Mustansiriyah University
Chemistry Department

Experiments in Clinical Biochemistry

(A hands-on approach)

by

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Created in 2003 and updated in 2019

Preface

Clinical Biochemistry brings together knowledge and skills from a variety of scientific disciplines including Biochemistry, Chemistry and Physiology. It applies these to detect, diagnose, treat and prevent diseases in human beings. On the basis, this Clinical Biochemistry handbook has been prepared for two main reasons:

1. To provide the required information about Biochemical basis and clinical significance of human disorders.
2. To supply accurate and specific methods for clinical determination of blood and urine contents. The results should offer clinical diagnosis and management of related diseases.

The present manual is designed for one-year course in Clinical Biochemistry for fourth year Science students at the Mustansyriah University, Department of Chemistry.

The availability of chemicals and the use of simple equipment were the main reason to the select experiments here. The Chemical and Biochemical principles are involved. These clinical tests are standard and done in clinical lab. in Iraqi hospitals.

This handbook has been firstly prepared in 2003 and authorized by the Chemistry Department-Mustansiriyah University.

Zahraa

Baghdad 2019

Biochemical specimens

The function of Clinical Biochemistry lab. is to perform quantitative and qualitative analysis of body fluids such as:

Blood, urine, cerebra spinal fluid (CSF), gastric juice, synovial fluid, duodenum, amniotic fluid, as well as saliva, faces and other materials.

The Blood:

Blood is a tissue circulates in a closed system of blood vessels. It consists of a cellular portion (red, white blood cells and the platelets), which forms (45%) of the blood volume and suspended in the cellular liquid medium plasma (55%) of blood volume.

Function of the Blood:

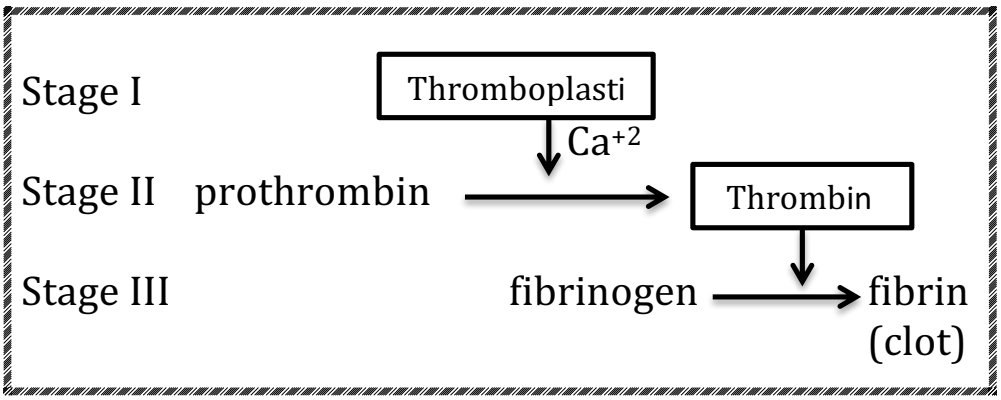
1. Respiration: Transport of oxygen from the lungs to the tissues and CO_2 from the tissues to the lungs.
2. Nutrition: Transport of absorbed food materials.
3. Excretion: Transport of metabolic wastes to the kidneys, lungs, skins, and intestines for removal.
4. Maintenance of normal acid-base balance in the body.
5. Regulation of water balance through the effect of blood on the exchange of water between the circulating fluid and the tissue fluid.
6. Regulation of the body temperature by the distribution of body heat.
7. Defense against infection by the white cells and the circulating antibodies.
8. Transport of hormones and metabolites.

The great majority of clinical chemistry analysis performed on blood and urine These analyses required the whole blood, serum or plasma to be collected from patients. When blood is drawn and allowed to clot, a clear liquid (serum) exuded from the clotted blood. Plasma on the other hand, separates from the cells only when blood is prevented from clotting (uncoagulated).

The clotting processes

The blood clot is formed by a protein (fibrinogen) which is present in a soluble form in the plasma and which is transformed to an insoluble network or fibrous material (fibrin).

The change of fibrinogen to fibrin is caused by thrombin, which in blood fluid exists as prothrombin. The conversion of prothrombin to thrombin depends on the cation of thromboplastin and Ca^{+2}. These stages may be diagrammed as follows:



Anticoagulants:

When the whole blood or plasma is required, an anticoagulant is required. The most commonly used are:

Heparin= is a normal blood constituent, but its physiological concentration is not high enough to prevent clotting in freshly down blood. it is a polysaccharide derivation and apparently function by inhibiting thrombin formation (anti-thrombin).
Oxalate= act by precipitating Ca^{+2} from the blood and prevents clotting. Potassium oxalate are used most commonly, also Li, Na and ammonium salts are used.
EDAT= acts similarly to oxalate, except that it chelates Ca^{+2} rather than precipitates it and therefore prevent clotting process.

Blood collection and handling

The general considerations relevant to the accuracy of subsequent chemical analysis are:

1. Stasis (stop the blood flow by a tourniquet) should be used for a minimum period of time since prolonged stasis may result in alteration of some chemical values.
2. Blood should not be taken while intravenous solutions are being administered, since these solutions may influence the chemical assay.
3. Syringes or evacuated tubes use to collect blood should be valid (not expire) to avoid contamination or hemolysis.

4. Some tests require anticoagulants therefore, gentle mixing is necessary to avoid clotting.

Centrifugation and removal of serum or plasma from the cells helps preserve the integrity of many constituents.

Refrigeration is perhaps the simplest and most reliable method for preservation of specimens for several days. Glycolysis and other enzymatic and bacteriological processes are slowed down considerably at lower temp. Refrigerated samples should be brought to the room temperature before they can be measured accurately.

Freezing the whole blood results in rupture of the red cells, but it does not injure plasma or serum. Therefore, storage of plasma or serum is helpful in preserving most enzymatic activities.

Calorimetric analysis

Most methods in clinical Biochemistry are based on quantitative measurement of a colored compound produced when a test sample is mixed with appropriate reagents. Such methods are referred to as **calorimetric** methods and measurements are made with instruments called **photoelectric calorimetric** or **spectrophotometer** in the visible region (400-750nm), where colors are normally visible**.** Furthermore, the amount of color produced should be proportional to the amount of substance being measured.

Light intensity and Beer's law:

Light intensity is of principle interest in spectrophotometry. When light is absorbed, its intensity is reduced, the amount of the absorbed light depends on the concentration of the absorbed substance and on the depth of the solution which the light pass through. This has been formalized into Beer's law:

$$A = a, b, c$$

Where:

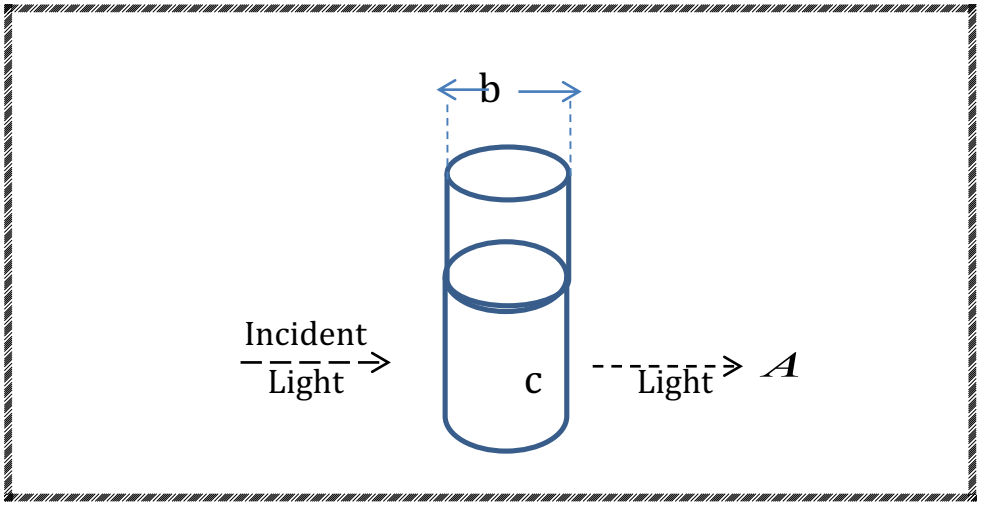
A= the absorbance of the light by the solution.

a= the molar absorptivity (constant).

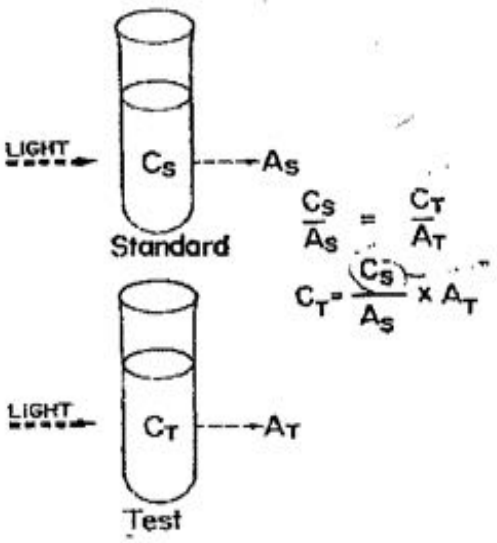
b= the thickness of solution in cm.

c= the molar concentration of the absorbing sub.

This is illustrated in the figure below:



It is reasonable that more concentration solution or longer light path should absorb more light, since in either case there are more absorbing molecules placed in the path of light. The quantitative application of Beer's is illustrated in the figure below:



Another measurement of change in light intensity is transmittance (T), which measures the amount of the light, passes through the solution. The (T) measurement is out of the scope of this handbook, however, the actual relationship between A and T is logarithmic one, $A = -\log T$.

Since some instruments are calibrated T in (%T) and

$T = 100 \times T$, then

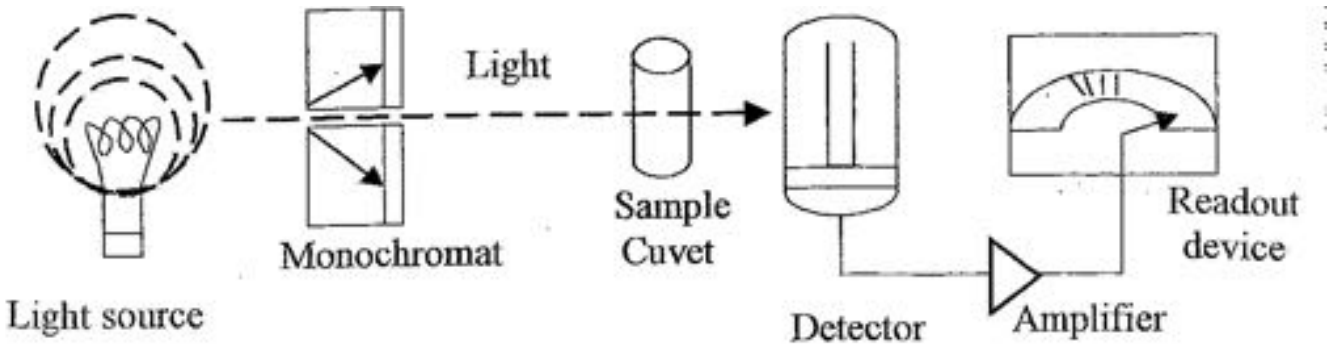
$$A = -\log\frac{\%T}{100} = -(\log \%T - \log 100) = \log \%T + \log 100$$

$$= 2 - \log \%T.$$

If readings are made in %T, they converted to A or used plotted values of %T against conc. on log.

Spectrophotometer

It is used to measure the absorbance of a solution at one or more wavelengths. The spectrophotometer produces light of a limited wavelength (monochromatic beam) to interact with the sample. This is done by prisms or diffraction grating. The components of the spectrophotometer are common to all, as illustrated below:



1. **Light source:** A tungsten filament lamp is used in the visible region (about 320 to at least 100nm) while a hydrogen-discharged lamp is used in the UV region (200-400nm).

2. **Wave length selector:** This device is used to isolate specific wavelengths or wavelength bands of light from the source. The principle component is either transmission or interference types, and monochormators are composed of either prisms or grating.
3. **Cuvette:** it is a transport container for samples which made of glass or plastic in a range of (320-1000nm), but quartz cuvette (silica) is for measurement below 320nm.
4. **Detector:** The detector measures light intensity by converting the light into an electrical signal. The more intense the light, the stronger the electrical signal. The most common detectors **are barrier layers** cells and **photomultiplier tubes**. Photo tubes and photoconductive tubes also are used.
5. **Readout device:** The electrical signal from the detector may be readout in terms of %Transmittance T or Absorbance A. this readout may be a digital display, a needle reading on a meter, galvanometer scale, or an ink signal on the chart paper of a recorder.

Experiment No.1

Determination of Blood Glucose

Glucose the Greek word for sweet, is one of the simple sugars product of carbohydrates or starches by digestive enzymes in the liver. Glucose is synthesized in the liver when dietary sources of glucose are not available from glycogen (glycogenolysis) or protein (gluconeogenesis). All cells use glucose as an important energy source. Some tissues (e.g. the central nrvous system, the brain and spinal cord) depend entirely on glucose. Glucose is stored in muscle and liver as glycogen by insulin hormone or it nay converted into keto acids, amino acids, and proteins or, it stored in adipose tissues, see Fig 1.

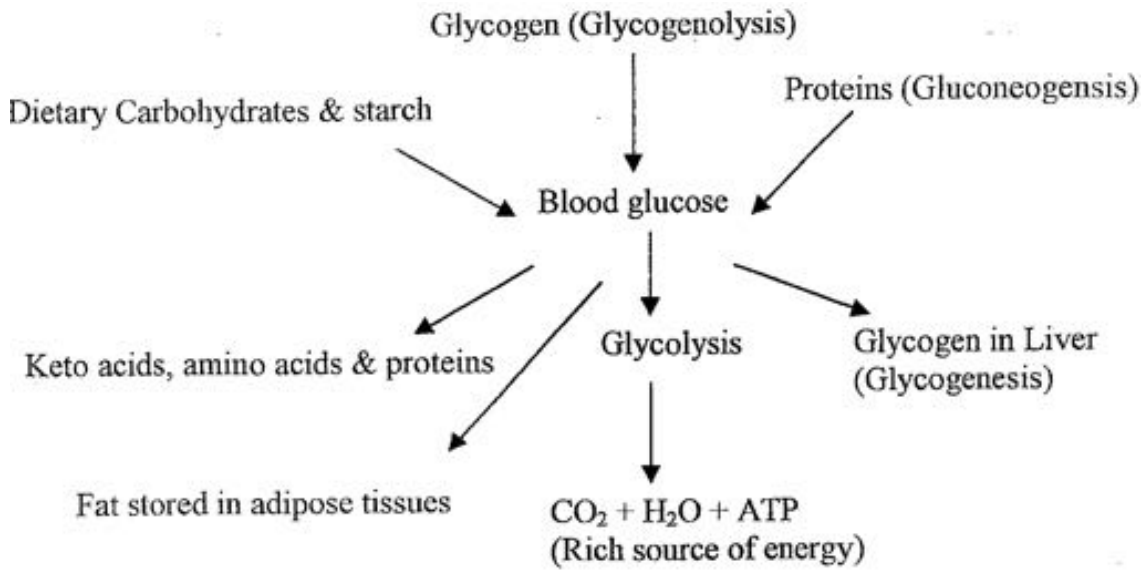


Fig 1. The glucose sources and metabolism pathways

There are many hormones affected blood sugar level, these include:

1. <u>Insulin</u>:

Produced by β- cells in the pancreas, reduces blood glucose by facilitating its entry into the tissue cells as glycogen form. A deficiency of insulin, increases blood glucose and causes Diabetes Mellitus.

2. Glycogen:

Produced by α- cells in the pancreas, its secretion is stimulated by hypoglycemia. Glycogen stimulates hepatic glycogenolysis and glycogenesis.

3. Growth hormones and Adrenocortico-trophic hormones :

These hormones are produced by anterior pituitary gland and caused increase in blood sugar level.

4. Hydrocortisone Hormone:

Secrete by adrenal cortex and stimulate gluconeogenesis.

5. Epinephrine and Thyroxine:

Epinephrine is secreted from adrenal medulla. Thyroxin is secreted by the thyroid gland. Both hormones stimulate glycogenolysis and raise blood glucose.

When plasma insulin level is law, for example during fasting, the hyperglycemic actions of glycogen, growth hormone, adrenocorticotrophic, hydrocortizon, epinephrine and thyroxin become apparently high to increase the glucose level.

Normal value of Blood Glucose: 70-120mg/dl.

It is a common practice to examine blood before breakfast, this is called **"fasting blood sugar" (F.B.S).** The fasting level of blood glucose expected in a normal individual

depends upon a large number of factors which are regarded as physiologic factors (not disease). These include age, how long it has been fast since the last meal, what sort of diet the patient has eaten for several days before the test, weather he has exercised gust before the test…etc.

After collecting the blood sample from patients, it is important to separate the serum from cells within a reasonable short time, since red and white blood cells continue to metabolize glucose after they have been removed from the body, which causes false results.

Plasma glucose is higher than whole blood glucose by roughly (12-13)% due to the lower water content of the cells(73%) than the water content of plasma(93%), although the concentration of glucose in both is the same.

Plasm sample collect when blood added to a tube containing an anticoagulant such as NaF (to avoid glycolysis) mix with potassium oxalate $K_2C_2O_4$ in a ratio (2mg oxalate/2.5mg florid per 1 ml of blood).

Clinical Significance of B. glucose

Hyperglycemia:

(a) **High values of F.B.S** cause diabetes mellitus, in which it may vary from normal up to 500mg/dl and over, according to the severity of the condition. As the β- glucose rises above 500mg/dl, there is an increasing possibility of proteins and lipids metabolism disturbance, ketosis, and some degree of diabetic coma will be present.

(b) **Gushing's Syndrome:** excess production of hydrocortisone, which increased gluconeogenesis.

(c) Acromegaly: over production of growth hormone (GH) from pituitary gland.

(d) Hyperadrenalinism: over production of adrenaline (epineph-rine) which causes glucogenolysis by the liver.

Hypoglycemia:

The hypoglycemia may be considered to be present when the β- glucose is below(40mg/dl), and this occurs most frequently as a result of:

(a) Over dosage with insulin in the treatment of diabetes.

(b) Starvation also tend to lower the β- glucose starving person do not die from hypoglycemia.

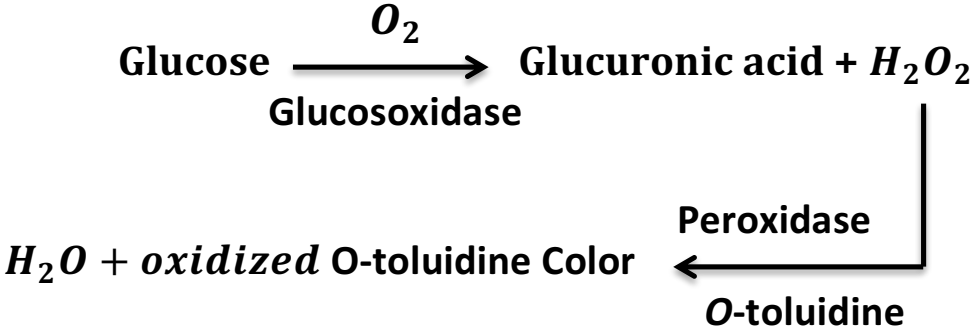
(c) Myxedema: low production of thyroxine gland hormones(hypothyroidism).

(d) Hypopituitarism: low production of pituitary gland hormones.

Methods used of B. glucose determination

Enzymatic method:

Principle:



Procedure:

Solution	Test (T)	Standard (S)	Blank (B)
Serum	10 uL	-	-
Standard solution	-	10 uL	-
Working solution (R1+R2)	1mL	1mL	1mL
Mix well, incubate at 37 C for 10 min or at 25 C for 30 min, then read the absorbance at 505 nm against blank solution			

Calculations:

Glucose concentration (mg/dl)$= \frac{Abs(T)}{Abs(S)} \times Standard\ Conc.$

$$= \frac{Abs(T)}{Abs(S)} \times 100$$

Significance of the solutions:

1. **Working solution:** R1 bottle contains phosphate buffer PB, pH 7 to maintain a suitable pH for enzyme activity. The bottle R2 contains peroxidase +GOD + 4-aminoantipyrine, which catalyze hydrolysis of glucose and oxidation of peroxide.

Reagents:

1. **Standard solution:** 100 mg/dL, ready to use.
2. **Working solution** (R1+R2): Tris buffer 100 mmol/L + phenol 0.3 mmol/L + GOD enzyme 10000 unit/L+ peroxidase 1 unit/L+ + 4-aminoantipyrine 2.6 mmol/L.

Experiment No.(2)

Glucose Tolerance test (G.T.T)

Following eating a carbohydrate male, there is a temporary rise in the blood glucose, where the extent and duration of increasing glucose depends on the type of food. Blood glucose does not increase in normal people more than (160-180) mg/dl and then return to the normal fasting levels within 2-3hr after taking the food.

This effect of carbohydrates can be studied under standard conditions by means of the glucose tolerance test G.T.T. The G.T.T is a body response to an oral administration of a standard amount of glucose. It is of considerable use in investigating the abnormalities of carbohydrate metabolism, where glucose is found in urine.

The natural rate of increasing blood glucose is determined by two forces, the rate of gastric emptying and the rate of intestinal absorption. A rise in blood glucose level normally stimulates the release of insulin from the pancreas, either directly or by intermediary gut hormones. The insulin response determines the rate of fall of the blood glucose.

The normal F.B.S is (6mmol/L) or less

The diabetic F.B.S is (8mmol/L) and more

If the glucose concentration after 2hr after an oral dosage of (75gm) glucose is (8mmol/L) or less, the result is normal.

If it is (11mool/L) or more, the result is diabetic. Between (8 and 11 mmol/L), the patient has impaired glucose tolerance.

***Oral G.T.T*:**

1. The patient should be fasting overnight (at least 10-16hr). Only water is permitted during the time course of the test (2.5-3hr). The patient should not smoke and remain seated.
2. Initially, a fasting blood sample and urine specimens are collected.
3. Fifty gm of 75 gm of glucose dissolved in (25-300) mL of water is then feed it to the patient (children 1.75gm/kg body weight). This is best followed by about (50-100)mL of water to take away the sweet taste and decrease the risk of the patient vomiting.
4. Blood samples are collected at 1/2 hour intervals for a period of 3hr (0.5, 1.0, 1.5, 2.0, 2.5, 3 hrs) after the glucose has been drunk. Urine specimens are collected at the similar intervals.

Table 1. A typical normal response of GTT.

Time/hrs.	**0**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
F.B.S(mg/dL)	70	130-150	120	90	70	80
Urine glucose	Negative tests throughout					

Table 1 showed the normal response of GTT, the initial blood glucose is within normal fasting limits (70mg/dl), The maximum level appeared after (0.5-1 hr) of taking the glucose (130-150mg/dL). Insulin will secret in a large amount and the blood glucose will return rapidly to the normal fasting levels which are often reached in 1.5.-2 hrs. Glucose should not appear in urine in at all time points, as glucose appears in urine only when its level in blood rise over (180mg/dL).

The following figure show the different types of G.T.T curves, where the typical normal curve is curve 3.

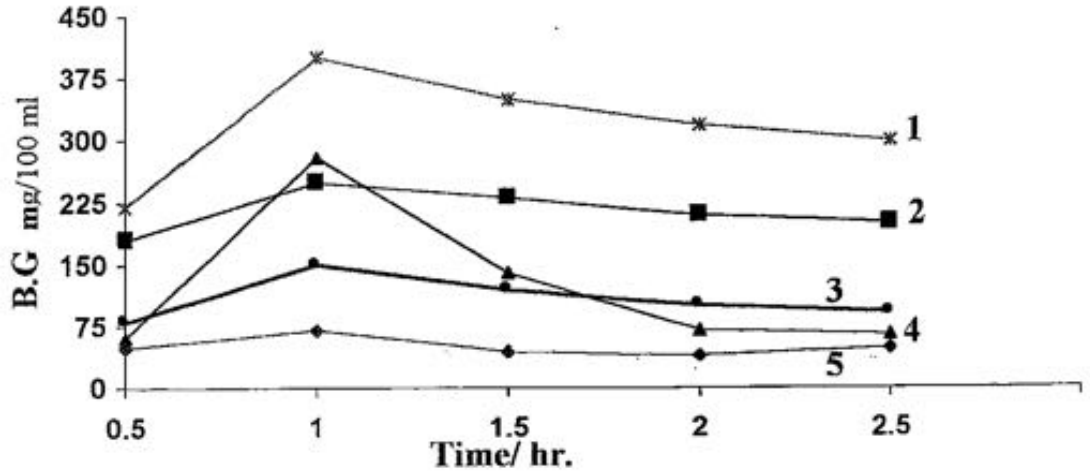


Fig 1. The G.T.T curves

***Curve (1): Sever diabetes G.T.T.*:**

Occurs in very sever liver diseases where the rate of glycogen formation and gluconeogenesis is reduced, therefore blood glucose reaches the higher levels than normal. Glucose appeared in urine at all time points.

***Curve (2): Mild diabetes G.T.T.*:**

The F.B.S. is higher than normal limits and there is a delay in the return of the blood glucose to a fasting level. Hyper activity of the thyroid, pituitary, and adrenals gland may also lead to some degree of glucose tolerance impairment comparable with that of a mild diabetes.

Curve (3): Normal.

***Curve (4): Lag-storage*:**

In this case: there is a rapid absorption of glucose after 0.5hr.(180mg/dl) followed by a rapid elimination of glucose

after 1hr of taking the glucose dosage(140mg/dl). After that, the glucose level is little raised over the normal limits. This curve is seen in patients with impaired absorption of carbohydrates.

***Curve (5): Flattened*:**

The F.B.S may sometime be under the usual normal limits, and there is a little rise in blood glucose. This curve is frequently observed in endocrine hypo activity as in hypothyroidism, hypoadrenocorticalism (Addison's disease), and hypopituitarism (Simmond's disease).

***Factors influence the G.T.T.*:**

1. **Previous diet.**: no special restrictions are necessary and a normal diet for (3-4) days. If, however, the test performed after a period of carbohydrate restriction, there may be abnormal G.T.T.
2. **Time of the test:** most G.T.T. are performed in the morning. There is an evidence that tests performed in the afternoon indicates higher plasma glucose level and that the accepted normal values may not be applicable.
3. **Drugs:** Drugs such as steroids, diuretics, hormones (ACTH, glucose, and thyroxine) may impaired G.T.T.

Experiment No.(3)

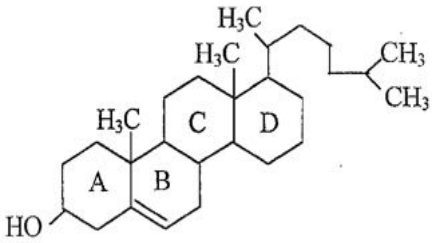
Determination of Lipid Profile

Lipid profile test includes serum total cholesterol, triglycerides, chylomicrons, high-density lipoproteins, low-density lipoproteins and very low-density lipoproteins.

1- Total Cholesterol:

Cholesterol is a derivative lipid has a chemical formula ($C_{27}H_{45}OH$) and its structural formula is shown below:

It is formed from steroids unit consist of 4 rings.



Cholesterol is **a** bile solid alcohol, where **Chole** means bile and **sterol** means solid. Cholesterol is found in all cells of the body. The brain and spinal cord contain large amount of cholesterol, which is form part of the lipid "insulation". It is a precursor of the adrenal and sex hormones.

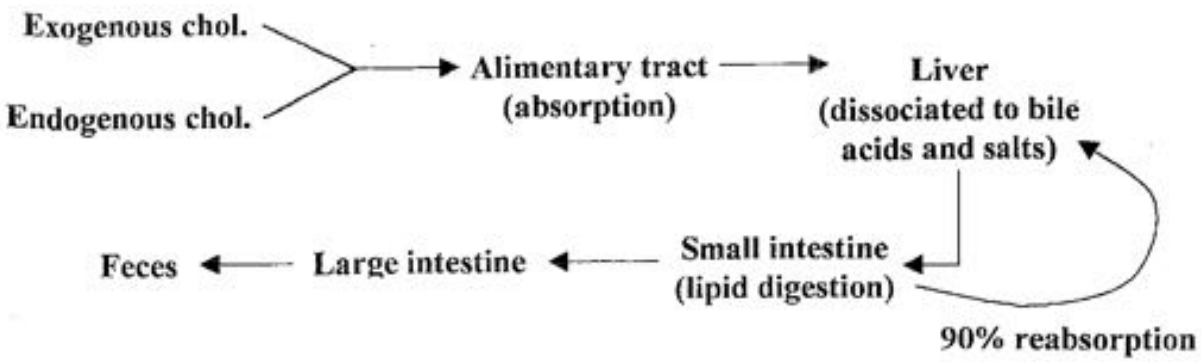
Cholesterol may be synthesized from 2-carbon units (acetyl CoA) in many body tissues, particularly liver, intestine, and skin. The liver is the main endogenous source of cholesterol, it synthesized about 1gm of cholesterol daily while other tissues synthesized only $^1/_2$gm.

Measurement of total cholesterol includes both the esterified and free forms of the steroid. In plasma or serum, 2/3 of the

total cholesterol exists in the esterified from, with the rest (1/3 of the total cholesterol) exists in the free forms.

The exogenous sources of cholesterol are:
Egg yolk, meat and animals' fats, milk, chess, butters.

Cholesterol is absorbed by alimentary tract, reaches the liver through blood stream. Liver cells oxidize the molecule by adding hydroxyl and carboxyl group to form cholic acid. These are excreted in the bile. (90%) of bile salt will reabsorbed to the liver via enterohepatic circulation, the remaining cholesterol is excreted with feces. These steps could be summarized as the following:



Normal value: 140-280 mg/dl.

Normal rang for total s. cholesterol in adults vary with age, sex and diet, physical activity and hormonal effect (GH. Thyroxine and glucagon decrease its level). A seasonal variation has also been observed, with levels higher in fall and winter than in spring and summer.

***Clinical significance*:**

The important of s. cholesterol measurement is related to atherosclerosis disease.

***Hypercholesterolemia*:**

1. **Atherosclerosis:** This disease refers to the deposition of fatty substances, largely but not entirely cholesterol, in the wall of the arteries, this will reduce the blood flow.
2. **Heart diseases:** Value between (300-400mg/dl) are rather frequent finding in coronary thrombosis and angina pectoris.
3. **Nephrotic syndrome:** When oedema is present, values up to(600-700mg/dl) due to the high level of lipoproteins which contains larger of cholesterol.
4. **Diabetes mellitus:** Values up to (400-500mg/dl) are found because of abnormalities in the lipids and proteins metabolism.
5. **Obstructive Jaundice:** Increases occur most commonly when there is obstruction in the large bile ducts.

***Hypocholesterolemia*:**

Cholesterol in the plasma tends to fall during starvation and after exercises.

1. Hyperthyroidism:

Excess thyroid gland activity reduces s. cholesterol value below(100mg/dl) in the servant cases, but this is not of diagnostic significance.

2. Hepatitis:

a- Infective hepatitis:

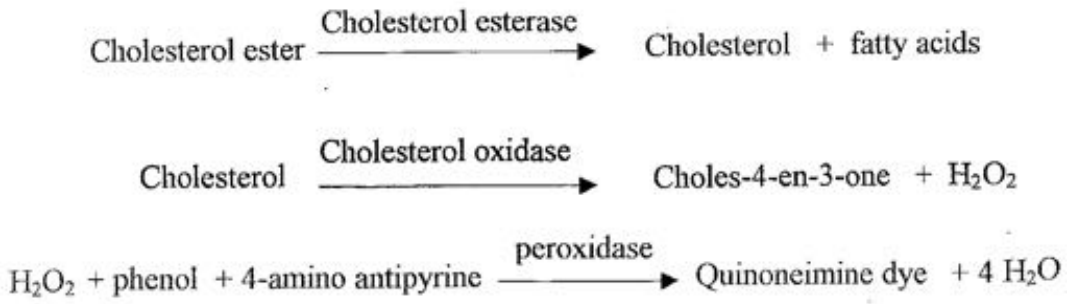
the tendency when liver cell damage is present, is that during the development of jaundice, the total cholesterol either remains within normal limits or falls a little while the

proportion of ester falls appreciably and may reach very low levels. As the condition improves, the total cholesterol are somewhat rested for a time after the jaundice has disappeared.

Method to measure s. cholesterol:

Enzymatic method (kit method):

Principle: Cholesterol esters is hydrolyzed by cholesterol esterase enzyme to free cholesterol and this will be oxidized by the catalytic activity of cholesterol oxidase and finally a color oxidation product produced could be estimated by spectrophotometer.



procedure:

Solutions	Standard	Test	Blank
Serum	-	30 ul	-
Std solution 200 mg/mL	30 ul	-	-
Working solution (R1+R2)	3 ml	3 ml	3 ml
Mix well, incubate at 37 C for 5 min or at 25 C for 10 min, then read the absorbance at 500 n			

Calculations:

T. cholesterol conc. (mg/dl)$= \frac{Abs.(T-B)}{Abs.(S-B)} \times Std.conc.$

$= \frac{T-B}{S-B} \times 200$

Significance of the solutions:

The working solution consists of 3 enzymes

1- **Cholesterol esterase:** break down the ester bond and liberate free cholesterol and fatty acids
2- **Cholesterol oxidase:** oxide the cholesterol molecule into diene and hydrogen peroxide.
3- **Peroxidase:** oxidize the hydrogen peroxide into ketone imine.

These three enzymes are solubilized in a buffer solution contains 4-amino antipyrine to color the product.

Reagents:

1- *Buffer solution R1*

Phosphate buffer solution (0.1 mole/L) + phenol (15mmole/L) + Na cholate (3.74 mmole/L)

2- *Working solution (R1+R2)*

*Dissolve contents of R2 bottle (*Cholesterol esterase 125 unit/L + Cholesterol oxidase 200 unit/L + peroxidase 1000 unit/L + 4-amino antipyrine (0.5 mmole/L) in R1 buffer solution.

2- Triglyceride TG :

Triglyceride TG forms by binding three fatty acids with glycerol. Triglyceride is useful to produce energy for the body when insufficient amount of carbohydrates. It shares cholesterol some of the physical properties, such as water insolubility, therefore, they need transporters to move in the blood circulation. These transporters are made of lipoproteins. About 90% of TG is transferred by chylomicron and the rest 10% is transferred by very low-density lipoprotein (VLDL). TG is synthesized in the liver and consumed by the body when needed as a rich source of energy instead of carbohydrates or lipids.

Normal value of TG is 10-150 mg/dL

Clinical significances

Hyperglyceridemia

Increasing level of TG occurs due to:

1- Over eating of carbohydrates
2- Kidney diseases
3- Diabetes mellitus
4- Sever pancreatitis
5- Gut
6- Liver cirrhosis

Hypoglyceridemia

decreasing level of TG occurs due to:

1- malnutrition
2- low level of low density lipoproteins

Method to measure serum TG:

Principle: TG is hydrolyzed into cholesterol as following:

$$TG \xrightarrow{\text{Lipase}} \text{Glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerokinase, } Mg^{+2}} \text{Glycerol-3-phosphate ADP}$$

$$\text{Glycerol -3P+ } O_2 \xrightarrow{\text{3-glycerol phosphate oxidase,}} \text{Dihydroxy acetone-P + } H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine + p-cholesterol} \xrightarrow{\text{peroxidase}} \text{quinonimine + } H_2O$$

Procedure:

Solution	Test (T)	Standard (S)	Blank (B)
Serum	10 uL	-	-
Standard solution	-	10 uL	-
Working solution (R1+R2)	1mL	1mL	1mL
Mix well, incubate at 37 C for 5 min or at 25 C for 10 min, then read the absorbance at 505 nm against blank solution			

Calculations:

$$\text{TG concentration (mg/dL)} = \frac{Abs(T)}{Abs(S)} \times Standard\ Conc.$$

$$= \frac{Abs(T)}{Abs(S)} \times 200$$

Significance of the solutions:

1. **Working solution:** R1 bottle contains phosphate buffer PB, pH 7.2 to maintain a suitable pH for enzyme activity. The bottle R2 contains peroxidase + 4-aminoantipyrine, which catalyze hydrolysis of TG and oxidation of peroxide.

Reagents:

1. **Standard solution:** 200 mg/dL, ready to use.
2. **Working solution** (R1+R2): PB buffer 100 mmol/L + phenol 0.3 mmol/L + peroxidase 1 unit/L+ + 4-aminoantipyrine 2.6 mmol/L.

3- Lipoproteins analyses

Lipoproteins are classified according to their intensity into:

Chylomicrons, very low-density lipoproteins VLDL, low-density lipoproteins LDL and high-density lipoproteins HDL.

The most important tests are HDL and LDL.

a) High-density lipoproteins HDL.

This type of lipoproteins called α-lipoprotein carries the cholesterol molecule from all tissues to the liver through the blood. It contains 25-45 % of cholesterol in addition to some phosphor lipids. HDL carries cholesterol to the liver where it metabolized, therefore, it is called good cholesterol and increasing level of HDL prevents atherosclerosis.

Normal value of HDL > 40mg/dL (0.83-2.5 mmol/L)

HDL level in female is higher than that in male because estrogen generates the protein that carries the cholesterol, therefore females are less likely to have atherosclerosis.

b) Low-density lipoproteins LDL

This type of lipoproteins called β-lipoprotein carries the cholesterol molecule from liver to all tissues through the blood. It contains 50-75 % of cholesterol, therefore, it is called bad cholesterol and increasing level of LDL is an indication of atherosclerosis.

Normal value of LDL < 180 mg/dL (0.5-3.88 mmol/L)

Measurement of LDL:

LDL is measured according to the equation

LDL cholesterol (mg/dL) = Total cholesterol - HDL cholesterol – TG/5

When serum TG is over 400 mg/dL, this equation should be applied:

LDL cholesterol (mg/dL) = Total cholesterol - HDL cholesterol – TG/22

Kidney function tests

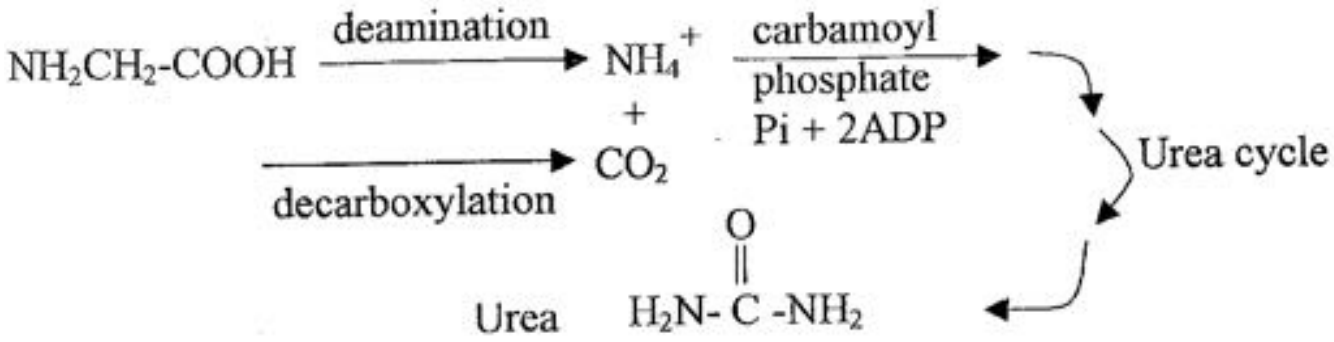
Determination of the non-protein nitrogen compounds:

Urea	creatinine	uric acid

Experiment No. (4)

Determination of **Blood Urea**

Urea is the detoxification product of ammonia derived from deamination of amino acids. Urea is therefore the most end product of protein catabolism.



Urea is synthezed from ammonia in the liver and it is then excreted by the kidneys. Urea is free to pass through all membranes of the body and is equally distribution in the body water.

The normal value of blood Urea : (15-45)mg/dl.

However, the concentration of urea inside red cells is slightly less than in plasma due to the presence of large amounts of Hb inside the cells. Whole blood urea concentration is therefore slightly less than in plasma or serum urea.

Usually serum is used instead of whole blood for the determination of urea, and the amount of urea is expressed in terms of its nitrogen content.

The concentration of urea depends upon the rate of production by the liver and the rate of removal by the kidneys. In most patients, the rate of production is a reflection of the protein intake. In sever liver disease, the ability of the liver cells to form urea is impaired: ammonia accumulate and urea level fall. The rate of removal depends upon urea conc. in the plasma, and capacity of the kidneys to remove urea from the plasma (kidneys function). In most clinical situations, changes in urea levels are more dependent upon kidney function than upon liver function.

Clinical significance:

Hyperuremia:

This case is seen not only in renal diseases but also in a number of other cases:

1. per-renal uremia cusses: Cardiac failure, vomiting, hemorrhage and shock.

2. Renal uremia cusses: Glomeruli nephritis, acute or chronic nephritis, tubular, and nephrosclerosis.

3. Post-renal uremia cusses: Mechanical obstruction to urine excretion, such as may occur in diseases of the ureters, bladder or urethra. The obstruction may be caused by stone or tumor.

Hypouremia:

Decreased serum values have been reported in protein malnutrition, pregnancy, and acute rehydration

Methods used for blood urea determination:

1. Enzymatic method:

Principle: a general method uses urease enzyme (found in soya and jack beans) to convert urea into ammonium carbonate, which is hydrolyzed in alkaline solution into ammonia and sodium carbonate and can be detected by addition of salicylate and hypochlorite. A green colored complex is formed.

$$H_2N - C - NH_2 + H_2O \xrightarrow[37^{\circ}C]{\text{Urease}} (NH_4)_2CO_3$$

Ammonium carbonate

$$(NH_4)_2CO_3 + 2NaOH \longrightarrow Na_2CO_3 + 2NH_3 + 2H_2O$$

$$2NH_3 + Na - nitroprosside \xrightarrow[\text{Na-salicylate}]{\text{Na-Hypochlorite}} \text{2,2-dicarboxyl indophenol}$$

Green compound

Procedure:

Solution	Test (T)	Standard (S)	Blank(B)
Serum	30 ul	-	-
Standard Solution (R1)	-	30 ul	-
Working solution(R2+R3)	3 ml	3ml	-
Mix well and let to stand for 5 min at 25 C.			
Alkaline solution (R4)	600ul	600ul	600ul
Mix well, incubate at 25 C for 10min, cool and read the absorbance at 580nm against blank solution.			

Calculations: Urea conc. (mg/dl) $= \frac{Abs(T-B)}{Abs(S-B)} \times Std.con$

$= \frac{T-B}{S-B} \times 50$

Significance of the solutions:

1. **Working solution**: This solution consists of urease solubilized in phosphate buffer (BP) pH 8, Na-nitroprusside, salicylate and EDTA, where urease hydrolyses urea and Na-nitroprusside acts as a color agent.
2. **Alkaline solution**: this solution is a mixture of Na_2CO_3 and Na-hypochlorite. It's used stop the enzymatic reaction and stabilize the color of the resulting product.

Reagents:

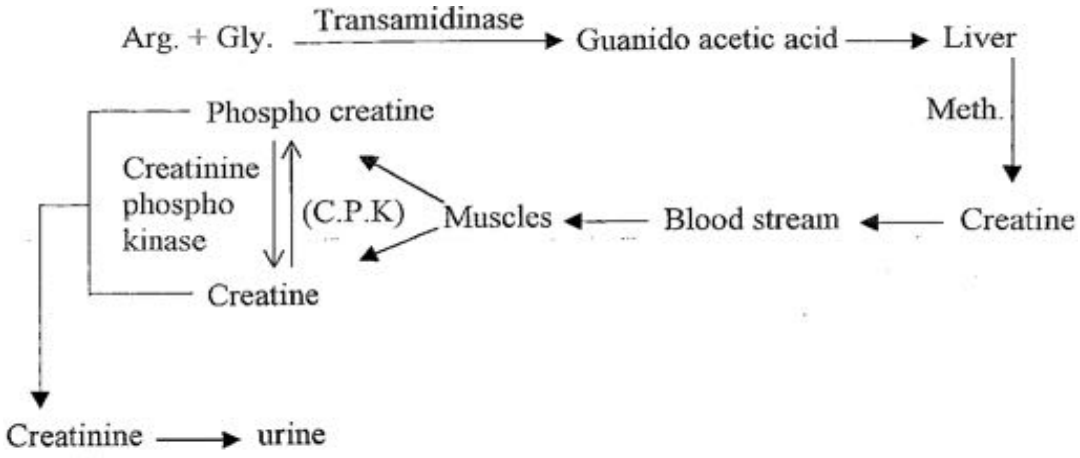
1. **Urea standard solution (R1)** : 50gm of urea in 100 ml of D.W.
2. **Urease solution (5000 unit/l)** : Dilute 10ml of stock to 250ml by D.W.
3. **Working solution (R2+ R3)** : Mix the content of R2 with the content of R3 (PB, pH 8 40 mmol/l+ Na salicylate 52mmol/l + Na-nitroprusside 2.83 mmol/l +EDTA 1 mmol/l).
4. **Alkaline solution** (R4): Na-carbonate 83 mmol/L + Na-Hypochlorite 3.75 mmol/L, ready to use.

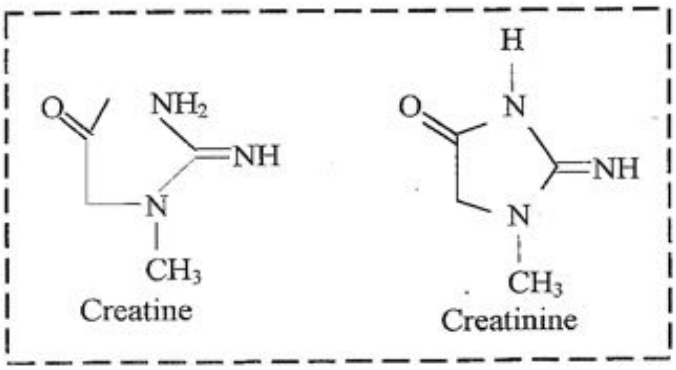
Experiment No. (5)

Determination of **Serum Creatinine**

Creatinine (or phosphor creatinine) is a catabolic end product, produced by loss of water (or phosphoric acid) from creatine in an irreversible reaction. Creatinine is not reutilized but it is excreted out of the body via the urine. It is formed at a nearly constant rate as shown below.

In kidney:





Creatine and phosphor-creatine is formed about 400mg of 100mg of muscle. Both compounds are converted non enzymatically into creatinine in a rate(2%)daily.

The normal value of s. creatinine: 0.1-1.4mg/dl depending on age. Sex and muscle mass.

Clinical significance of Creatinine :

Creatinine is generally regarded as the most useful naturally occurring substance to measure the diagnosis and follow up kidney disease and indicator of impaired extent of kidney damage.

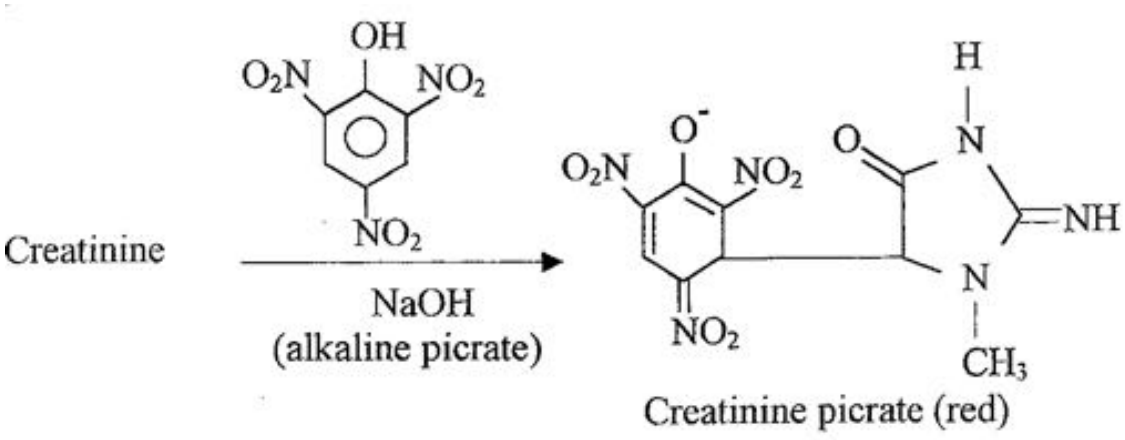
When s. creatinine is between 2-4mg/d a moderate to severe renal damage. This lack of creatinine sensitivity is managed by use of the creatinine clearance test(C.C.T) which gives information's specifically to the damage of filtration mechanism(clearance means the volume of plasma inter the creatinine).

Clinical significance of Creatine :

This substance level does not affect by the kidney damage and has no clinical significance about that. Its importance related to the muscular diseases: muscle distraction and muscular dystrophy where s. creatine will increased and there is another quantity in the urine.

Jaffe's reaction for s. creatinine estimation:

Principle: This method is based on a reaction between creatinine and alkaline picrate to give a red colour creatinine picrate which can be estimated by spectrophotometer.



Procedure:

In a centrifuge test tube add			
Solutions	**Test (T)**	**Standard (S)**	**Blank(B)**
Serum	0.5 ml	-	-
H_2O	3.5ml	1.5 ml	2.5 ml
Stand. Solution	-	1ml	-
Na- tungstate	0.5 ml	-	-
Add drop by with constant shaking:			
H_2SO_4	0.5ml	-	-
Allow to stand for 10min. centrifuge the solution of tube(T) only for 10 min.			
Supernatant	2.5ml	2.5ml	2.5ml
Na- picrate	2.5ml	2.5ml	2.5ml
Mix the contents and let stand for 15min. read the absorbance at 520nm.			

Calculations:

$$\text{Creatinine conc. (mg/dl)} = \frac{Abs(T-B)}{Abs(S-B)} \times Std.conc. \times \frac{100}{volume\ of\ serum}$$

$$= \frac{T-B}{S-B} \times 0.01 \times \frac{100}{0.25}$$

$$= \frac{T-B}{S-B} \times 4$$

Significance of the solutions:

Na- tungstate + H_2SO_4: Precipitate proteins present in the serum, which may react with picric acid and ultra the results.

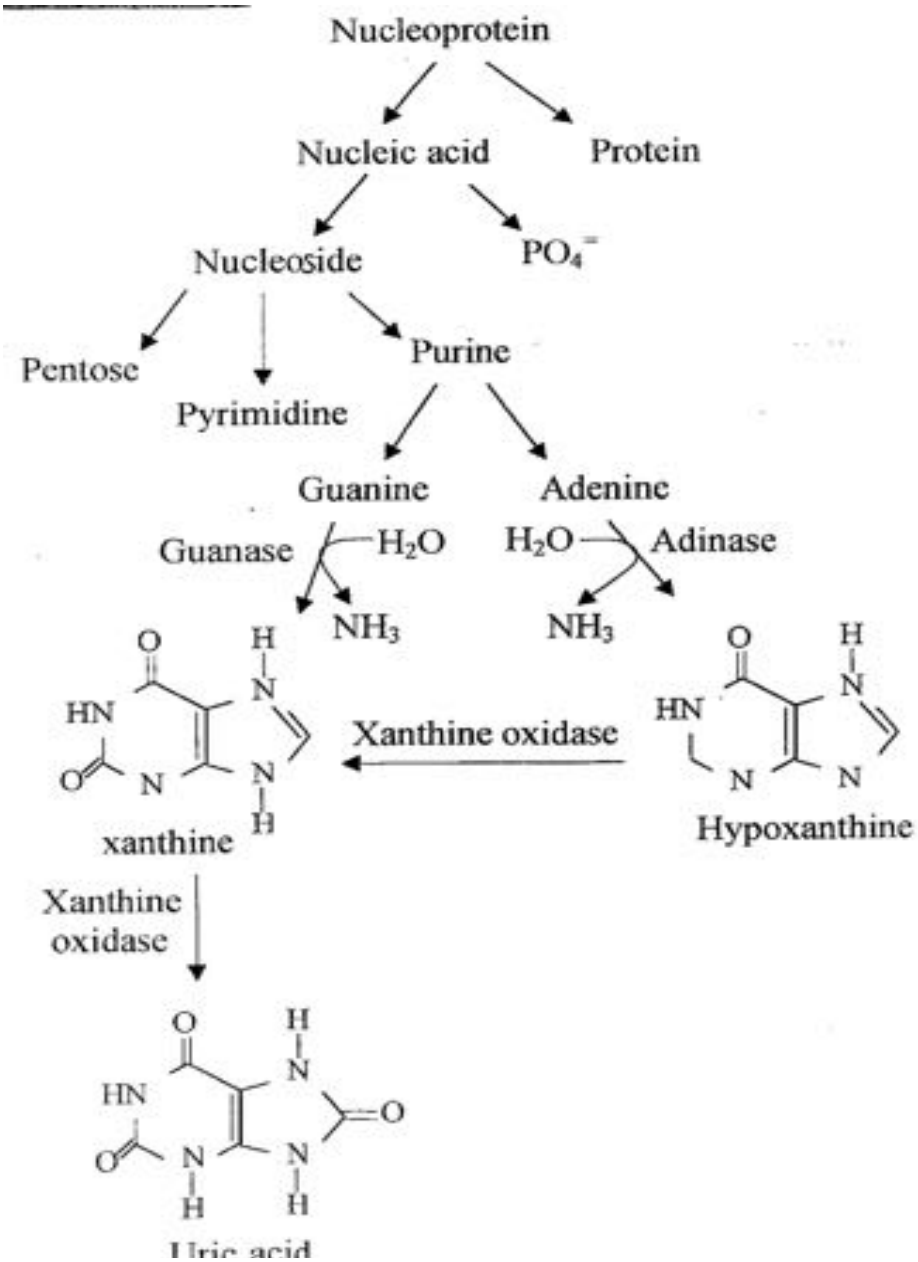
Reagents:

1. ***H_2SO_4 (2N/3) solution:*** 19 ml of conc. H_2SO_4 in 1$\mathcal{L}$ of D.W.
2. ***Sodium tungstate (10%) solution:*** Dissolve 100gm of Na_2Wo_4 in 1$\mathcal{L}$ of D.W.
3. ***Saturated picric acid:*** weigh 10gm of picric acid $C_6H_2OH(NO_2)_3$, crystals & dissolve in 1$\mathcal{L}$ of D.W.
4. ***Sodium hydroxide(10%)(w/v):*** Dissolve 20gm of NaOH in 200ml of D.W.
5. ***Alkaline picrate solution:*** mix 5 parts of solution 3+1 part of solution 4+30 part of D.W.
6. ***Stock creatinine standard:*** dissolve 1gm of creatinine in 1$\mathcal{L}$ *o*f (0.1N) HCl.
7. ***Working creatinine standard:*** dilute 10ml of stock in 1$\mathcal{L}$ of (0.1N) HCl.

Experiment No. (6)

Determination of **Uric acid**

Uric acid is a waste product of purine metabolism in humans. The purine basis adenine and guanine, formed in the course of nucleic acid catabolism and free nucleotide undergo oxidation to uric acid, as shown:



This scheme shows that uric acid sources are:

1. ***Exogenous*****:** red meat, liver, stimulants in coffee and tea.
2. ***Endogenous:*** nucleic acid catabolism. Liver is the main site of uric acid formation.

The normal values of s. uric acid

Men: 2.7-7.5 mg/dl, Women: 2.5-6 mg/dl, Children: 2.5 mg/dl,

Clinical significance:

Determination of s. uric acid is most helpful in the diagnosis of gout, where sodium urates are deposited in solid form in and about the joints.

Hyperuricemia:

Increased level of s. uric acid is found in Acute and chronic nephritis, urinary obstruction, high purine diet, diabetic keto acidosis, malignant tumors especially with extensive neurosis.

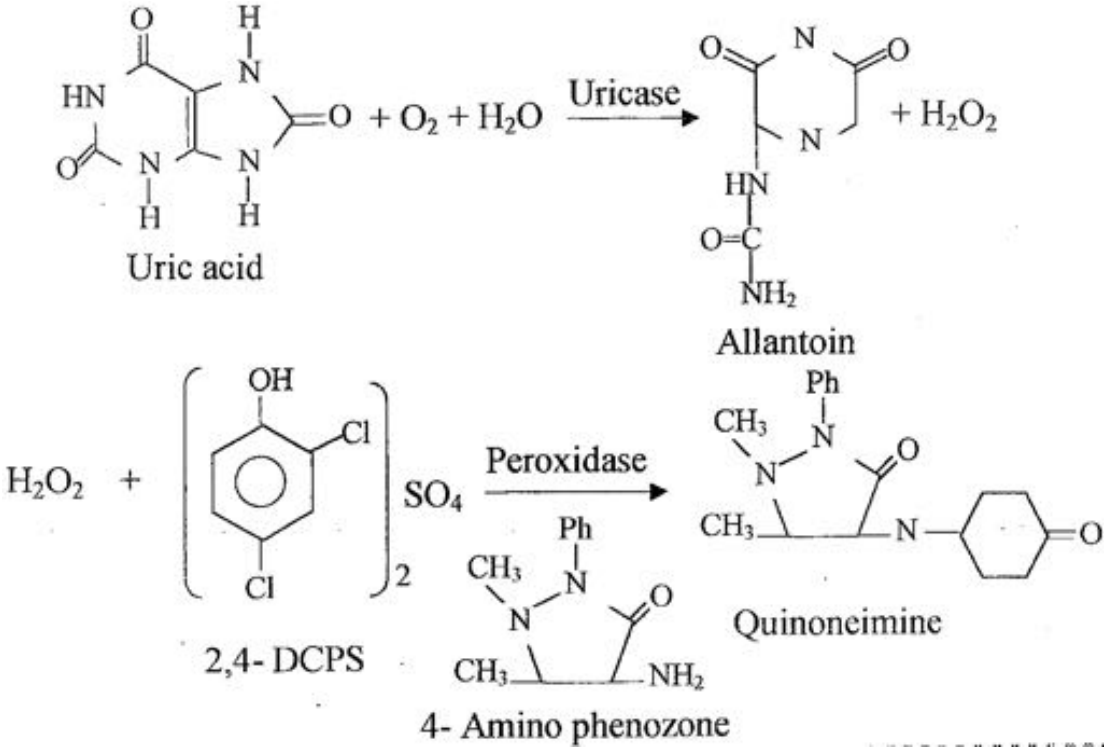
Hyperuricemia:

1. Proximal renal damage where urate reabsorption is reduced.
2. Xanthine oxidase deficiency.
3. Salicylate therapy.

Methods used for s. uric acid determination

Enzymatic method:

Principle: uric acid is oxidized by uricase enzyme to allantoin and hydrogen peroxide. The peroxide oxidizes 2.4. dichlorophenol sulfonate by peroxidase enzyme to give colored quinoneimine complex, according to the following equations:



Procedure:

In a centrifuge test tube add			
Solutions	**Test (T)**	**Standard (S)**	**Blank(B)**
Working solution **R1+R2**	3 ml	3 ml	3 ml
Standard solution **R3**	-	60 ul	-
Serum	60 ul	-	-
Mix well, incubate at 25 C for 10min and read the absorbance at 510nm against blank.			

Calculations:

Uric acid conc. (mg/dl) $= \frac{Abs(T)}{Abs(S)} \times Std.conc. \times$

$$= \frac{(T)}{(S)} \times 6$$

Significance of the solutions:

1. **Working solution:** contains a mixture of uricase +peroxidase + 4-aminoantipyrine in PB pH 7.4. Uricase oxidizes uric acid into allantoin and hydrogen peroxide and peroxidase oxidizes hydrogen peroxide to form quinoneimine complex

Reagents:

1. **Working solution (R1+R2):** Mix R2 bottle (uricase 70 unit/L + peroxidase 660 unit/L + 4-aminoantipyrine 1 mmol/L) with 30 mL of R1 bottle (PB, pH 7.4 50 mmol/L).
2. **Standard solution(R3):** Uric acid 6 mg/dL

Liver function tests

Liver tests are GPT& GOT, serum bilirubin, serum proteins and ALP, which may by classified according to the correlated liver damage into:

1. <u>Liver cell damage tests</u>:

This test includes the transaminase enzyme GOT & GPT which release when liver cells are damage. Therefore, these enzymes level is elevated in: viral hepatitis and liver cirrhosis or fibrosis.

2. <u>Liver dysfunction tests</u>:

a. The liver conjugation capacity tests:

the most important tests are total bilirubin, direct and indirect bilirubin. Bilirubin level is increase in jaundice disease, which caused by either by either acute liver disease or biliary duct obstruction.

b. The liver excretion capacity tests:

The usual test used in this field is Bromsulph Thalein Excretion test (BSP test). BSP is injected (5gm per Kg of body weight). In normal state, (5%) of injected does is remaining in blood stream after (45min), while the rest quantity is stored by liver.

b. The liver ability for proteins synthesis tests:

Total proteins, albumin, and globulins (except γ.)

3. <u>Cholestasis tests</u>:

Alkaline phosphatase ALP, and 5-Nucleotidase increase when there is intrahepatic or extrahepatic causes affected bile pigment transferring into the duodenum.

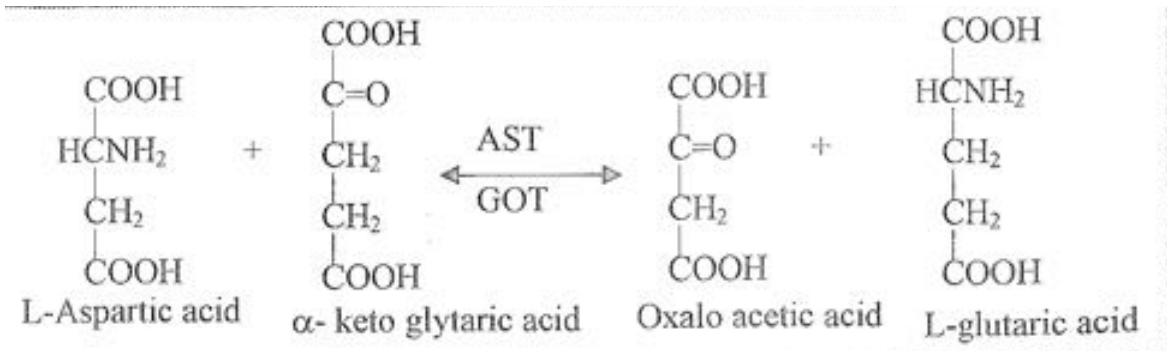
Experiment No. (7)

Determination of **Serum transaminase enzyme activities**

Transamination is the term given to the process in which the amino group is transferred from an α-amino acid. As a result, a different α-amino acid and α-keto acid are formed. All naturally occurring α-amino acids can take a part in such reactions. This process is catalyzed by enzymes referred to transaminase (or amino transferase), which plays a key role in intermediary metabolism as it provides a means for the synthesis and degradation of amino acid in living cells. Two clinically important examples of amino transferase enzymes are **GOT** and **GPT**.

(7-1) Glutamate oxaloacetate transaminase (GOT):

It is also called aspartate amino transferase (AST); catalysis the following reaction:



The enzyme properties:

1. It acts on the substrate; L-aspartic acid+ α-keto gutanic acid.

2. AST activity is widely distributed in human tissues: heart, liver, skeletal muscle, and kidney being the richest sources.
3. The optimum conditions of maximum enzyme activity are pH 7.4 at 37°C.
4. The enzyme activity inhibited anticoagulants, therefore, serum preferred to use rather than plasma.
5. The enzyme stability: 3 days in 25°C, 1 week in 5°C, and 1 month in 25°C.

The normal value of GOT : 2-20 I.U/L

: 2-23 μmol/ml. L

The enzyme GOT and (GPT) unit :

The enzyme activity is measured by international unit, which may be defined as:

The activity of enzyme that produced 1 μmol of oxaloacetic acid or(pyruvate) from aspartic acid or(alanine) respectively at 1 min per lit. of serum under certain conditions of temp (37°C) and pH (7.4).

Clinical significance of GOT :

The enzyme clinical usefulness is largely restricted to the diagnosis of heart and liver diseases. The most information is gained when the enzyme is measured simultaneously with other enzyme, particularly GPT, CPK, LDH & ALP. However, high level of GOT is observed in:

1. *Heart diseases*:

S. GOT was increased after myocardial infarction MI. GOT being to rise 6-8 hrs. after a MI occurs, and the peak

activity occurs at (24-36 hrs), then return to normal level usually follows in (4-5) days unless other attack occurs. The GOT activity is proportional with the cardiac muscle damage.

2. *Liver diseases*:

Large amount of GOT may be released into the blood, where very high levels are observed in acute liver disease, while lesser are seen in chronic liver disease. The most common entities are listed below:

a. Infection hepatitis.
b. Toxic hepatitis.
c. Bile duct obstruction.
d. Liver cirrhosis and cancer.

3. *Muscular diseases:*

S. GOT was increased in muscular dystrophy. Renal diseases do not significantly alter the s. GOT activity, but may instead appear in urine.

Methods used to measure s. GOT activity:

Principle: most methods of GOT activity determination employ a substrate containing L- aspartic acid and α-keto glutaric acid. The enzyme catalyzes the transfer of the amino group into keto group.

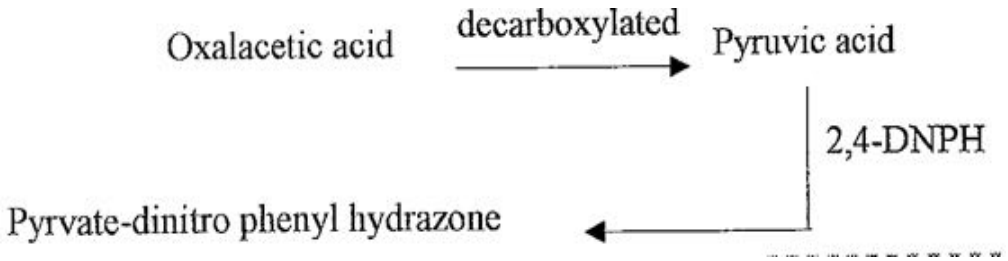
The amount of oxalacetic acid formed in this reaction is proportional to the GOT activity. The various methods differ from each other primarily in the manner in which the oxalacetic acid is measured.

1. The simplest version of the method involves the direct combination of oxalacetic acid with dinitrophenyl hydrazine

(DNPH) and measurement of the color in an alkaline solution:

oxalacetic acid $\xrightarrow{\text{2,4-DNPH}}$ oxalacetic–dinitrophenyl hydrazone (brown color)

2. In another method, the oxalacetic acid is decarboxylated to form pyruvic acid. The pyruvic acid then combines with DNPH to form a pyruvate dinitrophenyl hydrazine, which is then measured calorimetrically in alkaline medium.



Procedure:

a. The Kit method

transaminase activity could be measured by comparing the abs. (T-B) with I.U/L values by reference to the calibration table attached.

b. The manual method:

measure the pyruvate liberated in μmol/ml according to stand, pyruvate solution (0.4 μmol):

Solutions	***Test(T)***	***Blank of test(B_T)***	***Standard(S)***	***Blank of std.(B_S)***
Substrate	0.5ml incubate at 37°C for 3 min	0.5 ml	0.5 ml	0.5 ml
Serum std. pyruvate	-	-	0.1ml	-
H_2O	-	-	-	0.1ml
Mix well in each addition, incubate(T) only at 37°C for 60 min				
DNPH sol.	0.5 ml	0.5 ml	0.5 ml	0.5 ml
serum	-	0.1ml	-	-
Let to stand for 20 min				
NaOH sol.	5ml	5ml	5ml	5ml
Mix well, wait 10 min at 25°C , measure the abs. at 10nm				

Calculation:

GOT activity is measured in μmol/min/L as the following:

The oxalacetic acid formed by the serum is responsible for the abs. ($T-B_T$). The pyruvate in 0.1ml of the working standard (0.4 μmol) produces the abs. ($S-B_S$). So the pyruvate formed in μmol per min. per liter of serum is:

$$= \frac{(T - B_T)}{(S - B_S)} \times 0.4 \times \frac{1}{60} \times \frac{1000}{0.1}$$

$$= \frac{(T - B_T)}{(S - B_S)} \times 67$$

Then the calculated pyruvate is converted into (I.U/L) by reference to the calibration table attached.

Significance of the solutions:

1. **The substrate:** Prepared by mixing aspartic acid+ α-keto glutaric acid in a phosphate buffer(pH=7.4). this solution is which the enzyme act on.
2. **DNPH :** used to:

a. Combine with pyruvate to give color comp.
b. Act as enzyme reaction inhibitor.

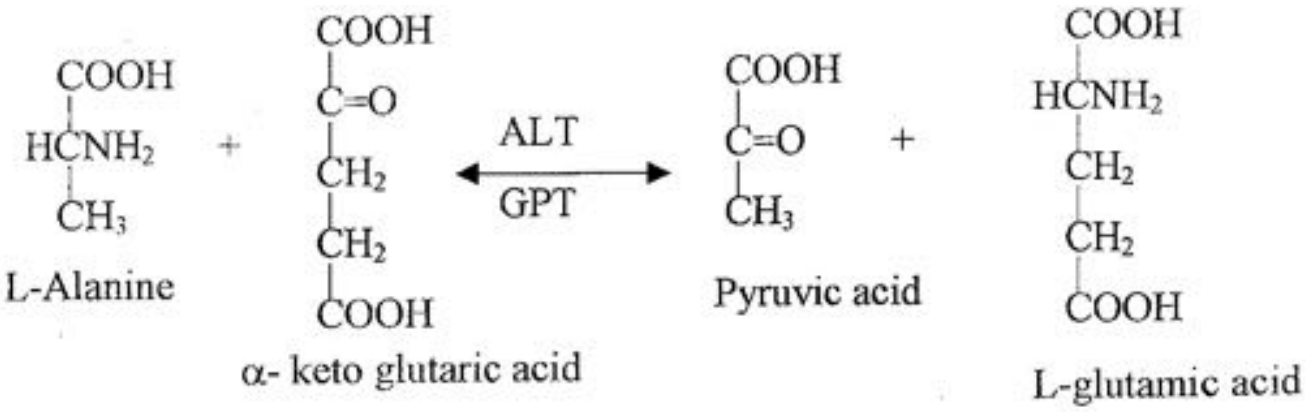
3. **NaOH**: After addition of NaOH, the color of solution stabilizes in 5 min , and remains constant for at least 30 min.

***Reagents*:**

1. **Phosphate buffer sol. (pH=7.4):** dissolve 11.3gm of Na_2HPO_4 and 2.7gm of KH_2PO_4 in 1L of D.W. store in a refrigerator.
2. ***Got substrate*:** dissolve 26.6 gm of Asp. Acid in 180ml of IN-NaOH(40gm of NaOH in 1L). add 0.292gm of α-keto glutaric acid (use IN NaOH to complete solubility), adijust the pH of the solution to 7.4$\pm$ 0.1 by adding IN NaOH dropwise, with stirring using a pH-meter. Dilute to 1L by using sol. (1), add 2ml of chloroform, and store in the refrigerator.
3. ***Stock pyruvate standard(20mM):*** dissolve 2 gm of Na-pyruvate in iL of sol.(1) transfer the solution to a plastic containers, store at- 15°C.
4. ***Working pyruvate standard:*** dilute sol.(3) 1:5 using sol.(1) [1bl of sol.(3) to 4ml of sol.(1)].
5. ***NaOH 0.4N:*** dissolve 16 gm of NaOH in 1L of D.W.
6. ***2,4-DNPH sol.*:** dissolve 198 gm of DNPH in 100ml of conc. HCl dilute to 1L by D.W. and store in a brown bottle.

(7-2)Glutamate pyruvate transaminase (GPT) enzyme:

It is also called alanine amino transferase (ALT); catalysis the following reaction:



The enzyme properties:

GPT properties are similar to these of GOT except:

1. GPT found in a highest concentration in the liver in spite of its active occurrence in skeletal muscles, heart & kidney.
2. The substrate of GPT is: L-alanine+ α-keto glutaric acid.

The normal value: 2-15 I.U/L

: 3-38 μmol/min/L

Clinical significance:

The GPT activity in tissues is generally less than GOT. Significant elevation of s. GPT occur in sever acute hepatitis where the enzyme is released into circulation. However, GPT level is increased in the following diseases:

1. Infection hepatitis.
2. Liver cirrhosis and biliary cirrhosis.

Some physicians use the ratio of GOT: GPT to diagnosing the nature of liver diseases, which is usually about 2.5 in liver cirrhosis.

3. Obstructive Jaundice:

Both GPT and GOT are moderately increased. The level of GOT in this case is about the magnitude as that seen after a myocardial infarct (coronary or heart attack), so an associated rise in GPT may help to differentiate these 2 conditions.

4. Liver Cancer.

Method used to measure S.GPT

Principle: The GPT activity is measured calorimetrically by determining the amount of pyruvic acid formed from alanine as said before. The pyruvate 2,4- dinitrophenyl hydrazine is formed by the addition of 2,4- DNPH, which is a color compound can be detected by spectrophotometer.

Pyruvic acid $\xrightarrow{\textbf{2,4-DNPH}}$ Pyruvate – 2,4 – dinitrophenyl Hydrazine(Red color)

Procedure:

The procedure is the same as for GOT except that the incubation time is 30 min rather than 60 min.

Calculation:

GPT activity in μmol/min/L in serum is:

$$= \frac{Abs(T - B_T)}{Abs(S - B_S)} \times 0.4 \times \frac{1}{30} \times \frac{1000}{0.1}$$

$$= \frac{(T - B_T)}{(S - B_S)} \times 133$$

Then the calculated pyruvate is converted into I.U/L by reference to the calibration table.

Significance of the solutions:

1. **The substrate:** Prepared by mixing alanine + α-keto glutaric acid in a phosphate buffer(pH=7.4). This solution is which the enzyme act on.
2. **DNPH :** used to:

c. Combine with pyruvate to give color comp.
d. Act as enzyme reaction inhibitor.

3. **NaOH**: After addition of NaOH, the color of solution stabilizes in 5 min , and remains constant for at least 30 min.

***Reagents*:**

1. All reagents are the same as for GOT except:

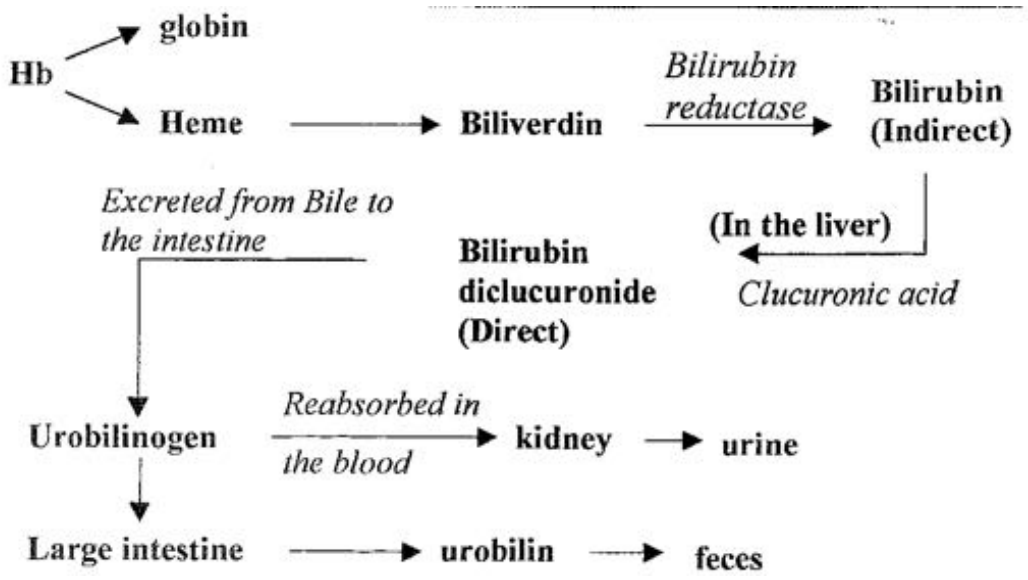
GPT substrate: Dissolve 18 gm of ala. In 180ml of D.W. add 0.292gm of α-keto glutaric acid(use 1N- NaOH to compete solubility), adjust the pH of the solution to 7.4$\pm$0.1 by adding 1N- NaOH drop wise, with stirring using a pH- meter. Dilute to 1L by using phosphate buffer (pH=7.4), add 2ml of chloroform, and store in the refrigerator.

Experiment No. (8)

Determination of **Serum Bilirubin**

Bilirubin is a waste product derived from the breakdown of hemoglobin in the reticuloendothelial system (spleen, bone marrow, Kupffer's cells in the liver). The bilirubin molecule formed in the spleen is non-polar and only a slightly soluble in water at the body pH, therefore, it's called free unconjugated or indirect reacting bilirubin. To transfer from the spleen to the liver, it must be soluble, therefore, free bilirubin form a complex with albumin and dissociates in the liver, where bilirubin conjugates with a glucuronic acid by glucuronly transferase enzyme (by forming an ester linkage between glucuronic acid and the propionic acid side chain of bilirubin).

The resulting polar water-soluble bilirubin is glucuronide which also known as conjugated or direct reacting bilirubin. Most of the conjugated bilirubin is actively excreted from the liver into bile duct into the intestine where it is reduced by bacteria to urobilinogen and urobilin. Urobilin is excreted with feces while urobilinogen is reabsorbed from the liver to the blood stream and excreted via the kidney, see the following figure.



The normal values of: s. Total bilirubin: 0.0-1 mg/dl.

(In serum): s. direct bilirubin: 0.0-0.2 mg/dl.

s. indirect bilirubin: 0.2-0.8 mg/dl.

(In Uring): Urobilinogen(+1).

(In feces): Urobilinogen(+2), Urobiline (+2).

Clinical significance:

The determination of s. bilirubin is to detect of subclinical or occult jaundice, which characterizes by founding orange dye on the skin, the sclera and the mucous membranes. Hyperbilirubinemia is frequently found in the following types of jaundice:

1. <u>Hemolytic jaundice</u>:

Caused by overproduction of bilirubin due to excessive hemolysis, occur in acute and chronic hemolytic anemia.

This condition is usually associated with increased value of s. indirect bilirubin.

2. Hepatic jaundice:

Caused by some type of intrahepatic obstruction which leads to cirrhosis of liver and hepatitis. Bilirubin accumulates and discharged back into the blood. In these conditions, the indirect bilirubin mainly increase in the early phase, but as the liver damage progresses, the direct form also become elevated.

3. Obstructive jaundice:

Caused by a post hepatic blockage of the larger bile channel, particularly the common bile duct, resulting in a reflex of bilirubin into the blood and only s. direct bilirubin elevates.

Methods of S. Bilirubin determination

Principle: Van Den Bergh and his co. workers applied the diazo reaction in 1913(first used by Ehrlich in 1883). The diazo reaction is:

Sulfanilic acid+ Na-nitrite ⟶ Diazotized sulfanilic acid

Both forms of the bilirubin(direct and indirect) give a pink azo- bilirubin with diazotized sulfanilic acid.

Conjugated bilirubin(direct) reacts in aqueous solution (direct reaction), while unconjugated bilirubin(indirect) requires an accelerator or solubilizer (such as alcohol) and therefore has been termed direct reaction.

Procedure:

1- Total bilirubin

Solutions	***Standard solution***		***Test solution***	
	Blank	Control	Blank	Control
Standard R4	150 ul	150 ul		
Serum	-	-	150 ul	150 ul
Diazo solution R1	3 ml		3 ml	
Working solution		3 ml		3 ml
Mix well after each addition, let to stand at 37 C for 5 min., and then read the absorbance through 10 min. at 555nm.				

2- Direct bilirubin

Solutions	***Standard solution***		***Test solution***	
	Blank B	Control C	Blank B	Control C
Standard R4	150 ul	150 ul		
Serum	-	-	150 ul	150 ul
Diazo solution R2 D	3 ml		3 ml	
Working solution D		3 ml		3 ml
Mix well after each addition, let to stand at 37 C for 5 min., and then read the absorbance through 10 min against their blanks. at 555nm.				

Calculation:

1- S. total or direct bilirubin(mg/dl) $= \frac{Abs.\ T}{Abs.\ S} \times$Std.conc.

$$= \frac{T}{S} \times 0.585$$

2- S. unconjugated bilirubin = total- conjugated

Significance of the solutions for total bilirubin:

1. **Diazo reagent R1:** Formed by mixing sulfanilic acid+ HCl +DMS to produce the intermediate compound. DMS is used to extract the unconjugated bilirubin.
2. **Working solution :** Formed by mixing R1+ Na-nitrite to give diazotized sulfanilic acid which reacts with bilirubin to form azo bilirubin .

Significance of the solutions for direct bilirubin:

3. **Diazo reagent R2 D:** Formed by mixing sulfanilic acid+ HCl only to produce the intermediate compound.
4. **Working solution D :** Formed by mixing R2+ Na-nitrite to give diazotized sulfanilic acid which reacts with bilirubin to form azo bilirubin .

***Reagents for total bilirubin*:**

1. Standard solution (R4): mix the contents of R4 bottle with 3 ml water and let to stand for 15 min.

2. Diazo solution(R1): Formed by mixing sulfanilic acid (30 mmol/l)+ HCl (150 mmol/l) +DMS (7 mmol/l).

3. working solution: Mix well 20 volumes of R1 with 1 volume of Na-nitrite (R3).

***Reagents for direct bilirubin*:**

1. Diazo solution(R2) D: Formed by mixing sulfanilic acid (30 mmol/l)+ HCl (150 mmol/l).

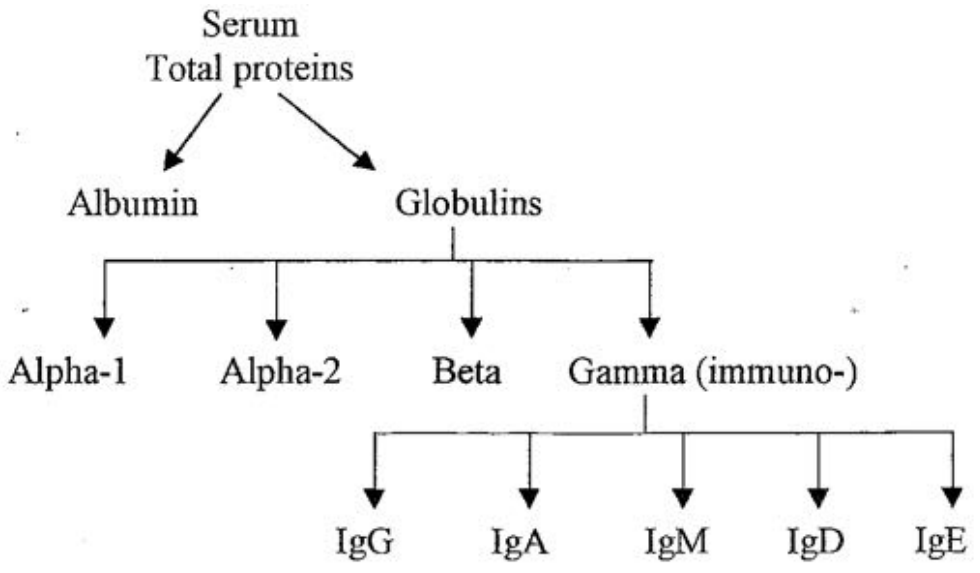
3. working solution D: Mix well 20 volumes of R1 with 1 volume of Na-nitrite (R3).

Experiment No. (9)

Determination of **Serum total proteins**

Proteins are polymers of α- amino acids occurring in both soluble and insoluble states in the body. In this handbook, only soluble proteins will be performed because of the emphasis on fluid rather than solid-tissue samples. The three-common fluids for such analyses are serum, plasma and cerebrospinal fluid(CSF).

Serum (as contrasted with plasma) is deficient in those coagulation. Therefore, serum protein will be approximately 0.25gm/dl lower than for plasma protein because of the absence of fibrinogen. Serum protein can be analyzed in total, in groups, and individually as illustrated in the figure:



Many proteins, including albumin, fibrinogen and most globulins are formed in the liver.

Clinical significance

Hyperproteinemia: occurs in

1. **Dehydration:** Dehydration may result from: a decrease in water intake, as occurs in frank water depletion or from excessive water loss, as occurs in: vomiting, severe diarrhea, and diabetic acidosis. Due to dehydration, total serum protein is increased, which increases all protein fractions.
2. **Multiple myeloma:** In this state, the total protein may increase over to 10 mg/dl due to over production of proteins.
3. **Cirrhosis of the liver.**
4. **Certain chronic disease:** In this case, the immunoglobulins are mostly increased.
5. **Artefactual stasis during venipuncture,** stasis induced by keeping the tourniquet on far too long during venipuncture causes fluid to escape into the extra vascular compartment, leading to hemoconcentration and falsely raised protein conc.
6. Drugs like, clofibrate, digitalis, epinephrine, steroids, progestin, insulin and thyroxine.
7. **Exercise:** An increase of (6-12%) occurs with vigorous exercise of short duration.
8. **Vertical vs horizontal position:** Total s. protein is reported to be lower by(0.4-0.8g/dl) with the subject supine than when in the erect position. This phenomenon is usually a scribed to hemoconcentration when in the erect position.

Hyperproteinemia:

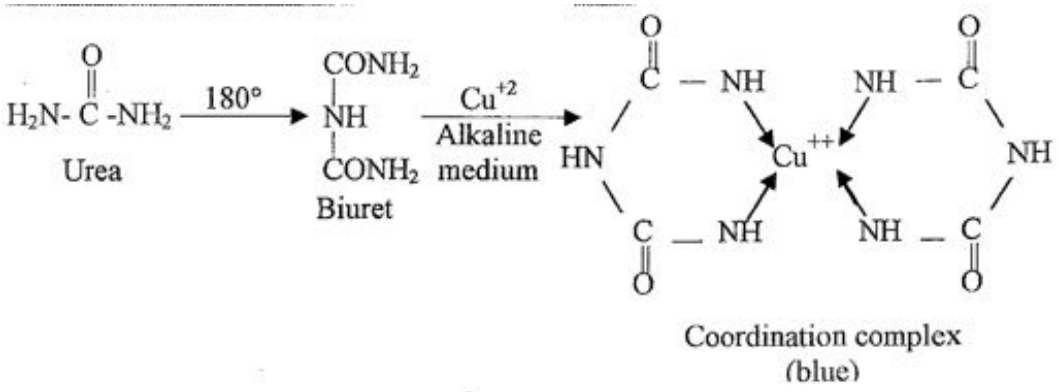
1. ***Overhydrating*:** The concentration of all fractions of protein is proportionately **decreased due to relative water excess.**

2. ***Kidney diseases as in the nephrotic syndrome*:** Large masses of albumin may be lost in the urine as a result of kidney damage.
3. ***Severe burns:*** Extensive bleeding, fever and necrosis where increased breakdown of protein.
4. Server protein deficiency(protein starvation).
5. Increased requirement, as in growth, pregnancy and hyperthyroidism.
6. ***Drugs*:** Estrogen has been reported to decrease the total protein.

Method for s. proteins estimation

Biuret method:

Substances contain two or more peptide bonds ($CO\text{-}NH_2$) give a blue to purple colored compound when react with alkaline copper solution. The reaction`s name "Biuret" means two urea molecules. When two urea molecules heated to about 180°C, it decomposes to give a product called "biuret", which in the presence of Cu^{++} in alkaline solution formed a violet colored complex which could be determined calorimetrically:



By means of the above reaction, peptide bonds of proteins are determined calorimetrically.

Procedure:

Solutions	***Test (T)***	***Standard (S)***	***Blank (B)***
Serum	20 uL	-	-
H_2O	-	-	20 uL
Standard albumin	-	20 uL	-
Biuret reagent	1ml	1ml	1ml
Mix , Place in water bath at 37°C for 10 min., allow to cool for 5 min. and read the absorbance at 450nm.			

Calculation:

$$\text{Total protein conc.(g/dl)} = \frac{Abs.(T-B)}{Abs.(S-B)} \times \text{Std.conc.}$$

$$= \frac{(T-B)}{(S-B)} \times 6$$

Biuret reagent: This solution is consisting of:

$$CuSO_4.5H_2O + NaOH + Na - K - tartarate + K1.$$

The alkaline cupper solution used to form the coordinated complex, but, about(3%) of the copper is precipitated as $Cu(OH)_2$ when added to protein solution at the alkalinity employed, therefore($Na - K - tartarate + KI$) solution is added to stabilize biuret reagent.

***Reagents*:**

1. **Standard Solution (0.5gm/100ml)**: Dissolve(0.5gm) of bovine albumin in 100ml of D.W.
2. **Biuret reagent**: Dissolve(4.5gm) of Na-K-tartarate in (250ml) of 0.1N-NaOH(4mg of NaOH in 1 L of D.W.). add 1.5gm of $CuSO_4.5H_2O$ +2.5gm of KI. Mix well and make it up to 500ml by 0.1N-NaOH.

Experiment No. (10)

Determination of **Serum Albumin and Globulin**

Albumin, the most prevalent protein (about 60% by weight), has a low molecular weight(66.000D), the biologic half time is 20 days and most of the protein functions done by albumin which are:

1. ***Regulatory function:*** Regulate the osmotic pressure of plasma and distribution of water between blood plasma and tissues.
2. ***Transport function:*** Albumin is a non-specific transporter for many physiologic substances such as drugs, antibiotics, various ions (Ca^{+2}, Mg^{+2},P^{+5}), amino acids, fatty acids, bilirubin, uric acid and hormones.
3. ***Catalytic function:*** The wide range of enzymes present in plasma are proteins.
4. ***Structural function:*** Alb also serves as a precursor for tissue proteins such collagen (fibrous protein found in connective tissues) and α- keratin.
5. ***Defense function:*** The immunoglobulins are antibodies, which provides a defense against infection such as thrombin and fibrinogen.

The normal value of:

Albumin: 2.5-5.6 gm/dl

Globulin: 1.3-3.2 gm/dl

Alb./Glob.=1.5-2.5.

Clinical significance of albumin & Globulin:

Table 1. The relationship between total proteins with diseases.

T. protein	Alb.	Glob.	Usual disorder
High	High	High	Dehydration
High	Normal	High	Multiple myeloma
Normal	Low	High	Hepatic damage, certain chronic infection
Low	Low	Normal	Renal diseases, in adequate diet, intestinal malabsorption, nephrotic syndrome
Low	Low	Low	3rd degree burns.

Methods used for serum albumin determination

1- DCG Method:

Albumin may be determined directly by dye binding, since some of the dyes undergo a change in color intensity upon protein binding.

Three examples of such dyes are:

1) Methyl orange.
2) 2-(4-hydroxyazo benzene)-benzoic acid(HABA).
3) Bromcresol green(BCG).

BCG is a pH indicator, which changes from yellow-blue in the pH range 3.8 to 5.4. At pH 4.2, albumin binds with BCG forming the dye-Alb-complex, which exhibit optical properties different from the free dye. Surfactants, such as Brij-35, decrease the color of the un-reacted dye and also prevents turbidity. These effects result in greater sensitivity and specificity of this method. Bilirubin, Hb or lipids, fatty acids and drugs, which are reversibly binding, do not affect the results.

2- Biuret Method.

Add serum(0.5ml)+ Na_2SO_3 solution(5.5ml)+ether(1ml) and mix by inverting it gently 20 times, centrifuge for 10 min and withdraw the supernatant.

Solutions	***Test (T)***	***Std.(S)***	***Blank (B)***
Supernatant	3ml	-	-
Std. albumin	-	3ml	-
H_2O	-	-	3ml
Biuret reagent	5ml	5ml	5ml
Mix , Place in water bath at 36°C for 10 min., cool the tubes and read the absorbance at 540nm.			

Calculation:

$$\text{S. albumin conc.(g/dl)} = \frac{Abs.(T-B)}{Abs.(S-B)} \times \text{Std.conc.} \times \text{dilution factor}$$

$$= \frac{(T-B)}{(S-B)} \times 0.5 \times 12$$

$$= \frac{(T-B)}{(S-B)} \times 6$$

Significance of the solutions:

1. **Sodium sulphite (Na_2SO_3) in ether:** to precipitate the globulin as a disc at the ether-water interface.
2. **Biuret reagent:** consists of alkaline cupper solution and used to form the coordinated complex

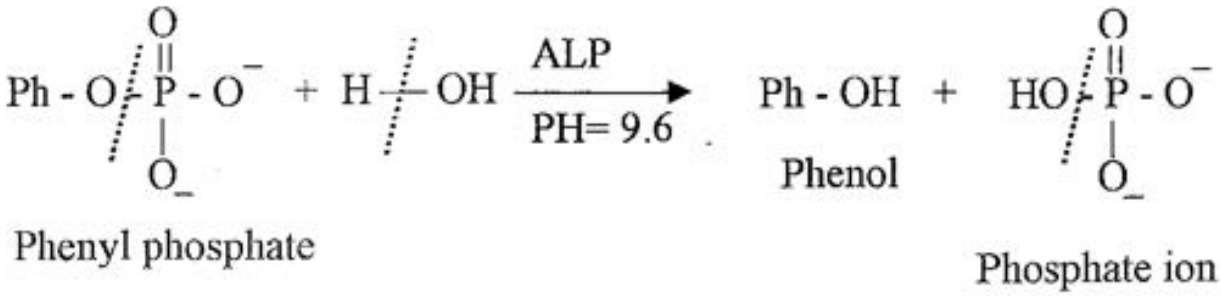
***Reagents*:**

All reagents are the same as for T. protein in addition to **Sodium sulphite (28%):** Dissolve (140gm) of unhydrated Na_2CO_3 in 500ml D.W. keep the solution at 37°C to remain soluble. If Na_2SO_3 does not available, Na_2SO_4(23%) may be used (11gm in 500 ml water).

Experiment No. (11)

Determination of **Serum alkaline phosphatase ALP**

The group of enzymes in the blood that catalyze the breakdown of phosphate esters bond, are known collectively as phosphatase. The clinically important phosphatase is divided into 2 groups depending on the pH at which they are most active. The group of phosphatase most active at pH 10 is called alkaline phosphatase(ALP) while those most active phosphatase at pH= 5 is called acid phosphatase(ACP)



Phenyl phosphate

Phosphate ion

ALP properties:

1. The optimal activity is exhibited at a pH of ~10.0 and temperature 37°C.
2. ALP requires Mg^{+2} , Mn^{+2} and Co^{+2} for activation.
3. ALP activity is exhibited by Ca^{+2}, PO_4^{-3}, $C_2O_4^{-2}$, EDTA, CN^- and F^-, therefore they should be avoided during preparation of plasma.
4. The substrate of ALP is p-nitrophenyl phosphate or phenyl phosphate or α-naphthyl phosphate.
5. ALP is found in the blood serum and plasma, bone, kidney, liver, mammary gland, intestine, lungs, spleen, leucocytes, and adrenal cortex. However, it is found chiefly in the bone and liver.

ALP helps the transference of metabolites through cells tissues, and bone calcification.

The normal value of ALP:

Adults: 3-13 KAU/dl

Children: 6-25 KAU/dl

⇒ ***King Armstrong unit of ALP activity:***

One unit being the mg of phenol liberated by 100ml of serum in 15 min under specified conditions.

Clinical Significance:

Increased S. ALP:

1- <u>Bone diseases</u>:

a. Paget's disease: increased 10-25 time than normal value.
b. Bone cancer.

<u>2- Hepatobiliary diseases:</u>

a. Obstructive jaundice.
b. Biliary Obstruction.

The enzyme activity is increased also in Pregnancy (increased bone formation) and growing children.

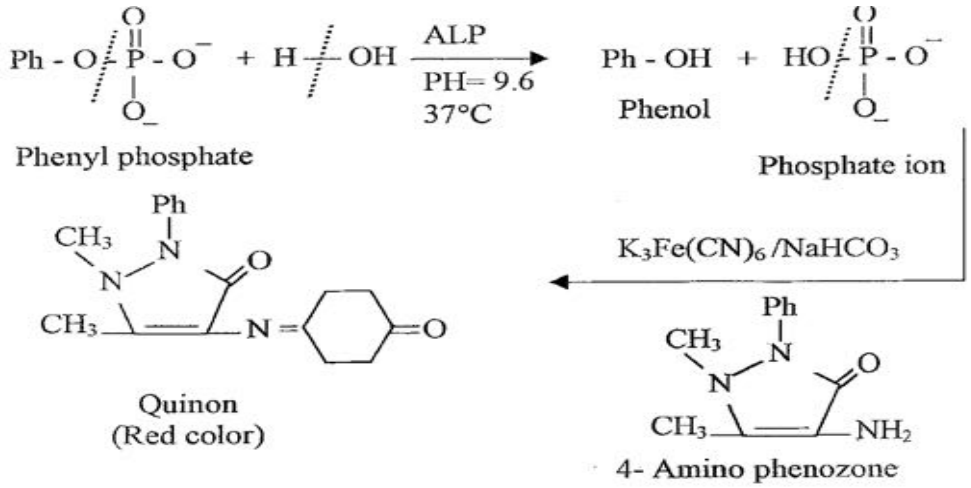
Decreased s. ALP level:

Hypoparathyroidism, chronic nephritis, and vit. C deficiency.

Methods of ALP activity determination

King- Armstrong method:

***Principle*:** This method is based on the addition of ALP`s substrate (phenyl phosphate) to the serum and incubate for 15 min. at 37°C. In the presence alkaline 4-amino antipyrine, a red or purple color is given with compounds containing a phenolic group. The intensity of the color is proportional to the enzyme activity.



Procedure:

Solutions	*Test (T)*	*Blank T*	*Standard (S)*	*Blank S*
Substrate solution **R1**	2ml	2ml	2ml	2ml
Std. solution **R2**	-	-	50 uL	-
Place test tubes at37°C for 5 min, then add:				
	0.1ml	-	-	-
Mix , gently and incubate the tubes in at 37°C for exactly 15 min., remove from the bath and add:				
4- amino antipyrine solution **R3**	0.5mL	0.5mL	0.5mL	0.5mL
$K_3Fe(CN)_6$	0.5mL	0.5mL	0.5mL	0.5mL
Serum	50 uL			
D.W		50 uL		50 uL
Mix well, settle for 10 min in dark and read the absorbance at 510nm.				

Calculation:

The amount of phenol present in the standard tube is $10\mu g$. Thus, the phenol produced in 15 min. by using 0.1ml serum is:

$$= \frac{Abs.(T-C)}{Abs.(S-B)} \times \text{Std concentration}$$

$$= \frac{Abs.(T-C)}{Abs.(S-B)} \times 20$$

Significance of the solutions:

1. NaHCO3+4- amino antipyrine+$K_3Fe(CN)_6$ solutions are used as oxidizing and coloring agent of the product of enzyme hydrolyses.

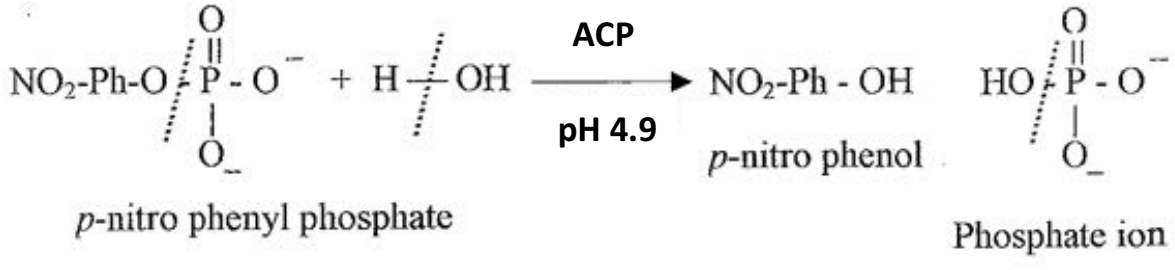
***Reagents*:**

1. **Buffer solution, pH=10, 50 mmol/L (R1):** Dissolve 6.3gm of unhydrated sodium carbonate+3.36gm of sodium bicarbonate in 1L of D.W. keep in the fridge.
2. **Substrate solution (0.01M)*:*** Dissolve 5mmol. of disodium phenyl phosphate in 1L of buffer, pH 10. Boil for 1 min to kill any organism if necessary. Cool and preserve with 2ml chloroform. Keep in the fridge.
3. **Standard phenol solution (R2)*:*** Dissolve 2gm phenol in 1L of 0.1 N-HCl(9ml of conc. HCl in 1L of D.W. store in a brown bottle and keep in the fridge.
4. ***4- amino antipyrine* (R3) :** Dissolve 6 gm of 4- amino antipyrine and 75 g of Na-arsenate in 1L of D.W. Keep in a brown bottle.
5. **Potassium ferricyanide solution:** Dissolve 24 gm of the substance/1L of in a brown bottle D.W. store in a brown bottle.

Experiment No. (12)

Determination of: **Serum acid phosphatase**

Acid phosphatase (ACP) refers a group of phosphatase that show a maximal activity near pH=5.0 and catalyzes the hydrolysis of an orthophosphoric monoester into alcohol and a phosphatase group, as shown in the biochemical reaction:



p-nitro phenyl phosphate

p-nitro phenol

Phosphate ion

Under the name of ACP, all phosphatase(isoenzyme) that have an activity at a pH below 7.0 are included. The greatest activity of ACP is present in liver, spleen, erythrocytes, platelets, bone marrow, the prostate gland, and the milk.

The prostate gland is the richest source, and contributes about 1/3 to 1/2 of the enzyme present in serum of healthy males.

Properties of ACP:

1- The optimal pH for individual ACP varies:

a) Depending on the tissue source. The prostatic acid phosphatase (PAP), which is of greatest clinical interest, has a well-action at optimum pH 4.8-5.1.

b) Depending on the substrate on which the enzyme acts: the more acidic the substrate, the lower the pH at which max, activity is obtained.

2- The optimal temperature for maximum enzyme activity is 37°C and the enzyme may by lost over 50% of its activity in an hour at room temperature.
3- The ACP activity is increased in the presence of Mg^{+2}, Co^{+2}, Mn^{+2} and inhibited by $C_2O_4^{-2}$, CN^-, PO_4^{-3}, … etc.
4- The enzyme acts on the substrate that ALP acts on.

The normal value of total ACP=1-3.5 KAU/dl.

Prostatic acid phosphatase (PAP)= 4 unit/L

⇒ ***King Armstrong unit of ACP activity:***

The enzyme unit that liberate 1mg of phenol through 1 hr. under certain conditions (pH=4.9 and 37°C).

Clinical Significance:

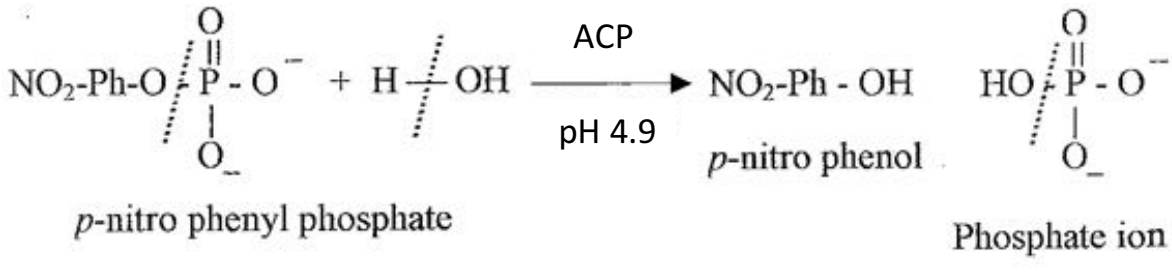
1. The main use of ACP estimation is in the diagnosis of prostatic carcinoma. Elevation of prostatic ACP (and generally, total ACP) are found in the sera of males with prostatic cancer associated with metastasis, where increased numbers of prostatic cells.
2. PAP is secreted into the semen and functions extra cellular. Its exact function is not known, but it is assumed to be important in fertilization. Testosterone increases its production and estrogen decreases.
3. The ACP level is increased also in:

a) Breast cancer.
b) Bone diseases like Paget's disease and bone cancer.
c) Various liver diseases.

Methods of ACP activity determination

1- Bessey, Lowry and Brock (BLB) method:

***Principle*:** This method is applied to measure the activity of total (ACP) and prostatic acid phosphatase (PAP) depends on the same principle of ALP activity measurement, as shown:



Where the substrate is hydrolyzed to p-nitrophenol which is yellow in alkaline solution. Absorbance of solutions at 410 nm.

Procedure:

Solutions	***Test T1 (Total ACP)***	***Test T2 (PAP)***	***Blank (B)***
Substrate solution (ACP) **R2**	0.5 mL	0.5 mL	0.5 mL
Substrate solution (PAP) **R3**	-	50 uL	-
Mix well, incubate at 37 C for 5 min, then add:			
Serum	100 uL	100 uL	100 uL
Mix well, incubate at 37 C for 2-3 min, then add:			
NaOH	5 mL	5 mL	5 mL
Serum	-	-	100 uL
Mix well and read the absorbance at 405 nm against blank			

Calculation:

Activity of total ACP (unit/L) = T1 * 0.101

Activity of PAP (unit/L) = T1 –T2 * 0.101

***Reagents*:**

1- **Buffer solution:** Dissolve 42 gm of citric acid in a little water, then add 376ml of (1N) NaOH and make the volume to 1L with D.W., check the pH in a pH-meter (4.9), preserve with few drops of chloroform and keep in the fridge.

2- **Buffered substrate (ACP**): 5.5 mmol/L of p-nitrophenylphosphate.

3- **Buffered substrate (PAP):** 200 mmol/L of Na-tartare, ready to use.

Experiment No. (13) Pancreatic function test

Determination of: ***Serum amylase activity***

Amylase are hydrolysis enzyme catalysis the hydrolysis of complex carbohydrate molecules into smaller components.

Human amylase is termed (α-amylase) or endo amylase because of its ability to split polysaccharide $\alpha(1-4)$ linkage. The end product of α- amylase reaction is the dextran, maltose, and some glucose molecules. β- amylase is found in plants and bacterial organism.

The amylase properties:

1. The enzyme is produced by the exocrine pancreas and the salivary glands to aid in the digestion of starch. It is also found in the liver and in the liming of the fallopian tubes.
2. The optimum enzyme activity at pH=6.9-7 at 37°C.
3. The enzyme is activated by Ca^{+2}, Cl^{-}, Br^{-}, NO_3, ClO_3, and HPO_4^{-}.
4. The enzyme has low M.Wt. (40.000-50.000D), therefore it removed from plasma by kidney and excreted into urine.
5. Its activity is stable for 1 week at 25°C and 2 months at(-4°C).
6. Serum is used instead of plasma because all anticoagulants are inhibited the enzyme activity(except heparin) .

The normal value: (60-180 somogyi/dl)

⇒ ***Somogyi unit of amylaese activity:***

The amount of enzyme that catalyze the hydrolysis of 5 mg of starch in 15 min. to a stage at which no color is given by iodine under optimum conditions (pH=7, 37°C).

Clinical Significance:

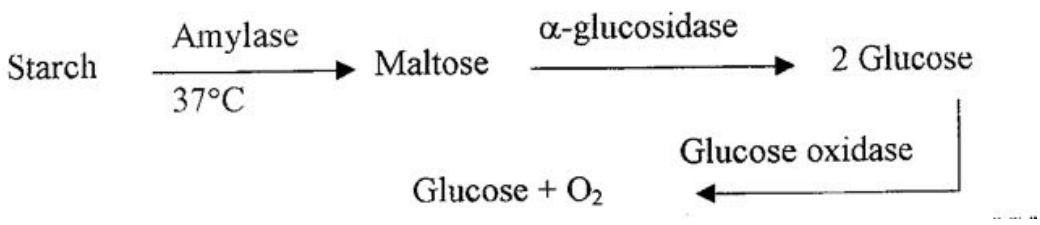
Determination of serum amylase is taken as an index or test of pancreatic function due to its large elevation level in pancreatitis.

1. **Acute pancreatitis:** The level reaches (1000-6000) somogyi/dl at(24-48hr.) after onset of the attack with a correspondingly large increase in urinary amylase. This rise starts within an hour of onset pain and remains so high for(3-4) days. After wards the level returns to normal in (4-8) days. Partly removed by renal excretion.
2. **Chronic pancreatitis:** Partial obstruction of the pancreatic duct system by fibrous tissue or tumor causes increase in amylase to 200 somogyi/dl.
3. **Mumps disease:** When the silvery gland secretion is turned off, serum amylase will rise to (200-600) unit.

Methods of determination of amylase activity

1. **Enzymatic method:**

***Principle*:** Starch is added to serum, amylase will hydrolyze starches to maltose, and by the addition of α- glucosidase enzyme maltose is degraded to glucose. The amount of glucose is a measure of amylase activity.



2. Caraway method:

***Principle*:** Starch is hydrolyzed by amylase in the sample to liberate smaller molecules. An iodine reagent is added, which forms a vivid blue color with remaining starch. The amylase activity is inversely proportional to the amount of color in the final solution. This method is cheap, simple, quick and sensitive, but it is limited by protein-iodine interaction requires blanks.

Procedure:

Note: Prepare a diluted serum(1:10) in a normal saline solution(0.9gm of NaCl in 100ml water).

Solutions	*Test (T)*	*Control(C)*	*Blank (B)*
Buffered Substrate	1ml	1ml	-
H_2O	-	0.1ml	1.1ml
Put test tube(T) only in a water bath at37°C for 3 min, then add the diluted serum			
Diluted serum (1:10 in normal saline)	0.1ml	-	-
Mix by swirling and return tubes to the bath for exactly 15 min, remove the tubes and immediately add:			
Iodine sol.	0.4ml	0.4ml	0.4ml
H_2O	8.5ml	8.5ml	8.5ml
Mix well after addition, and read the absorbance at 640nm.			

Calculation:

One amylase unit (Somogyi unit) catalyzes the hydrolysis of 5mg starch in 15 min. Since the substrate contains 0.4g/10mL starch, then 1mL amount used provides 4mg/mL of starch. Accordingly, 2mg of starch hydrolyzed in 15min. is equivalent to:

$$\frac{4}{15}\times\frac{15}{5}\times(unit)=\frac{0.8unit}{0.1ml\ serum}$$

$$=\frac{800unit}{dl}$$

Therefore,

$$\text{Amylase activity(unit/dL)}=\frac{Abs.(C-T)}{Abs.(C-B)}\times 800$$

The Abs of (C-T) represents the amount of substrate hydrolyzed by the serum sample. The Abs. of (C-B) represents 800 amylase unit.

Significance of the solutions:

1. **Buffered substrate sol.:** A mixture of Na_2HPO_4/ Benzoic acid, (pH=7) used to provide suitable medium for enzyme cation. Starch is added(0.4g/10ml) to the buffer sol. as a substrate.
2. **Iodine sol.:** Used to stop the enzyme reaction, forms a vivid blue color wthe remaining starch.

***Reagents*:**

1. **Buffered substrate solution:** Dissolve 26.6gm of sodium hydrogen phosphate+8.6gm of Benzoic acid in 500ml water, boil the solution and add soluble starch (0.4gm in 10ml water). Continue boiling the solution for 1 min., cool to room temp. and dilute to 1L by D.W.
2. **Stock iodine solution:** Dissolve 13.5gm of iodine +solution of KF (24gm/100ml water), dilute to 1L and store in an amber bottle at 4°C.
3. **Working iodine solution:** Take 100ml of stock sol.+ solution of KF (50gm in a little water) and dilute to 1L.

Experiment No. (14)

Determination of: ***Serum Calcium***

Calcium ion presents in the body in larger amounts than any cation. It mostly presents in bones and teeth. In the blood, essentially all the Ca exists in three forms:

(1) free Ca^{++}(50%).
(2) Bound to protein, primarily albumin (45%).
(3) a complex with certain organic compound, mainly citrate(5%).

The ionized form is the most important from a physiologic stand point, but its measurement is difficult. The percentage of total Ca depends on the amount of protein present and the pH of blood.

Calcium ion is important in:

1- Bone calcification.
2- The transmission of never impulses.
3- The maintenance of normal muscle contractility because it's a neuromuscular sedative.
4- As a cofactor in certain enzyme reaction.
5- The coagulation of the blood.
6- The transmission of organic and inorganic ions through the cells membranes.
7- Calcium and phosphorus tend to maintain equilibrium in the blood. they are usually considered together, since the disturbances of one quite often results in a disturbance of the other.

The normal value of s. calcium is: (9-11.5mg/dl):

The important dietary sources of Ca include milk, eggs yolk, chess and beans. The adult requires about 800mg Ca daily, but the growing child, pregnant and lactating women require more. Calcium is absorbed into blood stream from the small intestine.

Clinical Significance:

Hypercalcemia:

1. **Hyperparathyroidism**: One of the important diseases affecting Ca metabolism is the excessive and uncontrolled secretion of PTH (parathyroid hormone), which may occur in tumors of the parathyroid gland. Abnormally high level of this hormone result in excessive release of Ca from the bones and then elevate its levels. This may be related to phosphorus metabolism, which generally varies inversely with Ca.
2. Carcinoma metastatic to bone.
3. **Multiple myeloma:** It's a tumor of the plasma cells produce γ-globulin. Destruction of bone by the tumor and release of Ca in the blood side by side with elevated plasma protein (produced by the tumor itself) and increased binding calcium- protein will elevated s.Ca.

Hypocalcemia:

1. **Rickets**: decreased calcium and phosphorus levels caused abnormal bone formation due to:

a. Deficiency of vit. D.
b. Hypoparathyroidism.

This disease is characterized by convulsions and muscular twitching.

2. **Tetany:** a substantial reduction in Ca^{+2} conc. result by a state of neuromuscular excitability characterized by uncontrollable muscular cramps and tremors. In cases of suspected tetany, it is of a great value to the physician if Ca level can be determined rapidly so that therapy can be instituted immediately.
3. **Kidney disease:** Kidney failure quite regularly results in reduces the ability to excrete phosphorus causing a fall in the Ca level.

Methods used for Ca determination

There are many ways to measure the Ca, however, the widely used one is the calometric method:

Calorimetric method:

***Principle*:** The more widely used method. Anionic dyes as o-cresophthalein complexone form colored or florescent complexes with Ca^{++}. When the EDTA agent is added, the Ca is bound, instead, by this agent, and a color or florescence change results. Readings are made in spectrophotometer, which avoids the problem of determining a visual end point.

Procedure:

Solution	Test (T)	Standard (S)	Blank(B)
Serum	20 uL	-	-
Standard Solution (R1)	-	20 uL	-
Working solution(R2+R3)	1 mL	1mL	1mL
Mix well & read the absorbance at 572nm against blank			

Calculation:

$$\text{Ca conc. (mg/dL)} = \frac{Abs(T-B)}{Abs(S-B)} \times Std.con$$
$$= \frac{T-B}{S-B} \times 10$$

Significance of the solutions:

1. **Working solution: contains** cresophthalein-complexone to combine with Ca^{+2} and 8-hydroxyquinoline to eliminate the interference with Mg^{+2}

Normal value of Ca in this method is 8.1-10.4 mg/dL.

Experiment No. (15)

Determination of: ***Serum Inorganic phosphate***

The human body contains nearly one kg of phosphorous. Approximately 80% of this quantity are found in the calcium phosphate salt, which make up the inorganic substance of bone. About (10%) is combined with protein, lipids and carbohydrates and in other compounds in blood and muscle. The remaining (10%) is widely distributed in various chemical compounds.

The calcium-phosphate of bone gives them rigidity but also serve as a large storage depot for phosphate. The role of phosphate in other cells is important and complex. Much of the metabolic pathways involve esterification of metabolites with phosphate.

The phosphorous of blood is classified into:

1. Inorganic phosphate: Present as HPO_4^{-2} and $H_2PO_4^-$.
2. Organic phosphate or phosphate esters such as glycerol phosphate, nucleotides as ATP, hexo- phosphate.
3. Phosphate ester of fats (phosphor lipids), which are important in the structure and function of the membrane and transfer of certain substance in and out of the cells, ex: Lecithin, cephalin and sphingomyelin.
4. Phosphate is also an integral part of nucleic acid structure (RNA and DNA), which transfer genetic information.
5. Phosphoric acid makes up part of the prosthetic group of certain protein, such as casein of milk.

The whole phosphorous is referred as total phosphorous (Inorganic+ organic phosphorous). These parts

are acid soluble phosphate. Phosphate is excreted by the kidney following filtration and active tubular reabsorption. This process is inhibited by parathyroid hormone(PTH) which thereby increase phosphate excretion. This rate of renal excretion of phosphate controls the plasma Ca level. If the plasma Ca falls below normal level, the PTH causes the kidney to excrete more phosphate into the urine. The resulting fall in phosphate allows Ca to move from its storage depots in bone into the plasma, therefore increase level of Ca in plasma.

Normal values of s. inorganic phosphates :

Adults: 2.8- 5 mg/dL

Children: 4.5-6.5 mg/dL

Infants: 4.0-7.0 mg/dL

Clinical Significance:

Abnormal blood phosphate levels are most commonly seen in kidney, bone and parathyroid disease. Phosphate is usually measured along with Ca, since each measurement is useful in the interpretation of the other.

Hyperphosphatemia: Increased level of blood phosphate is seen in over does of vit D, renal failure, hypoparathyroidism, diabetic ketoacidosis, chronic nephritis, diarrhea, vomiting, acromegaly and in bone metastases.

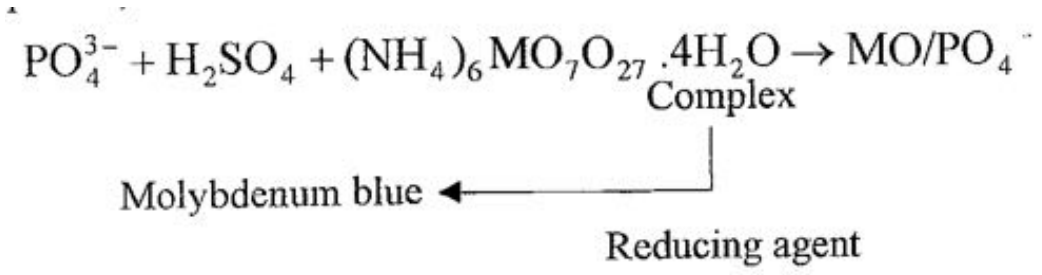
Hypophosphatemia Decreased valves are seen in rickets, hyperinsulinism, hypopitutarism, hypopara-thyroidism and malabsorption.

After carbohydrate meal, phosphate enters the cells with glucose (phosphorylated glucose), therefore, to estimate inorganic P, fasting blood should be collected from patients. Injection of insulin decreases the serum inorganic P during the treatment of diabetic coma.

Method of s. Inorganic phosphate determination:

(Fiske and Subbarow 1920):

***Principle*:** The oldest and still most commonly used methods which based on the reaction of phosphate ion with molybdic acid to form a yellow phosphor molybdate complex which can be measured directly or convert to molybdenum blue by a wide variety of reducing agents, such as "Sne12, Sne12+ hydrazine. Hydroquinone, 1-amino-2-naphthol-4- sulfonic acid.



***Procedure*:**

Solutions	*Test (T)*	*Control(C)*	*Blank (B)*
Serum	5ml		
Standard R1		5ml	
D.W			5ml
Working (R2+R3)	1ml	1ml	1ml
Mix well, let the tubes to stand 10 min. and then read the absorbance at 690 nm			

Calculation:

$$Inorganic\ phosphate(mg/dl) = \frac{Abs.\ T}{Abs.S} \times \text{Std. conc.}$$
$$= \frac{T}{S} \times 5\ mg/dL$$

Significance of the solutions:

Working reagent: Consist of the reducing agent (H_2SO_4, ammonium molybdate, ferric ammonium sulfate and ferrous nitrate) and the color reagent (H_2SO_4 + ammonium molybdate). This solution is used to from phosphor molybdate from the phosphorous present in the P.F.F

***Reagents*:**

1. Standard solution: 5 mg of P in 100 mL D.W with Na-azide

2. Reducing reagent R2 : contains H_2SO_4, ferric ammonium sulfate 100 gm/L, ferrous nitrate 2 gm/L.

3. Color reagent R3: contains ammonium molybdate 4.5 gm/L and H_2SO_4 1 N

4. Working solution :

Mix one volume of R2 with 2 volumes of R3