

EVALUATION OF A NEW METHOD FOR MEASUREMENT OF TOTAL BILIRUBIN USING BILIRUBIN OXIDASE

THESIS

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CONTENTS

INTRODUCTION AND AIM OF THE WORK	1
REVIEW OF LITERATURE	
<u>II) BILIRUBIN</u>	
A) Production and Metabolism of Bilirubin	
1. Sources of bilirubin	2
2. Transport of bilirubin	3
3. Hepatic metabolism of bilirubin	
a. Hepatocytic uptake	7
b. Conjugation	8
c. Excretion	11
4. Intestinal phase of bilirubin metabolism	11
5. Renal excretion of bilirubin	13
B) Chemistry of Bilirubin	
1. types	13
2. Structure	20
3. Biochemical characteristics	24
C) Serum Bilirubin	
1. Reference range and Physiological variations	26
2. Pathological variations	27
<u>III) METHODS OF DETERMINATION OF SERUM BILIRUBIN</u>	
A) Methods Based on the Diazo Reaction	
1. Van den Bergh reaction	36
2. Malloy and Evelyn method	39
3. Jendrassik and Grof method	41
4. Method of Powell	42
B) Determination of Bilirubin Using Direct-Reading Bilirubinometers	44
C) Determination of Bilirubin by the Use of HPLC	46
D) Methods Based on Oxidation of Bilirubin by Bilirubin Oxidase	49
E) Determination of Bilirubin Using Reflectance Spectrophotometry	50
F) Methods of Measurement of Free (Unbound) Bilirubin and the Reserve Bilirubin-Albumin Binding Capacity	
1. Methods of measurement of free bilirubin	
a. Kinetic spectrophotometric assay	52
b. The use of molecular sieve chromatography ...	53
c. The use of bilirubin oxidase	54

INTRODUCTION AND AIM OF THE WORK

INTRODUCTION:

Bilirubin is mainly an end product of hemoglobin metabolism. Its conjugation, transport and excretion occurs mainly through the liver. The measurement of serum bilirubin is one of the most frequently ordered liver function tests. It reflects the uptake, conjugation and the excretory functions of the liver.

There are several methods for the measurement of serum bilirubin including, the use of the diazo reaction, the direct spectro photometry of native bilirubin, and high performance liquid chromatography.

Recently, a new enzymatic method was introduced for the determination of serum bilirubin by the use of bilirubin oxidase.

AIM OF THE WORK:

Evaluation of the analytical performance of the new enzymatic method for the measurement of total serum bilirubin.

REVIEW OF LITERATURE

[I] BILIRUBIN

A) Production and Metabolism of Bilirubin:

1. Sources of bilirubin:

Bilirubin formation results from the catabolism of hemoproteins, primarily hemoglobin. Eighty to eighty five percent of plasma unconjugated bilirubin (UCB) is derived from degradation of hemoglobin heme and the remaining 15-20 % results from hepatic turnover of heme and hemoproteins such as the cytochromes, catalases and tryptophan pyrolase (Odell, 1980).

The catabolism of heme from all heme proteins appears to be carried out in the microsomal part of the reticuloendothelial cells by a complex enzyme system called hemoxygenase. By the time the heme reaches the hemoxygenase system the iron is oxidized to the ferric form constituting hemin. The hemin is then reduced with NADPH and with the aid of more NADPH, oxygen is added to the α -methenyl bridge between pyrroles I and II of the porphyrin forming α -hydroxyheme. The ferrous iron is again oxidized to the ferric form. With the further addition of oxygen, ferric iron is released, carbon monoxide is produced, and biliverdin IX- α results from the splitting of the tetrapyrrole ring. The heme itself participates in

this reaction as a catalyst. In birds and amphibia, the green biliverdin IX- α is excreted. In mammals, a soluble enzyme called biliverdin reductase reduces the methenyl bridge between pyrrole III and IV to a methylene group to produce the yellow pigment bilirubin IX- α (fig. 1) (Murray, 1988).

2. Bilirubin transport in serum:

UCB is specifically bound to albumin, albumin binding decrease bilirubin toxicity in vivo and in vitro (Mustafa et al., 1969). Binding to albumin is essential for transport because the solubility of unbound bilirubin at physiologic pH (7.4) is extremely low, averaging 0.4 mg/100 ml. Although bilirubin may bind to three sites on the albumin molecule, at plasma concentrations less than its molar equivalence with albumin (about 35 mg/dl) bilirubin is bound almost exclusively to a primary high-affinity site (Jacobsen, 1970). Albumin binding of bilirubin is reversible; albumin-bound bilirubin remains in equilibrium with free plasma bilirubin (Brown et al, 1980). The unbound or free bilirubin fraction in plasma, although exceedingly small (0.007 μ g/dl) is of great significance because only unbound bilirubin has the ability for transit across cell membrane, such as the blood brain barrier (Gollan and Schmid, 1979).

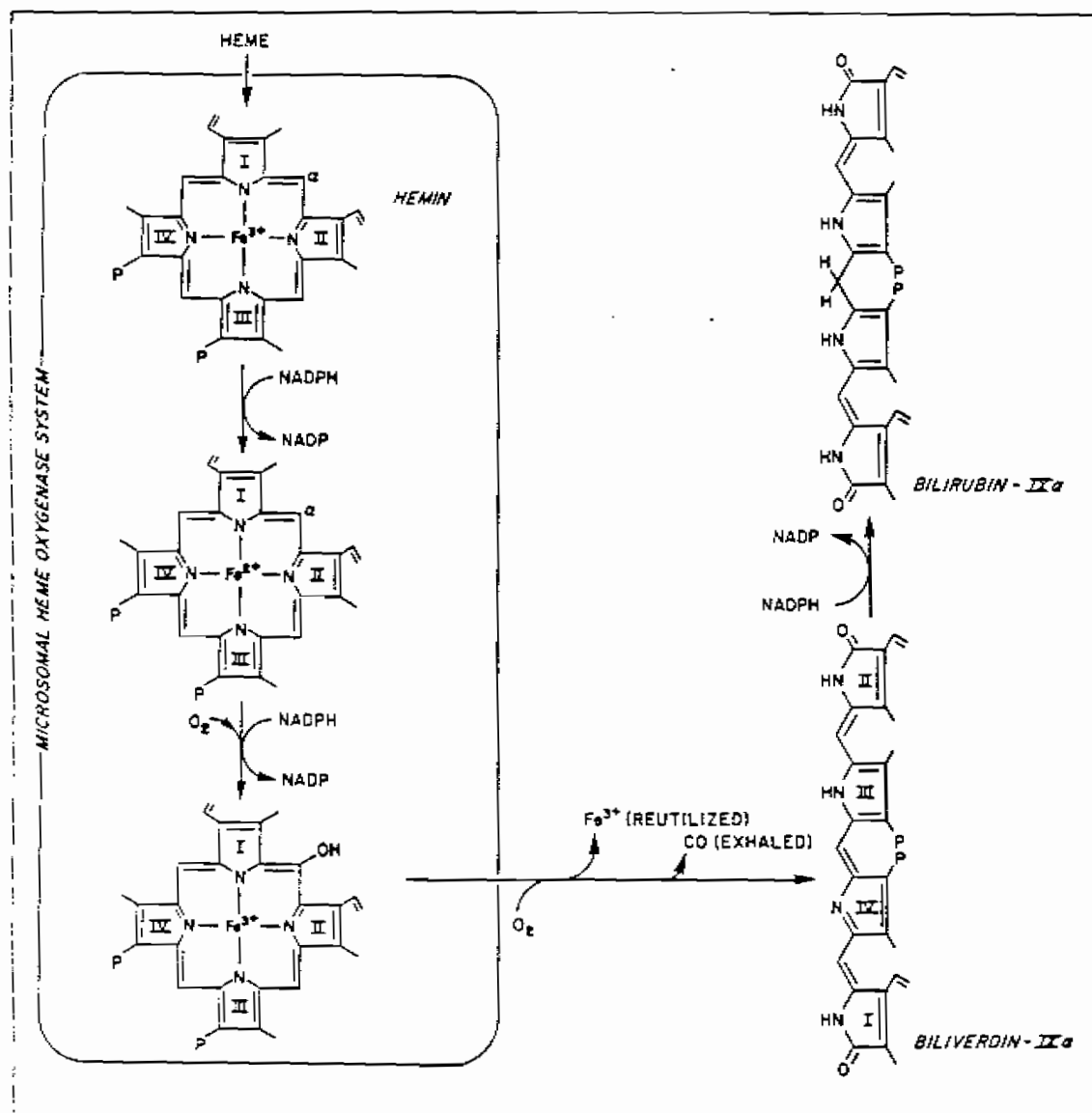


FIGURE (1): SCHEMATIC REPRESENTATION OF THE MICROSOMAL HEME OXYGENASE SYSTEM
 (Modified from Schmid, R. McDonough, A.F. IN: The Porphyrins. Dolphin, D (Editor), Academic Press, 1978)

This free (unbound) bilirubin is the cause of the neurological damage that occur to newborns with high plasma bilirubin (kernicterus) as it is toxic to the nerve cells (*Blondheim et al., 1972*).

The danger of kernicterus is determined by at least three factors: the concentration of unconjugated bilirubin, the patients' capacity to bind and metabolize bilirubin, and the presence of potential interfering chemical agents that can alter the bilirubin binding, such as chlorothiazide, diazepam, ethacrynic acid, Furosemide, oxytocin, phenobarbital, salicylate, sulfisoxazol and gentamycin. These substances are able to influence the "high affinity" binding sites, and may actually promote transfer of bilirubin into the brain (*Scheidt et al., 1977*). Infant serum binds bilirubin only a half to a third as effectively as adult serum (*Kapitulnik et al., 1975* and *Alayoff et al., 1980*). At birth, bilirubin binding by infant serum is equivalent at all gestational ages from 26-42 weeks (*Ritter et al., 1985*) but can be adversely affected during the first postnatal week by illness and certain drugs (*Brodersen, 1974*). *Ritter and Kenny (1986)* studied postnatal changes in albumin-bilirubin binding in sera from sick, extremely premature infants. There was a prolonged period of diminished binding capacity lasting for one month. A subsequent dramatic improvement occurred so that bilirubin binding was equal to that of

adult serum by three months (*Ritter and kenny, 1986*). The aetiology of the diminished infant albumin bilirubin binding remains unclear. It is not due to a difference in physical or immunologic properties of infant albumin (*Gitzelmann-Cumarasamy et al., 1979*). It is probably due to a factor that attaches to the bilirubin binding site or alters the conformation of the albumin molecule (*Ebbesen and Brodersen, 1982*).

The evidence that pregnant women also have diminished bilirubin binding suggests that the responsible factor might be transferred transplacentally (*Järnerot et al., 1981 and Ritter et al., 1985*).

It is important to estimate the level of either free bilirubin or the reserve albumin-binding capacity, which might serve as additional objective indices of the likelihood of kernicterus (*Porter and Waters, 1966*).

The safe upper limit of free bilirubin in neonates, beyond which kernicterus may occur is shown to be about 0.29 mg/dl. This level of free bilirubin is achieved when approximately 8.0% of the high affinity binding sites of albumin are saturated with bilirubin (*Jacobsen and Wennberg, 1974*).

Conjugated bilirubin also is transported in the plasma bound to albumin but its affinity for the protein is less than that of the unconjugated bilirubin (*Jacobsen, 1970*). This may explain the observation that the non-protein bound fraction of conjugated bilirubin (which accounts for less than one percent of total plasma conjugated bilirubin in jaundiced patients), and not the unconjugated bilirubin is freely filtered by the glomeruli, this filtered fraction is largely reabsorbed by the renal tubules. The residual non reabsorbed part gives rise to the bilirubinuria which is characteristic in hepatocellular and cholestatic jaundice (*Gollan and Schmid, 1979*).

3. Hepatic metabolism of bilirubin:

a. Hepatocytic uptake:

Many authors have claimed the existence of a specific albumin receptor in the membrane of the hepatocyte (*Berk et al., 1987; Sorrentino et al., 1987 and Tiribelli et al, 1988*).

On the other hand, the plasma membrane of the hepatocyte contains a number of proteins that reversibly bind organic anions and have been proposed as putative carriers for the hepatocytic uptake of these cholephiles (*Wolkoffs and Chung, 1980; Stremmel et al., 1983 and Berk et al., 1987*). Although the hepatocytes extract

bilirubin from sinusoidal blood without concomitant uptake of albumin, it is not known whether the small free fraction which is not bound to albumin or bilirubin-albumin complex, or both, interact with these plasma membrane patients (*Gollan and Knapp, 1985*). The hepatic uptake system operates far below saturation at normal plasma bilirubin concentrations, thus the uptake of bilirubin doesn't appear to be the rate limiting step in its secretion (*Tiribelli and Ostrow, 1990*). Kinetic studies indicate that the transfer of bilirubin across the hepatocyte plasma membrane is bidirectional. It has been calculated that more than 30 percent of unconjugated bilirubin initially taken up by the liver refluxes into the plasma, when it is presumably recycled to the liver (*Gollan and Knapp, 1985*). Once within the hepatocyte bilirubin again becomes protein bound to cytosol anion-binding proteins, primarily to ligandin (Y protein, glutathione S-S transferase) but also to other glutathione S-transferases and to Z-protein. This binding within the cell prevents back flow of bilirubin into the circulation. Binding to Z-protein occurs only after ligandin has become saturated (*Gollan and Schmid, 1979*).

b. Conjugation:

The biotransformation of bilirubin to more water soluble derivatives occurs within the endoplasmic reticulum of the microsomes (*Odell, 1980*). It is enzymatically bound with glucuronyl