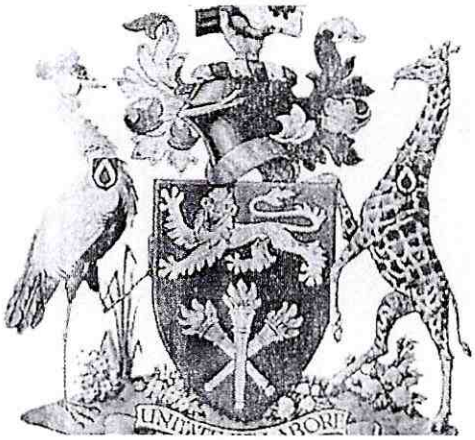


UNIVERSITY OF NAIROBI

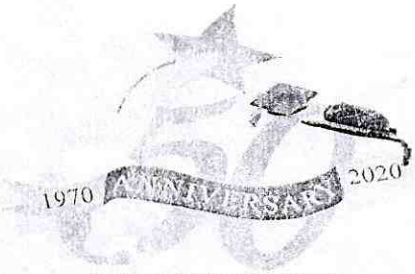
DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL

FOR

FIRST YEAR STUDENTS OF MBCHB, B.PHARM & BDS

ACADEMIC YEAR 2020/2021



UNIVERSITY OF NAIROBI
Celebrating 50 years of Academic Excellence

Contents

THE FOLLOWING MUST BE OBSERVED AT ALL TIMES:

1. **PUNCTUALITY** must be observed at all times, reporting late for practical lessons will not be tolerated.

2. For every practical done, a Lab Reports must be written and submitted for marking **NOT LATER THAN 7 DAYS** from the date the practical was done. (Drop your report in the **Box** next to room **B32**). Failure to observe the above schedule will attract a **PENALTY**.

3. Attend practical with your **DESIGNATED GROUP** unless prior permission is granted by the coordinator before the scheduled practical.

4. Practical are **NOT OPTIONAL**. Note that **NO** other practical(s) will be arranged for candidates who miss the same with their designated groups.

5. Students must write their original lab reports based on data obtained in the lab and research in library. **COPYING REPORTS IS UNETHICAL AND TOTALLY UNACCEPTABLE.** No mark will be awarded for copied report(s) and further disciplinary action may be instituted.

6. During the practical session students must **SIGN THE ATTENDANCE REGISTER** at the start and end of each practical session.

7. Knowledge gained from practical lessons will also be **EXAMINED** in **CATs** and **End of year exam.**

8. **CONSULT** team leaders for gp **A (Ms Agnes)** & gp **B (Ms Diana) (Room B18)** for any queries on Practical matters.

9. Please note that personal commitments **WILL NOT BE ALLOWED TO INTERFERE** with the smooth running of the academic programme.

10. Observe **HONESTY** at all times

AIMS AND OBJECTIVES OF "PRACTICAL BIOCHEMISTRY

The experiments described in this manual have been compiled for the students of medicine, pharmacy and dentistry by the Department of Biochemistry, University of Nairobi. The experiments will supplement the lectures in biochemistry that are given during the same period.

Each practical ends with questions that should be answered and submitted for marking. By answering the questions, you will obtain a better and longer-lasting understanding of biochemical techniques and the intricacies of biochemistry. When you have finished the course you should be able to:-

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

Revised and re-edited in 2013 by Prof. E. K. Nguu, Dr G. Juma, Thomas M Arani & Stephen M. Mutune

DIRECTIONS FOR PRACTICAL LABORATORY CLASSES AND GENERAL INSTRUCTIONS

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

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

Laboratory rules

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

Use of lab equipment for volumetric and gravimetric methods

1. **Volumetric flasks**: These are calibrated to contain a given volume at a specified temperature usually 20^{o} or 25^{o} C.

2. **Pipettes**: There are three basic types of pipettes used in biochemical work. These are:-
(i) Bulb (ii) Straight (iii) Micro

Transfer pipettes: (This type has a bulb in the middle). This is the most accurate type and is calibrated to deliver a certain volume. Such a pipette must be clean to deliver accurately. A test for cleanliness is to allow water to run out of it. If it drains smoothly, leaving no adhering drops within, it is satisfactory. If drops remain behind the pipette obviously it will not deliver the indicated volume.

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

3. **Burettes**

Macroburettes:

Fifty ml burettes are graduated to 0.1 ml but the levels of liquid can be estimated to 0.01ml. a burette must be absolutely clean to deliver accurately and must first be tested with water to make certain if drains without leaving droplets behind. Be sure the burettes do not leak when the stopcock is closed. Before performing titration ensure that there are no air bubbles trapped, especially near the stopcock or the drawn-out tip. Bubbles usually can be removed by allowing one or two ml to flow out. In reading a burette be sure your eye is on the same level as the meniscus to avoid parallax errors. If the burette is allowed to drain with stopcock open the film of solution remaining on the inner surface will not drain as fast as the level of the solution and a large error can ensure unless one waits for a few minutes after closing the stopcock before making a reading, to allow this film to come into equilibrium with the

column through surface tension effects. This is very important as many burettes are marked with a minimum emptying time which must be observed to get accurate readings. It is therefore advisable to allow drainage during a titration to occur at a rate considerably less than "wide open" to minimize such drainage errors.

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

Semi-microburettes:

These are frequently used in biochemical analysis. Microburettes have a total volume of 1.0, 2.0 or 5.0 ml and are graduated usually to 0.002 ml. They are used in the same manner as macro burettes, but drainage errors become much more serious. Remember that a single drop of an aqueous reagent may have a volume of about 0.05 ml. this magnitude in a semi-micro titration is of course very serious. Semi-microburettes are usually loaded from a permanently connected reservoir of reagent by means of pressure from a hand-bulb.

Cleaning Glass ware

Cleanliness of glassware is essential in all chemical manipulations, particularly in analytical work. Each student will clean his own equipment, and whenever possible this should be done immediately after use.

If precipitates and solutions are allowed to dry on the surface of glassware, the latter is much more difficult to clean. This is particularly true of dried deposits of fats, proteins, tissues etc. The best general cleanser for laboratory glassware is soap and warm water applied with still brush. Caution should be taken to avoid scratching the glassware with the metal handles of brushes. There are a wide variety of brushes available for different kinds of apparatus. Thorough rinsing with distilled water is required to obtain chemically clean glassware.

Cleaning of burettes and pipettes requires special attention since improper drainage of these due to greasy surface introduces serious errors in quantitative work. A general test for cleanliness of burettes and pipettes is to fill them with H_2O and allow them to drain at a normal rate. If any visible drops or rivulets of H_2O are left behind on the surface of the glass then the surface is not clean enough to drain reproducibly. If pipettes are clean to being with, they may require only copious rinsing with tap and then distilled water immediately after their use of preserve cleanliness. After pipetting biological materials such as blood, immediately rinse the pipette with water. Further cleansing with soap may be necessary.

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

Volumetric analysis and stoichiometric calculations

The concentrations of solutions are usually expressed in terms of ***molarity*** or ***normality***. A molar solution contains one gram-equivalent weight of the substance in one liter of the solution. Example: The molecular weight of NaOH is 40.01. A molar solution of H_2SO_4 contains 98.08 g of H_2SO_4.

A normal solution contains one-gram equivalent weight of the substance in one liter of solution. The equivalent weight of the substance depends on the nature of the reaction for which the solution is used. In acid-base reaction which yields or combines with exactly 1.0080 grams H^+ (the weight of one gram ion of H).

This process of quantitative combination, leading to neutralization of solution is called ***titration***. Example: The molecular weight of NaOH is 40.01. One molecule of NaOH can combine with one of H^+ ($H^+ + OH^-$), hence the equivalent weight of NaOH is 40.01 g and a normal solution of NaOH has the same concentration as a molar solution. HCL has a molecular weight of 36.47. A normal solution of HCL contains 36.47 g HCL per litre of the solution since one molecule of HCL yields one H^+. However, sulphuric acid contains two ionizable hydrogen atoms. The equivalent weight of H_2SO_4 is therefore the gram-molecular weight divided by 2 or 98.08 = 49.08g and a normal solution for H_2SO_4 therefore contains 49.04 g/2 H_2SO_4 per liter.

Other methods of expressing concentration

A molal solution is one containing 1.00 grams molecular weight of a solute dissolved in 100gm of solvent (note the distinction between a molar and a molal solution). Molality is properly used in calculations involving the colligative properties, such as osmotic pressure, freezing and boiling point depression and elevation, and certain other thermodynamic properties of water other solvents. Percent weight indicated grams of solute in 100 g of solution (w/w). This is the expression used for commercial available solutions. Percent volume indicates grams of solute in 100 mls of solution. This is by far the more commonly used expression involving per cent (w/w) Volume per cent.

This expression is commonly used to indicate concentration of a solution of the solute in a liquid. 50% alcohol for example means we have 50 mls in 100 ml solution

PRACTICAL 1

THE BEER-LAMBERT'S LAW

Why solutions are colored

Many biochemical experiments involve the measurement of a compound or group of compounds present in a complex mixture. Probably the most widely used method for determining the concentration of biochemical compounds is colorimetry, which makes use of the property that when white light passes through a coloured solution, some wavelengths are absorbed more than others. Many compounds are not themselves coloured, but can be made to absorb light in the visible region by reaction with suitable reagents. These reactions are often fairly specific and in most cases very sensitive, so that quantities of material in the region of millimole per liter concentrations can be measured.

The big advantage is that complete isolation of the compound is not necessary and the constituents of a complex mixture such as blood can be determined after little treatment. As discussed below, the depth of colour is proportional to the concentration of the compound being measured, while the amount of light absorbed is proportional to the intensity of the colour and hence to the concentration.

The Beer-Lambert Law

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

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

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

$I = I_o e^{-k3cl}$

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

Per cent $T = I / I_o x100 = e^{-k3cl}$

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

Extinction: if negative logarithms are taken on both sides of the equation instead of a ration then:

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

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

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

$A = k_3 cl$

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

Demonstration of beer's law using Bromophenol blue and Methyl orange
Principle
Colored compounds have their own characteristic absorption spectra and careful selection of the wavelengths where maximum absorption is found enables a mixture of two colored substances to be analyzed. In this experiment you will first determine the wavelengths at which the absorbance of bromophenol blue and methyl orange are maximum. You will then proceed to investigate the relationship between the absorbance and concentration of these two dyes at the wavelengths you will have determined.

Materials
1. Digital spectrophotometer
2. Bromophenol blue (10mg/litre)
3. Methyl orange (10 mg/liter)
4. An 'unknown' mixture of the two dyes

Method for absorbance curves

Determine the absorbance of each dye in turn against the range of wavelengths on the spectrophotometer. Remember, the instrument must be reset on zero absorbance with distilled water in the cuvette for each wavelength. Plot a graph of the absorbance recorded against the wavelength.

Carefully **note and record** the wavelength of maximum absorbance of each dye.

Method for demonstration of Beer's law

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

Tube no	1	2	3	4	5	6	7	8	9	10	11
Bromophenol blue (10mg/l)(ml)	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Dist. Water(ml)	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0

Set the wavelength which gave maximum extinction in the light path and zero the spectrophotometer with distilled water. Record the absorbance of each solution and plot this against the concentration of dye in each tube in µg/5 ml.

1. Repeat the above experiment using the wavelength which gave maximum absorption with the methyl orange. How do the curves of extinction against concentration conform to Beer's Law?

The Absorption spectrum of p-Nitrophenol

Materials

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

Method

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

Questions

1. Show the dissociation reactions of p-nitrophenol that explain the colour changes in acid and basic media.
2. Comment on the acid/base properties of p-nitrophenol.
3. Suppose the nitro group in p-nitrophenol was replaced by a carboxyl group, predict the effect of adding (a) HCL (b) NaOH to an aqueous solution as in the experiment above.
4. Explain the importance of p-nitorphenol in a medical biochemistry laboratory.
5. A solution of 10^{-5} moles/litre of substance X shows a transmittance of 70.2 percent at 260

nm in a 1 cm cuvette, calculate: (a) the absorbance (b) the transmittance in a 3 cm cuvette (c) the absorbance of 50 umole/litre of substance X in 1 cm cuvette.

6. A solution Y of 29.3 gm/litre has an absorbance of 0.25 at 260 nm. If the light path is 1 cm and the molecular weight of Y is 586, calculate (a) the molar extinction coefficient (b) the transmittance of 10 µmole/litre of solution.
7. Calculate the molar absorption coefficient, Σ, at 351 nm for aquocobalamin in 0.1 M phoshphate buffer, pH 7.0, from the following data, which were obtained in a 1 cm cell.

Solution	**concentration**	$\mathbf{I_o}$	**I**
A	2.23×10^5 M	93.1	27.4
B	1.90×10^5 M	94.2	32.8

PRACTICAL 2

HENDERSON - HASSELBALCH EQUATION AND PREPERATION OF BUFFERS

TITRATION OF ACID AND BASE

Introduction

Mole Chemistry

Methods of expressing the concentration of solutions

Molarity

The molarity is the number of moles of solute in 1000 mls of solution. The usual symbol for molarity is **M**. Equal volumes of the same molarity contain the same number of solute molecules. Remember Avogadro's number.

Molality

The molality of a solution is the number of moles of solute in 1,000 g of solvent. The usual symbol for molality is **m**. All solutions of the same molality contain the same number of solute molecules in equal weights of solution. Rarely used.

Equivalent weight or combining weight

Equivalent weight or combining weight of an element or ion is its atomic or formula weight divided by its valence. Elements entering to combination always do so in quantities proportional to their equivalent weights.

Normality

A normal solution contains one gram molecular weight of the dissolved substance divided by the hydrogen equivalent or valence of the substance per 1000 mls of solution. The symbol for normality is **N**.

In analytical chemistry involving chemicals between solutions, it is the practice to express concentrations in terms of Molarities or Normalities.

Reagents and Materials

1. Standard: Sodium carbonate (Na_2CO_3; M.Wt. 106) solution containing 0.08gm/10-ml)
2. Unknown Acid: 5.5 ml of concentrated Hydrochloric acid (HCl) diluted with 1000-ml of distilled water.
3. Unknown Alkali: Prepare a saturated solution of Sodium hydroxide (NaOH, M.Wt. 40.00) and allow it to stand for several days.
4. Dilute 5.0 ml of the clear supernatant to 1000 mls with distilled water.
5. Indicator solutions: Methyl red solution; Phenolphthalein solution.

Procedure

A. Into a clean 50-ml beaker, pipette exactly 10 ml of the Sodium carbonate solution (0.08 g/10 ml). Add 2 drops of Methyl red indicator.
   Titrate the base with the HCl solution from a 10-ml pipette at a rate of 1 ml while mixing. Just before the end point is reached, boil the solution to expel the CO_2, cool

and complete the titration. Accurately read and record the volume of the acid used. Run this determination in triplicate.

B. Using the HCl filled pipette, titrate 10-ml portion of the NaOH using Phenolphthalein solution as the indicator. Accurately read and record the volume of the acid used. Run this determination also in triplicate.

Results

Calculate

1. The Normality of the standard solution of Na_2CO_3.
2. The Molarity of this solution.
3. The Normality of the acid and the alkali used in the titration.

Questions

1. What is the molarity and normality of:
   a. acetic acid (60.032 g/litre)
   b. sulfuric acid (98.016 g/litre)
2. A student needs 200 ml of 0.1 M NaOH solution. What weight of solid is used?
3. How many grams of glucose are required to prepare 200 ml of 0.5-M solution?

Determination of pH without use of standard buffer solution

Introduction

Buffer solutions and pH

The animal body is composed of about 70 % water and 30 % dry matter and there are of course a great many dissolved salts or ions in the fluid, among the most important being "Hydrogen ion" H^+. First we need to be clear about what this term means. The common designation of H^+ suggesting a hydrogen atom which has lost its electron or more exactly a proton which carries a unit positive charge. Such a particle does not exist free in solution, but occurs as the strong acid of water, the hydronium ion, H_3O^+. So what we really mean when we speak of the hydrogen ion concentration is the hydronuim ion concentration.

$$H^+ + H2O \longleftrightarrow H_3O^+$$

The range of $[H^+]$ compatible with life is from 10^{-7} to 10^{-8} M and under normal conditions this is very closely controlled.

This experiment is designed to give experience in measuring hydrogen ion concentration and knowledge about the mechanism of control of $[H^+]$, which exists in tissues. However, we will not study them "in vivo" in the animal, but rather in the test tube or "in vitro".

First the Bronsted's definitions:

An acid is a substance that liberates or donates protons and a base is a substance that accepts protons.

Thus $HCl \longrightarrow H^+ + Cl^-$ (in water solution)
(acid) (base)

Or $H_2O \longrightarrow H^+ + OH^-$

NaOH is the sodium salt of the base OH^- or hydroxyl ion and becomes an alkali. This concept of acids and bases is important for it will be expanded later in an explanation of the mechanism of all chemical reactions that occur in the animal body. The concentration of ions in solutions is usually expressed as normality or molarity such as 1N or 0.1 M. The system becomes cumbersome when expressing the (H^+) of water or biological fluids such as 0.0000001 N or $1x10^{-7}$ N. This has led to the use of the term pH in which "p" signifies that the negative logarithm is used.

Thus:

pH = -log of the $[H^+]$

Or

pK = -log of K, the equilibrium constant.

Buffers

Water has the properties of both an acid and a base. Applying the Law of Mass Action to the equation:

$H^+ + OH^- = H_2O$, the equilibrium constant, K, may be expressed:

$$K = \frac{[H^+][OH^-]}{[H_2O]}$$

Since water is negligibly dissociated, the concentration of water is essentially constant and can be combined with K- Kwater.
Then

$$Kwater = [H^+][OH^-] = 10^{-14}$$

This is the ionic product at 22^0C. The value of 10^{-14} is derived from water conductivity measurements. When this expression is converted to the negative logarithm (p = -log).

$$pKwater = 14 = pH + pOH$$

This may be similarly applied to the dissociation of an acid in the terms of Bronsted's definition:

$$\underset{\text{(Acid)}}{Hac} = \underset{\text{(Base)}}{H^+ + Ac^-}$$

The equilibrium constant for the dissociation of the acid becomes:

$$Ka = \frac{[H^+][Ac^-]}{[HAc]}$$

or in terms of $[H^+]$ concentration:

$$[H^+] = \frac{Ka\,[HAc]}{[Ac]}$$

Expressing this in negative logarithms (p):

$$pH = pKa + \log \frac{[Ac^-]}{[HAc]}$$

This expression is known as the **Henderson-Hasselbalch** equation, and can be used in several ways. If the pKa is known for an acid, the ratio of the concentration of the salt to the acid can be used to prepare solutions of known pH as in the first part of the experimental work. An unknown pKa can be determined by measuring the pH of an acid as it is neutralized by a base, this you will do in the second part of this experiment.

As living cells are sensitive to changes in reactions of **pH** it is important that they be protected against such changes by the buffering action of the constituents of the tissue fluids, which provide the immediate environment.

Buffer solutions resist the effect of changes that might be expected by addition of a strong acid and a salt of that acid or alkali. They are solution of either a weak acid and a salt of that acid or a weak base and a salt of that base. Examples are:

Acetic acid + Sodium acetate
Ammonium hydroxide + Ammonium chloride

The reaction on the addition of a strong acid

$$\text{Acid} + \text{Buffer} \longleftrightarrow \text{Buffer} + \text{Salt}$$

$$H^{+}Cl^{-} + HAc + Na^{+}Ac \longleftrightarrow HAc + Na^{+}Ac^{-} + Na^{+}Cl^{-}$$

The H^{+} combines with a strong base, the acetate, to form a weak or undissociated acid.
The reaction of the addition of an alkali:

$$\text{Alkali} + \text{Buffer} \longleftrightarrow \text{Buffer} + \text{Water}$$

$$Na^{+}OH^{-} + HAc + Na^{+}Ac^{-} \longleftrightarrow HAc + Na^{+}Ac^{-} + H_2O$$

The hydroxyl combines with hydrogen from the acetic acid to form the weak acid water.
The pH of a buffer system is described by the Henderson-Hasselbalch equation and the "capacity" is determined by the concentrations.
Indicators are organic acids, which in solution exhibit one colour as the undissociated acid and a different colour as the base.

$$\underset{\text{Red}}{H^{+}IN^{-}} \longleftrightarrow \underset{\text{Yellow}}{Na^{+}IN^{-}}$$

The color change occurs at the point which the pH of the solution equals the pK and they are most commonly used to determine the end point of acid-base titrations. Since the colour change occurs gradually over a short range of pH, they can be used to measure the pH of an unknown solution by comparing colors.

Table of Indicators

Indicator (dye)	PK	pH range	Color change
Topfer's reagent	3.3	2.9 – 4.2	Red – Yellow
Bromophenol Blue	4.7	4.0 – 5.6	Yellow – Blue
Methyl red	5.1	4.2 – 6.3	Red – Yellow
Bromocresol purple	6.3	5.2 – 6.8	Yellow – Purple
Bromothymol blue	7.1	6.0- 7.6	Yellow – Blue
Phenol Red	7.9	6.8 – 8.4	Yellow – Red
Cresol Red	8.1	7.2 – 9.6	Yellow – Red
Thymol Blue (2^{nd} change)	8.9	8.0 – 9.6	Yellow – Blue
Phenolphthalein	8.7	8.3 – 10.0	Colorless-Pink
Thymol blue (1^{st} change)	1.65	1.2 - 2.8	Red – yellow
Bromocresol green	4.7	4.0 - 5.6	Yellow – blue

Reagents and Materials

1. Two solutions X and Y of unknown pH
2. 0.1 M NaOH solution
3. 0.1 M HCl solution
4. Alkaline indicator (dye)
5. Acid indicator (dye)
6. Test tube rack with 9 spaces in a row

Procedure

1. The first step in this procedure is to determine the approximate pH of the unknown solution so that a suitable indicator may be selected from the different indicators with which you are provided.To find out approximate pH range, first use wide range pH paper.

2. Treat a small portion of the unknown solution with 1m drop of indicator solution and compare the colour obtained with 0.1 N HCl and 0.1 M NaOH with the same indicator handled in the same way.

3. The same indicator is used for determining the pH of the unknown, if the colour obtained with the unknown is intermediate between the acid and alkali colours of the chosen indicator. If on the other hand the unknown shows either the full acid or the full alkaline colour with the indicator selected, it is unsuitable and another indicator must be tried in the same way, until the right indicator with the effective range has been found.

4. Set up 18 test tubes of approximately equal bore in 2 parallel rows of 9 in a rack, and add into the back row from left to right, 1, 2, 3, 4, 5, 6, 7, 8, and 9 drops of indicator respectively. Proceed in the same manner with the front row, but from right to left.

5. Add 1 drop of 0.1 M NaOH to each tube in the back row, and 1 drop of 0.1 M HCl to each tube in the front row.

6. Make each tube up to 10 mls with distilled water.
   Each tube contains the same amount of indicator but in different ratios of alkaline: acid from as shown in the table below

Tube pair	1	2	3	4	5	6	7	8	9
Drops in alk. Series	1	2	3	4	5	6	7	8	9
Drops in acid series	9	8	7	6	5	4	3	2	1
Ratio of alk:acid form	1/9	2/8	3/7	4/6	5/5	6/4	7/3	8/2	9/1
Log. Ratios	-.95	-.6	-.37	-.18	0	+.18	+.37	+.6	+.95

7. To determine the **pH** of an unknown, place 10 drops of indicator in a tube of the same bore as used for the standards and make up to 10 mls with the unknown solution. Match this tube with the standard pairs, to obtain the degree of ionization of the indicator, and then apply the equation,

$$pH = pK + \log \frac{\text{alkaline}}{\text{acid}} \text{ ratio}$$

   If the match is between 2 tubes the **pH** for each should be worked out and the average taken. The indicator chosen should have a **pK** near the **pH** of the solution under test. If the **pH** of a solution like urine is required compensation must be made for the colour by placing a tube of the solution behind the standard pair being examined and a tube of water behind the test.

PRACTICAL 3

SEPARATION OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

A mixture of low Mwt. Compounds may often be separated by means of partition chromatography. This method is based on the relative distribution o f the compound between two solvents in which it has different solubility. This partitioning effect may be carried out in solution (as in counter current distribution) or, more commonly, on an inert supporting medium.

The medium in paper chromatography consists of a network of hydrophilic cellulose fibres. The solvent system usually contains an organic alcohol, water and either a strong acid and a base. The cellulose binds to a layer of water, which represents *the stationary phase.* The *mobile phase* is the remaining solvent, which migrates up the paper by capillary action: the organic component having the greatest mobility. A compound applied as sample will distribute itself between the stationary water phase and the mobile organic phase depending on its relative affinity (solubility) for the two solvents. This partitioning effect is repeated many times and since the relative affinity is characteristic for a given compound, a separation is achieved. The more hydrophobic (non-polar) component migrate the furthest, the more hydrophilic (polar) remain near the origin.

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

Reagents and materials

1. 1 % Solution (1 mg/ml in water) of glycine, alanine, leucine, tryptophan, glutamic acid, lysine.
2. A mixture of three of the above (labeled X).
3. Casein hydrolysate (20 mg/ml) casein protein hydrolyzed in 5M H_2SO_4 at 100°C for 4h, then neutralized and deionized with Ba (OH) $_2$.
4. What man No. 1 paper (20 cm x 18 cm).
5. Chromatography solvent Ethanol: Ammonia: Water (8:1:1)
6. Ninhydrin reagent (1% in Ethanol).

Procedure

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

2.Using a pencil draw a line across each of two points on paper about 2 cm from the lower edge. Mark on the line 8 points at roughly 2.2 cm intervals, taking care to start well from the left hand edge. Avoid touching the paper with your fingers except by the extreme edges.

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

4. Repeat the above procedure with each of the other amino acids, the unknown mixture and the casein hydrolysate. Use the individual capillary tubes with each solution so as to avoid contamination. Allow the spots to dry completely. Then repeat a second time to concentrate the spot.
5. Form the paper into a cylinder and staple the edges together so that they do not Overlap. Place the cylinder, sample side down, in the chromatography bottle, taking care not to let the paper touch the glass walls. Replace the lid of tank firmly.
6. Allow to run for about one and half hours. Develop the chromatogram by Incubating at 100° C for a few minutes.
7. Spray the paper lightly with ninhydrin solution inside the fume cupboard. Do not allow the paper to become completely soaked. Develop the chromatogram by Incubating at 100° C for a few minutes.
8. Outline each spot in pencil and mark the centre of the spot. Calculate the RF value of each amino acid. Include the chromatogram (or a copy in your report).

$$Rf = \frac{\text{Distance from the origin to the centre of spot}}{\text{Distance from the origin to solvent front}}$$

Discussion

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

Questions

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

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

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

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

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

PRACTICAL 4

EXTRACTION AND THIN-LAYER CHROMATOGRAPHY OF SERUM LIPIDS

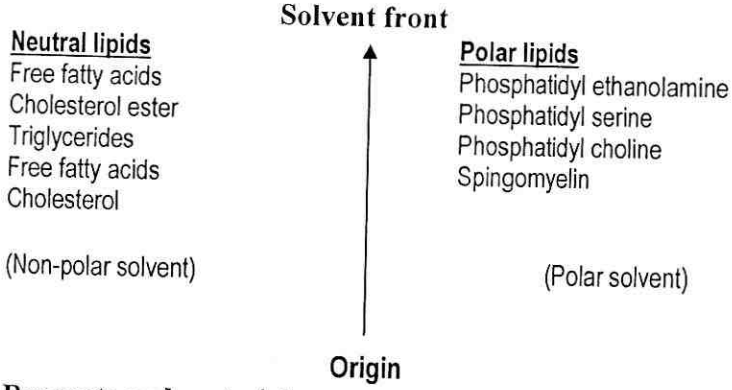
Lipids are compounds that are insoluble in water and soluble in a variety of organic solvents such as ether, chloroform and hexane. The major classes of lipids are triglycerides, phospholipids, non-esterified ("free") fatty acids and sterols (principally cholesterol).
In medicine, elevated serum lipid levels (*hyperlipidemia*) occur in such disease states as diabetes mellitus and hypothyroidism. In addition a correlation between serum *atherosclerosis* and *hyperlipidaemia* has been noted.
Most lipids are not found in free form in the blood or other tissues. They are associated with proteins to form lipoproteins. To analyze blood or tissue lipid content it is usually necessary to separate the lipids from the proteins and other water-soluble substances by extraction with organic solvents.
In today's practical you will extract the lipids from serum and then separate the different lipid classes by thin layer chromatography.

Principle of lipid extraction

The relative ease with which lipids may be extracted depends on the degree of their association with tissue proteins. The loosely-bound "neutral" lipids (e.g. triglycerides and cholesterol esters) are easily extracted with non-polar phospholipids, which are more tightly protein-bound, requires the addition of a polar compound (e.g. methanol) to the extraction solvent to dissociate the phospholipids – protein complex, in the extraction process the hydrophobic lipid molecules "dissolve" into the organic phase, leaving most of the water-soluble proteins, carbohydrates, amino acids and salts in the aqueous phase. The two phases are separated and the organic phase is analyzed for lipid content.

Principle of thin layer chromatography (TLC)

TLC is somewhat similar in principle to the paper chromatography technique that you used in an earlier practical. The main difference is that in place of the hydrated cellulose fibres of paper, the *adsorbent or stationery* phase in TLC is in the form of a very thin film of powdered material fixed on an inert rigid support such as glass plate. Techniques of sample application, "running", detection and RF calculation are all similar to those of paper chromatography.
The absorbent that you will use is silica gel, a form of silicon dioxide (SiO2) that has an affinity for lipid molecules, binding the highly polar phospholipids more tightly than the less polar compounds. An organic solvent moving up the plate will separate a mixture of lipids on the basis of the relative affinities of the lipids for the silica gel and for the solvent or mobile phase. A non-polar solvent (e.g. chloroform) will separate the neutral lipids leaving the phospholipids at the origin. More polar solvent mixtures (e.g. chloroform; methanol; acetic acid) will separate a mixture of phospholipids while the neutral lipids will move with the solvent front. In today's practical you will use both types of solvent system.



Reagents and materials

1. Fresh serum
2. Extraction solvent; chloroform: methanol (2:1)
3. Chromatography solvents (in chromatography jars);
   Chloroform: acetic acid (99.5:0.5)
   Chloroform: methanol: acetic acid water (25:15:4:2)
4. Methanol
5. Chloroform
6. TLC (2); silica gel on glass, 5 x 20 cm
7. Extraction tube
8. Centrifuge tube
9. Capillary pipette
10. Chromatography jars (2)
11. Iodine vapor chamber (under hood)

Procedures

Extraction of serum lipids

1. Place *1.0 ml of serum* in an extraction tube and add 7.0 ml of *chloroform: methanol (2.1)* mixture.
2. Shake vigorously for 3 min (a white precipitate will form).
3. Transfer the mixture to a centrifuge tube and centrifuge for 10 min. the aqueous and organic phase will separate into two layers with a dense white precipitate of protein at the interface.
4. *Taking care not to mix the two phase*, remove all of the upper aqueous phase and the white precipitate. If necessary discard a small portion of the organic layer to ensure complete removal of all water and protein.
5. Transfer the organic phase to a clean, dry 50 ml beaker *and carefully* evaporate to near dryness on a hotplate.
6. When nearly dry, add 1.0 ml methanol to aid in moving traces of water. (Repeat) a Technician will assist you in drying the samples.
7. When dry, add 0.5 ml of chloroform to beaker to dissolve lipids. The solution should be yellow, and there may be a small amount of insoluble residue.

TLC of lipid extract

1. Place two TLC plates on a clean dry work surface with coated side up. Handle plates only by edges and take care not to disturb the coating.
2. On each plate, make a single, faint pencil mark approximately 2 cm from one end and
3. cm from either side. This dot will be the origin where you will spot the sample.
4. Carefully apply the lipid extract to each plate at this spot. To do this, dip a capillary

pipette into the extract until the tip fills to a depth of about 1 cm. this volume is about 10µl (microlitres). Briefly and repeatedly touch the tip of the pipette to the pencil to and allow a small amount of the extract to flow onto the plate. Let the spot dry between each application and take care to keep the spot small, about *0.5 cm diameter*. Repeat until a volume of about 20µl has been applied to each plate.

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

Questions

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

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

Present your measurements in tabular form.

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

Study of some characteristics of fats and oils

No burner should be lighted

Reagents and Materials

1. Fat samples: glycerol; cottonseed oil, stearic acid, beef fat
2. Alcoholic NaOH
3. Na_2CO_3
4. Alcohol
5. Ether
6. Chloroform
7. Solid $KHSO_4$
8. Benedict's reagent
9. Wire loops
10. Dilute HCl, concentrated HCL
11. Absorbent paper

A) Grease spot test

Fats impart a translucent character to paper. This test is used to determine whether a fat is truly soluble in any specific solvent or not.

Procedure

Dissolve a small amount of fat in some ether. Place a drop of the solution with capillary tube on a piece of filter paper. Take the drop from the middle of the solution. Allow the solvent to evaporate and note the translucent stain on the paper. Repeat this test with the fat samples

provided using water, ether, chloroform, hot (warm in a water-bath!) and cold alcohol as solvents. Record the results in a table.

B) Emulsification

When oil or liquid fat is shaken with water it becomes finely divided, forming a rather transitory emulsion. In the presence of emulsifying agents, dispersion of fat globules is more complete and hence more permanent. Emulsifying agents such as soaps, proteins, or bile salts act by lowering the surface tension of the aqueous phase and are absorbed at the surface of the tiny oil globules. Thus they play a certain role in the solute-solvent interactions minimizing the tendency of the fat globules to coalesce. T he milk fat is stabilized by a hydrophilic protein envelope.

Procedure

Place about 0.5 ml of the corn oil in a test tube and 3 ml of water and shake vigorously to see if oil can be dispersed in water. Add 1 ml 0.5% Na_2CO_3 wait for 5 minutes.
Warm up and try again to disperse the oil. What is the effect? Repeat this test with beef fat first warming it in hot water so that the fats melt.

C) Glycerol

This important trihydric alcohol is a component of almost all fats and oils. It is a colourless, syrupy liquid with a boiling point at 290°C.

Procedure

1. Test the solubility of glycerol in water, hot and cold alcohol, ether, and chloroform, suing a grease spot test. If you get a positive test try washing the paper with water and dry it again.
2. Acrolein test for glycerol. (Use the burner in separate room). Acrolein has a characteristic pungent odour that arises from the dehydration of glycerol. Acrolein, CH_2 =CH = CHO, is an unsaturated aldehyde with the tendency to polymerise. Use two dry test tubes and place about 10 drops of vegetable oil in one and 10 drops of glycerol to the other. Add about an equal volume of solid potassium hydrogen sulphate. Heat the tubes gradually and note the odours.
3. Apply Benendict's qualitative test for sugars to glycerol. Record your observations.

Questions

1. What do you conclude from your results about the solubility of glycerol in ether, chloroform and water?
2. From your results above propose the possible effects of heating glycerol and vegetable oil with solid K_2HSO_4.
3. What do you conclude from Benedict's test about glycerol?
4. Propose possible medical use (s) for glycerol. Base your answer on its physical properties.

PRACTICAL 5

METHODS FOR PROTEIN ASSAY

The quantitative determination of protein can be achieved using a number of methods, each of which has its limitations. We consider three common methods below.

1. Biuret method

Proteins have a primary structure composed of amino acids linked by peptide bonds in a linear manner. This peptide backbone can interact with Cu (II) ions to form a purple complex. This complex is formed with any molecule containing two or more peptide bonds present. The name of the method comes from the simplest compound which gives the reaction, Biuret ($H_2NCONHCONH_2$). Relatively high protein concentrations (1-100mg/ml) are readily determined by the Biuret method.

In setting up a colorimetric proteins determination, a defined assay procedure must first be adopted. This procedure is then used with protein solutions of known concentration over a suitable range to establish a ***standard curve.*** This graph relates the final absorbance of the solution to the initial protein content of the sample. For this purpose you are given a standard solution of bovine serum albumin (concentration, 1mg/ml). In several subsequent experiments you will need to perform protein assays. Use the biuret method and determine the protein concentration by referring to your standard curve.

The Biuret method has the advantage that it can be used in the presence of inorganic ions. You will use this method to determine the protein concentration of normal human plasma and pathological human plasma. The protein concentration of normal plasma is in the range o 50-100mg/ml. After making a rough plot of your results for the standard curve, determine how much you will have to dilute the plasma samples so that their protein concentration then falls within the linear range of the standard curve.

In alkaline solution copper (II) ions combine with the peptide bonds of proteins to form a purple colour. The intensity of the purple colour is proportional to the protein concentration. Since the number of peptide bonds per unit mass is about the same for all proteins, the Biuret method is generally applicable. However, the assay is not sensitive below 0.25mg of protein. Also, some chemical and many biological compounds interfere with the method.

2. Lowry method

This colorimetric method depends on the Biuret reaction and on the reduction of arsenomolybdate reagent (Folin reagent E) by Tyr and Trp residues of the protein. Thus, the colour intensity given by an individual protein will depend not only on its mass but also on its relative content of Tyr and Trp. Some buffers and reducing compounds interfere with this method. However, it is highly sensitive with a lower limit of 5 microgram protein.

3. UV absorption method

This direct spectrophotometric method is based on the absorption of Tyr and Trp at 280nm. It suffers from the drawback as the Lowry method concerning the content of aromatic amino acids. For a pure protein the extinction coefficient can be determined and then used to measure absolute amounts of this protein. This is not possible for a mixture of proteins .But the method is still applicable provided a correction factor is applied to allow for absorption due to any contaminating nucleic acid. This method is rapid, sensitive and the protein sample is recovered after the measurement.

Reagent and materials

1. Bovine serum albumen (1mg/ml) solution of gamma- globulins in buffer
2. Biuret reagent
3. Folin reagent C and E
4. 0.01M phosphate b buffer, pH 7.0

Procedure

1. Prepare dilution of the protein solution as follow:

Volume of protein (ml)	0.0	0.2	0.4	0.6	0.8	1.0
Volume of distilled water (ml)	1.0	0.8	0.6	0.4	0.2	0.0

2. Add Biuret reagent (4.0 ml) to each tube. Allow to stand for exactly 30 min.
3. Measure the absorbance at 540nm. Plot the graph of absorbance at 540nm against quantity of protein (in mg) in the sample.
4. Decide on appropriate dilution factor for the plasma samples based on your rough plot of the standard curve.
5. Repeat steps 1 to 3 for the plasma samples appropriately diluted. By reference to the standard curve, determine the protein concentrations of the plasma samples.

Determine the Protein concentration of the gamma-globulin solution using each of the three methods described in the introduction.

1. Biuret method

Procedure

1. Add Biuret reagent (4.0 ml) to bovine serum albumin solution (1.0 ml), to gamma-globulin solution (1.0 ml) and to buffer (1.0 ml) in duplicate. Mix well.
2. After 30 min measure the absorbance at 540 nm. Calculate the protein concentration of the gamma-globulin solution.

2. Lowry method

Procedure

1. Dilute each protein solution ten times with buffer. To a sample of each diluted protein solution (1.0 ml) add folin reagent C (4.0 ml) and mix well.
2. After 15 min add Folin reagent E (1.0 ml) rapidly and mix well. After 30 min measure the absorbance at 600 nm. Calculate the protein concentration of the gamma-globulin solution.

3. UV method

Procedure

Dilute each protein ten times with buffer then determine the absorbance of the diluted gamma-globulin solution against a buffer blank at 280 nm and 260 nm. Calculate the protein concentration by applying the appropriate correction give the factor based on the ratio of A_{280} toA_{260}.

Questions

1. Compare the values for the protein concentration of the gamma-globulin solution Obtained by each of the three methods. Account for the variation of results.
2. Over what range of protein concentration (mg/ml in the assay mixture) is the Biuret Assay most useful?
3. Over what range of protein concentration is the Lowry method most useful.
4. What are the analytical disadvantages of (a) the Biuret method (b) the Lowry method
5. Which method is most appropriate for the determination of serum protein levels?
6. Which of the above method is most appropriate for automation in hospital Determination of serum proteins? Why?
7. Which other methods can be used in the assay for serum/plasma protein levels?
8. A sample (0.3 ml) was diluted with 0.9 ml water. An aliquot of the diluted extract (0.5 ml) was added to 2.5 ml biuret regent and gave an absorbance of 0.324 at 540 nm. Calculate the concentration.

PRACTICAL 6

SOLUBILITY OF PROTEINS: EFFECTS OF PH, SALT CONCENTRATION AND ORGANIC SOLVENTS

Many procedures for selectively separating mixtures of large quantities of proteins are based on the solubility properties of the protein, which vary with pH, salt concentration and the presence of organic solvents. This practical will examine the effect of these factors on protein solubility.

Isoelectric point precipitation of proteins

Principle

A protein molecule contains a number of amino acid residues that may be positive, negative or uncharged depending on the H^+ ion concentration in the solution. As with the individual amino acids, there is a pH at which the net charge on the protein equals zero, the **isoelectric** point. At this pH the protein is least soluble because, when there is no charge the repulsive forces between proteins molecules are minimized and the molecules can aggregate and precipitate.

Materials

Casein in 0.1 M Sodium acetate) Casein sodium acetate solution
Acetic acid, (0.01 M, 0.10M and 1.0M in water)

Procedure

1. Place nine test tubes in a rack and, using graduated pipettes, prepare the following dilutions of acetic acid as accurately as possible.

Tube No	1	2	3	4	5	6	7	8	9
Distilled water (mls)	8.38	7.75	8.75	8.50	8.00	7.00	5.00	1.00	7.40
0.01 M acetic acid (mls)	0.62	1.25	-	-	-	-	-	-	-
0.1 M acetic acid	-	-	0.25	0.5	1.0	2.0	4.0	8.0	-
1M acetic acid	-	-	-	-	-	-	-	-	1.6

2. To each tube, rapidly add 1.0 ml of casein in sodium acetate solution and mix at Once. (Note that the total volume in each tube is 10.0mls).
3. Observe the formation of turbidity in the solutions immediately after mixing and at 5 min and 30 min. record your observations in tabular form, indicating the degree of turbidity or precipitation by:

O - No turbidity,
X - Slight turbidity,
XX - Moderate turbidity
XXX - Dense turbidity

Questions: (to be answered in your report)

The mixtures of sodium acetate and varying amounts of acetic acid are buffers (i.e., a week acid and its salt). Calculate the **pH** of the buffer in each tube, using the **Henderson-Hasselbach** equation $\mathbf{pH} = pKa + \log \frac{[Ac^-]}{[HAc]}$

For acetic acid, pKa = 4.7. the concentration of **(HA)** ionized and unionized forms of acetic acid can be calculated from the information in the procedure, taking into account the final dilution. As a guideline, the pH of the buffer in tube 1 is 5.9.

1. From your observations, what is the **pH** of the isoelectric point of casein?
2. Assume that you have a mixture of three proteins in aqueous solution. Protein A has an isoelectric point at **pH** 3; Protein B at **pH** 5 and protein C at **pH** 9. Describe briefly how isoelectric precipitation could be used to separate these proteins.

Fractional precipitation of serum and plasma proteins using salts and organic solvents

Principle

Salts:

When a high concentration of salt ions is added to a protein solution, the ions attract water molecules surrounding the protein, withdrawing the water to such an extent that the protein molecules are "dehydrated" and thus precipitate. Varying the salt concentration allows selective precipitation of a protein mixture.

Ammonium sulphate $(NH_4)_2SO_4$, is most commonly used for protein precipitation because it is very soluble in water and has few harmful effects on proteins. The $(NH_4)_2SO_4$ results in "50% saturation", etc.

Organic solvents

Although proteins are readily denatured by organic solvents (e.g alcohols, acetone) at room temperature, when low temperatures (-5° to 2°C) are maintained, precipitation occurs without denaturation. The protein precipitates because the organic liquids lower the dielectric constant of the medium, thus increasing the attractive forces between protein molecules, which then aggregate.

NB: The two recommended precipitants for proteins are $(NH_4)_2SO_4$ and Na_2SO_4.

In the first part of the following experiment, you will precipitate the globulin fraction of serum (Na_2SO_4) and with methyl alcohol; the albumin will remain in solution. The amount of albumin relative to globulin (A/G ratio) is altered in certain pathological conditions and determination of the A/G ratio is therefore of clinical significance. Hence, after precipitation, the globulin (G) and albumin (A) fractions will be assayed calorimetrically by the Biuret method of protein analysis.

Note: *$(NH_4)_2SO_4$ react with Biuret reagent but Na_2SO_4 does not. In a final experiment you will examine the difference in precipitation of serum and plasma.*

Materials

1. Fresh serum
2. Oxalated plasma (Oxalate is an anti-coagulant)
3. Saturated ((Na_2SO_4))
4. Sodium acetate (0.04M).
5. Ice-cold methyl alcohol
6. Protein standard solution (10 mg casein/ml H_2O)

7. Biuret reagent
8. Ice bath

Procedures

A) Separation of serum globulin from albumin by Na_2SO_4 precipitation.

1. Pipette 1.0 ml of serum into a centrifuge tube and add 1.0 ml saturated Na_2SO_4
2. Mix and let stand for 5 min.
3. Centrifuge for 10 min.
4. Without disturbing the globulin pellet, transfer the Albumin i.e. clear supernatant fluid to a small graduate cylinder.
5. Dilute supernatant solution to 5.0 ml with distilled water. Use 2ml for assay.
6. Dissolve the globulin pellet in distilled water and also dilute to 5.0 ml. use 2 ml for the assay.
7. Assay both solutions as described in part C. **(Complete part B first).**

B) Separation of serum globulin from albumin by methyl alcohol precipitation

1. Pipette 4.5 mls of 0.04M sodium acetate into a test tube and add 1.0 ml serum.
2. Mix and cool in an ice bath at 0°C for 5 min.
3. Add 4.5 ml ice cold methanol, drop wise and with constant mixing. (If methanol is added too fast or without mixing, denaturation of proteins will occur).
4. Transfer to centrifuge tubes and centrifuge for 5 min.
5. Transfer supernatant solution to a clean test tube without disturbing pellet. Do not dilute the supernatant (i.e. Albumin) use 2 ml for assay.
6. Dissolve pellet (i.e. globulin in distilled water and dilute) in 10.0mls. Use 2 ml for assay.
7. Assay both solutions for proteins content as described in part C.

C) Biuret assay of protein content of serum fractions, using "standard curve" of protein concentration. Refer to previous experiment

For example, if the absorbance of an unknown protein sample is 0.15, the amount of protein in the sample is 3.0 mg).

1. Prepare 11 test tubes for protein analysis according to the following schedule (6 tubes are for the standard curve, and 5 for the serum samples)

Tube contents

Plot a standard curve

1-6 Standard casein (10 mg/ml) 0, 1, 2, 3, 4, 5, mls.
7 2 mls pellet)
8 2 mls supernatant) from Na_2SO_4 precipitation of serum
9 2 mls pellet)
10 2 mls supernatant) from methanol precipitation of serum
11 0.1 ml whole serum

2. Add distilled water to each tube to give a final total volume of 5.0mls. Mix well and add 3.0ml of Biuret reagent to each tube.
3. Mix again and let tubes stand for 20 min. for full colour development.
4. Read the absorbance of the samples in the spectrophotometer at 540 nm. Tube 1, containing no protein, is the blank and should be used to "zero" the instrument.

Plot a standard curve of absorbance Vs amount of protein (mg)
Use this graph to determine protein content of test tubes no. 7 – 11.
Report your results in mg/ml of the whole serum fraction

Serum protein fractionation assay

TUBES	Tube 7 (G) pellet (Na_2SO_4)	8 (A) supernatant (Na_2SO_4)	9 (G) pellet (CH_3OH)	10 (A) super (CH_3OH)	11 (Whole serum)
Na_2SO_4 (mls	2	2	-	-	-
(CH_3OH) (mls)	-	-	2	2	-
Whole serum (mls)	-	-	-	-	0.5
H_2O (mls)	3	3	3	3	4.5
Biuret mls	3	3	3	3	3
Absorbance (nm)					

3. For both the Na_2SO_4 and methanol precipitations, calculate the percentage of albumin and of globulin in the protein content of whole serum. With either precipitation, the sum of % G should equal 100%. If it does not, discuss the possible sources of error.

D) Comparison of protein precipitation of serum and plasma

1. Pipette 1.0 ml of serum into one test tube and 1.0 ml plasma into another.
2. Slowly add 1.0 ml of 50% Na_2SO_4 to each tube, mixing well.
3. Observe the difference in protein precipitation of the two preparations.

Questions to be answered in your report

1. Are there any differences between the protein content of the albumin and globulin fractions separated by Na_2SO_4 compared to methanol? For the type of serum you were using (bovine serum), the "normal" content of albumin is 3.2 + 0.3 g/100 ml, and for globulin, 4.0 + 0.4 g/100 ml. which precipitation method gave better agreement with normal values?
2. From your results calculate the A/G ratio. What is the clinical significance of an A/G ration that is higher than normal? Lower than normal?
3. After addition of 50% to serum and plasma, which sample has a precipitate? What protein has precipitated? (HINT: What is the main difference between serum and plasma?).

PRACTICAL 7

TESTS FOR VARIOUS CARBOHYDRATES

Theory

The principle carbohydrates in the animal organism are hexoses composed of six carbon atoms such as glucose and fructose and the pentoses or five carbon atom sugars. The hexoses supply a large part of the energy required by the cell and can be metabolized to pentoses which are an important part of nucleic acid molecules. The simple sugars are also known as monosaccharides (of varying chain length of 3 to 7 carbon atoms) and occur in many forms in nature. As the study of carbohydrate metabolism progresses examples of all of these will be found in the animal cell.

Carbohydrates can also be classified as polyhydroxy compounds having either an aldehyde group (aldoses) or a ketone group (ketoses). These groups will reduce certain metal ions with the sugar undergoing oxidation in the reaction forming a number of low molecular weight compounds. This reaction is the basis of Benedict's test for reducing sugars and for the quantitative determination of glucose in body fluids.

Both hexoses and pentoses form pyran and furan ring compounds in solution. Glucose, the most common hexose, forms a pyran ring with two isomeric forms.

Monosaccharides polymerize into large molecules as follows:-

Disaccharides, (2 units) example cane sugar

Oligosaccharides, (up to 10 units) Dialysable

Polysaccharides have many monocaccharide units. They include starch and glycogen. These are classed as macromolecules and are non-dialysable.

Another characteristic property of simple sugars is the optical activity due to the presence of asymmetric carbon atoms in the structure. This is manifested by the rotation of polarized light which will be discussed during your lecture sessions.

Experiment 1 **Carbohydrate tests**

Reagent and materials

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

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

A. Molisch Test

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

Experiment

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

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

Procedure

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

The formation of a purple ring at the interface indicates the presence of a carbohydrate. ***A green colour is not a positive reaction.*** In case of doubt, the test should be repeated on a more dilute solution.

B. Benedict's Test for reducing sugars

Alkaline copper reagents have been used for a long time as a sensitive test for reducing sugars which contains a potential aldehyde or ketone group. The group is designated as potential for the pyran of furan ring structure the group has the hemiacetal structure rather than an aldehyde or ketone structure. As already described this group will reduce certain metals forming oxidized low molecular weight compounds of the sugar.

Cu^{++} + Glucose ⟶ Cu^{+} (insoluble) + oxidized products

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

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

Procedure

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

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

C. Barfoed's Test

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

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

Procedure

1. Pipette 1 ml of each solutions into different test tubes
2. Add 5 ml of Barfoed's reagent to each test tubes

3. Mix and place all the tubes in the boiling water bath
4. Observe them carefully for the appearance of the ***red precipitate of Cu_2O*** and record the number of minutes required; remove the positive tube and allow it to stand
5. After 15 minutes remove any remaining tubes and allow them to stand and observe for any precipitate that may settle out. Note the time.
6. Record the observations in a table.

D. Seliwanoff's Test:

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

Procedure

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

E. Bial's Test

Bail's test is for pentose sugars and it depends on the rapid formation of the furfural from pentose under specific conditions of the test. In this test orcinol reacts with the furfural yielding a blue-green colour.

This is the last of the specific tests that will be used in the study of carbohydrates. It should be noted that two, Benedict's and Barfoed's depend on the reduction of copper and differences in the composition of the solution and the rate of reaction are used to obtain specific information. The other three tests depend on differential rates of dehydration to form furfural derivatives which have in turn reacted with phenolic compounds forming colored complexes.

Procedure

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

Experiment 2 **Iodine test for polysaccharides**

Theory

Iodine gives an intensive blue reaction when mixed with certain polysaccharide, but not with others. The 1-4-glycosidic linkage of polysaccharide form a helix, six pyranose groups forming one complete turn and the inner dimensions are such that an iodine molecule is suspended within a turn of the helix. The iodine behaves as an oscillator and absorbs all the energy of light except that of the short wave-length which gives the blue color of the reaction. Other polysaccharides with a(-4_ glycosidic linkage do not form a helix, and therefore, cannot form a red-violet colour with iodine.

Polysaccharides can be hydrolyzed by acids at about 100° C, with hydrogen ions acting as the catalyst.

Reagents and materials

1. 1% solution of starch
2. 1% solution of glycogen
3. 1% solution of cellulose
4. 1 M HCL
5. 0.1N Iodine solution

Procedure

1. Prepare a test plate by putting a drop of 0.1 N iodine solution in each depression.
2. Place 2 mls of each test solution in different test tubes.
3. Add 2 ml I M HCL to each tube, and stopper with cotton wool.
4. Test each polysaccharide at the start and at 3 min intervals for 30 minutes during hydrolysis by adding a drop of the solution to the iodine on the test plate.

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

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

Experiment 3

Quantitative determination of glucose in urine using Benedict's method

Theory

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

Procedure

1. Place 10 ml of the quantitative BENEDICT'S REAGENT with a pipette into the 100ml flask (flat bottomed flask) which is in your glass equipment.
2. Add the four or five glass beads provided. Place the flask on the hot plate gently bring to the boil.
3. On boiling, add drop wise to the solution (with 2.0 ml graduated pipette), your

Unknown glucose solution (urine). Start with your pipette filled to the 0.0 ml mark. Add drop wise till a precipitate forms, then continue to boil 1 or 2 minutes and add very slowly some further drops until the colour has entirely disappeared.

4. Make a note of the amount of sample used.
5. Repeat at least once or twice. Some students will have to continue adding their glucose solution patiently.

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

Questions

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

PRACTICAL 8

AN INVESTIGATION OF THE FACTORS WHICH AFFECT THE ACTIVITY OF ALKALINE PHOSPHATASE

Phosphatases (or phosphohydrolases) are enzymes which catalyse the hydrolysis of orthophosphate monoesters (the *substrate*) to inorganic phosphate and an alcohol (the *products*). They are found in all cells. You will use an alkaline phosphatase prepared from intestinal mucosa.

The **velocity** of the reaction is measure of the rate ***of disappearance of the substrate*** or ***of the appearance of the products.*** The amount of substrate or product present at any time during the reaction is usually determined colorimetrically. This is of course a simple procedure if either the substrate or products are naturally coloured substances, or if a synthetic substrate is used, which contains a coloured group. 4-nitrophenyl phosphate (4NPP) is a suitable synthetic substrate for phosphatases. The reaction it undergoes is:-

$$O_2N.C_6H_4.OPO^-_3 + H_2O \longrightarrow O_2N.C_6H_4OH + HPO_4^{2-}$$

4 NPP $\longrightarrow$ 4-nitrophenol (4NP)

Phosphate acts as an inhibitor of the enzyme-catalysed reaction.

The product, 4NP, is colorless, in its undissociated form, but ionizes in strongly alkaline solution to give the 4-nitrophenolate anion, which is yellow (absorption maximum 400 nm). Its concentration can therefore be measured directly, in a photometer. An added advantage is that addition of the alkali also stops the enzymes-catalyzed reaction.

The colour is stable, which means that you can continue with the experiments even if a spectrophotometer is not immediately available.

But make certain all your test-tubes are properly labeled. Cleanliness and efficiency are essential, if your experiments are to be successful.

Materials

Alkaline phosphatase (0.1 mg protein/ml, in water).
4-nitrophenyl phosphate (4.0 mM in water) 4NPP
4-nitrophenol (0.1 mM in 0.1 M NaOH) (4NP)
Glycine-NaOH buffer (0.1 M, pH 9.9)
Glycine-NaOH buffers (0.1 M, pH 8.4, 8.9, 9.4, 9.9, 10.4 & 10.9)
NaOH (0.1 M, in water)
Glycine-NaOH buffer (0.1 M, pH 9.9, containing 2.0 mM NaH_2PO_4)

Procedure

A) Preparation of standard curve for 4 nitrophenol

Prepare labeled test-tubes in single containing 5.0mls of: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 mM 4NP in 0.1 M NaOH. **(Note: 1.0mls of 4NP stock solution + 4mls of 0.1 M NaOH gives a final 4NP concentration 0.02mM/ml.)**

Read the absorbance of each tube in the photometer at 400 nm, against a **0.1 M NaOH blank**

Construct a standard curve by plotting the measured absorbance against the amount of 4NP present, as µmoles/ 5ml

B) The effect of pH on the activity of alkaline phosphates

1. Take 6 clean test tubes and label them with the pH values of the buffers you will use (8.4 – 10.9).
2. To each tube add: 0.5mls stock 4NPP solution (4mM in water) and 1.0mls of the appropriate buffer. Prepare a BLANK, in another tube by adding: 1.0mls of buffer (pH 9.9, 0.5mls of 4NPP and 0.5mls of water.
3. Place the tubes (in a rack labeled with your initials) into a water bath at 37°C, and leave to warm for a couple of minutes. E.g. 3 minutes.
4. Start the reaction by adding to each tube (except for the BLANK) 0.5mls of enzyme solution. Note the time.
5. After 30 minutes, stop the reaction in each tube (including the BLANK) by the addition of: 3.0 mls of 0.1 M NaOH.
6. Remove the tubes from the water-bath and measure the absorbance of each at 400 nm against the blank. Convert the absorbance into µmoles 4NP by use of your standard curve. Plot a graph of µmoles against absorbance using your standard curve. Plot a graph of µmoles 4NP produced, against pH. What is the optimum pH of alkaline phosphates?

C) The time course of the reaction

1. To a small conical flask, add:
   4.0 mls stock substrate solution and 8.0 mls buffer (pH 9.9).
   Place flask in a water bath at 37°C.
2. Number 6 test-tubes and add to each:
   3.0 mls of 0.1 M NaOH.
3. Start the reaction by the addition to the flask of:
   4.0mls of enzyme solution.
4. At intervals (0, 10, 20, 30, 40, and 60 minutes), remove 2.0 ml samples from the flask and add each to the appropriate test-tube. Mix thoroughly.
5. Read the absorbance of the tubes at 400 nm using the 0' minutes' tubes as the BLANK. Plot a graph of µmoles 4NP produced against time, in minutes.

Questions

1) What is the initial velocity of the reaction, in moles 4NP produced/minute?
2) Over what period of time is the reaction linear?

D. The effect of enzyme concentration on reaction rate

1. Set up the following tubes:

Tube number	1	2	3	4
Substrate (mls)	0.5	0.5	0.5	0.5
0.1M Gly-NaOH Buffer (mls)	1.0	1.0	1.0	1.0
Water (mls)	0	0.2	0.4	0.5

1. Incubate the tubes at 37°C for several minutes e.g. 3 minutes then begin the reaction by addition of the correct volume of enzyme to each tube.
2. Enzymes (mls) 0.5, 0.3, 0.1, 0.0
3. Stop the reaction after exactly 30 min. by the addition of 3.0 mls sodium hydroxide solution to each tube.
4. Read the absorbance of the tubes at 400 nm against tube 4. Convert the measured

absorbances to µmoles 4NP, using your standard curve. (This tells you the number of µmoles 4NP produced in 30 minutes).

5. Now calculate the initial velocity, V, of the reaction, in µmoles 4NP produced/minute. Work out by how many times the enzyme has been diluted in each tube and from this calculate the enzyme concentration, (mg/protein/ml) in the assay solution, for each tube.
6. Using these results, plot a graph of Vagainst (E).

Questions

1. Is the reaction dependent upon enzyme concentration, under conditions in which the substrate is 'saturating'? Illustrate your answer with the appropriate rate equation, and explanation of its meaning.
2. How many units/mls of alkaline phosphatase are present in the stock enzyme solution? (*1 unit* of enzyme activity is the amount of enzyme that will catalyse the transformation of *1µmole of substrate/minute*. Therefore, if 1 ml of enzyme transform 0.02 µmoles/minute the solution contains 0.02 units/ml).
3. If specific activity is defined as the number of *units of activity per mg of protein*, what is the specific activity of the stock phosphatase solution?

E) The effect of substrate concentration of reaction rate

1. To 5 labeled test-tubes, add: *1.0 mls* of buffer (pH 9.9). Then add water and substrate as follows:

Tube no.	1	2	3	4	5
0.1M Gly-NaOH Phosphate buffer (mls)	1.0	1.0	1.0	1.0	1.0
Stock substrate (mls)	0.5	0.4	0.3	0.2	0.1
Water (mls)	-	0.1	0.2	0.3	0.4

2. Incubate the tubes at 37°C for 3 minutes then start the reaction by the addition of: 0.5mls of enzyme solution.

Whilst the reaction proceeds, use the time to calculate the substrate concentration as moles/liter (M), *in the incubation mixture*, for each pair of tubes (tube 1 contain 10^{-3} moles/L substrate).

3. Stop the reaction after 30 min by addition of *3.0 mls of 0.1 m NaOH*.
4. Read the absorbance of the solution at 400 nm using 0.1 N NaOH as the blank. Plot graphs of V, the initial velocity (µmoles 4NP produced/min against (S), the substrate concentration and of 1/V against 1/[S]. Take care to have correct units. This second graph is called a Linewever-Burk plot.

Questions

1. From the second plot, estimate values of K_m and V_{max} for the alkaline phosphatase under the conditions specified in your assay system.
2. What is the enzyme concentration (mg/ml) in the assay system? What would be the value of V_{max} assuming an enzyme concentration of 1.0 mg/ml? Will the K_m also be different at this concentration?

3. How does the extrapolated value of V_{max} from your graph compare with the value obtained in section C, in which a saturating concentration of substrate was present? Explain the difference in values.

F) The effect of an inhibitor upon the reaction rate

1. This experiment is performed exactly as section E, but the buffer to be used contains 2.0 mM inorganic phosphate.
2. Calculate 1/V for each tube and plot your results on the same graph as in section E.

Questions

1. Is the inhibition by phosphate competitive or non-competitive? What does this mean in physical terms?
2. What is the inhibition constant, K_i, for phosphate? (units are moles l^{-1}). This can be calculated from the graph, since the point at which the line interests with the x-axis

$$= \frac{1}{K_m \frac{1+[I]}{K_i}}$$

Remember that the concentration of phosphate in the stock solution is 2.0 nM. It is diluted by addition of substrate and enzyme in the assay mixture, what is its concentration in the assay mixture? Use this value in calculating K_i.

G) Other experiments

If you complete the experiments given above before the end of the session, there are several others that you could try, such as the effect of temperature on the reaction rate, and the effect of inorganic ions such as Mg (II). Ask the lecturer in charge for details.

Revised and re-edited in 2013 by Prof. E. K.Nguu, Dr. G. Juma, Thomas M Arani & Stephen M. Mutune.

UNIVERSITY OF NAIROBI

DEPARTMENT OF BIOCHEMISTRY

PRACTICAL MANUAL FOR SECOND YEAR STUDENTS OF MBCHB, B.PHARM, AND BDS

ACADEMIC YEAR 2019/2020

AIMS AND OBJECTIVES OF "PRACTICAL BIOCHEMISTRY

The experiments described in this manual have been compiled for the students of Bachelor of medicine, pharmacy and dentistry by the Department of Biochemistry, University of Nairobi. The experiments will supplement the lectures in biochemistry that are given during the same period.

Each practical ends with questions that should be answered and submitted for marking. By answering the questions, students will obtain a better and longer-lasting understanding of biochemical techniques and the intricacies of biochemistry. When students have finished the course they should be able to:-

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

- *Revised by Prof. E. K. Nguu, Joseph Moro, D. Njau & Stephen M. Mutune.*

- *Approved for issue by Chairman, Department of Biochemistry*

---------- *Dated* 16/09/13

DIRECTIONS FOR PRACTICAL LABORATORY CLASSES AND GENERAL INSTRUCTIONS

Before starting any experiment student should read the instructions carefully, paying attention to all details, and should be quite certain about what he/she is trying to do. In the case of the qualitative tests, the quantities recommended are only approximate, but it should be appreciated that gross deviations from the recommended proportions of reagents and test materials may invalidate the test. In the quantitative experiments, the routine must be rigidly followed and quantities measured accurately. For this purpose a measuring cylinder is usually quite inadequate because the graduations near the base may be very inaccurate. Accurate measurements of volume may be made by either burette of pipette. These are most accurate when measurements are made between two calibration marks.
The results of an experiment and the conclusions to be drawn from it should be written in ink, in a practical notebook, as soon as possible after the conclusion of the experiment. The section on results should include a full account of all observations. If you are in any doubt about the significance of your findings, you should discuss them with your demonstrator.

Laboratory rules

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

Use of lab equipment for volumetric and gravimetric methods

Volumetric flasks: These are calibrated to contain a given volume at a specified temperature usually 20° or 25 ° C.

Transfer pipettes: (This type has a bulb in the middle). This is the most accurate type and is calibrated to deliver a certain volume. Such a pipette must be clean to deliver accurately. A test for cleanliness is to allow water to run out of it. If it drains smoothly, leaving no adhering drops within, it is satisfactory. If drops remain behind the pipette obviously it will not deliver the indicated volume.

There is a convention with regard to the use of the transfer pipette which must be observed for accurate delivery. The pipette is filled to about an inch above the calibration mark, the upper end being held closed with the forefinger. The solution is allowed to escape, until the bottom of the meniscus is level with the mark, which should be at eye level. The tip is then touched for a second or two with inner side of a clean beaker or test tube (not receiving vessel). The solution is then allowed to flow into the receiving vessel by gravity until flow ceases. The transfer is then complete, although you will note that a small column of liquid

still remains in the tip of the pipette. Do not blow this out into receiving vessel. The pipette is calibrated to deliver the specified volume if the above convention is observed in completing and delivery.

Burettes

Macroburettes:

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

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

Semi-microburettes:

These are frequently used in biochemical analysis. Microburettes, have a total volume of 1.0, 2.0 or 5.0 ml and are graduated usually to 0.002 ml. They are used in the same manner as macroburettes, but drainage errors become much more serious. Remember that a single drop of an aqueous reagent may have a volume of about 0.05 ml. this magnitude in a semi-micro titration is of course very serious. Semi-microburettes are usually loaded from a permanently connected reservoir of reagent by means of pressure from a hand-bulb.

Cleaning Glassware

Cleanliness of glassware is essential in all chemical manipulations, particularly in analytical work. Each student will clean his/her own equipment, and whenever possible this should be done immediately after use.

If precipitates and solutions are allowed to dry on the surface of glassware, the latter is much more difficult to clean. This is particularly true of dried deposits of fats, proteins, tissues *e.t.c.* The best general cleanser for laboratory glassware is soap and warm water applied with still brush. Caution should be taken to avoid scratching the glassware with the metal handles of brushes. There are a wide variety of brushes available for different kinds of apparatus. Thorough rinsing with distilled water is required to obtain chemically clean glassware.

Cleaning of burettes and pipettes requires special attention since improper drainage due to greasy surface introduces serious errors in quantitative work. A general test for cleanliness of burettes and pipettes is to fill them with H_2O and allow them to drain at a normal rate. If any visible drops or rivulets of H_2O are left behind on the surface of the glass then the surface is not clean enough to drain reproducibly. If pipettes are clean to being with, they may require only copious rinsing with tap and then distilled water immediately after their use of preserve cleanliness. After pipetting biological materials such as blood, immediately rinse the pipette with water. Further cleansing with soap may be necessary.

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

Volumetric analysis and stoichiometric calculations

The concentrations of solutions are usually expressed in terms of **molarity** or **normality**. A molar solution contains one gram-equivalent weight of the substance in one litre of the solution. Example: The molecular weight of NaOH is 40.01. A molar solution of H_2SO_4 contains 98.08 g of H_2SO_4 .

A normal solution contains one-gram equivalent weight of the substance in one litre of solution. The equivalent weight of the substance depends on the nature of the reaction for which the solution is used. In acid-base reaction which yields or combines with exactly 1.0080 grams H^+ (the weight of one gram ion of H).

This process of quantitative combination, leading to neutralization of solution is called titration. Example: The molecular weight of NaOH is 40.01. One molecule of NaOH can combine with one of H^+ ($H^+ + OH^-$), hence the equivalent weight of NaOH is 40.01 g and a normal solution of NaOH has the same concentration as a molar solution. HCL has a molecular weight of 36.47. A normal solution of HCL contains 36.47 g HCL per litre of the solution since one molecule of HCL yields one H^+. However, sulphuric acid contains two ionizable hydrogen atoms. The equivalent weight of H_2SO_4 is therefore the gram-molecular weight divided by 2 or 98.08 = $\underline{49.08}$g and a normal solution for H_2SO_4 therefore contains 49.04 g/2 H_2SO_4 per litre.

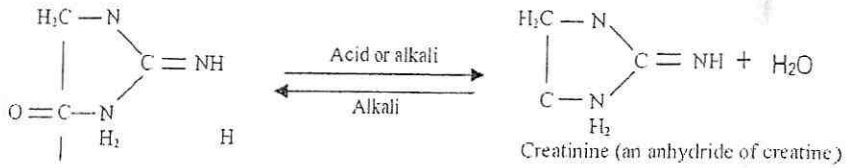
Other methods of expressing concentration

A **molal** solution is one containing 1.00 grams molecular weight of a solute dissolved in 100gm of solvent (note the distinction between a molar and a molal solution). **Molality** is properly used in calculations involving the colligative properties, such as osmotic pressure, freezing and boiling point depression and elevation, and certain other thermodynamic properties of water and other solvents. Percent weight indicated grams of solute in 100 g of solution (w/w). This is the expression used for commercially available solutions. Percent volume indicates grams of solute in 100 mls of solution. This is by far the more commonly used expression involving per cent (w/w). This expression is commonly used to indicate concentration of a solution of the solute in a liquid. 50% alcohol for example means we have 50 mls in 100 ml solution.

PRACTICAL 1

DETERMINATION OF CREATINE AND CREATININE SPECIMEN

Creatine and creatinine may be determined in any biological fluid, but plasma, serum, amniotic fluid, and urine are the specimens most commonly employed. Plasma and serum are preferred to whole blood since considerable amounts of noncreatinine chromogens are present in red cells. If kept for a few days, specimens for creatine and creatinine are best stored at refrigerator temperatures; if kept for longer periods, they should be frozen.
Aqueous solutions for creatine and creatinine very slowly approach a state of equilibrium with respect to each other, as indicated in the following:



Creatine

Although there are conflicting published reports about the speed with which this equilibrium is reached, it probably requires days or weeks at neutral pH. Creatinine however, is formed rather quickly from creatine in either alkaline or acid solutions. Although this reversible reaction is catalyzed in both directions by hydroxyl ions, hydrogen ions promote the reaction only toward the right. Because of the lability of creatine and creatinine, it is advisable to carry out analysis for these two substances on fresh specimens. When this is not possible, adjustment of pH to 7.0 or freezing, or both may delay the change for indefinite periods.

Urine contains only small amount of nocreatinine chromogens.

Principle

Creatinine

This substance is determined in diluted urine or in a protein-free filtrate of plasma or serum employing the Jaffe reaction which results in the production of a red tautomer of creatinine picrate after addition of an alkaline picrate solution.

Creatine: It is determined as the difference between the preformed creatinine and the total that results after the creatine present has been converted to creatinine by heating at an acid PH.

Reagents

1. Picric acid, 0.04 mol/litre. Dissolve about 9.3 g of picric acid (AR), in about 500 ml of water at 80^0 C. Cool to room temperature and dilute to 1 litre with water, and then titrate with 0.1 molar NaOH, suing phenolphalein as the indicator. Dilute as necessary to make 0.04 molar.
2. Sodium hydroxide, 0.75 mol/litre. Dissolve 30 g of sodium hydroxide, AR in water and when cool, dilute to 1 liter.

3. Creatinine stodk standard, 1 mg/ml. dissolve 0.100 g of creatinine, AR, in 100 ml of 0.1 molar HCL. Store in a refrigerator.
4. Creatinine working standard, 20 µg/ml. dilute 2 ml of the stock solution to 100 ml with water in a volumetric flask. Add a few drops of chloroform as preservative.
5. Sulphuric acid, 0.33 mol/litre. Add 18.8 ml of concentrated H_2SO_4, AR, to about 500ml of water. When cool, dilute to 1 litre.
6. Sodium tungstate, 5.0 g/100 ml. dissolve 50g of $Na_2WO_4\ 2H_2O$, AR, in water and dilute to 1 litre.

Standardization

1. Place the following in cuvettes, mixing after each addition:

Creatinine working standard (20 ug/ml) (ml)	Water	Picric acid 0.04 mol/1 (ml)	NaoH (ml)
0.25	3.75	1.0	1.0
0.5	3.5	1.0	1.0
1.0	3.0	1.0	1.0
2.0	2.0	1.0	1.0
3.0	1.0	1.0	1.0
4.0	0.0	1.0	1.0
0 (blank)	4.0	1.0	1.0

2. Allow tubes to stand for 15 min; then measure the absorbance of each at 500 nm, using the blank as a reference.
3. Construct an absorbance-concentration curve on rectangular coordinate paper, plotting absorbance as the ordinate.

Procedure

Creatinine in plasma, serum, or urine

1. Add 1.0 ml of sodium tungastate (5.0g/100 ml), 1.0 of 0.33 molar sulphuric and 1.0 ml of water to 1.0 ml of plasma or serum. Mix thoroughly and filter. If urine is analyzed make a 1/400 dilution of urine with water.
2. Make the additions to labeled tubes as indicated below:

	Unknown plasma (ml)	Unknown urine (ml)
Water	2.0	2.0
Creatinine working standard	0.0	0.0
Dilute urine (1:400)	0.0	2.0
Plasma (serum) filtrate	2.0	0.0
Picric acid (0.04 mol/l)	1.0	1.0
NaoH (0.75 mol/l)	1.0	1.0

3. Allow to stand for 15 min and measure the absorbance at 500 nm
4. Read the creatinine content from the preceding standard curve

Note. The standard prepared in this run is equivalent to 2.0 mg creatinine/100 ml plasma (serum) or 200 mg of creatinine/100 ml of urine.

Creatine in plasma, serum or urine

1. Determine the preformed creatinine according to the procedure just described.

2. Measure the following into three 12 ml graduated centrifuge tubes, mixing after each addition:

	Blank (ml)	Unknown plasma (ml)	Unknown urine (ml)
Water	4.0	2.0	2.0
Creatinine standard	0	0	0
Protein-free filtrate of plasma or serum	0.0	2.0	0.0
diluted urine (1:400)	0.0	0.0	2.0
Picric acid (0.04 mol/1)	1.0	1.0	1.0

3. Heat for 1 hour in a 100°C bath.
4. Cool and make up the volume to 5.0 ml.
5. Add 1 ml of 0.75 molar sodium hydroxide, mix and allow to stand for 15 min.
6. Measure the absorbance at 500 nm and read the values from the standard curve to obtain the total creatinine.
7. Subtract the preformed creatinine from the total and multiply the difference by 1.16 to obtain the concentration of creatine in mg/100 ml of plasma, serum or urine. The factor 1.16 is the ration of the molecular mass of creatine to that of creatinine. Some laboratories use a factor as 1.25 to allow for incomplete conversion of creatine to creatinine.

Normal values

Plasma or serum

	Men	Women
Creatinine*	0.9-1.5 mg/100 ml 0.08-0.13 mmol/1	0.8-1.2 mg/ 100 ml 0.07- 0.11 mmol/1
Creatinine	1.0-2.0 g/d	0.5-1.0 mg/100 ml
Creatine	0.17-0.50 gm/100 ml	0.35-0.93 mg/100 ml
Urine creatinine	1.0-2.0 g/day	0.8-1.8 g/day
Creatine	0-40 mg/day	0-80 mg/day

Questions

1. A 24-hour specimen of urine (950 ml) obtained by catheter, was brought to the laboratory. A 1 ml aliquot (pH 5.4) was diluted to 200 ml with distilled water. A standard containing 0.005 mg/ml creatinine was prepared. To each of three test tubes, containing 4 ml diluted urine, creatinine standard and water, respectively was added 2 ml of alkaline picrate solution. After 10 minutes, the absorbance readings at 530 nm were as follows: diluted urine 0.256, standard 0.485 and water 0. What was the total urinary creatinine excretion (mg/day).

The equilibrium constant for the reaction: AB $\rightleftharpoons$ A + B is 5×10^{-4} moles/L

The complex AB absorbs at 370 nm with a molar absorption coefficient of 300. Calculate the absorbance at 370 nm in a 1 cm cell of solution prepared by mixing 10 ml of 0.01 M solution A with 10 ml 0.01 M solution B.

PRCATICAL 2

ANALYSIS OF CALCIUM IN SERUM AND CHLORIDE IN URINE

Much clinical information is obtained from analysis of body fluids, especially blood (whole blood, serum or plasma) and urine. By common usage in medical biochemistry the term electrolyte refers to inorganic ions only. The principal body electrolytes are cations: hydrogen (hydronium), sodium, potassium, magnesium and calcium and the anions: bicarbonate phosphate, chloride and sulphate. Today you will perform titrimetric analyses of Ca^{++} in serum and CL^- in urine.

Notes on titrimetric analysis

In titration we measure the volume of a solution *(the titrant)* needed to react stoichiometrically with a definite quantity of a second solution. One of the solutions is a *"standard solution"* containing a precisely known amount of reactant while the other solution is the *"unknown"* sample, containing the substance whose concentration is to be determined. Usually the titrant is the standard solution, as in today's practical. However, the titrant may also be a solution whose concentration is known only approximately, but which is titrated against a standard solution of the same material as the constituent of the unknown sample. After determining the volumes of titrant required to react with both standard solution and unknown sample, the unknown quantity can be calculated by simple proportion. For example, if 10 mls of titrant react with 25 mg of substance in the standard solution, and 20 mls of titrant are required to titrant the unknown, then the unknown sample contains $\frac{20 \times 25}{10}$ or 50 mg of the substance.

Some essential requirements for titrimetric analysis

1. The reactions must be rapid and essentially irreversible under the conditions of the titration so that the titration has a definite stiochiometric point or END-POINT.
2. There must be some means of detecting the end point. The simplest method is by addition of an indicator which is a compound which changes colour near the end-point of the reaction.

Principle

The calcium is precipitated in ammonium oxalate directly from the serum, after washing, the precipitate is dissolved in acid and the oxalate content of the sample is titrated with permanganate ($KMnO_4$).

Materials and reagent

1. Fresh serum
2. Ammonium oxalate (4% w/v)
3. Ammonium hydroxide (2% w/v)
4. $KMnO_4$ (0.002 M)
5. H_2SO_4 (1 N)

Procedure

1. Pipette 2.0 ml of serum into a test tube and add 1.0 ml distilled water and 2.0 ml 4% ammonium oxalate.
2. Mix, and allow to stand for 5 min. shaking at frequent intervals.
3. Centrifuge for 10 min and carefully pour off the supernatant solution without disturbing the pellet of precipitate.
4. Invert tube on absorbent paper (e.g. paper towel) and allow to drain for 5 min. Wipe mouth of the tube to remove last traces for moisture.
5. Add 3.0 mls of 2% NH_4OH to tube and mix well with precipitate, using a thin glass rod.
6. Centrifuge again, remove supernatant and drain and wipe as in steps 3 and 4.
7. Add 4.0 ml H_2SO_4 to tube and mix with precipitate.
8. Heat tube in a hot water bath (near boiling) until all oxalate precipitate dissolves.
9. Keeping the solution hot (60-75°C), titrate with 0.002 M $KMnO_4$. (As you near the end point, the colour of added $KMnO_4$ will disappear more slowly. At this point, add the $KMnO_4$ drop-by-drop until the end point is reached (the end-point will be a very faint pink colour that persists for at least a minute.
10. Prepare a blank (4 ml of H_2SO_4).

Questions: - ***(To be answered in your report)***

1. (a) How many milliliters (mls) of MnO^-_4 were needed to titrate the serum sample? (Average your duplicate values).
2. How many millimoles (mmoles) MnO^-_4 were used? HINT: in a 0.002 M MnO^-_4 solution, 1.0 ml contains 0.002 mmoles).
3. Each mole of oxalate titrated had precipitaeted one mole of calcium ion in the serum sample. How many mmoles of Ca^{++} were present in your 1.0 ml serum sample?
4. The atomic weight of calcium is 40. What is the serum calcium concentration in mg/litre?
5. The normal adult human sera Ca^{++} concentration is about 5 meq/litre. What is this in (a) mg/100 ml? (b) mmoles/l.
6. What was the purpose of steps 5 and 6 of the procedure?

Determination of chloride in urine

Theory

Chloride is the major anionic component of the extracellular fluid of animal tissue and is preponderantly localized there. It is essential for water balance and osmotic pressure regulation as well as acid-base equilibrium in the body.

Principle

The chloride content of urine is titrated with mercuric nitrate solution, it forms unionized mercuric chloride, $HgCl_2$. At the end point the first excess of mercuric ions gives a violet or purple colour with the indicator, diphenylcarbozone.

Reagents and materials

1. Urine
2. Chloride standard (10 mM)
3. HNO_3 (1.0 M)

4. Diphenylcarbozone indicator solution
5. $Hg(NO_3)_2$ solution (in water)
6. Burette
7. Porcelain dish

Procedure (***Perform all operations in duplicate***)

Titration of standard chloride

1. Pipette 2.0 ml of Cl^- standard into a porcelain dish and add one drop of HNO_3 (1.0 M) and 3 drops of diphenylcarbozone indicator. MIX
2. Titrate with Hg (NO_3) until a TRACE of violet colour persists, indicating the presence of free Hg.
3. Pipette 0.2 ml of urine into porcelain dish and 1.8 ml of distilled water. Add HNO_3 (1 drop) and diphenylcarbozone as in 1 above.
4. Titrate to end point as above.

Questions

1. This chloride concentration in the standard solution was meq/litre. What is the concentration of CL^- in meq/litre in the unknown urine? Sample (REMEMBER: you used 2 mls of standard solution and 0.2 ml urine).
2. The standard solution of CL^- was made from NaCL. What weight of NaCL (M.W. 58.5) was required to make 1 litre of this solution?
3. What are the limitations of the method employed in this experiment?
4. Explain the circumstances under which the determination of CL^- in serum would be important.
5. What is the significance of:
   (a) High level of CL- in urine
   (b) Low level of CL- in urine
   (c) High level of CL- in serum

PRACTICAL 4

DETERMINATION OF SERUM TRANSAMINASES ACTIVITIES

Animal cells contain a variety of animal transferase. Aspartate aminotransferase (AST) is present in both the cytoplasm and mitochondria of cells. AST is also commonly referred to as glutamate aminotransferase (GOT). In conditions associated with mild degree of tissue injury, the predominant form serum is from the cytoplasm although some mitochondria enzyme activity is also present.

Both AST and alanine aminotransferase (ALT) are normally present in human plasma, bile, cerebrospinal fluid and saliva but one is found in urine unless a kidney lesion is present. The common name for ALT is glutamate pyruvate transaminase (GPT). In this practical session you will assay the activities of both AST and ALT in a serum specimen.

Glutamate pyruvate transaminase activity assay

Principle

L-Alanine: 2-Oxoglutarate aminotransferase catalyses the reaction:

L-Alanine + 2-Oxoglutarate $\longrightarrow$ L-glutamate + Pyruvate

Reaction of the pyrucate with 2,4-dinitrophenylhydrazine produces an intensely coloured hydrazone on the addition of sodium hydroxide. Measurement of optical density at 505 nm provides a measure of enzyme activity when compared with a standard graph.

Reagents

1. GPT Buffered substrate pH 7.4
   2-oxoglutaric acid 2 mM
   α-alanine 200 mm
   Phosphate buffer 0.1 M
2. 2,4-Dinitrophenylhydrazine reagent (1mM) in 1.0 N hydrochloric acid.
1. Pyruvate standard (2.0 mM) in distilled water.
2. Sodium hydroxide 0.4 M (carbonate free)
3. Aniline citrate reagent: Dissolve 5 g citric acid AR in 5 ml Aniline AP.

Procedure

Blood specimens should be carefully without haemolysis. The serum may be stored for short periods at 4°C. Haemolysed serum should not be used.

Calibration graph

Prepare seven tubes as follows:

Tube No	Water (mls)	Pyruvate std	Buffered substrate (mls)	International GPT units/L
1	0.2	0	1.0	0
2	0.2	0.05	0.95	6.5
3	0.2	0.10	0.90	12.5
4	0.2	0.15	0.85	21.0
5	0.2	0.20	0.80	30.5
6	0.2	0.25	0.75	39.5
7	0.2	0.30	0.70	56

Mix well and incubate at 37°C for 30 minutes and to each tube add:

- 1 ml 2, 4-Dinitrophenylhydrazine reagent (1 mM). Mix well and incubate for a further 20 minutes. Add to each tube.

- 10 ml sodium hydroxide solution 0.4 M (free from carbonate).

Mix well and allow tostand for 10 min

Read the optical density of each solution against test tube number one at 505 nm in 1 cm cuvette.

(International GPT unit is given in the above table. From the optical densities obtained plot a calibration curve against GPT international units per litre. The calibration graph is not linear).

For each serum to be assayed prepare two tubes as follows:

	TEST	CONTROL
Buffered substrate	1.0 ml	1.0 ml
Serum	0.2	0.0
Mix well incubate at 37°C for 30 min and Add 2,4-Dinitrophenyhydrazine reagent	1.0 ml	1.0 ml
Serum	0 ml	0.2 ml

Mix well and incubate for a further 20 min, add: 10 ml of 0.4M NaOH
Mix well and allow to stand for 10 min and read the optical density at 505 nm against a water blank. Serum GPT level in normal subjects lie within the limits 2-15 i.μ/l

Use of test in Diagnosis

The increase in serum L-alanine: 2-oxoglutarate aminotranferase (GPT) is considered a more sensitive indicator of hepatitis and liver cell damage than serum L-aspartate. 2-Oxoglutarate aminotransferase (GOT) as the former enzyme is found in higher concentrations in liver tissue than in heart muscle. Furthermore unless GOT is considerably increased, GPT levels do not usually increase in myocardial infarction. Hence GOT and GPT values can assist in the differential diagnosis of cardiac and liver diseases.

Determination of serum l-aspartate aspartate aminotransferase (GOT)

Principle

The enzyme also called L-aspartate: 2-oxoglutarate aminotransferase catalyses reaction

L-asparate + 2-oxoglutarate $\longrightarrow$ L-glutamate + oxaloacetate

The unstable oxaloacetate is quantitatively decarboxylated to pyruvate with aniline citrate reaction of pyruvate with 2,4-dinitrophenylhydrazine produces an intensely coloured hydrazone on the addition of sodium hydroxide solution: Measurement of optical density at 505 nm provide a measure of enzyme activity when compared with a standard graph.

Reagent

1. GOT Buffered substrate pH 7.4
   2mm α-oxoglutaric acid
   200 mM DL_Aspartic acid

0.1 M Phosphate buffer

2. 2, 4-Dinitrophenylhydrazine reagent (1mM) in 1.0 hydrochloric acid.
3. Pyruvate standard (2.0 mM) in distilled water.
4. 0.4 M Sodium hydroxide (Carbonate free)
5. Alanine citrate reagent

Dissolve 5 g citric acid, analytical reagent grade in 5 ml distilled water and add 5 ml aniline, analytical reagent grade.

Calibration graph

Prepare seven tubes as follows:

Tube No	Water (mls)	2 mM std Pyruvate (mls)	Buffered substrate (mls)	Internation al GOT units/L
1	0.2	0.0	1.0	0.0
2	0.2	0.05	0.95	6.0
3	0.2	0.10	0.90	13.0
4	0.2	0.15	0.85	22.0
5	0.2	0.20	0.80	30.5
6	0.2	0.25	0.75	39.5
7	0.2	0.30	0.70	51.0

Incubate at 37°C for 30 minutes and to each tube add:
1.0 ml of 2,4-dinitrophenylhydrazine reagent (1mM)
Incubate for a further 20 min, remove from water bath and to each tube add:
10.0 ml 0.4 M Sodium hydroxide. Mix well and allow to stand for 10 minutes.
Read the absorbance of each solution against test tube number one at 505 nm.
Subtract absorbance value obtained for tube 1 from the values obtained from the other tubes and from the absorbances obtained, plot a calibration curve.

Procedure

Serum or plasma should be separated from blood components. The serum may be stored for short periods at 4°C.
For each serum to be assayed prepare two tubes as follows:

	TEST	CONTROL
Buffered substrate place in water bath and leave for 3 minutes to reach 37°C	1.0 mls	1.0 mls
Serum	0.2 mls	0.0 mls
Incubate at 37°C for 1 hr and add; Aniline Citrate reagent	1 drop	1 drop
Serum	0.0 mls	0.2 mls
Leave in water for 5 minutes then add 2.4 dinitrophenylhydrazine	1.0 mls	1.0 mls
Leave in the water bath for 15 minutes then remove from the bath and add 0.4M NaOH	10 ml	10 ml

Mix the contents of each tube and allow to stand for 10 minutes, then read the absorbances at 505 nm against a water blank.
Serum GOT in normal subjects lie within the limits 4-20 IU per liter.

Use of test in diagnosis

The level of the enzyme L-aspartate: 2-oxoglutarate aminotransferase (GOT) increases significantly over the normal in myocardial infarction, hepatic necrosis and other diseases such as active cirrhosis. In a sample, the ratio of GOT to alkaline phosphatase is useful in the differential diagnosis of jaundice.

Questions

1. Write the reaction between pyruvate and 2, 4-dinitrophenylydrazine. Is the reaction specific? What are the compounds in serum that could possibly interfere with the enzyme assay?
2. What are some of the experimental causes of
   (a) False elevated activities of ALT and AST
   (b) False decreased activities of ALT and AST
3. Explain the role of cofactors in the above two experiments
4. Why should Haemolysed serum not be used for ALT and AST assay?
5. Explain briefly why the method you have used above is now no longer of the utilized in a diagnosis. Give the principle of the more appropriate method.
6. Outline the clinical disease conditions that are associated with elevated levels of
7. Serum transaminases.

PRACTICAL 5

Genomic DNA Extraction, Restriction Enzyme Digestion and Agarose Gel Electrophoresis

Overall Objective of this practicum

By the end of this topic, the student will be able to:

1. Extract genomic DNA from human adenovirus (HAdV) sample
2. Measure DNA concentration
3. Digest a DNA sample with a restriction endonuclease (enzyme) [R.E].
4. Analyze the R.E-digested DNA using agarose gel electrophoresis and interpret the banding patterns observed

Background: Deoxyribonucleic acid (DNA) is the genetic material of all organisms (except RNA viruses). It is the master molecule whose structure has all the information needed to create and direct chemical machinery of life. DNA is composed of units called nucleotides. DNA is a polymer found in all living cells except RBCs. The nucleotides join together to form very long chains. The DNA is organized as double stranded chain in which the two strands are connected by hydrogen bonds formed between bases where C pairs with G and A with T. The two DNA strands are negatively charged as they are rich in phosphate groups. In a typical eukaryotic cell, the long DNA stands are packed in the very small nucleus; this is done by its compactly folding it around positively charged (glue) Histone proteins.

DNA is the largest known molecule. A single unbroken strand of animal or plant DNA contains millions of atoms. The DNA from a single (diploid) human cell, if the 46 chromosomes were connected end-to-end and straightened, would have a length of approximately 2 meters and a width of approximately 2.4 nanometers.

Purification of Human Adenovirus Genomic DNA from Cell Culture Fluids (QIAamp DNA Mini Spin Protocol)

Experiment Objectives:

Students will use spin column techniques to isolate DNA from a DNA virus, HAdV. Further, students should relate the molecule of DNA as common to all plants and animals as the chemical that holds genetic information aiming to use for:

1. Genetic testing for different gene mutations (Electrophoresis, PCR, etc)

2. Forensic evidence (Body identification, Paternity testing, crimes)

Equipment and reagents needed

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

1. DNA source
2. QIAamp® DNA Mini
3. Ethanol (96–100%)*
4. 1.5 ml microcentrifuge tubes
5. Pipet tips with aerosol barrier
6. Microcentrifuge (with rotor for 2 ml tubes)
7. Vortexer
8. Water bath or heating block at 56°C

DNA Extraction Protocol (Adapted from QIAamp® DNA Mini and Blood Mini Handbook)

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

2. Add 200 µl virus sample to the microcentrifuge tube.

If the sample volume is less than 200 µl, add the appropriate volume of PBS. QIAamp Mini spin columns copurify RNA and DNA when both are present in the sample. RNA may inhibit some downstream enzymatic reactions, but not PCR. If RNA-free genomic DNA is required, 4 µl of an RNase A stock solution (100 mg/ml) should be added to the sample before addition of Buffer AL. Note: It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.

To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample

will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively. Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C for 10 min.

DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each spin column to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini

spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield. For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency. For long-term storage of DNA, eluting in Buffer AE and storing at –20°C is recommended, since DNA stored in water is subject to acid hydrolysis. A 200 µl sample of whole human blood (approximately 5 x 10^6 leukocytes/ml) typically yields 6 µg of DNA in 200 µl water (30 ng/µl) with an *A*260/*A*280 ratio of 1.7–1.9.

Questions:

1. What does DNA look like?
2. Where would you likely find DNA in a living organism?
3. The buffers used to in extracting DNA variably contain the following components: (a) soap [detergent] solution, (b) alcohol, (c) NaCl-EDTA. What do you think was the specific purpose of adding each of these in the buffers?
4. Why might be important to be able to isolate DNA in a lab?

Restriction Enzyme Digestion of Human Adenovirus Genomic DNA

The purpose of this procedure is to digest, or cut up, a quantity of DNA in preparation of for separation of the fragments by gel electrophoresis. DNA digests are carried out by numerous different kinds of restriction enzymes, molecules isolated from bacteria that cut DNA molecules at specific locations identified by a particular sequence of nucleotides. The source of the DNA is the human adenovirus and has a total length of 48,502 base pairs. In this procedure, you will cut lambda DNA using two different restriction enzymes (called single digests) and then cut the DNA with the two enzymes together (called a double digest) and compare the pattern and sizes of the resulting DNA fragments. You will also run uncut lambda DNA as a control group as well as set of DNA markers and a collection of pre-cut DNA fragments of known length that is used as

a reference when you are trying to determine the size of the fragments cut by restriction enzymes.

Materials needed

1. Purified genomic HAdV DNA solution
2. Restriction enzyme(s)
3. Restriction enzyme buffer(s)
4. Nuclease-free distilled and deionized water
5. microcentrifuge tubes
6. Pipet tips with aerosol barrier
7. Microcentrifuge (with rotor for 2 ml tubes)
8. Vortexer
9. Water bath or heating block at 37°C

Protocol for HAdV DNA digestion

1) Gather three 1.5 ml microtubes and label each accordingly: DNA ladder, uncut DNA, DNA + Restriction enzyme A). Also include your group's identification.

2) Transfer 15 uL of HAdV DNA to each of the 2 labeled microtubes. Use the following chart to guide you in the addition of the enzymes and buffers. All units are uL. Make the addition of the enzyme the last substance that you add to the tube.

TUBE	DNA	R. Enzyme	FastDigest buffer	dH2O	Total volume
Uncut DNA	15			15	30
DNA+ RE (_____)	15	1	3	11	30

Note: A specific buffering solution is added along with each enzyme to provide a chemical environment in which the enzyme works best. Fermentas sends FastDigest buffer along with their enzymes – all of the enzymes we are using work well with this buffer

3) Place all three tubes in the heat block (or water bath) set at 37°C (human body temperature) for 15 minutes.

Note: The uncut DNA is serving as a control in this experiment and as a result is being heated even though it has no restriction enzymes.

4) Inactivate the enzyme by heating for 5 min. at 65oC (optional).

5) After incubation, remove all three tubes from heat source and place in the freezer(-20°C) until ready to add to your electrophoresis gel.

Agarose Gel Electrophoresis of RE-Digested HAdV DNA

Background:

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose: To determine the presence or absence of HAdV DNA fragments and quantify the size (length of the DNA molecule) of the product.

Materials needed:

1. Agarose
2. TAE Buffer
3. 6X Sample Loading Buffer
4. DNA ladder standard
5. Electrophoresis chamber
6. Power supply
7. Gel casting tray and combs
8. DNA stain
9. Staining tray
10. Gloves

11. Pipette and tips

Recipes:

TAE Buffer

4.84 g Tris Base

1.14 ml Glacial Acetic Acid

2 ml 0.5M EDTA (pH 8.0)

- bring the total volume up to 1L with water

Add Tris base to ~900 ml H_2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1 L graduated cylinder and add H_2O to a total volume of 1 L.

Note – for convenience a concentrated stock of TAE buffer (either 10X or 50X) is often made ahead of time and diluted with water to 1X concentration prior to use.

6X Sample Loading Buffer

1. 1 ml sterile H_2O
2. 1 ml Glycerol
3. enough bromophenol blue to make the buffer deep blue (~ 0.05 mg)

-for long term storage, keep sample loading buffer frozen.

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

1. Measure 1.25 g Agarose powder and add it to a 500 ml flask
2. Add 125 ml TAE Buffer to the flask. (the total gel volume will vary depending on the size of the casting tray)
3. Melt the agarose in a microwave or hot water bath until the solution becomes clear. (if using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask)
4. Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.

5. Seal the ends of the casting tray with two layers of tape.
6. Place the combs in the gel casting tray.
7. Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
8. Carefully pull out the combs and remove the tape.
9. Place the gel in the electrophoresis chamber.
10. Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

1. Add 6 µl of 6X Sample Loading Buffer to each 25 µl PCR reaction
2. Record the order each sample will be loaded on the gel, including who prepared the sample, the RE used to digest the DNA - what organism the DNA came from, controls (Uncut HAdv DNA) and molecular weight ladder.
3. Carefully pipette 20 µl of each sample/Sample Loading Buffer mixture into separate wells in the gel.
4. Pipette 10 µl of the DNA ladder standard into at least one well of each row on the gel.

Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

Questions and Conclusions:

1. In your notebook, calculate the number and sizes of the different DNA fragments that will be produced by your digest.
2. What special property of DNA allows for gel electrophoresis and staining adhesion?
3. Describe two precautions you take when preparing and running a gel?
4. What is the purpose of loading a ladder beside the samples?

Experiment 6: Polymerase Chain Reaction (PCR)

History of PCR

The polymerase chain reaction (PCR) was developed in 1983 by Dr. Kary Mullis while working for Cetus Corporation. In 1993, he received the Nobel Prize in Chemistry for this important contribution that revolutionized molecular biology. The technique can be used to amplify DNA sequences from any type of organism. It has been adapted over the years to allow amplification of RNA samples, as well as quantification of the amount of DNA or RNA in a sample. The isolation of a thermal stable DNA polymerase (Taq) from an archaebacteria isolated from a geothermal vent in Yellowstone National Park allowed the reaction to be carried out in a single closed tube driven by varying temperatures. Today, the technology has been revolutionized and is applicable in many important application including modern medicine, agriculture etc.

Theory

The Polymerase Chain Reaction, (PCR), is an extremely efficient and sensitive tool that can be used to make millions of copies of any specific DNA sequence, even when the sequence is in minute amounts or in a complex mixture. For example,a single hair root, or a microscopicblood stain left at a crime scene, can provide enough DNA sample for PCR.

PCR is based on the way cells replicate their DNA. During DNA replication, the double stranded DNA molecule separate and DNA polymerase enzyme, assemblesdeoxyribonucleotidesto form two new partner strands for each of the original strands. The original strands serve as templates for the new strands. The new strands are assembled such that each nucleotide in the new strand is determined by the corresponding nucleotide in the template strand. The nucleotides adenine (A)base pairs with thymine (T) while guanine (G) always base pairs with Cytosine (C) on the opposite strand to form a double stranded DNA helix. Because of this base-pairing specificity, each newly synthesized partner strand has the same sequence as the original partner strand, and replication produces two identical copies of the original double-stranded DNA molecule.

In PCR, the "target" DNAsequence, that need to be amplified, undergoes about several rounds of replication in a small reaction tube. During each replication cycle, the number of molecules of

the target sequence doubles, since the products and templates of each round of replication all become the templates for the next round. After n rounds of replication, 2n copies of the target sequence are theoretically produced. After thirty cycles, PCR can produce 230 or more than ten billion copies of a single target DNA sequence. Thus this is called a polymerase chain reaction because DNA polymerasecatalyses a chain reaction of replication.DNA amplification makes it possible to obtain ample quantities of specific DNAsequences to perform a variety of analyses.

A typical PCR reaction consists of the following components, mixed together in a solution with a total volume of between 25 and 100 microliters. The solution must include the template DNA, the primers, nucleotides to serve as building blocks for the newly forming DNA, DNA polymerase to catalyze the synthesis, and buffers and salts, usually including magnesium ions, which are required for optimal activity of the DNA polymerase. The template can be an unpurified mixture of DNA, such as DNA extracted from a swab of a cheek cells from a patient or crime suspect.

To perform the PCR reaction, the tube containing the solution is placed into a machine called a DNA thermal cycler. Thermal cyclers are basically programmable heating blocks or specialized equipment that is customized to fluctuate between specifically timed temperature variations. They usually contain a thick aluminum block with holes in which PCR reaction tubes can fit snugly. The block can be rapidly cooled or heated to specific temperatures, for specific lengths of time, under programmable computer control. Each cycle in a PCR reaction is controlled by changing the temperature of the block and, therefore, of the reaction mixture.

The first step in PCR is to heat the mixture to a high temperature, usually 94 to 95 °C. for about five minutes. In a process known as **denaturation** the hydrogen bonds that hold together the two strands of a double helix are broken at these temperatures, and the DNA separates into single strands.

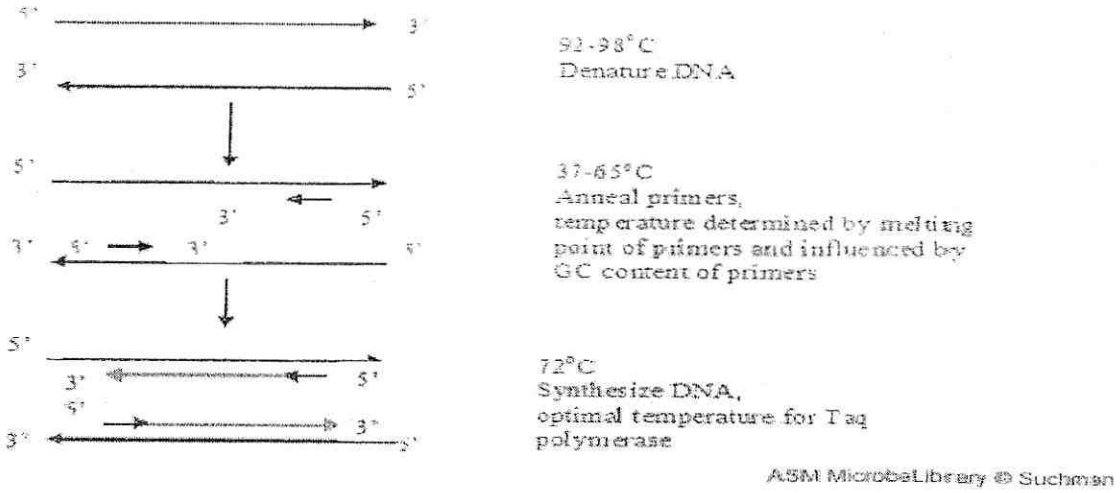
In the second step,**Annealing**, the PCR mixture is cooled to a lower temperature, typically between about 50 °C and 65 °C. This allows the primers to anneal to their specific complementary sequences in the template DNA. The temperature for this step is chosen carefully to be just low enough to allow the primers to bind, but no cooler. A lower annealing

temperature might allow the primers to bind to regions in the template DNA that are not perfect complements, which could lead to the amplification of non-specific sequences.

The optimal annealing temperature for a set of primers can be determined by a formula that is based on the nucleotide composition of the primers, but it is often a matter of trial and error to find the best annealing temperature. The annealing step usually takes about fifteen to thirty seconds, an amazingly short time considering that the primers must "scan" through the template DNA to find their proper binding sites.

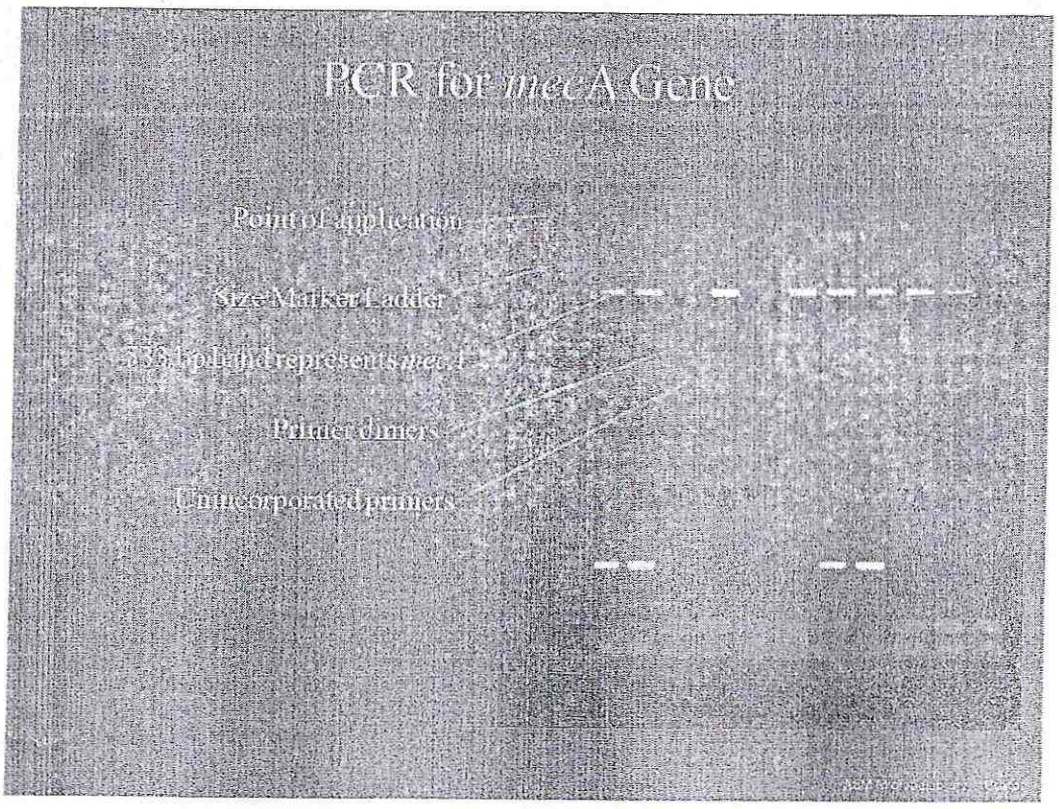
In the third step,**Extension**, the reaction is heated again, usually to about 72 °C, the temperature at which the DNA polymerase is most active. At 72 °C, Taq DNA polymerase adds nucleotides to the 3′ ends of annealed primers at the rate of about two thousand nucleotides per minute. Therefore, to amplify a sequence that is one thousand nucleotides long, the primer extension step must last about thirty seconds at 72 °C. By the end of this step, each template strand has a new complementary strand. This completes the first cycle of the PCR reaction.

The cycle can be repeated, at that point, by restarting the denaturation step. In the next cycle, the original two DNA strands will serve again as templates, as will the two newly synthesized strands. In this way, the number of templates has doubled, and it will double again with each successive cycle.At the end of the reaction, the tube contains DNA fragments that are almost solely copies of the target DNA. The original template DNA mixture is still present, but for the purpose of most applications (with the exception of subsequent PCR experiments), it is present in negligible amounts compared to the PCR product.



After amplification, the PCR product, sometimes called an amplicon, is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain and compared to known-sized molecular markers for production of bands of the correct size.

Example results



Example PCR gel electrophoresis agarose gel demonstrating a 533 bpamplicon as well as primer dimers and unincorporated primers. (Rebecca Buxton, University of Utah)

PCR.is therefore, a rapid, inexpensive, and a relatively easy way of producing a large number of copies of a specific DNA sequence. This is particularly advantageous when there is very little or poor quality DNA. RNA, which is converted from DNA into protein, can also be amplified in the same manner as DNA, however, DNA is much more stable and is easier to isolate.

In this experiment Human adenovirus DNA extracted from an earlier experiment will be amplified by conventional PCR using primers set that amplify the part of the hexon gene of the virus.

Materials for PCR

1. Human adenovirusDNA sample
2. 50µl PCR tubes
3. P10 –P1000Micropipeter
4. Box of micropipeter tips
5. Beaker of ice
6. 0.5µMof each Forward andReverse primers
7. Mixture of 50 µMdeoxynucleotidestriphosphates (dNTPs)mixture
8. 10X PCR buffer
9. 25mM $MgCl_2$,
10. *Taq* DNA polymerase
11. PCR Thermocycler
12. Mineral oil (if thermocycler does not have hot lid to reduce condensation)

Procedure

1. Prepare a PCR reaction mixture (Master Mix) of the following composition:
   - 5 µl of 1X PCR Buffer
   - 3µl of 3µM MgCl
   - 1µl of 200µM concentrations of each deoxynucleotide triphosphates (dNTPs)
   - 2.5 µl of 0.5µM concentrations Forward primer)
   - 2.5µl of 0.5µM concentrations of Reverse primer
   - 0.25µl of 2.5 U of *Taq*DNA polymerase High Fidelity
   - 30.75 µl of H_2O (Nuclease free) to bring volume to 50 µl

2. To the master mix add the 5 µL DNA template per reaction
3. Tap tube gently to mix and spin briefly in microcentrifuge to get all contents to bottom, then place on ice until ready to load in thermocycler.

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Polymerase Chain Reaction (PCR)

1. Programme the thermocycler using the following parameters
   - ❖ An initial denaturation at 94°C for 2 minutes, followed by 30 cycles of
   - ❖ Denaturation at 94°C for 1 minute,
   - ❖ Annealing at 54°C for 1 minute,
   - ❖ Elongation step at 72°C for 3 minutes,
   - ❖ Final extension at 72°C for 7 minutes.
   - ❖ Hold samples at 4°C.upon PCR completion
2. Insert you PCR mixture in a PCR tube into the thermocycler and carry out a PCR run using the above programme.

   Note: If thermocycler does not have a heated lid, layer thin film of mineral oil over mixture to prevent evaporation during cycling.

Gel Electrophoresis

1. Set the electrophoresis gel casting chamber appropriately by inserting the combs (ask for assistance)
2. Prepare a 1% agarose gel electrophoresis by measuring1g of agarose and mixing with 100mls TAE running buffer.
3. While swirling (or use magnetic stirrer) heat the agarose to boil
4. Cool to about 40°C and add 1µL of ethidium bromide to agarose mix
5. Pour the mixture onto the assembled gel casting chamber and allow the gel to solidify
6. Transfer the solidified gel onto the gel electrophoresis chamber
7. Fill the chamber with running buffer (TAE/TBE) until the gel is submerged
8. Prepare 1kb DNA ladder by mixing 5 µL of the provided ladder with 5 µL of the loading dye and carefully load on the first well of the solidified gel
9. Prepare the sample for loading by mixing 5µL of DNA with 5µl of loading dye and carefully load at the bottom of each well on the solidified gel.
10. Put on the power and let the gel run for approximately 30 minutes at 70 Voltage (Keep checking to avoid an overrun)
11. Stop the electrophoresis by putting off the power after the run

12. Analyze the bands in the gel by visualizing under ultra violet light in a Transi Illuminator Alpha Imager HP

Questions

1. Outline five potential problems associated with PCR experiment
2. Explain why Taq polymerase is preferred PCR than other bacterial polymerases
3. Describe the role of *Taq*polymerase in amplifying the DNA.
4. Explain the role of the DNA ladder in a PCR experiment
5. Outline the applications of PCR technology
6. Explain five ways in which PCR can be applied in medicine

Master mix for 20 groups

Reagent	1 Reaction 50 µl Volume	30 Reactions µl
1X PCR Buffer	5	150
3µM MgCl	3	90
200µM concentrations of each deoxynucleotide triphosphates (dNTPs)	1	30
0.5µM concentrations Forward primer)	2.5	75
0.5µM concentrations ofReverse primer	2.5	75
2.5 U of *Taq*DNA polymerase High Fidelity	0.25	7.5
DNA template	5	
H_2O (Nuclease free) to bring volume to 50 µl	30.75	922.5

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