Section 2.1

Bilirubin

2.1.1 Chemical properties of bilirubin

Molecular formula \( C_{33}H_{36}N_4O_6 \)

Molar mass \( 584.66 \)

Solubility Chloroform, DMSO + Na\(_2\)CO\(_3\), DMSO + NaOH

Physical status Orange Crystalline Solid

IUPAC Name: \((5Z,5'S')-5,5'-\text{methylenebis}(1H\text{-pyrrole-5,2-diyl})\text{bis}(\text{methan-1-y1-1-ylidene})\) \text{bis}(1H\text{-pyrrol-2(5H)-one})
2.1.2 Introduction

Bilirubin is the breakdown product of heme moiety of hemoglobin; other hemeoproteins include cytochromes, catalase, peroxidase, tryptophan pyrrolole and a small pool of free heme. In humans, 250–400 mg of bilirubin are produced daily, of which approximately 20% is produced from non-hemoglobin sources [1].

Increase in concentration of direct reacting bilirubin in blood causes hyperbilirubinaemia [2], which is toxic under certain conditions inducing jaundice, hyperbilirubinemia-induced auditory dysfunction and neurotoxicity resulting in brain damage [3]. On the other hand, mild unconjugated hyperbilirubinaemia behaves as mild antioxidant [4] and might offer protection against cardiovascular diseases [5] and tumor development. Recent research survey has reported that low concentration of direct reacting bilirubin induces stroke in body [6] and sometimes causes cardiac problems too. Serum bilirubin levels are often enhanced under a variety of clinical conditions. Bilirubin has attracted the attention of physicians since antiquity. Its chemistry, metabolism and disposal have been studied systematically during the last two centuries as a model for hepatic disposal of biologically important organic anions of limited aqueous solubility [7].

In the circulation of blood, bilirubin is bound to serum albumin, which prevents its potential toxicity thought to be caused by free bilirubin [8]. Despite its high-affinity of binding to albumin, bilirubin is rapidly and selectively taken up by the liver [9], biotransformed upon conjugation with glucuronate [10], and secreted into bile. Thus bilirubin is converted into bilirubin glucuronic acid in the liver and excreted along with bile. The metabolism of bilirubin starting from heme proteins to
its final excretion is represented schematically in Figure 2.1.1. Obstruction to bile flow results in the appearance of direct reacting bilirubin in blood.

2.1.3 Bilirubin Metabolism

From the formation of bilirubin until its elimination from the body as a waste product of heme catabolism it undergoes a series of metabolic alterations and transport processes. Partial or complete failure at any point in this sequence can give rise to jaundice [11]. Initially enzyme heme oxygenase converts heme to biliverdin. The biliverdin reductase converts biliverdin to bilirubin. This bilirubin is unconjugated, indirect, and insoluble in water, therefore it can be detected in the urine. Unconjugated bilirubin bound to albumin is transported to the liver. In the liver, bilirubin gets conjugated to glucuronic acid by bilirubin glucuronosyltransferase. This conjugated bilirubin is direct, and soluble in water. It is then actively transported into bile channels. Conjugated bilirubin drains into the duodenum and passes through the proximal small bowel. It is not absorbed in the intestine. In the distal ileum and colon, it is converted back to unconjugated bilirubin by normal gut bacteria; 80-90% of this product gets excreted along with the feces, known as stercobilinogen, which imparts feces its brown color. Ten to twenty percent is passively absorbed, enters the portal venous blood, and is re-excreted by the liver. A small amount escapes hepatic uptake, filters across the glomerulus and is excreted in the urine. A small fraction is reabsorbed into the portal circulation and excreted in the urine as urobilinogen. The schematic representation of bilirubin metabolism is shown in Figure 2.1.2.
Figure 2.1.2: Schematic representation of the formation and excretion of bilirubin
2.1.4 Formation of bilirubin

Bilirubin is formed from heme by opening the heme ring at α carbon bridge [12]. This cleavage is catalysed by the enzyme heme-oxygenase (HO) (Figure 2.1.3). Three molecules of O₂ are consumed in this reaction in presence of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), a reducing agent. The α-methene bridge carbon is eliminated as CO and the iron molecule is released producing biliverdin IXα [13]. This is reduced to bilirubin by the action of cytosolic biliverdin reductases [14], which require NADH or NADPH for activity. Cleavage at non-α sites is possible; it is probably non-enzymic and occurs only to a minor extent. This results in the formation of other isomers; some can be detected in body fluids, but in small amounts or under special conditions. Because of the asymmetry in the arrangement of the side chains around the heme molecule, the methene-bridge carbon atoms, α, β, γ and δ [15] are not equivalent thus four isomeric bilirubins IX are therefore possible depending on which attacks methene bridge. The bilirubin found in mammalian bile consists almost exclusively of α - isomer, designated as bilirubin IXα.
2.1.5 Bilirubin fractions

Bilirubin in plasma is not a single homogeneous substance but represents a variable mixture of isomers and fractions. The various ‘‘bilirubins’’ differ, sometimes considerably, in their physiological behaviour and their chemical reactions *in vitro*.

2.1.5.1 Free bilirubin (Bf)

Free bilirubin is the breakdown product of hemoglobin (Hb) of aged erythrocytes in the reticuloendothelial cells of the spleen [16]. This free bilirubin is not bound to albumin and its toxic effect is believed to occur even at a concentration...
of 0.005 mg/dL. So far, there has been no really reliable method has been developed for measuring free bilirubin content in plasma (or alternatively for measuring the free binding capacity of albumin for free bilirubin).

### 2.1.5.2 Unconjugated bilirubin (Bu)

The free bilirubin bound to albumin is called unconjugated bilirubin [17]. The splitting of heme ring at different positions (α, β, γ or δ) leads to the formation of its various isomers which cannot form hydrogen bonds, and are therefore more readily water-soluble and get excreted through the urine.

### 2.1.5.3 Conjugated (glucuronated) bilirubin (Bc)

The free or unconjugated bilirubin bound by albumin [18] is carried to liver, where it is conjugated with glucuronic acid by the enzyme glucuronyltransferase. The enzyme, glucuronyltransferase transforms the albumin-bound bilirubin to monoglucuronide or diglucuronide conjugated bilirubin.

### 2.1.5.4 Delta-bilirubin (Bd)

Delta-bilirubin [19] arises through a non-enzymatic covalent coupling reaction between glucuronated bilirubin and albumin, which is nontoxic and excreted neither in urine nor in bile but is slowly metabolized with a half-life of 20 days.

### 2.1.6 Reference ranges of bilirubin in serum [20, 21]

Only conjugated form and/or direct bilirubin is filtered by the kidneys and is also excreted into the urine (called urine bilirubin) [22]. Normal values may vary from lab to lab. The reference range of bilirubin in blood is mentioned in Table 2.1.1.
Table 2.1.1. Reference range of bilirubin in serum

<table>
<thead>
<tr>
<th>Type of bilirubin</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg / dL</td>
</tr>
<tr>
<td>Conjugated or Direct bilirubin</td>
<td>0.1 to 0.3</td>
</tr>
<tr>
<td>Unconjugated or Indirect bilirubin</td>
<td>0.2 to 0.8</td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>0.3 to 1.0</td>
</tr>
</tbody>
</table>

2.1.7 Clinical Significance

Any increase in formation or retention of bilirubin (hyperbilirubinemia) [23] by the body may result in a diseased condition called jaundice, which is characterized by an increase in the bilirubin level in the serum and the presence of a yellowish pigmentation in the skin. Jaundice may be classified as.
2.1.8 Analytical significance of bilirubin measurement

Bilirubin is the potentially toxic catabolic product of heme metabolism. There are elaborate physiologic mechanisms for its detoxification and disposition. Bilirubin is poorly soluble in water at physiologic pH [28], because of internal hydrogen bonding that engages all polar groups and gives the molecule an involuted structure. The intramolecular hydrogen bonding shields the hydrophilic sites of the bilirubin molecule resulting in a hydrophobic structure.

Water-insoluble, unconjugated bilirubin is associated with all known toxic effects of bilirubin [29, 30]. Thus, the internal hydrogen bonding is critical in producing bilirubin toxicity and also in preventing its elimination. Conversion of unconjugated bilirubin to a water-soluble form by disruption of the hydrogen bonds is essential for its excretion by the liver and kidney. This is achieved by glucuronic acid conjugation of the propionic acid side chains of bilirubin. Bilirubin glucuronides are water-soluble and are readily excreted in bile. Bilirubin is primarily excreted in normal human bile as diglucuronide.

A low concentration of bilirubin is found in normal plasma, almost all of which is indirect. The sum of the direct and indirect forms (or conjugated and unconjugated) is termed as total bilirubin. Routine analytical procedures exist for the determination of total bilirubin and for the measurement of direct bilirubin. The indirect fraction is obtained by subtracting the direct value from the total value. Measurement of total bilirubin and determination of direct and indirect fractions are important in routine screening and also for the differential diagnosis of jaundice.
2.1.9 **Reference method (CLSI)**

Doumas et al. (1985) described a “Candidate Reference Method” for the measurement of total bilirubin in serum which has been recognized as the reference method by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) [31, 32].

The basis of this method was the Jendrassik-Grof procedure [33, 34, 35], which was optimized with respect to the concentration of the reagents, the reaction time and a range of marginal conditions. For testing sample and blank, 0.5 mL of serum is mixed with 4.0 mL of caffeine reagent. After 10 min, 1.0 mL diazo reagent (sample) or 1.0 mL sulfanilic acid (blank) is added, and after further 10 min 3.0 mL of tartrate solution are added. After 10 min, photometry is performed at 598 nm. The method is calibrated with the Standard Reference Material SRM 916a of the National Institute of Standards and Technology (NIST), which contains unconjugated bilirubin at a purity of 99%. The material must be dissolved (by means of dimethyl sulfoxide and Na$_2$CO$_3$) in human serum or in 40 g/L solution of albumin. The method is linear up to 25 mg/dL.

The interference through hemoglobin [36] that is typical for the diazo methods is only apparent in the sample at Hb concentration of 2 g/L. The addition of ascorbic acid [37, 38] (0.1 mL per 40 g/L solution) before addition of tartrate reagent is recommended to overcome the interference. Addition of ascorbic acid to every sample is, however, not recommended, since ascorbic acid destroys the diazo reagent.
2.1.10 Methods other than diazo methods

2.1.10.1 Enzymatic measurement

Bilirubin oxidase (BOX) catalyses the oxidation of bilirubin to biliverdin in the presence of molecular oxygen [52, 53, 54]. At pH values of 5.0–8.5, the biliverdin is further oxidized to purple products that finally become colourless. Total bilirubin is measured with the enzymatic method at pH 8.2 after the addition of sodium dodecyl sulfate and sodium cholate; the two additives required to release the bilirubin that is bound to albumin, which then, just like the unbound bilirubin, can be rapidly converted by BOX. All bilirubin fractions of the serum (including the conjugated fractions) get oxidized to biliverdin. The total bilirubin can be quantitatively determined either on the basis of the decrease in the absorption of bilirubin at 425 nm or through the increase in the absorption of the purple pigment at 450 nm [55]. An advantage of the enzymatic method is the low amount of sample required. However, the values obtained for total bilirubin are somewhat lower than those obtained by the diazo-procedure.

2.1.10.2 Bilirubinometer

Bilirubinometer is a simple filter photometer, which measures the absorption of plasma at 454 nm near the absorption maximum of bilirubin. Since neonate plasma contains no lipochromes it likewise absorbs around this wavelength (e.g., carotene), bilirubin can be determined quantitatively if the spectral interference by hemeoglobin is compensated. This can be achieved through an additional measurement at 540 nm. Since the molar extinction coefficient of the hemeoglobin is identical at 455 nm and 540 nm [56, 57], the bilirubin concentration can be calculated from the difference

\[ \Delta \varepsilon = \varepsilon_{455} \text{nm} - \varepsilon_{575} \text{nm}. \]
2.1.10.3 Transcutaneous bilirubinometry

Transcutaneous bilirubinometry measures the intensity of yellow color in the skin [58, 59, 60] and subcutaneous tissue and correlates it with the serum bilirubin concentration in newborn infants.
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Section 2.2

Creatinine

2.2.1 Chemical properties of creatinine

Creatinine exists in two forms as shown in Figure 2.2.1

Figure 2.2.1: Structure of creatinine

IUPAC name : 2-imino-1-methylimidazolidin-4-one
Solubility : Water soluble
Molecular mass : 113.118
Colour : White amorphous solid
Molecular formula : C₄H₇N₃O
Density : 1.09 g cm⁻³
Melting point : 300 °C
2.2.2 Introduction

Creatinine is a break-down product of creatine phosphate present 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.

In the body, creatinine is formed by a spontaneous and irreversible conversion of creatine and creatine phosphate [1], which is the source of high-energy phosphate bonds for the immediate reformation of ATP during muscular contraction. Creatinine is normally synthesized from arginine, S-adenosyl-methionine and glycine, primarily by the kidney and liver [2], but recent evidences suggest that the brain and testis may also be involved in the synthesis of Creatinin [3]. Creatinine is consumed along with the diet predominantly in meat, fish and other animal products [4]. A proportion of creatine and phosphocreatine entering the cell is phosphorylated to form Creatine phosphate, which subsequently provides an immediate source of ATP for energy requiring processes. Finally, creatine is converted to creatinine and is excreted completely through urine without any secretion or adsorption.

Creatinine is assumed to be metabolically inert [5]. Conversion of creatinine into creatine is not possible under the normal physiological conditions; neither it is reabsorbed nor actively secreted in renal tubules [6]. Blood collects the creatinine from various parts of the body and enters the kidney, where the creatinine is filtered and excreted completely through kidneys.

Creatinine is present not only in serum and erythrocytes but is also found in all bodily secretions, such as sweat, bile, and gastrointestinal fluids. Being ultrafiltrable, it is also present in the cerebrospinal fluid [7].
2.2.3 Creatinine metabolism

Synthesis of creatinine in the body starts with the reaction of two amino acids, Arginine and Glycine [8] in the kidney and in liver [9]. This reaction is catalyzed by the enzyme L-Arginine-glycine amidinotransferase (AGAT) producing guanidinoacetic acid and ornithine. Ornithine as a by product is utilized to increase muscle growth by stimulating the release of anabolic hormones such as growth hormone and insulin [10]. The guanidinoacetic acid is catalyzed by the enzyme S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT) leading to the formation of creatine. This creatine is then transported to the skeletal muscles through the bloodstream. The phosphate of adenosine tri phosphate (ATP) gets transferred to creatine, generating creatine phosphate, through the action of creatine phosphokinase. Creatine phosphate is stored in the muscle as high energy phosphate. The reaction is reversible and when the energy demand is high creatine phosphate donates its phosphate to adenosine di phosphate (ADP) to yield adenosine tri phosphate [11]. Creatinine is formed in muscle tissues from creatine phosphate by a nonenzymatic dehydration process and by the loss of phosphate.

Creatinine is transported through the bloodstream to the kidneys. About 1000-1200 ml of blood passes through kidneys per min. From this volume 120 ml of plasma/min are filtrated by glomerules of normal kidneys. The kidneys filter out most of the creatinine and dispose it off along with the urine. The schematic representation of creatinine metabolism is presented in Figure 2.2.2.
Figure 2.2.2. Schematic representation of creatinine metabolism in the body
2.2.4 Formation of creatinine

Creatine (Cr) and phosphocreatine (PCr) have been known to play important roles as components of an energy buffer mechanism in animal cells. In this system, ADP and ATP get interconverted (Figure 2.2.3) with the participation of creatine and phosphocreatine [12]. Catalyzed by creatine kinase, this reversible reaction helps the cells to maintain adequate ATP levels during large fluctuations in energy demand.

![Figure 2.2.3: Interconversion of creatine to phosphocreatine](image)

Creatine is a non-essential dietary molecule; its degradation rate within human body system is about 1.6% (2 g) per day [13]. The degradation of creatine pool involves the non-enzymatic conversion (Figure 2.2.4) of creatine to creatinine [14], most of which is subsequently excreted from the body via the kidneys. A small proportion of creatinine may get converted into other compounds such as arginine and guanidinobutyrate [15].
2.2.5 Reference range of creatinine in serum and urine sample

Creatinine level in urine is affected by many factors such as age, sex, height, weight, muscle mass, intake of food, medicines used and many other factors. Normal urine creatinine concentration ranges from 40 to 300 mg/dL in males and 37-250 mg/dL in females [16]. Lower creatinine values of 17 mg/dL have also been reported in rare instances. Since creatinine concentration is directly related to creatine in muscle, excretion through urine varies invariably. The reference ranges of creatinine [17, 18] in urine, blood and creatinine clearance are presented in Table 2.2.1.
Table 2.2.1: Reference ranges of creatinine in blood and urine samples

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th></th>
<th>Female</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dL</td>
<td>mM/L</td>
<td>mg/dL</td>
<td>mM/L</td>
</tr>
<tr>
<td>Urine creatinine</td>
<td>40–300</td>
<td>3.5–26.5</td>
<td>37–250</td>
<td>3.3–22</td>
</tr>
<tr>
<td>Blood creatinine</td>
<td>0.6–1.2</td>
<td>0.05–1.06</td>
<td>0.5–1.1</td>
<td>44–97</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>90–140 ml min⁻¹</td>
<td></td>
<td>87–107 ml min⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

2.2.6 Clinical significance of creatinine

Formation of creatinine is fairly constant [19], about 2% of the whole body creatine being transformed per day. Creatinine thus formed cannot be reutilised and is considered as a waste product. Creatinine from various parts of the body is collected and carried by blood to kidney. The glomeruli in kidney play an important role in filtering and excretion of creatinine from blood.

Blood carrying creatinine and various other metabolites enters the semipermeable walls of the glomerular capillaries which are almost impermeable to proteins and large molecules. The filtrate is thus virtually free of proteins and other cellular elements. The glomerular filtrate is formed by squeezing fluid through the glomerular capillary. About 20% of renal plasma flow is filtered every minute, (125 ml.min⁻¹). This is the glomerular filtration rate (GFR). GFR helps in regulating the creatinine ratio in blood and urine.

If the filtering rate of the glomerular capillaries of kidney is deficient, then creatinine level rises in blood. Therefore, creatinine levels in blood and urine are used to assess the creatinine clearance, which reflects the GFR. The failure of filtration by glomerular capillaries of kidney indicates the chronic kidney disease [20].
Any disease that affects the filtration process of the kidneys will tend to raise serum creatinine levels in blood over time. Doubling of serum creatinine levels in blood is an indicative of 50 percent reduction in the filtration rate of the kidneys.

Diseases associated with increased levels of creatinine include glomerulonephritis, pyelonephritis, diabetic nephropathy [21] and conditions such as shock and congestive heart failure that reduce blood flow through the kidneys.

2.2.7 Analytical significance of creatinine measurement in serum and urine

Since creatinine is the non toxic waste metabolite, it is neither absorbed nor adsorbed or secreted in body and is completely filtered out through kidneys. Measurement of creatinine concentration in blood and urine plays an important role in identifying many diseases. This test is adopted as routine blood and urine test.

Serum or plasma can be used to measure creatinine concentration in blood. Increase or decrease in blood creatinine is usually a sign of problems associated with kidney function which directly reflects on GFR [22]. Other than GFR, increase in the creatinine concentration in blood relates to many other diseases which directly or indirectly affects the kidney functioning. Analytical measurement of creatinine concentration in blood helps in preliminary identification of certain diseases like, cancer of testis or uterus [23], cholangitis, congestive heart failure, diabetes mellitus, hypertension, malaria and such other diseases. A recent study suggests that lower-serum creatinine level is associated with an increased risk for the development of type 2 diabetes [24].

Creatinine excretion in urine is constant and is proportional to total muscle mass. Urinary excretion of creatinine varies due to many factors especially intake of creatinine rich foods (e.g., roasted meat). Analytical measurement of creatinine
concentration in urine helps in preliminary identification of certain diseases like hypothyroidism, Alzheimer-type Dementis, hyperthyroidism, muscular dystrophy and protein malnutrition.

Measurement of the ratio of creatinine concentration in blood to that of urine gives an idea of creatinine clearance. The creatinine clearance provides an estimate of the amount of plasma that must have flowed through the kidney glomeruli per minute with complete removal of its content of creatinine to account for the creatinine per minute actually appearing in the urine. This test needs both blood sample and 24-hour urine collection from the patient.

Higher or low creatinine clearance by the kidney indicates the malfunctioning of kidney. Thus creatinine clearance is an indirect way to analyse the chronic kidney disorder via GFR. Increase or decrease in creatinine clearance by the kidney indicates the prevalence of certain major diseases like acromegaly, burn, acute/ or chronic pyelonephritis, heart failure, extrahepatic biliary obstruction, glomerulonephritis, gout, growth hormone deficiency, liver cirrhosis and many others.

2.2.8 Reference method (CLSI)

Most of the routine serum creatinine assays that are in current use have evolved based on the reaction initially described by Jaffe in 1886. Over the years, Jaffe used to report that a red color is produced when creatinine reacts with alkaline picrat. Folin in 1904 used this “Jaffe’s reaction” to determine creatinine content in urine. Since then it has been recognized that in addition to creatinine, a number of other compounds [25] also give the so called Jaffe’s reaction. Jaffe’s assay has since passed through many phases. Early methods used deproteinised blood. To improve specificity, creatinine was isolated from common interfering substances by adsorption.
on to aluminium silicates such as Lloyd’s reagent \[26\], followed by elution into alkaline picrate after centrifugation and decanting. Cation exchange resins were also in use for this purpose \[27\].

Boyne and his group of innovative biochemists in Australia developed an interesting solution to detect the false high results produced by the Jaffe assay method \[28\]. Their procedure involved incubating approximately 100 μL of serum for 1 hour in a sample cup containing a mixture of a dried solution of creatininase and creatinase. By assaying the sample before and after enzymatic treatment, they found that they could obtain similar accuracy as that of HPLC. The enzymes used in the assay have now formed the basis for most of the specific routine assays currently in use which underlies the reaction sequence as shown below:

\[
\begin{align*}
\text{Creatinine} & \quad + \quad \text{H}_2\text{O} \quad \xrightarrow{\text{creatinase}} \quad \text{Creatine} \\
\text{Creatine} & \quad + \quad \text{H}_2\text{O} \quad \xrightarrow{\text{creatinase}} \quad \text{Sarcosine} \quad + \quad \text{Urea} \\
\text{Sarcosine} & \quad + \quad \text{O}_2 \quad + \quad \text{H}_2\text{O} \quad \xrightarrow{\text{sarcosine oxidase}} \quad \text{Formaldehyde} \quad + \quad \text{Glycine} \quad + \quad \text{H}_2\text{O}_2
\end{align*}
\]

The hydrogen peroxide generated in the above reaction sequence can be measured spectrophotometrically using a Trinder’s reaction acceptor, producing a quinoneimine with high molar absorptivity (eg Roche Creatinine Plus assay), using a leuco dye (Vitros dry chemistry system), or with blood gas analyser electrodes (eg new Radiometer dual electrode system which corrects for creatine). Other enzymatic systems using creatinine deaminase, which convert creatinine to N-methylhydantoin and ammonia (with various options for measuring ammonia), and creatininase, (with NADH measured at 340 nm after a creatine kinase reaction sequence), have found little acceptance and application in routine analysis.
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Section 2.3

Hemoglobin

2.3.1 Introduction

Hemoglobin or haemoglobin symbolized as Hb or Hgb is the iron-containing oxygen-transporting metalloprotein found in red blood cells (RBC) or erythrocytes of all vertebrates [1] (except Channichthyidae fish family) [2] and also in the tissues of some invertebrates. Hemoglobin is present in the RBC of the body. Each RBC contains approximately 280 million hemoglobin molecules. Chemically hemoglobin is a tetrameric globular protein consisting of two each of alpha and beta chains [3]. The alpha and beta chains are fixed by different loci and are differentially regulated during human development. Of the four chains of hemoglobin each encloses an iron-containing heme co-factor, which binds the oxygen. It is the main function of hemoglobin to capture oxygen from the lungs and deliver the same to all tissues for respiration, and in turn transport carbon dioxide back from tissues to lungs. One hemoglobin molecule can transport up to 4 oxygen molecules. In addition, hemoglobin also plays vital role in regulating blood flow and blood pressure.

Hemoglobin is a conjugated protein having a molecular weight of nearly 64,500 D. Hemoglobin exhibits characteristic features of both tertiary and quaternary structures of proteins [4]. Most of the amino acids in hemoglobin form alpha helices, connected by short non-helical segments. Hydrogen bonds stabilize the helical sections inside this protein causing attractions within the molecule. Hemoglobin's
quaternary structure is derived from its four subunits in roughly a tetrahedral arrangement [5].

2.3.2 Structure of hemoglobin

Hemoglobin was discovered by Hühnfeld in 1840 [6] and its molecular structure was depicted by X-ray crystallography in 1959 by Max Perutz [7]. Ever since the discovery of the relation between its structure and function by Max Perutz in 1978 after almost 20 years of research, hemoglobin is presently the most thoroughly understood protein thus far.

The common hemoglobin molecule is the assembly of four globular protein consisting of two subunits, two alpha chains, each with 141 amino acids [8] and two beta chains, each with 146 amino acids [9]. Each subunit is composed of a protein chain tightly associated with a non-protein heme group. Each protein chain arranges into a set of alpha-helix structural segment connected together in a globin fold arrangement; this folding pattern contains a pocket that strongly binds the heme group. In this protein molecule, the globin portion is approximately 94% while the heme comprises of 6%.
A heme group consists of an iron (Fe) ion (charged atom) held in a heterocyclic ring, known as porphyrin. This porphyrin ring consists of four pyrrole molecules cyclically linked together by methene bridges with the iron ion being bound at the center. The iron ion, which is the site of oxygen binding, coordinates with four nitrogens atoms in the center of the ring, all of which lie in one plane. The iron is bound strongly to the globular protein via the imidazole ring of the F8 histidine residue [10] (also known as proximal histidine) below the porphyrin ring.
Figure 2.3.2: Proximal histidine F8 which holds the heme group with globin, Phorphyrin structure of heme.

The heme molecule contains the iron atom in ferrous (Fe$^{2+}$) state to allow the binding of oxygen. But if the iron is in ferric (Fe$^{3+}$) state then there is no binding of oxygen and it is known as oxyhemoglobin [11]. In this case the molecule is referred to as “methemoglobin or ferrihemoglobin”. When O$_2$ binds to the ferrous ion, all the forces get balanced equally and the iron is pulled back to the center.

Hemoglobin exists either in relaxed state (R-state) or intense state (T-state) and in these forms they differ both in their structure and affinity for oxygen [12]. They also differ in the number and energy of the interactions between hemoglobin subunits. In the T-state, constraints between subunits oppose the structural changes resulting in ligand binding, whereas in the R-state, these constraints are released, thereby enhancing ligand-binding affinity. Both T and R states of hemoglobin are shown in Figure 2.2.3.
2.3.3 Synthesis of hemoglobin

Synthesis of hemoglobin involves a complex series of steps. The heme part and globin part are synthesized separately [13]. The heme part is synthesized in a series of steps in the mitochondria and the cytosol of immature red blood cells, while the globin protein parts are synthesized by ribosomes in the cytosol.

2.3.3.1 Heme synthesis

Heme is synthesized in a complex series of steps involving multi enzymes in the mitochondrion and in the cytosol of the cell. Synthesis of heme starts in mitochondrion with the condensation of succinyl CoA and glycine by aminolevulic acid (ALA) synthase [14] to form 5-aminolevulic acid. This molecule is transported to the cytosol where a series of reactions produce a ring structure called coproporphyrinogen III. This molecule returns to the mitochondrion where by an addition reaction protoporphyrin IX is produced. The enzyme ferrochelatase inserts iron into the ring structure of protoporphyrin IX to produce heme. This heme is strongly bound to the globin groups by histidine as shown in Figure 2.3.4.
Two distinct globin chains (each with its individual heme molecule) combine together to form hemoglobin [15]. One of the chains is designated as alpha. The second chain is called "non-alpha". The human globin genes undergo an orderly ontogenetic program of expression during their development from embryo to adult stage. Specific genes for the globin chains are switched on and off to produce different hemoglobin types [16].

### 2.3.4 Hemoglobin variants

Normal hemoglobin contains four globin groups 2α and 2β which are associated with a heme group. Other than α and β globins there are many other globins are also present. Based on the type of globins several variants of hemoglobin are present as listed in Table 2.3.1.
In humans, there are 5 types of normal hemoglobins are present; others are of abnormal types. Two types of normal hemoglobins are present in embryos: Hb Gower-1 and Hb Gower-2. Normal hemoglobin in the fetus is Hb F. The 2 normal types of hemoglobin in adults are Hb A and HbA2.

Table 2.3.1: Hemoglobin variants

<table>
<thead>
<tr>
<th>Form</th>
<th>Chain</th>
<th>Fraction of total Adult Hb</th>
<th>Clinical state</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td>α₂β₂</td>
<td>90%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>HbA2</td>
<td>α₂δ₂</td>
<td>2-5%</td>
<td>Normal</td>
<td>[17]</td>
</tr>
<tr>
<td>HbA1C</td>
<td>α₂β₂-glucose</td>
<td>3-9%</td>
<td>Normal</td>
<td>[18]</td>
</tr>
<tr>
<td>HbF</td>
<td>α₂γ₂</td>
<td>&lt;2%</td>
<td>Normal</td>
<td>[19]</td>
</tr>
<tr>
<td>Gower 1</td>
<td>ζ₂ε₂</td>
<td>Embryonic</td>
<td>Normal</td>
<td>[20]</td>
</tr>
<tr>
<td>Gower 2</td>
<td>α₂ε₂</td>
<td>Embryonic</td>
<td>Normal</td>
<td>[21]</td>
</tr>
<tr>
<td>Hb portland</td>
<td>ζ₂γ₂</td>
<td>Embryonic</td>
<td>Normal</td>
<td>[22]</td>
</tr>
<tr>
<td>HbH</td>
<td>β₄</td>
<td>0 %</td>
<td>αThalassaemia</td>
<td>[23]</td>
</tr>
<tr>
<td>Hb Barts</td>
<td>γ₄</td>
<td>0 %</td>
<td>αThalassaemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α chain aggregates insoluble</td>
<td>0 %</td>
<td>βThalassaemia</td>
<td></td>
</tr>
<tr>
<td>HbS</td>
<td>α₂β₂</td>
<td>0 %</td>
<td>Sickle cell disease</td>
<td>[25]</td>
</tr>
</tbody>
</table>
2.3.5 Clinical significance of hemoglobin

The primary function of hemoglobin is to bind the oxygen from lungs and transport it to various parts of the body and in turn bring back carbon dioxide to lungs. Hemoglobin in its tetrameric state has got 4 iron molecules attached to globin group via a histidine molecule. Each iron molecule can bind with either one oxygen molecule or one carbon dioxide molecule. Thus each hemoglobin molecule at a time can transport four oxygen or carbon dioxide molecules. Hemoglobin combines with oxygen and carbon monoxide cooperatively.

Very high hemoglobin concentration causes high blood viscosity, which results in compromised oxygen delivery to tissues and also creates cerebrovascular complications. Studies have also shown that a high maternal hemoglobin concentration leads to increased risk of poor pregnancy [26].

2.3.6 Reference ranges of hemoglobin

The hemoglobin level is measured in grams per deciliter. The normal ranges of hemoglobin levels are dependent on the age and gender [27].
### Table 2.3.2: Reference range of hemoglobin in human blood

<table>
<thead>
<tr>
<th>Age</th>
<th>Male (g / dL)</th>
<th>Female (g / dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New born</td>
<td>14.7–18.6</td>
<td>12.7–18.3</td>
</tr>
<tr>
<td>6 months – 2 years</td>
<td>10.3–12.4</td>
<td>10.4–12.4</td>
</tr>
<tr>
<td>2 - 12 years</td>
<td>11.0–13.0</td>
<td>10.7–13.3</td>
</tr>
<tr>
<td>12 - 18 years</td>
<td>11.0–13.3</td>
<td>10.9–13.3</td>
</tr>
<tr>
<td>&gt; 18 years</td>
<td>10.9–15.7</td>
<td>10.7–13.5</td>
</tr>
</tbody>
</table>

#### 2.3.7 Analytical significance

Total hemoglobin count is a routine blood test conducted which helps in diagnosing many abnormalities in the body system. The transport of oxygen in blood is undertaken by hemoglobin, the largest component of red blood cells. This protein collects oxygen from respiratory organs, mainly the lungs, and releases it to tissues in order to generate the energy necessary for cell survival. Hemoglobin is one of the most refined proteins because its evolution and small mutations in its structure can produce anemia [28] and other severe pathological conditions. Hemoglobin test is considered as an important blood test as it is a tool for determining many types of disorders including certain kinds of blood related diseases.

If the hemoglobin level in blood is lower than the required normal range, then it is usually indicative of anemic condition [29], which may have many implications, mainly the anemic person will have a tendency to have low levels of oxygen in the body. Others include nutritional deficiency, chemotherapy drugs, bone marrow disorders, loss of blood, abnormal hemoglobin, and kidney failure.
On the other hand, abnormally high levels of hemoglobin have varying causes and implications. Smokers usually will have high hemoglobin levels. This condition may also true for people living in high altitude areas. Dehydration of body may also result into a temporary rise in hemoglobin levels. Other possible causes for an abnormally high hemoglobin level are the abuse of the drug erythropoietin, some tumors, and advanced lung disorders such as emphysema.

A notable disease that can be indicated by an abnormal hemoglobin level is sickle cell anemia [30]. This is a genetic disorder in which the type of the hemoglobin is faulty that also imparts a different shape to the red blood cells. As the name implies, the red blood cells assume the shape of sickles. The difficulty with these sickle-shaped red blood cells is in passing through the small blood vessels, which results in medical problems including anemia. Another blood disorder associated with hemoglobin is thalassemia in which the globulin molecules themselves are defective.

2.3.8 Analytical methods for the determination of hemoglobin in human blood.

The method of choice for hemoglobin determination is the cyanmethemoglobin method also called as Drabkin’s method and that is the standard method approved by clinical and laboratory standard institute (CLSI) [31]. This method has been adapted widely to date as an international standard method. The principle behind this method is that when blood is mixed with a solution containing potassium ferricyanide and potassium cyanide, the potassium ferricyanide oxidizes iron to form methemoglobin. The potassium cyanide then combines with methemoglobin to form cyanmethemoglobin, which is a stable color pigment read photometrically at a wave length of 540 nm. The color is related to the concentration
of hemoglobin in the blood. All forms of hemoglobin except sulfhemoglobin can be measured by this method. The reagent has high stability.

Another method accepted for the measurement of hemoglobin concentration by CLSI is hemoglobinometry [32]. Multiwavelength laboratory oximeters by the use of spectrophotometric absorption of a blood specimen determine the percentage of hemoglobin saturated with oxygen and the percentage of dyshemoglobins. Pulse oximetry is a noninvasive method of estimating the arterial oxygen saturation and pulse rate from pulsatile absorption signals derived from a sensor placed on the skin. The principle is based on the fact that oxy- and deoxyhemoglobin have different absorption spectra (see Figure 2.3.1), at the commonly used wavelengths of 660 nm (red light) and 905 to 940 nm (infrared light).
Literature cited


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Introduction, Hemoglobin


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