

I. INTRODUCTION

A. Basic knowledge on bilirubin

Bilirubin metabolism

Bilirubin is the major metabolic product of heme from hemoglobin. In healthy adults, the breakdown of heme-containing proteins generates about 250 to 350 mg of bilirubin per day (Muraca *et al.*, 1988). The porphyrin ring, which is a metabolized product of heme, (Figure 1) is oxidized by a microsomal heme oxygenase (Burtis and Ashwood, 1996) to form a biliverdin product. The biliverdin is then reduced to bilirubin by the NADPH dependent biliverdin reductase enzyme in the reticuloendothelial cells. For each mole of heme broken down, 1 mole of carbon monoxide and ferric iron is liberated. Carbon monoxide is toxic and excreted out of the body *via* lung.

Approximately 80 to 85% of bilirubin is derived from hemoglobin which release from aged or damaged erythrocytes and are destroyed in the reticuloendothelial cells of the liver, spleen and bone marrow. The remaining 15% of bilirubin is produced from red blood cell precursors destroyed in the bone marrow and from the catabolism of other heme-containing proteins such as myoglobin, cytochromes and peroxidases, which are distributed and conjugated throughout the body.

Bilirubin transportation

After production in peripheral tissues, bilirubin released into the blood stream is tightly, but reversibly, bound to albumin (Martin, 1981). Transport of bilirubin in the bound state prevents it from crossing cell membranes and entering tissue cells where it would exert toxic effects. Simultaneously, bilirubin may be bound to a number of anionic drugs, such as sulfonamide, antibiotics, barbiturates and salicylates and other compounds which may occasionally presented in serum and thus effect the formation of the glucuronide that can be excreted into the small intestine (Johnson, 1995).

Bilirubin-albumin complex transported to the liver is taken up rapidly by hepatocytes *via* the hepatic sinusoids and sinusoidal microvilli

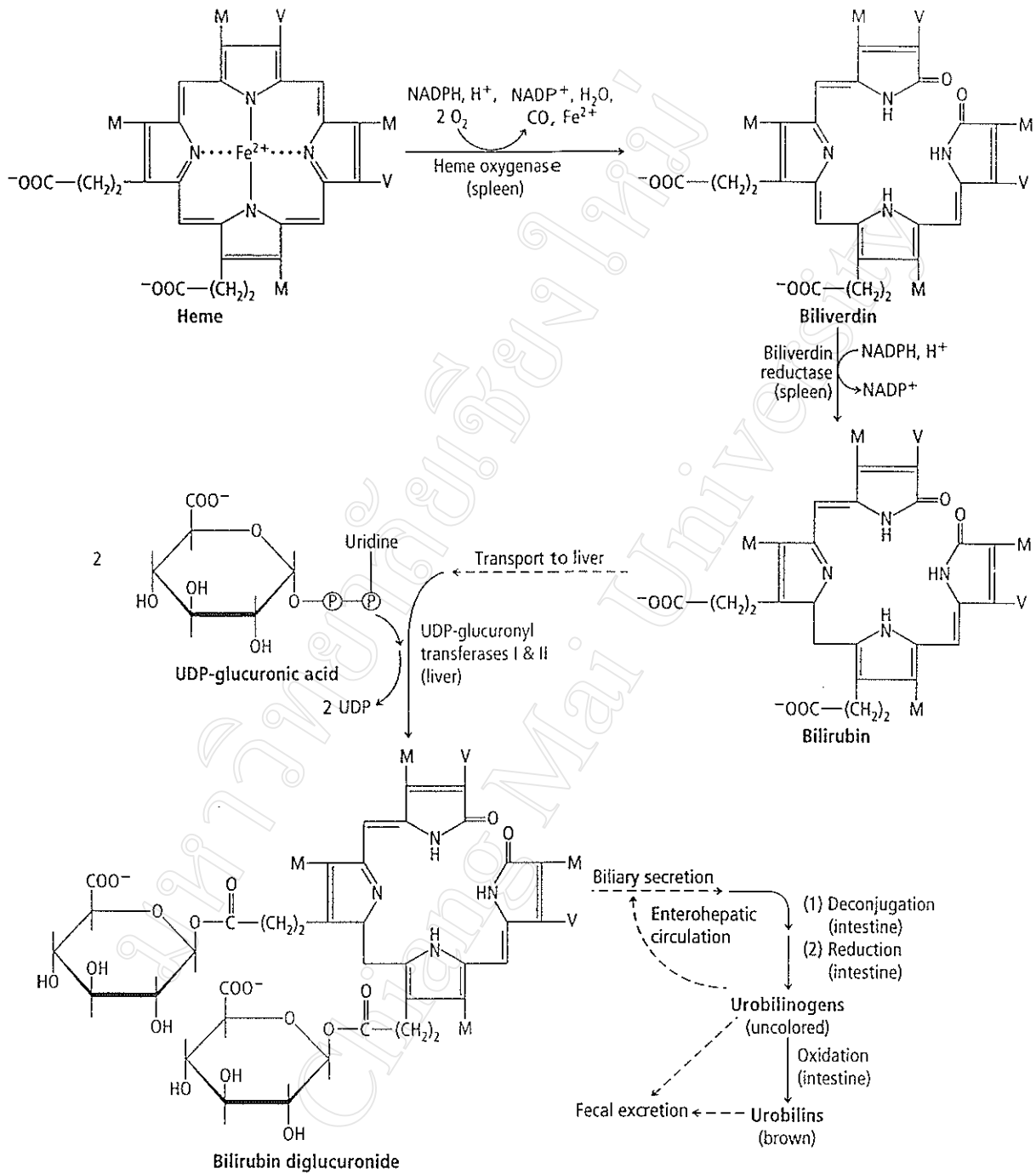


Figure 1 Schematic representation of heme degradation and bilirubin metabolism (Meisenberg and Simmons, 1998).

(Pincus and Schaffner, 1996). Inside the hepatocytes, bilirubin becomes the substrate of the UDP-glucuronyl transferase enzyme which catalyzes the conjugation of propionic acid side chains of bilirubin with glucuronic acid to form mainly bilirubin diglucuronide (Figure 1). Small amount of mono-and tri-glucuronide forms are also produced (Arias *et al.*, 1988).

Bilirubin excretion

In normal adults, the majority of about 85 to 90% of bilirubin excreted into bile is diconjugated and the remainder 10 to 15% is monoconjugated. The conjugated bilirubin is transported to the membrane surface opposite the sinusoidal face, which is in contact with bile canaliculi (Smith *et al.*, 1988). Conjugated bilirubins which directly excrete into bile canaliculi, pass through the hepatic common bile ducts and enter the upper small intestine where they are minimally reabsorbed. In the upper small intestine, bilirubin glucuronides are hydrolyzed in the alkaline pH by the enzyme β -glucuronidase to form the unconjugated pigment. The unconjugated bilirubin is further reduced by anaerobic intestinal microbial flora in large intestine to form stercobilinogen, mesobilinogen and urobilinogen, a group of three colorless tetrapyrroles that are known collectively as urobilinogens (Awad, 1997).

Up to 20% of urobilinogens produced daily are reabsorbed from the intestine to the liver via the enterohepatic circulation. The majority of the reabsorbed urobilinogen is taken up by the liver and is re-excreted into the bile. Only 2 to 5% enter the general circulation and appear in the urine (Billing *et al.*, 1957). In lower intestinal tract, the three urobilinogen tetrapyrrole products are spontaneously oxidized to produce the corresponding orange-brown bile pigments, stercobilin, mesobilin and urobilin and are excreted in the feces (Harper, 1977).

B. Clinical significances of bilirubin

Diseases or conditions that interfere with bilirubin metabolism may cause a rise in serum bilirubin concentration. Jaundice, a condition characterized by an increase of bilirubin in the blood and a brownish-yellow pigmentation of the skin, sclera and mucus membranes appear when the serum bilirubin concentration reaches about 2.5 mg/dL. By itself, hyperbilirubinemia is usually not a threat to health, since adequate mechanisms exist for its binding and detoxifying. However,

hyperbilirubinemia indicates an abnormality in the production or subsequent metabolism of bilirubin. Five mechanisms that can lead to hyperbilirubin or jaundice are listed as follows (Schreiber, 1996).

- 1) Overproduction
- 2) Impaired uptake by liver cells
- 3) Defects in the conjugation reaction
- 4) Reduced excretion into bile
- 5) Obstruction to the flow of bile

The first three mechanisms caused by an increase in unconjugated serum bilirubin, whereas the latter two mechanisms produce an elevation of both unconjugated and conjugated bilirubin in serum. The elevation of bilirubin in plasma or jaundice, could be classified as follows (Zimmerman, 1984) :

1) Prehepatic jaundice (Hemolytic jaundice)

The prehepatic type of jaundice is commonly caused by hemolytic anemia. The increased destruction of red cells brings a larger load of bilirubin-protein complex to the liver than the organ can handle. When this additional bilirubin is converted to the glucuronide and excreted into the intestinal tract, an increased amount of urobilinogen is formed in the colon. A portion of this is excreted in the stool ; the other portion is reabsorbed and returned to the liver. The liver cannot pick up the large quantities of urobilinogen and therefore the increased amounts escape into the general circulation and are excreted into the urine by the kidneys. The increased breakdown of hemoglobin also caused an increase in the amount of unconjugated bilirubin in the blood without corresponding increase in conjugated bilirubin.

2) Hepatic jaundice (Hepatocellular jaundice)

The causes of hepatocellular jaundice involve the liver cell directly. There may be specific defects, or there may be diffuse damage such as in viral hepatitis, drug toxicity and intrahepatic obstruction. In those conditions, the injured cells lose their ability to remove bilirubin from serum and to conjugate bilirubin with glucuronate, thus resulting in an increase of unconjugated bilirubin in serum. In addition, the damaged cells are not capable of removing all of the urobilinogen from the portal blood in the enterohepatic circulation.

3) Posthepatic jaundice (Obstructive jaundice)

The obstructive jaundice is commonly caused by the conditions such as gallstone, spasm or neoplasm. The block of bile passage to the small intestine cause regurgitation of bile into the sinusoids. The bilirubin glucuronides cannot reach the intestine and therefore, no urobilinogen is produced for recirculation to the liver or excretion in the stool. Since there are conditions in which the posthepatic blockage is intermittent or incomplete, the stools may contain decreased amounts of urobilinogen and small quantities of this compound may appears in the urine. The blood contains increased amounts of bilirubin and bilirubin glucuronides caused by regurgitation and impairment of liver cell function.

4) Jaundice caused by genetic diseases.

4.1 Gilbert's syndrome

Gilbert's syndrome is a familial type nonhemolytic jaundice. It caused by a transport deficit in the sinusoidal membrane of the hepatocytes, resulting in an elevation of unconjugated bilirubin (indirect bilirubin) in serum. Gilbert's syndrome is most frequently diagnosed in young adults ranging in age from 20 to 30 years (Rosenthal *et al.*, 1983).

4.2 Crigler-Najjar syndrome

Crigler-Najjar syndrome is a lower than normal or absent activity of UDP-glucuronyl transferase. The condition results in low or no conjugation of bilirubin to glucuronic acid so that bilirubin cannot be excreted into the canaliculi. Unconjugated bilirubin builds up within hepatocytes and then back-diffuses into the sinusoids before entering the circulation. Much more serious is the syndrome which resulted from an inborn error of metabolism (pathological jaundice). Newborns with this disease become jaundiced (bilirubin levels > 5 mg/dL). Patients may die in infancy owing to the development of kernicterus as described by bilirubin staining of basal ganglia of the brains (Frisell, 1982).

4.3 Dubin-Johnson syndrome

Dubin-Johnson syndrome is a rare and benign genetic disorder of bilirubin excretion. In this disorder, hepatic uptake and storage are normal but bilirubin excretion into bile is markedly impaired due to the abnormal liver cell mechanisms. In patients with this disorder, serum

total bilirubin concentrations are usually elevated to the range of 2 to 5 mg/dL. Conjugated bilirubin which is the predominant form, accumulated within the hepatocytes, is eventually back-diffuses into the circulation (Whitby *et al.*, 1988).

5) Physiological jaundice in newborn

This type of jaundice is ordinarily short-lived which happens in premature infants. The level of bilirubin in a neonatal blood may sometime reach 20 to 25 mg/dL. The accumulation of bilirubin in blood stream are caused by nonpermanent lack of the enzyme UDP-glucuronyl transferase. This condition leads to the deposition of the unconjugated form in the skin which becomes yellowish. The white parts of the eyes may also look yellow (Orten and Neuhaus, 1975). The neurological problems can be occurred with some early signs of abnormal hearing function. High level of prolonged accumulation may lead to severe brain damage (Meisenberg and Simmons, 1998).

C. The usefulness of serum bilirubin measurement

(Pesce and Kaplan, 1987)

Measurement of serum bilirubin is useful in differentiating a wide variety of pathological states. As mentioned earlier, bilirubin concentrations are elevated in the blood either by increased production, decreased uptake by the liver, decreased conjugation, decreased secretion from the liver or obstruction of the bile duct. The first important step in the diagnosis of jaundice is to measure both conjugated and unconjugated bilirubin fractions in serum. In cases of increased production, decreased liver uptake or decreased conjugation, the unconjugated or so-called indirect bilirubin will be primarily elevated. In case of decreased secretion from the liver or bile duct obstruction, the conjugated or so-called direct bilirubin will be primarily elevated. In various liver diseases, as well as conditions other than liver diseases (e.g. increased production by enhanced red blood cell destruction), can cause serum bilirubin concentration to be elevated. Elevation of direct bilirubin fraction is observed in serum of most adult acquired liver diseases with impairment in bilirubin secretion from liver cells. In chronic, acquired liver diseases, the serum bilirubin concentration is usually normal until a significant amount of liver damage has occurred and cirrhosis is present.

In acute liver disease, bilirubin concentration is usually increased in relative to the severity of the acute process. In bile duct obstruction, or diseases of the bile ducts such as primary biliary cirrhosis or sclerosing cholangitis, the direct bilirubin fraction is often elevated along with serum alkaline phosphatase and GGT activities.

D. Classification of bilirubin fractions in serum

Bilirubin is a family of bile pigments, all classes contain a common asymmetric tetrapyrrole structure. There are three major classes of bilirubin in blood : unconjugated bilirubin (Bu), sugar-conjugated bilirubin (Bc), and bilirubin covalently linked to albumin, also called δ -bilirubin (B δ) or biloprotein (Doumas and Wu, 1991).

1) Unconjugated bilirubin

Unconjugated bilirubin is extremely apolar and practically insoluble in water at physiologic pH and temperature. It was suggested that the main form of Bu (the IX- α isomer) is not a linear tetrapyrrole, but a tightly internally folded structure in which the propionate groups are linked to the pyrrole nitrogens (Fog and Jellum, 1963). The folded conformation of Bu stabilized by six intramolecular H-bonds is the native Z,Z-configuration (native Bu). The H-bonds can be partially ruptured by irradiation to form the open chain Z,E and E,Z configuration or completely destroyed to form the unfolded E,E isomer (Figure 2). The radiated forms are more polar than that of the poorly solubilized native Bu form.

2) Sugar-conjugated bilirubin

Sugar-conjugated bilirubin is formed by the enzymatic addition of one to two molecules of sugar (principally glucuronic acid) onto either one or both of the propionic acid side chains of Bu (Figure 3). The resulting mono-(mBc) and di-(dBc) sugar conjugated bilirubins are more polar than Bu, nontoxic to cells, and are excreted against a concentration gradient across the canalicular membrane of the hepatocyte in the bile. It appears that while dBc is the predominant bilirubin in human bile, mBc is often the chief conjugated in jaundiced sera in which the direct fraction is greater than 50% of total bilirubin (Wu and Sullivan, 1982).

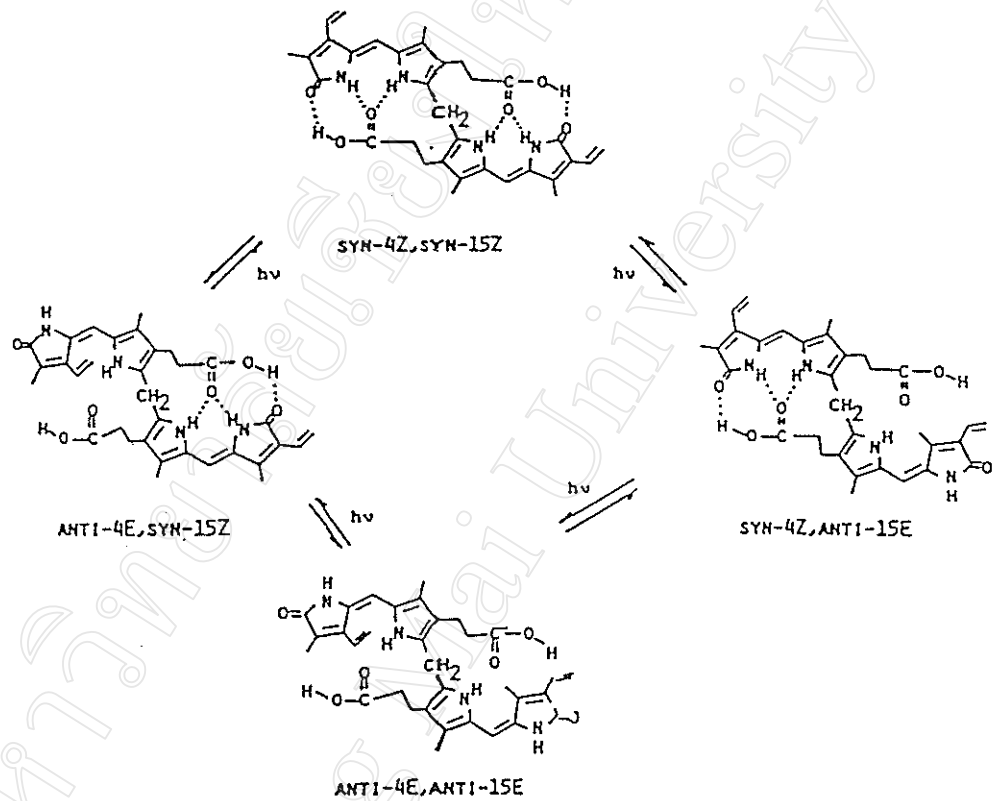


Figure 2 Photochemical reaction cycle for bilirubin IX- α showing $Z \rightleftharpoons E$ isomerization and concomitant disruption (or formation) of intramolecular hydrogen bonds (Doumas and Wu, 1991).

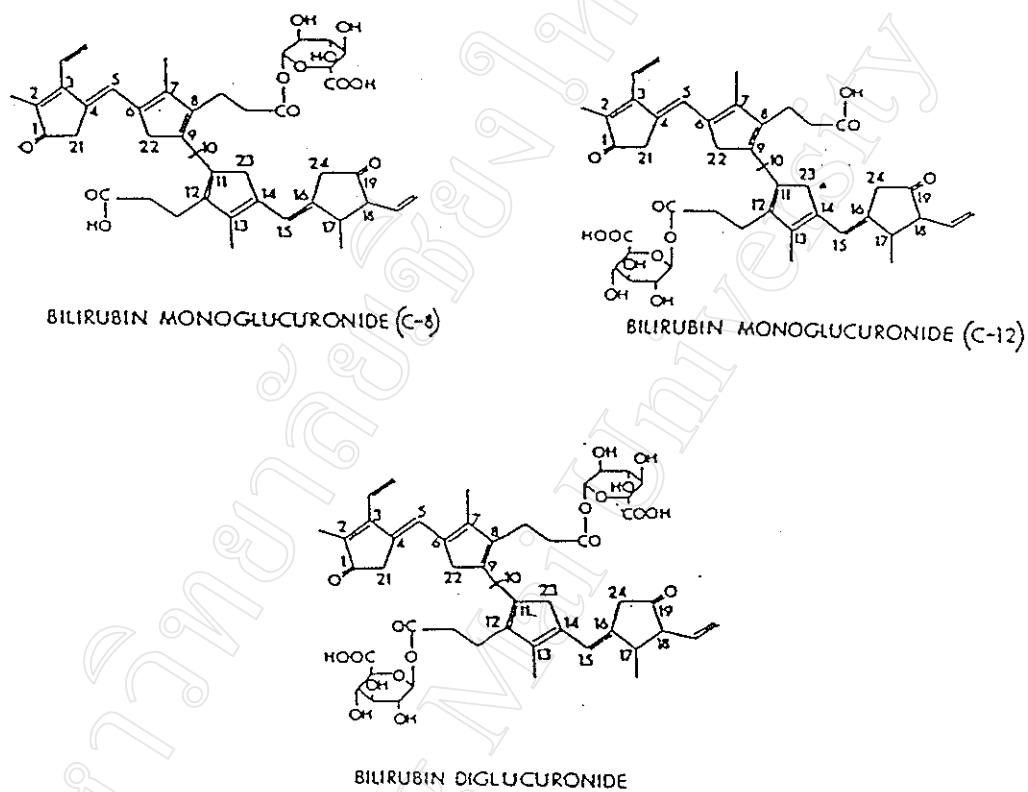


Figure 3 Structures of bilirubin glucuronides. Monoglucuronides can exist as two molecular species, depending on whether the C-12 or C-8 propionic acid side chain is esterified (Doumas and Wu, 1991).

3) Bilirubin covalently linked to albumin (δ -bilirubin)

$B\delta$ is a bilirubin covalently linked to albumin through an amide bond between one of its two propionic acid side chains and an ϵ -amino group of lysine residue on albumin (Wu *et al.*, 1981). Formation of $B\delta$ *in vivo* appears to be largely nonenzymatic, and it involves acyl migration of bilirubin glucuronic ester to a nucleophilic site on albumin. Unconjugated bilirubin does not react with albumin to form δ -bilirubin. Further evidence for the formation and structure of $B\delta$ is as follows :

- 1) In unconjugated hyperbilirubinemia (hemolytic jaundice) there is no more than a trace of $B\delta$ in serum (Brett *et al.*, 1984).
- 2) In obstructive hepatobiliary disease, the increase in concentration of $B\delta$ in serum is paralleled to the increase in Bc (Lauff *et al.*, 1983).
- 3) δ -bilirubin is the slowest fraction to clear from serum following resolution of severe hepatobiliary disease, presumably owing to the half-life of albumin which is bound to the conjugated bilirubin molecule (Reed *et al.*, 1988).
- 4) In diazotization reaction of $B\delta$ with diazo reagent, one half forms azopigment whereas the other half is still bound to albumin (Lauff *et al.*, 1982; Doumas *et al.*, 1987).

E. Methods for measurement of bilirubin fractions in serum

The preference methods used for measuring of total bilirubin and bilirubin fractions in serum are summarized as followed.

1) Direct spectrophotometry (Doumas and Wu, 1991)

This method used for determining total bilirubin in serum. The principle is the absorbance of bilirubin at 454 nm is proportional to its concentration. The method is relatively insensitive to hemolysis, which is often present in specimens obtained from infants, due to difficulty in skin puncture technique. Absorbance values observed at 454 and 540 nm represent the sum of absorbances at each wavelength of bilirubin and

hemoglobin present in the specimen. Because the absorbance due to hemoglobin is essentially at both wavelengths, the difference in absorbance between the two wavelengths ($A_{454}-A_{540}$) represents absorbance of bilirubin only (Sherwin and Sobenes, 1996). The serum of newborn infants does not contain carotene, lipochromes and other pigments that increase the absorbance at 454 nm. However, these pigments may be present in serum from older children and adults, and use of the direct spectrophotometric method should therefore be restricted to newborns (Hertz *et al.*, 1974 ; Watkinson *et al.*, 1982).

2) Diazotization method (Doumas and Wu, 1991)

The reaction involved the coupling bilirubin (both forms) with the diazo reagent (Ehrlich's reagent). Since unconjugated bilirubin is poorly dissolved in water, therefore, compounds such as methanol, caffeine, sodium benzoate or urea etc. have been used to "accelerate" the coupling reaction of its molecule with the diazotized sulfanilic acid. The azopigment color product has indicator properties at different acid or alkaline pH condition, and thus are absorbed spectrophotometrically at the wavelength varied from 550-600 nm (Doumas *et al.*, 1985; Landis and Pardue, 1978). It has been demonstrated that hemoglobin, turbidity, indican, metal ions and the drugs L-dopa and α -methyl-dopa interfere with the diazotization reaction (Lo and Wu, 1983 ; Young *et al.*, 1975).

The method which is based on the Jendrassik-Grof principle is the reference method recommended by the Committee on Standards of the American Association for Clinical Chemistry and "credentialed" by the National Reference System for the Clinical Laboratory (National Committee for Clinical Laboratory Standards, 1989).

For total bilirubin determination, serum or plasma is added to a solution of sodium acetate and caffeine-sodium benzoate, which is then added to diazotized sulfanilic acid to form a purple azobilirubin. This reaction is terminated by the addition of ascorbic acid, which destroys the excess diazo reagent. A strongly alkaline tartrate solution is then added to convert the purple azobilirubin to blue azobilirubin, and the intensity of the color is read at 600 nm (Novros *et al.*, 1979).

In determination of conjugated bilirubin in serum or plasma, the reaction is carried out in a dilute hydrochloric acid solution. The diazotization is performed in the absence of accelerator in a one-minute reaction. The addition of the tartrate solution is required for developing a strong alkaline pH condition which provides a stable blue color of

azopigment that can be measured at the same absorbance as total bilirubin (Fody, 1996).

Value for unconjugated (indirect) bilirubin is obtained by subtracting conjugated (direct) bilirubin from the total bilirubin value.

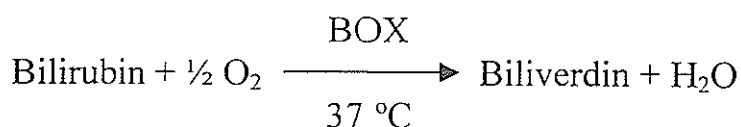
3) High-performance Liquid Chromatography (HPLC) method (Adachi *et al.*, 1988)

In this method, serum is treated with saturated sodium sulfate to precipitate most of the globulins, but not albumin, and the bilirubins are resolved on a reversed-phase column. The bilirubin species are eluted in order of decrease polarity. Bilirubin species was fractionated into four elution peaks : δ -fraction (delta bilirubin), γ -fraction (bilirubin diglucuronide), β -fraction (bilirubin monoglucuronide) and α -fraction (unconjugated bilirubin), respectively (Lauff *et al.*, 1981). The concentration of each fraction is calculated from the peak areas by use of a calibration curve. Total bilirubin values (the sum of four fractions : α , β , γ and δ fraction) in serum measured by this method agreed well with those obtained by the reference Jendrassik-Grof method (Lauff *et al.*, 1983).

HPLC, which effectively separates and quantitates the four bilirubin fractions, is the method of choice. However, the method is limited by (1) the need to pretreat serum to remove the globulins, which may entail a variable loss of delta bilirubin ($B\delta$) ; (2) errors in measurement of each fraction may be cumulative, and may result in a large total error; (3) the method is relatively insensitive at total bilirubin concentrations below 1 mg/dL ; (4) the procedure is laborious and impractical for routine use.

4) Enzymatic methods (Doumas and Wu, 1991)

An enzymatic method for the measurement of bilirubin depends upon a bilirubin oxidase (EC 1.3.3.5) isolated from *Myrothecium verrucaria*. Bilirubin oxidase (BOX) catalyzes the oxygen-dependent conversion of bilirubin to biliverdin, as shown by the equation below:



The decrease in absorbance owing to the disappearance of bilirubin is linearly related to its concentration. The oxidation rates for the various bilirubin depend on pH of the reaction mixture and on the presence of surface active agents (surfactants).

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