino acids, chloride, ammonia, calcium phosphates, sulphates, sodium and potassium and they are present as inorganic substances.

Pathological Constituent

Appearance: If the fresh urine is turbid, it may be due to

(a) Presence of fat globules as in lipuria.
(b) Presence of pus cells in urinary tract infection.

Colour: Dark amber- Concentrated urine or presence of pigments.

Red- Coporphyrins, uroporphyrinshaemoglobin.

Greenish yellow- Bile pigments

Brown to black- Met-haemoglobin, homogentisic acid

Odour: Due to ingested foods like garlic is not pathological smell of acetone but due to chronic starvation, diabetic ketosis.

Reaction: Decrease urinary pH: metabolic acidosis increases; increase urinary pH: metabolic alkalosis increases.

Specific gravity: In diabetic mellitus, the specific gravity is high due to glycosuria.

Volume: Changes in 24hr urine volume are seen in:

(a) Polyuria: Diabetes mellitus and insipidus certain nervous disorders.
(b) Oligouria: Acute nephritis, fever, loss of fluids as in diarrhoea.

The major abnormal constituents of urine are:

1. Blood e.g haemoglobin, erythrocytes.
2. Protein e.g albumin, globins globulins.
3. Acetone bodies e.g. acetone, acetoacetic acid.
4. Sugar e.g. sugar lactose.
5. Bile salt pigments and pigments.
6. Porphobilinogen (increased or decreased).
7. Urobilinogen (increased or decreased).

The presence of any of these three substances suggests disease conditions. Clinistix can be used to detect these abnormal constituents of urine.

Experimental procedure

Carry out the following tests on the urine sample provided.

1. examine the physical characteristics with respect to
    i. appearance
    ii. odour
    iii. reaction

    Inference

2. **Proteins (albumin)**
    a. **Coagulation test**
    Acidify 10ml of the urine in a test tube with3ml of 2% acetic acid, mix and heat the upper portion. A precipitate in the heated liquid denotes albumin compare with the lowest portion.
    b. **Heller's test**
    Pour 3ml conc. nitric acid down the sides of a test tube containing 3ml urine. A fluffy white ring at the junction of the liquid is positive for protein.
    c. **Sulphosalicylic acid test**
    Add 0.5ml of sulphosalicylic acid to 3ml urine, protein are precipitated. Globulin proteoses disappear on boiling and reappear at about 50°C.

3. **Blood**
   Add a few drops of O-tolidine reagent (0.5%in glacial acetic acid) to an equal volume of 30% hydrogen peroxide in a test tube. Add about 1ml of urine (boiled and coloured). A deep green or blue colour indicates the presence of blood.
4. **Acetone bodies**
   Rethora's test
   Add ammonium sulphate crystals to saturate 5ml urine. Add 1ml conc. ammonia and a few drops of sodium nitroprusside solution, mix, allow to stand and note the colours formed.
5. **Bile salts and pigments**
   Hay's test for bile salts
   Take half a test tube of dilute bile (test urine sample) and half a test tube of water (control). Gently sprinkle sulphur powder into both tubes. Sulphur sinks in the bile but floats on water why?

Gmeline test for bile pigment

Pour 3ml conc. nitric acid down the sides of a test tube containing 3ml urine so as to form an interface. A green ring is positive for bile pigments.

Fouchet's test for bile pigment

To 5ml bile in a test tube, add a few crystal of magnesium sulphate and dissolve. Add sufficient $BaSO_4$, filter and dry the precipitate using filter papers. The bile pigments get absorbed to $BaSO_4$. Place one drop of Fouchet's reagent on the dry precipitate. A green/bluish green colour develops on the precipitate showing the presence of bile pigments.

6. **Sugar**
   To 5ml benedicts add 1ml of urine, put in a boiling water bath for 5 minutes. A reaction indicates the presence of reducing sugar.

7. **Urobilinogen and porphobilinogen**

Add 5ml Ehrlich's diazo reagent to 5ml urine mix and stand for 10 minutes. Add 5ml chloroform, shake vigorously for a few seconds and allow layers to separate. If the chloroform layer is pale pink, it indicates urobilinogen. If the aqueous layer shows pale pink, there is presence of porphobilinogen.

Question

1. In what diseased state would the following be positive in urine
a. Protein
b. Blood

c. Acetone bodies
d. Sugars
e. Bile pigments
f. Urobilinogen and porphobilinogen

EXPERIMENT 25: TEST FOR DETECTION OF KETONES (ROTHERA'S METHOD)

Introduction

The formation of acetoacetic acid (3-hydroxybutyric acid and acetone, usually called acetone bodies) in quantity in the blood may be dangerous or even fatal. Apart from any intrinsic toxicity, two of the substances are fairly strong acids which have to be neutralized before excretion. The organism may be unable to supply alkali for this without seriously depleting the food alkali necessary for respiration. Man has no rapid adaptation against this type of poisoning (acidosis). It is consequently important that attention should be given to this aspect of fatty acid catabolism.

The disease condition occurs only where there is lack of available carbohydrate and will always be associated with ketosis (acetone bodies in blood) and ketonuria (excretion of acetone body in urine).Small amount of acetone bodies are excreted normally eg in 24 hours acetoacetic acid- 9mg, β-hydroxybutyric acid- 25mg and acetone- 3mg. Pathologically the amounts are greatly increased, especially that of β-hydroxybutyric acid of which 75g per day may be excreted in severe diabetic mellitus.

Principle

The acetoacetic group, or acetone reacts with sodium nitroprusside at the optimum alkaline pH provided by the buffer system to give purple colour.

Materials

1. Ammonium sulphate
2. Sodium nitroprusside
3. Concentrated ammonia

Procedure:

1. Fill about ½ inches of a 6" x 5/8'' test tubes with crystalline ammonium sulphate.
2. Add approximate 5ml urine and one crystal of sodium nitroprusside.
3. Shake well to mix. Add 1 to 2ml of concentrated ammonia.
4. Mix by inversion and allow to stand for a few minutes. Nitroprusside in alkaline solution reacts with a ketone group to form a purple colour. Therefore a permanganate colour indicates the presence of acetone, acetoacetic acid or both.

Sample		Test	Observation	Inference
Urine	**A**			
	B			
	C			
	D			

Conclusion/ Summary

EXPERIMENT 26: FOUCHETS TEST FOR BILIRUBIN

Introduction

The amount of bilirubin found in blood is normally very small (0.1 – 0.5mg) per 100ml. If the bilirubin content is abnormally high, the pigment diffuses through the capillaries and gives the skin and mucous surfaces the characteristic yellow appearance of jaundice. This may be caused by several alterations including excessive haemolysis (physiologically in the Jaundice of new born and pathologically in haemolytic jaundice), hindrance of excretion due to injury of the polygonal cells of the liver, which excrete the pigment (infective jaundice) and prevention of excretion due to obstruction of the bile duct (obstructive jaundice). In the last condition, bilirubin is excreted in the urine in appreciable amount.

The colour of bile is mainly due to bilirubin and biliverdin which are golden yellow and green respectively. Bilirubinuria is usually accompanied by excretion of bile salts. Bile salts may be excreted in urine without bile pigment in certain stages in liver diseases. Traces of bilirubin without bile salts may be excreted in condition of excessive haemolysis.

Principle

Barium chloride combines with sulphate radicals in the urine forming a precipitate of barium sulphate. Any bile pigment present adheres to these larger molecules and is detected by the oxidation of bilirubin (yellow) to biliverdin (green) with ferric chloride in the presence of trichloroacetic acid.

Materials:

1. Glacial acetic acid
2. Barium chloride
3. Fouchet reagent

Procedure:

1. Test the reaction of the urine and if alkaline, acidify with 2 drops of glacial acetic acid.
2. Add 5ml of 10% of barium chloride to 10ml urine and mix well.
3. Filter to remove the precipitate.
4. Unfold the filter paper and add one drop of Fouchet’s reagent into the precipitate if bile is present, a green or blue colour develops.

Sample		Test	Observation	Inference
Urine	**A**			
	B			
	C			
	D			

Conclusion/ summary

EXPERIMENT 27: EHRLICH'S TEST FOR UROBILINOGEN/ PORPHOBILINOGEN

Introduction

After excretion from the gall bladder into the duodenum, biliverdin and bilirubin pass to the large intestine where they are reduced by the action of bacteria to a colourless chromogen called urobilinogen. Urobilinogen are isolated from pathological urine. In the condition of excessive haemolysis e.g haemolytic jaundice or pernicious anaemia, part of the bile pigment formed by break down of the haemoglobin is excreted in urine as urobilinogen (and possibly urobilin and urobilinus) formed from colourless urobilinogen when the urine is exposed to air, giving the urine an orange colour. Abnormal amount of urobilin may be found in liver disease or temporary in constipation.

Materials

1. Ehrlich reagent (p-dimethyl amino benzaldehyde).
2. Hydrochloric acid.
3. Sodium acetate.
4. Chloroform or amyl alcohol.

Method

To 2ml of urine add 2ml of Ehrlichs reagent (p-dimethylamino benzaldehyde in hydrochloric acid).

1. To 2ml of urine add 2ml of 6N HCl to act as control.
2. Allow to stand for 10 minutes.
3. Add 4ml of saturated sodium acetate.

A pink or red colour is given by urine with increased urobilinogen content. Porphobilinogen is also detected by Ehrlich test. To distinguish from urobilinogen; urobilinogen colour is soluble in chloroform whereas any porphobilinogen remains in the aqueous layer and is not extracted by the organic solvent.

NB: Chloroform may be substituted with amyl alcohol. The urobilinogen is always extracted by the organic solvent and porphobilinogen remain in the aqueous phase.

Sample		Test	Observation	Inference
Urine	**A**			
	B			
	C			
	D			

Conclusion/ Summary

EXPERIMENT 28: LIVER FUNCTION TESTS

There are several tests to assess the functions of the liver. The serum alanine amino transferase activity (ALT) or the aspartate amino transferase activity (AST) is widely utilised. The levels of these enzymes are elevated in liver damage, obstructive jaundice, infective hepatitis and liver cancer.

1. Alanine amino transferase activity (ALT)
   Alanine amino transferase or serum glutamate pyruvate transminase (SGPT) catalyzes the reversible conversion of alanine to pyruvate

$$\text{Alanine} + \alpha\text{- ketoglurate} \xrightleftharpoons{\text{ALT}} \text{glutamate} + \text{Pyruvate}$$

The rate of formation of pyruvic acid is proportional to the activity of the ALT. The normal value of ALT is 10-35 IU/L.

Principle of the reaction

Pyruvate is converted to lactic acid by the enzyme lactate dehydrogenase (LDH). Each molecule of pyruvic acid reduced by the LDH requires an equimolar amount of $NADH_2$ to be oxidised to NAD. This is measured by the decrease in absorbance at 340nm. The amount of $NADH_2$ oxidized per unit time is proportional to ALT activity.

Experimental procedure

1. You have been provided with a serum sample of unknown concentration. Into a cuvette, pipette the following solution
   Alanine 3.0ml
   NADH 0.05ml
   LDH 0.05ml
   Serum 0.05ml
2. Cover the cuvette with parafilm and mix thoroughly by gentle inversion. Place the cuvette in a 25°C water bath for 5 minutes. Add 0.10ml α-ketoglutarate to the mixture.
3. Mix thoroughly and read the absorbance immediately at 340nm for zero time. Return the cuvette to the 25°C water bath and read again at exactly 5, 10, 15, 20, 25 and 30 minutes. Keep the cuvette in-between readings in the water bath.

Exercise

1. Calculate the IU (activity) of ALT in the serum sample. It is defined as one IUL/mole NADH oxidized at 25°C at pH 7.4.

Clue

i. Calculate the mean decrease in absorbance at 340nm per minute.

$$\text{Activity} = \frac{\Delta E/\text{min} \times 1000 \times V_2 \times f}{6.22 \times 10^5 \times V_1 \times d}$$

V_1 = Volume of the enzyme

V_2 = Final reaction mixture volume

6.22 = the extinction coefficient of NADH ($6.22 \times 10^5 cm^2$)

1000 = factor to convert enzyme activity to µmole/min/ml or nM/min/ml

f = dilution factor

d = the light path length (1cm).

2. Explain clearly how serum bilirubin and urinary urobilinogen levels give a clear picture of an infected liver.
3. Explain the roles of liver in amino acid deamination and detoxification .

EXPERIMENT 29: ASPARTATE AMINO ACID TRANSFERASE ACTIVITY (AST)

AST catalyzes the reversible transfer of an amino group between aspartic acid and α-ketoglurate to oxaloacetate.

$$\text{Aspartic acid} + \text{a-ketoglutarate} \xrightleftharpoons{\text{AST}} \text{Oxaloacetate} + \text{glutamate}$$

The rate of formation of oxoaloacetate is proportional to the activity of the AST.

The normal value of AST is 8 – 20 IU/L.

Principle

The rate of formation of oxaloacetate is estimated by converting it to malate by the enzyme malate dehydrogenase (MDH). For each molecule of oxaloacetate reduced to malate, one mole of $NADH_2$ is oxidised to NAD. The rate of $NADH_2$ oxidized is therefore directly related to the SGOT activity. It is measured by the decrease in absorbance at 340nm over a standard time interval.

Experimental procedure

1. In a cuvette, pipette the following solution:

Solution	1	L-Aspartate	3.00ml
Solution	2	NADH	0.005ml
Solution	3	MDH	0.05ml
		Serum	0.05ml

   (The solutions are in 100nM phosphate buffer at pH 7.4).
2. Cover the cuvette with parafilm and mix thoroughly by gentle inversion.
   Place the cuvette in a 25°C water bath for approximately 5mins to allow temperature equilibrate

   Solution 4 (α- ketoglutarate) 0.1ml
3. Mix thoroughly and immediately read the absorbance at 340nm. At the same time you read the absorbance (zero time), start the clock. Return the cuvette to the 25°C water bath at exactly 2, 4, 6, 8, 10, 15, 20 and 30 minutes.
   Record the absorbance. Keep the cuvette at 25°C between readings. The absorbance at 340nm decreases as NADH is oxidized.

Exercise

1. Calculate the IU AST in the serum sample. One IU is defined as IU/mole

NADH oxidized/minute at 25°C at pH 7.4.
Hint: as in ALT calculations

2. Explain the biochemical events leading to the clinical condition called jaundice
3. What is kernicterus?
4. Why is urine darkly pigmented and stools abnormally pale in obstructive jaundice?

DISCUSSION

EXPERIMENT 30: ESTIMATION OF LACTATE DEHYDROGENASE (LDH)

Human sera contain several LDH isoenzymes and their relative portions change significantly in certain pathologic conditions. High level of the enzyme is caused by damage to the tissues. Myocardial infarction, infection hepatitis and muscular dystrophy results in elevated levels.

LDH catalyzes the reaction of pyruvate to lactate

$$\text{Pyruvate} + \text{NADH} + H^{+} \longrightarrow \text{Lactate} + \text{NAD} + \text{LDH}$$

The normal value of serum LDH at 30°C is 55 – 140 IU/L.

Principle

Pyruvate is reduced to lactate in the presence of NADH. The decrease in absorbance at 340nm as the coenzyme is oxidized to NAD provides a measure of enzyme activity. This favours lactate formation.

Experimental procedure

1. In a cuvette, pipette the following
   Solution (Pyruvate in 50nM = phosphate buffer pH 7.5) 3.00ml
   Place the cuvette in a 25°C water bath for approximately five minutes to equilibrate and add

SolnII	NADH	0.05ml
Serum		0.1ml
	Total Volume	3.15ml

Cover the cuvette with paraffin and mix thoroughly by gentle inversion. Read the absorbance at 340nm using spectrophotometer. Start the clock (zero time), return the cuvette to the water bath (20°C) at 1, 2, 3, 4, 5, 6, 8, 10, 15 and 20 minutes.

Record the absorbance at 340nm. Keep the cuvette in the water bath 25°C between readings and it is imperative to be exact in the time measurements.

Exercise

1. Calculate the IU/L in the sample. One IU is defined as IU mole of NADH oxidized/min at 25°C pH 7.5.

   Hint:

   $$\text{Activity} = \frac{\Delta E/\text{min} \times 1000 \times V_2 \times f}{6.22 \times 10^5 \times V_1 \times d}$$

   V_1 = Volume of the enzyme

   V_2 = Final reaction mixture volume (3.15)

   6.22 = the extinction coefficient of NADH ($6.22 \times 10^5 cm^2$)

   1000 = factor to convert enzyme activity to µmole/min/ml or nM/min/ml

   f = dilution factor

   d = the light path length (1cm).

2. Why is LDH referred to as a kidney marker enzyme?

DISCUSSION

EXPERIMENT 31: DETERMINATION OF SERUM CHOLESTEROL

Cholesterol is one of the most important animal sterols. Highest concentrations are found in nerve and glandular tissue. It is an important precursor of many steroids.

Normal serum total cholesterol is in the range of 150 – 200mg/dl. 1/3 of the total cholesterol is in the free form and 2/3 is esterified. High concentration of cholesterol in blood is considered one of the risk factors for coronary heart disease and atherosclerosis. Total cholesterol in serum is increased in diabetes mellitus, chronic alcoholism and hyperlipoproteinemias.

Cholesterol is determined clinically by treating it with Liebermann– Burchard reagent which produces a blue green colour.

Experimental procedure

1. Prepare the following tubes by pipetting 1-5ml of the standard cholesterol solution. Pipette in duplicate 2ml of the unknown sample in tubes 7 and 8.
2. Make the volume equal to the (5ml) with chloroform.
3. Prepare the Libermann – Burchard reagent freshly by
   a. Pipetting 20ml of acetic anhydride and cooling it on a chilled water bath.
   b. To this cooled acetic anhydride, add 1ml of previously cooled concentrated H_2SO_4 while keeping the mixture in a chilled water bath. Mix the solution thoroughly.
   c. Add 2ml of Liebermann – Burchard reagent to each tube and mix thoroughly. Keep the tubes in a dark cupboard and read after 30 minutes at 660nm.

Result

Tube No	1	2	3	4	5	6	7	8
Standard cholesterol solution (ml)	-	1	2	3	4	5	-	-
Conc. Of solution (ml)								
Unknown solution (ml)							2	2
Chloroform (ml)	5	4	3	2	1	0	3	3
Libermann – Burchardreagent (ml)	2	2	2	2	2	2	2	2
O. D at 660nm								

Exercise

1. Prepare the calibration curve and calculate the concentration of the unknown solution.

2. Prepare a diet regimen for a patient with high cholesterol serum levels. What is the biochemical basis of this regimen?
3. What is cholecystitis?

GRAPH

DISCUSSION

EXPERIMENT 32: IODINE VALUE LIPIDS

The iodine number of a lipid is defined as the number of grams of iodine which can be absorbed by 100grams of the lipid. It is a measure of the degree of unsaturation of the fatty acids present in the lipid.

Principle

Since iodine does not react readily with iodine bonds in fatty acids, bromine 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 standard solution of potassium thio-sulphate.

Experimental procedure

1. Determine the iodine equivalent of the blank by pipetting 5ml of chloroform into a conical flask. Add 5ml of dam's iodine into a burette. Cork and shake thoroughly, then place the flask in a dark cupboard for 10 minutes.
2. Add 5ml of 10% potassium iodine solution and add 20ml water. Mix thoroughly.
3. Titrate the liberated iodine with the standard thiosulphate provided. Towards the end of titration (when the solution has a pale straw colour), add 1ml of a starch solution. Continue the titration until the blue colour disappears.
4. Shake the conical flask throughout the titration to ensure that all the iodine is removed from the chloroform layer.
5. Repeat the entire procedure using in place of chloroform, 5ml of 0.5% chloroform solution of the test sample provided i.e dissolve the test samples in 0.5% chloroform.

Exercise

i. Calculate the iodine value of various lipids.
ii. Briefly explain the importance of the unsaturated fatty acids in medicine.
iii. Draw the structure of γ – linolenic acid.
iv. Name two polyunsaturated fatty acids obtained from linolenic acid.

CALCULATION

DISCUSSION

EXPERIMENT 33: ACID VALUE OF LIPIDS

The amount of free fatty acid present is therefore an indication of the age quality of the oil.

The acid value is defined as the number of mg potassium hydroxide or sodium hydroxide required to neutralize the free acid present in 1.0g of the lipid. It is a relative measure of rancidity as free fatty acids are normally formed during decomposition of oil glycerides.

Principle

The acid value is determined by directly titrating the oil/fat in an alcoholic medium against potassium hydroxide or sodium hydroxide to neutralization.

Experimental procedure

1. Weight exactly 10.0g of the lipid and add 1.0ml phenolphthalein solution, mix thoroughly and titrate with 0.1M KOH/NaOH from a burette until the faint pink colour persists for 20 – 30 seconds. Note the volume of alkali used.

Exercises

1i Calculate the acid value of the lipid provided.

ii Which fatty acids are important for the maintenance of good health? How do they perform their roles in the human body?

iii Are phospholipids? Name the acidic phospholipids.

2a Distinguish between saturated and unsaturated fatty acids.

b. What is rancidity?

$$AV = \frac{56.1\ VN}{W}$$

V= Volume of KOH used.

N= Normality (Molarity) of KOH

W= Weighty of sample in gram, 56.1= Constant

CALCULATION

DISCUSSION

EXPERIMENT 34: EFFECT OF ELECTROLYTE ON SALIVARY AMYLASE

Digestion of food in the mouth by the action of the enzyme salivary amylase on starch is split into maltose by α-amylase in saliva. The optimum pH for amylase action is 6.6.

Saliva is a viscous fluid secreted by three pairs of glands in the mouth; the parotid, submaxillary and sublingual glands. Saliva is about 99.5% water; the rest is glycoprotein, certain organic constituents and inorganic salts including thiocyanate.

When salivary amylase acts on its substrate (starch), a stage of digestion is reached when no colour is obtained with iodine solution. This stage is called the 'achromic point'. The time taken to reach this point is known as the chromic period. The determination of the chromic period serves as an index of the enzyme activity.

Experimental Procedure

1. Prepare the tubes as in the table, without the saliva. Pre-incubate all mixture at 37°C in the water bath for 5 minutes.
2. At zero time add 2.0ml of saliva.
3. At 1 minute interval, test 3 drops aliquot of each solution in the tube with 2 drops of 0.01 in iodine solution using a microtitre 1 spot plate.

   Note the colour change, when no colour is obtained with iodine, note the time, this is the "achromic point"

Tube No. (ml)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1% starch	10	10	10	10	10	10	10	10	10
Distilled water	1	--	--	--	--	--	--	--	--
0.1M NaCl	--	1	--	--	--	--	--	--	--
0.1M Na_2SO_4	--	--	1	--	--	--	--	--	--
0.1M $CaSO_4$	--	--	--	1	--	--	--	--	--
0.1M KCl	--	--	--	--	1	--	--	--	--
0.1M $AgNO_3$	--	--	--	--	--	1--	-	--	--
0.1M KBr	--	--	--	--	--		1	--	--
Saliva	2	2	2	2	2	2	2	2	2
The chromic period									
Chromic time									

QUESTION

1. Report the achromic point and chromic period of each tube.
2. What is the effect of electrolytes on the salivary amylase enzyme?
3. What can you conclude about the property of this enzyme from this experiment.

DISCUSSION

DISCUSSION

CASE STUDY IN HEALTH AND DISEASE

1. Oral glucose tolerance test was performed on a 48 year old person and the results are given below. What is your opinion

Time (hour)	Blood glucose (mg%)		Urine Sugar (Benedicts test)	
	Patient	Normal	Patient	Normal
0 (Fasting)	80	75	Blue	Blue
0.5	130	130	Blue	Blue
1.0	160	140	Blue	Blue
1.5	142	120	Blue	Blue
2.0	120	70	Blue	Blue

2. The following are the findings in a patient brought to the hospital in the coma state. What is your diagnosis?

Blood Sugar	Patient	Normal
(fasting)	270mg%	65-110mg%
Benedict's test with Urine	Orange	Blue
Rothera's test with Urine for ketone bodies	Positive	Negative
Serum Bicarbonate	16mEq/L	21-28mEq/L
Plasma pH	7.25	7.35-7.45

3. A child was brought to a doctor with a complaint that the child was not growing well and milestones of the child was delayed.

The child was often suffering from diarrhoea. On examination, the child was found to have cataract in the eye. Urine examination showed reduction with Benedict's reagent but not with the glucose oxidase method. What is the probable diagnosis in this case?

4. A young man came to the doctor complaining of diarrhoea. His eyes were sunken and the physician noted additional signs of dehydration. His temperature was normal. He explained that the episode had occurred following intake of lots of ice cream. Prior episodes of similar nature were observed following intake of significant amount of dairy products. Explain the biochemical reason for such type of clinical picture.
5. A 40 year old man on his annual biochemical investigations is found to be hypercholesterolemic with fasting plasma cholesterol level of 8.5mmol/l (327mg/dl). He was treated with a synthetic cholesterol dropped for this slight decrease despite being the patient on cholesterol free diet?
6. In the above case, suggest the possible successful drug treatment for lowering fasting cholesterol.
7. Lipid profile of a patient shows high LDL concentration. Explain how elevation of LDL concentration is a risk factor in the development of myocardial infarction.

DISCUSSION

DISCUSSION

DISCUSSION

DISCUSSION

DISCUSSION

APPENDIX

NORMAL LEVELS OF IMPORTANT CHEMICAL CONSTITUENTS

1. Blood

(pH = 7.4; Volume 4.5 to 5.0 litres in adults)

Constituent	**Unit**	**Normal Level**
Acid phosphate (serum)	K – A units/dl	13.5
Alkaline phosphate (serum)	K – A units/dl	4 – 11
Alkali reserve (plasma)	Volumes/dl	50 – 70
	meq/l	22 – 30
Amylase (serum)	Somogyi units/dl	80 – 180
Bilirubin, total (serum)	mg/dl	0.2 – 0.8
Calcium, total (serum)	mg/dl	9 – 11
	meq/l	4.5– 5.5
Chloride, as NaCl (serum)	meq/l	95 - 110
Cholesterol, total (serum)	mg/dl	150 – 250
Cholesterol, ester (serum)	mg/dl	100 – 170
Copper (serum)	µg/dl	75 – 160
Creatinine (serum)	mg/dl	1.0 – 1.8
Fibrinogen (plasma)	mg/dl	200 – 400
Haemoglobin (blood)	g/dl	14 – 16 (men)
		13 – 14 (women)
GOT (serum)	m.IU/ml	6 – 18
GPT (serum)	m.IU/ml	4 – 16
Iron (serum)	µg/dl	65 – 175
LDH, total (serum)	m.IU/dl	70 – 240
Lactic acid (blood)	mg/dl	6 – 18
NPN(blood)	mg/dl	20 – 40
Potassium (serum)	meq/l	3.5 – 5.5
Phosphorus, inorganic (serum)/	mg/dl	3.0 – 4.5
	g/dl	6.5 – 7.5
Protein, total (serum)	g/dl	3.5 – 5.0
Albumin (serum)	g/dl	
Globulins, total (serum)		
Proteins, differential by paper electrophoresis	% of total proteins	Albumin 55.0 α1-globulins 5.3 α2-globulins 8.7
	µg/dl	β-globulins
PBI (serum)	mg/dl	13.4
Pyruvic acid (blood)	meq/l	ϒ-globulins
Sodium (serum)		17.5
Sugar, fasting (blood)	mg/dl	3.5 – 7.0
Folin-Wu	mg/dl	0.5 – 1.0

Nelson-Somogyi	mg/dl	136 – 149
Glucose oxidase	mg/dl	
Triacylglycerols, as triolein V		80 – 100
	mg/dl	70 – 90
Urea (blood)	mg/dl	60 – 70
Urea nitrogen (blood)	mg/dl	50 – 155 (men)
Uric acid (serum)	mg/dl	40 – 115 (women)
Vitamin A (serum)		15 – 35
		9.5 – 20
		3.0 – 5.0
		19 – 36

2. CSF

(Appearance: colourless, clear fluid; pH + 7.4; volum = 125mlin adults

Constituent	**Unit**	**Normal Level**
Chloride, as NaCl	meq/I	120 -130
Globulins	Qualitative	Nonne-Apelt test: - -ve
		Pandy's test : -ve
Proteins	mg/dl	15 – 40
Sugar (Folin- Wu)	mg/dl	50 - 80

3. Urine

Constituent	Unit	Normal Level
Amino acid nitrogen	g/24 hours	0.08 – 0.15
Ammonia nitrogen	g/ 24 hours	0.5 – 1.0
Amylase (diastatic index)	wohlgemuth unit/ml	6 – 30
Calcium	mg/24 hours	100 – 300
Citric acid	mg/24 hours	300 – 800
Chloride as NaCl	g/24 hours	10 -12
Creatinine	g/24 hours	1.0 – 1.2
17 – ketosteroids	mg/24 hours	10 – 20 (men) 6 – 16 (women)
Phosphate, inorganic as	mg/24 hours	600 – 100
phosphorus sulphur, total	g/24 hours	0.7 – 1.5 Neutral = 10% Ethereal = 10% Inorganic = 80%
Titrable acid	meq/24 hours	15 - 50
Urea	g/24 hours	15 - 25
Uric acid	g/24 hours	0.5 – 0.7

Plasma proteins	Normal values g/100ml
Total	6.3 – 7.8
Albumins	3.2 – 5.1
α1–globulins	0.06 – 0.39
α2–globulins	0.28 – 0.74
β-globulins	0.69 – 1.25
immunoglobulins (or ϒ-globulins)	0.8 – 2.0
IgA	0.15 – 0.35
IgG	0.8 – 1.8
IgM	0.08 – 0.018
IgD	Approximately 0.003
Fibrinogen	0.2 – 0.4
Mucoprotein	Approximately 0.135
haptoglobulin	0.03 – 0.19