Course: Practical Clinical Biochemistry, 5th stage
College of Pharmacy, University of Anbar
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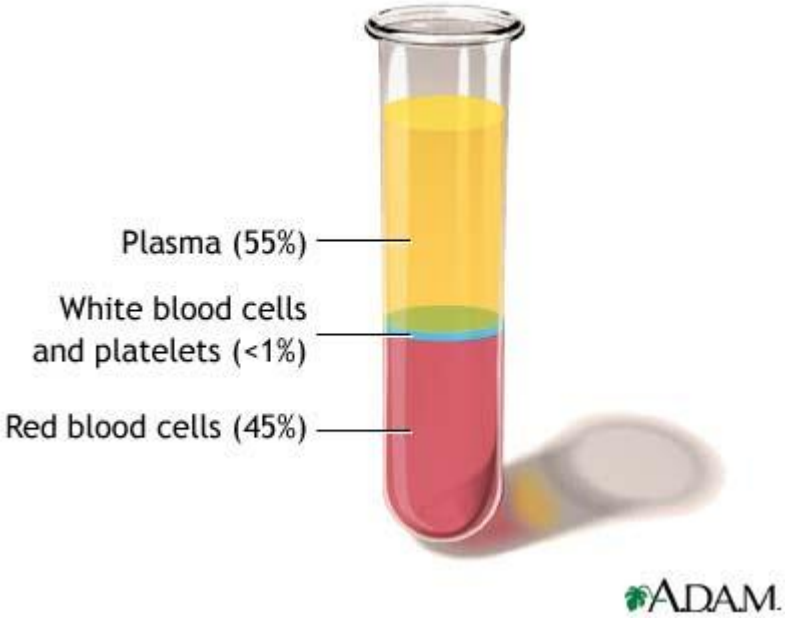
Practical No: 1

TYPES OF SPCEMIN

There are three types of blood specimens- serum, plasma and whole blood. Each different specimen is collected for various reasons. When blood is removed from the body, typically, it will coagulate or clot within 30 to 60 minutes. Serum can be separated from blood by centrifugation. Centrifugation is a process that spins the blood at high speeds in a machine called a centrifuge. This spinning separates the serum from the blood cells enmeshed in blood clot. Blood serum looks pale-yellow and has a similar composition to plasma. However, serum does not contain fibrinogen. Laboratory tests, like chemistry and immunology test are commonly performed on serum. Coagulation tests cannot be performed on serum because the coagulation factors are separated out of the serum during the centrifuge process.

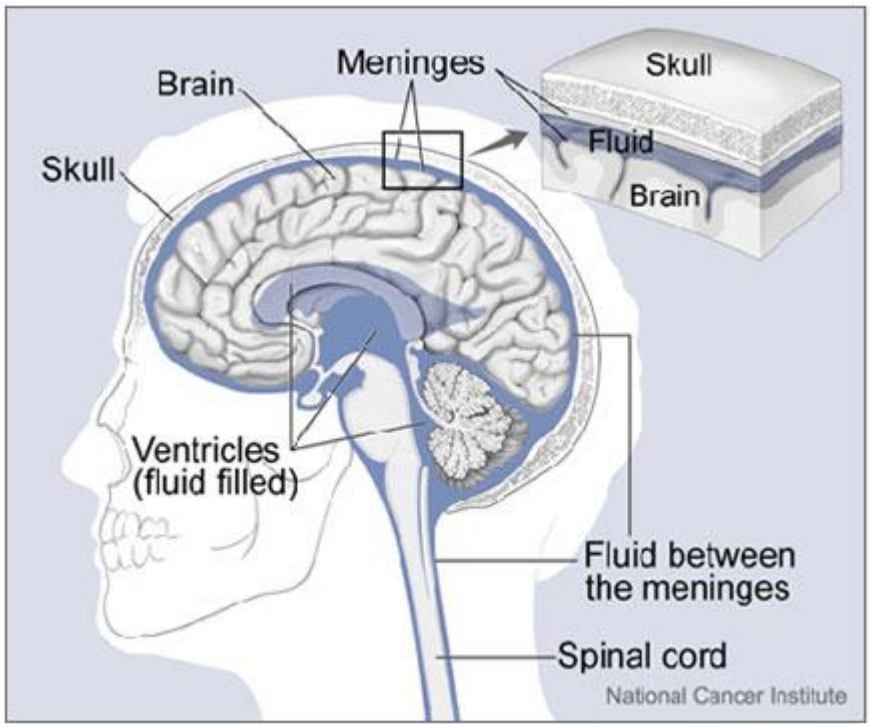
1- Whole blood specimens are usually required for hematology tests. These types of tests require the blood to remain in the same form as it is in the bloodstream. It is important that the blood specimen does not clot or separate. An anticoagulant must be added and the specimen should be mixed for at least 2 minutes immediately before performing the test.



2- Urine has a long, rich history as a source for measuring health and well-being and remains an important tool for clinical diagnosis. The clinical information obtained from a urine specimen is influenced by the collection method, timing and handling.

3- Saliva testing is a diagnostic technique that involves laboratory analysis of saliva to identify markers of endocrine, immunologic, inflammatory, infectious, and other Types of conditions. Saliva is a useful biological fluid for assaying steroid hormones such as cortisol, genetic material like RNA, proteins such as enzymes and antibodies, and a variety of other substances. Saliva testing is used to screen for or diagnose numerous conditions and disease states, including Cushing's disease, anovulation, HIV, cancer, parasites, hypogonadism, and allergies.

4- Cerebrospinal fluid (CSF) is a clear watery liquid that is formed and secreted by the choroid plexus, a special tissue that has many blood vessels and that lines the small cavities or chambers (ventricles) in the brain. A bout 17 ounces (500 mL) are produced each day. This rate of production means that all of the CSF is replaced every few hours. A CSF analysis is a group of tests that evaluate substances present in CSF in order to diagnose conditions affecting the central nervous system.



Anticoagulant

An **anticoagulant** is a substance that prevents coagulation (clotting) of blood. A group of pharmaceuticals called anticoagulants can be used *in vivo* as a medication for thrombotic disorders. Some anticoagulants are used in medical equipment, such as test tubes, blood transfusion bags, and renal dialysis equipment.

Heparin is a biological substance, usually made from pig intestines. It works by activating antithrombin III, which blocks thrombin from clotting blood. Heparin can be used *in vivo* (by injection), and also *in vitro* to prevent blood or plasma clotting in or on medical devices.

Anticoagulants outside the body

Laboratory instruments, blood transfusion bags, and medical and surgical equipment will get clogged up and become nonoperational if blood is allowed to clot. In addition, test tubes used for laboratory blood tests will have chemicals added to stop blood clotting. Apart from heparin, most of these chemicals work by binding calcium ions, preventing the coagulation proteins from using them.

- EDTA is denoted by mauve or purple caps on Vacutainer(A **vacutainer** blood collection tube is a sterile glass or plastic tube with a closure that is evacuated to create a vacuum inside the tube facilitating the draw of a predetermined volume of liquid. Most commonly used to draw a blood sample directly from the vein, these also are used to collect urine samples) brand test tubes. This chemical strongly and irreversibly binds calcium. It is in a powdered form.
- Citrate is usually in blue Vacutainer tube. It is in liquid form in the tube and is used for coagulation tests, as well as in blood transfusion bags. It binds the calcium, but not as strongly as EDTA. Correct proportion of this anticoagulant to blood is crucial because of the dilution. It can be in the form of sodium citrate or ACD.
- Oxalate has a mechanism similar to that of citrate. It is the anticoagulant used in fluoride (grey top) tubes.

Practical No: 2

Spectrophotometry

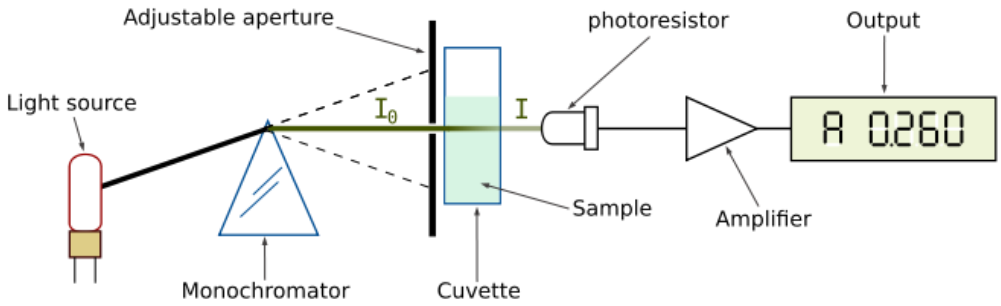
In chemistry, **spectrophotometry** is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared.

Spectrophotometry involves the use of a spectrophotometer. A spectrophotometer is a photometer that can measure intensity as a function of the light source wavelength. Important features of spectrophotometers are spectral bandwidth and linear range absorption or reflectance measurement

In short, the sequence of events in a modern spectrophotometer is as follows:

1. The light source is imaged upon the sample
2. A fraction of the light is transmitted or reflected from the sample
3. The light from the sample is imaged upon the entrance slit of the monochromator

The monochromator separates the wavelengths of light and focuses each of them onto the photodetector sequentially



Beer-Lambert Law

Introduction

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as:

$A = a(\lambda) * b * c$

where A is the measured absorbance, $a(\lambda)$ is a wavelength-dependent absorptivity coefficient, b is the path length, and c is the analyte concentration. When working in concentration units of molarity, the Beer-Lambert law is written as:

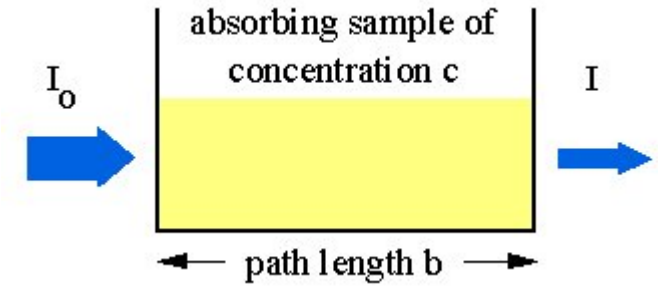
$A = \varepsilon * b * c$

where ε is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}.

Instrumentation

Experimental measurements are usually made in terms of transmittance (T), which is defined as:
$T = I / I_o$
where I is the light intensity after it passes through the sample and I_o is the initial light intensity. The relation between A and T is:
$A = -\log T = -\log (I / I_o)$.

Absorption of light by a sample



Modern absorption instruments can usually display the data as either transmittance, %-transmittance, or absorbance. An unknown concentration of an analyte can be determined by measuring the amount of light that a sample absorbs and applying Beer's law. If the absorptivity coefficient is not known, the unknown concentration can be determined using a working curve of absorbance versus concentration derived from standards.

Practical No: 3

BLOOD SUGAR

Blood sugar concentration, or **glucose level**, refers to the amount of glucose present in the blood of a human or animal. Normally, in mammals the blood glucose level is maintained at a reference range between about 3.6 and 5.8 mM (mmol/l). It is tightly regulated as a part of metabolic homeostasis.

Normal values

Fasting blood sugar=80-120mg/dl.

Random blood sugar=110-180mg/dl.

The homeostatic mechanism which keeps the blood value of glucose in a remarkably narrow range is composed of several interacting systems, of which hormone regulation is the most important.

There are two types of mutually antagonistic metabolic hormones affecting blood glucose levels:

catabolic hormones (such as glucagon, growth hormone, cortisol and catecholamines) which increase blood glucose;

and one anabolic hormone (insulin), which decreases blood glucose.

Blood glucose laboratory tests:

- ☒ Fasting blood sugar (ie, glucose) test (FBS)
- ☒ Urine glucose test
- ☒ Two-hr postprandial blood sugar test (2-h PPBS)
- ☒ Oral glucose tolerance test (OGTT)
- ☒ Intravenous glucose tolerance test (IVGTT)
- ☒ Glycosylated hemoglobin (HbA_{1C}) .
- ☒ Random blood glucose.

Hyperglycemia

Hyperglycemia or **Hyperglycæmia**, or **high blood sugar**, is a condition in which an excessive amount of glucose circulates in the blood plasma. This is generally a glucose level higher than (200 **mg/dl**). Reference ranges for blood tests are 11.1 **mmol/l**, but symptoms may not start to become noticeable until even higher values such as 250–300 mg/dl or 15–20 mmol/l. A subject with a consistent range between 100 and 126.

Signs and symptoms

- Polyphagia - frequent hunger, especially pronounced hunger
- Polydipsia - frequent thirst, especially excessive thirst
- Polyuria - frequent urination
- Blurred vision
- Fatigue (sleepiness)
- Weight loss
- Poor wound healing (cuts, scrapes, etc.)
- Dry mouth

In diabetes mellitus, hyperglycemia is usually caused by low insulin levels (Diabetes mellitus type 1) and/or by resistance to insulin at the cellular level (Diabetes mellitus type 2), depending on the type and state of the disease.

Low blood sugar

If blood sugar levels drop too low, a potentially fatal condition called hypoglycemia develops. Symptoms may include lethargy, impaired mental functioning; irritability; shaking, twitching, weakness in arm and leg muscles; pale complexion; sweating; paranoid or aggressive mentality and loss of consciousness. Brain damage is even possible.

Principle of glucose measurement:

Glucose is determind after enzymatic oxidation by the enzyme glucose oxidase,the formed hydrogen peroxide reacts under the catalysis of peroxidase withphenol and 4-aminophenazone to give the red-violet quinoneimine dye as indicator.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4\,H_2O$$

Procedure:

Standard	Test	Reagents
	10µl	Serum
10 µl		Standard
1ml	1ml	Enz&dye reagent

Mix, incubate for 10 minutes at 20-25 **C or for 5 minutes at 37C.Measure the absorbance for each tube ,read at 500 nm.**

Calculation:

$$\text{Concentration of serum glu} = \frac{\text{Absorption of sample}}{\text{Absorption of standard}} \times \text{Conc of Standard}$$

(mg /dl or mmol /L)
Concentration of Standard is 100mg/dl or 5.5 mmol/L.

Practical No: 4

LIPID TESTES

Triglyceride

Triglyceride is a glyceride in which the glycerol is esterified with three fatty acids It is the main constituent of vegetable oil and animal fats.

Triglycerides, as major components of very low density lipoprotein (VLDL) and chylomicrons, play an important role in metabolism as energy sources and transporters of dietary fat.

Reference ranges for blood tests, showing usual ranges for triglycerides (increasing with age)

Interpretation	**Level** mmol/L	**Level** mg/dL
Normal range, low risk	<1.69	<150
Borderline high	1.70-2.25	150-199
High	2.26-5.65	200-499
Very high: high risk	>5.65	>500

Please note that this information is relevant to triglyceride levels as tested after fasting 8 to 12 hours. Triglyceride levels remain temporarily higher for a period of time after eating.

Causes Of *hypertriglyceridemia*

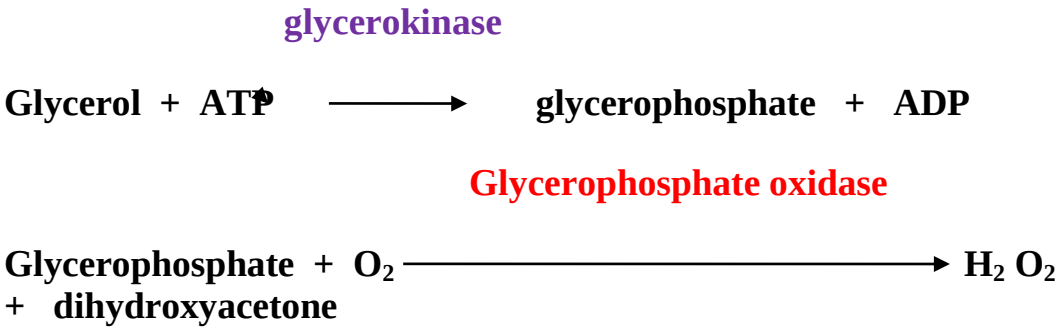
- Idiopathic (constitutional)
- Obesity
- High carbohydrate diet
- Diabetes mellitus.
- Excess alcohol intake
- Nephrotic syndrome
- Hypothyroidism (underactive thyroid)

Low triglyceride levels may be due to:

- Low fat diet
- Hyperthyroidism
- Malabsorption syndrome
- Malnutrition

Principle of triglyceride measurement;

Enzymatically; lipase hydrolyzes TG to glycerol and fatty acids. Glycerol is then phosphorylated in the following reaction:



H $_2O_2$ is measured in a peroxidase catalyzed reaction that forms a colored dye. And then we measure by spectrophotometer.

Cholesterol

About 20–25% of total daily cholesterol production occurs in the liver; other sites of high synthesis *rates include the intestines, adrenal glands, and reproductive organs. Synthesis within the* body starts with one molecule of acetyl CoA and one molecule of acetoacetyl-CoA. Since cholesterol is insoluble in blood, it is transported in the circulatory system within lipoproteins.

Blood cholesterol levels and risk for heart disease:

Interpretation	**Level** mmol/L	**Level** mg/dL
Desirable level corresponding to lower risk for heart disease	< 5.0	< 200
Borderline high risk	5.2–6.2	200–240
High risk	> 6.2	> 240

However, as today's testing methods determine LDL ("bad") and HDL ("good") cholesterol separately, this simplistic view has become somewhat outdated. The desirable LDL level is considered to be less than 100 mg/dL (2.6 mmol/L).

Clinical significance

Hypercholesterolemia

Hypercholesterolemia is the presence of high levels of cholesterol in the blood. It is not a disease but a metabolic derangement that can be secondary to many diseases and can contribute to many forms of disease, most notably cardiovascular disease(CVD). Familial hypercholesterolemia is a rare genetic disorder that can occur in families, where sufferers cannot properly metabolized cholesterol.

abnormally high cholesterol levels (hypercholesterolemia); that is, higher concentrations of LDL and lower concentrations of functional HDL are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This disease process leads to myocardial infarction (heart attack)(MI), stroke, and peripheral vascular disease.

Hypocholesterolemia:

Abnormally low levels of cholesterol are termed *hypocholesterolemia*. Research into the causes of this state is relatively limited, and while some studies suggest a link with depression, cancer and cerebral hemorrhage

Lipoproteins

The largest lipoproteins, which primarily transport fats from the intestinal mucosa to the liver, are called chylomicrons. They carry mostly fats in the form of triglycerides and cholesterol. In the liver, chylomicron particles release triglycerides and some cholesterol. The liver converts unburned food metabolites into very low density lipoproteins (VLDL) and secretes them into plasma where they are converted to intermediate density lipoproteins(IDL), which thereafter are converted to low-density lipoprotein (LDL) particles and non-esterified fatty acids, which can affect other body cells.

LDL molecules, therefore, are the major carriers of cholesterol in the blood, and each one contains approximately 1,500 molecules of cholesterol ester. These LDL molecules are oxidized and taken up by macrophages, which become engorged and form foam cells. These cells often become trapped in the walls of blood vessels and contribute to artherosclerotic plaque formation. These plaques are the main causes of

heart attacks, strokes, and other serious medical problems, leading to the association of so-called LDL cholesterol (actually a lipoprotein) with "bad" cholesterol.

High-density lipoprotein (HDL) particles transport cholesterol back to the liver for excretion, but vary considerably in their effectiveness for doing this having large numbers of large HDL particles correlates with better health outcomes, and hence it is commonly called "good cholesterol". In contrast, having small amounts of large HDL particles is independently associated with atheromatous disease progression within the arteries.

LIPID PROFIL TEST:-

What is a lipid profile?

The lipid profile is a group of tests that are often ordered together to determine risk of coronary heart disease. They are tests that have been shown to be good indicators of whether someone is likely to have a heart attack or stroke caused by blockage of blood vessels or hardening of the arteries (atherosclerois). The lipid profile typically includes:

- Total cholesterol
- High density lipoprotein cholesterol (HDL-C) — often called good cholesterol
- Low density lipoprotein cholesterol (LDL-C) —often called bad cholesterol
- Triglycerides

Estimated LDL = [total cholesterol] − [total HDL] − [estimated VLDL].

Estimated VLDL=**TG/5**

Recommended range:-

Interpretation	**Level** mmol/L	**Level** mg/dL
Low HDL cholesterol, heightened risk for heart disease	<1.03	<40 for men, <50 for women
Medium HDL level	1.03–1.55	40–59
High HDL level, optimal condition considered protective against heart disease	>1.55	>60

A low HDL level may also be associated with:

- Familial combined hyperlipidemia
- Noninsulin-dependent diabetes (NIDD)
- Use of certain drugs such as anabolic steroids, antipsychotics, beta blockers, corticosteroids, and protease inhibitors.

LDL Cholesterol

Optimal: Less than 100 mg/dL (2.59 mmol/L)
Near/above optimal: 100-129 mg/dL (2.59-3.34 mmol/L)
Borderline high: 130-159 mg/dL (3.37-4.12 mmol/L)
High: 160-189 mg/dL (4.15-4.90 mmol/L)
Very high: Greater than 190 mg/dL (4.90 mmol/L)

Risk factors include:

- Cigarette smoking.
- Age (if you are a male 45 years or older or a female 55 years or older)
- Low HDL cholesterol (less than 40 mg/dL (1.04 mmol/L))
- Hypertension (Blood Pressure of 140/90 or higher or taking high blood pressure medications)
- Family history of premature heart disease.
- Diabetes

Practical No: 5

Bilirubin

Bilirubin is a breakdown product of heme (a part of hemoglobin in red blood cells). The liver is responsible for clearing the blood of bilirubin. It does this by the following mechanism: Bilirubin is taken up into hepatocytes, *conjugated* (modified to make it water-soluble), and secreted into the bile, which is excreted into the intestine.

Increased total bilirubin causes jaundice, and can signal a number of problems:

1. Pre hepatic: Increased bilirubin *production*. This can be due to a number of causes, including hemolytic anemia and internal hemorrhage.

2. Hepatic: Problems with the liver, which are reflected as deficiencies in bilirubin *metabolism* (e.g., reduced hepatocyte uptake, impaired conjugation of bilirubin, and reduced hepatocyte secretion of bilirubin). Some examples would be cirrhosis and viral hepatitis.

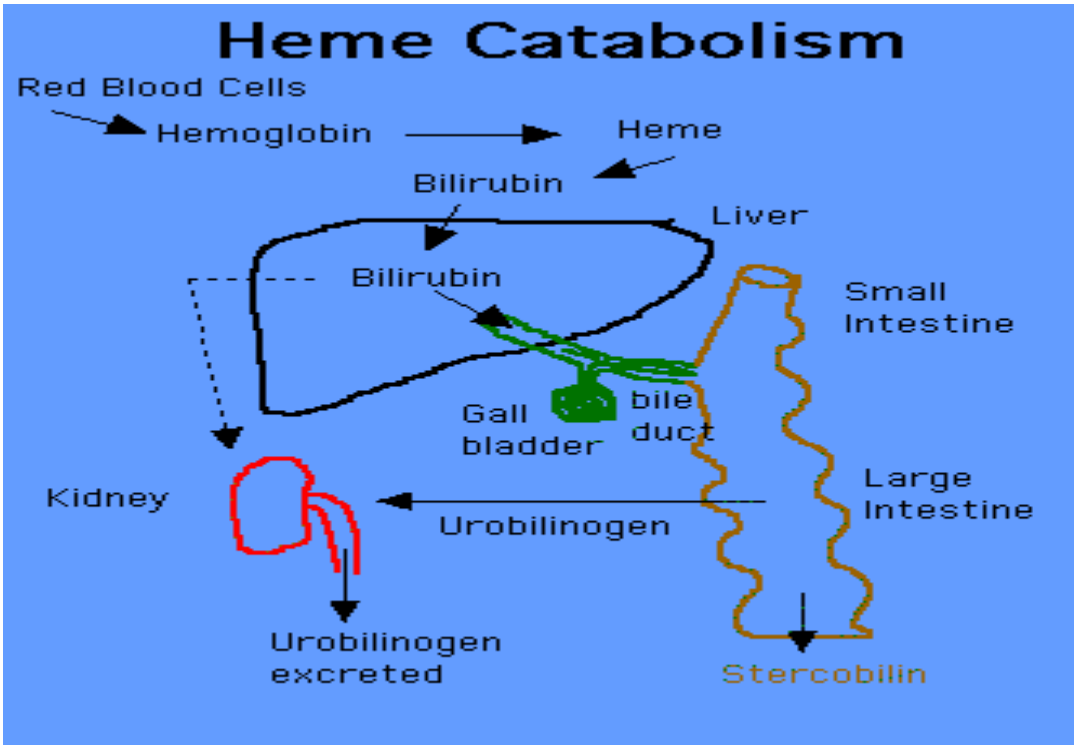
3. **Post hepatic:** Obstruction of the bile ducts, reflected as deficiencies in bilirubin *excretion*. (Obstruction can be located either within the liver or in the bile duct).

Direct bilirubin (conjugated bilirubin)

The diagnosis is narrowed down further by looking at the levels of direct bilirubin.

- If direct (i.e. conjugated) bilirubin is normal, then the problem is an excess of unconjugated bilirubin (indirect bilirubin), and the location of the problem is upstream of bilirubin excretion. Hemolysis, viral hepatitis, or cirrhosis can be suspected.

If direct bilirubin is elevated, then the liver is conjugating bilirubin normally, but is not able to excrete it. Bile duct obstruction by gallstones or cancer should be suspected



Large amounts of bilirubin in the blood can lead to jaundice. Jaundice is a yellow color in the skin, mucus membranes, or eyes.

Jaundice is the most common reason to check bilirubin levels.

- Most newborns have some jaundice. The doctor or nurse will often check the newborn's bilirubin level. See: Newborn jaundice
- The test may also be done in older infants, children, and adults who develop jaundice.

A bilirubin test will also be done if your doctor thinks you may have liver or gallbladder problems.

Normal Results

It is normal to have some bilirubin in your blood. Normal levels are:

- Direct (also called conjugated) bilirubin: 0.2 to 0.4 mg/dL
- Total bilirubin: 0.3 to 1.2 mg/dL

Note: mg/dL = milligrams per deciliter

Normal value ranges may vary slightly among different laboratories. Talk to your doctor about the meaning of your specific test results.

The examples above show the common measurements for results for these tests. Some laboratories use different measurements or may test different specimens.

What Abnormal Results Mean

In newborns, bilirubin levels are higher for the first few days of life. Your child's doctor must consider the following when deciding whether your baby's bilirubin levels are too high:

- cHow fast the level has been rising
- Whether the baby was born early
- How old the baby is

Jaundice can also occur when more red blood cells than normal are broken down. This can be caused by :(pre-hepatic)

- Hemolytic anemia
- Transfusion reaction

The following liver problems may also cause jaundice or high bilirubin levels (hepatic):

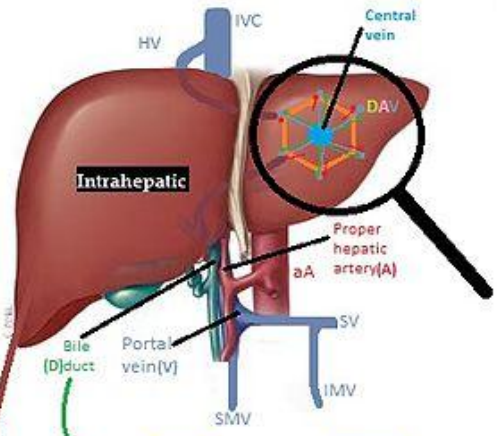
- Cirrhosis (scarring of the liver)
- Hepatitis
- Gilbert's disease

The following problems with gallbladder or bile ducts may cause higher bilirubin levels (post-hepatic):

- Biliary stricture
- Cancer of the pancreas or gallbladder
- Gallstones

Prehepatic

Excessive hemolysis(Malaria etc.)--more heme---goes to spleen---converts into unconjugated bilirubin(binds to albumin as non soluble)----Rise in unconjugated bilirubin (Indirect)



Cholestasis- **ALP** raised and **GGT** +ve
(Extrahepatic) Conjugated bilirubin **(Direct)** raised

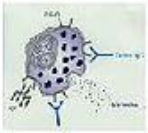
Intrahepatic damage- **PT time** also raised

because Coagulation factors(All except factor 8) are synthesized in liver.

Icterus- sign of Hyperbilirubinmia

Raised ALT/AST in -:

A-Autoimmune disease



B- Hepatitis B

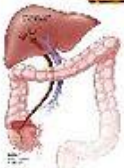
C- Hepatitis C

D- Drugs(eg. paracetamol)

E- Ethanol(Alcohol)

F- Fatty liver(Non alcoholic steatohepatitis)

G- Growth(Tumor)



H- Hemochromatosis

I- Iron, Copper(Wilson's disease)

Determination of bilirubin

Principle:

Sulfanilic acid reacts with sodium nitrite to form diazotized sulfanilic acid. In the presence of Dimethyl sulfoxide, total bilirubin reacts with diazotized sulfanilic acid to form azobilirubin. In the absence of Dimethyl sulfoxide only direct bilirubin reacts with diazotized sulfanilic acid to form azobilirubin

Procedure:

Total bilirubin

	Reagent Blank	Test
Total bilirubin reagent	1000 µL	1000 µL
Activator Total	20 µl	20 µl
Serum / calibrator	-	50 µL

Mix well and incubate exactly 5 minutes at 37 C. Measure the absorbance of calibrator and test against reagent blank at 546 /630 nm.

Calculation

Total bilirubin= OD of test – OD of reagent blank × 29.0 (Factor)

Direct bilirubin

	Reagent Blank	Test
Direct bilirubin reagent	1000 µL	1000 µL
Activator Direct	20 µl	20 µl
Serum / calibrator	-	50 µL

Mix well and incubate exactly 5 minutes at 37 C. Measure the absorbance of calibrator and test against reagent blank at 546 /630 nm.

Calculation

Direct bilirubin= OD of test – OD of reagent blank × 20.0 (Factor)

Note: Sometimes you find different procedure for each biochemical test when you use different kit.

Renal Function Test

Renal function, in nephrology, is an indication of the state of the kidney and its role in renal physiology. **Glomerular filtration rate** (**GFR**) describes the flow rate of filtered fluid through the kidney. **Creatinine clearance rate** ($\mathbf{C_{Cr}}$) is the volume of blood plasma that is cleared of creatinine per unit time and is a useful measure for approximating the GFR. Both GFR and C_{Cr} may be accurately calculated by comparative measurements of substances in the blood and urine.

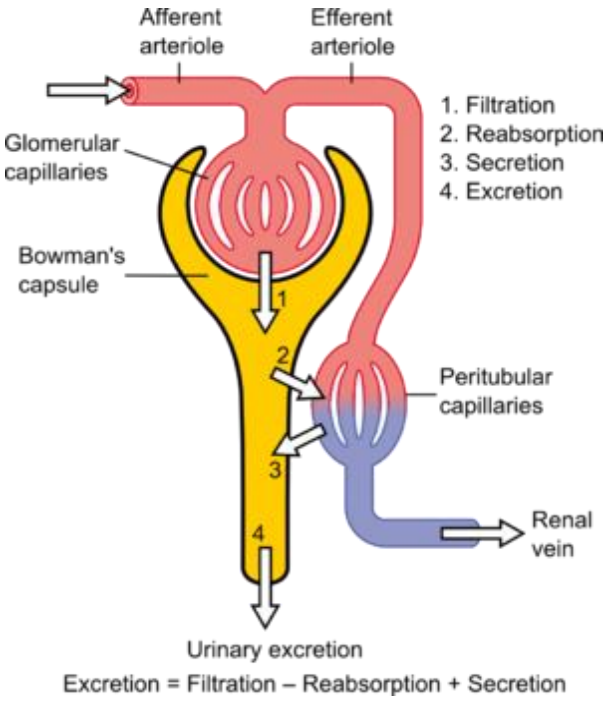


Diagram showing the basic physiologic mechanisms of the kidney

Blood urea nitrogen

The blood urea nitrogen (BUN) test is a measure of the amount of nitrogen in the blood in the form of urea, and a measurement of renal function. Urea is a substance secreted by the liver, and removed from the blood by the kidneys.

Physiology:

The liver produces urea in the urea cycle as a waste product of the digestion of protein. Normal human adult blood should contain between (15-40) mg/dl.

The most common cause of an elevated B.Urea (azotemia) is poor kidney function, although a serum creatinine level is a somewhat more specific measure of renal function

A greatly elevated BUN (>60 mg/dL) generally indicates a moderate-to-severe degree of renal failure. Impaired renal excretion of urea may be due to temporary conditions such as dehydration or shock, or may be due to either acute or chronic disease of the kidneys themselves.

An elevated Blood Urea

Decrease of blood flow to the kidney (as seen in heart failure or dehydration) without indicating any true injury to the kidney. However, an isolated elevation of B.Urea may also reflect excessive formation of urea without any compromise to the kidneys.

Increased production of urea is seen in cases of moderate or heavy bleeding in the upper gastrointestinal tract (e.g. from ulcers). The nitrogenous compounds from the blood are resorbed as they pass through the rest of the GI tract and then broken down to urea by the liver. Enhanced metabolism of proteins will also increase urea production, as may be seen with high protein diets, steroid use, burns, or fevers.

A low BUN usually has little significance:-

1) Liver problems.
2) Malnutrition (insufficient dietary protein).
3) Excessive alcohol consumption.
4) Over hydration.
5) During pregnancy.

Determination of serum urea concentration:

Principle

Determination of urea is by the indirect method using the urease-modified Berthelot reaction. Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide as in the following equation:

Urea + H_2O $\xrightarrow{\text{urease}}$ 2 NH_3 + CO_2

In an alkaline medium, the ammonium ions react with the salicylate and hypochlorite to form a green colored dye the absorbance of which is proportional to the urea concentration in the sample.

Procedure

Wavelength: 600 nm
Temperature: 37° C or (20-25°C)
Conc. Of Standard: 50 mg/dl or 13.3 mmol/

Working reagent: Mix 1 volume of **R1**+ 24 volumes of **R2**. Stable for 4 weeks at 2- 8 ° C. and 7 days at 15 -25 ° C.

Tubes	Blank	Sample	Cal.Standard
Working reagent	**1.0 ml**	**1.0 ml**	**1.0 ml**
Sample	**-**	**10 µl**	**-**
Cal. Standard	**-**	**-**	**10 µl**

- Mix and incubate the tubes for 5 minutes at 37 ° C. or for 10 minutes at room temperature (16 -25 ° C.)
- **Pipette**

R3	**1.0 ml**	**1.0 ml**	**1.0 ml**

- Mix and incubate the tubes for 5 minutes at 37° C. or for 10 minutes at room temperature (16 -25 ° C.)

- Read the absorbance A of the samples and the standards at 600 nm against the reagent blank.

Calculation

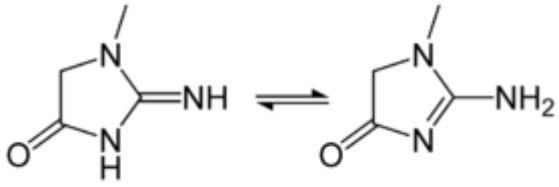
$$\text{Urea conc.} = \frac{A\ \text{test} - A\ \text{blank}}{A\ \text{standard} - A\ \text{blank}} \times \text{conc. of standard}$$

Normal Values:

Newborns <10 Days = 6.4 -53.5 mg/dl (1.1-9.0 mmol/L)
Adult (12 - 60 Years) = 15 - 40 mg/ dl (2.5 -6.6 mmol/L)

Serum Creatinine

Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). Chemically, creatinine is a spontaneously formed cyclic derivative of creatine. Creatinine is chiefly filtered out of the blood by the kidneys,



reference ranges:

The typical human reference ranges for serum creatinine are:

For women (0.5 to 1.0) mg/dL (about 45-90 µmol/L).

For men (0.7 to 1.2) mg/dL (60-110 µmol/L).

What causes kidney failure?

Kidney failure can occur from an acute situation or from chronic problems.

In acute renal failure, kidney function is lost rapidly and can occur from a variety of insults to the body. The list of causes is often categorized based on where the injury has occurred.

Prerenal causes:

Causes are due to decreased blood supply to the kidney. Examples:

- Hypovolemia (low blood volume) due to blood loss
- Dehydration from loss of body fluid (vomiting, diarrhea, sweating, fever
- Poor intake of fluids
- Medication, for example, diuretics ("water pills") may cause excessive water loss.
- Loss of blood supply to the kidney due to obstruction of the renal artery or vein.

Renal causes:

(Damage directly to the kidney itself) include:

- Multiple Myeloma
- Acute glomerulonephritis or inflammation of the glomeruli, the filtering system of the kidneys. Many diseases can cause this inflammation.

Post renal causes:

Due to factors that affect outflow of the urine:

- Obstruction of the bladder or the ureters can cause back pressure when there is no place for the urine to go as the kidneys continue to work. When the pressure increases enough, the kidneys shut down.
- Prostatic hypertrophy or prostate cancer may block the urethra and prevents the bladder from emptying.
- Tumors in the abdomen that surround and obstruct the ureters.
- Kidney stones

Chronic renal failure develops over months and years. The most common causes of chronic renal failure are related to:

- Poorly controlled diabetes
- Poorly controlled high blood pressure
- Chronic glomerulonephritis

***Determination of creatinine*:**

1. *Chemical method (Jaffe reaction):*

Principle: Creatinine in protein-free filtrate of serum or diluted urine in alkaline solution reacts with picric acid to form a red-orange chromogen the absorbance of which is proportional to the creatinine concentration in the sample.

Deproteinization procedure:

Trichloroacetic acid (TCA)	1.0 ml
Serum	1.0 ml

Mix well & Centrifuge at 2500 rpm for 10 min, and then collect the supernatant which can be stored up to 7 days at +2 to +8˚C.

Procedure

Prepare three test tubes and add reagents according to the table:

Wavelength: 520 nm
Temperature 25˚C

Blank	Standard	Sample	
–	–	1.0 ml	Supernatant
–	0.5 ml	–	Standard
0.5 ml	0.5 ml	0.5 ml	NaOH
0.5 ml	0.5 ml	0.5 ml	Picric acid
0.5 ml	0.5 ml	–	TCA
0.5 ml	–	–	Distilled water
Mix well, let stand for 15 min at 25˚**C** then measure the absorbance of sample and standard against blank.			

Calculation

$$\text{Creatinine conc.} = \frac{A\ \text{test} - A\ \text{blank}}{A\ \text{standard} - A\ \text{blank}} \times \text{conc. of standard}$$

2. *Kinetic method (Modified Jaffe reaction):*

Procedure

Wavelength:	510 nm
Temperature:	37 °C
Conc. Of Standard:	2 mg/dl or 176.8 µmol/L
Zero adjustment	*distilled water*

Working reagent : Mix 1 volume of R1 + 1 volume of R2 . Stable for 1 week at room temperature, stored tightly closed and protected from light.

Working reagent	1.0 ml
Sample or standard	100 µl

- Mix gently. Insert cuvette into the temperature –controlled instrument and start stopwatch.
- Record absorbance at 510 nm after 30 seconds A1 and after 90 seconds A2 of the sample or standard addition.

Calculation

$$\frac{(A2 - A1)\ \text{sample}}{(A2 - A1)\ \text{standard}} \times C\ \text{standard} = \text{mg/dl creatinine}$$

Interfering factors:

- Acetone, vitamin C, glucose, and cephalosporins falsely↑ creatinine readings.
- Bilirubin and hemoglobin falsely↓ creatinine readings.

THE END

Practical No: 7

Uric acid

- **Uric acid** (or **urate**) is a heterocyclic compound of carbon, nitrogen, oxygen, and hydrogen with the formula $C_5H_4N_4O_3$.

  Uric acid is formed from the breakdown of nucleic acids and is an end product of purine metabolism.

Biology

- Uric acid is produced by xanthine oxidase from xanthine and hypoxanthine, which in turn are produced from purine. Uric acid is more toxic to tissues than either xanthine or hypoxanthine. Uric acid is released in hypoxic conditions.

Medicine

- In human blood plasma, the reference range of uric acid is between

  (3.6 - 7.2) mg/dL (214-494)µmol/L.

Clinical Significance

- Disease states with increased plasma uric acid:
  - - Gout
  - - Increased catabolism of nucleic acids
  - - And renal disease
  -
- In Gout increased serum levels of uric acid lead to formation of **monosodium urate crystals** around the joints. This painful condition is the result of needle-like crystals of Uric Acid precipitating in joints and capillaries.
- Uric acid test is useful to assess for gout and to monitor patients with renal failure.

There are an other diseases as:

- Diabetes.
- Metabolic syndrome
- Uric acid stone formation
- *Cardiovascular disease*

Determination of uric acid:

- **Principle**

Uric acid is oxidized by uricase to allantoin and hydrogen perioxide, according to the following equation:

- Uric acid + O_2 + H_2O $\xrightarrow{\text{Uricase}}$ Allantoin + CO_2 + H_2O_2

- The formed H_2O_2 reacts under catalysis of peroxidase with 3,5-dichloro-2-hydroxybenzeze-sulphonic acid (DCHBS) and 4-aminophenazone (PAP) to give red-violet quinoneimine dye as indicator.

$$2H_2O_2 + \text{DCHBS+PAP} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + HCl + 4\,H_2O$$

Procedure

Wavelength: 510 nm
Temperature: 37° C or (20-25°C)
Conc. Of Standard: 6.0 mg/dl

Sample	Standard	Blank	
1 ml	1 ml	1 ml	Working reagent
—	20 µ	—	Standard
20 µ	—	—	Sample
Mix, incubate 5 min. at 37° C or 10 min. at 20-25° C. The color is stable for 30 min.			

Calculation

- Uric acid conc. (mg/dl) = $\frac{A\ \text{test} - A\ \text{blank}}{A\ \text{standard} - A\ \text{blank}} \times$ conc. of standard

(6 .0 mg/dl)

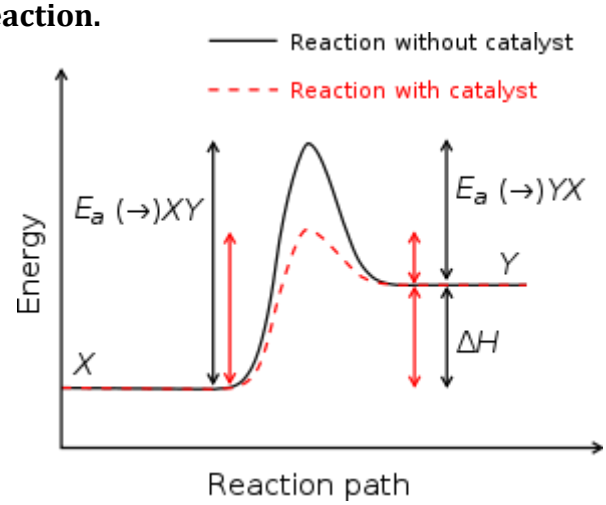
Practical No: 8

Enzyme specificity

Introduction - Enzyme Characteristics:

A living system controls its activity through enzymes. An enzyme is a protein molecule that is a biological catalyst with three characteristics. First, the basic function of an enzyme is to increase the rate of a reaction. Most cellular reactions occur about a million times faster than they would in the absence of an enzyme. Second, most enzymes act specifically with only one reactant (called a substrate) to produce products. The third and most remarkable characteristic is that enzymes are regulated from a state of low activity to high activity and vice versa. Gradually, you will appreciate that the individuality of a living cell is due in large part to the unique set of som e 3,000 enzymes that it is genetically programmed to produce. If even one enzyme is missing or defective, the results can be disastrous.

Catalysts: A catalyst is a substance that accelerates the rate of a chemical reaction but remains chemically unchanged afterwards. The catalyst increases rate reaction.



The presence of the catalyst opens a different reaction pathway (shown in red) with a lower activation energy. The final result and the overall thermodynamics are the same.

Enzyme Parts List:

The activity of an enzyme depends, at the minimum, on a specific protein chain. In many cases, the enzyme consists of the protein and a combination of one or more parts called cofactors. This enzyme complex is usually simply referred to simply as the enzyme includes:

Apoenzyme:
Cofactors: A cofactor is a non-protein substance which may be organic, and called a coenzyme.

The overall enzyme contains a specific geometric shape called the active site where the reaction takes place. The molecule acted upon is called the substrate

Urease occurs in many bacteria, several species of yeast and a number of higher plants. The enzyme is important in assaying for urea.

Specificity: Urease is specific for urea and hydroxyurea.

Factor Effect of Enzyme

(1) Enzyme Concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount;

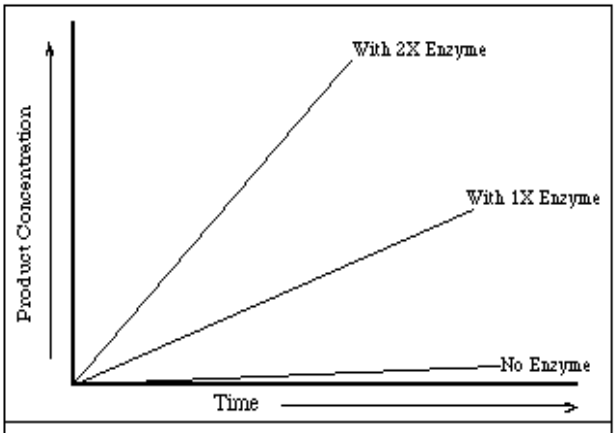


FIGURE 4
"Zero order" reaction rate is independent of substrate concentration.

These reactions are said to be "zero order" because the rates are independent of substrate concentration, and are equal to some constant k. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero order kinetics, allowing the assay to run for double time results in double the amount of product.

Procedure

We take 4 test tubes and add the same amount of substrate for the first 3 test tubes but change the amount of enzyme leads to change of color

As follow:

1- Add (1) drop of phenol oxidase enz.(potato extract) + 14 drops of D.W. in order to complete the volume to 1 .5 ml.
2 - Add (3) drops of potato extract + 12 drops of D.W.
3 - Add (7) drops of potato extract +8 drops of D.W.
4 - Add (14) drops of potato extract

Then add 2 drops of 0 .01 M of catechol at 37C and wait for 5 minutes in water bath , the following changes can be noticed :

1 st yellow
2 nd. Light brown
3 rd. brown
4 th dark brown

So we can say when increasing the amount of enzyme, the rate of reaction is increasing also.

(2) Substrate Concentration

It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum. After this point, increases in substrate concentration will not increase the velocity (delta A/delta T). This is represented graphically in Figure 2.

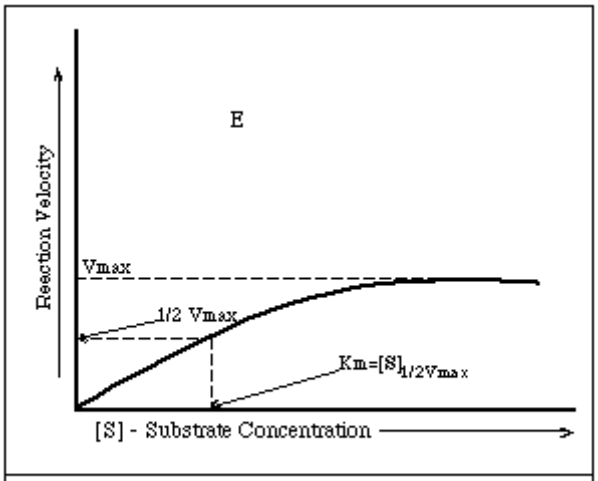


FIGURE 8
Effect of substrate concentration

Procedure:

- 0.5 ml of 0 .01M of catechol solution + 2.5 ml of D.W. in order to

Complete the volume to 3 ml

- 1ml of 0 .01M of catechol solution + 2ml of D.W.
- 2ml of 0 .01M of catechol solution +1 ml of D.W.
- 3 ml of 0 .01M of catechol solution

Then add 0.5 ml of potato extract and put the test tubes at 37C and Wait for 5 minutes in the water bath, the following changes can be Noticed:

1 st . Very light brown
2 nd . light brown
3 rd . brown
4 th . Dark brown
So the activity increased as the amount of substrate is increased until We reach to the maximal velocity (V_{max}) at which any increase because There is no enzyme is available to react.

(3) Temperature Effects

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. As shown in Figure 13, the reaction rate increases with

temperature to a maximum level, then abruptly declines with further increase of temperature. Because most animal enzymes rapidly become denatured at temperatures above 40°C, most enzyme determinations are carried out somewhat below that temperature.

Over a period of time, enzymes will be deactivated at even moderate temperatures. Storage of enzymes at 5°C or below is generally the most suitable. Some enzymes lose their activity when frozen.

Procedure:

Take 3 test tubes and put in each one 8 drops of potato extract +8
Drops of catechol and then :

- put the 1st one in ice bath(0C)
- put the 2nd one in your hand (37C)
- put the 3rd one in boiling water (70C)

The following changes can be noticed
1 st. no change
2 nd .brown
3 rd . colorless.

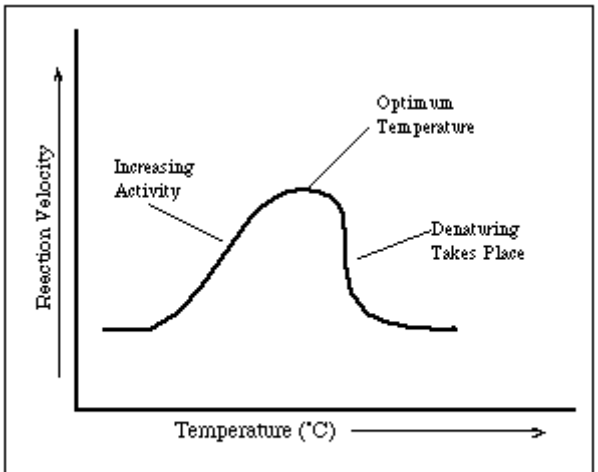


FIGURE 13
Effect of temperature on reaction rate

(4) Effects of pH

Enzymes are affected by changes in pH. The most favorable pH value - the point where the enzyme is most active - is known as the optimum pH. This is graphically illustrated in Figure.

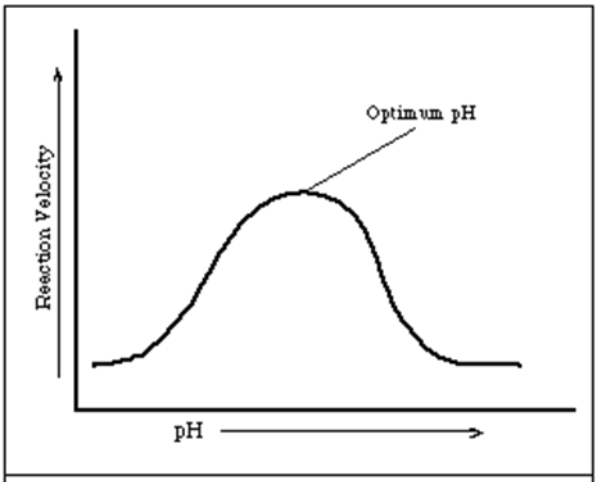


FIGURE 14
Effect of pH on reaction rate

Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability.

Procedure
In this experiment, the amount of
Enzyme and substrate are constant
And using the buffer solutions with varying PH values:

- 1 ml of PH (HCL) at value (1)
- 1ml of PH at value(5)
- 1ml of PH at value(7)
- 1ml of PH at value(11)

Liver function tests LFTs

Liver function tests (LFTs or LFs), are groups of clinical biochemistry laboratory blood assays designed to give information about the state of a patient's liver. The parameters measured include albumin, bilirubin (direct and indirect) and others. According to some, liver transaminases (AST/ALT (SGOT/SGPT) are *not* liver function tests, but are biomarkers of liver injury in a patient with some degree of intact liver function. Other sources include transaminases. Most liver diseases cause only mild symptoms initially, but it is vital that these diseases be detected early. Hepatic (liver) involvement in some diseases can be of crucial importance. This testing is performed by a medical technologist on a patient's serum or plasma sample Some tests are associated with functionality (e.g., albumin); some with cellular integrity (e.g., transaminase) and some with conditions linked to the biliary tract (gamma-glutamyl transferase and alkaline phosphatase). Several biochemical tests are useful in the evaluation and management of patients with hepatic dysfunction. These tests can be used to (1) detect the presence of liver disease, (2) distinguish among different types of liver disorders, (3) gauge the extent of known liver damage, and (4) follow the response to treatment. Some or all of these measurements are also carried out (usually about twice a year for routine cases) on those individuals taking certain medications- anticonvulsants are a notable example- in order to ensure that the medications are not damaging the person's liver.

- ## Albumin (Alb)

  Albumin is a protein made specifically by the liver, and can be measured cheaply and easily. It is the main constituent of total protein; the remaining froglobulins). Albumin levels are decreased in chronic liver disease, such as cirrhosis. It is also decreased in nephrotic syndrome, where it is lost through the urine.

  Reference Range: 3.5- 5.3 g/dl

Albumin levels may be **elevated** in:

1) Congestive heart failure
2) Poor protein utilization
3) Congenital

Albumin levels may be **decreased** in:

a. Dehydration
b. Hypothyroidism

c. Malnutrition - Protein deficiency
d. Dilution by excess H_2O (drinking too much water, which is termed "polydipsia," or excess administration of IV fluids)
e. Kidney losses (Nephrotic Syndrome)
f. Protein losing-enteropathy (protein is lost from the gastrointestinal tract during diarrhea)
g. Skin losses (burns, exfoliative dermatitis)
h. Liver dysfunction (the body is not synthesizing enough albumin and indicates very poor liver function)

Alanine transaminase (ALT)

Alanine transaminase (ALT), also called serum glutamic pyruvate transaminase (SGPT) or alanine aminotransferase (ALAT) is an enzyme present in hepatocytes (liver cells).

Reference range :
Up to 49 IU/L

Aspartate transaminase (AST)

Aspartate transaminase(AST) also called serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (ASAT) is similar to ALT in that it is another enzyme associated with liver parenchymal cells. It is raised in acute liver damage, but is also present in red blood cells and cardiac and skeletal muscle and is therefore not specific to the liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage. Elevated AST levels are not specific for liver damage, and AST has also been used as a cardiac marker.

Reference range : Up to 46 IU/L

- ***Alkaline phosphatase (ALP)***

Alkaline phosphatase (ALP) is an enzyme in the cells lining the biliary ducts of the liver. ALP levels in plasma will rise with large bile duct obstruction, intrahepatic cholestasis or infiltrative diseases of the liver. ALP is also present in bone and placental tissue, so it is higher in growing children (as their bones are being remodeled) and elderly patients with Paget's disease.

Reference range : 30- 120 IU/L

In humans, alkaline phosphatase is present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct,kidney, bone, and the placenta. Humans and most other mammals contain the following alkaline phosphatase isozymes:

- ALPI – intestinal
- ALPL – tissue non-specific (liver/bone/kidney)
- ALPP – placental (Regan isozyme)

Diagnostic use

The normal range is 20 to 140 IU/L. High ALP levels can show that the bile ducts are blocked Levels are significantly higher in children and pregnant women. Also, elevated ALP indicates that there could be active bone formation occurring as ALP is a byproduct of osteoblast activity (such as the case in Paget's disease of bone). Levels are also elevated in people with untreated Celiac Disease.

Lowered levels of ALP are less common than elevated levels.

Elevated levels

If it is unclear why alkaline phosphatase is elevated, isoenzyme studies using electrophoresis can confirm the source of the ALP. Heat stability also distinguishes bone and liver isoenzymes ("bone burns, liver lasts"). Placental alkaline phosphatase is elevated inseminomas and active form of Rickets.

Lowered levels

The following conditions or diseases may lead to reduced levels of alkaline phosphatase:

- Hypophosphatasia, an autosomal recessive disease.
- Postmenopausal women receiving estrogen therapy because of osteoporosis.
- Men with recent heart surgery, malnutrition, magnesium deficiency, hypothyroidism, or severe anemia.

- Wilson's disease.
- Oral contraceptives.

Gamma glutamyl transpeptidase (GGT)

Although reasonably specific to the liver and a more sensitive marker for cholestasis damage than ALP, Gamma glutamyl trans peptidase (GGT) may be elevated with even minor, sub-clinical levels of liver dysfunction. It can also be helpful in identifying the cause of an isolated elevation in ALP (GGT is raised in chronic alcohol toxicity.

Reference range
(0 – 42) I U/L

Determination of Liver function

Procedure:

General system parameter

	GPT	**GOT**
Mode of reaction	Kinetic	Kinetic
Wavelength	340 nm	340 nm
Factor	1745	1745
Reagent volume	1 ml	1m l

Working reagent: Mix 4 volume of R1 with the volume of R2. The working reagent is stable for 30 days at 2-8 °C.

Sample : serum or plasma (Free hemolysis)

Working reagent	Sample	standard
1000	100 µl	-----
1000	-----	100 µl

Mix and incubate at 37 °C for 1 minute. Measure the change in absorbance per minute (Δ OD/ min) during 3 minutes.

Calculation:

GPT, GOT = (Δ OD/ min) × 1745

PHOSPHATE

Phosphorus is a mineral found in a wide variety of foods. When you eat these foods, your body takes their phosphorus content and creates a related substance called phosphate. Most of this phosphate is held in your bones; however, a small percentage of it, called serum phosphate, circulates in your bloodstream.

SERUM PHOSPHATE:

Phosphate is formed in your body when phosphorus combines with oxygen. Only 1 percent of your body's phosphate supply circulates in your bloodstream, according to a July 7, 2010 article in the Journal of the American Academy of Physician Assistants. Roughly 90 percent of this circulating phosphate floats free, while the remaining 10 percent is bound to proteins in the blood. Serum phosphate plays a vital role in helping the body properly regulate its electrical status, as well as its acid-base balance, or pH. Typically, blood levels of phosphate range anywhere from 3.0 to 4.5 mg/dL.

Phosphorus tests are most often ordered along with other tests, such as those for calcium, parathyroid hormone (PTH), and/or vitamin D, to help diagnose and/or monitor treatment of various conditions that cause calcium and phosphorus imbalances.

While phosphorus tests are most commonly performed on blood samples, phosphorus is sometimes measured in urine samples to monitor elimination by the kidneys.

When is it ordered?

Since mildly abnormal phosphorus levels usually cause no symptoms, phosphorus testing is typically performed as a follow up to an abnormal calcium test and/or when symptoms of abnormal calcium such as fatigue, muscle weakness, cramping, or bone problems are present. Phosphorus testing may also be ordered along with other tests when symptoms suggest kidney and gastrointestinal disorders.

When conditions causing abnormal phosphorus and/or calcium levels are found, testing for both may be ordered at regular intervals to monitor treatment effectiveness. When someone has diabetes or signs of an acid-base imbalance, a doctor may sometimes monitor phosphorus levels.

What does the test result mean?

Low levels of phosphorus (hypophosphatemia) may be due to or associated with:

- Hypercalcemia, especially due to hyperparathyroidism
- Overuse of diuretics
- Malnutrition
- Alcoholism
- Severe burns
- Diabetic ketoacidosis (after treatment)
- Hypothyroidism
- Hypokalemia
- Chronic antacid use
- Rickets and osteomalacia (due to Vitamin D deficiencies)
-

Higher than normal levels of phosphorus (hyperphosphatemia) may be due to or associated with:

- Kidney failure
- Hypoparathyroidism
- Diabetic ketoacidosis (when first seen)
- Increased dietary intake (phosphate supplementation).

- Phosphate levels are normally higher in children than in adults because their bones are actively growing. Low phosphate levels in children can inhibit bone growth.
- Blood and urine levels of phosphorus may be affected by the use of enemas and laxatives containing sodium phosphate, excess dietary Vitamin D supplements, and by intravenous glucose administration.

Determination of PHOSPHORUS

Procedure:

Working reagent: Mix 1 volume of R1 + 1 volume of R2. Stable for 8 hr at 2-8 °C.

Cal Concentration : 5 mg/dL

Tubes	Sample	Standard
Working reagent	1.0 ml	1.0 ml
Sample	50 µl	--
Cal	--	50 µl

Mix , let stand the tubes for 1 minute and then pipette:

R3 Developer	0.5 ml	0.5 ml

Mix, and let the tubes stand 10 minutes at room temperature.

- Read the absorbance A of the sample and the standard at 740 nm against the reagent blank.

Calculation:

$$\frac{\textbf{A sample}}{\textbf{A standard}} \textbf{ X C standard = mg/dl}$$

Normal ranges:

Children	**4.0 – 7.0 mg/dl (1.29 -2.26 mmol/L)**
Men	**2.5 - 4.5 mg/dl (0.81 -1.45 mmol/L)**
Women	**1.5 – 6.8 mg/dl (0.48 – 2.19 mmol/L)**

THE END