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Review on "Evolution of Methods of Bilirubin Estimation"

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Abstract: *Bilirubin is an end product of heme metabolism, which is under disposal of tissues like liver, intestine and kidney. Clinically hyperbilirubinemia appears as jaundice or icterus. Jaundice can usually be detected when the serum bilirubin level exceeds 2.0 to 2.5 mg/dl.*
Determination of total and direct bilirubin levels helps the physician to know the underlying pathogenic mechanism. Hence accurate and unbiased estimation of bilirubin becomes mandatory. The problem of finding an accurate and specific method of bilirubin assay has been a challenge for over 50 years for many workers.
Historically, Ehrlich in 1883 treated bilirubin in urine with diazo reagent and described formation of a red – blue coloured pigment. Later, Van den Bergh and Muller found that bilirubin in normal serum reacted with Ehrlich's diazo reagent (diazotized sulfanilic acid).
We shall discuss here the further evolution of methods of bilirubin estimation till the modern era and also will cover the various aspects of interpretation and quality control measures.
Key Words- *Bilirubin, Biliverdin, Urobilinogen, Diazo, hepatitis.*

I. Introduction

Jaundice word is derived from Latin word glabinus and French word jaunisse. Jaundice or icterus is the yellow pigmentation of the skin and sclera. Jaundice can usually be detected when serum bilirubin level exceeds 34 to 43µmol/L (2.0 to 2.5 mg/dl) ([1]).Neonatal jaundice is the most common reason for readmission to hospital in the first week of life. Mostly, neonatal jaundice is benign and does not need any intervention. However, the possibility that jaundice may be a sign of serious underlying illness warrants close consideration. Hence accurate and unbiased estimation of bilirubin is thus mandatory . The problem of finding an accurate and specific method of bilirubin assay has for 50 years occupied the attention of many workers. In this article we would review the methods of bilirubin estimation. The literature search was carried out through journal articles on internet, one review article , and few text books but no content has been copied and pasted.

1.1 *Chemistry of bilirubin*-

The structure of bilirubin was originally established by Noble Laureate, Hans Fischer, in 1942.
After researching the primary literature, the following information about bilirubin was uncovered:

- The decomposition of hemoglobin in the body results in the formation of the Z,Z-isomer of bilirubin.
- Phototherapy results in the conversion of the Z,Z-isomer of bilirubin into the E,E-isomer.
- Two terminal rings of bilirubin favour lactam form ([2])

A cyclic system containing the grouping —CONH— is called a lactam, and the isomeric form, —COH & bond; NH—, a lactim(Fig-1 & Fig-2). However both the forms are available and are interchangeable.

1.2 *Physiology of bilirubin*:

Free heme can be toxic, so nature evolved a family of microsomal heme oxygenase enzymes to degrade heme([3,4]), and their blockade leads to greatly increased excretion of unmetabolized heme in the bile ([5]). These enzymes cleave the heme ring to form biliverdin, iron, and carbon monoxide (CO; Fig 3).CO is excreted via lungs and iron is reutilized . Biliverdin is a green coloured pigment which is subsequently reduced to bilirubin by the reduced form of nicotinamide adenine dinucleotide phosphate(NADPH) dependent cytosolic enzyme biliverdin reductase([6]) (Fig-3) .

Bilirubin is an end product of heme metabolism, which is under disposal of tissues like liver, intestine and kidney (Fig-4).The daily amount of bilirubin originating from destruction of red blood cells is around 250 ~ 300 mg ([7]). Following four bilirubin fractions have been isolated from serum: 1) unconjugated bilirubin(α-bilirubin)

[1]. monoconjugated bilirubin (β-bilirubin)
[2]. diconjugated bilirubin (γ-bilirubin)
[3]. A fraction irreversibly bound to protein (δ-bilirubin)

Bilirubin IXα is produced from protoporphyrin by microsomal heme oxygenase . 99.9% of unconjugated bilirubin in the circulation is bound to albumin. According to ROC curve Bilirubin/Albumin ratio cut off value for predicting acute Bilirubin induced neurologic dysfunction was 8 mg/g with a sensitivity of 100% and specificity of 94%([8]).

Studies have shown that the unbound bilirubin levels associated with kernicterus increase as birth weight and gestation increases([9]).

Weight-based unbound bilirubin reference value (the level of unbound bilirubin at which exchange transfusion should be considered) is 1.3µg/dL/kg up to a maximum of 4µg/dL and the solubility limits of unbound bilirubin at pH 7.4 is approximately 4µg /dL ([9-13,14-16]) .

1.3) ***Hepatic metabolism of Bilirubin*** **-(Fig-4).**

This process can be divided into three distinct phases.

1.3.1) Hepatic uptake: of bilirubin is by organic anion transporting polypeptides, liver-specific transporter (rlst/HLST), and/or by organic anion transporters (OAT2, OAT3 ([7]).

1.3.2) Conjugation - In the ER, bilirubin is conjugated by bilirubin uridine diphosphate (UDP)-glycosyltransferase to form mono- and diglucuronides of bilirubin which is soluble & called as conjugated bilirubin ([7]).

1.3.3) Excretion – At the canalicular membrane, bilirubin glucuronides are excreted into bile by multidrug resistance-associated protein 2 (MRP2), a member of the ATP-binding cassette transporter family ([7]).

1.4) ***Intestinal metabolism of Bilirubin :*** Bilirubin glucuronides are hydrolyzed by the catalytic action of β-glucuronidase from the liver, intestinal epithelial cells, and bacteria and then reduced by anaerobic intestinal microbial flora to form urobilinogens (Fig-4) which spontaneously oxidize to produce stercobilin,mesobilin,and urobilin, which are the major pigments of stool ([6]).

1.5) ***Renal Excretion of Bilirubin***: The presence of bilirubin in urine is evidence of conjugated Hyperbilirubinemia ([17]) (Fig-4) .

II. Physiological Role Of Bilirubin In Our Body-

In birds, reptiles, and amphibians, biliverdin is the predominant end product of heme degradation([18]).For reasons that until now have seemed obscure, in mammals, biliverdin undergoes additional metabolism ([19]) (Fig-3).

2.1) ***Extraordinary roles of bilirubin***:

An uncoupler is a compound that impedes ATP generation but has no effect on electron transport chain. The energy released by electron transport is mostly dissipated .Some portion of it generates heat, this explains the thermogenic effect of uncouplers. Bilirubin levels well above physiological range can act as an physiological uncoupler and thus helps maintain body temperature in infants ([20])**.**

Bile pigments such as biliverdin naturally possess significant anti-mutagenic and antioxidant properties and therefore fulfill a useful physiological function ([21]).Biliverdin and bilirubin have been shown to be potent scavengers of peroxyl radicals ([21,22]). They have also been shown to inhibit the effects of polycyclic aromatic hydrocarbons, heterocyclic amines, and oxidants—all of which are mutagens. Studies have even found that people with higher concentrations levels of bilirubin and biliverdin in their bodies have a lower frequency of cancer and cardiovascular disease ([22]).

In vitro experiments showed that biliverdin and bilirubin competitively inhibited HIV-1 proteases at low micromolar concentrations, reducing viral infectivity. However, when tested in cell culture with micromolar concentrations, it was found that biliverdin and bilirubin reduced infectivity by blocking viral entry into cells. Results were found to be similar for HIV-2 and SIV ([23]).

Why have mammals evolved **an** energetically expensive **an**d apparently unnecessary enzymatic step to converting the relatively innocuous biliverdin to the more toxic **bilirubin**?

Moreover, why would nature develop a system that generates "elevated" **bilirubin** levels in a high proportion of all neonates?

In 1965, Wishingrad and associates ([24,25]) argued that hyperbilirubinemia of premature infants is not as deleterious as previously thought. Furthermore, some individuals with the impaired bilirubin glucuronidation system of type 2 Crigler-Najjar syndrome maintain bilirubin levels of 19 mg/dL for 50 years without detectable damage to the nervous system ([26]). Some authors have suggested that unconjugated

bilirubin is physiologically useful, because it can cross the placenta, moving from the fetal to the maternal circulation easier that biliverdin ([27,28]).

2.1.1) Bilirubin as an antioxidant-

As early as the 1950s, bilirubin was reported to protect against the oxidation of lipids such as linoleic acid and vitamin A ([29,30,31]).

In the late 1980s, Ames and colleagues ([32,33]) demonstrated that the antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation. Serum concentrations of bilirubin are high enough to account for a substantial portion of the total antioxidant capacity of serum ([34,35]). During the oxidant stress associated with the intracellular environment, cell is exposed to high concentrations of reactive oxygen species. Remarkably, as little as 10 nanomolar bilirubin can protect cultures from the oxidant stress of 10000 times higher concentrations of hydrogen peroxide ([36]).

How might one explain this paradox?

One possibility would involve cycling between biliverdin and bilirubin (fig-3). The tissues have low endogenous levels of bilirubin**,** as the micromolar levels necessary for direct antioxidant actions would be toxic([37]) .

Heyman et al([38]) and Yeo et al ([39]) observed a diminished incidence of retinopathy of prematurity due to treatment with supplemental oxygen, in infants with elevated serum bilirubin, although some studies fail to detect such relationships ([40-45]).

Certain studies have monitored the rate of rise in serum bilirubin in the first few days of life in infants with illnesses that are associated with free radical production, such as circulatory failure, neonatal asphyxia, aspiration, and sepsis ([46,47]). The rate of bilirubin rise was less in patients than in a control group, suggesting that bilirubin is consumed to cope with oxidative stress. Others have found that bilirubin levels correlate with total antioxidant capacity in blood of neonates ([34,35,48,49]) and children with sickle cell disease ([50]).

Higher bilirubin levels, ~15.4 μM (0.9 mg/dL), were associated with lowered risk of myocardial infarction and other cardiovascular disease events compared with individuals with lower bilirubin levels of ~8.6 μM (0.5 mg/dL) ([51,52]).

III. Diagnostic Importance of Bilirubin-

Clinically hyperbilirubinemia appears as jaundice or icterus. Jaundice can usually be detected when the serum bilirubin level exceeds 2.0 to 2.5 mg/dl. When the level of bilirubin is between 1 to 2 mg/dl ,it is known as latent jaundice. In most cases hyperbilirubinemia itself has little pathophysiologic effect. However, unconjugated plasma bilirubin that is not bound to albumin can cross the blood brain barrier. In conditions such as neonatal jaundice or type-I or type-II Crigler-Najjar syndrome extremely high concentrations (>20mg/dl) of unconjugated bilirubin can accumulate, and the resulting diffusion of bilirubin into the central nervous system can cause encephalopathy and permanent impairment of nervous function ([17]).

Conjugated Hyperbilirubinemia (Table-1) with elevated direct and indirect reacting material indicates impairment of secretion into bile, while unconjugated hyperbilirubinemia (Table-1) reflects impaired conjugation.

Determination of total and direct bilirubin levels, helps the physician to know the underlying pathogenic mechanism leading to increased bilirubin levels ([17]).

The reference value measured by auto analyzer differs somewhat by the reaction time, temperature, and promoter of the diazotization.

Total bilirubin: 0.3 ~ 1.2 mg/dl , Direct bilirubin: 0.0 ~ 0.3 mg/dl

Direct type bilirubin actually does not exist in the serum, however, a small portion of indirect reacting bilirubin may present as direct reaction, thus the result of direct bilirubin may show the maximum value of 0.3 mg/dl, but never above. It is about 20 ~ 30 % of the total bilirubin concentration.

In some liver diseases, the total bilirubin will be within 1.2 mg/dl, but when the direct bilirubin concentration is above 0.3 mg/dl, the existence of liver disease should be considered.

3.1) *Clinico-pathological classes of Jaundice*

3.1.1) Pre – hepatic

Excessive destruction of red blood cells increases bilirubin formation. However, with normal hepatic function, the liver is able to remove the excess bilirubin fairly rapidly so this jaundice is usually mild. As there is no hepatic or post hepatic obstruction bile is able to enter the gut normally so the stools appear normal. As the amounts of bile pigments entering the gut are greater than normal, the amount of urobilinogen reabsorbed from the gut is also increased, this raises it's levels in the urine (Table-2). There is often also a small increase in the serum conjugated bilirubin.

The only causes of pre-hepatic jaundice other than increased haemolysis are some genetic conditions causing congenital hyperbilirubinaemias.

3.1.2) Hepatic

This type of jaundice results either because of defective uptake or conjugation. Flavaspidic acid, used in the treatment of tape worm infestation, competes with bilirubin for binding to ligandin, may cause unconjugated Hyperbilirubinemia during its administration. The jaundice readily subsides with cessation of the drug.

Damaged liver cells are less able to transfer bilirubin from the blood into the bile. This form of the condition is usually referred to as hepatocellular jaundice. Liver cell function may be embarrassed in infections of the liver such as viral hepatitis, failure of liver cell function in primary liver cancer, or damage caused by poisons or drugs. Hepatic jaundice may also be caused by conditions which lead to intrahepatic obstruction.

In neonate because of immature hepatic enzyme glucuronosyl transferase and lack of intestinal bacteria preventing conversion of bilirubin into urobilinogen, unconjugated hyperbilirubinemia results([53]).

Hereditary glucuronosyl transferase deficiency is seen in -

I. Gilbert's Syndrome-Table-2

II. Type I Crigler-Najjar syndrome-Table-2

III. Type II Crigler-Najjar syndrome- Table-2

Acquired deficiency of glucuronosyl transferase- It is susceptible to inhibition by variety of agents such as chloramphenicol, novobiocin, vitamin K, breast milk , pregnanediol, free fatty acids, hypothyroidism ([53]).

3.1.3) Post hepatic

3.1.3.1) Familial defects in hepatic excretory function- (Table-2)

a) Dubin-Johnson syndrome- The serum contains more diconjugated than monoconjugated bilirubin , just the reverse of what is seen in acquired hepatobiliary disease and Rotor syndrome. This reversed ratio is believed to be characteristic and diagnostic for homozygous patients.

b) Rotor syndrome- Serum conjugated bilirubin has more monoconjugates than diglucuronide conjugates.

3.1.3.2) Acquired defects of hepatic excretory function-

a) Drug induced and post operative. Obstruction of the bile ducts after they have left the liver, sometimes referred to as extrahepatic Cholestatic jaundice.. As there is no bilirubin in the gut none is reabsorbed into the blood to be excreted by the kidneys as urobilinogen. This means the urine contains little or none of this pigment(Table-2). However as the levels of bile pigment in the blood continue to rise it is excreted in the urine, once a renal threshold is reached, causing dark colored urine ([53]) (Table-2).

b) Hepatitis and Cirrhosis: Table-2

Thus the main purpose of the initial fractionation of the serum bilirubin is to distinguish hepatic parenchymal and biliary obstructive disease from the diseases associated with predominantly unconjugated hyperbilirubinemia.

Role of bilirubin in Neonate - Jaundice is considered pathologic if it presents within the first 24 hours after birth. Newborns produce bilirubin at a rate of approximately 6 to 8 mg per kg per day. This is more than twice the production rate in adults, primarily because of relative polycythemia and increased red blood cell turnover in neonates (54]). Bilirubin production typically declines to the adult level within 10 to 14 days after birth ([55]).

3.2) ***Physiologic Jaundice***

The average total serum bilirubin level usually peaks at 5 to 6 mg per dL (86 to 103 μ mol per L) on the third to fourth day of life and then declines over the first week after birth ([55]).

Bilirubin elevations of up to 12 mg per dL, with less than 2 mg per dL (34 μ mol per L) of the conjugated form, can sometimes occur. Infants with multiple risk factors may develop an exaggerated form of physiologic jaundice in which the total serum bilirubin level may rise as high as 17 mg per dL (291 μ mol per L) ([56]).

3.3) ***Jaundice and breast feeding***

It is due to relative caloric deprivation in the first few days of life ([57]). In moderate jaundice (total serum bilirubin level above 12 mg per dL) or in severe jaundice (total serum bilirubin level above 15 mg per dL [257 μ mol per L]) ([57,58]).

This may develop in up to one third of healthy breastfed infants ([59]). Total serum bilirubin levels vary from 12 to 20 mg per dL (340 μ mol per L) and are nonpathologic. Substances in maternal milk, such as β-glucuronidases , and nonesterified fatty acids, may inhibit normal bilirubin metabolism ([60,54,61,62]).

3.4) Pathologic Jaundice

Features of this includes appearance of jaundice within 24 hours after birth, a rapidly rising total serum bilirubin concentration (increase of more than 5 mg per dL per day), and a total serum bilirubin level higher than

17 mg per dL in a full-term newborn ([56,60]) and elevation of the serum conjugated bilirubin level to greater than 2 mg per dL ([55, 56,63]).

IV. Methods Of Determination:

In clinical laboratory where problems of human disease confront the chemist most urgently, simplicity and rapidity of analytical procedures have always been at a premium ([64]). For this reason, a comparison of yellow color of serum with that of a yellow standard was for many years much used clinically as a measure of serum bilirubin.

Early methods for bilirubin estimation were based on measurement of its oxidation product, biliverdin or on assessment of the icteric index. However pigments in serum other than bilirubin such as, carotene, xanthophylls,& haemoglobin also may contribute to the icteric index, limiting it's usefulness. Ehrlich in 1883 treated bilirubin in urine with diazo reagent and found that a red –blue coloured pigment was formed. Introduction of the diazo reaction for serum bilirubin by van den Bergh in 1918 led to its widespread adoption for quantitating the pigment in serum. Van den Bergh and Muller found that bilirubin in normal serum reacted with Ehrlich's diazo reagent (diazotized sulfanilic acid) when alcohol was added. Their observation that bile pigment reacted with the diazo reagent without the addition of alcohol led to the recognition that some change in bilirubin had been affected by the liver. Bilirubin that reacts with the diazo reagent without the addition of alcohol was called "direct" or conjugated while the form that reacts only in the presence of alcohol was called "indirect" or unconjugated. 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 total bilirubin. The indirect fraction is obtained by subtracting the direct value from the total value. The determination of direct as well as total bilirubin is used in differentiating certain types of jaundice ([65]).

4.1) S*pectrophotometric method*

Higher total bile pigment levels for adult plasma were obtained by spectrophotometer by White et al than by diazo coupling by method of Lathe and Ruthevan. White et al using a 1:51 dilution of serum, in phosphate buffer measured the optical densities at 455 and 575mμ.By difference they obtained a value considered proportional to bilirubin content and independent of any haemoglobin present. This was then multiplied by a factor derived from the molar extinction coefficients of the two substances dissolved in human albumin, and from dilution of the serum under test, in order to obtain the bilirubin concentration. One source of error in spectrophotometric assays of serum colour with respect to bilirubin determination is that due to non bilirubin yellow pigments (66]). In a well controlled study of 185 icteric and 55 non icteric sera, Fog found poor correlation between bilirubin assayed by colour and bilirubin assayed by diazo coupling. Yellow non bilirubin pigments accounted for 0-40 percent of total yellow color measured 460mμ with a correction for hemoglobin and turbidity. He also examined sera from patients of hemolytic and nonhemolytic jaundice by spectrophotometric and Diazo coupling(Jendrasik and Grof) procedure and concluded that appreciable amounts of non bilirubin yellow pigments are frequently present in the sera of adult patients with hepatic or post hepatic jaundice. This is also Fog's experience in regard to jaundiced infants with Biliary obstruction or cytomegalic inclusion disease ([67]). Doubt as to the reliability of spectrophotometer assays originates also in the finding that a small displacement of the entire absorption spectra of bilirubin can be brought about by the therapeutic level of severaldrugs ([68]). Limitations of Spectrophotometer method should therefore be borne in mind. Because the plasma of new born babies contains very small amount of carotenoids (the colour of which is equivalent to less than 0.1 mg of bilirubin/100 ml), the use of a spectrophotometric method offers a rapid and convenient means of assessing Hyperbilirubinemia. Unconjugated bilirubin alone can be similarly determined in sera of infants, after first precipitating conjugated pigment with 80% acetone, as described by Mertz and West ([69]). Bile pigment level obtained by diazo method and spectrophotometer showed good agreement in plasma obtained from infants(both icteric & nonicteric) in first week of life. An exception was an infant with inspisated bile syndrome as his blood consisted mainly of conjugated bilirubin. Since infant plasma is almost devoid of lipochromes,if appears that estimation by White et al includes in addition to lipochromes,if present, a measure of non bilirubin pigments which are exclusively found in plasma reacting "directly" by the method of Lathe and Ruthevan([70]) .

4.2) *Diazo method's*

Although there are many kinds of methods for the determiantion of bilirubin, most of the methods are according to the principle of Diazo reaction method reported by Hijmans van den Bergh .

- Van den Bergh, Malloy and Evelyn Reaction — In an aqueous solution, Ehrlich's diazo reagent reacts with the direct bilirubin in the serum to form a pink to reddish-purple colored compound (azobilirubin). It was read at one minute. In a 50% methyl alcohol solution, Ehrlich's diazo reagent reacts with the total bilirubin in the serum to form a pink to reddish-purple colored compound. (Read at 30 minutes.) Malloy & Evelyn used diazo reagent for the colorimetric estimation of bilirubin which was read at 540 nm ([65]).

These methods require preliminary step of protein precipitation in alcoholic solution at about pH 4.This leads to low & poorly reproducible results because of co precipitation of bilirubin esters along with protein ([70]).

- Later in 1938 Jendrassik & Grof modified the diazo method by using caffeine benzoate as an accelerator which splits the unconjugated bilirubin protein complex releasing the bilirubin so that it can react with diazotised sulphanilic acid. A plasma blank was carried out. The reagent blank was zero. The tartrate buffer makes the mixture alkaline and converts the red acid bilirubin to a green coloured compound which was read in EEL colorimeter using Ilford 607 filter. At this wavelength the absorbance due to haemoglobin or carotene is minimal. This was the only departure made from original method of Jendrassik & Grof who employed a pulfrich photometer with filter S61.The method was republished in English by fog (1958a) ([70]).
- Powell(1944) added conventional diazo reagent to the plasma followed by sodium benzoate urea solution. In this method a plasma blank test is carried out in which HCL replaces diazo reagent. Colour densities were read in EEL colorimeter at Ilford filter 625.This method was used for plasma containing bilirubin conc. upto 5mg/dl. Analyses on more deeply pigmented plasma were made with 0.5 ml of 1 in 5 saline dilutions of plasma ([70]).
- Method of King & Coxon(1950) modified in a manner as suggested by Perryman et al(1957)-Ammonium sulphamate(0.15%) was incorporated in diazo reagent and few crystals of sodium azide were added after the addition of ethanol (85%v/v).Readings were made at 530mμ and 425mμ,and the azo pigment extinction was then corrected for interference by haem pigments by means of a simple formula.
- Method of Lathe & Ruthven Synthetically prepared taurobilirubin was used as a model for direct bilirubin**.** It was found that extinction and absorption maximum values depend on pH, alcohol & albumin. Most important for practical purposes is the observed rise of azo dye extinction in serum and in albumin caused by alcohol. This means, if the Malloy and Evelyn method for the quantitative colometric estimation of direct bilirubin in serum is used, that part of the directly reacting taurobilirubin is estimated as indirect bilirubin. Revision of colorimetric methods for direct bilirubin estimation in serum is recommended ([71]). Following conclusions were reached about the reaction of bilirubin with diazo reagent, with the help of Malloy and Evelyn method:

All the bilirubin in human plasma of high bilirubin content is bound to the plasma albumin .

- The plasma bilirubin which gives a direct reaction in 10 minutes when a procedure of Malloy and Evelyn is used is attached to the plasma albumin as a dissociable complex.
- That which does not give a direct reaction is attached to a fraction of plasma albumin precipitated by ammonium sulphate at pH 6.8 between 61 and 72.5 percent saturation-probably by a valence bond.
- The role of methyl alcohol in causing all the bilirubin to react is purely catalytic.

The diazo method of bilirubin estimation is not very accurate especially in detecting low levels of bilirubin. Direct bilirubin over estimates bilirubin esters at low bilirubin levels and under estimates them at high concentration. Thus slight elevation of unconjugated bilirubin not detected, which is of value in detecting conditions like Gilbert syndrome. A newer highly accurate method of estimation involves alkaline methanolysis of bilirubin followed by chloroform extraction of bilirubin methyl esters and later separation of these esters by chromatography and spectrophotometric determination at 430 nm. Assays based on dry reagent chemistry have also been reviewed. One method used in many clinical chemistry laboratories is based on photographic film technology, and uses actachem dry chemistry slides. (Schiff's diseases of the liver) ([70])
Linearity limit of diazo methods is 20 mg/dl. Difficulties for using diazo method arise from two main causes: the use of unsatisfactory artificial standards for matching the colour; and the presence of extraneous coloured substances.

4.3) Peroxidase method-

The peroxidase method ([72]) obtains the unbound conjugated bilirubin (*b*c) and unbound unconjugated bilirubin (*b*u) using horseradish peroxidase (HRP, EC 1.11.17)-catalyzed oxidation of *b*c and *b*u by a peroxide (usually hydrogen or ethyl hydrogen peroxide) to colorless products at sample dilutions of about 1:40. Bilirubin bound to albumin (*A*:*b*c and *A*:*b*u) is protected from oxidation ([73]), but the rate of dissociation of the bound complexes must be significantly faster than the rate of bilirubin oxidation or the oxidation will proceed at a steady-state unbound bilirubin concentration that is below the equilibrium unbound bilirubin concentration ([74, 75]). The unbound bilirubin concentration is calculated by dividing the reaction velocity (rate of change in bilirubin light absorption at 460 nm) by *K*p z [HRP], where *K*p (min21) is the first-order rate constant for the oxidation of bilirubin by peroxide in an albumin-free solution containing 1 mg/ml HRP. Since only unconjugated bilirubin is neurotoxic, *b*u is the relevant fraction of the unbound bilirubin and *A*:*b*u 1 *b*u the

relevant fraction of the total bilirubin concentration. The peroxidase method without modification is therefore only applicable when the total bilirubin is predominantly unconjugated bilirubin.

4.4) Peroxidase Diazo method-

Add a small volume of HRP and peroxide to an aliquot of sample (usually 25 ml) and allowing the reaction to proceed 0 or *t* min before initiating the diazo test. The sulfanilic acid denatures the HRP, stopping the bilirubin oxidation reaction. Since the bilirubin
oxidation products are diazo negative , only nonoxidized bilirubin will form diazo derivatives. The volumes of HRP and peroxide added determine the sample dilution at which the unbound bilirubin is measured. For example, if 25 ml of HRP and 10 ml of
peroxide are added to 25 ml of sample, the unbound bilirubin is measured at a dilution of 1:2.4. The reaction would be stopped at the selected times with 0.5 ml of sulfanilic acid solution and the diazo test completed by adding 25 ml of nitrite followed by 0.5 ml of diluted methanol ([76]).

4.5) *High pressure liquid chromatography-*

HPLC methods allow for relatively rapid separation and quantification of the four bilirubin fractions. In 1916 Vandenberg and Muller noted that the bilirubin from patients with obstructive jaundice reacted "directly" whereas that from hemolytic jaundice reacted "indirectly", that is accelerator was required for its reaction. The direct reacting pigment was later identified as conjugated bilirubin and indirect reacting as unconjugated bilirubin. Although the distinction was found to be useful clinically, but it soon became apparent that there is not in fact, a precise relationship between indirectly reacting bilirubin and unconjugated bilirubin, and directly reacting bilirubin and conjugated bilirubin, respectively. In particular direct measurements overestimate conjugated bilirubin at low concentrations and underestimate it at higher concentrations. To overcome these limitations alkaline methanolysis method was developed. The bilirubin mono and diglucuronide conjugates are converted to mono and dimethyl esters by treatment with alkaline methanol. Unconjugated bilirubin is not affected by the reaction and is extracted into chloroform with the methyl ester derivatives. The pigments can then be separated and quantified by high performance liquid chromatography(HPLC) or thin layer chromatography and detected spectrophotometrically in the effluent. Use of an internal standard and calibration of the method with crystalline reference bilirubin and bilirubin methyl esters permit direct measurement of the individual pigment fractions in the sample ([77]).

4.6) S*imple colorimetric method for the estimation of plasma biliverdin-*

A new colorimetric method for the assay of biliverdin in biological fluids is described. The method, based upon the reaction of biliverdin with barbituric acid, offers improved sensitivity and selectivity when compared to direct spectrophotometric measurements. Using this method biliverdinaemia was observed in two patients with obstructive jaundice of malignant origin ([78]).

V. Newer Methods Of Bilirubin Estimation:

New enzymatic assay- for total (TBil) and direct bilirubin (DBil), the principle of which involves measuring the decrease in absorbance at 450 nm produced by bilirubin oxidase from Myrothecium verrucaria. Since TBil and DBil are oxidized at pH 7.2 and 3.7, respectively, the degree of bilirubin oxidation is measurable in each case. An analysis of bilirubin by high-performance liquid chromatography, before and after the enzymatic reaction with bilirubin oxidase, verified the specificity of the enzyme. The results obtained using this method varied linearly with TBil and DBil concentrations up to at least 250 mg/L and 150 mg/L, respectively. Reducing substances, commonly used anticoagulants and hemoglobin showed no apparent interference. The degree of day-to-day precision (CV) for TBil and DBil ranged from 1.2% (206.2 mg/L) to 10.6% (3.5 mg/L) and from 1.8%(84.3 mg/L) to 12.4% (2.1 mg/L), respectively. Values measured using this new method correlated well with those obtained by Malloy-Evelyn's method and the slide method employing the Kodak Ektachem analyzer ([79]) .

5.1) *Non invasive method-*

Neonatal jaundice occurs in nearly 70% of term and 80% of preterm babies. Management of jaundiced neonates often requires measurement of total serum bilirubin (TSB). Total serum bilirubin (TSB) is commonly determined by spectro-photometric methods by analyzing plasma or serum sample. Such techniques require drawing of blood causing pain and trauma to the neonate. In addition, there is a wide ange of intra- and inter-laboratory variability in the performance of the bilirubin analyzers. These problems have led to search for a non-invasive, reliable technique for estimation of TSB.

A large number of studies have demonstrated the possibility of prediction of serum bilirubin in neonates by measuring the yellowness of the skin in the jaundiced neonate using transcutaneous bilirubino-meters.

5.1.1) Principle of transcutaneous bilirubinometers – ([80])

High correlation between cutaneous bilirubin and total serum bilirubin (TSB) form the basis of transcutaneous Bilirubinometry. These meters work by directing light into the skin of the neonate and measuring the intensity of specific wavelength that is returned. The number of wavelengths, used is variable in different transcutaneous bilirubinometers. The meter analyzes the spectrum of optical signal reflected from the neonate's subcutaneous tissues. These optical signals are converted to electrical signal by a photocell. These are analyzed by a microprocessor to generate a serum bilirubin value. The major skin components, which impart the spectral reflectance in neonate, are

(i) melanin, (ii) dermal maturity, (iii) hemoglobin, and (iv) bilirubin.

Earlier, the transcutaneous bilirubinometers utilized only a few wavelengths. In these meters, there was no provision to overcome the impact of dermal maturity and melanin content. Therefore, separate analysis for each patient population (different ages and races) was required one had to refer to different conversion tables for each population. However, a new product, BilicheckTM (Specter, Inc) performs a spectral analysis at more than 100 different wavelengths. By subtracting the spectral contribution of the known components, the bilirubin absorbance is quantified. The available meters can be divided into 2 categories:

(i) Multi wavelength Spectral Reflectance meters (Bilicheck) TM

(ii) Two-wavelength (460 nm, 540 nm) Spectral Reflectance meters (Minolta, Bili-test)

Although BILIRUBIN is one of the most commonly performed laboratory measurements in the newborn, the measurement of TSB (total serum bilirubin) concentration remains remarkably inaccurate.

Repeated surveys over the last 3 decades have disclosed a high level of interlaboratory variation in the measurements of both total and direct-reacting serum bilirubin concentrations in neonatal sera.

In a recent study, a sample with a known TSB concentration of 14.8 mg/dL (243 μmol/L) was analyzed in 14 different university hospital laboratories. The mean (± SD) measured TSB was 15.2 ± 2.5 mg/dL (coefficient of variation 16.4%) and the range was 12.1 to 18.5 mg/dL (207 to 316 μmol/L). This remarkably wide range of values in skilled hands suggests that considerable caution is necessary before generalizing bilirubin levels from one institution to the wider universe of newborns. Within-laboratory variation generally is considered to be lower, but in the study of Vreman and associates ,over time, the coefficient of variation for repeated measurements of the same sample was as high as 17.2% in one laboratory([81]).

These variations between laboratories might explain the frequent occurrence in clinical practice of an infant being admitted for treatment of hyperbilirubinemia because an outside laboratory found a high TSB level, but when the test is repeated in the hospital laboratory, the TSB level is 5 or 6 mg/dL (85 to 103 μmol/L) lower. Of course, there is no way of knowing which value is correct. A 16.4% coefficient of variation between laboratories means that if the true serum bilirubin value is 20 mg/dL (342 μmol/L), the 95% confidence limits of a repeat measurement at another laboratory could fall anywhere between 14.4 and 26.6 mg/dL (246 to 455 μmol/L). Because our followup, surveillance, and intervention in jaundiced infants are based on TSB values, spurious underestimation of the TSB concentration might lead to withholding of necessary therapy, and overestimation will produce unnecessary clinical intervention ([81]).

VI. Conclusion -

CLIA recommendation for acceptable performance in total bilirubin is Target value ± 0.4mg/dl or ± 20% greater. Following such guideline and adhering to multirule control measures such analyte of clinical significance should be reported and a great deal of clinico-chemical correlation is needed to manage the cases ([82]).

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Table 1 – Causes of hyperbilirubinemia

PredominantlyUnconjugated Hyperbilirubinemia	Predominantly Conjugated Hyperbilirubinemia
I. Overproduction A. Hemolysis (intra and extravascular) B. Ineffective erythropoesis **II. Decreased hepatic uptake** **III. Decreased bilirubin conjugation (decreased hepatic glucuronosyl transferase activity)** A.Hereditary transferase deficiency 1. Gilbert's syndrome 2. Crigler-Najjar type II (moderate transferase deficiency) 3. Crigler Najjar type I (absence of transferase) B. Neonatal jaundice (transient transferase deficiency) C. Acquired transferase deficiency 1. Drug inhibition (e.g.chloramphenicol ,pregnanediol) 2. Breast milk jaundice (transferase inhibition by pregnanediol and fatty acids in breast milk) 3. Hepatocellular disease (hepatitis, cirrhosis) D. Sepsis	**I. Impaired hepatic excretion** A. Hereditary disorders 1. Dubin-Johnson syndrome 2. Rotor syndrome 3. Recurrent (benign) intrahepatic cholestasis 4. Cholestatic jaundice of pregnancy B. Acquired disorders 1. Hepatocellular disease (e.g.,viral or drug induced hepatitis,cirrhosis) 2. Drug induced cholestasis (e.g.,oral contraceptives,androgens,chlorpromazine) 3. Alcoholic liver disease. 4. Sepsis 5. Postoperative state 6. Parenteral nutrition 7. Biliary cirrhosis **II. Extrahepatic Biliary obstruction** 1. Gallstones 2. Biliary malformation . 3. Infection 4. Malignancy 5. Hemobilia (trauma,tumor) 6. Sclerosing cholangitis 7. Malignancy 8. Inflammation (pancreatitis)

Table-2 ,Serum and Urine Bilirubin levels in various pathological conditions.

Bilirubin disorder	Serum Bilirubin Unconjugated Conjugated	Urine Bilirubin	Comments
Overproduction: I. Hemolysis (intra and extravascular) II. Ineffective erythropoesis	Increased Normal	Nil	↑bilirubinturnover,serum bilirubinrarelyexceeds 4mg/dl, ↑Urobilinogen in Urine.
Defective hepatic uptake: I. Some drugs(e.g.,flavaspidic Acid,novobiosin) II .Gilbert's syndrome(some cases)	Increased Normal	Nil	
Defective Conjugation: I.Neonatal Jaundice II.Gilbert's syndrome III.Crigler,Najjar syndrome(typesIandII) Type-I Type-II	Increased Low Increased Low [21 to 51 µmol/L (1.2-3mg/dl)] Increased Low Increased [340-770 µmol/L (20 to 45mg/dl)] Increased [103 to 340 µmol/L (6 to 20 mg/dl)].	Nil Nil Nil	↓Glucuronosyltransferas ↓conversion of bilirubin to urobilinogen. ↓ Glucuronosyltransferase and ↓ bilirubin uptake TypeI=Absenceof transferaseType II=deficiency of transferase.
Defective excretion: I.Intrahepatic obstruction A.Familial syndromes 1.Dubin-Johnson 2.Rotor B.Drugs(eg,chloramphenicol,methyltestesterone) C.Benign recurrent intrahepatic Cholestasis	Increased Increased [51 to 257 µmol/L(3 to 15 mg/dl)] Increased Increased Increased Increased Increased Increased	+ + + +	↑ Urinary coproporphyrin typeI ↑ Alkaline phosphatase but other function tests usually normal. ↑ Alkaline phosphatase. ↓or absent Urobilinogen in urine.
Hepatocellular disease I.Hepatitis II.Cirrhosis	Increased Increased Increased Increased	+ 	Conjugated or total serum bilirubin>50-70%.

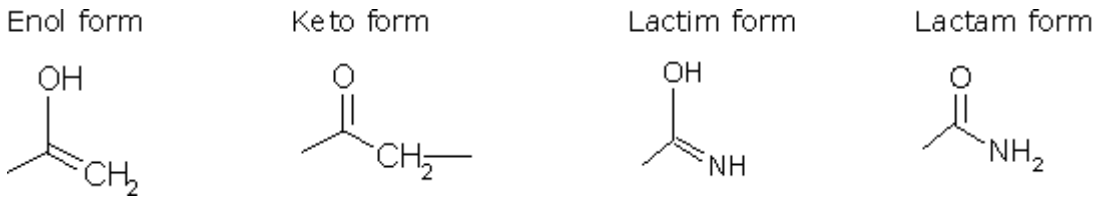


Figure1 -Various forms of bilirubin. Both the forms are available and are interchangeable.

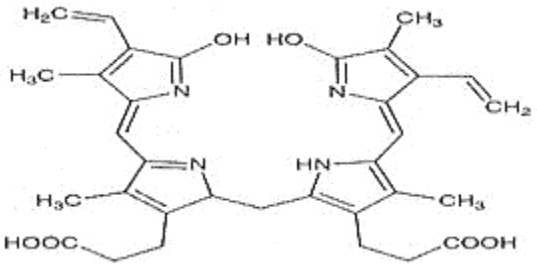
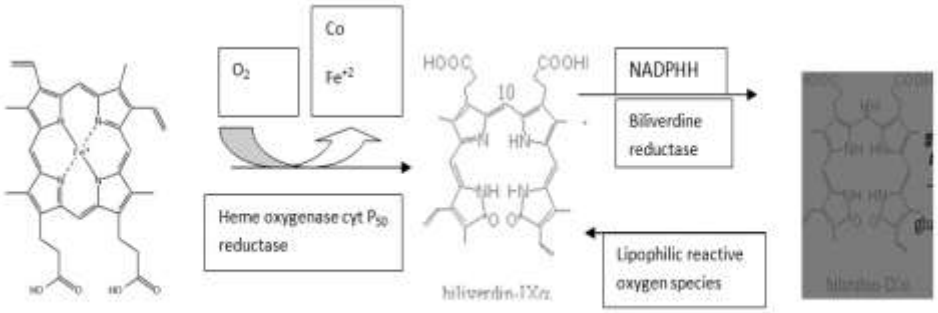


Figure 2:Structure of bilirubin



reactive oxygen species

Glutathione (GSH) —glutathione peroxidase, glutathione reductase, NADPH→ Oxidised Glutathione (GS-SG)

Figure3: Role of bilirubin as an Antioxidant

Synthesis of bilirubin from haemoglobin and its role as an Antioxidant.

[Haemoglobin is dissociated into heme and globin. Globin is degraded to its amino acids which are reused. Heme in presence of heme oxygenase, NADPH and O_2, forms green pigment biliverdin which is reduced to bilirubin.

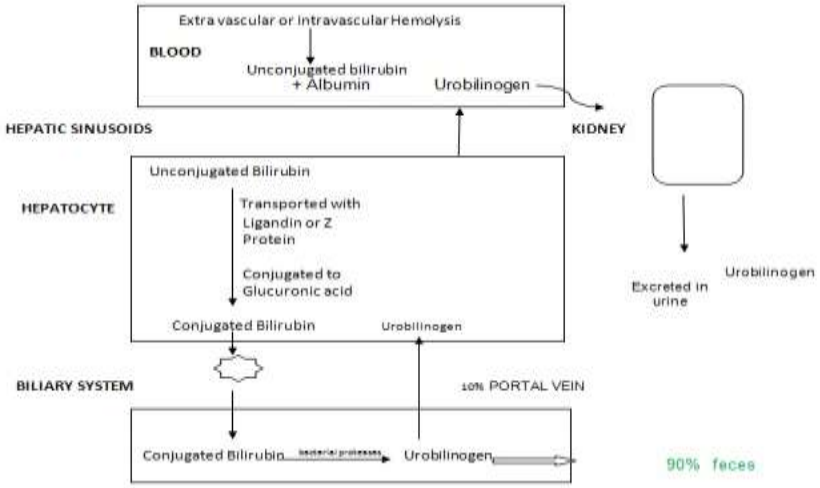


Figure4- Metabolism of bilirubin

Bilirubin formed in peripheral tissues is transported to the liver by binding noncovalently to plasma albumin where it gets conjugated and enters the intestine through bile duct . There it is converted to urobilinogen , which in turn is excreted through feces and urine.