METABOLISM-2

BIOC-312

Lab Manual

يقول مايكل هارت الذي اختار نبينا محمد عليه أفضل الصلاة و السلام كأعظم شخصية في التاريخ:

إن إختياري محمداً، ليكون الأول في أهم وأعظم رجال التاريخ، قد يدهش القراء، ولكنه الرجل الوحيد في التاريخ كله الذي نجح أعلى نجاح على المستويين: الديني والدنيوي.

فهناك رُسل وأنبياء وحكماء بدءوا رسالات عظيمة، ولكنهم ماتوا دون إتمامها، كالمسيح في المسيحية، أو شاركهم فيها غيرهم، أو سبقهم إليهم سواهم، كموسى في اليهودية، ولكن محمداً هو الوحيد الذي أتم رسالته الدينية، وتحددت أحكامها، وآمنت بها شعوب بأسرها في حياته. ولأنه أقام جانب الدين دولة جديدة، فإنه في هذا المجال الدنيوي أيضاً، وحّد القبائل في شعب، والشعوب في أمة، ووضع لها كل أسس حياتها، ورسم أمور دنياها، ووضعها في موضع الانطلاق إلى العالم أيضاً في حياته، فهو الذي بدأ الرسالة الدينية والدنيوية، وأتمها.....فليكن قدوتنا ولنكمل رسالته الدينية والدنيوية.

Contents

EXPIREMENT-1

Quantitative Determination of Protein by Biuret Reaction

Principle:

- **Biuret reaction**: is a method that can be used to determine the amount of soluble protein in a solution.

- It is used in the detection and estimation of proteins and peptides.

- The reaction is characterized by a blue-violet color upon the addition of cupper sulfate to any compound containing more than 2 peptide bonds.

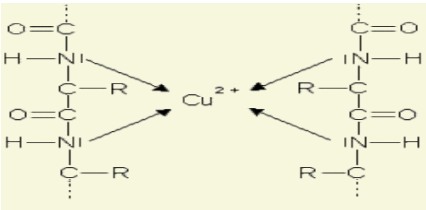
- Biuret reagent (dilute copper sulphate in strong alkali) reacts with all compounds that contain two or more peptide bonds.

- The name of the reaction is derived from the organic compound biuret.

- Dipeptides and free amino acid (except serine and threonine) do not give this reaction.

- The blue color produced is believed to be due to a co-ordination complex between the Cu^{++} and four nitrogen atoms, two from each of two adjacent peptide chains.

- The spectrophotometer can then be used to measure the intensity of the color produced. The more protein presents the darker the color.



Stability of the color:

The color formed is stable for few hours but will increase slowly with longer standing.

Concentration range for the assay -----→0.25 to 1mg/ml

Method:

- Prepare the tubes according to the following table:

Tube	B	1	2	3	4	UNK
Standard (BSA)	-	0.25ml	0.5ml	0.75ml	1ml	-
Unknown protein	-	-	-	-	-	1ml
H_2O	1ml	0.75ml	05ml	0.25ml	-	-
Biuret reagent	1.5ml	1.5ml	1.5ml	1.5ml	1.5ml	1.5ml

- Vortex all tubes and incubate at 37C˚ for 15 min.

- Read the absorbance at 540nm.

Comments:

- Protein level is usually measured in urine to evaluate renal diseases.

References:

-Plummer D.T., An introduction to Practical biochemistry,Tata McGraw - Hill Edition 1988.

Calculations

A- Plot absorbance versus protein concentration

B- Calculate the concentration of the unknown using the standard curve (conc. of std. = 2mg/ml).

C- Calculate the conc. of unk. using the equation.

D- Calculate the unknown concentration in mg% and in mg/250ml

Result Sheet

Tube	Conc.	Absorbance
B		
1		
2		
3		
4		
Unk1		
Unk2		

EXPIREMENT-2

Quantitative Determination of Protein by Dye-Binding method

Principle:

- **Bromocresol green (BCG):** is a dye used for the measuring of albumin concentration.
- Albumin binds to BCG selectively at $_{P}$H 4.2

The dye-binding method is known to be specific and sensitive for the determination of protein concentration (albumin) because:

1- The reagent has the ability to displace most substances which may initially be bound to the protein.

2- It is able to detect low concentration of protein about 50µg.

Dye + protein $\xrightarrow{\text{pH 4.2}}$ dye-protein complex

(green) (blue)

-There is a tendency for the protein-dye complex to precipitate at pH 4.2 which is very near the iso-ionic pH of albumin.

-There is some variability in the intensity of the color produced with albumins from different sources.

Method (1):

Tube	1	2	3	4	Blank
BSA	2ml	-	-	-	-
UnK_1	-	2ml	-	-	-
UnK_2	-	-	2ml	-	-
H_2O	-	-	-	-	2ml
Dye reagent	2ml	2ml	2ml	2ml	2ml

- Vortex and stand for 10 min
- Read at 630nm

Method (2):

- Prepare the tubes according to the following table:

Tube	1	2	3	4	5	6	Unk	B
BSA	0.1ml	0.3	0.5	0.7	0.9	1	-	
H_2O	0.9ml	0.7	0.5	0.3	0.1	-	-	1ml
UnK_1	-	-	-	-	-	-	1ml	
Dye reagent	2ml	2ml	2ml	2ml	2ml	2ml	2ml	2ml

- Vortex and stand for 10 min
- Read at 630nm

Comments:

- Albumin is measured to evaluate:

1- Liver disease.

2- Nutritional status.

3- Renal disease.

- In healthy renal and urinary tract system, the urine contains no protein or only traces amounts.
- 1/3 of normal urine protein is albumin.
- Albumin is usually the abundant protein in pathological conditions-----→ Albuminuria ~~ = Proteinuria
- Normal value (NV):
- Qualitative = -ve.
- Quantitative ---→ male = 1-14mg / dl

  ----→ Female = 3-10 mg/ dl

References:

Plummer D.T., An introduction to Practical biochemistry,Tata McGraw - Hill Edition 1988.

Calculations

Method (1):

-Calculate the concentration of the unknown proteins by using the equation

$$\frac{A_{unk}}{C_{unk}} = \frac{A_{st}}{C_{st}}$$

$$C_{unk} = A_{unk} / A_{st} \quad X\ C_{st}$$

-Calculate the concentration of the unknown proteins in:

(mg/ml),(µg/25ml),(µg/50ml),(g/0.5ml)-

Concentration of standard=2mg/ml

Method (2):

- Plot the standard curve to determine the concentration of unknown.

- Calculate the concentration of the unknown proteins in:

(mg/ml), (µg/25ml), (µg/50ml), (g/0.5ml)-

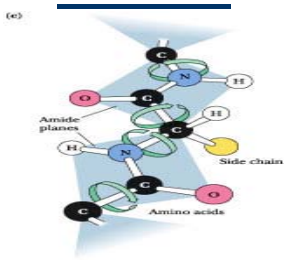
Concentration of standard=2mg/ml

Result Sheet

Tube	Conc.	Absorbance
1		
2		
3		
4		
5		
6		
Unk		
B		

EXPIREMENT-3

Quantitative Determination of Protein by Lowry Method



Principle:

- This is the classical and one of the most sensitive methods for measuring the concentration of proteins as little as 0.2 µg.

- A reagent for the detection of phenolic groups known as the Folin and Ciocalteu is used in the quantitation of protein by this method.

-The reaction involves 2 steps:

1- In the 1st step the reagent detects tyrosine residues due to their phenolic nature but the sensitivity is considerably improved by the incorporation of cupric ions. The product of this step is the copper-protein complex. This called a Biuret chromohpore.

2- In the 2nd step the copper-protein complex causes the reduction of the phosphotungstic and phosphomolybdic acids (the main constituents of Folin reagent) to tungsten blue and molybdenum blue. Approximately 75% of the reduction occurs by copper-protein complex and tyrosine and tryptophan are responsible for the remainder. The reduced Folin is blue and thus detectable with a spectrophotometer in the range of 500-750nm.

- Advantage:

1- Sensitive over a wide range.

2- Can be performed at room temp.

3- 10-20 times more sensitive than UV detection.

- Disadvantage:

1- Many substances interfere with the assay (K. Mg, EDTA, Tris buffer…).

2- Amount of color varies with different proteins. (WHY?)

3- The assay is photosensitive so illumination during the assay must be kept consistent foe all samples.

4- Takes a considerable amount of time to perform.

Note: Phenolic compounds present in the samples react also so this is must be take in consideration.

Reagents:

Reagent A: 2g NaOH , 10g Na_2CO_3, 0.1g Na-K tartarate per 500ml water.

Reagent B: 0.5g $CuSO_4$.5 H_2O/ 100ml water

Reagent C: Mix 10ml Solution A+ 0.2ml Solution B (prior to use).

Reagent D: Mix 1Part of phenol (2N) with1 part water (prior to use).

Method:

Tube	**1**	**2**	**B**
BSA	1ml	-	-
UnK	-	1ml	-
H_2O	-	-	1ml
Reagent C	8ml	8ml	8ml
Shake and stand 30 min			
Reagent D	1ml	1ml	1ml
Shake and stand for minimum 20 min but not longer than 2 hour **Read at 750nm**			

References:

-Plummer D.T., An introduction to Practical biochemistry,Tata McGraw - Hill Edition 1988.

Calculations

A-Calculate the concentration of the unknown proteins by using the equation

$$\frac{A_{unk}}{C_{unk}} = \frac{A_{st}}{C_{st}}$$

$C_{unk} = A_{unk} / A_{st} \times C_{st}$

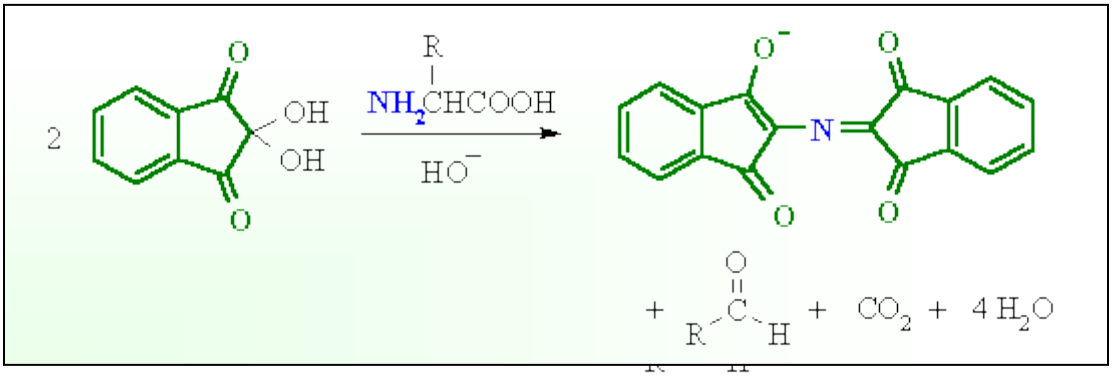
B- Calculate the concentration in mg/ml,g/l,mg/dl

Result Sheet

EXPIREMENT-4

Quantitative Estimation of Amino Acids by Ninhydrin

-The reaction between alpha-amino acid and ninhydrin involved in the development of colour is described by the following mechanism



-A ninhydrin test is a colour reaction given by amino acids and peptides on heating with the chemical ninhydrin.

-The technique is widely used for the detection and quantitation (measurement) of amino acids and peptides.

-Ninhydrin is a powerful oxidizing agent which reacts with all amino acids between pH 4-8 to produce a purple colored-compound.

-Ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple colour that is detected in this method.

-The reaction is also given by primary amines and ammonia but without the liberation of Co_2

-The amino acids proline and hydroxyproline also reacts but produce a yellow color.

- Note that since ninhydrin is a strong oxidizing agent, proper caution should be exercised in handling this compound. It is especially potent at the elevated temperature under which the reaction is carried out.

- The ninhydrin reagent will stain the skin blue and cannot be immediately

washed off completely if it comes in contact with the skin. However, as in any other stain on the skin, the colour will gradually rub off after about a day.

Method:

Prepare the tubes according to the following table:

Tube	**1**	**2**	**3**	**4**	**5**	**6unk**	**B**
Std. glycine	0.1ml	0.3	0.5	0.7	0.9	-	-
DW	0.9ml	0.7	0.5	0.3	0.1	-	1
Unk	-	-	-	-	-	1	-
Phosphate buffer	0.1ml	0.1	0.1	0.1	0.1	0.1	0.1
Ethanol-acetone	1.5ml	1.5	1.5	1.5	1.5	1.5	1.5
Ninhydrin	**2ml**	**2ml**	**2ml**	**2ml**	**2ml**	**2ml**	**2ml**

- Place the tubes in boiling water bath for 10min.
- Cool in ice
- Add water to each tube to raise the volume to 10ml
- Mix well by vortex
- Read the absorbance at 580 nm

References:

-Lee, Y.P.; Takahashi, T. *Anal. Biochem.* **1966**, *14*, pp 71-77.

-Robyt, J.F.; White, B.J. *Biochemical Techniques Theory and Practice*; Waveland: Prospect Heights,IL, 1990.

Calculations

- Plot the standard curve.
- Std. conc. = 100µg/ml
- Calculate the concentration of unknown. in µg/ml, mg/ml, mg%, g%.

Result Sheet

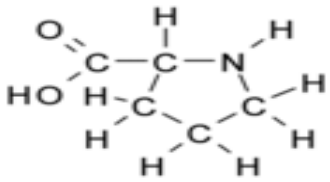
tube	Conc.	Absorbance
1		
2		
3		
4		
5		
UnK		
B		

EXPIREMENT-5

Quantitative Estimation of Proline by Ninhydrin

-The α-imino acids proline and hydroxyproline react with ninhydrin but not produce purple colour, instead, they form a yellow colour which can be measured by spectrophotometer.

-Proline and hydroxyproline also differ from other amino acids in that, they don't produce CO_2 when they react with ninhydrin.



Method:

Prepare the tubes according to the following table:

Tube	**1**	**2**	**3**	**4**	**5**	**6unk**	**B**
Std. proline	0.1ml	0.3	0.5	0.7	0.9	-	-
DW	0.9ml	0.7	0.5	0.3	0.1	-	1
Unk	-	-	-	-	-	1	-
Phosphate buffer	0.1ml	0.1	0.1	0.1	0.1	0.1	0.1
Ethanol-Acetone m	1.5ml	1.5	1.5	1.5	1.5	1.5	1.5
Ninhydrin	2ml	2ml	2ml	2ml	2ml	2ml	2ml

- Place the tubes in boiling water bath for 10min.
- Cool in ice
- Add water to each tube to raise the volume to 10ml
- Mix well by vortex
- Read the absorbance at 440 nm

References:

-Lee, Y.P.; Takahashi, T. *Anal. Biochem.* **1966**, *14*, pp 71-77.

-Robyt, J.F.; White, B.J. *Biochemical Techniques Theory and Practice*; Waveland: Prospect Heights,IL, 1990.

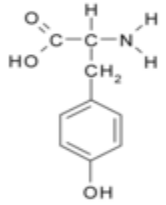
Calculations

- Plot the standard curve.
- Std. conc. = 100µg/ml
- Calculate the concentration of unknown. in µg/ml, mg/ml, mg%, g%.

Result Sheet

tube	Conc.	Absorbance
1		
2		
3		
4		
5		
UnK		
B		

EXPIREMENT-6
Quantitative Estimation of Tyrosine by Pauly`s Method



- Tyrosine is non-essential amino acid (AA) or more accurately it is semi-essential AA because it is synthesized from essential AA phenylalanine.
- It is formed from phenylalanine in a reaction catalyzed by phenylalanine hydroxylase enzyme.

Phenylalanine hydroxylase

Phenylalanine----------→Tyrosine

- The reaction is not reversible so tyrosine can not replace the nutritional requirement for phenylalanine.
- Phenylketonuria (PKU): is a genetic disease characterized by the absence of phenylalanine hydroxylase enzyme.
- Tyrosine can be estimated by using **Pauly`s method**
- ***Principle of Pauly`s reaction:***
- This reaction occurs in 2 steps:

1- **Diazotation:**

When primary aromatic amines are reacted with nitrous acid (HNO_2) which generated from $NaNo_3$ and HCl, a reaction occurs which makes a diazonium ion. The reaction takes place under freezing conditions.

Sulfanic acid + sodium nitrate+ hydrochloric acid------→ Diazonium compound

2- **Coupling:**

The diazoted Sulfanic acid then couples with phenol group in tyrosine (it reacts also with amine or imidazol) to form a red azo dye.

-The depth of the color can then be determined by spectrophotometer.

in cold

Diazoted Sulfanic acid + Tyrosine----------→ Azo dye (Red)

Warning: Azo dye is toxic and may cause genetic mutation.

Method:

Tube	blank	1	2	3	4	5	6 (unk)
Std tyrosine	-	0.5 ml	0.6	0.7	0.8	0.9	-
Unknown	-	-	-	-	-	-	1 ml
DW	1ml	0.5	0.4	0.3	0.2	0.1	-
Sulfanic acid	1ml	1	1	1	1	1	1
Sodium nitrate	1ml	1	1	1	1	1	1
***Wait 3 min.**							
***add $NaCo_3$**	2ml	2	2	2	2	2	2
***wait at room ter for 3 min.**							
Read abs at 540n							

- wait at room temp for 3 min.
- Read absorbance at 540.

References:

Holme, DJ and Peck H. Analytical Biochemistry. Addison Wesley Longman Limited 1998.

Calculations

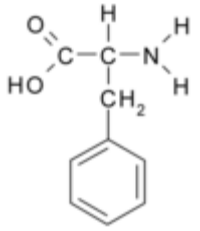
- Plot the standard curve.
- Calculate the conc. in each tube in mg/ml
- Standard conc. =0.1 g/l = 0.1 mg/ml
- Calculate the conc. of Unk in mg/ml , mg% , g%, µg/ml

Result Sheet

tube	Conc.	Absorbance
B		
1		
2		
3		
4		
5		
Unk		

EXPIREMENT-7

Flurimetric Determination of Phenylalanine



- The flurimetric methods often offer improved specificity and sensitivity over colorimetric procedures and the quantitative assays for the aromatic amino acids.
- Phenylalanine reacts with ninhydrin in the presence of a dipeptide (usually L-glycyl-L-leucine or L-leucyl-L-alanine)to form a fluorescent product.
- The fluorescent is enhanced and stabilized by the addition of an alkaline copper reagent to adjust the pH to 5.8 and the resulting fluorescence is measured at 515 nm after excitation at 365nm.

Reagents:

1-Copper reagent:

-Sodium carbonate (1.6g/L)

-Sodium potassium tartrate (65g/L)

-Copper sulphate (60mg/L)

2-Buffer:

Sodium succinate 0.3M (pH=5.8)

3-Dipeptide reagent:

L-glycyl-L-leucine **OR** L-leucyl-L-alanine (5mM)

4-Ninhydrin reagent:

5- Standard

Phenylalanine (1mM)

Method:

1- Mix

- 20 µl sample (standard or unknown).
- 20 µl succinate buffer.
- 80 µl ninhydrin reagent.
- 40 µl dipeptide reagent.

2- Heat at 60^0C for 2 hours and then cool to room temperature.

3- Add 2ml copper reagent.

4- Measure the fluorescence at 515nm after excitation at 365nm.

References:

Holme, DJ and Peck H. Analytical Biochemistry. Addison Wesley Longman Limited 1998.

Calculations

-Use the resulting fluorescence to calculate the concentration of unknown.

EXPIREMENT-8

Quantitative Determination of Ammonia Level

Ammonia is the end product of protein metabolism.

Sources of Ammonia:

1- From Amino Acids

- Many tissues, particularly liver, form ammonia by aminotransferase and glutamate dehydrogenase.

A- Aminotransferases---→ acts by transferring the amino group from AA to α- keto acid.

1- Alanine aminotransferase (ALT) = (SGPT)

Alanine ←----------→ Pyruvate

α ketoglutarate glutamate

2- Aspartate aminotransferase (AST) = (SGOT)

Aspartate ←----------→ Oxaloacetate

α ketoglutarate glutamate

B- Glutamate Dehydrogenase (Oxidative deamination)

NH_3^+

Glutamate ←------ ----------→ α ketoglutarate

- This reaction results in liberation of free ammonia.
- Therefore it is the pathway whereby the amino group of most AA can be released as free ammonia.

2- From Glutamine

The kidneys form ammonia from glutamine by the action of renal glutaminase.

H_2O glutaminase NH_4^+

Glutamine glutamate

-Most of ammonia is excreted in the urine in the form of $NH4^+$ which is important mechanism for maintaining of acid-base balance.

3- From bacterial action in the intestine on protein

- Bacterial action on intestinal protein------→leads to liberation of ammonia.

4- From amines

- Amines obtained from diet.

- Monoamines that serve as hormones and neurotransmitters by the action of amine oxidase.

5- From purines and pyrimidines

- Amino groups attached to the rings are released as ammonia.

Removal of Ammonia:

Removal of ammonia is an essential process because high ammonia level affects acid-base balance and brain function.

The ways responsible to remove ammonia:

1- Urea cycle
2- Glutamine Formation----→ it is the major way for removal of ammonia mainly in the brain.

Why glutamine is found in plasma at higher conc. than other AAs?

Because of its transport and storage function.

Ammonia Blood level:

Although ammonia is constantly produced in tissues, it is present at very low levels in blood because of:

1- Rapid removal of ammonia from the blood by the liver.
2- Many tissues, mainly muscle, release AA nitrogen in the form of glutamine or Alanine rather than as free ammonia.

Ammonia toxicity:

Ammonia is neurotoxic since it can pass through blood brain barrier and in sever cases leads to brain damage.

Estimation of Ammonia:

Principle:

Oxidation
Phenol + sodium hypochlorite ----------→blue complex

Ammonia + sodium nitroprusside

- In the presence of ammonia and of sodium nitroprusside acting as catalyst, phenol is oxidized by Na-hypochlorite to form a blue dyestuff.
- The conc. of blue dye is directly proportional to that of ammonia and can be measured photomerically.
- TCA is added to blood to ppt protein.
- It is very difficult to determine the conc. of ammonia in blood because it presents at very low conc.
- The samples must be collected fresh to avoid false results due to the formation of secondary ammonia by deamination of AAs.

Method:

Prepare the tubes as you see in this table

Tube	**1**	**2**	**3**	**4**	**5**	**B**	**Unk**
Std. ammonia	0.1ml	0.2	0.3	0.4	0.5	-	0.5
DW	0.4	0.3	0.2	0.1	-	0.5	-
Phenol	5	5	5	5	5	5	5
Hypochlorite	5	5	5	5	5	5	5

- Mix by vortex
- Store for 5-10 min at 37^0 C
- Read the absorbance at 623 nm.

References:

Champe PC , Harvey RA and Ferrir, DR Lippincott's Illustrated Reviews Biochemistry.Lippincott Williams &Wikins.2005

Calculations

- Calculate the conc. in each tube.
- Std. conc. = 20 mg %
- Draw the standard curve to determine the conc. of unk. In mg%, mg/50ml

Comments:

-Normal value (NV) ---→ adults --→15-56 µg / 100ml

-Normal value (NV) ---→ birth-10 days --------→109-182 µg / 100ml

- Ammonia level is increased in cases of liver and renal diseases and in certain cases of inborn error of metabolism
- Ammonia level may vary with protein intake.
- Exercise may increase ammonia level.
- Haemolysed blood gives falsely elevated levels.

Result Sheet

tube	Conc.	Absorbance
B		
1		
2		
3		
4		
5		
Unk		

EXPIREMENT-9

Quantitative Estimation of Uric Acid

Uric acid (UA) is an end product of purine (A & G) metabolism.

- It is transported by the plasma from the liver to the kidney, where it is filtered and where about 70% is excreted. The remainder is excreted in GIT and degraded.
- UA is not very soluble in aqueous medium, so there are clinical conditions in which elevated levels of UA results in deposition of Na^+ urate crystals primary in joints.
- The basis for this test is that an overproduction of UA occurs when :

1- There is excessive destruction of cells (as in leukaemia).

2- Inability to excrete the substance produced (renal failure).

3- There is excessive cell breakdown and catabolism of nucleic acids (as in gout).

Estimation of UA in solution

Principle:

This test depends on the reduction of Mn^{+7} (pink) to Mn^{+4} (brown) by uric acid and further reduction from Mn^{+4} to Mn^{+2} (colourless) by sulphuric acid.

$$N_4C_5O_3H_4 + H_2SO_4 + KMnO_4 \rightarrow K_2SO_4 + MnSO_4 + \text{oxalate} + NH_3 + CO_2$$

- $KMnO_4$ is a strong oxidizing-self indicator reagent.
- In the presence of UA and acidic medium, with mild heating (to prevent reversible reactions by the liberation of $CO_{2)}$, the $KMnO_4$ is colourless, but

when all the UA is oxidized, the excess of $KMnO_4$ gives its pink colour, indicating the end point.

- The colour persists only 30s since the oxalic acid formed is also a reducing agent (very weak) and will reduce the pink $KMnO_4$ after some time (30s).

Method:

1- In a conical flask, place 10ml UA + 2ml con. H_2SO_4

2- Warm the solution to 60-70^0 C in water bath for 5 min.

3- Titrate using N/20 $KMnO_4$ until a faint pink colour is formed, which persists on shaking for 30s.

4- Repeat twice and take the average.

Calculations

- Calculate the percentage of uric acid in the sample

Calculations:

1ml N/20 $KMnO_4$ = 0.00375 g uric acid

Zml (volume) = Y g uric acid

$$Y = \frac{Zml \times 0.00375}{1} \times \frac{100}{\text{volume of uric acid}}$$

Expirement-10

Quantitative Determination of Creatinine

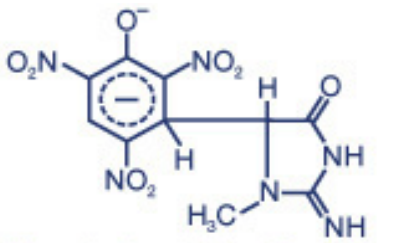
- Creatinine is a substance that, in health, excreted by the kidney.

- It is the by-product of muscle energy metabolism and is produced at a constant rate according to the muscle mass of the individual.

- Endogenous creatinine production is constant as long as the muscle mass remains constant. Because all creatinine is filtered by the kidneys in a given time interval is excreted in urine, creatinine levels are equivalent to the glomerular filtration rate (GFR).

Principle:

- Jaffé reaction is based on the observation that at an alkaline pH, creatinine reacts with picrate to form a red-orange adduct.



Picrate-Creatinine Complex

Method:

Tube #	1	2	3	4	B	UnK
Creatinine standard	1ml	2ml	3ml	4ml	-	2ml
DW	3ml	2ml	1ml	-	4ml	-
Alkaline Picrate	**2ml**	**2ml**	**2ml**	**2ml**	**2ml**	**2ml**

- Leave for 15min at room temp.

- Read the absorbance at 520nm.

References:

Calculations

A- Plot absorbance versus protein concentration

B- Calculate the concentration of the unknown using the standard curve (conc. of std. = 0.06mg/5ml).

C-Calculate the conc. of unk. using the equation.

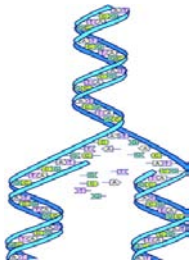
D-Calculate the unknown concentration in mg% and in mg/250ml

Result Sheet

Tube #	Conc.	Absorbance
1		
2		
3		
4		
B		
Unk		

EXPIREMENT-11

Chromatographic identification of Nucleotides



Chromatography: is a technique of separating a mixture of 2 or more different components based on distribution between 2 phases.

1- Stationary phase ----→ such as solid matrix in column.

2- Mobile phase----→liquid or gazes.

Chromatographic methods depend on:

Differential solubilities (partition) & adsorptives of substances to be separated with respect to the 2 phases.

- The rate of migration of the various substances is affected by their relative solubilities in the polar stationary phase and non-polar mobile phase.

- The distribution of a given solute between 2 phases depends on the partition coefficient (Kp) of the solute

$$Kp = \frac{\text{conc. in stationary phase}}{\text{conc. in mobile phase.}}$$

Therefore, the molecules are separated according to their polarities with non-polar molecules moving faster than polar ones.

- Thin-layer chromatography (TLC) is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction. It also permits the optimization of the solvent system for a given separation problem. In comparison with column chromatography, it only requires small quantities of the compound (~ng) and is much faster as well.

Stationary Phase:

- As stationary phase, a special finely ground matrix (silica gel, alumina, or similar material) is coated on a glass plate, a metal or a plastic film as a thin layer (~0.25 mm). In addition a binder like gypsum is mixed into the stationary phase to make it stick better to the slide. In many cases, a fluorescent powder is mixed into the stationary phase to simplify the visualization later on.

Preparing the Plate:

Do not touch the TLC plate on the side with the white surface. In order to obtain an imaginary start line, make two notches on each side of the TLC plate. You can also draw a thin line with pencil. **Do not use pen**. ***Why?*** The start line should be 0.5-1 cm from the bottom of the plate.

Solvent system:

n-Butanol: Water: conc. NH_4

10 : 1 : 0.5

Application:

The thin end of the spotter is placed in the dilute solution; the solution will rise up in the capillary (capillary forces).

Touch the plate briefly at the start line.

Allow the solvent to evaporate and spot at the same place again. This way you will get a concentrated and small spot. Try to avoid spotting too much material, because this will deteriorate the quality of the separation considerably (‘tailing’). The spots should be far enough away from the edges and from each other as well.

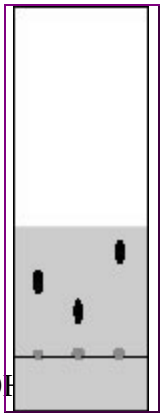
Developing a Plate:

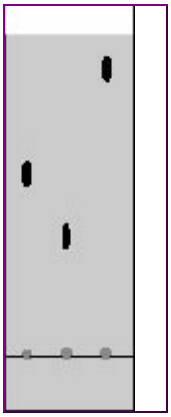
A TLC plate can be developed in a beaker or closed jar. Place a small amount of solvent (= mobile phase) in the container. The solvent level has to be below the starting line of the TLC, otherwise the spots will dissolve away. The lower edge of the plate is then dipped in a solvent. The solvent (eluent) travels up the matrix by capillarity, moving the

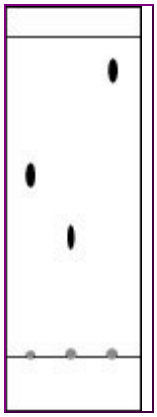
components of the samples at various rates because of their different degrees of interaction with the matrix (=stationary phase) and solubility in the developing solvent.

-Allow the solvent to travel up the plate until ~1 cm from the top. Take the plate out and mark the solvent front **immediately**. Do not allow the solvent to run over the edge of the plate. Next, let the solvent evaporate completely.

OR







TLC chamber for development e.g. beaker with a lid or a closed jar	after ~5 min	after ~10 min	after drying

Comment:

Non-polar solvents will force non-polar compounds to the top of the plate, because the compounds dissolve well and do not interact with the polar stationary phase.

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www.wikipedia.org

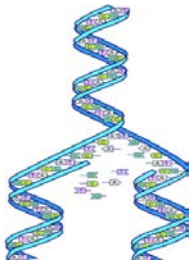
Result Sheet

- Calculate R_f and identify the unknown

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

EXPIREMENT-11

Chromatographic identification of Nucleotides



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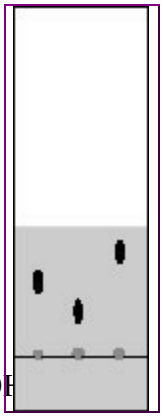

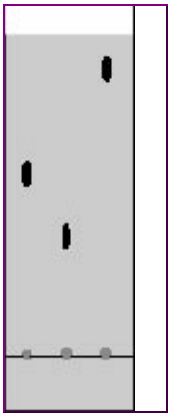

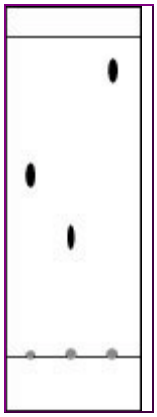

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Result Sheet

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$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

EXPIREMENT-12

Quantitative Determination of Phosphorus

Phosphorous is an essential mineral since it is required by every cell for normal function.

- The majority of phosphorous in the body is found as phosphate (Po_4).
- 85% of the body's P is found in bone and teeth in the form of Ca-P- salts and the remainder resides within the cells.

Phosphate Functions:

1- Required for generation of body tissue (why?)----→ involves in the protein synthesis that help all of us to reproduce.
2- Component of metalloprotein.
3- Function in metabolism of glucose and lipid.
4- Storage and transfer of E from onsite to another.
5- Helps stimulate glands to secret hormones.
6- Helps in the maintenance of acid-base balance.
7- A number of enzymes and hormones and cell signalling molecules depend on phosphoryaltion for their activation.

- Dietary P is absorbed in small intestine and any excess is excreted by kidneys.
- Regulation of blood calcium and P levels is regulated through the actions of parathyroid hormone (PTH) and vitamin D.

Principle:

Acids like Po_4 that contain more than one acidic (ionizable) proton (H^+) are called poly-protic or poly-basic acids.

The dissociation of these acids occurs in a stepwise fashion i.e. one H^+ lost at time.

H_3Po_4 is tribasic acid:

H_3Po_4----------→$H^+ + H_2Po_4^-$	$_pH$=4.4 (MO)
$H_2Po_4^-$---------→$H^+ + HPo_4^{--}$	$_pH$=9.2 (Ph.Ph.)
HPo_4^{--}---------→$H^+ + Po_4^{---}$	$_pH$=2.6 (no indicator)

Method:

1- Fill the biuret with 0.1 N NaOH

2- In a conical flask put 10ml phosphoric acid + 2gm Nacl + 3 drops of MO indicator.

3- In another conical flask put 10ml phosphoric acid + 2gm Nacl + 3 drops of Ph.Ph. indicator.

4- Titrate the 2 flasks with 0.1N NaOH until the end point (colorless in MO and faint pink in ph.ph).

Calculations

- Calculate the concentration of P in the sample and then determine its weight.

$$N \times V \text{ (acid)} = N \times V \text{ (base)}$$

$$N \times 10 \text{ ml} = 0.1 \times \text{volume of NaOH}$$

$$N_{(H3Po4)} = 0.1 \times V_1 \text{ or } V_2 \ / \ 10$$

$$\text{Normality} = \text{weight} / \text{Eq wt} \times 1000 / V_{ml}$$

$$\text{Weight} = N \times Vml \times \text{Eq wt} / 1000$$

$$\text{Eq wt} = \frac{\text{Mol. weight}}{\text{Valance}}$$

MO indicator---→ $\text{Eq wt} = \frac{\text{Mol.wt}}{1}$

Ph.Ph indicator---→ $\text{Eq wt} = \frac{\text{Mol.wt}}{2}$

Result Sheet