UNIVERSITY OF NAIROBI
FACULTY OF MEDICINE

DEPARTMENT OF BIOCHEMISTRY

PRACTICALS MANNUAL FOR FIRST YEAR STUDENTS OF BSc.(Biochemistry)

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Introduction to Laboratory Equipments and Procedures II

Exercise 1: Common Biochemical equipments

This section deals with the proper use and safety procedures involved in dealing with a variety of Lab equipments.
Students must have a good knowledge of the use and working of the following major biochemical equipments, their main components, use and care

- Weighing balance
- pH Meter
- Spectrophotometer
- Centrifuges
- Autoclave
- Water baths
- Water Distiller
- Incubator/oven

Question: Describe how you would use and care for a pH Meter
What safety precautions would you take while working with a centrifuge?

Exercise 2: Accuracy and Precision

The accuracy and precision of the assay for copper sulphate
Biochemical experiments are prone to error caused by inaccurate diluting, pipetting, variable times of incubation and the operator's inexperience. The precision is also limited by the quality of the volumetric apparatus and the efficiency of the measuring equipment. These errors accumulate and the results show some variability and are inaccurate
In this experiment, the precision and coefficient of variation of the method is determined by each pair of students repeating the assay five times.

Materials:
Stock Copper Sulphate solution: 5 mg/ml Copper Sulphate.
Working Sulphate solution;, dilute x5 (1:5)

Method:
Measure the absorbance of Copper Sulphate solution provided at 540 nm. Repeat the measurement five times.
What is the accuracy and Coefficient of variation of the results obtained using this Method.
{Coefficient of variation is the standard deviation expressed as a percentage of the mean value = (standard deviation/mean value) x 100%}

HBC 112: LABORATORY PRACTICE AND INSTRUMENTATION

Introduction to Laboratory Equipments and Procedures I

This exercise is an introduction to the use and care of major biochemical equipments; it provides a guide to good laboratory practice, general lab safety, safe handling and prudent storage of all chemicals used in a Biochemistry laboratory. It introduces new students to good laboratory practices and provides a meaningful approach to lab safety for the inexperienced laboratory student.

Exercise 1: Types of Laboratory materials commonly used in a Biochemistry Lab, their uses, cleaning and care.

This section deals with the different uses, proper cleaning, care and safe handling of glassware to prevent injury in the lab. Students must clearly identify and be able to handle, safely clean, and care for the following Labware. Students must be able to safely wash and sterilize laboratory glassware

- Beakers, pipettes including Pasteur and Micropipettes, test-tubes, Volumentric Flasks, Measuring cylinders, Burettes, Jars, Wash Bottles, Cuvettes, Funnels, Washing Brushes, Ice Buckets, stand and Clamps, Crucibles, Forceps, Tubes and Tubing, Vials etc

Question: Describe the procedure you would use to clean pipettes after use

Exercise 2: Safe handling of chemicals

This section defines chemical hazards associated with many chemicals used in the Lab. It covers safe handling practices of hazardous chemicals needed to avoid accidents. Students must be able to identify the following types of Chemical hazards and know the meaning of safety signs;

- Flammable chemicals (e.g Acetone, benzene, ether, methylated spirit)
- Toxic Chemicals (Chromic acid, Chloroform, Phenol, Methanol, Toluene
- Explosive e.g. Picric acid
- Corrosive and irritant substances (Strong acids/Alkalis)
- Radioactive Compounds
- Biohazard
- Oxidizing Chemicals
- Harmful Chemicals

Students must know the proper uses and procedures for using fume hoods to prevent laboratory accidents and injuries

Question: What rules should a student observe when working/handling flammable organic solvents? Describe the proper precautions and measures to take for fire prevention in the laboratory.

Exercise 3: Spillage and waste disposal

This section demonstrates the correct procedures for dealing with spillages and waste materials from labs. Students must be able to clean spilt chemicals and to properly dispose off solid waste material, infected material and animal carcasses

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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 data
9. predict which biochemical technique might be useful in solving a particular problem later in your profession.

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 or pipette. These are most accurate when measurements are made between two calibration marks.

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

LABORATORY RULES

1. Do not replace any solution in a reagent bottle, take only the minimum amount required

2. Replace the stopper immediately a solution has been taken from a bottle and take care not to mix stoppers

3. Do not put pipettes into bottles of quantitative reagents

4. Do not remove a bottle of special reagents from side shelves to your own benches

5. Never pipette a corrosive fluid, always use a measuring cylinder or burette

6. Good results are only obtained with clean apparatus: many erroneous results are due to dirty apparatus

7. When you have finished work, leave your bench clean and dry, just as you should expect to findit. See that all waste materials is put in the waste boxes provided and not into sinks.

USE OF LAB EQUIPMENT FOR VOLUMETRIC AND GRAVIMETRIC METHODS

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

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

TRANSFER PIPETTES: (This type has a bulb in the middle). This is the most accurate type and is calibrated to deliver a certain volume. Such a pipette must be clean to deliver accurately. A test for cleanliness is to allow water to run out of it. If it drains smoothly, leaving no adhering drops within, it is satisfactory. If drops remain behind the pipette obviously it will not deliver the indicated volume.
There is a convention with regard to the use of the transfer pipette which must be observed for accurate delivery . The pipette is filled to about an inch above the calibration mark, the upper end being held closed with the forefinger. The solution is allowed to escape, until the bottom of the meniscus is level with the mark, which should be at eye level. The tip is then touched for a second or two with inner side of a clean beaker or test tube (not receiving vessel). The solution is then allowed to flow into the receiving vessel by gravity until flow ceases. The transfer is then complete, although you will note that a small column of liquid still remains in the tip of the pipette. Do not blow this out into receiving vessel. The pipette is calibrated to deliver the specified volume if the above convention is observed in completing and delivery.

BURETTES

Macroburettes:

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 it drains without leaving droplets behind. Be sure the burettes do not leak when the stopcock is closed. Before performing titration ensure that there are no air bubbles trapped, especially near the stopcock or the drawn-out tip. 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 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 own equipment, and whenever possible this should be done immediately after use. If precipitates and solutions are allowed to dry on

the surface of glassware, the latter is much more difficult to clean. This is particularly true of dried deposits of fats, proteins, tissues etc.

The best general cleanser for laboratory glassware is soap and warm water applied with still brush. Caution should be taken to avoid scratching the glassware with the metal handles of brushes. There are a wide variety of brushes available for different kinds of apparatus. Thorough rinsing with distilled water are required to obtain chemically clean glassware.

Cleaning of burettes and pipettes requires special attention since improper drainage of these due to greasy surface introduce serious errors in quantitative work. A general test for cleanliness of burettes and pipettes is to fill them with H_2O and allow them to drain at a normal rate. If any visible drops or rivulets of H_2O are left behind on the surface of the glass then the surface is not clean enough to drain reproducibly. If pipettes are clean to begin with, they may require only copious rinsing with tap and then distilled water immediately after their use 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 NaOH contains 40.01 g of NaOH for litre. The molecular weight of H_2SO4 is 98.08. 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 neutralisation of solution is called titration.

Examples : The molecular weight of NaOH is 40.01. One molecule of NaOH can

combine with one of H^+ ($H^+ + OH^-$), 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 = $\underline{49.08g}$ 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 100 gm 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

THE BEER-LAMBERT'S LAW

WHY SOLUTIONS ARE COLOURED

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

of the compound being measured, while the amount of light absorbed is proportional to the intensity of the colour and hence 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 the absorbing medium increases. These two laws are combined together in the Beer-Lambert law:

$I = I_o e^{-k3cl}$

Transmittance

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

Per cent $T = I/I_o \times 100 = e^{-k3cl}$

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

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

$\log_e I_o/I = k_3cl$

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

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

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 transmitttance.

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

Principle

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

Materials

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

Method

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

Tube No)	1	2	3	4	5	6	7	8	9	10	11
Bromophenol(10 mg/litre)(ml)	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0

Distilled water (ml)	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0

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

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

THE ABSORBANCE CURVES OF BROMOPHENOL AND METHYL ORANGE

Materials

These are as in the previous experiment.

Method

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

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

QUESTIONS

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

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

THE ABSORPTION SPECTRUM OF P-NITROPHENOL

Materials

1. p-Nitrophenol (10 mM)
2. HCl (10 mM)
3. NaOH (10 mM)
4. Spectrophotometer

5. Volumetric flasks(100 mls)

Method

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

QUESTIONS

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

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

Buffer Preparation

Introduction

The regulation of internal pH is a physiological function of major importance for all organisms. It is accomplished in part by the presence, in cells and physiological fluids, of buffer systems, i.e., weak acids and their conjugate bases in concentration ratios consistent with the desired pH. The principal intracellular buffer is the phosphate system and that of blood is the bicarbonate system. The relationship between pH and the ratio of the concentrations of the buffer components is given by the Henderson-Hasselbach equation.

$$pH = pKa + \log [A^-]/[HA] \text{ where } pH = -\log H+ \text{ and } pKa = -\log Ka$$

The weak acid dissociation concepts shown below are the basis for derivation of this equation.

$$HA \text{ (weak acid)} \rightleftharpoons H^+ + A^- \text{ (conjugate base)}$$

$$Ka \text{ (equilibrium constant)} = \frac{[H^+][A^-]}{[HA]}$$

Biochemists and other life scientists also need to control pH in their experiments and frequently prepare aqueous buffer solutions for enzyme assays, extraction solvents, incubation media, etc. Usually the biochemist knows the desired pH and the volume of buffer needed, but must make decisions about the buffer components and the buffer concentration. The buffer used depends in part on the desired pH. A weak acid with pKa = pH ± 1 is usually selected. The buffer concentration is the sum of the concentrations of the weak acid and its conjugate base and depends on how closely the pH must be controlled, the expected amount of acid or base to be released, and the desired ionic strength.

A buffer may be prepared in a number of ways, three of which are given below:

1. Neutralization of a portion of the weak acid with sufficient strong base to give the desired ratio, $[A^-]/[HA]$:

$$HA + NaOH \rightarrow Na^+ + A^- + H2O$$

2. Neutralization of a portion of the weak base with sufficient strong acid to give the desired ratio, $[A^-]/[HA]$:

$$Na^+ + A^- + HCl \rightarrow HA + Na^+ + Cl^-$$

3. Combining the weak acid and the conjugate base in amounts necessary to give the desired ratio, $[A^-]/[HA]$.

The three methods will all give solution solutions with the same pH and buffer ion concentrations, if appropriate experimental techniques are used for buffer preparations. It should be noted, however, that in the case of method 2, the buffer solution will also contain the salt of the strong acid used to neutralize the conjugate base.

Experimental Procedure

In this experiment, you are asked to make buffer solutions of phosphate using three different methods. You will be assigned the desired pH for each of your buffer solutions in class. Make 100 mL of solution containing 0.1 M final concentration of the buffer. When you have finished your calculations, prepare the solutions and check the pH of your buffers using a pH meter.

Materials

1 M NaOH: 40.01 g/L of solution
1 M HCl: 83 mL conc. HCl/L of solution
1 M Acetic acid: 57 mL conc. acetic acid/L of solution
Sodium acetate, $NaC_2H_3O_2$
Potassium phosphate, dibasic, K_2HPO_4
Potassium phosphate, monobasic, KH_2PO_4

Procedure

1. Standardize the pH meter at $25^{O}C$ using the standard buffers provided.
2. Prepare an acetate buffer (20 ml) of pH 5.1by mixing the appropriate volumes of 1 M acetic acid and 1 M sodium acetate, as calculated from the Handerson-Hasselbalch equation.
3. Check the pH of the acetate buffer using the pH meter.
4. Dilute a small portion of the acetate buffer by serial dilution one thousand times with water and measure the pH of the diluted buffer.
5. Prepare a phosphate buffer (20 ml) of pH 6.9 by mixing the appropriate volumes of 1 M potassium dihydrogen phosphate and 1 M dipotassium hydrogen phosphate, as calculated from the Handerson-Hasselbalch equation.
6. Check the pH of the phosphate buffer.
7. To a portion (5ml) of the phosphate buffer, add 1 M HCl (1.0 ml) and measure the pH of the solution. Repeat using 1 M NaOH (0.1ml).
8. Incubate the acetate buffer at 50^{O} C for about 10 minutes. Measure the pH of the heated buffer.

Analysis of Results

In your report, describe exactly how you proceeded, show calculations, and give the assigned and measured pH value of your assigned buffers. Include in your discussion the buffer capacity of your solutions.

Discussion

1. Comment on any discrepancy observed between the calculated value of the pH and the measured value for the acetate and phosphate buffers.
2. What was the effect on dilution of the acetate buffer? Explain.
3. Was the phosphate buffer more resistant to cid or alkali?
4. What was the effect of increase temperature on the acetate buffer? Explain.

Questions

1. You are given sodium acetate, hydrochloric acid, sodium bicarbonate and sodium carbonate. Determine which of these you would need to make a buffer with pH = 4.7 by method 2. You do not need to do calculations.

2. You are again given sodium acetate, hydrochloric acid, sodium bicarbonate and sodium carbonate. Determine which of these you would need to make a buffer with pH = 9.9 by method 3. You do not need to do calculations.

3. By going to a buffer table, suggest a buffer system (not phosphate or carbonate) and calculate acid/conjugate base ratio to get a buffer pH = 6.6.

4. Did the pH values for the solutions you prepared agree with the expected pH you were assigned? If not, discuss possible reasons for the difference you observed.

5. The pKa given for the phosphate buffer is often under question. Based upon your results, do you think the given pKa is correct? Using the data from your experiment (assuming you used excellent lab technique), calculate the actual value of the phosphate pKa.

Useful Information

Substance	MW	pK_1	pK_2	pK_3
K_2HPO_4	174.18	2.15	6.85	12.33
KH_2PO_4	136.09	2.15	6.85	12.33
$NaC_2H_3O_2 \cdot 3H_2O$	136.08	4.74		

* pKa values in the table were taken from: "Buffers for pH and metal ion control" D.D. Perrin and B. Dempsey (1974).

Protein Assays

Experiment 1

Absorbance Assay (280 nm)

Introduction

Absorbance assays for the determination of proteins concentrations in a solution are fast and convenient, since no additional reagents or incubations are required. No protein standard need be prepared. The assay does not consume the protein. The relationship of absorbance to protein concentration is linear. Because different proteins and nucleic acids have widely varying absorption characteristics *there may be considerable error*, especially for unknowns or protein mixtures. Any non-protein component of the solution that absorbs ultraviolet light will interfere with the assay. Cell and tissue fractionation samples often contain insoluble or colored components that interfere. The most common use for the absorbance assay is to monitor fractions from chromatography columns, or any time a quick estimation is needed and error in protein concentration is not a concern.

Principle

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum.

Equipment

- Phosphate buffer (PBS)
- Mixed-protein sample dissolved in PBS
- Pure protein sample dissolved in PBS
- Protein standard contaminated with nucleic acid
- Non-protein standard contaminated with nucleic acid
- Pure non-protein standard NOT contaminated with nucleic acid
- A spectrophotometer with UV lamp and
- Quartz cuvette

Procedure

Carry out steps 1-4 (280 nm only) for a very rough estimate. Carry out all steps if nucleic acid contamination is likely.

1. Warm up the UV lamp (about 15 min.)
2. Adjust wavelength to 280 nm
3. Calibrate to zero absorbance with buffer solution only
4. Measure absorbance of the protein solution

5. Adjust wavelength to 260 nm
6. Calibrate to zero absorbance with buffer solution only
7. Measure absorbance of the protein solution

Post Lab Analysis

1. *Unknown proteins or protein mixtures.*
   Determine the concentration of the unknown proteins or protein mixtures.

Use the following formula to roughly estimate protein concentration. Path length for most spectrometers is 1 cm.

Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm.)

2. *Pure protein of known absorbance coefficient.*
   Determine the concentration of the pure protein sample.
   Use the following formula for a path length of 1 cm. Concentration is in mg/ml, %, or molarity depending on which type coefficient is used.
   Concentration = Absorbance at 280 nm divided by absorbance coefficient
   To convert units, use these relationships:
   Mg protein/ml = % protein divided by 10 = molarity divided by protein molecular weight

3. *Unknowns with possible nucleic acid contamination.*
   Determine the concentration of the unknown protein/nucleic acid sample.
   Use the following formula to estimate protein concentration:
   Concentration (mg/ml) = (1.55 x A280) - 0.76 x A260)

Experiment 2

Hartree-Lowry Protein Assays

Introduction

The Lowry assay is an often-used protein assay. For some time it was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. The bicinchoninic acid (BCA) assay is based on the same principle. However, the modified Lowry is done entirely at room temperature. The Hartree version of the Lowry assay, a recent modification that uses fewer reagents, improves the sensitivity with some proteins, is less likely to be incompatible with some salt solutions, provides a more linear response, and is less likely to become saturated.

Principle

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of

tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

Equipment

- Standard liquid handling supplies
- A spectrophotometer with infrared lamp and filter (650nm capability)
- Glass or polystyrene (cheap) cuvettes.
- Water bath at 50^{O} C
- Buffer (PBS?)
- Protein solution(s) of unknown concentration(s)
- Bovine Serum Albumin (various concentrations of between 30 to 150 µg/ml)

Procedures

Reagents

1. Reagent A consists of:
   - 2 gm sodium potassium tartrate x 4 H20
   - 100 gm sodium carbonate
   - 500 ml 1N NaOH,
   - Top Up with H_20 to one liter (that is, 7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration). Keeps 2 to 3 months.
2. Reagent B consists of:
   - 2 gm 2 gm sodium potassium tartrate x 4 H20
   - 1 gm copper sulfate (CuSO4 x 5H20)
   - 90 ml H20
   - 10 ml 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulfate). Keeps 2 to 3 months.
3. Reagent C consists of:
   - 1 vol Folin-Ciocalteau reagent diluted with 15 vols water.

Procedure

1. Prepare a series of dilutions of 0.3 mg/ml bovine serum albumin in the same buffer containing the unknowns, to give concentrations of 30 to 150 micrograms/ml (0.03 to 0.15 mg/ml).
2. Add 1.0 ml each dilution of standard, protein-containing unknown, or buffer (for the reference) to 0.90 ml reagent A in separate test tubes and mix.
3. Incubate the tubes 10 min in a 50 degrees C bath, then cool to room temperature.
4. Add 0.1 ml reagent B to each tube, mix, incubate 10 min at room temperature.
5. Rapidly add 3 ml reagent C to each tube, mix, incubate 10 min in the 50 degree bath, and cool to room temperature. Final assay volume is 5 ml.
6. Measure absorbance at 650 nm in 1 cm cuvettes.

Post Lab Analysis

1. Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts of the unknown(s) from the curve.
2. Determine concentrations of original samples from the amount of protein, volume/sample, and dilution factor, if any.

Experiment 3

Biuret Protein Assay

Introduction

The principle of the Biuret assay is similar to that of the Lowry, however it involves a single incubation of 20 min. There are very few interfering agents (ammonium salts being one such agent), and displays fewer deviations than with the Lowry or ultraviolet absorption methods. However, the Biuret consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. The Bradford assay is faster and more sensitive.

Principle

Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent.

Equipment

- Standard liquid handling supplies
- A visible light spectrophotometer (transmission in the region of 550 nm)
- Glass or polystyrene (cheap) cuvettes.

Procedure

Reagent

- Biuret reagent, consisting of:
  - 2.25 gm Sodium potassium tartrate
  - 0.75 gm Copper sulfate x 5 H2O
  - 1.25 gm Potassium iodide, all dissolved in order in 100 ml 0.2 M NaOH (0.8 gm/100 ml). Bring volume to 250 ml with distilled water. Discard if a black precipitate forms.

Assay

1. Warm up the spectrophotometer 15 min. before use.

2. Dilute samples to an estimated 1 to 10 mg/ml with buffer. Add 1 ml to each assay tube. Duplicate samples are recommended, and a range of dilutions should be used if the actual concentration cannot be estimated.
3. Prepare a reference tube with 1 ml buffer.
4. Prepare standards from 10 mg/ml bovine serum albumin, preferably calibrated using absorbance at 280 nm and the extinction coefficient. Range should be from 1 to 10 mg protein.
5. Add 9 ml Biuret reagent to each tube, vortex immediately, and let stand 20 min.
6. Read at 550 nm.

Post Lab Analysis

1. Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve.
2. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.
3. Briefly describe other methods that can be used to determine protein concentrations in a given sample

Appendix

Comments about determination protein concentration by the absorbance (280nm) Assay

Cold solutions can fog up the cuvette, while warm solutions can release bubbles and interfere with the readings. For concentrated solutions (absorbance greater than 2) simply dilute the solution.
Absorbance coefficients of some common protein standards:

- Bovine serum albumin (BSA): 63
- Bovine, human, or rabbit IgG: 138
- Chicken ovalbumin: 70

Comments about determination protein concentration by the Hartree-Lowry Assay

Recording of absorbances need only be done within 10 min. of each other for this modified procedure, whereas the original Lowry required precise timing of readings due to color instability. This modification is less sensitive to interfering agents and is more sensitive to protein than the original. As with most assays, the Lowry can be scaled up for larger cuvette sizes, however more protein is consumed. Proteins with an abnormally high or low percentage of tyrosine, tryptophan, or cysteine residues will give high or low errors, respectively.

Comments about determination protein concentration by the Biuret Assay

The color is stable, but all readings should be taken within 10 min. of each other. As with most assays, the Biuret can be scaled down for smaller cuvette sizes, consuming less protein. Proteins with an abnormally high or low percentage of amino acids with aromatic side groups will give high or low readings, respectively.

TESTS FOR VARIOUS CARBOHYDRATES

Theory

The principle carbohydrates in the animal organism are hexoses composed of six carbon atoms such as glucose and fructose and the pentoses or five carbon atom sugars. The hexoses supply a large part of the energy required by the cell and can be metabolized to pentoses which are an important part of nucleic acid molecule's. The simple sugars are also known as 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 the **Benedict's** test for reducing sugars and for the quantitative determination of glucose in body fluids.

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

Monosaccharides polymerize into large molecules as follows:

Disaccharides, 2 units, example cane sugar)
Oligosaccharides, upto 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 polarised light which will be discussed during your lecture sessions.

EXPERIMENT I

REAGENT AND MATERIALS:

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

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

A*Molisch Test*

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

Experiment

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

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

Procedure:

1. Pipette 2 ml of the sugar solution into separate test tubes
2. Add 2 drops of the Molisch's reagent

3. Mix thoroughly, incline the test tube, and put 1 to 2 ml of Concentrated H_2HO_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 $\rightarrow$ 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 test tubes
3. Place all the tubes simultaneously in a boiling water bath and heat for 3 minutes
4. Cool and observe.

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

C. *Barfoed's Test*

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

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

Procedure:

1. Pipette 1 ml of each solutions into different test tubes
2. Add 5 ml of Barfoed's reagent to each test tube
3. Mix and place all the tubes in the boiling water bath
4. Observe them carefully for the appearance of the red precipitate of Cu_2O and record the number of minutes required; remove the positive tube and allow it to stand
5. After 15 minutes remove any remaining tubes and allow them to stand and observe for any precipitate that may settle out. Note the time.
6. Record the observations in a table.

DSeliwanoff's Test:

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

Procedure:

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

E. Bial's Test:

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

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

PROCEDURE

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

IODINE TEST FOR POLYSACCHARIDES

THEORY:

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

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

REAGENTS AND MATERIALS

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

PROCEDURE:

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

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

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

QUALITATIVE ANALYSIS OF LIPIDS

Introduction

Lipids are defined as those compounds that are soluble in organic solvents (such as ether, hexane or chloroform), but are insoluble in water. This group of substances includes triacylglycercols, diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, sterols, caretonoids and vitamins A and D. The major functions of lipids are as storage and transport of metabolic energy and as components of biological membranes. The lipid fraction of a fatty food therefore contains a complex mixture of different types of molecule. Even so, triacylglycerols are the major component of most foods, typically making up more than 95 to 99% of the total lipids present. Triacylglycerols are esters of three fatty acids and a glycerol molecule. The fatty acids normally found in foods vary in chain length, degree of unsaturation and position on the glycerol molecule. Consequently, the triacylglycerol fraction itself consists of a complex mixture of different types of molecules. Each type of fat has a different profile of lipids present which determines the precise nature of its nutritional and physiochemical properties. The terms fat, oil and lipid are often used interchangeably by food scientists. Although sometimes the term *fat* is used to describe those lipids that are solid at the specified temperature, whereas the term *oil* is used to describe those lipids that are liquid at the specified temperature. In the following experiments, qualitative tests will be carried out for triacylglycerols and unsaturated lipids.

Experiment 1

QUALITATIVE ANALYSIS OF TRIACYLGLYCEROLS

A: The Grease Spot Test for triacylglycerol

Materials and reagents

Test lipid samples
Methylene chloride
Pestle and mortar

Experimental procedure

1. Dissolve ~0.1 g of the test sample in 2 ml of methylene chloride. If solid material remains, grind it in a mortar and pestle and then filter to remove the solid from the test solution. If a liquid sample is insoluble, take the test solution from the methylene chloride layer.

2. Place several drops of the test solution onto a sheet of paper and then allow the solvent to evaporate.

3. A positive test is indicated by the formation of translucent spot on the paper.

B:Determination of Saponification Number

The *saponification number* is a measure of the *average molecular weight* of the triacylglycerols in a sample. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by treatment with alkali:

Triacylglycerol + 3 KOH $\longrightarrow$ Glycerol + 3 Fatty acid salts of potassium

The saponification number is defined as the mg of KOH required to saponify one gram of fat. The lipid is first extracted and then dissolved in an ethanol solution which contains a known excess of KOH. This solution is then heated so that the reaction goes to completion. The unreacted KOH is then determined by adding an indicator and titrating the sample with HCl. The saponification number is then calculated from a knowledge of the weight of sample and the amount of KOH which reacted. The smaller the saponification number the larger the average molecular weight of the triacylglycerols present.

Materials and reagents

Fat samples in 4% w/v solution in ethanol

0.3 N KOH dissolved in 90% ethanol

0.3 N HCl

Phenolphthalein solution

Reflux air condenser

Experimental procedure

1. Add 1 g of fat, 25 ml of 0.3M alcoholic KOH and a few glass beads to 250 ml flask
2. Boil for 30 min under reflux condenser
3. Cool then add 2-3 drops of phenolphthalein
4. Tiltrate with 0.3 N HCl
5. Carry out a blank tiltration of 25 ml of alcoholic KOH with 0.3 N HCl

Post Laboratory questions

1. Explain the biological functions of triacylglycerols
2. Define the characteristic feature of saponifiable lipids

3. Explain how the saponification number of monoacyl-, diacyl- and triacyl-derivative of oleic acid will differ

Experiment 2

QUALITATIVE ANALYSIS OF UNSATURATED LIPIDS

A: The halogenation test for unsaturated compounds

Reagents and materials

Test lipid samples
Methylene chloride
5% Bromine solution in Methylene chloride

Experimental procedure

1. Dissolve 0.1 g of the test sample in 5 ml of methylene chloride. If solid material remains, grind it in a mortar and pestle and then filter to remove the solid from the test solution. If a liquid sample is insoluble, take the test solution from the methylene chloride layer.

2. To 3 ml of the test solution add dropwise, 5% solution of bromine in methylene chloride (a dark brown solution) .

3. A positive test for unsaturated compounds is indicated by a colorless solution. The sample solution remains colorless if an unsaturated. The solution turns red/brown if only saturated compounds are present.

The degree of unsaturation of a compound can be determined by slowly adding bromine solution until the first red/brown color is observed. The more bromine solution required, the greater the unsaturation.

B: Determination of Iodine Value in edible oils

The iodine value (*IV*) gives a measure of the average *degree of unsaturation* of a lipid: the higher the iodine value, the greater the number of C=C double bonds. By definition the iodine value is expressed as the grams of iodine absorbed per 100g of lipid. One of the most commonly used methods for determining the iodine value of lipids is "Wijs method". The lipid to be analyzed is weighed and dissolved in a suitable organic solvent, to which a known excess of iodine chloride is added. Some of the ICl reacts with the double bonds in the unsaturated lipids, while the rest remains:

$$\text{R-CH=CH-R} + \text{ICl}_{\text{excess}} \longrightarrow \text{R-CHI-CHCl-R} + \text{ICl}_{\text{remaining}}$$

The amount of ICl that has reacted is determined by measuring the amount of ICl remaining after the reaction has gone to completion ($ICl_{reacted} = ICl_{excess} - ICl_{remaining}$). The amount of ICl remaining is determined by adding excess potassium iodide to the solution, and then titrating with a sodium thiosulfate (Na2S2O3) solution in the presence of starch to determine the concentration of iodine released:

$$ICl_{remaining} + 2KI \longrightarrow KCl + KI + I_2$$

$$I_2 + \text{starch} + 2Na_2S_2O_3 \text{ (blue)} \longrightarrow 2NaI + \text{starch} + Na_2S_4O_6 \text{ (colorless)}$$

Thus the concentration of C=C in the sample can be calculated by measuring the amount of sodium thiosulfate needed to complete the titration. The higher the degree of unsaturation, the more iodine absorbed, and the higher the iodine value. The iodine value is used to obtain a measure of the average degree of unsaturation of oils, and to follow processes such as hydrogenation and oxidation that involve changes in the degree of unsaturation.

Materials and reagents required:

Chloroform,

Oils (eg coconut oil, olive oil, sunflower oil)

Hubls iodine: dissolve seperately 26g iodine and 30 g mercuric chloride in about 250 ml of ethanol each, mix the two and make to 1 litre with ethanol

Burete, pipettes and porcelain dishes

Experimental procedure;

1. Fill a 50 ml burette with Hubls solution, note the initial reading
2. To 500 mg of of oil in a dry porcelain dish add 5 ml of chloroform. Dissolve the oil by gentle swirling
3. Add Hubls solution solution to the dissolved oil until the colour of iodine appears. Compare the resulting colour with control containing 5ml chloroform and 2-3 drops of Hubls iodine
4. Calculate the iodine number for the various oils taking into consideration the fact that 1ml of Hubls iodine contains 26 mg of iodine.

Post laboratory questions

1.What are the names and structures of the nutritionally important polyunsaturated fatty acids

2. What is the nutritional significance of polyunsaturated fatty acids

3. Which polyunsaturated fatty acid is required for prostaglandin synthesis

Qualitative tests for Nucleic Acid (DNA)

Introduction

Deoxyribonucleic acid (DNA) is perhaps the most important molecule in living things. Its main functions are:

i) Genetic code to pass genetic information to next generation (inheritance) and

ii) Code for making proteins (determines bioactive molecules).

It is a long, thin fiber with a deoxyribose sugar/phosphate backbone with purine & pyrimidine bases protruding from the sugar and contains the information necessary to control the chemistry of life. What does DNA look like? What are some of its many unique properties?

Experiment 1

Purpose

In this investigation, you will have the opportunity to precipitate and spool DNA onto a glass rod. The DNA has already been isolated from the nuclei of salmon sperm cells.

Materials

(i) Salmon sperm DNA in a buffered solution, 2 mL

(ii) Strong NaCl solution [5M], less than 1 mL

(iii) Ethyl alcohol, 4 mL

(iv) Test tube

(v) Glass rod or Pasteur pipette

(vi) Eye dropper

(vii) TE buffer

(viii) Sterile 15 mL tube

(ix) P-1000 Sterile blue tips

Procedure

1. Obtain 2mL of herring (or salmon) sperm DNA in a test tube. Examine it closely. **Describe its appearance - color and viscosity.**

2. Add 4 or 5 drops of strong NaCl solution using an eyedropper, and MIX.

3. *Slowly* trickle 4 mL of ethyl alcohol into the test tube containing the DNA and NaCl. DO *NOT* MIX.

4. **Observe the *interface* between the two solutions.** You should see a layer of alcohol form on top of the layer containing the DNA and NaCl. *Do not mix the two layers...*yet.

5. Place the glass rod or pipette all the way to the bottom of the test tube containing the two-layered solution.

6. **Watch the interface as you rotate the rod/pipette.** Wind (spool) the DNA that comes out of solution on to the rod/pipette. These are not single DNA molecules, but thousands of molecules. If you have a partner, be sure to take turns spooling.

7. Examine and touch the DNA on the rod. **Record the appearance of DNA -- color and texture.**

8. When finished, ask lecturer/technician what to do with your **spooled DNA** and glassware.

Lab Activity

PRELA; 1. Why is it important to study and understand DNA?

OBSERVATIONS; 2. Describe in words and with a drawing the appearance of your isolated DNA:

POST LAB; 3. Describe a follow-up activity that you could do using your isolated DNA:

Experiment 2

Purpose

When students "spool" DNA, they are told that the white stuff is DNA without any proof that it is indeed DNA. In this investigation, you will have the opportunity to determine (indirectly) that what was spooled is actually DNA.

How can you prove that the spooled white stuff is DNA? Possible ways include:

- Analyze composition chemically for A, T, C and G's and sugar-phosphate backbone
- Use to change an organism's genotype (and phenotype?)
- Other?

One way to indirectly prove a substance is DNA, is to mix the DNA with a chemical that reacts with DNA specifically. The chemical we choose to use is ETHIDIUM BROMIDE.

When ethidium bromide is mixed with DNA in solution (not precipitated DNA), the dye slips into the double helix between the stacking bases. Since RNA will fold to form secondary structure with a double helix in most cases, then ethidium bromide may also slip in into this RNA double helix. In that location both in RNA and DNA, the ethidium bromide, when exposed to ultraviolet light, fluoresces an orangey-pink color. Therefore, ethidium bromide reacts specifically with nucleic acids.

METHOD:

Identify what needs to be tested:

1) *The spooled DNA.*

Re-suspended the spooled DNA in solution to allow the ethidium bromide to slide into the helix by dissolving it DNA into a sterile test tube with 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and letting it to re-suspend overnight (or maybe sooner - watch to see if some of it re-dissolves during the class period).

Discuss controls:

1) Positive control: known DNA sample (e.g. Salmon Sperm DNA).

2) Negative control: water (or TE buffer)

3) Negative control: another substance that could be co-purified with DNA from cells such as protein: egg white.

- **Materials/Equipment:**

(i)	UV transilluminator
(ii)	EtBr (5μg/mL)
(iii)	P-20
(iv)	Sterile yellow tips
(v)	Plastic wrap (or substitute)
(vi)	TE buffer
(vii)	Egg white
(viii)	Salmon sperm DNA
(ix)	Gloves
(x)	Goggles
(xi)	Student samples of re-suspended DNA.

- **Procedure:**

1. Place a piece of clear plastic wrap, plastic baggie, or a transparency on the surface of the UV monitor (to protect the surface and for easier clean-up).
2. Wearing gloves and goggles, pipette FOUR samples of 10 μL of ethidium bromide (5 μg/mL) in a row on the plastic wrap.
3. Into the first 10 μL "drop", mix 10 μL water or TE buffer.
4. Into the second 10 μL "drop", mix 10 μL egg white.