UNIVERSITY OF NAIROBI

FACULTY OF MEDICINE

DEPARTMENT OF BIOCHEMISRTY
FST II PRACTICAL PROGRAMME

Practical course for Second year students of Bsc. Food Science and Technology
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TABLE OF CONTENTS

LIST		PAGE
Aims and objectives of Practical Biochemistry		iii
Direction for practical, laboratory classes and general instructions		iii
Laboratory rules		iv
Use of laboratory equipments for volumetric and gravimetric methods		iv
Theoretical introduction to methods of expressing concentrations in volumetric analysis and stociometric calculations		vi

PRACTICALS

Practical 1	Colorimetry and spectrophotometry	1
Practical 2	Determination of total protein by biuret method	6
Practical 3	Protein assay by Folin-phenol method	8
Practical 4	Comparison of protein assay methods	9
Practical 5	Determination of ascorbic acid in fruit juices	11
Practical 6	Comparison of Glucose assay methods	13
Practical 7	Thin layer chromatography (TLC) of sugars	16
Practical 8	Gel-filtration	18
Practical 9	Ion-exchange chromatography of proteins	21
Practical 10	Electrophoresis of enzymes by polyacrylamide gel (PAGE)	23
Practical 11	Nitrate reductase activity by invivo assay methods	26

AIMS AND OBJECTIVES OF PRACTICAL BIOCHEMISTRY

The experiments described in this manual have been compiled for the students of second year B.Sc. Food Science and Technology. 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 long-lasting understanding of biochemical techniques and the intricacies of Biochemistry. When you have finished the course you should be able to: -

1. Follow Biochemical procedures to obtain relevant data.
2. Interpret data from a Biochemical experiment.
3. Write a concise logical report based on laboratory findings.
4. Assess the application and limitation of common Biochemical tests in quality control.
5. Correctly measure the volume of liquids and operate simple equipments.
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 whish 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 quire 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 find it. See that all waste materials is put in the waste boxes provided and not into sinks.

USE OF LAB EQUIPMENT FOR VOLUMETRIC AND GRAVIMETRIC METHODS

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

Pipettes: There axe 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 mi 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 mi 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 les3 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 mI. 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 mI and are graduated usually to 0.002 mI. They are used in the same manner as microburettes, but drainage errors become much more serious. Remember that a single drop of an aqueous reagent may have a volume of about 0.05 mI. 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 is required to obtain chemically clean glassware.

Cleaning of burettes and pipettes requires special attention since irnproper 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 water and allow them to drain a normal rate. If any visible drops or rivulets of water 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 water 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 9 of NaOH for litre. The molecular weight of H_2SO_4 is 98.08. A molar solution of H_2SO_4 contains 98.08 9 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. 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 40.019 and a normal solution of NaOH has the same concentration as a molar solution. HCl has a molecular weight of 36.47. A nonnal solution of HCl contains 36.479g 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 (98.08) divided by 2 (= 49.08g) and a nonnal 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 100rnls of solution. This is by far the more commonly used expression involving percent (w/v). Volume percent, this expression is commonly used to indicate concentration of a solution of the solute in a liquid (v/v), 50% alcohol for example means we have 50mls in 100mI solution.

PRACTICAL 1

COLORIMERTY AND SPECTROPHOTOMETRY

Theory

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 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/ foods e.t.c. can be determined after little treatment. As discussed below, the depth of the colour is proportional to the concentration of the compound being measured, while the amount of light absorbed is proportional to the intensity of 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 transmitted light **I** is less than **Io.** There is some loose 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 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 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:

$$\mathbf{I = I_0 e^{-k3cl}}$$

Transmittance

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

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

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

Extinction

If negative logarithim are taken on both sides of the equation instead of the ratio then:

$$\log_e I_o/I = k_3cl$$

$$\log_{10} I_o/I = k_3cl/ 2.303$$

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

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

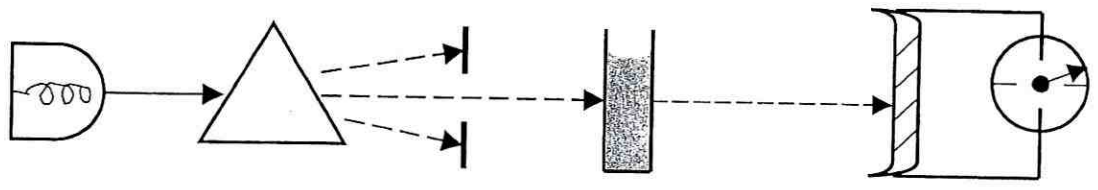
$$A = k_3cl$$

Where,

A = absorbance
k_3 = molar extinction coefficient
C = concentration of absorbing medium
L = cuvet/ SP tube/ cell/ path length

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 transmittance curve.

It is now customary to use a photoelectric device to measure the intensity of transmitted light, which to the most elaborate spectrophotometer, but irrespective of the complexity of the instrumentation, the basic principle is the same. The mechanism of the spectrophotometer is illustrated in the diagram.



1	2	3	4	5	6
White Light Source (Bulb)	Prism/ wavelength Selector	Slit	Cuvet	Photo Cell	Meter

Note:

1. This is normally any electric bulb, operated by a battery or on a regulated main to control the fluctuations.
2. A prism is used to split the white light into its various wavelengths.
3. The Slit is used to isolate the wavelength of light that is maximally absorbed by the solute increasing the sensitivity.
4. The known or unknown solutions are placed in a cuvet/ cell/ SP tube.

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

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

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

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

Theory

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

Materials and reagents

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

Method

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

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

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

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

THE ABSORBANCE CURVES OF BROMOPHENOL AND METHYL ORANGE

Materials and reagents
These are as in the previous experiment.

Method

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

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

Questions

1. What is the wavelength that gives maximum absorbance for each dye?
2. How does mixing the dyes affect the absorption spectrum?
3. Draw the structures of bromophenol blue and methyl orange. Comment on their key functional groups and their common laboratory uses.

THE ABSORPTION SPECTRUM OF P-NITROPHENOL

Materials and reagents
1. p-Nitrophenol (10 rnM)
2. HCl (10 mM)
3. NaOH (10 mM)
4. Spectrophotometer
5. Volumetric flask (100ml)
6. Volumetric flasks (100 mls)

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

Questions
1. Show the dissociation reactions of p-nitrophenol and explain the colour changes in acid and basic media.
2. Comment on the acid/base properties of p-nitrophenol.
3. Suppose the nitro group in p-nitrophenol was replaced by a carboxyl group, predict the effect of adding (a) HCl (b) NaOH to an aqueous solution as in the experiment.

4. Explain the importance of p: nitrophenol in a medical biochemistry laboratory and food industries.
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 50 mmol/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 and the molecular weight of Y is 586, calculate (a) the molar extinction coefficient (b) the transmittance of 10 mμmol/litre of solution.
7. Calculate the molar absorption coefficient, ε at 351 nm for aquocobalamin in 0.1 M phosphate buffer, pH 7.0, from the following data, which were obtained in a l-cm cell. Solution Concentration Io I

Solution	Concentration	Io	I
A	2.23×10^{-5}M	93.1	27.4
B	1.90×10^{-5}M	94.2	32.8

PRACTICAL 2

PROTEINS – DETERMINATION BY THE BIURET METHOD

Theory

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 t form a purple complex. This complex is formed with any molecule containing two or more peptide bonds and the colour intensity s proportional to the number of peptide bonds present. The name of the method comes from the simplest compound, which gives the reaction, **biuret** ($H_2NCONHCONH_2$). Relatively high protein concentration (1 – 10 mg/ml) is readily determined by the Biuret method.

In setting up a colorimetric protein 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 (BSA) (concentration, 10 mg/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 concentrations of food samples. The protein concentration of rough plot of your results for the standard curve, determine how much you will have to dilute the food samples so that their protein concentration then falls within the linear range of the standard curve.

You will have some time available during periods of waiting. Use this time effectively by making models of peptides and polypeptide helices using the Orbit molecular building system.

Reagents and Materials

Standard Protein Solution (BSA) 10mg/ml
Distilled water
Test tubes
Biuret Reagent, food samples X_1 and X_2
Spectrophotometer, SP tubes (cuvets)
Straight pipettes

Procedure (Perform all operations in duplicate)

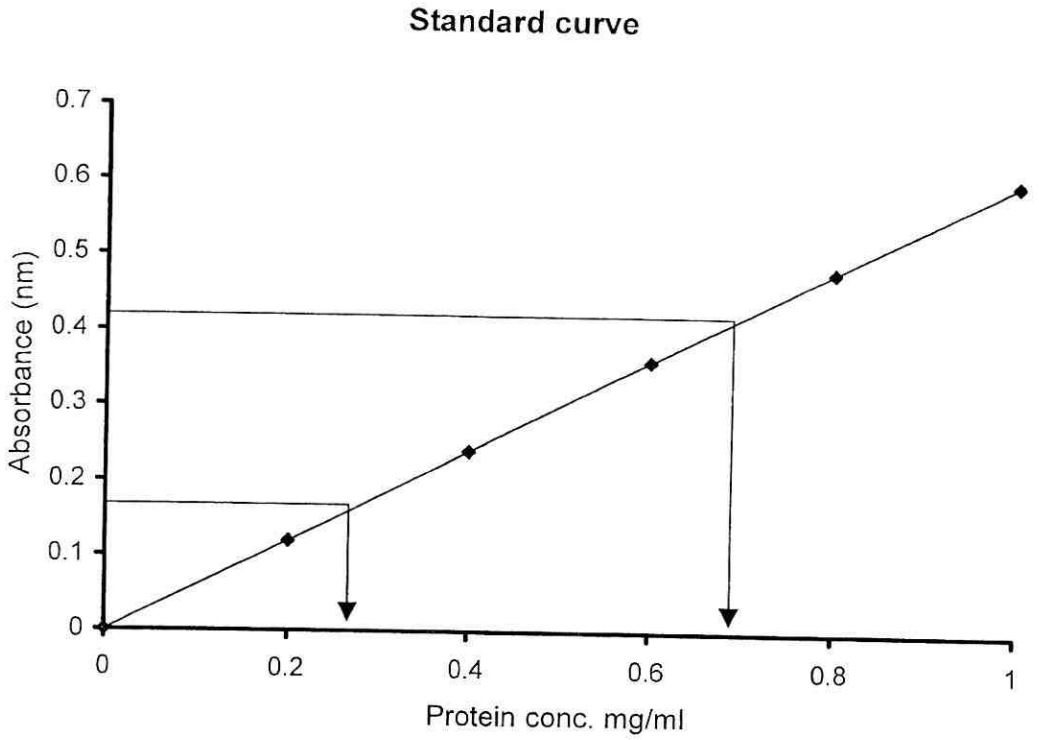
1. Prepare dilutions of the standard protein solutions as follows.

Test tube No.	1	2	3	4	5	6	X_1	X_2
Volume of protein solution (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		

Prepare duplicates at each dilution.

2. Add Biuret reagent (4.0 ml) to each tube. Shake well Allow to stand for exactly 30 min.
3. Measure the absorbance at 540 nm against quantity of protein (**mg**) in the sample.

4. Decide on an appropriate dilution factor for the food samples based on your rough plot of the standard curve unknown A and B.
5. Repeat steps 1 to 3 for the food samples, appropriately diluted. By reference to the standard curve, determine the protein concentrations of the food samples.



Discussion

Comment on your results, indicating the range of protein quantity for which the Biuret assay is most useful.

Questions

PRACTICAL 3

PROTEINS– DETERMINATION BY FOLIN-PHENOL METHOD

Theory

This procedure was developed by Lowry *et al.* (1951), and has the advantage of being about 10 times more sensitive (5-15mg) than Biuret method. The colour formed by Folin-ciocalteau reagent is due to the reaction of the protein with alkaline copper as in the case of Biuret test and also due to the reduction of phosphomolebdate- phosphotangstate salts in the reagents by tryptophan.

Reagents and materials

Reagent A: 2g NaOH, 10g Na_2CO_3 and 0.1g Na-K Tartarate in 500 ml water

Reagent B: 0.5g $CuSO_4$ 5 H_2O in 100 ml of water

Reagent C: Mix 100ml of reagent A and 2.0ml of reagent B prior to use

Reagent D: Dilute Folin- ciocalteu-Phenol reagent to bring make it to one normal (1N) with Std protein solution (BSA) (1mg/ml) respect to its acid concentration (Acid conc. of commercial preparations usually 2N)

Food samples A and B

Procedure

1. Prepare dilutions of standard protein solution as follows:

Tube No.	1	2	3	4	5	6	**7(unk)**
Volume of protein solution (ml)	0	0.2	0.4	0.6	0.8	1.0	**0.5**
Volume of distilled water (ml)	1	0.8	0.6	0.4	0.2	0.0	**0.5**

Prepare duplicates of each dilution

2. Add 5 ml of reagent C, shake all tubes well. Allow to stand for 10 in at roomtemperature.
3. Add 0.5 ml of Folin's-Coicalteau reagent (D) to all tubes and mix immediately. Allow to stand for 30 minutes at room temperature.
4. Measure absorbance at 750 nm.
5. Plot **absorbance** against **quantity of protein** in the sample.
6. Decide an appropriate dilution factor for the food samples based on your plot of the standard curve.
7. Repeat steps **2-4** for food samples after its appropriate dilution. By reference to the standard curve determine the protein concentrations of food samples.

Discussion

Comment on sensitivity of Biuret's and Folin-Phenol methods for determination of proteins.

Questions

PRACTICAL 4

A COMPARISON OF THREE METHODS FOR THE ASSAY OF PROTEIN

Theory

The quantitative determination of protein can be achieved by using a number of methods, each of which has its limitations.

a) **Biuret method:** In alkaline solution copper (II) ions complex 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 0.25 mg of protein. Also, some chemicals and many biological compounds interfere with the method.

b) **Lowry method:** This calorimetric 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 compound interfere with this method. However, it is highly sensitive with a lower limit of 5 microgram protein.

c) **UV absorption method:** This direct spectrophotometric method is based on the absorption of Tyr and Trp at 280nm. It suffers form the same 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 contamination nucleic acid. This method is rapid, sensitive and the protein sample is recovered after the measurement.

Reagent and Materials

Bovine serum albumin (BSA) (1 mg/ml) in 0.01m phosphate buffer, pH 7.0
Solution of gamma – globulins in buffer
Biuret reagent
Folin reagents C and E
0.01M phosphate buffer, pH 7.0

Procedure

Determine the protein concentration of the gamma-globulin solution using each of the three methods described in the Introduction.

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.
3. 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.
4. After 15 min. add Folin reagent D (1.0 ml) rapidly and mix well. After 30 min measure the absorbances at 600 nm. Calculate the protein concentration of the gamma globulin solution.
5. Determine the aborbance of the diluted gamma-globulin solution (4.0 ml) against a buffer blank(4.0 ml) at 280 nm and 260 nm. Calculate the protein concentration **by applying the appropriate correction factor based on the ration of A280 to A260.** (Consult the lecturer).

Discussion

Compare the values for the protein concentration of the gamma-globulin solution obtained by each of the three methods. Which methods is (a) the most sensitive (b) the most accurate (c) the most rapid (d) the least wasteful of protein samples? Justify your answers.

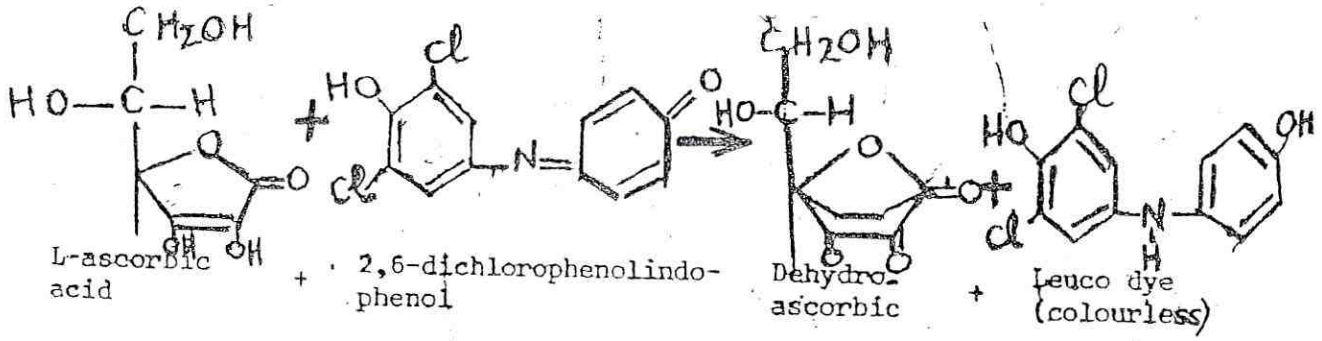
PRACTICAL 5

DETERMINATION OF ASCORBIC ACID (VITAMIN C) IN FRUIT JUICE USING 2:6 DICHLOROPHENOLINDOPHENOL (DCPIP)

Introduction

Titration of 2:6 dischlolophenolindophenol is one of the methods most frequently used for the determination of ascorbic acid in acid solution. Ascorbic acid is oxidized by the coloured dye to dehydroascorbic acid. At the same time, the dye is reduced to a colourless compound (leucobase) so that the end point of the reaction can be easily determined.

Reaction formula:



The dye is decolourised by other compounds as well as ascorbic acid but the specificity can be increased to some extent by carrying out the reaction in an acid solution where interfering substances react only slowly. The dye is obtained in powder form, or commercially tablet form so that 1 tablet is equivalent to 1 mg of ascorbic acid.

Reagents and materials

1. Glacial acetic acid
2. Solution of 2:6 dichlorophenolindophenol – either 1 tablet dissolved in 100ml of distilled water, or 20 mg of the powder in 100 ml of distilled water. **1 ml of the dye= 0.1mg of ascorbic acid.**
3. Fruit juice – Various brands
4. Standard ascorbic acid solution – 40 mg of pure ascorbic acid in 100 ml of 10% acetic acid.

   Therefore, concentration = 40 mg/100 ml (standard stock solution)
   Dilute 5 ml to 100 ml with 10% acetic acid
   Therefore concentration = 2mg/100 ml (working standard)
   1 ml of the dye should be neutralized by 5 ml of this solution.

Procedure

Pipette 0.5 ml of the dye into a test tube and add 1 ml glacial acetic acid. Run in the fruit juice slowly, with constant shaking, until the red colour has been discharged. Note the amount (**v ml**) of the fruit juice require.

Calculations

V ml of fruit juice = 0.5 ml of dye
= 0.05 mg of ascorbic acid

100 ml of fruit juice = $\frac{0.05 \times 100}{V}$ mg of ascorbic acid.

Discussion

Questions

1. Compare the levels of ascorbic acid in commercial fruit juices and other given natural fruit extracts.
2. Comment on the level values of ascorbic acid in commercial juices and natural preparations

PRACTICAL 6

COMPARISON OF GLUCOSE ASSAY METHODS

Theory

Your are familiar with the Folin-Wu method for the assay of reducing sugar in the range 0-200 nanomoles. An alternative method for assaying glucose involves the use of **glucose oxidase reagent**. The sequence of reactions in this method is:

$$\text{D- glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{peroxidase}} H_2O + {}^1/_2O_2$$

$${}^1/_2O_2 + AH_2 \longrightarrow A\ (\text{coloured}) + H_2O$$

When A is a redox dye which is colourless in the reduced state but coloured in the oxidized state. Each of these two methods has its advantages and disadvantages, some of which will become clear in this experiment. The glucose oxidase reagent is expensive. Another factor which is not considered in other glucose assay methods is interference in the assay method by contaminating substances. You will investigate the effect of the non-reducing sugar sucrose and glucose-6- phosphate on the assay of glucose by each method. One factor which is not considered here is reproducibility,. The glucose oxidase method is the more reproducible of the two.

Reagents and materials

Standard glucose solution (0.5 mM in water)
Sucrose solution (1 mg/ml in water)
Glucose-6-phosphate (G6P) solution (1 mg/ml in water)
Glucose oxidase reagent
Folin - Wu alkaline copper reagent
Phosphomolybdic acid reagent.

Procedure

Construct a standard curve for glucose using the Folin-Wu method as follows:
To individual test tubes add standard glucose solution at suitable intervals over the range 0-1.0 ml and make each volume up to 1.0 ml with water. Do this in duplicate.

1. Add the alkaline copper reagent (2.0 ml) to each tube.
2. Mix well and heat in a boiling water bath for 10 min. then cool
3. Add phosphomolybdic acid reagent (1.0 ml) to each tube and mix well. After 30 min measure the Absorbance at 540 nm.
4. At the same time construct a standard curve for glucose using the glucose oxidase method as follows. To individual test tube add standard glucose solution at suitable intervals over the range0-1.0 ml and make each volume up to 1.0 ml with water. Do this in duplicate.
5. Add glucose oxidase reagent (3.0 ml) to each tube. Mix well and leave the tubes in a closed locker for about 30 min. before reading the absorbance at 435 nm.

6. Using a suitable volume of standard glucose solution, carry out the Folin-Wu assay and the glucose oxidase assay in the **absence** and **presence** of sucrose solution (0.1 ml) and glucose-6- phosphate solution (0.1 ml).

Draw standard curves for glucose as measured by;

a) The Folin-Wu method and
b) The glucose oxidase method.

Express your results as **amount of glucose** in micromoles (on the x-axis) against **absorbance**.

Discussion

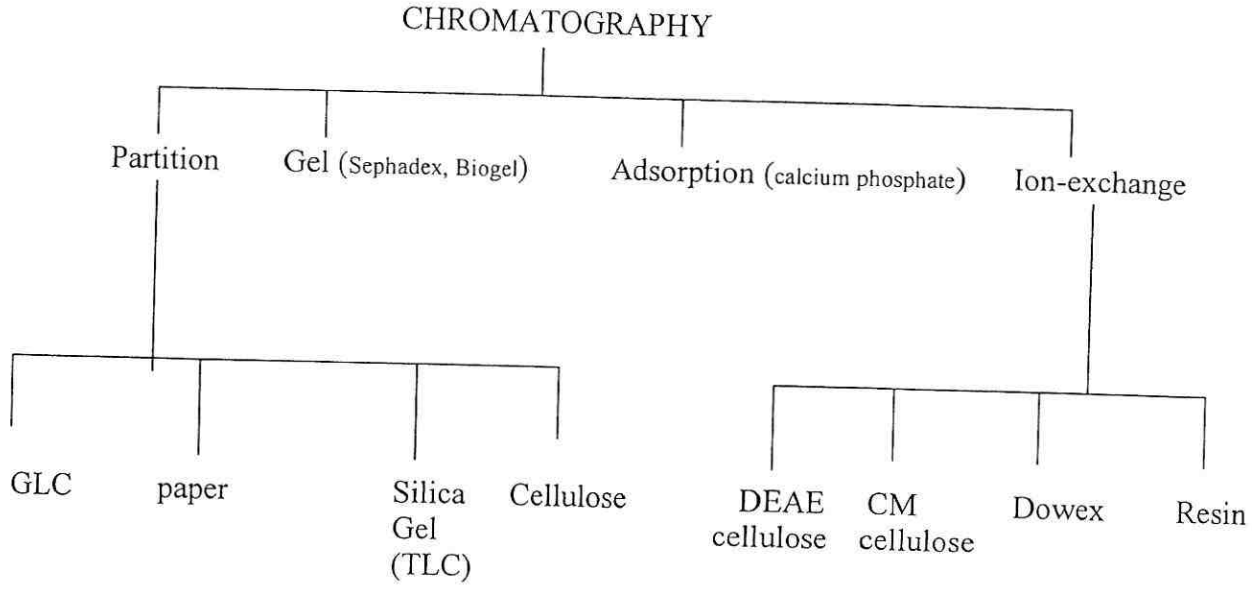
Compare the Folin-Wu method and the glucose oxidase method with respect to:

(a) Sensitivity (b) specificity (c) simplicity (d) reproducibility (e) effect of contaminating substances.

Questions

1. Which of the two assay methods would you use to assay;
   a) L-glucose
   b) Glucose in the presence of galactose.
   Justify your answer.
2. Suggest a method for assaying sucrose, giving brief details.
3. Which method would probably be used in the laboratory for measuring glucose concentration in the fruit juices and food? Justify your answer.

CHROMATOGRAPHY



Terminology

Adsorbent: Stationary phase (solid or liquid) or support (solid)
Solute: Sample or component
Solvent: Mobile phase or developer
Solvent front: Forward edge of solvent
Origin: Position of applied sample
Rf value: Position of solute-on chromatogram given by

$$Rf = \frac{\text{Distance traveled by solute}}{\text{Distance from origin to solvent front}}$$

Under specified conditions Rf is a constant for each solute.
Detection: Most solute are not coloured and so they must be visualized in some way.
Elution: Process whereby solvent carries the separated solutes off the chromatogram so that they can be collected individually.
Effluent: The eluted mobile phase.

PRACTICAL 7

THIN LAYER CHROMATOGRAPHY OF SUGARS

Theory

Thin layer chromatography (TLC) is based on the same partitioning effect as you encountered with paper chromatography. However, it is performed with glass plates coated with thin layer (usually 0.25 mm) of silica gel. It has certain advantages over paper chromatography, including a shorter time to develop the chromatogram.

Coffee bean contains a wide variety of carbohydrates, including small amounts of the free monosaccharides (e.g. glucose) and disaccharides (e.g. starch) and sugars bound to other organic, non-carbohydrates groups (glycosides). These components may be extracted with distilled water and analyzed by TLC. Extracts of natural materials usually contain many other substances (particularly proteins), which may interfere with the TLC. However, the protein content of coffee bean is relatively low and no further purification is required.

Disaccharides, polysaccharides and glycosides are hydrolysed by dilute acid to the constituent monosaccharides. Before TLC the acid must be removed to avoid "tailing" (compare paper chromatography of the casein hydrolysate) for this experiment the coffee bean extract has been hydrolysed with dilute HCl, hydrolysis products are then separated and identified by TLC followed by staining. Two solvent systems are provided for comparison.

Reagents and materials

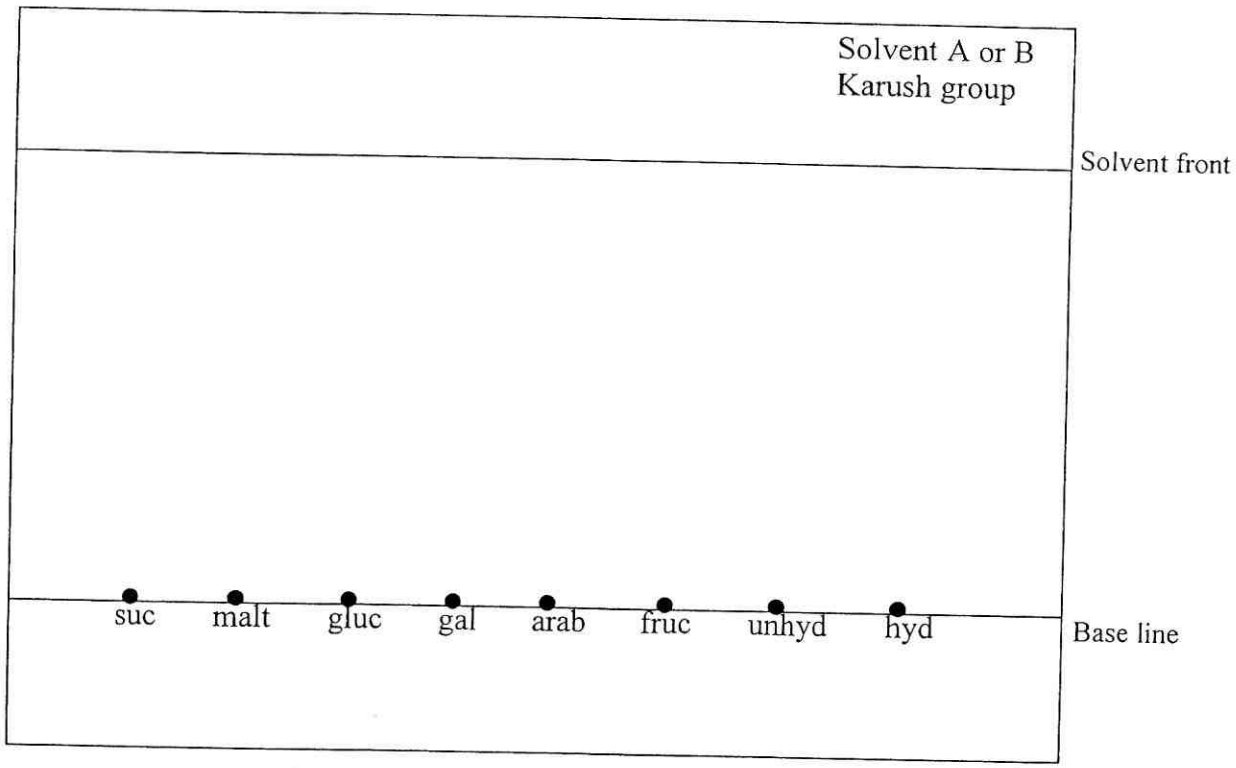
Unhydrolysed and hydrolysed extracts of coffee bean
TLC plates (25cm X 25cm) coated with silica gel G in 0.02M-borate buffer, pH 8.0
TLC tanks with lids
Standard solutions of sucrose, maltose, glucose, glactose, arabinose, fructose (1% in water).
Solvent A: Isopropanol: ammonia: water (9:1:2)
Solvent B: Butanol: acetic acid: water (4:1:5)
Aniline phthalate reagent (for reducing sugars)
Anisaldenyde reagent (for carbohydrates)

Procedure

1. Apply samples of the standard sugar solutions to each of the TLC plates using the capillary tubes provided. The spots should be about 2cm apart and applied in a straight line about 2cm from the bottom of the plate. Also apply samples of the unhydrolysed and hydrolysed extracts to each plate.
2. When the spots are dry, mark the plate with your name and sugar samples applied and the solvent systems to be used.
3. Place the plate in the appropriate solvent systems, cover the tank and develop the chromatogram for about $1^1/_2$ h, or until the solvent front has risen a suitable distance.
4. Remove the plate, mark the solvent front and allow to dry.
5. Spray one plate with aniline phthalate reagent and heat at 100° for about 10 min. Mark on the plate those spots, which appear and calculate the corresponding Rf values.
6. Then spray the same plate with anisaldehyde reagent, heat at 100° for about 10 min and note the new spots corresponding to non-reducing sugars.

7. Spray the second plate with anisaldehyde reagent and heat at 100° for about 10 min. calculate the Rf values for the visualized components.

Separation of sugars by TLC



Discussion

1. Comment on your results. Account for any differences in the sugar components of the hydrolysed and unhydrolysed extracts.
2. Does TLC enable you to identify conclusively any of the sugar components?
3. How would you improve the separation?

Questions

PRACTICAL 8

GEL FILTRATION

Theory

In the following experiment you will study gel filtration, a technique based only on molecular size. Sephadex consists of linear polysaccharide chains of alpha-glucose residues joined by chemical cross-links. It takes the form of very small spherical bead, which absorb a characteristic amount of water when placed in aqueous solution. The degree of swelling depends on the extent of the cross-linking. The swollen beads contain holes or pores of which are smaller than the pores can penetrate the swollen beads but larger molecules are excluded. The Sephadex G-100 used here excludes molecules of mol. wt. greater than about 100,000.

The swollen Sephadex is normally packed into vertical columns for chromatography. After application of the sample the column is eluted with buffer at a suitable flow rate and fractions of the eluate of a convenient volume are collected. Normally the fractions are analyzed for protein, enzyme, ions etc. Molecules, which are, totally excluded form the Sephadex beads appear in the eluate at a characteristic point depending on the type of Sephadex and the size of the column; this is referred to as the **void volume, Vo**. Smaller molecules will be retarded to a greater or lesser extend depending on their size; they have a characteristic **elution volume, Ve**. The elution volume of a very small molecule, such as an inorganic ion, marks the **internal volume, Vi**, of the column.

Gel filtration is versatile technique with several uses. The major applications are in protein purification, determination of mol. wt. of proteins, removal of a salt from a protein solution (desalting) and buffer exchange.

In this experiment you will study the ability of a highly porous Dextran gel, Sephadex G-100, to fractionate a mixture of proteins on the basis of molecular size. Such a procedure is widely applied in the purification of enzymes and in the determination of their molecular weights. The latter application is simpler than most other procedures for estimation of molecular weights of enzymes, since the technique is applicable to an unpurified preparation of the enzyme. Purification by gel filtration is usually a mild technique resulting in little loss of enzyme activity.

For determination of molecular weights. A Sephadex gel is chosen which is known to have an exclusion limit greater than the mol. wt. of the enzyme concerned. A column of the swollen gel is prepared in a suitable buffer and sample containing proteins of known mol. wt. is applied to the gel. The sample also normally contains markers for the void volume, **Vo** (Blue Dextran 2000) and for the internal volume, Vi (sucrose or inorganic ion) of the column. Elution is performed with buffer at a suitable flow rate and fractions of appropriate volume are collected and analyzed for absorbance, protein or enzyme activity. The peak of enzyme activity corresponds to the elution volume, Ve, of that enzyme. A graph of log mol. wt. against elution volume should be linear within the effective fractionation range of the gel used. Alternatively, Kav may be plotted against log mol. wt., where **Kav =(Ve – Vo)**.

(Vt – Vo) and **Vt** is the total volume of gel bed. In this way the column of Sephadex gel is **calibrated** and the molecular weight of an uncharacterized enzyme can be determined by referring its elution volume on the same column to the calibration curve. Alternatively, and less accurately, the mol. wt. may be calculated from an equation relating Veto **Vo**. For Sephadex G-100 is: log molecular weight = 5.941 (Ve/Vo).

Reagents and materials

0.01M Na phosphate buffer, pH 7.0 containing 0.1M NaCl
Sephadex G-100, equilibrated in the above buffer
Alkaline phosphatase solution (1 mg/ml in buffer)
Blue Dextran 2000 solution (5 mg/ml in buffer
Cytochrome c solution (5 mg/ml in buffer)
0.2M glycine-NaOH buffer, pH 10, containing 0.1mM $MgCl_2$
5mM 4-nitrophenyl phosphate (4NPP) in water
0.1M NaOH
Chromatography tube with rubber tubing and clamp

Procedure

1. Prepare a column of Sephadex G-100 of about 10 cm bed height. Allow the column to settle by passing one bed volume of buffer through it. Measure the diameter of the column and its height, and calculate the total volume (**Vt**) of the gel bed
2. Prepare a sample mixture containing **Blue Dextran** (0.1 ml) and **Cytochrome c** (0.1 ml). Apply the sample to the top of the column and elute it at a suitable flow rate (about one drop per second) with buffer.
3. When all the Cytochrome c has emerged, stop the elution. Measure the volume of any one fraction. NB:There is a clear separation between Blue Dextran and Cytochrome c during elution, Blue Dextran elutes first.
4. Determine the elution profile of the Blue Dextran by measuring the absorbance of each fraction at 600 nm. As a blank use a fraction eluted before the Blue Dextran. Determine Ve for Blue Dextran – this represents the void volume, Vo, of the column.
5. Determine Ve for Cytochrome c similarly but estimate it by its absorbance at 410nm. Calculate Kav for cytochrome c and compare its known mol. Wt. (12,400) with that derived from the Pharmacia chart (Kav against log molecular weight) and from the equation given in the introduction (consult the instructor).
6. Apply a sample (0.2 ml) of alkaline phosphatase to the column, eluting and collecting fractions as before. Assay enzyme activity in the fractions by incubating a sample (0.1 ml) with glycine buffer (0.2 ml) and 4NPP (0.3 ml) at 37°C for 15 min. Stop the reaction with 0.1M NaOH (2.5 ml) and measure the absorbance at 420 nm.
7. Determine Ve for alkaline phosphatase and its Kav value. Estimate the mol. wt. of the enzyme by reference to the Pharmacia chart and by calculation using the equation given in the Introduction. **Do not discard the Sephadex gel.**

Discussion

How closely did your derived values for the mol. wt. of cytochrome c agree with its known mol. wt.? Could you use your column to separate alkaline phosphatase from cytochrome c? How might you improve the determination of the mol. wt. of alkaline phosphatase by gel filtration?

Questions

1. Which type of Sephadex might you use for determining the mol. wt. of each of the following proteins: insulin ($6x10^3$), haemoglobin (68x10), urease (4 x 105) and glutamate dehydrogenase ($1.2x10^6$)?

2. Why is 0.1M NaCl included in the eluting buffer?
3. Would you expect the gel filtration behaviour of a fibrous protein like fibrinogen to resemble that of a typical globular protein?
4. Some enzymes, particularly α– glucosidase and dextranase, bind to Sephadex gel and are not eluted. Why might this be so? How could you determine the mol. wt. of α – glucosidase by gel filtration?

PRACTICAL 9

ION –EXCHANGE CHROMATOGRAPHY OF PROTEINS

Theory

Proteins in solution bear an overall charge contributed by the total of the acidic and basic groups of the appropriate amino acids on the surface of the protein. Each protein has a characteristic **isoelectric point, pI,** at which pH it is electrically neutral. At pH values above the pI the protein has an overall negative charge, below the pI an overall positive charge.

DEAE – cellulose is an anion exchange material. It consists of an insoluble but hydrophilic cellulose matrix bearing diethylaminoethyl $(C_2H_5)_2\ NC_2H_5^-$ groups on its surface. Over the pH range 2 – 9 these groups can accept a proton and become positively charged. The fixed positive albumin is negatively charged and binds to DEAE - cellulose; globulin is positively charged and remains in solution 1. The albumin is released from the ion-exchanger with salt solution or by changing the pH. CM – cellulose is a certain exchange material, which bears carboxymethyl ($-CH_2COOH$) groups.

When purifying a protein by ion-exchange chromatography it is necessary to choose certain important operating variables, including: operating temperature, type of separation (column of batchwise), dimensions of column, pH and composition of elution buffers, and elution flow rate. For all experiments it is useful to know the capacity of the chosen exchanger and the likely time course of absorption and desorption.

Where high resolution is the major factor, ion-exchange chromatography should be with a linear salt or pH gradient. One problem associated with the packing of columns is that "classification" of the ion-exchange particles may occur. This problem may be largely overcome by the use of thick slurry to pack the column. The flow rate used for elution should be less than that used in column packing. The flow rate is directly proportional to the pressure drop across the column and its cross-sectional area, and inversely proportional to its length.

Samples should be equilibrated with the starting buffer and excess salts should be removed by prior dialysis. **Application of the sample should be such as not disturb the top layer of ion-exchanger.** Elution may be carried out in one of three ways:

(a) Using the starting buffer if the component interest is not adsorbed at that pH;
(b) Using gradient elution in which the pH or salt concentration is varied linearly over a suitable range;
(c) Using stepwise elution, so as to produce a sharp change in eluting power, which moves down the column as a front. Gradient elution has a higher resolving power than stepwise elution and is less likely to cause denaturation of labile proteins.

In this experiment you will perform ion-exchange column chromatography of the proteins of egg white using CM-cellulose and eluting with a stepwise increase in salt concentration.

Reagents and materials

CM-cellulose (Whatman CM 11) in 0.01M
Sodium phosphate buffer, pH 6.0
Egg white solution (10% in 0.01M phosphate buffer, pH 6.0)

NaCl solutions (0.05M, 0.1M, 0.2M, 1.0M in 0.01M phosphate buffer pH 6.0)
Standard ovalbumin solution (10 mg/ml)
Biuret reagent
Column fitted with glass wool plug, rubber tubing and clamp
Filter paper circle (same diameter as column)
0.01M sodium phosphate buffer, pH 6.0

Procedure

1. Stir the suspension of CM- cellulose well and pour the slurry into the column. Allow the excess liquid to drain off. Add more slurry until the height of the packed bed is about 8 cm. **Do not allow the ion-exchanger to become dry at any time.**
2. Add egg white solution (10 ml) to the column. Adjust the flow rate to about 1 ml/min (**about one drop every 3 sec.**) and collect the effluent.
3. Wash off any unabsorbed protein with an aliquot (10 ml) of starting buffer. Collect the effluent and combine it with the previous effluent.
4. Elute the bound protein by adding successive 10 ml portions of buffered0.05M, 0.1M, 0.2M and 1.0M. NaCl, collecting the effluent at each separate salt concentration. Measure the volume of each fraction.
5. Dilute a sample of the egg white solution ten times. Determine the protein concentration of the diluted egg white solution, the ova albumin standard and each fraction by the Biuret assay.
6. Calculate the amount of protein (**mg**) recovered in each fraction and the overall yield of protein.

Discussion

Draw a histogram showing the amount of protein recovered at each salt concentration. Account for your chromatographic separation and the yield of protein.

Questions

1. How else might you have eluted the bound proteins?
2. Draw the likely pattern of protein bands if you subjected the fractions to electrophoresis at pH6.0. What precaution would you take before electrophoresing the fractions containing bound proteins?
3. How might you improve the resolution of the protein components of egg white by cation-exchange chromatography.
4. Which components of the NaCl solution actually elutes the bound protein?

PRACTICAL 10

ELECTROPHORESIS OF ENZYMES IN POLYACRYLAMIDE GEL

Theory

You are familiar with the separation of proteins by ion-exchange chromatography. All types of electrophoresis are governed by the single set of general principles i.e. charged particles in solution migrate to the electrode of opposite charge when an electric field is applied. The mobility of a molecule is determined by following factors.

$$\text{Mobility of a molecule} = \frac{\text{Applied voltage (x) X Net charge on the molecule (g)}}{\text{Friction of the molecule (f)}}$$

In this experiment you will separate the various isoenzymes of lactic acid dehydrogenase by electrophoresis in a gel medium.

Agar is a natural product extracted from seaweed. It is an anionic polysaccharide containing galactose and galactose sulphate residues. The sugar residues are joined by β-glycosidic linkages. A neutral fraction containing no sulphate can be isolated from agar. This friction is agarose and it is also used as a gel medium for electrophoresis. When dissolved in hot aqueous solution both agar and agarose form a three-dimensional lattice of polysaccharide chains, which, on cooling, sets to form a suitable gel.

Polyacrylamide is made by the polymerization of acrylamide (CH_2=CH-C-NH_2) into long chains in the presence of an appropriate catalyst. These chains can be "crosslinked" by including methylene-**bis** (Acrylamide) CH_2 =CH-C-NH-CH_2-NHC-H. To the initial acrylamide solution (before polymerization). If sufficient methylene-bis (acrylamide) is present, all of the polymer chains in a solution will be connected and be part of a single, covalently bonded molecule. When the chains become linked into one molecule, most independent movement of the chains is lost, and the solution becomes a gel. The carboxamide groups of the acrylamide residues are chemically stable, have a high affinity for water and yet are non-ionic. The gel is very stable and an ideal support for electrophoresis.

The properties of the isoenzymes of lactate dehydrogenase are well documented and most tissues have been shown to contain up to 5 electrophoretically distinct forms. The amount and distribution of these isoenzymes are characteristically of the tissue of origin and are a useful way of "finger printing the tissue". Electrophoresis of the LDH isoenzymes can be carried out on a variety of supporting media but the polyacrylamide gel has the advantage of giving separation of the various forms in a relatively short period of time.

Materials and methods

1. Apparatus for polyacrylamide – gel electrophoresis
2. Power pack
3. Crude LDH preparation from Rat heart
4. Crude LDH preparation from Rat liver
5. Standard Heart LDH
6. Standard Muscle LDH

Reagents

1. .05 M Tris-glycine buffer (pH 8.3)
2. 100 ml Reagent A
   INHCI 48 ml
   Tris 36.6gm
   TEMED 0.23ml (N,N,N`,N` Tetramethyelthylenediamine)
   H_2O to 100 ml (pH 8.9)
3. 100 ml Reagent B:
   INHCI 48 ml
   Tris 5.98 gm
   TEMED 0.46 ml
   H_2O to 100 ml (pH 6.7)
4. 100 ml Reagent C;
   Acrylamide 28.0 gm
   Bis 0.735 gm (N,N` - methylene bis acrylamide)
   Water to – 100 ml
5. 100 ml Reagent D
   Acrylamide 10.0 gm
   Bis 2.5 gm
   H_2O to 100 ml
6. 100 ml Reagent E
   Riboflavin 4.0 mg
   H_2O to 100 ml
7. 200 ml Reagent F
   40% sucrose solution

Working solutions

These solutions may be prepared the day they are used.

1. Small-pore solution 1
   1 part reagent A
   2 parts reagent C
   1 part H_2O
2. Small-pore solution 2
   Ammonium persulfate (Analar) 0.14%
3. Large-pore solution
   1 part reagent B
   2parts reagent D
   1 part reagent E
   4 parts reagent F
4. Staining medium
   1 ml D-L-sodium lactate 0.5M, pH 8.3
   3 mg NAD^+
   8 mg NBT (Nitroblue tetrazolium dye)
   1.4 mg PMS (Phenazine methosulphate)
   4.3 ml Tris-HCI buffer O.2M, pH 8.3

Procedure

1. Prepare the small-pore gel solution by mixing small-pore solution 1 and 2 in the ratio of 1:1.
2. Place the gel tubes in their stands and add the small-pore gel solution to about 80% volume of the tube. Also add a few drops of water on top of the gel solution without disturbing the gel layer. Let the tubes stand for about 30 minutes for polymerization. Decant the water and blot the tubes.
3. Prepare the solution of spacer-gel or larger-pore gel in the given ratio and add about 0.15 ml to the top of each separation gel (already polymerized). Similarly add about 1-2 mm water layer and polymerize for 20 min, under the fluorescent tube.
4. After polymerization of the spacer gel, decant the water from the top of the tube and put the gel tubes in the grooves of the upper chamber without damaging the gel tubes.
5. Add bout 200 ml of tris-glycine buffer to the lower chamber and place the upper chamber over it. Carefully remove any air bubbles from the bottom of the gel tubes.
6. Add 0.1 ml of one of the LDH samples to 0.1 ml of the 40% sucrose solution. Place this solution in the gel-tubes and then fill the tubes with tris-glycine buffer (mixed with 0.001% bromphenol blue in water as the tracking dye) up to the brim of the tubes. Fill the upper chamber with tris-glycine buffer so that the electrode dips into the buffer.
7. Make the electrical connections. The cathode (negative) must be connected to the upper chamber. First use 2mA current per tube until the dye enters the spacer-gel. Then increase the current to 5mA per tube. Run it until the dye moves about 3 cm distance (about 1-1.5 hours)
8. Take the gel from the tubes by injecting water into the tubes with a syringe. Wash the gels with cold tris-HCI buffer (0.1ml, pH 8.3) and then place them in the staining medium for at least 30 min. at 37°.

Discussion

Make an accurate diagram of your stained gel. What conclusions can you make concerning the LDH isoenzymes of muscle, Liver and heart?

After every 10 min. withdraw 0.5 ml of the sample form each of these flasks and determine the amount of NO_2 produced as follows: add 0.5 of water to each of these tubes and then 1 ml of 1% sulfanilamide solution. After mixing them thoroughly add 1 ml of 0.02% N-1 Naphthylethylene diamine dihydrochloride. Allow the colour to develop for a at least 20 min. The colour is stable for several hours. Record the absorbance at 540 nm on spectronic 20. Also prepare a standard curve for nitrite by using 0, 20, 40, 50, 80 and 100 nmoles of $NaNO_2$. Note that the final volume of the solution in the tubes or standard curve should be the same as that in the sample tubes.

Calculation

From the standard curve for NO_2^-, determine the amount of NO_2^- in each sample tube. Then calculate the amount of NO_2^- produced in each flask as follows:

$$\text{nmoles of } NO_2^- \text{ produced by 1.0 of the tissue} = \frac{X \times Y}{0.5 \times 1.0}$$

Where X = amount in nmoles of NO_2^- in 0.5 ml of the aliquot used to develop colour.
Y = total volume of the medium remaining in the flask

Plot the amount of nitrite produced per g. tissue against the time of incubation.

Questions

1. Is nitrite produced both by the leaf disks kept either in light or dark? If not, explain why nitrite does not accumulate in one of these treatments.
2. Why is it necessary to provide the seedlings with nitrate solution several hours before determining nitrate reductase activity? What results would you expect if the seedlings water grown on only distilled water?

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