WRITE UP

SMALL GROUP DISCUSSION

Topic: Biochemical Laboratory Tests

I11.1 *Describe commonly used laboratory apparatus and equipments, good safe laboratory practice and waste disposal.* ***Lecture, Small group discussion***

Lab Hazards:

Biological Hazards

To operate a clinical laboratory safely, it is essential to prevent the exposure of laboratory workers to infectious agents (hepatitis B virus, HIV) by:

i. accidental puncture with needles
ii. spraying of these materials by syringe or spilling on benchtop or floor
iii. centrifuge accidents
iv. cuts or scratches from contaminated vessels

It is essential that specimens should be handled using universal precautions such as:

1. Handle all specimens with care by using barrier's protection (glove, gown, lab coats, face shield, eye protection)
2. Never mouth pipette or blow out pipettes that contain potentially infectious materials
3. Dispose all sharps appropriately.
4. Hepatitis B vaccination should be offered to all employees at risk of potential exposure (technician, phlebotomist, etc.)

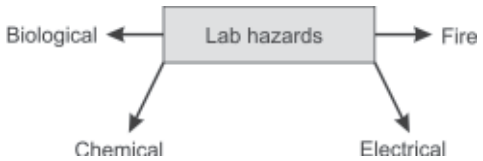

Fig. 2.1: Lab hazards

Nine classes of hazardous materials
(UN classification)

1. Explosives
2. Compressed gases
3. Flammable liquids
4. Flammable solids
5. Oxidizer materials
6. Toxic materials
7. Radioactive materials
8. Corrosive materials
9. Miscellaneous materials.

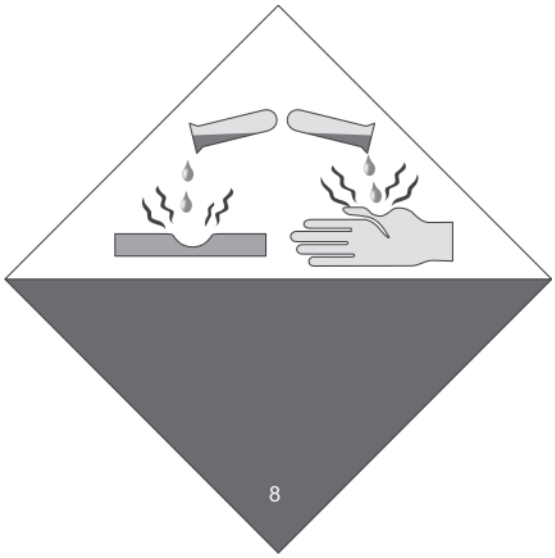

Fig. 2.2: Label for corrosive

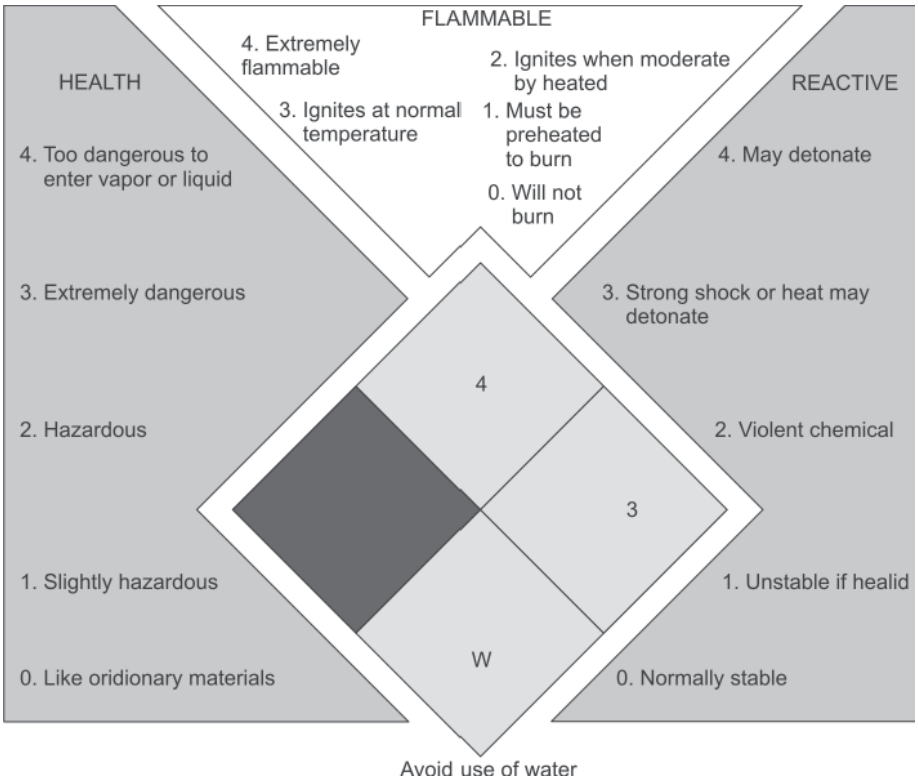

Fig. 2.3: Labeling identification system

Safety in the Laboratory

A clinical chemistry laboratory should have formal safety program.

*Aspects of Safe Operations of a Clinical Chemistry L*ab:

• Formal safety program

• Mandated plans:

Chemical hygiene plans

Blood-borne pathogen plans

• Identification of lab hazards:

Biological

Chemical

Electrical

Fire

• OSHA, CDC approved hazard

• Communication standards

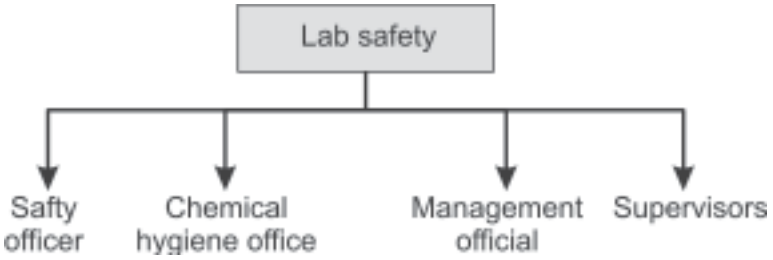

Fig. 3.1: Lab safety network material

Guidelines for Lab Safety

1. Each new employee should get general lab safety material, undergo orientation program.

2. Each employee should have knowledge of location of available evacuation routes, firefighting equipments and their operation.

3. Employee should undergo continuing education programs on lab safety.

Supervision of lab safety measures in terms of:

– proper labelling, handling and disposal of chemicals in terms of hazards

– fire extinguisher

– hoods

– earthing of electrical equipment

– proper handling and disposal of patient specimens.

5. Inspection: Periodic and scheduled safety inspections should be the routine.

6. Accreditation of lab with Health Care Organization.

7. Safe and scientific waste disposal and management.

BI11.2 Describe the preparation of buffers and estimation of pH. ***Lecture, Small group discussion***

Buffers

Buffers resist change in pH on addition of small quantities of acid or alkalis. Buffers are made up of a weak acid and a salt of a strong base. E.g. acetate buffer is mixture of acetic acid and sodium acetate. When a drop of strong acid is added to this buffer, H^+ ions combine with $CH_3 COO^-$ ions to form, a weak acid (CH_3 COOH) and thus their ions cause net increase $[H^+]$. On addition of a strong base, release OH^- ions react with acetic acid to form water and acetate ions resulting change in pH.

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Exercise –

Preparation of buffer

Buffers of different pH may be prepared by using Henderson Hasselbach equation.

$$pH = pka + \log \frac{[Salt]}{[acid]}$$

pka = - log Ka.

Preparation of 1l 0.1 M acetate buffer of pH 5.76 (given pka = 4.76)

Steps –

Prepare 1l solution of 0.1M acetic acid and 0.1M sodium acetate

pH = pka +(〖[CH〗_3 〖COO〗^-])/(〖[CH〗_3 COOH])

If we substitute pH as 5.76, pka as 4.76, ratio of slat and acid for desired pH is as under-

5.76 = 4.76 + log [Salt]/[acid]

5.76-4.76 = log [Salt]/[acid]

1 = log [Salt]/[acid]

[Salt]/[acid] = 10

[Salt] = [acid] = 10 = 1

So total value is 1l, take 909 ml of salt solution (0.1M) and 90.9 ml of acetic acid (0.1M) and mix to prepare buffer of pH 5.76.

Concept of acid-base reactions

The reaction between an acid and a base to form salt and water is called neutralization. A strong acid and strong base produce a neutral solution and a strong acid plus weak base gives an acidic solution and the reaction does not go to completion. A weak acid plus strong base gives a basic solution. A weak acid plus a weak base gives an acidic or basic or neutral solution depending on relative strength of acid and base.

Applications of buffers

Buffer systems are used to study enzyme activity and other living processes e.g. pH of human blood of 7-4. Phosphate buffer maintains pH in cell cytoplasm and is used in study of cell biology and microbiology.

BI11.3 Describe the chemical components of normal urine. ***Lecture, Small group discussion***

Characteristics of Normal Urine

Examination of urine is now mostly performed

1. to screen for unsuspected disease
2. to confirm or eliminate diagnosis
3. to assist in treatment.

Physical Appearance

Normal urine is pale, straw-yellow because of presence of pigment urochrome mainly and uroerythrin and urobilin in small amounts. Highly concentrated urine has dark amber color, and its causes are sweating, diarrhea, heart failure. Pale urine is observed when concentration of solid decreases and volume is large. It is seen after excessive fluid intake, alcohol, diuretics, chronic renal failure, diabetes insipidus (specific gravity low). Pale urine of high specific gravity occurs in diabetes mellitus.

Constituent	*Color*

Bile	greenish yellow
Blood	red, reddish brown, smoky
Hemoglobin	deep red
Melanin, alkapton, porphyria	darken on standing
Myoglobin	dark red
Rifampicin and phenadione	orange

Odor (Smell)

- Normal odor of urine is aromatic (ammonical). In infants, it is strong ammonical. Following infection with *E. coli* it has, fishy odor. Ketone bodies in urine give it a fruity odor. In conditions like maple syrup disease, urine has a characteristic smell.

Specific Gravity

In general, specific gravity is a reflection of state of hydration and functional ability of kidney. Normal specific gravity of urine is 1.015 to 1.025. Specific gravity of urine increases as concentrati on of main constituents increases (e.g. Na, Cl, urea, creatinine, sugar, protein).

Low fixed (< 1.010) is observed in diabetes insipidus (DI), (Chronic renal failure) CRF.

High (> 1030) occurs in DM, albuminuria, radiopaque dye.

pH

Normal pH of urine is 4.7 to 7.5

- pH of urine is alkaline after meals, vegetarian diet known as alkaline tide.
- After non-veg diet pH of urine is acidic.
- Alkaline pH also occurs when urine contains bacteria in large number.
- pH is a function of need to eliminate fixed acids and urinary pH reflects status of the pH of the blood.

Need to manipulate pH

- In case of acidic urine, prevention of calcium carbonate and magnesium–ammonium phosphate kidney stones. Alkaline urine is produced to prevent oxalate, uric acid stones. Alkalinization of urine is
- occasionally used to treat drug overdose (e.g. Salicylate) or hemolytic transfusion reaction and for optimization of dosage of antibiotics.

Volume

Volume of urine is influenced by fluid intake and diet. Normal urine volume is 1 to 1.5L/24 h. Polyuria occurs when urine volume is more than 1.5 L/24 hrs and is observed in DM, DI and late stage of CRF. The term oliguria is used when urine volume is less than 400 ml. Oliguria occurs in diarrhea, acute nephritis, early CRF, liver disease, congestive cardiac failure (CCF). When urine volume is less than 400 ml, it is termed as *anuria.* Anuria occurs in mercury poisoning, bilateral renal stone, shock.

Deposits

Urine is usually transparent and presence of phosphate and urate results in cloudiness of urine. Presence of phosphate gives rise to white/buff colored deposits which disappear on acidification of urine.

Whereas, presence of urate results in buff pink colored deposits which disappear on heating. Pathological deposits in urine are pus, blood, bacteria resulting turbidity or thick deposits.

Chemical constituents

Normally, 50 to 60 g of solid constituents are present in urine over a 24 hours period.

Organic substances are 30 to 35 g and are composed of urea, uric acid, creatinine mainly. Others include amino acids namely cysteine, leucine, tyrosine, drugs and their metabolites for instance bilirubin, cholesterol, indigo, xanthine.

*Inorganic constit*uents are 20 to 25g. They include Ca, PO , NH , SO , Cl

1. *Urea*: 25 to 30 g/24 hr. Normally urea appears in urine.

High protein diet, catabolic states result in increased urea excretion and excretion is reduced in impaired liver function, kidney disease.

2. *Creatinine*: Normally 0.5 to 1.0 g/24 hrs of creatinine is excreted in urine and rate of creatinine excretion depends on muscle mass, increased muscular activity, kidney disease, muscular dystrophy.

3. *Amino acids:* 150 to 200 mg

4. *Uric acid:* 0.5 to 1 g

   Excretion depends on diet

   Increased in gout, leukemia

5. *Chloride:* 10 to 15 g

Chief inorganic constituent

Decreased in fasting, increased perspiration

Increased in chronic nephritis, fever, burn, diarrhea, vomiting

6. *Phosphate:* 1 g

Depends on diet

Increased in acidosis, rickets, osteomalacia

Decreased in pregnancy, renal disease, hyperparathyroidism

7. *Sulphate:* 1 g

8. *Ammonia:* 0.5 to 0.7 g, formed by tubular cells from glutamine

Ammonia excretion decrease in kidney disease

Increase in diabetic ketoacidosis.

VIVA

1. Specific gravity of urine in:
(a) diabetes mellitus
(b) diabetes insipidus
2. Test for fructose
3. Name ketone bodies
4. Test for ketone bodies
5. Following ingestion of cherry and plums, which sugar appears in urine?
6. Site of ketone body production
7. In renal glycosuria, blood glucose levels are
8. Can ketone bodies appear in urine on fasting?

KEY

1. Raised, Low, may be fixed at 1.010
2. Seliwanoff ’s
3. Acetone, acetoacetate, 3 OH butyrate
4. Rothera’s
5. Pentose
6. Liver
7. Normal
8. Yes

BI11.4 Perform urine analysis to estimate and determine normal and abnormal constituents ***Lecture, Small group discussion***

BI11.20 Identify abnormal constituents in urine, interpret the findings and correlate these with pathological states

Examination of Urine for other Abnormal Constituents

Sugar

Sugar in urine is known as *Melituria.*

Various sugars may be found in urine but glucose is by far *most common* (lactose, pentose, fruct ose may be present). Excretion of glucose in urine is termed as *Glycosuria.*

Causes of glycosuria

1. *Renal glycosuria:* Proximal tubular defect—normal blood glucose concentration.
2. *Overflow glycosuria:* Diabetes mellitus—when glomerular filteration exceeds maximum tubular reabsorptive capacity.

Renal threshold for glucose is 180 mg% and glucose starts appearing in urine when the levels exceed this value. Physiological glycosuria can occur during first six months of pregnancy.

Other sugars in urine

Fructose: After ingestion of honey
Pentoses: After cherry, plums
Lactose: In lactating women can also appear in urine.
Other causes of appearance of sugar in urine:

- Rapid absorption
- IV dextrose
- Fanconi's syndrome
- Renal tubular defect

Ketones

Ketone bodies:

Acetoacetate (20%)

β-hydroxybutyrate (78%)

Acetone (2%),

are ketone bodies (KB) produced as an end product of fat breakdown.

Any condition in body which limits carbohydrate utilization with resultant increased fat utilization leads to ketone body production by liver.

Excessive fat metabolism will occur in

Starvation

DM

Ketonemia

Accumulation of KB in blood is ketonemia.

Ketonuria

Excretion of KB in urine is ketonuria

Proteins

Normal urine contains upto 150 mg/24 hrs of proteins (2/3 globulin, 1/3 albumin). Protein in urine at a greater output than > 0.1 g protein in 24 hours is considered abnormal and proteinuria > 0.5 g/d should be investigated fully.

Causes of proteinuria

1. *Renal*

Glomerular loss of proteins (common):

Toxin

Infection

Vascular disorder

Immunological reaction

(Nephrotic syndrome, glomerulonephritis)

Tubular loss of proteins:

Mild secondary to proximal tubular damage:

Heavy metal intoxication

Post-transplant

Acute tubular necrosis (ATN)

2. *Pre-renal*

Infections

Fever

Dehydration

Shock

3. *Postrenal*, due to obstruction to outflow of urine, lower urinary tract infection

4. *Asymptomatic proteinuria or orthostatic proteinuria (postural)*:

Individuals with this condition do not excrete protein into urine after they have been lying down (first morning specimen), but, after standing for 2 hours or more, will routinely display a small amount of proteinuria. This is because of pressure of lardotic spine on kidney.

Another form of asymptomatic proteinuria is *exercise proteinuria*.

Blood

Intact red blood cells may be present in urine.

Presence of RBCs in urine is hematuria, and presence of Hb in urine is *Hemoglobinuria.*

Causes of hematuria

Renal: GN, stone tumor, TB, trauma, acute tubular necrosis

Lower tract: Infection, stone, tumor, stricture.

Extra-renal: Blood dyscrasias, scurvy, drugs sulfa, salicylate cyclophosphamide, anticoagulants, fever,

SABE, appendicitis, diverticulitis, tumor colon, rectum.

Presence of Hb in Urine—Hemoglobinuria

Following intravascular hemolysis, Hb is picked up by hemopexin and recycled to liver. When the capacity to bind and remove Hb is exceeded by the rate of Hb release, Hb passes through glomerulus and appears in urine. Also, bleeding from lower urinary tract is a frequent cause of *Hemoglobinuria*.

RBC may pass through glomerulus in glomerular disease.

Causes of Hemoglobinuria

PNH

Unaccustomed exercise

G-6-PD deficiency

Autoimmune hemolytic anemia

Incompatible blood transfusion

Falciparum malaria

Drugs: Sulfas, quinine

Urobilinogen

Important constituent of normal urine.

Increased in hemolytic anemia

Urinary bile pigments (bilirubin and biliverdin) and bile salts may be excreted in urine in obstructive jaundice.

Bilirubin: Normally almost none of conjugated bilirubin enters circulation. With biliary tract obstruction, conjugated bilirubin reflux back into circulation and being water-soluble passes easily through glomerulus and appears in urine.

Myoglobin

Myoglobinuria occur following, crush injuries, heavy exercise, seizures, myopathies.

Microscope Examination (M/E)

M/E of sediment of urine:
Following centrifugation, urine shows following urinary deposits:

1. *Chemical substances*
    i. *Inorganic*

        Ca, PO salts

        Calcium oxalate crystals are envelope in shape, seen in primary hyperoxaluria

        Triple phosphate crystals: Prism- shaped crystals.

        Calcium carbonate crystals: Amorphous/dumb-bell crystals seen in infected urine

    ii. *Organic:* Uric acid crystals: Insoluble in acid and dissolve on alkalinization or warming to 60°C variable in shape: rhombic, plate, barrel-shaped.

        Cystine: Hexagonal plates appear in cystinuria

2. Cells

    RBC: 0 to 3 RBC/HPF in urine is abnormal. Seen in GN, stone, trauma, drug induced hematuria

    WBC: > 3 to 5/HPF point towards infection, easy to identify by cytoplasmic granules and nuclei

    Epithelial cells can be squamous, transitional and renal tubular cells and are of no medical significance

    Organisms: Provided contamination or overgrowth has not occurred, presence of bacteria correlates to urinary tract infection (UTI)

    Casts: Represent as collection of protein and cellular debris.

    Hyaline cast: Cylindrical, seen after exercise, proteinuria

RBC and WBC cast: RBC cast: in GN.

WBC cast in UTI.

Epitheloid cast: Have round central nucleus, seen in GN

Others: Fatty, waxy, granular casts.

Method for Glucose Estimation in Urine

Benedict's test

Take 5 cc of Benedict's reagent, add 8 drops urine to it.

Boil it for 2 minutes and see color of deposits

Benedict's reagent contains copper sulfate, sodium/ potassium citrate, sodium carbonate. It is copper reduction reaction.

Color		*Concentration*
Green	+	0.5 g%
Yellow	++	1 g%
Orange	+++	1.5 g%
Brick red	++++	2 g%

Other reducing substances in urine:

Urate

Creatinine

Salicylate

Glucuronates

Fructose

Galactose

Lactose

Pentose

Other tests for detecting glucose in urine:

Phenylhydrazine (osazone formation)

Chromatography

Dry chemistry strips

Dry Chemistry Strips

Diastix

It contains GOD-

POD enzyme, KI and blue background dye. I liberated on the final reaction blends with blue color to produce a sense of color changes 30 sec after wetting the strip with urine.

In a neonate or infant, presence of a reducing substance in urine should be further investigated to determine its nature.

Ketone Bodies (KB)

They are

Acetoacetate

β-hydroxy butyrate

Acetone

They are produced as end product of fat breakdown.

Excessive fat metabolism occurs in starvation, DM

Ketonemia: Accumulation of KB in blood.

Ketonuria: KB excretion in urine

Normally small amounts of ketone bodies are produced in liver.

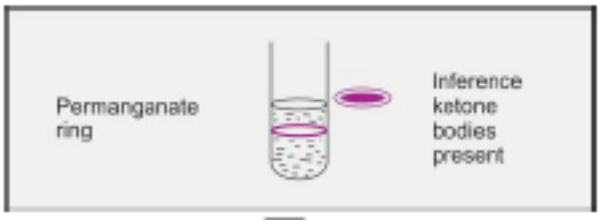


Fig. Rothera's test

Rothera's Test

Take 2 ml urine, saturate it with ammonium sulfate (till further addition does not dissolve in solution).

Add few drops/a pinch of sodium nitroprusside. Then, add liquid NH along the side of the tubes. A permanganate ring is formed inferring that ketone bodies are present.

DISCUSSION

Most important consequence of increased metabolism of fat in diabetes is accumulation of KB in blood. Unless adequate intracellular glucose is available, FFA released from adipose tissue are oxi dized to acetyl CoA except brain. Much of acetyl CoA is condensed to acetate which in turn gets reduced to 3-OH butyrate.

Liver is main site of ketogenesis. When KB begin to accumulate in body they also appear in the urine. Although diabetics are able to metabolize ketones normally, their increased production may exceed the capacity for catabolism.

Ketosis following starvation or vomiting occurs in the same way as ketosis of diabetes and small amounts of ketone may occasionally be detectable in the early morning urine specimens of normal persons.

Rothera's test:

Positive for acetoacetate and acetone, but, not with 3-OH butyric acid in routine. First oxidize it to acetoacetate by adding H_2O_2, then add acetic acid and boil to remove acetone. Then perform Rothera's test or ketostix for detection available as stick or tablet.

Ketostix: Plastic strips impregnated with sodium nitroprusside and glycine. Lavender or purple color develops if KB are positive.

Other Tests

1. Fluorometric, enzymatic assay
2. 3-OH butyrate dehydrogenase test
3. Spectrophotometry

Urobilinogen

After bile is excreted into intestine, conjugated bilirubin is reduced by (β-glucuronidase) bacterial action to mesobilirubin, stercobilinogen and urobilinogen. Urobilinogen and stercobili nogen are colorless and get oxidized to colored pigment stercobilin and urobilin.

The reduced products of bilirubin are absorbed into portal circulation to be re excreted by liver:

Enterohepatic circulation (EHC).

Parts of sterco – and uro-bilinogen reabsorbed by EHC are excreted in urine.

Normal values of urobilinogen in urine:

0.5 to 2.5 mg/24 hrs average 0.64 mg

Any pathology in GIT increases urobilinogen in urine. They include:

Liver and biliary tract disease

Toxemia of pregnancy

Alcoholic intoxication

Urinary and fecal urobilinogen are increased in:

Hemolytic anemia (because of increased production),

i.e. in urine bilirubin is negative and urobilinogen is markedly increased.

Urine Urobilinogen

Absent in complete obstruction of CBD (common bile duct) e.g. CA head of pan creas (because bilirubin is unable to get into intestine to form urobilinogen).

Urine Picture in Obstructive Jaundice

Urine bilirubin positive
Urine urobilinogen negative.

Tests for Estimation of Urobilinogen (UB)

Ehrlich's test (para-di-methyl amino benzaldehyde in concentrated HCl).

Procedure

Take 2.5 cc fresh urine, add 2.5 cc Ehrlich's reagent.

Modification

Add 2 drops sodium acetate and then 2.5 cc of Ehrlich's to reduce acidity after reaction of UB with Ehrlich's reagent inhibits color formation with indole and skatole and take 2.5 cc urine sample for comparison.

Salient Features of Ehrlich's Test

1. Ehrlich's reagent reacts with urobilinogen to form a colored urobilinogen aldehyde.
2. It can't detect urobilin.
3. *Watson's modification:* Ferric hydroxide can reduce urobilin to urobilinogen and then Ehrlich's reagent is added to detect urobilin in urine.

Bile Salts

Sodium Glycocholate

Sodium Taurocholate

Bile acids are formed in liver and excreted in bile, enter intestine. Then bile acids enter liver by enterohepatic circulation. Main function of bite salts is fat digestion because they reduce surface tension and act as emulsifying agent.

Primary bile acids are formed from cholesterol and secondary bile acids are formed in intestine. Bile has alkaline pH and has Na and K. Bile acids exist as corresponding amino acid salts. Usually exist as sodium glycocholate and taurocholate, hence they are called *bile salts.*

Bile salts are formed from cholesterol in liver as cholic acid and excreted as glycholic acid. Taurine salt is excreted as *taurocholic acid.*

Bile salts have lower surface tension (ST) and on adding sulfur, ST is decreased and sulfur settles down. If bile salts are absent, then sulfur floats on surface.

Bile salts are present in urine in obstructive jaundice.

Hay's Sulfur Test

Fill test tube till brim, sprinkle sulfur powder over it. If bile salts are present, sulfur sinks down due to lowering of surface tension by bile salts, otherwise it remains on surface.

Bile Pigments

Bilirubin is breakdown product of Hb. Unconjugated bilirubin is not able to pass through glomerular barrier of kidney, while conjugated bilirubin is excreted in urine and is water-soluble.
Bilirubin is oxidized to differently colored compounds such as, biliverdin (green), bilicyanin (blue) which are known as bile pigments.

Test for Bile Pigment

Fouchet's Test

Reagent: 10 percent FeCl in 25 percent TCA

Procedure: Take 5 cc of urine and add 2 ml 10 percent BaCl to it and a few crystals (drops of saturated) ammonium sulfate, precipitates (ppt) of barium sulfate, appear. Dry the ppt. Pour few drops of Fouchet's reagent and greenish blue color confirms presence of bile pigments.

Bilirubin is found in urine in obstructive jaundice and in liver cell jaundice (infective and toxic) and absent in prehepatic jaundice.

Sources of Bilirubin

Hemolysis

Heme metabolism

Catabolism of hemoproteins

Characteristics of Bilirubin

1. Formation of bilirubin occurs in: spleen (80%) liver, and bone marrow
2. Transported bound to albumin to liver
3. Uptake by Y-protein ligandin and Z-proteins into hepatocytes
4. Intrahepatic transport in microsomes
5. Conjugation by UDP glucuronyl transferase I and II
6. Secretion of bilirubin by active transport into blood
7. Enterohepatic metabolism of urobilinogen:

Bilirubin in intestine is converted to urobilinogen which is partially reabsorbed and excreted into urine and partly excreted in feces giving characteristic color to stools.

Table: Urine picture in various types of jaundice

	Prehepatic	*Hepatic*	*Posthepatic*
Urobilinogen	+++	+ or n	-
Bile salt	-	-	++
Bile pigment	-	n	+++

Other tests: obstructive jaundice: ALP
Hepatic: SGOP/SGPT
PTT

VIVA

1. Urobilinogen is absent in urine in ….
2. Bilirubin is absent in urine in …..
3. Ehrlich's test detects …… in urine
4. Bile salts give …… test
5. Bile salts are present in …….. in urine
6. Clay colored stools are seen in ……..

KEY

1. Obstructive jaundice
2. Hemolytic jaundice
3. Urobilinogen
4. Hay sulfur test
5. Obstructive jaundice
6. Obstructive jaundice

Inherited Metabolic Disorders IMD

Inborn metabolic disorders are caused by genetically determined abnormalities that lead to a block in one of many interrelated biochemical pathways. The block frequently leads to accumulation of substrate and secondary metabolites or to deficiencies of downstream products.

Features

1. IMD are categorized by type of metabolic pathways that are impaired, such as disorders of carbohydrates, fats or amino acids.
2. These disorders may involve pathways of specific cellular organelles, including lysosomes, mitochondria, peroxisomes.

When to Suspect an IMD?

When there are unusual, unexplained clinical features or abnormal lab findings in infancy or childhood, the possibility of an IMD should be considered. Also, history of consanguineous marriage or more than one infant in family affected by same abnormality.

Cause of presentation can be due to (i) direct or enzyme abnormality and (ii) can be demonstrated indirectly.

Disorders of Intermediary Metabolism

It occurs as a result of three basic mechanisms:

1. *Enzyme deficiency* with defective substrate conversion.
2. *Membrane transport defect* resulting in failure of absorption or excessive excretion of a compound.
3. *Defects in receptors* involved in mediating metabolism

Biochemical Basis of Resulting Disease

2. Deficiency (due to lack of production) or excessive loss of desired compound (↓B)
3. Conversion of accumulated compound to altered/toxic metabolite (↑C)

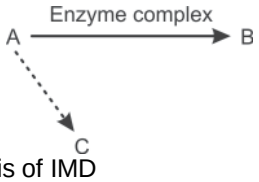


Fig. Basis of IMD

Table Inherited metabolic diseases

α, antitrypsin deficiency
Cystic fibrosis
Classic galactosemia
Familial hypercholesterolemia
Glycogen storage disease
Hereditary fructose intolerance
Lesch-Nyhan syndrome
Liver: (disorders of conjugation and excretion of bile:) Gilbert, Crigler-
Najjar, Dubin Johnson, Rotor syndrome
Hemochromatosis:
Lysosomal storage disease
Maple syrup urine disease
Phenylketonuria
Porphyrias
Renal glycosuria
Steroid 21-hydroxylase deficiency
Tyrosinemia type 1
Vitamin D- dependent and resistant rickets
Wilson’s disease

Inherited metabolic diseases are the result of gene mutations causing production of an abnormal protein or preventing synthesis of a protein.

***Table** IMD*

Diseases (a)	*Inheritance (b)*	*Defect (c)*	*Clinical Features (d)*	*Diagnostic approach/ detection (e)*
Glycogen Storage Disease				
Muscle forms				
II, IIIa	Hereditary	acid maltase, glycogen debranching enzyme	exercise intolerance	myoglobinuia, increased CK
Dubin-Johnson	AR.	decreased hepatic excretion of bilirubin	mild, fluctuant conjugated hyperbilirubinemia hepatic pigment deposition	↑S-bilirubin ↑U-bilirubin ↑ copropropor phyrinogen I/III ratio in urine
Mucopolysaccaharidosis (MPS)	AR	α-L-Iduronidase	progressive mental and physical debilitation	Increased urinary MPS (dermatanSO_4, heparan SO_4)
Maple syrup- disease (MSVD)	AR.	Branched chain decarboxylase defect	characteristic odor of urine: Maple syrup or burnt sugar	urine DNPH
Phenylketonuria PKU	AR.	Phenylalanine hydroxylase deficiency	mental retardation if untreated	high phenylamine in serum, urine, CSF
Porphyria ATP	AD	PBG deaminase	Abdominal pain, psychia-teric disturbance, peripheral neuropathy, Photosensitivity liver damage	ALA, PBG in urine
Renal glycosuria	?	Part of Fanconi's syndrome	Failure to thrive, rickets, polyuria with dehydration eventually renal failure	Blood glucose normal, positive for reducing sugars
Argininosuccinate	AR	AS lyase deficient	growth retardation neurolo-gical dysfunction	↑Argininosuccinate citrulline in blood, urine

Table Renal amino acid transport defects (autosomal recessive)

Disorder	*Defect*	*Findings*	*Urine analysis*

Hartnup disease	Defect in intestinal and renal transport of	Asymptomatic pellagra-like signs due to impaired neutral amino acids and tryptophan Increased urinary excretion of cystine, lysine, arginine, ornithine Increased urinary excretion of amino acids, along with rickets Ornithine, lysine, arginine lost in urine neutral amino acids and tryptophan Increased urinary excretion of cystine, lysine, arginine, ornithine Increased urinary excretion of amino acids, along with rickets Ornithine, lysine, arginine lost in urine	↑neutral amino acids
Cystinuria	Increased urinary excretion of	Cystine stones	Urine cyanide-nitroprusside

	cystine, lysine, arginine, ornithine		
Fanconi syndrome			Urine amino acid, bicarbonate, phosphate, glucose, potassium, uric acid Urine amino acid
Dibasic amino aciduria			

Describe the principles of colorimetry

Colorimetry

Methods for quantitative analysis of blood, urine and other biological materials are based upon the production of coloured compounds in solution, the intensity of which is used as a measure of concentration.

Laws of Absorption of Light

Two common methods of expressing the amount of light absorbed by a solution are:

1. By % transmittance

2. By optical Density (O.D), Absorbance (A) or Extinction (E) of the solution

OPTICAL DENSITY-

LOG T%=Log 100/1

Lambert Beer's Law:

Lambert Beer law states that light absorption is proportional to the number of molecules of absorbing material through which light passes. Absorbance changes with the thickness of the solution and with the concentration of absorbent in a manner characteristic for each absorbing material of a given wavelength.

$$\frac{IE}{Io} = e^{-Kct}$$

The mathematical expression at a given wavelength is

Where-

IE = Intensity of emergent light.

Io = Intensity of incident light.

K = A constant.

C = Concentration of coloured substance.

t = Thickness of the layer of solution.

e = Base of natural logarithm (2.718)

IE/IO is known as transmittance

Beer Lambert's law is applied for:

a) Only monochromatic Light

b) There should be no changes in ionization, dissociation association or solvation of the solution with concentration

When A (absorbance) is plotted against C (concentration), a straight line passing through the origin should be obtained as absorbance is directly proportional to concentration. With the help of a standard curve the concentration of an unknown solution can be readily determined.

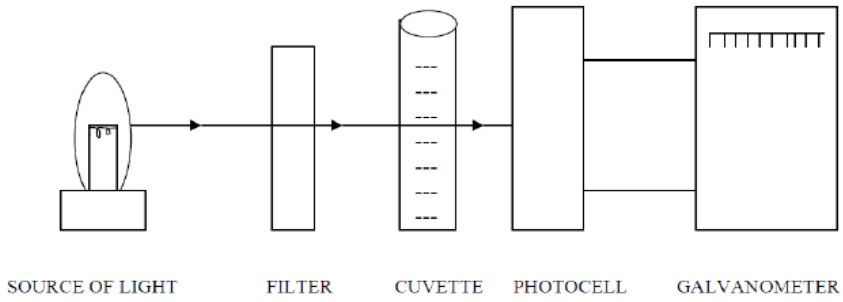


Fig. photoelectric colorimeter

Demonstrate the estimation of serum creatinine and creatinine clearance ***Practical***

Creatinine

Creatine, methylguanidinoacetic acid is synthesized in the liver, passes into circulation and is taken up almost entirely by skeletal muscle for conversion to creatine phosphate.

Creatine is synthesized from three amino acids namely, glycine, arginine and methionine. Firstly, glycine and arginine undergo transamination in kidney to guanidoacetate and ornithine. Guanidoacetate undergoes methylation by S-adenosyl methionine in liver to form creatine. Creatine is stored in muscle as creatine phosphate.

About two percent of creatine is converted to creatinine. Creatinine is anhydride form of creatine. 24 hours excretion of creatinine is constant in an individual and is proportional to muscle mass and does not depend on dietary intake. Free creatinine is a waste-product of creatine and is present in all body fluids and creatinine is freely filtered by glomerulus.

Determination of Creatinine

1. Chemical method
2. Enzymatic method
3. Autoanalyzer
4. HPLC

Chemical Method:

Jaffe's Method

Principle

Creatinine reacts with saturated alkaline picrate solution to produce a yellowish red colored complex, intensity of which is read at 520 nm.

This test is not specific because other non-creatinine chromogens in blood react similarly with alkaline picrate solution which are 20 percent in serum and 5 percent urine and raise the true value of creatinine by 0.2 to 0.4 mg percent.

Non-creatinine chromogens: Acetone, acetacetate, pyruvate cephalosporins, guanidine.

Substances such as glucose, ascorbate, aspartate, histidine cause fading of color of test.

Modified Jaffe's Reaction

Lloyd's reagent—hydrated aluminium silicate (Fuller's earth)

Protein is precipitated with tungstic acid and supernatant fluid is mixed with Lloyd's reagent which adsorbs creatinine in acid condition. After centrifugation, pellet is formed which is resuspended in alkaline picrate to elute creatinine.

Procedure

PFF	ml	ml	ml
Standard (0.01 mg/ml)	2	-	-
Distil water	-	1	-
5% NaOH	2	3	4
Saturated picric acid	1	1	1
	1	1	1

Mix, keep at room temperature for 15 minutes and read at 520 nm.

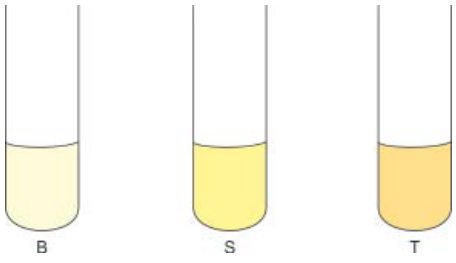


Fig. : Estimation of S. creatinine

Calculation:

T/S × 2 mg percent

4 ml PFF has 1 ml serum

1 ml of PFF has ¼ ml

2 ml in test has $\frac{1}{1} \times 2 = 0.5$ ml sera

$$\frac{Tx\ 0.01}{S\ \ 0.05} \times 100 \text{ mg/dL}$$

Concentration of standard being 0.01 mg per ml

Normal value: 0.8–1.5 mg percent

Urine Creatinine

Normal values:

100-200 mg/dL

1–2g/24 hours

Serum creatinine estimation follows Lambert-

Beer's law till 15 mg percent

In case of urine, creatinine, we cannot apply it to the same concentration, so 1:100 dilution is done.

Procedure: Take three test tubes, label them as test (T), standard (S) and blank (B)

	T	S	B
Urine	ml	ml	ml
Distil water	3	-	-
Standard	-	-	3
5% NaOH	-	3	-
Saturated picrate	1	1	1
Mix, take reading at 520 nm	1	1	1

Calculation:

100 ml of solution contains 1 ml urine

and 3 ml will contain:

$$\frac{1}{100} \times 3 = 0.03 \text{ ml}$$

Concentration of test in mg/dL

$$\frac{0.01x3}{0.03} \times 100$$

$$= \frac{Tx100}{S}$$

Interpretation

Creatinine is endogenously produced and released into body fluids at constant rates and its plasma levels are maintained within normal limits. Its clearance can be measured as an indicator of GFR.

Increased Creatinine Levels:

1. Renal disease
2. Due to:
    - Changes in muscle mass
    - Starvation
    - Muscle wasting diseases
    - After surgery
    - Corticosteroids administration
    - Physiological increase in GFR occurs in pregnancy

Plasma creatinine is most reliable simple bbiochemical test of glomerular function. Normal creatinine levels do not imply normal renal function, although raised creatinine usually indicates

Enzymatic Methods
Creatininase Method

$$\text{Creatine} \xrightarrow{\text{Creatininase}} \text{Sarcosine} + \text{Urea}$$

Sarcosine
Sarcosine ——→ Formaldehyde + Glycine + H_2O_2
$+H_2O+$ O + colored complex

H_2O_2 + O_2 acceptor ——→ H_2O + colored complex

1. Creatininase method
2. Creatinine deaminase
3. Dry chemistry

Autoanalyzer
Based on Jaffe's reaction, enzymatic methods
HPLC

Creatinine Clearance (Cl)

Definition: ml of plasma cleared off creatinine in one minute by both the kidneys.

Normal range 95 to 105 ml/min.

Creatinine Clearance

1. Creatinine levels depend on muscle mass
2. Preferred over urea clearance
3. Does not depend on rate of urine flow
4. Excretion is constant over 24 hours, while urea clearance depends on diet, increased metabolism.

Calculation:

$$Cl = \frac{uv}{p}$$

where u = urine creatinine
v = rate of urine flow
p = plasma creatinine

Advantages of Creatinine Clearance

1. Used as prognostic parameter in renal failure
2. Has no diagnostic value
3. Used in CRF for prognosis, dosage adjustment.

Creatinine Clearance Decreased in:

1. Chronic GN
2. Diabetic nephropathy
3. Chronic interstitial nephritis

VIVA	KEY
1. Name three amino acids required for the synthesis of creatinine	Glycine, arginine, methionine
2. How creatine is converted to creatinine?	Spontaneously
3. How creatine is stored in muscle?	As creatine phosphate
4. Role of creatine phosphate in muscle	source of energy
5. Non-creatinine chromogens	Acetone, pyruvate cephalosporin, glucose, ascorbate

6. In modified Jaffe's reaction which reagent is used?
7. Normal range of serum creatinine
8. Creatinine is a biochemical test of ……. function
9. In muscle wasting disease serum creatinine levels are …….
10. S. creatinine levels are …….. constant/fluctuating over 24 hours
11. S. creatinine levels start rising when ……. renal mass is functional
12. Creatinine clearance is …… GFR
13. Creatinine clearance is measured over …….. hours
14. Is creatinine secreted and reabsorbed in kidney?
15. Does rate of urine flow affect creatinine clearance?

BI11.8 Demonstrate estimation of serum proteins, albumin and A:G ratio ***Practical***

Fundamental Role of Proteins in Life

The nature of living organisms depends essentially on two classes of substances: Proteins and nucleic acids. While nucleic acids contain blue prints for hereditary characteristics, the structures of proteins enable them to act as the catalysts (enzymes), hormones, antibodies, coagulation factors, serving as building block units for subcellular, cellular and organic structures, carriers (of gases, metals, hormones vitamins, etc.), contractile elements (in muscles), maintenance of acid-base balance and osmotic pressure, etc.
In order to understand how these substances play such vital roles, understanding of the relationship between their structures and their biological functions is important.

General Properties of Proteins

All proteins are polymers of amino acids and all except two have an amino group attached to the carbon atom next to the carboxyl group (the α-position); i.e. they are α-amino acids.

□□□□□ Experiments on Proteins

a. Composition tests
b. Color reaction
c. Precipitation reaction

Proteins occur in both soluble and insoluble states in the body. In routine, tests on soluble proteins are performed in clinical chemistry laboratory in serum, urine and CSF samples.

Composition Tests

In addition to C, H and O, the proteins invariably contain nitrogen and generally sulfur. Elements such as I, Fe, Cu, and Zn are also occasionally present.
Some proteins yield only amino acids when hydrolyzed (simple proteins), whereas others produce amino acids plus other types of molecules (conjugated proteins).

General Composition Test

Heat some powdered egg albumin in a dry test tube in which a moist strip of red litmus paper and a piece of filter paper moistened with lead acetate solution is suspended.
As powder chars on heating, fumes of ammonia are evolved, turning the red litmus blue (indicating presence of N and H) and lead acetate paper is blackened (indicating presence of S).
Sulfur is present in proteins as cystine, cysteine, or methionine. Majority of proteins contain more methionine sulfur than cystine plus cysteine sulfur. Exceptions are keratins, insulin, certain serum albumins which contain all their sulfur in the form of cystine and cysteine.

Color Reaction of Proteins and Amino Acids

Because of their peptide structure and the presence of different amino acid groups in their molecules, proteins react with a variety of agents to form colored products.
Several of these color reactions of proteins are of importance in the quantitative detection and qualitative estimation of proteins and their constituent amino acids.
Proteins used in these tests are the following:
1. Albumin: The albumin is soluble in water, coagulated by heat, and deficient in glycine. They are products of both plants and animals. Examples include egg albumin, serum albumin, lactalbumin of milk. Albumins may be prercipitated from solution by saturation with ammonium sulfate.
2. Globulin: The globulins are insoluble in pure water, but they are soluble in dilute neutral solution of salts and are heat coagulable. They are precipitated by half saturation with ammonium sulfate.
Examples – ovoglobulin of egg yolk, serum, globulin, myosin of muscle.
3. Albuminoids or scleroproteins: They are the least soluble of all the proteins and constitute a very diverse groups of proteins. They are entirely animal proteins and are chief constituents of exoskeleton such as hair, horn, hoofs, nails, as well as of supporting and connecting fibrous tissues, and of the organic material of cartilage and bone.
Examples – keratin of hair, horn, hoof, nail, elastin of connective tissue and ligaments, collagen of bone, cartilages.

Gelatin

It results from treatment of collagen with boiling water or dilute acids and is sometimes classified as scleroproteins. Gelatin is a protein derivative and differs from collagen and keratin in having a much simpler physical structure and is being easily soluble and digestible.
It lacks tryptophan and lacks certain other amino acids like tyrosine and cystine. It is rich in arginine.

Casein

It is a phosphoprotein, and makes up a third of protein of human milk and five-sixth of protein of cow's milk. This is precipitated by half saturation with ammonium sulfate. It lacks sulfur-containing amino acids.

Peptones

They are secondary derived proteins. They are hydrolytic products of simpler proteins. They are soluble in water, are not coaguable by heat, and are not precipitated by saturation with ammonium sulfate. They are precipitated by phosphotungstic acid. They lack sulfur containing amino acids.

Complete hydrolysis of a natural protein:

Protein → protein → metaprotein → proteose → peptone → peptides → amino acid

Mixtures of proteoses, peptones and peptides are prepared commercially and used for bacterial culture media.

Mucoproteins

Mucoprotein present in saliva is termed as mucin. Mucoproteins are conjugated proteins consisting of proteins combined with mucopolysaccharides such as hyaluronic acid and chondroitin sulfate. Watersoluble mucoproteins have been obtained from serum, egg white and human urine.

Also, mucoproteins are important constituents of ground substance of connective tissue. Several gonadotropic hormones FSH, hCG, are mucoprotein.

Color Reactions

These reactions are due to a reaction between some one or more of the constituents radicals or groups of the complex protein molecule and the chemical reagent or reagents used in any given test.

Ninhydrin Test

Principle

The amino groups of amino acids are very resistant to hydrolysis but may be easily removed by oxidation. When amino acids are heated with ninhydrin, they are quantitatively deaminated.

This test is given by α-amino group containing compounds, based on the principle of oxidative deamination. During this reaction CO_2, NH_3, aldehyde with one carbon less than the amino acid is produced.

Ninhydrin (triketohydrindene hydrate) is a powerful oxidizing agent which reacts with all amino acid between pH 4 and 8 to give a purple colored compound. This reaction is also given by primary amines, NH_3 but without liberation of CO_2. Amino acids, proline and hydroxy proline react to produce yellow color. This reaction is very sensitive, ideal for detection of amino acids on chromatograms and their quantitation.

Procedure

To 1 ml of protein solution, add two drops of freshly prepared ninhydrin reagent. Heat to boiling for one to two minutes and allow to cool. A blue color develops, if the test is positive. This is known as Ruhemann's color complex.

Application

Ninhydrin reaction is carried out for qualitative detection of proteins and their hydrolytic products in biological fluid such as urine.

□□□□□ Biuret Test

Principle

When urea is heated to 180° C, it decomposes to form biuret. If a strongly alkaline solution of biuret is treated with very dilute copper sulfate, a violet color is obtained.

This reaction is given by substances containing two carbamyl (CONH2) groups joined either directly or through a carbon or nitrogen atom i.e. at least two peptide bonds/tripeptide should be present.

Color Produced

Proteins—purplish violet

Peptones—pink

Gelatin—blue color

Peptide—pink color.

Procedure

Take 6 cc of 5% NaOH, add two drops 1% CuSO4 mix, divide it into two parts:

Part I : Add 3 mol solution - Violet

Part II : Add distil water (control).

Application

Biuret test is used as test for detection/estimation of proteins in biological system.

Excellent method for quantitative determination of proteins in blood and other fluids.

□□□□□ Millon's Reaction

Principle

Millon's reagent is a solution of mercurous and mercuric nitrates containing nitric acid. When Millon's reagent is added to a protein solution, a white precipitate is generally first formed, which turns red upon heating. This reaction is not specific for proteins, since this test is given by phenols in general.

Since tyrosine is the only phenolic amino acid in proteins, a positive Millon's test indicates the presence of tyrosine.

Principle

Mercury being heavy, metal precipitates proteins and NaNO2 is changed to HNO2 which oxidizes Hg(NO3)2 to HgNO2 which is red in color.

$NaNO_2 \rightarrow HNO_2$,
$HNO_2 + Hg(NO_3) \rightarrow HgNO_2 + HNO_3$
(Red)

Procedure

To 1 ml protein solution, add few drops millon's reagent.
Boil it for 30 sec – white porecipitate appear.
Cool, add 1 drop 1% $NaNO_2$, warm gently, appearance of red precipitate gives this test positive.

□□□□□ Xanthoproteic Reaction

Principle

Test for aromatic nucleus (benzoid radicals). This test is given by proteins with phenyl group with which HNO_3 forms certain nitro-modification to give yellow colored compound which on addition of alkali turns orange. This test is given by tyrosine, tryptophan.

Procedure

Take 3 cc protein solution, add 1 cc concentrated nitric acid and heat. A yellow precipitate forms and on cooling, add NaOH or NH_4OH to it in excess results in formation of orange color.

Note -

This test is not given by phenylalanine under ordinary condition.
It is not satisfactory for urine examination.

□□□□□ Hopkins-Cole (Glyoxylic Acid Reaction) Test

Principle

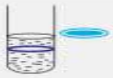
Given by indolic ring which condenses with aldehyde to give violet color in the presence of mild oxidizing agent and strong acid (tryptophan gives this test).

Procedure

To freshly prepared formalin (1:500), add 1 to 2 ml concentrated H_2SO_4 along the side of the tube. A violet ring at the junction of two is formed showing presence of indole ring.

Note -

Pure H_2SO_4 should be used.
Gelatin does not give this reaction.

Test	Observation	Inference
Ninhydrin test	Violet ring 	α-amino acid
Biuret test	Violet color  Control test	Two peptide bonds (tripeptide)
Xanthoproteic test	Orange color 	Aromatic amino acid (tyrosine tryptophan)
Hopkins-Cole test	Violet ring 	Indole ring (tryptophan)
Lead acetate		Sulfur-containing amino acid (cysteine, cystine)
Sakaguchi	Cherry red 	Guanidino group (arginine)

Procedure
Take 3 cc protein solution, add 1 cc 10 percent NaOH and 2 drops α-naphthol.
Add one drop of NaOBr, cherry red colored solution is obtained.

□□□□□ Biological Value (BV) of Proteins

BV is a measure of nitrogen retained for growth and/or maintenance and is expressed as:
percentage of N absorbed.
Definition
Percent of absorbed N that is retained in the body.
It provides a reasonably good index for the nutritive value of proteins.
Factors Affecting BV
1. Amount and relative proportions of their constituent amino acids (essential amino acids).
2. Nutritional availability: Rate of liberation and absorption of amino acids.
Classification and Quality of Proteins
1. Higher quality (1st class/complete protein): Animal protein
2. Incomplete protein: Vegetable protein
3. Whole egg and milk proteins especially lactalbumin rank highest percentage of essential amino acids.
4. Meat, fish, poultry occupy next position in the scale.
5. Cereals, legumes and nuts are generally poor, because they lack essential amino acids and thus they are incomplete proteins and are of poor quality.
= retained N ×
BV 100
absorbed N
during a specific interval.
Table

Table 37.1: BV of various food stuff

Food	BV
Egg	90
Milk	84
Meat	80
Fish	85
Rice	64

Drawback of BV
It cannot take into the account the nitrogen that might be lost during the digestion process.
VIVA Key: Answer
1. What is the principle of ninhydrin test Oxidative deamination
2. Which test detects α-amino group Ninhydrin

3. Test for detecting aromatic aminoacid Xanthoproteic
4. Hopkins-Cole test is used for detection (Tryptophan) Indole ring

of which amino acid?
5. T/F: Phenylalanine gives xanthoproteic test F
6. Test for guanidine group Sakaguchi
7. Test for tyrosine Millon's
8. Test for proteins in biological systems Biuret
9. Egg has (low/high) BV High
10. Peptones give ———— color with biuret Pink
11. Amino acid with anti-oxidant property Arginine
Reactions given by peptones: Ninhydrin, Biuret, Millon's, Xanthoproteic, Hopkins-Cole, but not lead acetate.
Reactions given by gelatin: Biuret, Sakaguchi (because it is rich in arginine).

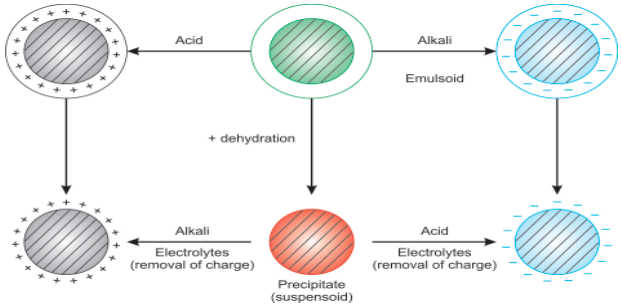
Experiment:
To perform protein precipitation reactions
Methods
1. Heavy metal
2. Alkaloidal reagent
3. Fractional precipitation
4. Strong acids and alkali
5. Precipitation by alcohol and heat
1. Heavy metals: $HgCl_2$, $AgNO_3$, $CuSO_4$, $Pb(C_2H_3O_2)_2$
Principle:
Heavy metals neutralize negative charge on proteins to form metal proteinate, e.g. mercuric proteinate, silver proteinate, etc.
Procedure:
Take 2 cc of protein solution, add a few drops of Millon's reagent ($HgSO_4$ in concentrated H_2SO_4), white precipates are formed.
Application: Egg albumin is used as an antidote for mercury or lead poisoning.
2. Alkaloidal reagents: Picric acid, phosphotungstic acid, tannic acid, metaphosphoric acid, phosphomolybdic acid, sulfosalicylic acid, Esbach's reagent.
Principle: Proteins behave as positively charged in presence of alkaloidal reagents to form precipitates of protein picrate and protein tannate, etc.
Procedure – Take 2 cc of protein solution and add a few drops of alkaloidal reagent.
Reagent Precipitate
Sulfosalicylate White
Picric acid Yellow
Esbach's reagent Yellow
Tannic acid Orange/brown
Application: Protein precipation is carried out in quantitative exercises, e.g. urea estimation.

3. Fractional precipitation: Ammonium sulfate, sodium sulfate, sodium chloride.
Principle: All proteins except peptone are precipitated by saturating their solution with ammonium sulfate. Globulin are precipitated by half saturation and albumin by full saturation.
Salts dehydrate the protein solution, dehydration and denaturation of molecules occur and molecular aggregation in solution results.
Procedure: Take 3 cc ammonium sulfate and add 3 cc serum protein solution and mix, white turbidity appears.
Globulins precipitate at half saturation. Filter the turbidity and to the filtrate add ammonium sulfate slowly and mix it to get a saturated solution. The resulting turbidity is due to albumin precipitating at full saturation.
Concentration of salt required for precipitation of proteins depends on pH of protein solution i.e., charge on protein complex.
4. Strong acids and alkali
HCI, HNO_3, H_2SO_4, NaOH
Principle: In the presence of acid, proteins carry positive charge to produce protein chloride, protein nitrate, etc. In the presence of alkali, proteins carry negative charge to produce sodium proteinate, etc.
Procedure: Take 5 cc of concentrated nitric acid in a tube, add 3 cc protein solution drop-wise. A white ring appears at the junction of two layers because of protein precipitation.
Application: Heller's test – to detect protein in urine.

5. Precipitation by alcohol and heat:
i. Heat coagulation: Fill a test tube .th full of protein solution, heat upper one-third portion, white turbidity or opalescene appears. Lower portion acts as control. Proteins get denatured on heating and produce turbidity.
ii. Alcohol: Alcohol is a dehydrating agent. Take 5 cc protein solution and add 2 cc alcohol, turbidity results due to precipitation of proteins. Precipitation is effective at isoelectric pH and cause intramolecular changes which affect solubility and other properties of proteins. This is known as denaturation. Prolonged contact with alcohol produces irreversible coagulation, e.g. fixing of tissues for histological examination.

?????? Theory of Protein Precipitation

Proteins form colloidal solution of the type known as EMULSOIDS. Charge and hydration are two stability factors which keep the particle in solution. For every protein, there is definite characteristic pH, known as isoelectric pH.
Chemical or physical agents which act on chemical bonds to produce irreversible changes in proteins, whereas residual valencies respond to changes in salt concentration, pH, temperature in reversible manner.
For every protein, there is a definite characteristic pH known as isoelectric point pI at which particles are electrically neutral and cease to migrate to poles of an electric field. Proteins are least soluble at pI except: gelatin and ovalbumin remain in solution at pI.



Proteins exhibit both chemical and colloids properties. Former is due to presence of reactive groups (COOH, NH_2) in protein molecule and latter being dependent upon changes in charge and dehydration of particles in solution. Proteins exist as Zwitter ion at pI and addition of acid results in formation of protein salt (i.e., protein chloride, which ionizes into positive protein and negative acid ion.
Addition of alkaline at pI of protein result in metal proteinate salt which ionizes to positive metal ion and negative protein ion.
So, in acid solution, protein exists as positive ion and in alkaline solution as negative ion.
Other properties of protein at pI:
Solubility: Minimum/negligible at pI
Viscosity, osmotic pressure are minimum at pI.
These are dependent upon Donnan equilibirium setup between particles and surrounding dispersion medium.

Protein Denaturation

Protein is called a native protein if its amino acid composition and stereochemical structure are unchanged from natural state.
These properties control all functions of a protein i.e., solubility, proteolytic activity, oxygen carrying capacity, etc.
When proteins undergo changes in structure or composition these characteristics are altered and proteins are said to be denatured and primary structure of proteins is preserved and hence it is a reversible process.
Certain proteins especially those which are fibrous or highly elongated molecule in solution, e.g. myosin are easily denatured. While others: Carbohydrate-rich glycoproteins seem to be resistant to usual physical agents (e.g. ovomucoid).

Denaturation may be caused by heating, neutral acid and alkali, shaking, stirring, grinding, UV radiation (split peptide bond adjacent to ring). Ultrasonic waves (disrupt aromatic ring), neutral chemical agents, e.g. urea. Proteins can be renatured on warming. Proteolytic action of pepsin goes on heating which is restored on cooling.

Coagulation

Coagulation is a irreversible process, e.g. egg white coagulates on heating.

Salting out
Precipitation by ammonium sulfate at high concentration results in salting out of proteins due to dehydration: Decrease in activity of water which diminishes the solubilizing interaction between water and polar protein groups. Here native structure of protein is preserved and it is a useful method for isolation and purification of proteins.
Salting in
Globulin precipitates at half saturation, while at full saturation it is in solution. This is salting in. Globulins which are insoluble in pure water become soluble in the presence of small amounts of salts. This phenomenon is known as salting in. It is due to force of attraction between protein ions and salt ions.
At high concentration of very soluble salts, e.g. ammonium sulfate, sodium sulfate and phosphate, buffer proteins are salted out of the solution. This is due to a dehydration: A decrease in activity of water which diminishes solubility interactions between water and polar protein groups.

Significance of Protein Precipitation

1. Egg albumin is antidote in heavy metal poisoning, e.g. Pb, Hg.
2. Alcohol in fixation of histopathology slides.
3. Heller's test in precipitation ion of protein in urine and CSF.
4. Esbach's reagent can be put in urine bag and precipitates get collected at bottom which can be measured.

BI11.9 Demonstrate the estimation of serum total cholesterol and HDL cholesterol ***Practical***

Cholesterol

Cholesterol present in the blood occurs both as free and as cholesterol esters.
Methods for Determination of Total Cholesterol
i. Direct method: Utilizes serum or plasma.
ii. Indirect method: Involves treating sample with solvent extraction or other isolation procedure. They include: chemical, colorimetic and enzymatic methods.

Chemical Methods

Liebermann Burchard's Reaction

i. Uranyl acetate
ii. Zlatkis method
Principle
Cholesterol in acidic media reacts with acetic anhydride and sulfuric acid to form green colored complex which is read colorimetrically at 570 nm. Sulfuric acid is a dehydrating and oxidizing agent, while, iron enhances the reaction. Acetic anhydride sulfuric acid is corrosive.
Zlatkis Method
Uses acetic acid and ferric chloride.
Uranyl Acetate Method
Procedure
UA (uranyl acetate) for protein free filtrate has uranyl acetate, acetic acid, $FeCl_3$ and is known as acetate ferric chloride precipitating (ppting) reagent. Take three test tube and label them as test (T), standard (S) and blank (B).

Mix well, keep in dark for 10 min (red color appears)
Read at 560 nm.
Calculation
Cholesterol =
T x 200 mg/dL
S

5 ml solution has 0.05 ml serum
3 ml will have 0.05/5 Å~ 3 = 0.03
1 ml standard has 2 mg cholesterol
0.03 ml will have 2 Å~ 0.03 = 0.06 mg/ml cholesterol
[std = 2 mg/ml = 200 mg percent]
Precautions
1. Glass wares should be completely dry.
2. Reagents are corrosive, use dispensers.

3. $FeCl_3$—acetate should be stored at 2 to 8°C, but should be brought to room temperature before estimation.
Normal Range: Cholesterol
150 to 250 mg/dL
Conversion of mg/dL to nmol/L ÷ 38-7 or Å~ 0.026.
Enzymatic Method
Advantage
1. More specific and sensitive
2. Linear up to 700 mg percent
3. Low sample volume required

Applied Aspects

1. Raised cholesterol levels (hypercholesterolemia)
CHD (coronary heart disease)
Angina
DM
Hypothyroidism
Obstructive jaundice
(Because decreased excretion of bile salts increases cholesterol levels)
NS (nephrotic syndrome)
Hyperlipidemia (familial)
2. Hypocholestemia
MAS (malabsorption syndrome)
Severe wasting severe malnutrition
Hyperthyroidism
Hemolytic jaundice
Anemia
Pernicious anemia

Cholesterol Metabolism

Cholesterol is synthesized from acetyl CoA (90 percent in liver, gut) and key enzyme is HMG CoA reductase.
HMG CoA reductase enzyme is regulated by:
i. Hormone (insulin, thyroxine)
ii. Diet
iii. Feedback inhibition
iv. Synthesis

Acetyl CoA can enter Kreb's cycle, utilized for ketone body, cholesterol synthesis and lipogenesis.
In DM, (insulin deficiency), PDH (pyruvate dehyrogenase) enzyme is inhibited. So, Kreb's cycle and lipogenesis are inhibited. Other routes which are stimulated are:
i. cholesterol synthesis
ii. KB (ketone body) synthesis.
Insulin increases HMG CoA reductase activity and glucagon and glucocorticoides decrease HMG CoA reductase activity.
High levels of cholesterol and its metabolites control cholesterol biosynthesis by feedback inhibition and decrease synthesis of translation of mRNA, decrease its rate of degradation.
HMG CoA reductase is also inhibited by: Lovastatin, compactin.
In hyperthyroidism, usually T_4 stimulates HMG CoA reductase increasing cholesterol synthesis and degradation. In hypothyroidism, predominant effect is decreased degradation, hence, cholesterol levels are increased.
HMG COA reductase: Salient features
Decreased by: fasting
Inhibited by: feedback inhibition
Mevalonate
Statins
Glucocorticoides
Glucagon
Bile acid
Cholesterol
Increased by:
Insulin
Thyroxine
No direct inhibition of the enzyme occurs by cholesterol, but its metabolite is considered to act by repression of transcription of HMG CoA reductase gene.

□□□□□ Salient Features of Cholesterol

1. Cholesterol is synthesized in the liver, is transported to the tissues in LDL, derived from VLDL.
2. Dietary cholesterol reaches liver in chylomicron remnant.
3. HDL acquires cholesterol from peripheral cells and other lipoproteins and this is esterified by enzyme LCAT.
4. CE is transferred to remmant particles which are taken up by liver, whence the cholesterol is excreted.

□□□□□ Uses of Cholesterol

1. Synthesis of:
Vitamin D
Steroid hormone
2. Bile salt metabolism
3. Present in cell membrane and maintains membrane fluidity

Treatment
Exercise
Low fat diet
Increase fiber intake
Decrease stop smoking

□□□□□ Drug Therapy to Lower Cholesterol Levels

1. Interrupt enterohepatic circulation of bile acids by:
Cholestyramine
Ileal resection
2. HMG CoA inhibitors (statins):

Mevastatin
Lovastatin
3. Block absorption of cholesterol from GIT:
Sitosterol
Cholestyramine resin
4. Fibrates:
Gemofibrozil
5. Others:
Niacin
Probucol
LDL aphoresis
Nicotinic acid
Source of cholesterol in diet: Meat, egg yolk, seafood, dairy products.
Cholesterol is in unesterified (free) form in intestine, and esterified cholesterol is hydrolyzed by cholesterol esterase to free cholesterol and FFA. Esterified cholesterol and FFA cholesterol is hydrolyzed by cholesterol esterase to free (secreted from pancreas and small intestine). Free cholesterol is solubilized by bile acids to form mixed micelle which are absorbed in intestine. About 30 to 60 percent of dietary cholesterol is absorbed daily.

□□□□□ Synthesis of Cholesterol

300 to 1800 g derived from diet, it can be synthesized endogenously by liver and other tissues from acetate. 90 percent synthesis takes place in liver and gut. Peripheral cells and other organs depend on cholesterol delivery from circulation.

□□□□□ Esterification of Cholesterol

LCAT is responsible for formation of all of the CE (cholesterol ester). LCAT is synthesized in liver, released in circulation, enzyme continues to esterify plasma cholesterol even after the blood sample is drawn and stored at room temperature. So, analyze sample for Cholesterol and CE immediately or keep it at 0°C.

Catabolism

CE (cholesterol ester) cells is hydrolyzed by acid lipase (lysosome). Cholesterol on reaching the liver is either secreted unchanged into bile or metabolized to bile acids (about 400 mg/d cholesterol is converted to bile acids), i.e. cholic and chenodeoxy cholic acid.

VLDL

VLDL is principal transport form of endogenous TG. Initially, they share a similar fate to chylomicrons, TG being stripped off by action of LPL (lipoprotein lipase). As VLDL particles become smaller, free cholesterol and apolipoproteins are released from their surfaces and taken up by HDL, thus coverting VLDL to IDL and finally LDL. Further, triglycerides are removed by hepatic lipase forming LDL – cholesterol.
LDL – Principal carriers of cholesterol (CE)
HDL – Serves two functions:
i. Source of apoproteins for chylomicrons and VLDL.
ii. Mediates reverse cholesterol transport.
HDL takes up cholesterol from senescent cells and other lipoproteins and transferring it to remnant cholesterol particles which are taken up by liver.
Cholesterol is present in tissues and plasma lipoproteins as free cholesterol and CE. It is synthesized in many tissues from acetyl CoA and is eliminated from the body in bile as cholesterol or bile salts. Cholesterol is precursor of all steroids in the body, e.g. vitamin D, corticosteroid, sex hormones, bile acids.
Cholesterol is amphipathic lipid and is an essential structural component of membrane and outer layer of plasma lipoproteins. CE is storage form of cholesterol found in most tissues. Free cholesterol is removed from tissues by HDL and transported to liver for its conversion to bile acid in the process known as reverse cholesterol transport.
Cholesterol is major constituent of gallstones and its chief role is in genesis of atherosclerosis of vital arteries causing cerebrovascular disease (CVD), coronary heart disease (CHD) and peripheral vascular disease. Coronary atherosclerosis correlates with high plasma LDL : HDL cholesterol ratio. 700 mg/d of cholesterol arises by synthesis and rest is provided by diet. Liver accounts for 10 percent of total cholesterol synthesis in humans, the intestines account for about another 10 percent. Virtually all tissues containing nucleated cells are capable of synthesizing cholesterol.

Serum cholesterol is correlated with incidence of atherosclerosis and CHD. And inverse relation exists between HDL-C and CHD, LDLC/HDLC ratio is predictive of coronary artery disease risk.

VIVA KEY

1. Reaction involved in chemical Liebermann-Burchard estimation of cholesterol reaction
2. Normal range of serum cholesterol 150 to 250 mg/dL
3. Cholesterol is synthesized from Acetyl CoA
4. In hyperthyroidism, serum cholesterol Low levels are
5. Effect of insulin on HMG CoA reductase Stimulates
6. Mevastatin/statins act on which enzyme? HMG CoA reductase (inhibit)
7. Mechanism of action of fibrates Increase VLDL (e.g. clofibrate) catabolism
8. Low cholesterol levels are seen in MAS, hyperthyroidism
9. High cholesterol is risk factor for Atherosclerosis

BI11.10 Demonstrate the estimation of triglycerides ***Practical***

Triglycerides:

Form of lipids formed by combining of fatty acid with glycerol in intestinal mucosa and liver

Functions:

Storage form of energy

Component of biological membranes

Thermal insulation etc.

PHYSIOLOGIC BASIS

Dietary fat is hydrolyzed in the small intestine, absorbed and resynthesized by mucosal cells, and secreted into lacteals as chylomicrons

Triglycerides in the chylomicrons are cleared from the blood by tissue lipoprotein lipase

Endogenous triglyceride production occurs in the liver.

These triglycerides are transported in association with β-lipoproteins in very low-density lipoproteins (VLDL)

Methods of triglyceride estimation:

1. Chemical methods:

a). Solvent extraction with solid phase adsorption

b). Liquid phase partition

2. Enzymatic methods

Chemical methods:

Require solvent extraction of plasma to solubilize triglycerides and to denature and remove protein

For solid phase adsorption:

Extract must be treated with adsorbent material to remove phospholipids and other substances

isopropanol extracts treated with a zeolite mixture or with alumina,

chloroform extract treated with sialic acid

For liquid phase partition method:

Extracted with nonane and isopropanol to separate triglycerides into:

Upper nonane layer

Lower layer containing contaminants such as glucose, phospholipids

PROCEDURE FOR SOLID PHASE ADSORPTION

Blank, standard and samples diluted with distilled water

Extraction of lipids with isopropanol

Addition of **Zeolite** mixture and agitation to promote phospholipid adsorption

Tubes left overnight at 0 to 5 degrees to increase reproducibility of method

Reagitation with **Zeolit**e and production of clear supernatant by centrifugation

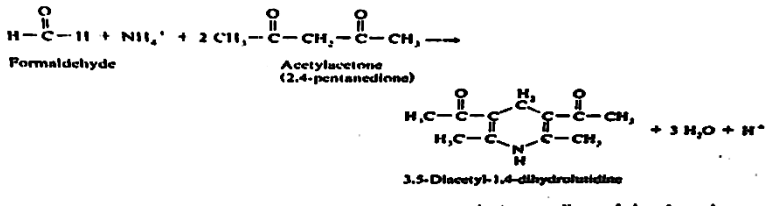
Incubation of supernatant with **potassium hydroxide** solution to hydrolyse triglycerides to glycerol + FFA

Incubation of hydrolysed supernatant with **periodate** and **acetyl acetone** in ammonium acetate to facilitate **yellow lutidine formation**

Measure O.D at 570nm

Reaction: solid phase adsorption

Glycerol + Periodic acid → Formaldehyde



Blank, standard and sample acidified with dilute sulphuric acid

Extraction of triglycerides into nonane with nonane/isopropanol mixture

Incubation of nonane extract with NaOH solution to hydrolyse TG→glycerol

Oxidation of glycerol with periodate to produce formaldehyde (and formic acid)

Incubation with acetylacetone in ammonium acetate lutidine formation

O.D. measured 570nm

Calculation: sample triglyceride concentrations determined by comparing sample O.D. with standard O.D at 570nm

ENZYMATIC METHOD

SAMPLE COLLECTION:

Sample should be taken after 10-14hrs of fasting in EDTA tube

Sample can be stored at 4 degrees for up to 7 days

Warm samples at 37 degrees before analysing the stored sample

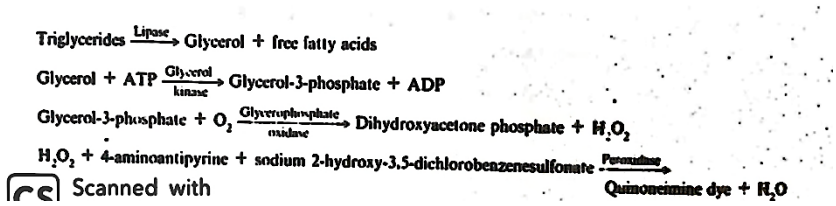
PRINCIPLE

Triglycerides hydrolysed with lipase to form glycerol and free fatty acids

Glycerol undergoes further enzymatic reactions to form red coloured quinoneimine dye that is read at 510nm

ENZYMIC ASSAY SEQUENCE—TRIGLYCERIDES

- 1. Triglyceride H2O-- Bacterial lipase→ Fatty acid Glycerol
- 2. Glycerol + ATP –Glycerol kinase→ Glycerophosphate + ADP
- 3. Glycerophosphate + O2-- Glycerophosphate oxidase→ Dihydroxyacetone + H2O2
- 4. H2O2 + Dye -- Peroxidase→ Color



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COMPOSITION: *REAGENTS*

Buffer containing:

Pipes buffer

4- Chlorophenol

Magnesium ions

Sodium azide

Enzyme reagent containing:

4- Amino phenazone

ATP

Lipases

Glycerol kinase

Glycerol-3-phosphate oxidase

Peroxidase

PROCEDURE

	Blank	Standard	Test
Reagent	1mL	1mL	1mL
Distilled water	10ul	-	-
Standard	-	10ul	-
Serum	-	-	10ul

Mix and incubate for 15 minutes at 37 degrees

Measure OD at 510 nm after setting blank at zero

CALCULATION

$$\text{Concentration of TG (mg/dL)} = \frac{200 \times \text{OD of test}}{\text{OD of standard}}$$

Conversion factor (TG): mg/dL x 0.0113= mmol/L.

CONTRIBUTION OF ENDOGENOUS FREE GLYCEROL

Enzymatic TG reaction sequences react with endogenous free glycerol

Endogenous free glycerol:

Universally present in serum

Can be a significant source of interference

Contributes a 10–20 mg/dL overestimation of triglycerides in most specimens

About 20% of specimens will have higher glycerol

Glycerol levels increased in certain conditions:

Diabetes, liver disease

From glycerol containing medications

Reagents are available that correct for endogenous free glycerol

Most common correction: “Double-cuvet blank ” method:

With a second parallel measurement using TG reagent without lipase enzyme to quantify only the free glycerol blank

Or “single-cuvet blank,” begins with lipase-free reagent

When is it ordered?

Adults: A lipid profile, which includes TG recommended every 4 to 6 years to evaluate risk of heart disease in healthy adults

Children should have a lipid profile screening at least once between the ages of 9 and 11 and once again between the ages of 17 and 21.

Testing may be ordered more frequently when people have identified risk factors for heart disease

Some risk factors for heart disease include:

Cigarette smoking

Being overweight or obese

Unhealthy diet

Being physically inactive—not getting enough exercise

Age (men 45 years or older or women 55 years or older)

High blood pressure (hypertension—blood pressure of 140/90 or higher or taking high blood pressure medication)

Family history of premature heart disease (heart disease in an immediate family member—male relative under age 55 or female relative under age 65)

Pre-existing heart disease or already having had a heart attack

Diabetes or prediabetes

Test Preparation Needed?

Current standards recommend that testing be done when you are fasting

For 9 to 12 hours before the test, only water is permitted

In addition, alcohol should not be consumed for 24 hours just before the test

A clinician may decide if a subject may be tested without fasting

One should Follow any instructions given and inform the person drawing blood whether or not he has fasted

COMMENTS

Proper calibration and standardisation should be done

Blood collection tubes lubricated with glycerol should not be used

Plasma samples should be analysed on the day of collection

Stability of reagents should be monitored

INTERFERING SUBSTANCES FOR TG MEASUREMENT

Plasma ascorbic acid:

Can interfere with the oxidation/reduction reactions involved in the measurement of TG

Bilirubin:

Can also cause interference both spectrally, chemically

Significant plasma hemolysis:

Can spectrally interfere with TG measurements and may cause dilution of lipid constituents

REFERENCE RANGE:

Normal: 60-160 mg/dL

Borderline high 160- 199 mg/dL

High 200-499 mg/dL

Very high >500 mg/dL

VALUES

If serum is clear: serum triglyceride level generally < 350 mg/dL

Triglycerides proposed to be a weak independent risk factor for coronary artery disease

Triglycerides >1000 mg/dL can be seen:

When a primary lipid disorder is exacerbated by alcohol or fat intake or by corticosteroid or estrogen therapy

INTERPRETATION

Increased in:

Hypothyroidism
Diabetes mellitus,
Nephrotic syndrome
Chronic alcoholism (fatty liver)
Biliary tract obstruction
Stress
Familial lipoprotein
Lipase deficiency
Familial dysbetalipoproteinemia
Familial combined hyperlipidemia
Viral hepatitis
Cirrhosis
Pancreatitis
Chronic renal failure

Gout

Pregnancy

Glycogen storage diseases types I, III, VI

Anorexia nervosa

Dietary excess

Obesity

Drugs:

Beta blockers

Cholestyramine

Corticsteroids

Diazepam

Diuretics

Estrogens

Oral contraceptives

INTERPRETATION

Decreased in:

Tangier disease (α- lipoprotein deficiency)

Hypo- and abetalipoproteinemia

Malnutrition

Malabsorption

Parenchymal liver disease

Hyperthyroidism

Intestinal lymphangiectasia

Drugs:

Ascorbic acid

Clofibrate

Nicotinic acid

Gemfibrozil

CLINICAL CORRELATION

HYPERTRIGLYCERIDEMIA:

Usually asymptomatic till levels are >1000 mg/dL

Causes:

High carbohydrate diet

High fat diet

High alcohol intake

Inactivity

Obesity

Diabetes mellitus

Lysosomal diseases

Familial disorders

Signs and symptoms:

Epigastric pain, nausea, vomiting

Dyspnoea

Xanthomas

Eye related like corneal arcus etc.

Also lead to various cardiovascular diseases, acute pancreatitis etc.

Treatment:

Omega-3 fatty acids

Fibric acid derivatives(fenofibrate etc.)

Niacin

HMG-CoA reductase inhibitors(atorvastatin, lovastatin etc.)

CAUSES OF SECONDARY HYPERTRIGLYCERIDEMIA

Lifestyle	Diseases	Medications
Excess calories	Poorly controlled diabetes	Corticosteroids
Excess dietary fat intake	Hypothyroidism	Oral estrogen
Excess simple sugars	Renal disease	Retinoic acid derivatives
Overweight/Obesity	HIV infection	Beta adrenergic blockers
Alcohol intake	Cushing's syndrome	Thiazide diuretics
Pregnancy	Acromegaly	Protease inhibitors
	Growth hormone deficiency	Bile acid sequestrants
	Lipodystrophy	Anti-psychotic drugs
	Paraproteinemia	Cyclosporine/tacrolimus
	Nephrotic Syndrome	L-asparaginase
		Interferon alpha 2b
		Cyclophosphamide

HYPOTRIGLYCERIDEMIA

Causes:

Low fat diet

Malabsorption

SLE

Celiac disease
Crohn's disease
Malnutrition
Hyperthyroidism

What strategies can be used to lower triglyceride levels?

In many people, high triglycerides are caused by another disorder, such as diabetes, obesity, renal failure, or alcoholism

With these conditions, the strategy is to treat the primary cause

When high triglycerides are not caused by another disorder, they are often seen together with high cholesterol and treatment is directed toward lowering both cholesterol and triglycerides

Lifestyle changes, such as a healthy diet and increased exercise, are usually the primary strategy for lowering levels

If these fail, lipid-lowering medications such as statins are generally recommended

What strategies can be used to lower triglyceride levels? RISKS!

Usually, most people with high triglyceride levels have no symptoms and the only means of discovering a high level is with blood test

However, in rare cases, a person may have an extremely high level of triglycerides (well above 1000 mg/dL) sustained over time and the individual may experience repeated bouts of acute pancreatitis

Some of the signs and symptoms include pain in the upper half of the stomach area that develops suddenly and then gradually gets worse, fever, nausea, vomiting, and sometimes jaundice

A person with severely high levels may develop lesions on skin: xanthomas

These typically appear as several small, round, solid, yellow bumps mostly on the back, chest, buttocks, shoulders and thighs.

CASE

A 15-year-old woman presented to surgical unit with acute pancreatitis. Some of her laboratory results were as follows:

Plasma(fasting)

Cholesterol 33.4mmol/L (3.5-5.0)

Triglyceride 69.1mmol/L (0.3-1.5)

HDL cholesterol 0.9mmol/L (1.0-1.8)

Amylase <20U/L (<200)

On examination, she had eruptive xanthoma on her arms and thighs and fundoscopy revealed lipaemia retinalis.
The patient has grossly elevated lipid concentrations with severe hypertriglyceridemia

The blood would be lipaemic and some plasma sodium assay (indirect ion electrode) may show pseudohyponatraemia.
She was found to have lipoprotein lipase deficiency when this enzyme was measured before and after heparin administration, which releases the enzyme from capillaries into the circulation.
The lipoprotein lipase deficiency can result in chylomicron syndrome and eruptive xanthomas may be present.
Plasma amylase concentration is normally elevated in acute pancreatitis but , due to gross lipaemia, assay was unsatisfactory, giving low value.
The latter is an important point for preference of spot urinary amylase or assay of plasma amylase after separation from lipid fraction.

BI11.11 Demonstrate estimation of calcium and phosphorous. ***Practical***

Measurement of Total Calcium

Principle

O-cresolphthalein complexone method (OCPC)
Metal complexing dye CPC [3', 3" – bis (biscarboxy methyl) amino methyl 5', 5" – dimethylphenophthalein] forms a red chromophore with calcium ions in alkaline solution, it is generally measured at 578 nm (wavelength between 570 and 580 nm).

Chromogen reagent has:

1. 8 hydroxyquinoline to mask interfering cations mainly Mg.
2. Urea to eliminate turbidity of lipemic serum and to enhance complex formation.
3. Ethanol to prevent color development in blank.

Chromogen Reagent

Prepare fresh reagent by taking equal amount of OCPC and diethamolamine.

Procedure

Take three tubes label them T, S, B

	T	S	B
OCPC reagent (ml)	5	5	5
Standard (10 g%)	-		
Serum	50 ul	-	-
Mix			
Read the absorbance at 570 nm.			

Calculation

S. calcium = OD /OD x 10 in mg percent
Normal range = 9 to 11 mg percent

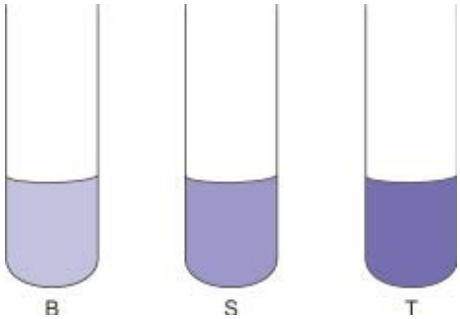


Fig.: Estimation of S. calcium

Precautions

1. Do not use hemolyzed sample.
2. All glassware used in the determination should be acid-washed and thoroughly rinsed with distilled water.
3. Avoid venous stasis during venipuncture.

Other Methods

1. *Titration method (Clark and Collip):* Calcium is precipitated as oxalate directly from the serum and after washing, the precipitate is dissolved in acid and titrated with permanganate.
   Disadvantage
   Time consuming (end-point is not clear).
2. *Iodometric titration:* An excess of permanaganate is used, potassium iodide is added and iodine liberated from this excess is titrated with 0.025 N thiosulfate using starch as indicator.
   Drawback: Not very accurate.
3. *Titration with EDTA:* In alkaline medium Ca is titrated ammonium phosphate.
   Drawback – End point is vague.
4. *Flame photometer:* Ca and Mg are more difficult to be excited in flame than Na and K. Consequently, a higher temperature or flame is required.
5. *Autoanalyzer:* Using O-CPC method.
6. *Atomic absorption spectrophotometry:* Used as a reference method

Physiology and Biochemistry of Calcium

Calcium is fifth most common element and most prevalent cation found in the body. Average human body contains approximately 1 kg of calcium.

Calcium is found in three main compartments –

Skeleton – 99 percent
Soft tissue – 1 percent
Extracellular fluid < 0.2 percent

In blood, all of the calcium is in the plasma and exists in three physiochemical states:
Free or ionized 50 percent
Bound to plasma proteins 40 percent
Complexed with small anions 10 percent
Free or ionized calcium is the biological active form. Its concentration in plasma is tightly regulated by parathyroid hormone and decreases 1,25 dihydroxy vitamin D.
Of the protein bound calcium fraction, 80% is associated with albumin, 20% with globulin.

Interpretation

Reduced Serum Calcium

- Lowest values are found in hypoparathyroidism
- Hypoalbuminemia is most common cause of reduction in concentration of total serum calcium
- Chronic liver disease
- Nephrotic syndrome
- Congestive heart failure
- Chronic renal failure
- Pseudohypoparathyroidism.

Hypercalcemia

- Primary hyperparathyroidism: Most common cause of hypercalcemia
- Malignancy with skeletal involvement
- Vitamin D overdose
- Granulomatous disease: Sarcoidosis, tuberculosis
- Renal failure: Acute, chronic.

Phosphorus

Estimation

Measurement of inorganic phosphorus in serum is done by Fisk and Subbarow method.

Principle

In acidic medium, acid molybdate reagent reacts with inorganic phosphorus to form phosphomolybdic acid and hexavalent phosphomolybdic acid is reduced by ANSA (1,2, 4-amino naphthol sulfonic acid) to give blue compound which is estimated colorimetrically.

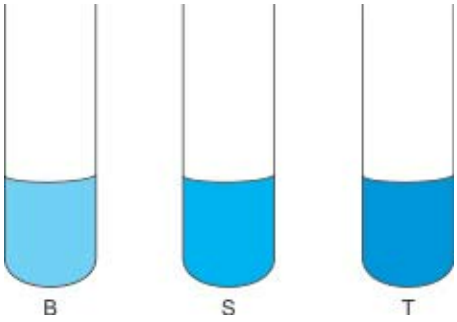


Fig.: Estimation of S phosphorus

Procedure

Preparation of protein free filtrate (PFF):

To 4 ml of 5 percent TCA, add 0.2 ml serum and centrifuge.
Take three test tubes and label them as T, S, B.

	T (ml)	S (ml)	B (ml)
DW	1.8	1.8	1.8
PFF	2.5	-	-
Standard (8 mg%)	-	2.5	2.5
DW	0.5	-	-
Molybdic acid	0.2	0.5	0.5
ANSA		0.2	0.2

Concentration of serum phosphorus = ODT/ODS x 8 mg percent

Precautions

1. Molybdic acid should be clear solution.
2. Test tubes should be dry, clean.

Normal values: 2.4 to 4.5 mg percent

Biochemistry and Physiology of Phosphorus

Phosphorus in the form of inorganic or organic phosphate is an important and widely distributed element in the body.

Adult human has 600 g of phosphate of which (i) 85 percent is in skeleton (ii) rest in soft tissues.

Phosphate has both extracellular (substrate for bone mineralization) and intracellular functions (as high energy phosphate bond in ATP, constituent of important enzymes).

Interpretation

Hyperphosphatemia

1. Usually secondary to inability of kidneys to excrete phosphate:
   CRF, ARF
   Hypoparathyroidism and pseudo-hypoparathyroidism which increase tubular reabsorption of phosphate.
2. Increased phosphate intake
3. Intracellular phosphate shift:
   Lactacidosis
   Respiratory acidosis
   Untreated diabetic ketoacidosis
4. Cell lysis
   Rhabdomyolysis
   Leukemia

Lymphoma

Manifestations

Most acute problem associated with rapid elevation of phosphate levels is hypocalcemia with tetany, seizures.
If hyperphosphatemia is chronic, secondary hyperparathyroidism, soft tissue calcification occurs.

Causes

1. Shift of phosphate from extracellular to intracellular spaces due to:
   Glucose
   Insulin
   Respiratory alkalosis
2. *Renal phosphate wasting*:
   Fanconi syndrome – lowered Tm for phosphate
   Primary or secondary hyperparathyroidism
3. Intestinal phosphate loss:
   a. Increased loss:
      Vomiting
      Diarrhea
      Phosphate binding antacids
   b. Decreased absorption
      Malabsorption syndrome
      Vitamin D deficiency
4. *Cellular phosphate loss*:
   Acidosis
   Diabetic ketoacidosis
   Lactacidosis

Manifestations

Muscle Weakness

Acute respiratory failure due to muscle weakness
Impaired mineralization of bones, rickets, or osteomalacia

BI11.12 Demonstrate the estimation of serum bilirubin ***Practical***

Bilirubin

Bilirubin is formed from Hb in RES (reticuloendothelial system) and circulates attached to plasma albumin, in low concentration in the blood.

Bilirubin is insoluble in water and conjugated bilirubin is excreted in bile as diglucuronide and thus made water-soluble.

Experiment: Estimation of Total Serum Bilirubin

Principle

Bilirubin reacts with diazotized sulfanilic acid to give a purple color at neutral pH, absorbance of which is measured at 540 nm
Methanol accelerates diazotization process.
This reaction is termed as *van den Bergh reaction*

i. *Direct-acting bilirubin* or *conjugated bilirubin* is water-soluble and reacts with diazo reagent within one minute.
ii. *Indirect-acting bilirubin* or *unconjugated bilirubin* is non-polar, insoluble in water and does not react with diazo reagent.

iii. Indirect bilirubin = Total bilirubin – Direct bilirubin.

···Reagents

Diazo A—sulfanilic acid in HCl
Diazo B—NaNO
Diazo reagent—10 ml of A and 0.3 ml of B should be prepared freshly
Diazo blank—HCl (0.1 N)

···Procedure

Total Bilirubin

Take 2 test tubes, label them as test (T) and control (C)

	T(ml)	C (ml)
	1.8	1.8
	0.2	0.2
	0.5	-
	-	0.5
	2.5	2.5

(Addition of methanol dissolves unconjugated bilirubin)
Mix and keep item dark for 30 min
Read at 530 nm and take OD of standard as 0.24

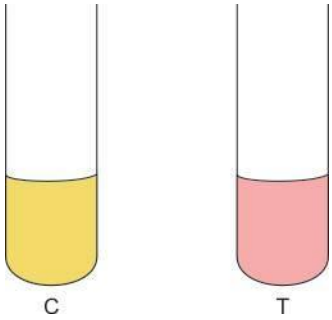


Fig. : Estimation of S. bilirubin

···Calculation

$$\text{Serum bilirubin (mg/dl)} = \frac{ODT - ODC}{ODS} \times 8$$

$$= \frac{T - C}{0.24} \times 8 \text{ mg/dl}$$

···Bilirubin Standard

Artificial standard: Methanol red in glacial acetic acid is used.
OD of methyl red is 0.24 which corresponds to 8 mg percent of bilirubin.

Precautions

1. Hemolyzed sample should not be used.
2. Color is photosensitive, so keep it in dark, take reading immediately.

3. Interfering substances are:
    - Vitamin B complex
    - Carotene
    - Hemolysis

Other Methods

Direct Bilirubinometer

Absorbance of bilirubin in serum at 454 nm is proportional to its concentration.
(Here second filter at 540 nm is chosen as oxy Hb has same absorbance at 454 nm. (Oxy Hb gives absorbance at 454 and 540 nm while bilirubin reads at 454).
This method is used for neonates.

Reflectance Spectrophotometry

This is a thin film technique where sodium benzoate and caffeine is present in top layers. The second layer has gelatin to trap serum protein and third layer has cationic polymer called a *mordant* that binds bilirubin. It is read at 400 nm. It is useful in neonates with physiological jaundice.

HPLC

Interpretation

Jaundice—It is yellow discoloration of tissues due to bilirubin deposition with raised S bilirubin (BR) levels.

- *Normal values of bilirubin*: 0.2 to 0.8 mg percent
- *Latent jaundice*: When serum bilirubin levels are 1 to 2 mg/dl
- *Clinical jaundice*: When levels are >2 mg/dl

Classification of Jaundice

- Prehepatic/hemolytic
- Hepatic
- Posthepatic/obstructive

Prehepatic

- It is due to increased breakdown of Hb
- Liver cells are unable to conjugate all the increased BR formed.

Causes

- Hemoglobinopathies
- Hereditary sphereocytosis

- Incompatible blood transfusion
- Hemolytic disease of newborn
- Malaria
- Drugs, e.g. sulphonamides.

···Hepatic

In hepatic jaundice, there is disease of parenchymal cells of liver.

Causes

a. *Defective conjugation*: Decreased number of functional cells.
   - Chronic hepatitis defect in conjugation:

     Criggler Najjar Syndrome

b. *Infective and toxic jaundice* causing intrahepatic cholestasis.
c. *Cholestatic jaundice*: Due to drugs, e.g. chlorpromazine, steroids causing intrahepatic obstruction.

···Posthepatic

Obstruction to flow of bile in extra-hepatic ducts.

Causes

- CA head of pancreas
- Gallstones
- Stricture of bile duct.

···Neonatal Jaundice

High values (30 to 40 mg percent) can be encountered in newborn especially in babies from mothers who have developed Rh antibodies in case of Rh incompatibility. Here, bilirubin may get deposited in brain (basal ganglia) causing *kernicterus*. It crosses blood brain barrier in infant causing damage to brain may be fatal sometimes.

Treatment—exchange transfusion of blood.

Neonatal jaundice is due to deficiency of glucuronyl transferase enzymes which conjugate bilirubin. It is usually seen in premature infants.

···Physiological Jaundice

Activity of hepatic conjugating enzymes is usually low at birth, but increases rapidly (2 to 15 d) thereafter. Transient jaundice usually less than 5 mg/dl is observed.

Treatment: Phototherapy

It results in formation of more soluble isomers of bilirubin which are more easily excreted in urine.

VIVA	KEY
1. Source of bilirubin	Heme
2. Site of bilirubin synthesis	RES (Spleen, bone marrow, Kupfer cells of liver)
3. Conjugated bilirubin is	Water soluble (direct-acting)
4. Normal range of serum bilirubin	0.2–0.8 mg/dl
5. Latent jaundice when bilirubin is	1–2 mg/dl
6. Direct reacting bilirubin	Conjugated BR (water-soluble)
7. Why tubes after adding diazo reagent are kept its dark	Color is photosensitive
8. Color of stool in obstructive jaundice	Clay colored
9. ALP levels rise in which type of jaundice	Obstructive jaundice
10. Bilirubin is absent in urine of …… type of jaundice	Hemolytic
11. Urobilinogen in urine is absent in ……	Obstructive jaundice
12. Gilbert's syndrome is due to	

13. Incompatible blood transfusion causes
14. Rh-incompatibility causes in newborn
15. Glucuronyl transferase deficiency is seen in
16. Kernicterus

17. Carcinoma head of pancreas causes

Decreased conjugation of
bilirubin
Hemolytic/prehepatic jaundice

Neonatal jaundice

Crigler-Najjar syndrome

Deposition of bilirubin in basal
ganglia in neonates
Obstructive jaundice

BI11.13 Demonstrate the estimation of SGOT/ SGPT ***Practical***

BI11.14 Demonstrate the estimation of alkaline phosphatase ***Practical***

Alkaline phosphatase is found in many tissues including bone; liver, intestine, kidney, placenta.

Estimation of ALP

□□□□□ Principle

4 nitrophenyl phosphate in presence of ALP is converted to 4 nitrophenoxide (colorless) at pH 10.
Phenol released by enzymatic hydrolysis from phenyl phosphate is estimated colorimetrically.

□□□□□ Procedure

Take 4 test tubes, label them
test (T), control (C), standard (S), blank (B)

	T (ml)	C (ml).	B (ml)
Buffer (pH 10)	1	1	1
PP substrate (Disodium phenyl phosphate)	1	1	1

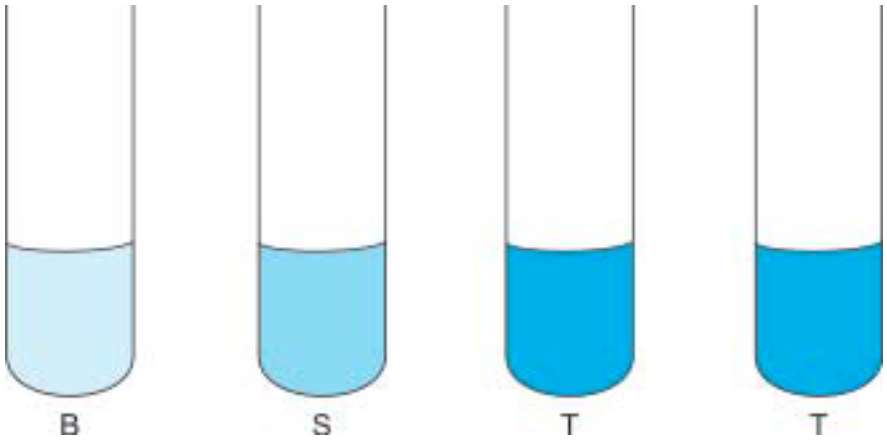


Fig. : Estimation of ALP

Incubate in water bath at 37°C for 3 min

	T	C	S	B
Phenol standard (10 ug/ml)	-	-	1	-
DW	-	-	-	1
Serum	0.1	-	-	-
Mix gently, incubate at 37°C for 15 minutes				
Serum	-	0.1	-	-
0.5 NaOH	0.8	0.8	0.8	0.8
0.5 N $NaHCO_3$	1.2	1.2	1.2	1.2
Aminoantipyrine	1	1	1	1
Potassium ferricyanide	1	1	1	1

Read the color at 510 nm

NaOH – to stop the reaction

$NaHCO_3$ – provides alkaline medium

Aminoantipyrine – gives color

□□□□□ Calculation

Amount of phenol in standard tube is 10 mg. Thus, phenol produced in 15 minutes in test will be

T–C/S–B x 10 mg

= T–C/S–B x 100/0.1 x 100 μg/100 ml

Hence, 100 ml of serum would liberate

T–C/S–B x 10 mg of phenol

Since 1 KAU is the production of mg of phenol in 15 minutes.

Under the condition of test therefore serum ALP = T–C/S–B x 10 KAU/100 ml

Normal range = 3–13 KAU/100 ml

□□□□□ Clinical Significance

ALP is elevated in:

Cholestatic liver diseases

Paget's diseases of bone

Cirrhosis

Bone tumors

□□□□□ Isoenzymes

Placental – heat-stable

Hepatic – heat-labile

Bone – heat-labile

(details are discussed under chapter: Liver Function)

Separation of isoenzymes:

1. Electrophoresis

2. Immunological techniques

BI11.15 Describe & discuss the composition of CSF

Cerebrospinal fluid is usually obtained for diagnostic purposes by lumbar puncture. CSF should be submitted separately for different studies namely:
a. Chemistry and immunology studies
b. Microbiological examination
c. Total and differential cell count
d. Cytology
The four major categories of diseases in which CSF examination is required are:
1. Meningeal infection
2. Subarachnoid hemorrhage
3. CNS malignancy
4. Demyelinating diseases
The following chemical tests are commonly carried out on CSF: Determination of glucose, chlorides and proteins, quantitative test for globulin

Appearance

Normal CSF is clear, colorless and gives no coagulum or sediment on standing under sterile conditions.

Color

Presence of blood is main cause of abnormal color. Normally, no RBCs are present in CSF and some
may be introduced as a result of trauma while obtaining the fluid. Pathologically, blood may be present
in subarachnoid hemorrhage and in hemorrhage into the ventricles.

Turbidity

It is seen when there is marked increase in the number of cells or when organisms are present in CSF.
Commonly seen in streptococcal infection.

Coagula

In pathological fluid, where there is increase in proteins, in CSF, fibrin clots may form.
In tuberculous meningitis, a fine web-like coagulum is sometimes formed when fluid is allowed to
stand overnight.

Chemical Examination

□□□□□ Glucose

The sugar content of normal lumbar fluid is usually between 50 and 80 mg per 100 ml and is slightly
lower than the blood sugar (i.e. 65% of blood glucose levels)
Most important pathological change is decrease in glucose levels which occurs in meningitis.

□□□□□ Estimation of Sugar

This may be carried out by any of the blood sugar methods in use.

Proteins

The protein content of normal lumbar fluid lies between 15 to 45 mg per 100 ml. The protein present
is almost entirely albumin.

Increase in total protein is the commonest abnormality found in polyneuritis, meningitis, tumors.
Estimation of Protein
By turbidimetry method
Principle
Proteins are precipitated by 3 percent TCA and the resulting turbidity is read colorimetrically.
Procedure
Take 3 test tubes, and label them T, S, B.

	T (ml)	S (ml)	B (ml)
CSF	1	-	-
Standard	-	1	-
DW	-	-	1
3% TCA	3	3	3

Mix well, keep at room temperature for 10 minutes.
Read absorbance at 420 nm

Calculation

OD_T/OD_S Å~ 50 mg/100 ml

Chlorides

Chloride content of normal CSF is 120 to 130 meq/L and is appreciably higher than plasma chloride. Most important alteration is in meningitis where a decrease is observed, and lowest values are seen in tubercular meningitis.
Estimation of Chloride
Methods used for plasma chloride can be used for CSF chloride estimation.

BI11.16 Observe use of commonly used equipments/techniques in biochemistry

Laboratory including: pH meter

Paper chromatography of amino acid

Protein electrophoresis

TLC, PAGE

Electrolyte analysis by ISE

ABG analyzer

ELISA

Immunodiffusion

Autoanalyser

Quality control

DNA isolation from blood/ tissue

Demonstration

BI11.17 Explain the basis and rationale of biochemical tests done in the following conditions: ALREADY IN BOOK

CASE HISTORIES, SPOTTING, VIVA Qs, PICTURES

- diabetes mellitus

- dyslipidemia

- myocardial infarction

- renal failure

gout

- proteinuria

- nephrotic syndrome

- edema

- jaundice

- liver diseases

pancreatitis,

disorders of acid- base balance

thyroid disorders.

BI11.18 Discuss the principles of spectrophotometry.

ALREADY IN BOOK

BI11.19 Outline the basic principles involved in the functioning of instruments commonly used in a biochemistry laboratory and their applications. ***DOAP sessions***

ALREADY IN BOOK

BI11.20 Identify abnormal constituents in urine, interpret the findings and correlate these with pathological states

see: **BI11.4**

BI11.21 Demonstrate estimation of glucose, creatinine, urea and total protein in serum.

see: **BI11.7, 8**

Urea

Urea is the main end product of protein metabolism in the body. Removal of amino groups from amino acids, occurs from which urea is formed. Process of urea formation takes place in the liver. Determination of blood urea is important not only in many diseases of the kidneys but in a wide range of conditions which are not primarily renal.

Experiment

□□□□□ Estimation of Blood Urea by Diacetyl Monoxime Method

Principle

Diacetylmonoxime (DAM) method: Urea reacts with DAMO (diacetylmonoxime) to give azo compound which is yellow in color and stabilizes color intensifies by adding ferric alum and color is read at 430 nm.

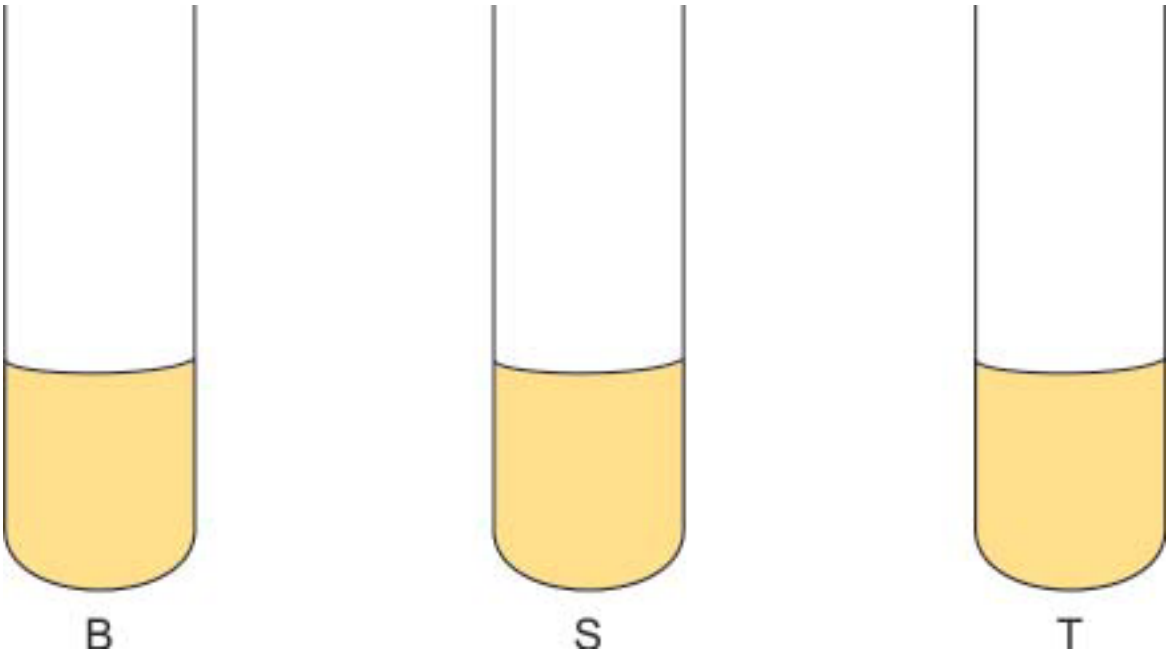
Procedure: Prepare protein free filtrate by taking 0.2 ml of oxalated blood, 1.9 ml 5 percent Zn SO_4

and 1.9 ml 0.3 NaOH and centrifuge.

	T (ml)	S (ml)	B (ml)
PFF	2	-	-
Working urea standard (0.025 mg/ml)	-	2	-
Distil water	-	-	2
DAM	2	2	2
Ferric alum	2	2	2

Mix

Keep in boiling water bath for 5 minutes. Cool it under tap water and take absorbance at 420 nm.



Reaction

DAMO + water → hydroxylamine + Diacetyl

↓

Diazine + water derivative

Calculation

Concentration of Test

= ODt/ODs x 0.05/0.01 x 100 in dl

= ODt/ODs x 50 mg percent

4 ml (total vol PFF) has 0.02 ml blood

1 ml has 0.02/4

2 ml has 0.02/4 x 2 = 0.1 ml blood

Working standard concentration is 0.025 mg/ml

i.e. 1 ml has 0.025 mg urea

2 ml has 0.025 x 2 = 0.05 mg urea

100 ml has 0.05 x 100 = 0.05 mg urea

Normal urea levels: 15 to 35 mg percent

Merits of this method:
Urea reacts readily with DAMO
It is quick, DAMO is stable
Demerit:
Color developed is photosensitive
Not specific

Other Methods

Urease Method

Urea + H_2O urease NH_3 + NH_2COOH (Carbamic acid)

$\longrightarrow$

$\downarrow$

$NH_3 + CO_2$

This ammonia is measured
Urostat dry chemistry: Strips for urea estimation in serum or blood.

□□□□□ Berthelot Reaction

NH_3 formed by urease reaction reacts with phenol in presence to hypochlorite to form an indophenol which with alkali gives blue colored complex and sodium nitroprusside acts as catalyst.

□□□□□ Glutamatate Dehydrogenase Method (GDH)

Urease
Urea + water $\longrightarrow$ $NH_3 + CO_2$
GDH
α-ketoglutarate + NH_3 + NADH $\longrightarrow$ L-glutamate + H_2O

□□□□□ Nesselerization

NH_3 formed by urea reaction (reacts with Nessler's reagent) is determined colorimetrically.
Drawback of Methods Measuring Ammonia
They are susceptible to contamination of ammonia from lab and endogenous ammonia in the specimen.
Uremia
Uremia is rise in urea levels (in body)
Classified as Prerenal
Renal
Postrenal
Prerenal
Decreased plasma volume:
• Salt water depletion, e.g. protracted vomiting (pyloric obstruction intestinal obstruction), paralytic ileus
• Prolonged diarrhea
• Shock due to hemorrhage/burns/toxemia
• High protein-diet
– Muscle wasting
– Starvation
– Increased protein catabolism
• Reabsorption of blood following GI hemorrhage
• Cortisol treatment
– Liver disease
– Decreased renal perfusion
Renal
Glomerulonephritis
Intrinsic renal disease

Renal failure
Pyelonephritis
Hg poisoning
Postrenal
Obstruction to outflow of urine leads to back pressure on renal pelvis and decreases GFR of urine:
Benign enlargement of prostate (BEP)
Urinary calculi
Stricture urethera
Malignant tumor invading ureter
Causes of Low (Blood) Urea
Hemodilution
Low protein intake
Liver disease: destruction of cells
Leading to impairment of urea cycle
Urea represents 45 to 50 percent of NPN (non-protein nitrogen) of blood and 80 to 90 percent of total urinary N (nitrogen) excretion.

Blood Urea Nitrogen (BUN)
BUN = 0.467 x blood urea (mg/dl)
Urea levels are directly proportional to protein intake and inversely proportional to rate of excretion.
□□□□□ Urine Urea
Normal levels (15-35 g/l)
Urea is filtered from blood at the glomerulus, but, passive tubular resorption occurs at a significant extent, especially at low rates of urine flow.
Urea Production Increased:
High protein intake
Catabolic states
Increased absorption of amino acids and peptides after GI hemorrhage
Liver disease
Urea Production Decreased:
Low protein intake
Plasma urea concentration increases during dehydration as a consequence of tubular reabsorption even when renal function is normal.
Catabolism of proteins and nucleic acid results in formation of so called non-protein nitrogen compounds (NPN) which are derived from exogenous (dietary) or endogenous (tissue) proteins.
Proteins undergo proteolysis to form amino acids which undergo transamination releasing ammonia.
More than ninety percent of urea is excreted through kidneys with losses through GIT and skin accounting for most of all the remaining minor fraction.
Factors Affecting Plasma Levels of Urea
1. Increase protein intake
2. Increased endogenous protein breakdown:
• Fever
• Cushing's syndrome
• Intestinal bleeding
3. Increased urea reabsorption in tubule:
Prerenal acute renal failure (ARF)
4. Postrenal ARF:
Reabsorption from tubular fluid above the level of obstruction.
VIVA KEY
1. Type of anticoagulant used for Oxalate
sample for blood urea

2. Function of ferric alum in DAM method Intensifies color
3. Define clearance
4. Unit of clearance ml/min
5. Normal range of blood urea 15–35 mg/dL
6. Normal range of standard urea clearance 40–65 ml/min
7. Urea clearance depends on Protein intake, rate
of urine flow
8. Urea clearance is — of GFR 3/5th
9. T/F: urea is affected by rate of urine flow T
10. What happens to blood urea levels after meals Increase
11. Clearance has …..value : diagnostic/ Prognostic
prognostic
12. Urea is synthesized in Liver
13. Causes of raised urea level in blood
14. Which is better index of clearance: Creatinine

Estimation of Blood Sugar O-toluidine Method

Experiment

Principle

Glucose condenses with o-toluidine in glacial acetic acid when heated at 100°C, produces an equilibrium mixture of n-glycosylamine and corresponding Schiff 's base to produce a blue green product (chromogen),
absorbance of which is measured at 630 nm.

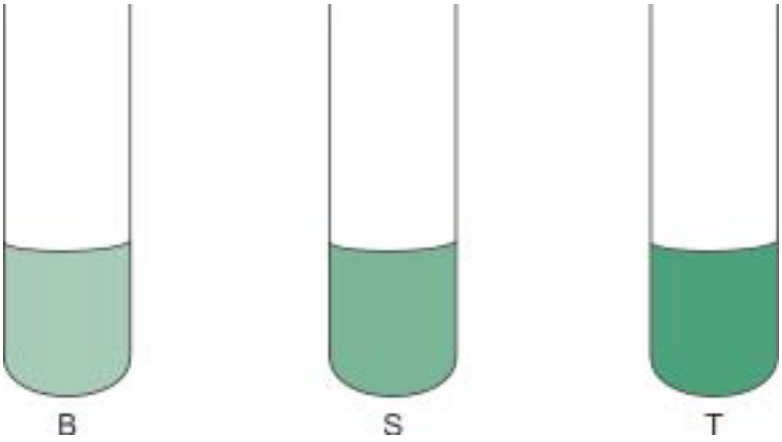
Sugar + O–toluidine ⟶ glycosylamine
↑↓
Green chromogen ⟵ Schiff 's base

Procedure

Take 0.2 ml serum sample and add 1.8 ml of 4 percent TCA. Mix, centrifuge. Take 0.5 ml of supernatant (PFF). Take three test tubes label them as test (T), standard (S) and blank (B).

	T ml	S ml	B ml
PFF	0.5	-	-
Sugar standard (0.1 mg/ml)	-	0.5	-
Distil water	-	-	0.5
O-toluidine reagent	2.5	2.5	2.5

Keep the tubes in boiling water bath for 9 minutes and cool it under cold water for 5 minutes. Then read OD at 630 nm (color is stable for 1 hr).



Calculation
Standard concentration:
1 ml standard has 0.1 mg sugar
0.5 will have 0.1 Å~ 0.5 = 0.05 mg/ml sugar
PPF: 0.2 ml serum is taken in 2 ml of PFF
0.5 ml will have $\frac{2 \times 0.5}{0.2}$ = 5 mg/ml

Concentration of sugar in T =

$$\frac{ODT \times 0.05 \times 100}{ODS \times 0.05} = \frac{T \times 100}{S} \text{ mg/dL}$$

Precautions
1. O-toluidine reagent is highly corrosive and should not be pipetted by mouth
2. Store O-toluidine in amber colored bottle
Advantages
1. Sensitive and only galactose produce absorbance comparable with that of glucose which is present in negligible amount in blood normally.
2. Hemolysis does not interfere with the test.
Disadvantages
1. Bilirubin contributes to some increase in glucose values and should be read with caution in neonatal jaundice.
2. In uremia, higher values are obtained.
3. Thymol preservatives inhibit color formation.
4. Dextrin causes turbidity in final color
5. Presence of EDTA >1 mg/ml or NaF >5 mg/ml cause increase in color.

Other chemical methods - 1. Folin Wu
2. Nelson Somoyogi
3. Inverse colorimetry

Enzymatic Methods

Glucose Oxidase Method – (GOD – POD Method)

Principle
Glucose oxidase
Glucose + H_2O + O_2 ———→ Gluconic acid + H_2O_2
Peroxidase

$H_2O_2 \longrightarrow H_2O + [O]$
$[O]$ + acceptor $\longrightarrow$ colored chromogen
Acceptor
O – dianisidine
O – toluidine
4 – aminophenazone (used commonly)
4 – aminoantipyrine.
Advantages
1. No interference by reducing agents such as uric acid, creatinine, GSH, Hb
2. Less interference by drugs like INH, paracetamol, hydralamine, tolazamide
3. Ascorbate retards the color to a lesser extent
4. Minor interferences are caused by xylose, galactose, L-dopa.

Hexokinase Method

Principle
Hexokinase
D-glucose + ATP $\longrightarrow$ G-6-P + ADP
G6PD
G-6-P + NAD $\longrightarrow$ 6 gluconolactone + NADH + H^+
Increase in absorbance of NADH is measured at 340 nm which is proportional to amount of glucose.
Advantage
It is unaffected by uric acid, bilirubin, vitamin C, lipemia, hemolysis.
But, thimerosal (anticoagulant) interferes with this test.

□□□□□ Glucose Dehydrogenase Method

Principle

G6PD

Glucose + NAD + $\longrightarrow$ Gluconolactone + NADH + H+

Increase in absorbance of NADH is measured at 340 nm. It serves as reference method for standardization of method.

Ion Selective Electrode (ISE)

O2 consumed in reaction of glucose oxidase can be measured polarographically using ISE.

Dry Chemistry

It uses glucose oxidase method on dry chemistry strips.

Procedure

1. 10 microliter sample is poured over the strip.

2. Glucose diffuses into second layer containing reagent and glucose oxidase reaction produces colored end product.

3. Color change is compared with charts or intensity of product is measured by reflectance densitometer.

Strips Available Commercially

1. *Dextrostrip*—blood glucose estimation.

2. *Urostrix*—urine glucose estimation.

Infrared Detectors: They are also available lately.

Interpretation

Normal Values

Fasting 60–100 mg percent (O-Toluidine method)

60–80 mg percent (Enzymatic method)

Fasting up to 140 mg percent

Postprandial (PP) after 2 hours of glucose load <200 mg percent

Causes of Raised B Sugar Levels

DM: Primary:

IDDM

NIDDM

Secondary:

Pancreatitis

Hormonal — Cushing's

Conn's syndrome

Acromegaly

Drugs or chemicals

Insulin receptor abnormality

Genetic syndrome

Others

Classification of DM and Other Types of Glucose Intolerances:

Idiopathic:

IDDM

NIDDM (non-obese)

Gestational (GDM)

IGT (impaired glucose tolerance)

Previous abnormal GT (Prev AGT)

Potential abnormal GT (Pot AGT)

Glucose intolerance associated with certain condition and syndromes

Venous whole blood concentration are 15% lower than plasma. Capillary values are 7–8% higher than
venous values.

GTT (Glucose Tolerance Test)

Preparation of Patient

1. In a overnight fasting (10 to 16 hrs) person, after 3 days of unrestricted diet and activity, perform this test.
2. Omit medications known to affect glucose tolerance, e.g. thiazides furosemide, clonidine, lithium carbonate.
3. Avoid undue exercise and smoking and alcohol intake.

GTT (WHO Expert Committee Recommendations)

In adults, 75 g of glucose dissolved in 250 to 300 ml of water is consumed in 5 to 15 min or 1 g/kg body/wt is recommended.

In children 1 g/kg up to 75 g maximum is given.

Sampling

Take fasting blood sample, then every 30 minutes for 2 hours after an oral glucose load.

Interpretation

F ≥140 mg percent and/or

2 hours ≥200 mg percent indicates DM.

F <140 mg percent (80 to 140) and

2 hours between 140 to 200 indicates impaired GT

GRAPH

Flat curve: Observed in hypopituitarism, MAS, myxedema, Addison's disease

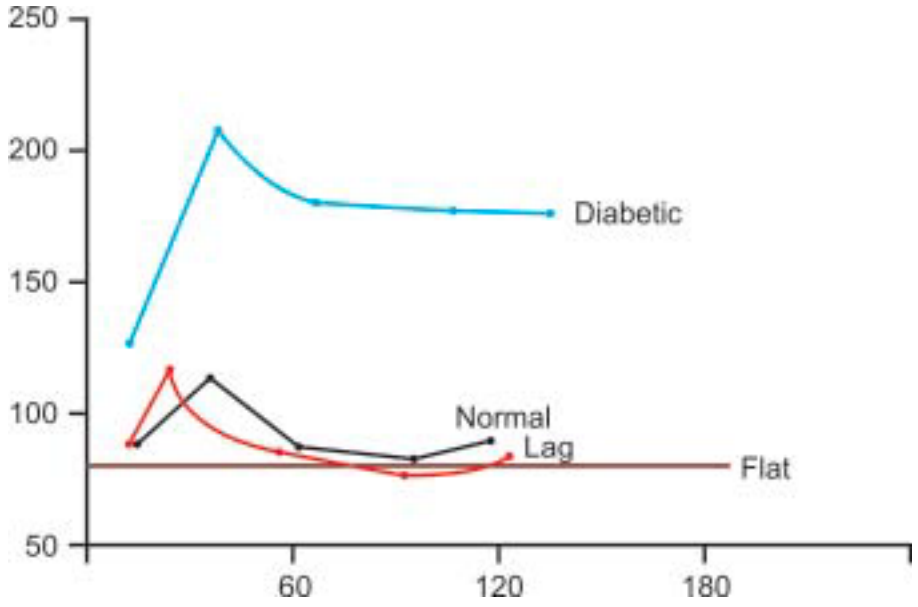


Fig.: Oral glucose tolerance

Increased GTT

Lag Curve

Exaggerated rise in blood glucose occurs after an oral load of glucose, but, levels quickly fall and 2 hours concentration is normal. Also, transient glycosuria occurs. It is seen following rapid gastric emptying in some hyperthyroid patients and after partial gastrectomy.

VIVA KEY

1. Normal range of fasting B glucose 60–90 mg/dL (enzymatic levels is method)
2. Renal threshold for glucose is 180 mg/dL
3. Dose of glucose given for GTT 1g/kg body weight
4. Role of NaF/fluoride in blood Inhibits glycolysis glucose sampling (enolase step)
5. Principle of inverse colorimetry Yellow potassium ferricyanide is reduced to colorless potassium ferrocyanide by glucose
6. Flat curve on GTT is seen in Hypopituitarism
7. Oxygen acceptor used in GOD-POD 4-amino-antipyrine method
8. Measure to assess long-term control Glycosylated Hb of glucose levels
9. Which type of diabetes is ketosis prone? IDDM

10. Hypoglycemia in diabetic patient If patient takes insulin occur when or oral hypoglycemic agents but skips the meal

DOAP session – Demonstrate, Observe, Assess, Perform

DOAP sessions

BI11.22 Calculate albumin: globulin (AG) ratio and creatinine clearance

ALREADY IN BOOK

BI11.23 Calculate energy content of different food Items, identify food items with high and low glycemic index and explain the importance of these in the diet

ALREADY IN BOOK

BI11.24 Enumerate advantages and/or disadvantages of use of unsaturated, saturated and trans fats in food

ALREADY IN BOOK

DOAP session – Demonstrate, Observe, Assess, Perform