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THE UNIVERSITY OF ALBERTA

Mechanism of Bilirubin Toxicity in a Neural Cell Line.

BY

Yair Amit.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF **Doctor of Philosophy**

IN

MEDICAL SCIENCES

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EDMONTON , ALBERTA

SPRING 1990.

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled -

Mechanism of Bilirubin Toxicity in a Neural Cell Line.

submitted by - **Yair Amit**

in partial fulfillment of the requirements for the degree

of - **Doctor of Philosophy**

in - **Medical Sciences**

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Dedicated to my wife Ada

and my children Itai, Ido, and Yorai

ABSTRACT

The mechanism of bilirubin toxicity to the central nervous system has been the subject of numerous investigations over the past decade. The results of several clinical and experimental studies suggest that bilirubin is toxic to various cellular functions with reversibility of early stages of bilirubin encephalopathy. Yet, the major biochemical defect underlying bilirubin toxicity has not been completely elucidated. The difficulties in analyzing the results and the inability to point to a primary target of bilirubin toxicity stem from variables in experimental design, the use of different animal models and cell systems, and the use of unstable bilirubin mixtures. Spectrophotometric measurements demonstrated that bilirubin in tissue culture media, at concentrations of 35-125 μM and at bilirubin-to-albumin [B/A] molar ratios up to 3, is stable over a 24-hour period. The use of a neural cell line and the presence of appropriate albumin concentrations are advantageous. We have measured the interaction and toxic effects of bilirubin to N-115 cells, a murine neuroblastoma cell line. The results obtained point to a multistep interaction process between bilirubin and the plasma membrane. Bilirubin binding is dependent on bilirubin concentration, B/A molar ratios, temperature and pH conditions, and is partially reversible with the addition of albumin. Under appropriate B/A molar ratios, bilirubin was found to affect Na^+/K^+ ATPase activity, [^{3}H]-thymidine uptake, L-[^{35}S]-methionine incorporation into protein, and mitochondrial functions. The toxic effects seem

to be dependent again on B/A molar ratio, bilirubin concentration, and length of exposure. However, it is not possible to single out the primary target for bilirubin toxicity conclusively. In N-115 cells, once toxicity appeared, it was irreversible. Moreover, toxicity appeared long after removal of the bilirubin-containing media following a short-term exposure to bilirubin, during which toxicity was not manifest. We conclude that, under appropriate experimental conditions, the binding interaction between bilirubin and the cell plasma membrane is complex, and that bilirubin is toxic to several cellular functions in N-115 cells in a progressive and irreversible process.

Acknowledgments

In 1984 I first came to Edmonton for a fellowship program in Neonatology. Shortly thereafter I became involved with Dr. Schiff's research on bilirubin. This thesis is the result of an intense laboratory work that would never have been completed without the help of many, to whom I wish to express my thanks and gratitude.

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B/A	-	Bilirubin to albumin molar ratio.
DMEM	-	Dulbecco's modified Eagle medium.
FCCP	-	Carbonyl cyanide -*p* - trifluoromethoxyphenylhydrazone.
HEPES	-	N-2-Hydroxyehtylpiperazine-N-2-ethanesulfonic acid.
HPLC	-	High performance liquid chromatography.
HSA	-	Human serum albumin.
MTT	-	3-(4,5-dimethylthiazol-y-yl)-2,5-diphenyl tetrazolium bromide.
PBS	-	Phosphate buffered saline.
PFM	-	Protein Free Medium.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Hyperbilirubinemia is a common occurrence in the newborn period. Bilirubin encephalopathy (kernicterus) is a major complication of the toxic effect of bilirubin on brain cells. Originally described in jaundiced newborns, it has been seen in recent years in premature infants suffering from a mild degree of hyperbilirubinemia.

The protection of the newborn's brain from bilirubin has been attributed to a number of factors, among them the capacity of albumin to bind bilirubin, the integrity of the blood brain barrier, and the integrity of the neural cells.

The mechanism of bilirubin encephalopathy has been extensively studied over the past decade. However, the primary target, the toxic manifestation and the nature of the interaction between bilirubin and neural cells remains unclear. Studies conducted on neural and on non-neural cells and tissues demonstrate that bilirubin may impair a large number of cellular functions. However, the use of bilirubin concentrations higher than those usually encountered in clinical situations, and the use of varying albumin concentrations may account for the multiplicity of effects and inconclusive results.

The use of bilirubin without the addition of albumin or at high bilirubin-to-albumin molar ratios causes rapid aggregation and precipitation, auto-oxidation, and decomposition of the pigment, as well as photoisomerization of the natural occurring

bilirubin IX-α isomer. Since bilirubin may be poisonous to cells, clearly it is important to establish appropriate experimental conditions under which bilirubin is maintained in solution throughout the time the cells are exposed to bilirubin.

The purpose of the work herein described was to establish the appropriate experimental conditions for studies related to bilirubin and its cellular interaction and to define the following:

1) The interaction between bilirubin and the neural cell.
2) The target and mechanism of bilirubin toxic effects.

In the following sections a number of subjects will be reviewed:

1) Neonatal jaundice.
2) Bilirubin metabolism, structure, and binding properties.
3) Bilirubin toxicity.
4) Kernicterus and bilirubin encephalopathy.
5) Studies on bilirubin toxic effects.

2. Neonatal Jaundice

Hyperbilirubinemia is a common occurrence during the neonatal period. Clinical hyperbilirubinemia is defined as a serum bilirubin concentration that exceeds 26 µmol/L, and is common to most newborn infants during their first week of life. In 10 to 15% of all normal-term babies, hyperbilirubinemia becomes sufficiently high to be visible as jaundice [1]. Although the majority of jaundiced full-term babies appear completely healthy, standard textbooks of newborn medicine mandate diagnostic investigation to rule out pathologic causes of jaundice in those infants whose serum bilirubin concentrations exceed a level of 170 to 220 µ mol/L [2,3]. The incidence of serum bilirubin concentrations above 220 µmol/L ranges from 4.5% to 20% during the first week of life [4,5]. Although the presence of hyperbilirubinemia engenders some concern, 56% of infants whose serum bilirubin concentrations exceed the above levels show no cause for the jaundice [6].

There are many causes for neonatal hyperbilirubinemia unique to the fetus and the newborn. During the last stages of fetal life, removal of erythrocytes provides an increasing load of hemoglobin for catabolism. This results in an increase in bilirubin production [7]. The normal newborn produces more than double the bilirubin production of 3.6 mg/kg/day observed in the adult. Moreover, no rate-limiting step in hemoglobin catabolism and unconjugated bilirubin formation is recognized in the mammalian fetus [8,9,10,11].

The disposal mechanism for bilirubin in the fetus involves two pathways. The vast majority of unconjugated bilirubin is cleared via the placental circulation into the maternal circulation, where it is disposed of by the maternal liver [8,10]. The second pathway involves excretion by the fetal liver. This pathway is limited due to several factors. Foremost among these is a marked deficiency in hepatic uridine diphosphate glucuronyltransferase, noted in human as well as other mammalian fetuses [7]. As a result, the conjugating capacity of fetal liver is almost undetectable. Other factors associated with decreased hepatic clearance of bilirubin in the fetus are reduced hepatic blood flow and low levels of bilirubin binding proteins [9,11]. However, as a result of the different disposal processes, unconjugated hyperbilirubinemia is rarely evident at birth, even in severe cases of hemolytic anemia in the fetus.

The newborn infant, like the fetus, has several impairments in bilirubin metabolism and transport. These include increased bilirubin production [7], deficiency of hepatic bilirubin binding proteins and decreased glucuronyltransferase activity [12,13], as well as increased enterohepatic circulation of bilirubin [14]. Taken together, these factors usually result in the occurrence of increased concentrations of serum unconjugated bilirubin during the first days of life. Clinically, this is usually defined as "physiologic jaundice of the newborn" [15]. Yet, in certain groups of infants this phenomenon is exaggerated and the jaundice becomes pathological. A variety of conditions may result in unconjugated

hyperbilirubinemia : hemolytic disorders, polycythemia, increased extravasation of blood, increased enterohepatic circulation of bilirubin, defects in bilirubin metabolism, breast feeding, inherited metabolic disorders and prematurity [2,3].

There are two functionally distinct periods in physiologic jaundice of the newborn . The first is observed during the first 5 days of life and is characterized, in the full term infant, by a rapid rise in serum unconjugated bilirubin concentration to a peak of 100-120 μmol/L on the third day of life, and a rapid decline until the fifth day. In the premature infant, the peak value is higher and does not occur until the fifth to seventh day of life. The second period of physiologic jaundice is characterized by a relatively stable serum unconjugated bilirubin level of about 35 μmol/L that lasts until the end of the second week, in term infants, or for more than a month in preterm infants. After the second stage, serum unconjugated bilirubin concentrations decline to levels observed in normal adults [3,15,16,17,18,19].

Many studies of serum bilirubin concentrations in normal-term and in premature babies have provided guidelines for the diagnosis of "physiologic" and pathologic jaundice [4]. Pathologic jaundice is suspected whenever the following criteria are present:

1) Clinical jaundice in the first 24 hours of life.
2) Total serum bilirubin concentration increasing by more than 85 μmol/L per day.
3) Total serum bilirubin concentration exceeding 220 μmol/L in term infant and 255 μmol/L in prematures.

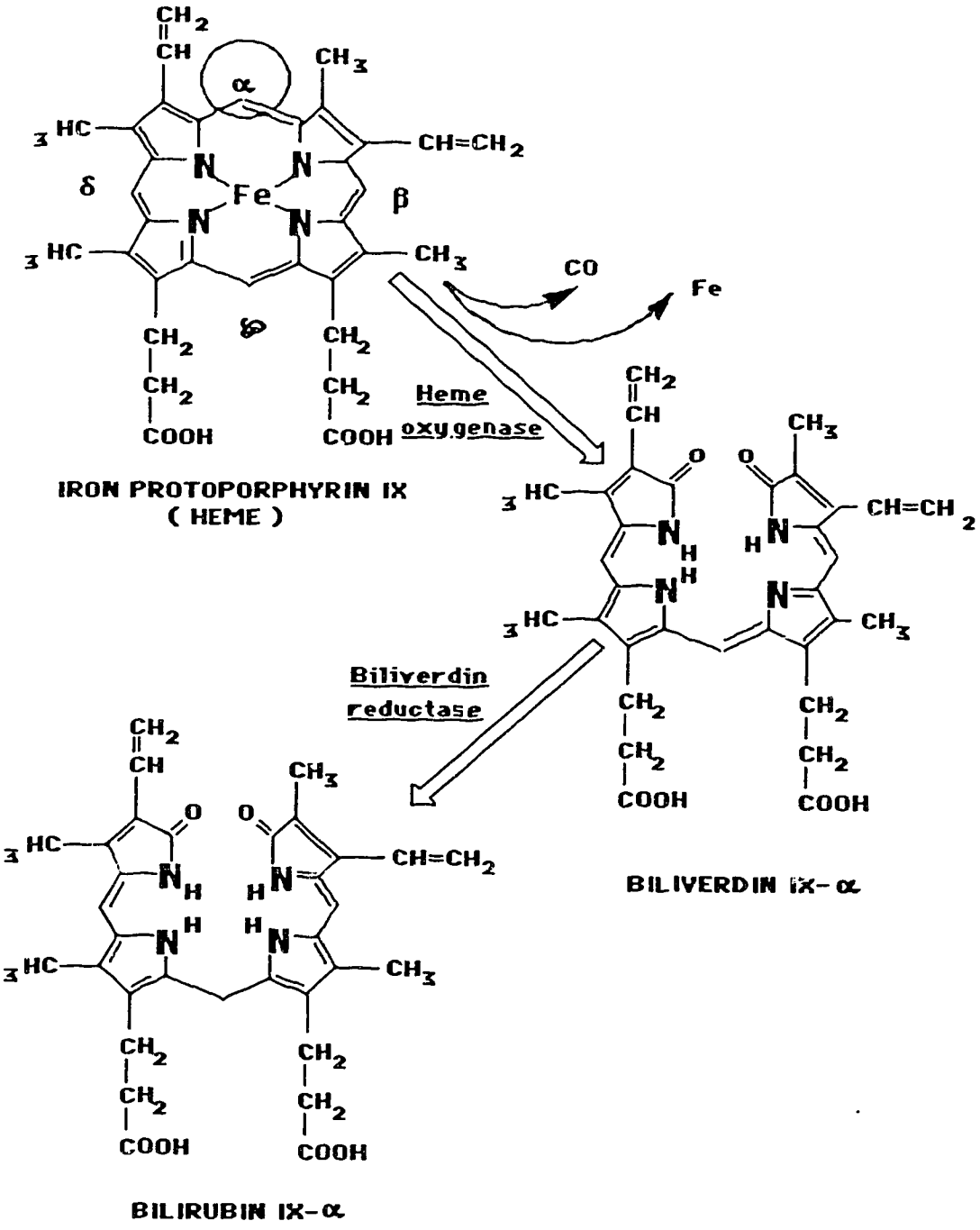
4) Direct serum bilirubin levels over 25-34 μmol/L.
5) Clinical jaundice lasting more than a week in a term baby, or two weeks in a premature infant [3].

3. Bilirubin Metabolism

Bilirubin is formed by the catabolism of different heme proteins including hemoglobin, myoglobin and heme-containing enzymes such as cytochromes, catalases and pyrolases [20]. Hemoglobin is the principal source of bile pigment in mammals, accounting for approximately 80% of the daily bilirubin production [21].

The metabolic pathway of heme catabolism has been clarified to a considerable extent [22]. Heme (Plate 1) is catabolized by a microsomal heme oxygenase localized primarily in the reticuloendothelial system [23], in tissue macrophages, and in the intestinal brush border membranes [24]. Inside the microsome, the porphyrin iron - located within the cyclic tetrapyrrole - is reduced, and an oxygen radicale is generated. Radicale attack and subsequent oxidation of the carbon atom at the α-methene carbon position, break the porphyrin ring. As a result, biliverdin IX-α is formed with loss of the iron atom and release of carbon monoxide. In mammals, biliverdin undergoes further reduction to bilirubin IX-α (Plate 2) [23]. The conversion is catalyzed by biliverdin reductase located in the cytosol [23].

Plate 1. Enzymatic oxidation of heme.



Bilirubin is a waste product and has no apparent function. Although the concentration of bilirubin in the serum is generally low, its concentration in the bile is significantly higher [20]. Five steps are involved in the transport of bilirubin from its sites of formation to the intestinal tract:

1) Transport in the plasma firmly bound to albumin [25].
2) Carrier-mediated transfer of bilirubin into the hepatocyte and binding to acceptor proteins located in the cytosol [26,27].
3) Hepatic conjugation that renders the pigment polar and water soluble [28].
4) Excretion of conjugated bilirubin into the bile [28]
5) Transport and elimination in the intestine [29,30].

Once inside the liver cell, bilirubin is transported to the smooth endoplasmic reticulum where the insoluble pigment is conjugated, thus converted into a water-soluble monoglucuronide pigment [29]. The final step in bilirubin metabolism, within the hepatocyte, is a second glucuronidation which takes place in the cytosol by a plasma membrane-bound enzyme [29]. Bilirubin mono- and diglucuronide are then excreted into the bile. When conjugated bilirubin reaches the sterile newborn intestine, the normal reduction of bilirubin to fecal stercobilinogen does not occur. Instead, a large proportion of the bilirubin is hydrolyzed by β-glucuronidase located in the brush border of the small intestine [29,30]. The resultant unconjugated bilirubin is reabsorbed in the gut and taken up by the portal system to start the disposal process

again [29,30], giving the so called enterohepatic circulation of bilirubin.

Disorders of bilirubin metabolism affect human beings from birth. The detrimental effects appear to arise chiefly from the virtual insolubility and instability of the pigment in aqueous solution at physiologic pH. Several bilirubin IX-α polar groups, namely, two carboxyl, two lactam, and two pyrrol groups render the substance soluble in water (Plate 2). The actual insolubility is explained by intra-molecular hydrogen bonding. In the hydrogen-bonded molecule (Plate 3), the hydrophilic polar COOH and NH groups are intimately associated and unavailable for interaction with polar groups in the environment. The insolubility of bilirubin-acid, with its two protonated carboxyl groups (Fig. 4), is considered the basis for its neurotoxicity. Understanding the conditions of bilirubin-acid formation is important for understanding the mechanism of its toxicity [31,32,33].

Bilirubin forms a saturated aqueous solution containing a very low concentration of the acid and a higher concentration of the dianion (Plate 4 & 5) [31,32]. Due to negative charges, the dianion is present in equilibrium with its dimer. The degree of dimerization is independent of pH, since hydrogen ions are not involved. However, with increasing hydrogen ion concentration some of the dimers and dianions take up protons from the medium, forming acid anions with fewer negative charges. The decrease in electric repulsion is followed by formation of large aggregates. During this aggregation the solution usually remains clear and

Plate 2: Bilirubin IX- α

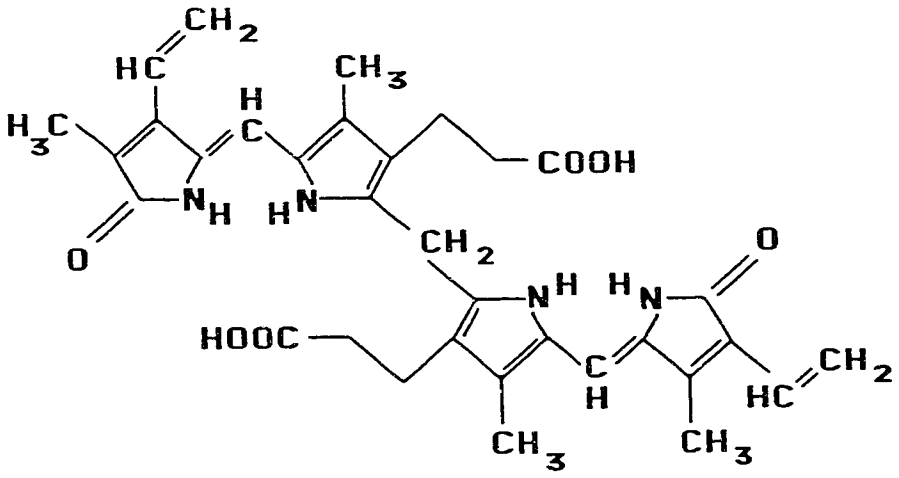


Plate 3: Bilirubin IX-α acid, intramolecularly hydrogen bonded.

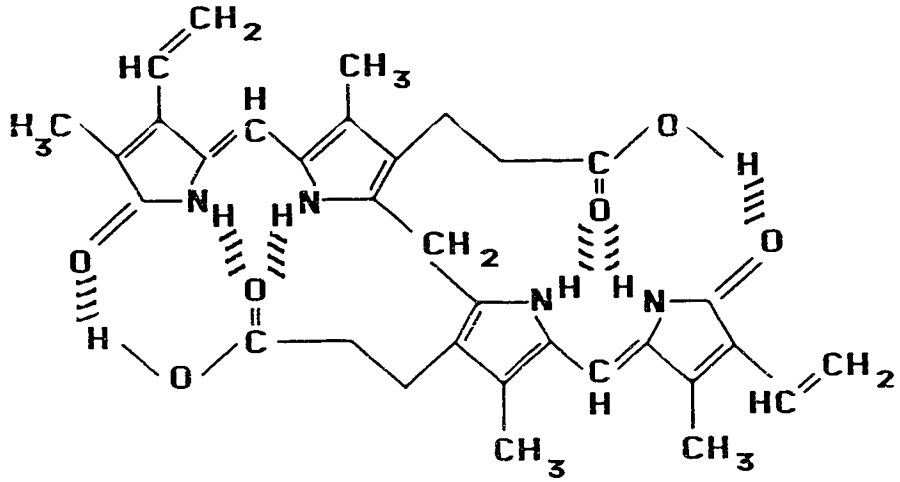


Plate 4: Bilirubin IX-α acid.

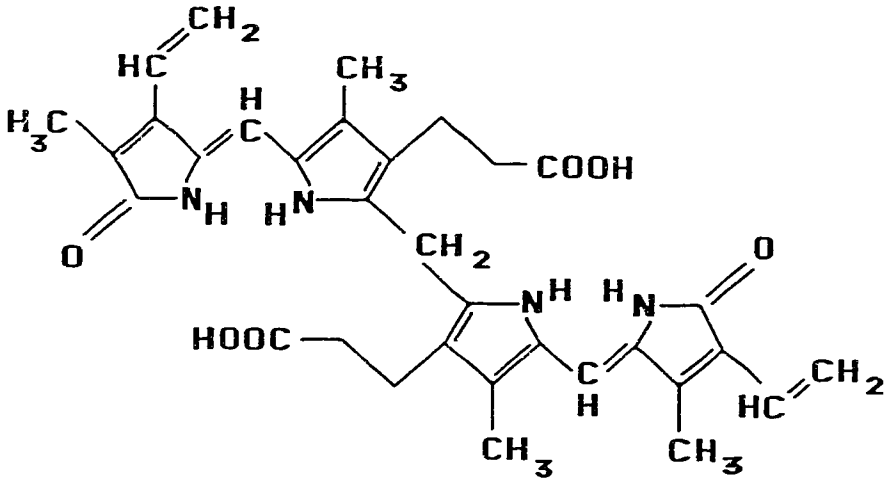
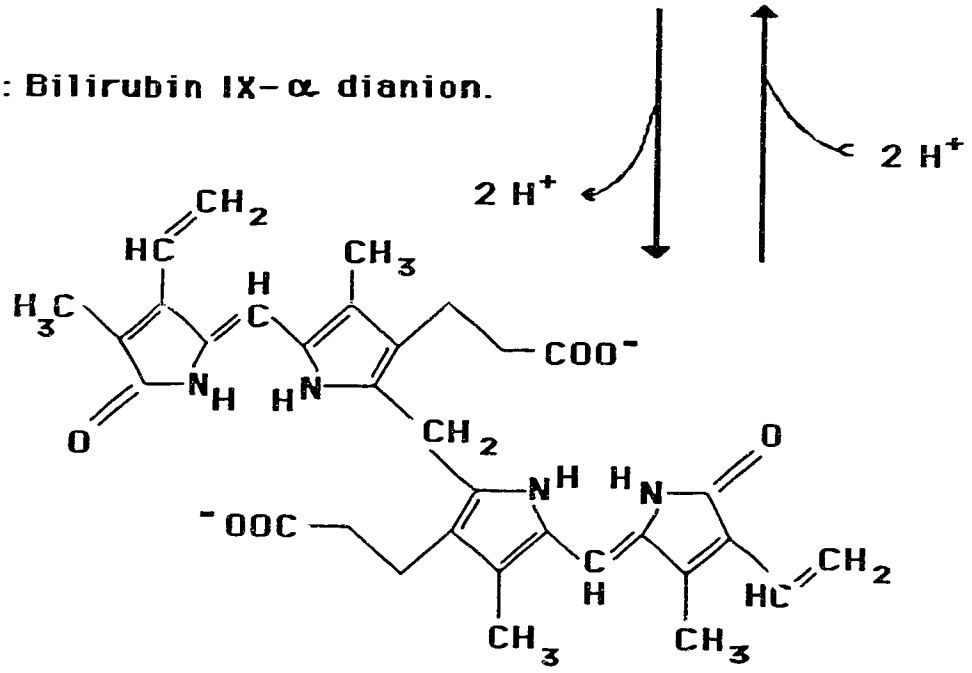


Plate 5: Bilirubin IX-α dianion.



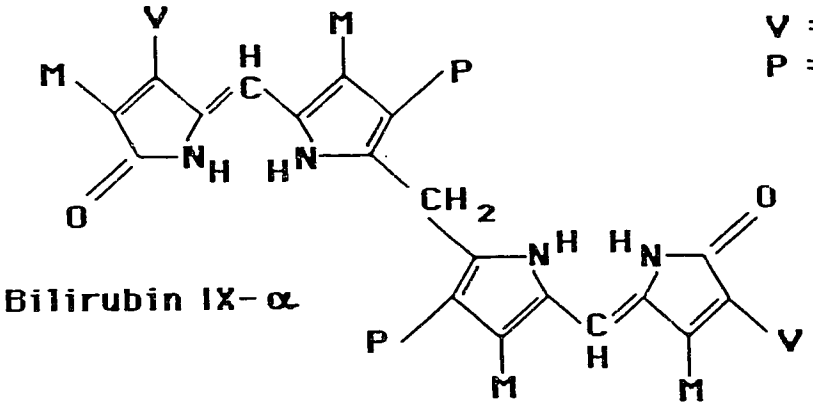
bright yellow when observed by the naked eye. The presence of strong light scattering indicates that a colloid suspension, and not a genuine bilirubin solution, is present [31,32,33,34].

When colloid formation is expected but fails to take place, the bilirubin solution is said to be in a supersaturated state. Under acidic pH conditions, supersaturation with extensive aggregation and precipitation of the insoluble protonated acid will occur [33]. Phospholipids accelerate the aggregation of bilirubin at acidic pH conditions as well as co-precipitation of bilirubin and phospholipids [34]. When a neutralized supersaturated solution of bilirubin is mixed with a suspension of erythrocyte membranes or mitochondria, and with liposomes or phospholipids ***in vitro*** , a process of binding and aggregation of bilirubin starts immediately and proceeds rapidly [33]. The end result is similar to colloid aggregates with the aggregates remaining attached to the membranes [34]. Since the same cellular structures are present in intact cells, it is reasonable to assume that the same process will occur ***in vivo.***

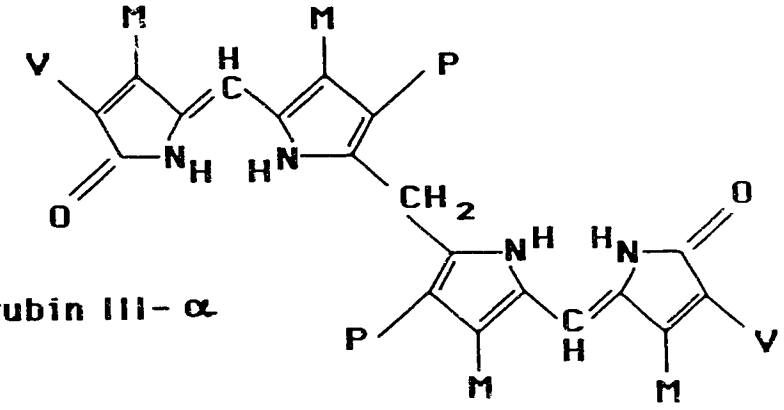
Besides its insolubility and tendency to aggregate and form colloids, bilirubin is unstable in solution and tends to auto-oxidize and decompose. Hydrogen-bonded bilirubin, dissolved in oxygenated alkaline aqueous solution, is unstable and may undergo rearrangement and auto-oxidation [35]. Furthermore, over a pH range from 7.4 to 12 [36] or in the presence of acid [37], bilirubin IX-α is cleaved at the central methylene bridge with subsequent rearrangement of the separate units to give a mixture of bilirubin III-α, and bilirubin XIII-α in addition to the natural IX-α isomer (Plate 6) [36,37].

Plate 6: Bilirubin isomers.

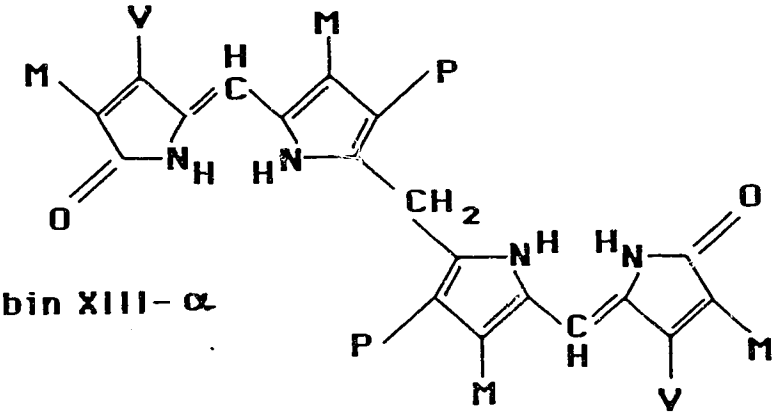
M = CH_3
V = $CH{=}CH$
P = CH_2CH_2COOH



Bilirubin IX-α



Bilirubin III-α



Bilirubin XIII-α

4. Bilirubin Binding to Albumin

In order to prevent bilirubin precipitation and toxicity, it is necessary to bind the pigment to a carrier. Serum albumin serves as a universal carrier, reversibly binding a large number of substances including bilirubin [32,38,39]. The importance of the interaction between bilirubin and albumin was demonstrated by several investigators. Bowen et al, demonstrated the protective role of albumin against unconjugated bilirubin injected into puppies [40]. Mustafa et al, found that one mole of albumin binds one mole of bilirubin and detoxifies it [41]. Odell [42] and Silverman et al [43] described increased bilirubin toxicity as a result of a dissociation of the pigment from its albumin binding site, caused by the use of different drugs.

In the blood, unconjugated bilirubin dianion is bound to a high affinity binding site on albumin with smaller amounts located at one or two lower affinity sites [44]. The binding process is fast, occurring in a matter of milliseconds [45], and is pH-independent within a pH range between 7 and 9.

Nevertheless, the distribution of bilirubin, ***in vivo***, between serum albumin and tissues is highly sensitive to pH changes, where acidosis favors a shift of the pigment from albumin to fat [46]. The shift is readily explained by a change in the solubility of the unbound pigment, formation of aggregates with lipoid membranes and lipids, and a shift in the bilirubin-albumin binding equilibrium [46]. Formation of the bilirubin-albumin complex is

reversible and is associated with protection of the pigment from degradation by various processes - e.g. photochemical degradation, isomerization, auto-oxidation and enzymatic reduction [47,48,49].

The same type of binding takes place ***in vitro*** , when a solution of albumin, at a slightly alkaline pH condition, is mixed with a solution of bilirubin dissolved in sodium hydroxide. Another type of binding occurs if a solution of albumin, ***in vitro*** , is mixed with a molar excess of bilirubin at pH 7.4 or below. Under such conditions a slow process of association takes place whereby large aggregates, consisting of large numbers of albumin and bilirubin acid molecules, are formed. This process results in co-crystallization of albumin and bilirubin with little bilirubin left in the solution [50]. Binding of bilirubin acid to albumin is pH-dependent since a high number of hydrogen ions are involved. Increasing the pH conditions of the solution towards an alkaline pH disintegrates most of the aggregates, and an equilibrium of binding of the anion is re-established [32,50].

5. Bilirubin Binding to Other Proteins

Although the only plasma protein with strong affinity for bilirubin is albumin, bilirubin can also bind to other blood components [51]. Binding to non-albumin proteins in the serum is important only when bilirubin concentrations exceed those of albumin and when the available primary binding sites on albumin are saturated. Bilirubin can bind to proteins such as serum β-lipoproteins and α-globulin, but when bilirubin is present in

the serum, the pigment distribution is always in favor of albumin [51,52,53,54].

Of physiological importance is bilirubin binding to proteins located in the cytosol, especially in hepatocytes. These proteins probably function as carriers for bilirubin within the cell and facilitate the uptake of the pigment [13]. Whether the binding is necessary for protection of the cell content against the pigment is unknown .

Other cellular and tissue components such as erythrocyte membrane [55,56,57], pulmonary hyaline membrane [58,59], mitochondria from heart and brain cells [60,61], glycolipids [62] , lipids and phospholipids [61,63,64] have been shown to bind bilirubin. Binding of the pigment to non-albumin proteins and other cellular components is of lesser importance and negligible when albumin exceeds bilirubin molar concentrations.

6. Bilirubin Interaction With Lipids .

The interaction of bilirubin with lipids has been studied by several investigators. Mustafa and King [61] suggested that bilirubin, in supersaturated solutions, is capable of binding to a variety of native membrane lipids as inferred by spectral changes. The changes observed were rapidly reversed by washing the liposomes with albumin, suggesting a loose binding of bilirubin to lipids. Weil and Menkes [62] have demonstrated that bilirubin interacts with gangliosides ***in vitro*** . In another study [63] bilirubin, at physiologic pH conditions, quenched a fluorescent

probe located within the lipid bilayer of membranes. Talafant [64] has found different binding qualities between the pigment and different phospholipids.

Of major importance is the knowledge of the interaction between bilirubin and the lipid bilayer. Eriksen et al [34] and Cestaro et al [65] demonstrated that bilirubin may be incorporated within the hydrophobic hydrocarbon domains of the bilayers, but migrate to the surface as equilibrium is achieved. On the other hand, Tipping et al [66] and Hayward et al [67], in a more recent study, were able to demonstrate that bilirubin is capable of passive diffusion across the lipid bilayer into liposomes. However, since no complete extraction of bilirubin from the liposomes was demonstrated, an interaction between the lipid bilayer and the pigment cannot be excluded.

The properties of bilirubin with regard to its effect on monolayers were demonstrated in two studies . In 1939 Stenhagen and Rideal [68] explored the interaction between bilirubin and various lipids and proteins. The results obtained suggest an interaction of the carboxyl groups of the pigment with the primary amide groups of lipids and proteins. Another series of monolayer experiments was carried out by Notter et al [69], exploring the effect of bilirubin on dynamic surface tension forces. Under acidic pH conditions, bilirubin-acid intercalates with the phospholipid acyl-fatty acid chains. At higher pH values, the more soluble bilirubin interacts with water away from the hydrophobic core of the lipid bilayer. Overall, it was shown that under acidic pH

conditions bilirubin is a highly surface-active material at the interface, and is capable of influencing the spreading behavior of membrane lipids [69].

7. Bilirubin Interaction With Membranes

The interaction of bilirubin with the central nervous system should consist of three steps: a) the entry of bilirubin into the brain from blood, b) the binding of bilirubin to the neural cell surface with or without subsequent internalization, and c) the interaction of bilirubin with plasma membrane, leading to alteration of membrane properties, or with intracellular targets.

Studying the interaction of bilirubin with synaptosomal plasma membrane, Vazquez et al [70] proposed a three step model for the interaction : 1) a rapid initial complex formation between anionic forms of bilirubin and the polar lipid head groups on the membrane surface, 2) a slow inclusion of bilirubin into the hydrophobic core of the lipid bilayers, and 3) the formation of bilirubin acid aggregates, by the remaining bilirubin molecules, on the surface of the plasma membrane [70]. While Vazquez demonstrated a multi-step interaction between bilirubin and the synaptosomal membrane, Leonard et al [71] suggested a different model for interaction. According to their results the interaction of bilirubin with model or biological membranes depends on the sizes of the free volumes, located within the membrane. These pools of free volumes varied according to the lipid composition and the presence or absence of proteins in the membranes. Bilirubin

appears to interact with neither the polar nor the apolar regions of the membrane but to partition with the free spaces in the apolar region of the lipid bilayer [71].

8. Bilirubin Toxicity

Despite the extensive knowledge of the chemical and biochemical properties of bilirubin, the question whether bilirubin is poisonous or only potentially toxic to the living organism has not been completely elucidated. While adults produce up to 250 mg of bilirubin daily without any harm and large doses have been injected intravenously into adults [72] and newborn babies [73] with no apparent ill effects, hyperbilirubinemia in newborn infants [44] and newborn rats [74] may cause bilirubin encephalopathy. Furthermore, studies in experimental animals indicate that unconjugated hyperbilirubinemia impairs liver mitochondrial function [41]. In contrast, no toxic hepatic effects have been seen in humans or Gunn rats suffering from prolonged unconjugated hyperbilirubinemia due to hepatic glucuronyltransferase deficiency [75,76].

A variety of pathologic conditions may result in severe or prolonged jaundice characterized by increased serum concentration of unconjugated bilirubin [77]. In several studies, bilirubin has been shown to be poisonous to neural and non-neural cells and tissues both ***in vitro*** and ***in vivo*** [78,79,80]. Bilirubin toxicity usually manifests as central nervous system damage which occurs almost exclusively during the early neonatal period [77]. Passage of

unconjugated bilirubin from the intravascular space - across the blood brain barrier - into the brain is thought to be the cause of kernicterus and bilirubin encephalopathy (see below).

9. Kernicterus and Bilirubin Encephalopathy

In 1903 Schmorl [81] coined the term kernicterus to describe the characteristic yellow staining of subcortical nuclei of the brain, that was commonly observed in jaundiced infants who died from severe erythroblastosis fetalis. The term was selected specifically to differentiate it from a more diffuse yellow staining of periventricular tissues and hemisphere surfaces, a condition considered secondary to passive diffusion of bilirubin following tissue necrosis [83]. Kernicterus, originally used as a pathologic term, is now associated with a particular clinical picture which varies from subtle neurologic changes such as high tone deafness to more extreme forms of severe choreoathetosis, mental retardation and, in some cases, to immediate death of the infant [82,83,84,85,86]. Moreover, in infants who survive the acute stages of hyperbilirubinemia but subsequently die, the staining may no longer be present, yet the basal ganglia display microscopic evidence of cell injury, neuronal loss and glial replacement [87,88,89]. Bilirubin encephalopathy is a more appropriate term to describe the clinical picture associated with the diffuse staining of the brain, the neuronal damage and the neurological picture associated with hyperbilirubinemia.

It is generally accepted that unconjugated bilirubin deposited in the brain is responsible for the yellow staining and the neurologic dysfunction characterizing bilirubin encephalopathy. To be toxic to the nervous system, unconjugated bilirubin has to cross the blood-brain barrier and specifically interact with vulnerable neural cells. The blood-brain barrier is a complex structure consisting of tight junctions cementing brain capillary endothelial cells plus adjoining foot processes of astroglial cells. Soon after contact with the astrocytes, continuous tight junctions seal the endothelial cells together and polar molecules no longer readily enter the brain by simple diffusion. Essential molecules such as glucose, organic acids and amino acids, therefore, require specific transporters to mediate their passage into the brain. Functionally, the blood-brain barrier comprises a series of carriers and transport mechanisms for various substances [90]. Permeation of the blood-brain barrier may result from changes in the anatomy and/or the function of its constituents.

The blood-brain barrier of the neonate is immature and thus may be more permeable [91]. Whether immaturity and increased permeability are responsible for the passage of free bilirubin into the neonatal brain is not clear. Many different factors, besides immaturity of the blood-brain barrier, account for the development of kernicterus. Among them are relative hypoalbuminemia, hypoxia, acidosis, hyperosmolarity, hypothermia, sepsis and drugs competing for bilirubin binding sites on albumin [92,93,94]. Endothelial cells of brain capillaries, as other cells, are

susceptible to injury by toxins and other abnormal metabolic conditions. Most evidence supports a passage of free bilirubin across the blood-brain barrier but a transfer of the albumin-bilirubin complex has not been excluded [95]. In normal infants, the restrictive nature of the blood-brain barrier is very well preserved despite immaturity of the endothelial cells composing the barrier. In infants with an intact blood-brain barrier, bilirubin will leave the blood to enter the brain only when the pigment is uncoupled from albumin and other plasma proteins. However, if brain endothelial cells are damaged, the altered barrier will then permit bilirubin, uncoupled from or complexed with albumin, to enter and damage the brain cells [95].

Another factor, the selective affinity of bilirubin for specific brain sites, complicates the picture of bilirubin encephalopathy. The vulnerability of specific brain areas to bilirubin toxic effects may be patterned by the blood flow to the brain [96,97,98] or affected by the different bilirubin binding affinities to various brain phospholipids [62,63,71].

Brodersen has suggested the possible existence of a bilirubin oxidase enzyme within the neural cells, which might play a role in protecting the cells by oxidizing the unbound bilirubin [99]. The presence of such an enzyme remains speculative.

Thus, protection of the newborn's brain from bilirubin may be attributed to a number of factors :

1) The interaction of bilirubin with albumin and /or different phospholipids [37,38,62,63,100].
2) The integrity of the blood brain barrier and of the brain cell membrane [95,98,101,102,103,].
3) The possible presence of a bilirubin oxidase enzyme [99].

The classical form of bilirubin encephalopathy , which was generally observed in term infants with hemolytic diseases, is virtually unknown today. This is a result of an improved and aggressive therapy directed at controlling hyperbilirubinemia with phototherapy, exchange transfusion, and prenatal management of the mother and fetus [104,105].

Unfortunately, kernicterus is still being observed at autopsies [105]. Small premature babies are the population at greatest risk for the development of bilirubin encephalopathy. In these infants, kernicterus has been found at bilirubin levels that are considered to be within the normal and "safe" range for the mature newborn [106,107,108]. Several potentiating factors that affect albumin binding of bilirubin or enhance tissue uptake of bilirubin have been suggested. Among these are low birth weight, hypothermia, asphyxia, acidosis, hypoalbuminemia, sepsis, meningitis and the use of drugs that displace bilirubin from its albumin binding sites [109,110]. To date, there .s no proof for a direct relationship between the potentiating factors and the presence or absence of kernicterus and bilirubin encephalopathy [110,111,112]. The question as to what is affecting the newborn infant, still remains open. Is hyperbilirubinemia <u>per se</u> toxic, or is hyperbilirubinemia

an associated factor with the compounding effect of the other risk factors [113] ? Despite the uncertainty, measures have been taken to reduce the risk of bilirubin encephalopathy by adjusting the critical bilirubin concentrations to birth weight, gestational age and clinical situations at which medical intervention is indicated [114].

10. Studies on Bilirubin Toxicity

That bilirubin might be toxic to neural cells stems from the clinical association between the neurological picture and hyperbilirubinemia. However, despite a fairly detailed understanding of the chemistry and biochemistry of bilirubin there have been very few studies designed to define the interaction between bilirubin and the central nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions [78]. Specific kinetic studies carried out in hepatocytes [115,116,117,118,119,120] and human erythrocytes [121], have suggested the existence of saturable bilirubin binding sites. In other studies, the effects of pH and albumin on bilirubin binding to endothelial cells [122], fibroblasts [48,123], and isolated mitochondria [124] have been demonstrated. Our understanding of the interaction between bilirubin and neural cells is based on studies in which either the brain was exposed to bilirubin through opening of the blood brain barrier [95,98], or brain slices were exposed directly to bilirubin [125]. Both approaches present a relatively crude assessment of this interaction. To have a clear understanding of the mechanism of

bilirubin toxicity to the neural cell, knowledge of the interaction between bilirubin and the cell is critical.

Results of several studies indicate that bilirubin interferes with various cell functions [78,79,80]. Bilirubin toxicity to non-neural cells has been investigated extensively over the past years in fibroblasts [126,127,128,129], hepatocytes [130,131], erythrocytes [132,133,134,135], leukocytes [136,137], platelets [138] and Ehrlich ascites cells [139,140]. Toxic manifestations of bilirubin were demonstrated by non specific effects on cell viability and growth [126,127,128,129], cell morphology [135], and cell behavior [137,138]. More specific effects were observed when ATP synthesis [127] and membrane enzymes [133,134,139,140] were investigated.

Studies conducted on neural tissue demonstrated that bilirubin may impair a large number of cell functions such as changes in energy metabolism [41,141,142], alteration in the physical structure and function of cell membranes [61,62,63,64,65], changes in key intracellular enzymes [143,144,145,146,147], inhibition of both DNA [148,149] and protein synthesis [150,151,152,153], changes in carbohydrate metabolism [154,155] and modulation of neurotransmitter synthesis [156] and release [157]. Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, Gunn rats which suffer from hereditary unconjugated hyperbilirubinemia, served as a model [145,148, 150,151,152,153,155]. In the other, brain cells from normally developed animals were used

[144,146,147,154,155,157]. There is a major difference between the two. The use of the Gunn rat as a model for bilirubin encephalopathy is based on the assumption that the damage seen is primarily due to bilirubin. Although extensive damage to the nervous system in the Gunn rat can be attributed to bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded [158,159].

Bilirubin toxicity of the central nervous system is thought to occur in two stages : 1) an early reversible stage, sometimes referred to as subclinical and transient bilirubin-induced neurotoxicity, and 2) a later stage initiated when the sequelae become irreversible [80,160,]. Clinical studies in hyperbilirubinemic neonates have shown reversibility of the acute toxic bilirubin-induced changes in auditory nerve and brainstem responses [161,162,163]. Cowger demonstrated that bilirubin toxicity in an L-929 cell line was reversible with the addition of albumin [127]. Recently, Hansen et al demonstrated a similar phenomenon in hippocampal slices [157], and Wennberg provided evidence for the reversibility of bilirubin toxicity and mitochondrial uptake of bilirubin in erythrocytes [164]. On the other hand, working in a cell free system, Sano et al demonstrated that bilirubin inhibition of protein kinase C activity is irreversible [147].

A major concern when experimenting with a bilirubin-to-albumin molar ratio that exceeds one, is the instability of bilirubin

leading to the formation of bilirubin aggregates and co-aggregates of bilirubin and albumin [33,37,38,165]. Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been fully addressed in experiments dealing with bilirubin toxicity ***in vitro***. The frequent use of non-physiological bilirubin concentrations in ***in vitro*** studies, the addition of varying albumin concentrations with alteration of bilirubin-to-albumin molar ratios, and variations in the cells investigated, are among the major reasons for inconclusive results.

To date, few studies have been carried out in cultured neural cells. The question as to whether bilirubin is indeed toxic to the brain cell or whether the yellow staining of the brain is a coincidental finding has been raised. Schiff et al [149], reported recently that bilirubin toxicity in N-115, a murine neuroblastoma cell line, was dependent on bilirubin concentration, bilirubin to albumin molar ratio and time of exposure to bilirubin.

The present work will define the specific ***in vitro*** conditions under which bilirubin, when added to cells in media, is stable and remains so during the entire experiment. Working under these conditions and using N-115, a murine neuroblastoma cell line in culture, the present studies will attempt to characterize the following:

1) The interaction between bilirubin and the cell.
2) The target and the mechanism of bilirubin toxicity at the cellular level.

3) The possible reversibility of the toxic effects.

4) The delayed bilirubin effects after short-term bilirubin exposure during which no evidence of toxicity is manifested.

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CHAPTER 2

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Bilirubin - Neural Cell Interaction: Characterization of Initial Cell Surface Binding Leading to Toxicity in the Neuroblastoma Cell Line N-115.

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Introduction

Hyperbilirubinemia and bilirubin encephalopathy are well known occurrences in the newborn period [1,2]. It has been suggested that the protection of the newborn's brain to bilirubin toxicity may be due to a number of different factors. These include: a) the interaction of bilirubin with albumin and/or different phospholipids [3-9] and b) the integrity of the blood-brain-barrier and the brain cell membrane [10-14]. The fact that bilirubin might be toxic to neural cells stems from the clinical association of the neurologic picture that has emerged and the associated hyperbilirubinemia [15].

In spite of a fairly detailed understanding of the chemistry and biochemistry of bilirubin, there have been very few studies designed to define the interaction of bilirubin with the nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions. Specific binding and kinetic studies carried out on hepatocytes [16-21] and human erythrocytes [22] have suggested the existence of saturable bilirubin binding sites. Other studies have demonstrated the effect of pH and albumin on the binding of bilirubin to L-929 cells [4], endothelial cells [23], fibroblasts [24] and isolated mitochondria [25].

The interaction of bilirubin with the central nervous system should consist of three steps, (i) the entry of bilirubin into the brain from the blood, (ii) the binding of bilirubin to neural cell surface with or without a subsequent internalization, and (iii) the interaction of bilirubin with intracellular targets (in the case of

internalization) or the alteration of plasma membrane properties leading to the toxic effect. There are two different views as to the mechanism of bilirubin entry into the brain. Some studies suggest that though bilirubin exists as a complex with albumin in the blood, only free bilirubin crosses the blood-brain-barrier (free bilirubin hypothesis) whereas other studies suggest that under certain conditions such as hyperosmolality, the blood-brain-barrier will be opened and bilirubin enters the brain as a bilirubin-albumin complex [10,11]. Once bilirubin enters the brain, the toxic effects will be determined by the interaction of bilirubin with the individual neurons.

Different approaches have been made to study the interaction of bilirubin with neural cells. There are studies that exposed either the whole brain [10,11] or brain slices to bilirubin [26]. These studies give a relatively crude assessment of the interaction because the exposure as well as the washing after the exposure will not be complete in a tissue and data are expressed in terms of total bilirubin uptake per gram of brain tissue. Another approach has been to characterize the interaction using membrane fractions and lipids of nervous tissue including components like sphingomyelin and gangliosides [9,27,28]. But these systems are far removed from the actual physiological situation with respect to the target as well as the form of bilirubin solution used. These studies employ supersaturated solutions of bilirubin, whereas in plasma, bilirubin is believed to be present predominantly as a complex with albumin. A better approach to the problem is to use a neural cell line under

the normal conditions of tissue culture in the presence of albumin as a model system. Such studies are almost lacking in the literature.

The present study examines the nature of the interaction of bilirubin with the murine neuroblastoma cell line N-115. The cells were exposed to bilirubin at different concentrations and different bilirubin to albumin molar ratios (B/A). The cellular uptake of bilirubin was characterized in terms of the kinetics, apparent equilibration (limiting values) and the effect of pH and temperature on the equlibration. The results indicate that the "free" form of bilirubin is the reactive species, and it interacts with the plasma membrane through a multistep binding process.

Materials and Methods

Materials. All reagents were of analytical grade and were purchased from Sigma Chemical Co. (USA). Bilirubin purity was verified by high performance liquid chromatography (HPLC), as indicated below, and was found to contain 92% IX-α isomer, 4.8% XIII-α isomer, and 2.8% III-α isomer. No other bile pigments were detected. Since all measurements of bilirubin extracted from cells were performed on HPLC, no further purification was carried out. [^{3}H]-bilirubin was prepared by ***in vivo*** labelling in rats using δ-amino [3,5(N)-^{3}H] levulinic acid (New England Nuclear) as the precursor [8]. [^{3}H]-bilirubin was purified from the bile as described by McDonagh [3], and was found to contain more than 98% bilirubin IX-α by HPLC (absorption at 454 nm), with specific activity of 1710 CPM/nmole bilirubin.

HSA (fraction V, Essentially Fatty Acid Free) obtained from Sigma Chemical Co. (St. Louis, MO), Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline (PBS) and fetal calf serum (FCS) were obtained from GIBCO (Canada). Solvents used were of HPLC grade (JT Baker Chemical Co.).

Bilirubin treatment of cells. The murine neuroblastoma cell line N-115 was seeded at a density of 3 x 10^6 cells/plate in 10 cm culture dishes (Falcon) and grown in standard DMEM plus 10% FCS, pH 7.4 at 37°C in a 5% CO_2 humidified atmosphere for 10-12 hours. The media was then removed, the cells washed twice with sterile PBS, and reincubated in 10 mL of protein-free media [29] containing human serum albumin plus 25 mM N-2-hydroxy-ethylpiperazine-N-2 ethanesulfonic acid (HEPES) to maintain a pH of 7.4 for another 12 hours, before the experiments with bilirubin were started. The albumin concentration was varied in different experiments to meet the required final B/A ratios. Three or four culture dishes were used in each experimental condition. These dishes were seeded with cells as above, with bilirubin being added to two or three of them. The remaining dish contained experimental media plus bilirubin, but no cells - a measure of non-specific binding of bilirubin to the plate.

A stock solution of bilirubin was made by dissolving 2 mg bilirubin in 1 mL of N_2-purged 0.1 N NaOH. Bilirubin was added to the culture media to achieve the appropriate experimental conditions, followed immediately by the addition of an amount of 0.1 N HCl equivalent to the amount of NaOH added to restore the pH of the

culture media to 7.4. Under the experimental conditions bilirubin-albumin mixtures were found to be stable when measured spectrophotometrically for a minimum of three hours and is reported elsewhere [30].

All procedures involving addition, incubation and extraction of bilirubin were carried out in a dimly lit room to avoid bilirubin photodegradation.

Measurement of bilirubin uptake by cells. At the end of the incubation period, the media was removed and saved for pH measurement. The cells were washed four times with ice cold PBS and then dislodged from the plate with a rubber policeman in 1.5 mL PBS and transferred into an Eppendorf Test tube. The cell suspension was then vortexed and 0.1 mL aliquots were taken for DNA analysis [31] and cell viability as measured by the nigrosin exclusion technique [32]. The remainder was spun down in a microfuge (Eppendorf) for 5 minutes and the supernatant removed. Bilirubin was extracted from the pellet by adding 0.9 mL of methanol:chloroform (1:2, v/v) followed by sonication for 10 minutes, and centrifugation for 10 minutes in an Eppendorf microfuge. The supernatant was dried under N_2 and kept at -20°C until HPLC analysis was performed [33].

Bilirubin extracts from the cells were analyzed by reverse-phase HPLC (Beckman Altex Ultrasphere IP, 5 µm, C-18, 25 x 0.46 cm column with Beckman Altex IP precolumn 4.5 x 0.46 cm) using 0.1 M di-n-octylamine acetate in methanol, pH 7.7, as eluant with a flow

rate of 1.0 mL/min - and the detector set at 454 nm [33]. Quantitation of peak areas was performed on a Gilson Data Master reporting integrator, using preweighed bilirubin (Sigma, Lot No. 25F-0584) as external standard.

The experiments with [^{3}H] bilirubin were also performed as described above except that instead of extracting cell-bound bilirubin with organic solvents and analysis by HPLC, the cells were suspended in 0.1 mL of 0.2 N NaOH and neutralized with 0.1 mL of 0.2 N HCl. The radioactivity was measured by liquid scintillation counting after adding 15 mL of aqueous counting scintillant (Amersham).

Results

In this study, uptake is defined as the total amount of bilirubin associated with the cells including both surface bound and internalized bilirubin. The results are the mean of the net uptake (total minus non-specific) of the two or three experimental dishes. The non-specific uptake was always less than 1.7% of the total uptake. If not mentioned otherwise, the bilirubin concentration refers to the total (input) concentration. The term "free bilirubin" is used to denote the bilirubin remaining after saturating the high affinity primary binding sites of albumin and as such include both "free bilirubin" in solution and the bilirubin loosely bound to albumin. The bilirubin-albumin solution were found to be stable for the time periods used in this study [31] and the isomeric composition of the bilirubin extracted from the cells was found to

be the same as the input bilirubin within error limits (1-2% increase in photoisomers).

Fig. 1 gives the time course of bilirubin uptake by N-115 cells when the cells are incubated with 100 μM bilirubin at different B/A ratios. The bilirubin uptake at a B/A ratio of 3 in 10 min is 80 pmole/μg DNA and plateaus at 125 pmole/μg DNA in 40 min. , whereas at a B/A ratio of 0.8 the rate is much slower and levels achieved are much less, < 5 pmole/μg DNA in 90 minutes. Thus, there is a sharp increase in the initial rate as well as the extent of uptake with increasing B/A ratio even though the input (total) concentration of bilirubin is held constant. The results support the idea that the "free" rather than the albumin-bound form of bilirubin is responsible for toxicity. Since the stoichiometry of albumin-bilirubin is 1:1 the concentration of "free bilirubin" will increase drastically as the B/A ratio increases from 0.8 to 3.0.

The effect of varying the bilirubin concentration on the initial rate of uptake of bilirubin by the neuroblastoma cell is given in Fig. 2. At a B/A ratio of 3, increasing the bilirubin concentration from 12.5 μM to 100 μM shows no evidence of saturation. A similar result was obtained at a B/A ratio of 1.5 with concentrations ranging up to 250 μM bilirubin. The apparent absence of saturation kinetics in either case likely rules out the possibility of carrier-mediated transport across the plasma membrane implicated in the uptake of bilirubin by hepatocytes [17-22]. The concentration of "free bilirubin" can also be varied by varying the B/A ratio at a constant total bilirubin concentration. The initial uptakes under

these conditions are plotted in two different forms in Fig. 3. As expected, the initial rate decreases rapidly with increasing albumin concentration almost linearly (probably) up to B/A = 2 and then very slowly (Fig. 3A). The same data plotted as a function of "free bilirubin" concentration calculated from the bilirubin-albumin stability constant of $3.2 \times 10^7 M^{-1}$ [4] is given in Fig. 3B, and shows no saturation up to 80 μM of "free bilirubin". (The curve suggests the possibility of saturation at higher bilirubin concentrations and a possible explanation for this is that at high B/A ratios the free bilirubin concentration is so high that it might form small aggregates, the reactivity of which might be less than that of the monomeric form. The results in Figs. 2 and 3 along with the known binding of bilirubin to lipids such as sphingomyelin and gangliosides with the affinity in the range of 10^5 - 10^6 M^{-1} [9,27,28] argue against the notion of a bilirubin carrier in N-115 cells.

The apparent equilibrium uptake (limiting values in Fig. 1) as a function of bilirubin concentration at B/A ratios of 1.5 and 3 are shown in Fig. 4. The curves are neither linear, expected for passive diffusion, nor hyperbolic, expected for a normal receptor-ligand system. The curves are parabolic (or rather part of a sigmoidal curve) suggestive of cooperative binding of bilirubin to the cells (At B/A = 1.5, a reasonable linear fitting can be done as shown by the solid line. However, there is considerable deviation from a linear extrapolation of early points as shown by the dotted lines). The uptake of [^{3}H] bilirubin by N-115 cells given in Table 1, also agree

with the non linear behavior seen in Fig. 4 effectively rulling out a diffusion mechanism .

To further characterize the binding we tested reversibility of binding by trying to extract cell-bound bilirubin with fresh albumin. Extraction was performed after incubating the cells with bilirubin for different time intervals and the results are given in Table 2. The uptake is partially reversible and the fraction reversible (extracted) decreases with an increasing period of incubation of cells with bilirubin. This indicates that the binding cannot be described by a simple receptor ligand system. The effect of temperature on bilirubin binding is given in Table 3. The temperature insensitivity of bilirubin uptake at B/A ratio of 1.5 suggests a specific binding to the cell because non-specific binding is expected to increase with increasing temperature due to increased concentration of "free bilirubin" in equilibrium with albumin at higher temperatures [3]. The difference in behavior at B/A ratios 1.5 and 3 could be a reflection of a complex binding process.

The effect of pH on bilirubin uptake by N-115 cells at a B/A ratio of 1.5 is given in Fig. 5. The uptake increases rapidly with decreasing pH - almost a 10 fold increase in uptake as the pH of the medium is lowered by 1 unit from pH 8.0 to pH 7.0. Changes in pH are reported to affect bilirubin deposition in the brain, erythrocytes and mitochondria [13,14,16,26,34]. One of the factors likely to contribute to this pH effect is the increased concentration of "free bilirubin" resulting from the decreased affinity of bilirubin for albumin with decreasing pH. Decreasing the pH from 7.4 to 7 leads to

a 4 fold increase in bilirubin uptake by N-115 cells whereas the expected change in "free bilirubin" concentration is negligible (16.729 μM at pH 7.4 and 16.738 μM at pH 7 calculated from binding constants of 3.2 x 10^7 M^{-1} at pH 7.4 and 2.8 x 10^7 M^{-1} at pH 7 [4]) suggesting that factors other than "free bilirubin" concentration may be responsible.

Discussion

The mechanism of bilirubin toxicity to the nervous system has been the subject of numerous investigations over the last few decades, yet the area is dominated by speculation rather than concrete ideas. This is mainly due to the peculiar properties of the bilirubin molecule. The molecule is neither hydrophilic nor hydrophobic, as indicated by its very poor solubility in aqueous media at neutral pH and poor to moderate solubility in organic solvents [6]. This has given rise to considerable limitation in experimentation as well as the interpretation of experimental data. It has also led to the use of a variety of model systems consisting of bilirubin solutions of varying kinds from supersaturated solutions at alkaline pH to bilirubin-albumin mixtures of different ratios and a range of targets from pure lipids and proteins to the whole brain. Though these studies have provided valuable information on different aspects of bilirubin action, a complete picture is still lacking. An important piece of information missing is the nature of bilirubin interaction with the plasma membrane. Studies with purified proteins and subcellular fractions have shown that bilirubin at micromolar concentrations can affect the activity of many

enzymes of cytosolic, mitochondrial and microsomal origin [2]. The relevance of these findings in relation to bilirubin toxicity ***in vivo*** requires an understanding of whether bilirubin can cross the plasma membrane and if so, what intracellular concentrations can be achieved under clinically relevant conditions. An integrated approach consisting of the quantification of bilirubin uptake and the measurement of consequent changes in some biochemical parameters of toxicity in the same system is desirable. Using a neural cell line we have shown recently that bilirubin affects mitochondrial function, protein synthesis and DNA synthesis in intact N-115 cells and the toxicity is determined by the concentration of bilirubin, B/A ratio and the period of exposure [35,36]. The complementary studies on the cellular uptake of bilirubin are presented here. In the clinical situation it is assumed that a B/A of less than one is safe, as the majority of bilirubin is bound to the primary "tight" binding site of the albumin molecule. In order to assess bilirubin interaction with the cell, we have used a B/A greater than 1 which would make available free bilirubin and/or loosely bound bilirubin [36].

The results in Figs. 1-4 clearly indicate that the uptake of bilirubin by N-115 cells increases with increasing period of exposure, B/A ratio and bilirubin concentration at a given B/A ratio consistent with our earlier results on the measurements of toxicity parameters under the same experimental conditions [35,36]. While this suggests that bilirubin enters the cell, the data presented here are not consistent with a simple transport mechanism. The data can be explained in terms of a multistep binding with the plasma

membrane similar to that proposed for the interaction of bilirubin with rat brain synaptosomal plasma membrane vesicles [28]. According to this model the interaction occurs in three steps: (i) bilirubin binding to the polar head group region of the membrane, (ii) insertion of the surface-bound bilirubin into the hydrophobic core of the membrane, and (iii) membrane induced aggregation of bound bilirubin on the surface of the membrane.

The unusual rate curve for bilirubin uptake at B/A = 1.5 (Fig. 1) could be a reflection of the multistep binding process. The effect is seen at bilirubin concentrations of 50 and 100 μM. Similar rate curves have been reported for the interaction of bilirubin with synaptosomal plasma membrane vesicles and liposomes made of lipids and proteins extracted from these vesicles [28]. The very low concentration of free bilirubin at B/A = 0.8 and a much faster uptake due to a high concentration of "free bilirubin" at B/A = 3 might explain the apparent normal behavior under these conditions. A multistep binding mechanism is also supported by the concentration-dependence of limiting uptake given in Fig. 4 and Table 1. The parabolic or probably sigmoidal curve is indicative of a cooperative process reflecting the aggregation of bilirubin on the membrane at high concentrations. The partial reversibility of bilirubin uptake, as assessed by the extraction with albumin (Table 2) also favors a multistep mechanisms. The bilirubin displaced from N-115 cells by albumin mainly represents the bilirubin bound to the cell surface (polar head groups), the initial step, because the fraction reversed decreases with increasing period of exposure. The

remaining non-extractable portion need not be irreversible in the thermodynamic sense because the dissociation of bilirubin aggregates and the desorption of bilirubin from the hydrophobic core of the membrane could be very slow processes as in the case of some lipids. The half-life for the desorption of membrane components such as phospholipids and glycolipids is in the order of days [37,38]. The difference in the effects of temperature on uptake at B/A ratios of 1.5 and 3 (Table 3) could be a further reflection of a multistep mechanism. At 50 μM bilirubin and B/A = 1.5, the "free bilirubin" concentration will be low so that the cell-bilirubin interaction is likely to be dominated by the initial step(s) whereas at 100 μM bilirubin and B/A = 3 the aggregation step is likely to be dominant. The step(s) following the initial binding is entropy driven as suggested by the increase in uptake with increasing temperature at B/A = 3. The most probable explanation for this is the penetration of bilirubin into the hydrophobic interior of the bilayer causing a disordering of acyl chains (increasing the fluidity). A recent study has suggested that the entropy gain may be due to the partitioning of bilirubin into free spaces in the bilayer [39]. The increased uptake with decreasing pH (Fig. 5) is also suggestive of hydrophobic interaction. As the pH is decreased the concentration of bilirubin monoanion will increase at the expense of bilirubin dianion and because of the reduced charge on the monoanionic form, penetration into the hydrophobic interior of the membrane will be favoured. A multistep binding mechanism including an aggregation of bilirubin on the surface has been suggested earlier for the interaction of

bilirubin with lipid vesicles and erythrocyte ghosts [22,27,28 40,41].

It is difficult to conclude from the present data on the question whether bilirubin crosses the plasma membrane and reaches intracellular targets. Some of the possibilities to be considered follow. Bilirubin may be confined to the plasma membrane and elicit the intracellular response by membrane-mediated transduction of information. Another possibility is that a fraction of the (plasma) membrane-bound bilirubin is transported into the cytosol by partitioning into a cytosolic carrier molecule. The ability of albumin to extract partially the cell-bound bilirubin (Table 2) and our earlier finding that bilirubin trapped in lipid vesicles can be extracted with albumin [42] support the idea. However, the presence of such carrier molecules for bilirubin has not yet been demonstrated in the nervous system though proteins such as Z-protein, glutathione-S-transferase and ligandin have been implicated to have such a role in the liver [20,43]. Finally, the possibility that bilirubin in the plasma membrane reaches intracellular membranes through membrane recycling or aqueous diffusion of the monomer as proposed for phospholipids and cholesterol [37,44] should also be considered. Experiments including subcellular fractionation of bilirubin-treated cells are in progress to obtain further insight into the mechanism of bilirubin transport across the plasma membrane.

Table 2-1. [^{3}H] bilirubin uptake by N-115 cells at 37°C. Cells were incubated with the indicated concentrations of bilirubin containing a constant amount of [^{3}H] bilirubin (28,000 CPM/dish) for the indicated periods and the cell-bound radioactivity was measured. The values are given as Mean ± S.E. of three dishes of cells.

B/A	Conc. of Bilirubin (μM)	Period of Incubation (min.)	[^{3}H]bilirubin Uptake (CPM/μg DNA)
1.5	5	5	7.86 ± 0.59
1.5	150	5	12.32 ± 1.26
1.5	5	60	12.78 ± 0.28
1.5	150	60	49.66 ± 1.12
3.0	5	60	19.46 ± 2.91
3.0	50	60	58.61 ± 1.80
3.0	75	60	68.58 ± 1.62
3.0	100	60	67.84 ± 2.03

Table 2-2. Reversibility of bilirubin uptake by N-115 cells at 37°C. For each case, 6 dishes of cells were treated with 100 μM bilirubin (B/A=3) at 37°C for the indicated period. Three dishes were subjected to uptake measurements by HPLC as usual (Control). To the remaining three dishes after a bilirubin washout, 33 μM of HSA was added and incubated at 37°C for 30 min. and then bilirubin remaining bound to the cell was measured as usual and this represented the uptake after extraction with albumin (Residual). The difference between control and residual uptakes gives the bilirubin extracted with albumin which represents the readily reversible portion of uptake. All uptake values are Mean ± S.E. from three dishes.

Period of Bilirubin Treatment	Bilirubin Uptake (pmole/μg DNA)		Bilirubin Extracted with Albumin	
(min.)	Control	Residual	pmole/μg DNA	% Control
3	26.8 ± 1.0	11.3 ± 1.6	15.5	57.8
10	80.5 ± 3.1	48.0 ± 1.8	32.5	40.4
20	72.5 ± 5.2	43.3 ± 4.7	29.2	40.3
40	124.6 ± 5.9	104.6 ± 3.4	20.0	16.1
60	132.8 ± 8.7	93.0 ± 4.6	39.8	30.0

Table 2-3. Effect of temperature on bilirubin uptake by N-115 cells.The cells were maintained at the indicated temperature for 2 hours and pH was maintained at 7.4 by adding appropriate amounts of 40 mM HEPES to the media. Bilirubin (100 µM at a B/A=3 and 50 µM at a B/A=1.5) was then added to the cells and incubated at the respective temperature for an additional 60 min. (B/A= 3) and 90 min. (B/A=1.5). Cell-bound bilirubin was extracted and measured by HPLC. The values at B/A= 3 are Mean ± S.E from three dishes while the values at B/A=1.5 are means from two dishes. The values in parenthesis give the pH of the medium at the end of incubation with bilirubin.

Temperature	Bilirubin Uptake (pmole / µg DNA)	
°C	B/A = 1.5	B/A = 3
4	15.4 (7.68)	30.7 ± 1.8 (7.84)
15	9.2 (7.70)	52.1 ± 10.4 (7
25	15.9 (7.75)	59.6 ± 4.4 (7.98)
37	14.4 (7.70)	94.0 ± 10.7 (7.88)

Figure 2-1. Time course for the uptake of bilirubin by N-115 cells at 37 C. Cells were incubated with bilirubin for different time intervals at 37 C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells. 100 μM bilirubin at B/A=0.8 (x), B/A=1.5 (o), and B/A=3 ( ), 50 μM bilirubin at B/A=1.5 (Δ). The curves for B/A=0.8 & 3 are drawn as rectangular hyperbolas, whereas the curves for B/A=1.5 are drawn as smoothed interpolations because the fitting to rectangular hyperbola results in a straight line and the deviations are considerable.

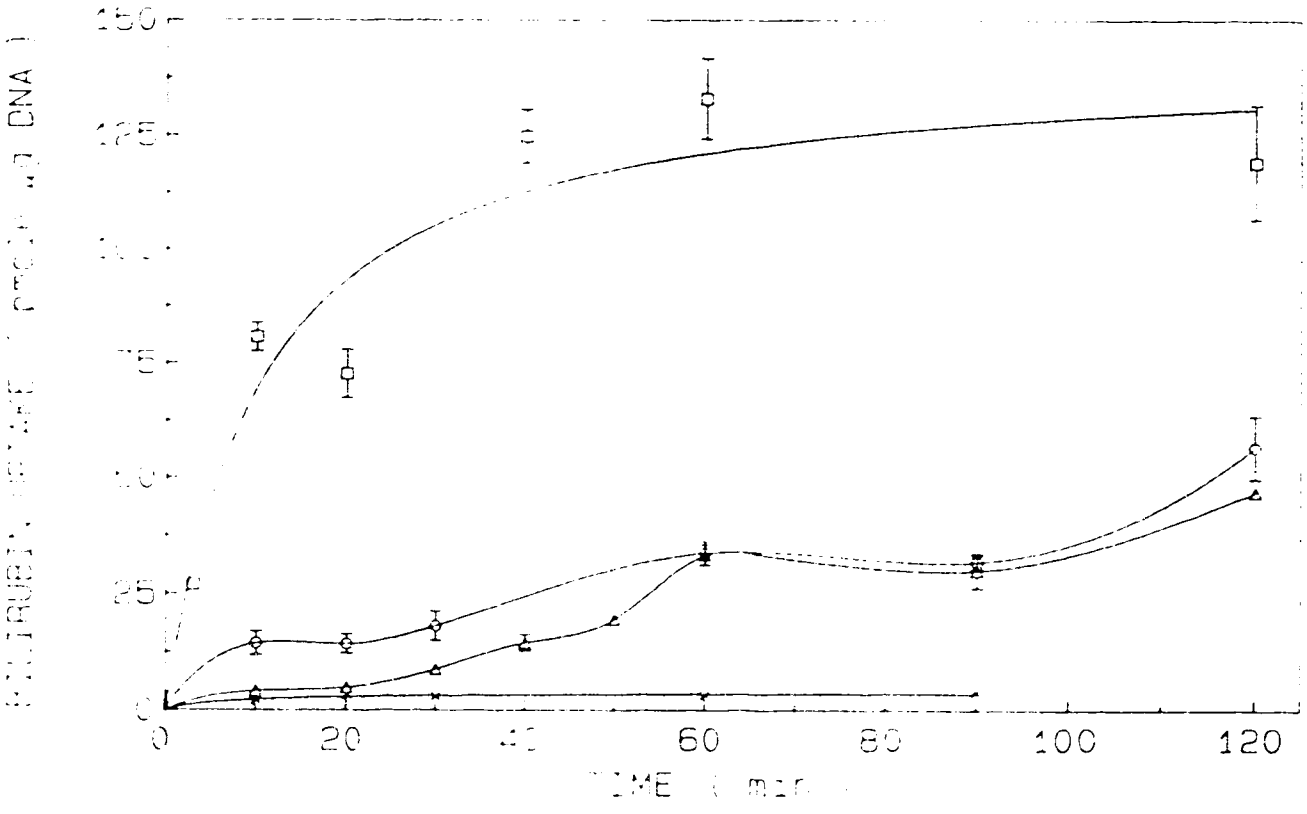


Figure 2-2. Initial rate of uptake of bilirubin by N-115 cells as a function of bilirubin concentration at constant B/A ratio. Cells were incubated with indicated concentrations of bilirubin for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=3 (Δ), and the mean of duplicates (which differ by <18%) for B/A=1.5 (□).

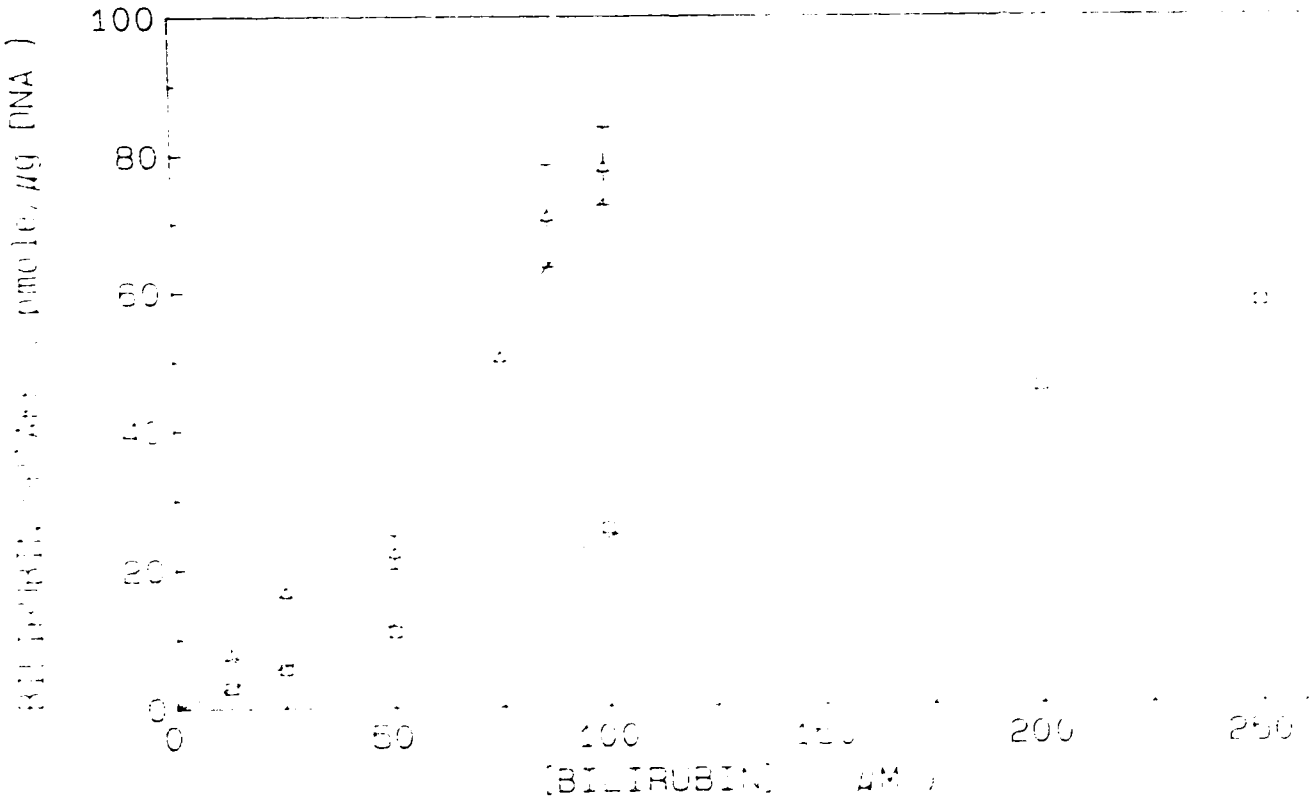


Figure 2-3. Initial rate of uptake of bilirubin by N-115 cells as a function of B/A ratio at a constant concentration of bilirubin. Cells were incubated with 100 μM bilirubin (and varying albumin concentration) for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Cellular uptake of bilirubin is plotted as a function of albumin concentration (A) and as a function of free bilirubin concentration (B). Concentration of free bilirubin was calculated assuming a bilirubin-albumin binding constant of 3.2 x 10^7 M^{-1} [4]. Each point represents the Mean ± S.E. from three dishes of cells.

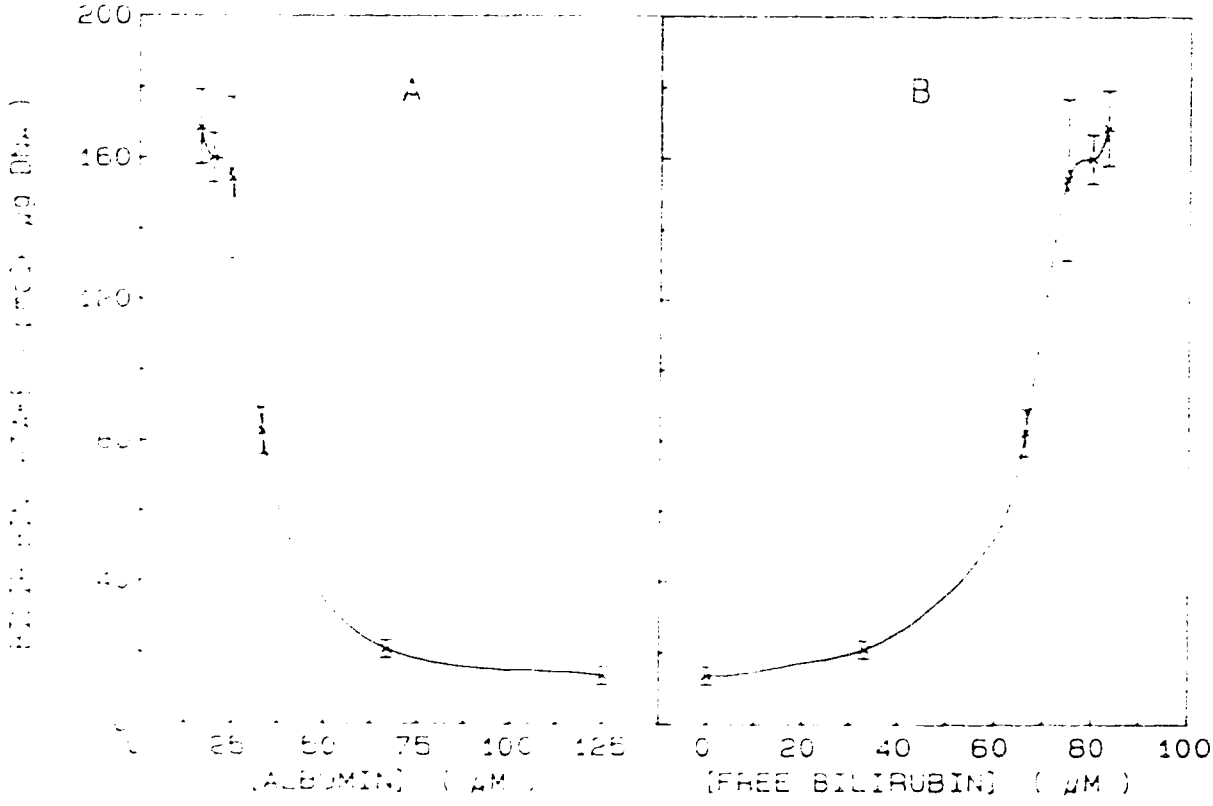
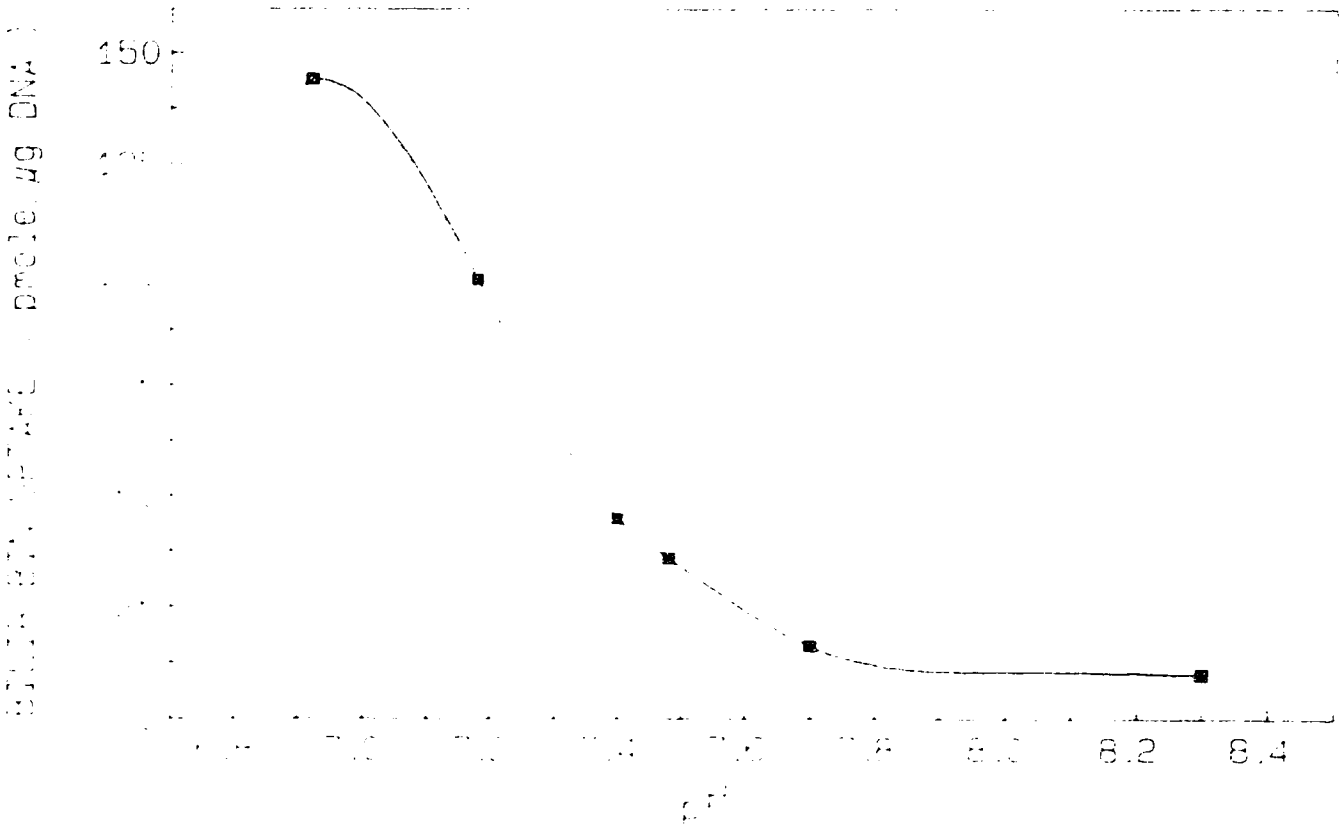


Figure 2-4. Limiting (apparent equilibrium) uptake of bilirubin by N-115 cells as a function of bilirubin concentration. Cells were incubated with indicated concentrations of bilirubin for 2 hours at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=1.5 (x) and the mean of duplicates (which differ by < 14%) for B/A=3 (⊜). The dotted lines are linear extrapolations from points with bilirubin concentrations < 50 μM.

Figure 2-5. Effect of pH on bilirubin uptake by N-115 cells. Cells were grown as usual, the media was aseptically removed, 100-200 µL of sterile 1N HCl or 1N NaOH was added to achieve the desired pH and the media was gently poured back into the culture dish. Cells were incubated for 1 hour at 37°C and then 50 µM bilirubin at B/A=1.5 was added. After an additional 90 min. of incubation, cell-bound bilirubin was extracted and measured by HPLC. Each point represents the mean of duplicates (which differ by < 13%).



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CHAPTER 3

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Mechanism of Bilirubin Toxicity in a Neural Cell Line.

BY

Yair Amit.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF **Doctor of Philosophy**

IN

MEDICAL SCIENCES

DEPARTMENT OF PEDIATRICS

EDMONTON , ALBERTA

SPRING 1990.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled -

Mechanism of Bilirubin Toxicity in a Neural Cell Line.

submitted by - **Yair Amit**

in partial fulfillment of the requirements for the degree

of - **Doctor of Philosophy**

in - **Medical Sciences**

Dr. D. Schiff

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Dr. W. Schneider

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Dr. M. Poznansky

Date: April 25, 1990

Dedicated to my wife Ada

and my children Itai, Ido, and Yorai

ABSTRACT

The mechanism of bilirubin toxicity to the central nervous system has been the subject of numerous investigations over the past decade. The results of several clinical and experimental studies suggest that bilirubin is toxic to various cellular functions with reversibility of early stages of bilirubin encephalopathy. Yet, the major biochemical defect underlying bilirubin toxicity has not been completely elucidated. The difficulties in analyzing the results and the inability to point to a primary target of bilirubin toxicity stem from variables in experimental design, the use of different animal models and cell systems, and the use of unstable bilirubin mixtures. Spectrophotometric measurements demonstrated that bilirubin in tissue culture media, at concentrations of 35-125 μM and at bilirubin-to-albumin [B/A] molar ratios up to 3, is stable over a 24-hour period. The use of a neural cell line and the presence of appropriate albumin concentrations are advantageous. We have measured the interaction and toxic effects of bilirubin to N-115 cells, a murine neuroblastoma cell line. The results obtained point to a multistep interaction process between bilirubin and the plasma membrane. Bilirubin binding is dependent on bilirubin concentration, B/A molar ratios, temperature and pH conditions, and is partially reversible with the addition of albumin. Under appropriate B/A molar ratios, bilirubin was found to affect Na^+/K^+ ATPase activity, [^{3}H]-thymidine uptake, L-[^{35}S]-methionine incorporation into protein, and mitochondrial functions. The toxic effects seem

to be dependent again on B/A molar ratio, bilirubin concentration, and length of exposure. However, it is not possible to single out the primary target for bilirubin toxicity conclusively. In N-115 cells, once toxicity appeared, it was irreversible. Moreover, toxicity appeared long after removal of the bilirubin-containing media following a short-term exposure to bilirubin, during which toxicity was not manifest. We conclude that, under appropriate experimental conditions, the binding interaction between bilirubin and the cell plasma membrane is complex, and that bilirubin is toxic to several cellular functions in N-115 cells in a progressive and irreversible process.

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B/A	-	Bilirubin to albumin molar ratio.
DMEM	-	Dulbecco's modified Eagle medium.
FCCP	-	Carbonyl cyanide -*p* - trifluoromethoxyphenylhydrazone.
HEPES	-	N-2-Hydroxyehtylpiperazine-N-2-ethanesulfonic acid.
HPLC	-	High performance liquid chromatography.
HSA	-	Human serum albumin.
MTT	-	3-(4,5-dimethylthiazol-y-yl)-2,5-diphenyl tetrazolium bromide.
PBS	-	Phosphate buffered saline.
PFM	-	Protein Free Medium.

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Hyperbilirubinemia is a common occurrence in the newborn period. Bilirubin encephalopathy (kernicterus) is a major complication of the toxic effect of bilirubin on brain cells. Originally described in jaundiced newborns, it has been seen in recent years in premature infants suffering from a mild degree of hyperbilirubinemia.

The protection of the newborn's brain from bilirubin has been attributed to a number of factors, among them the capacity of albumin to bind bilirubin, the integrity of the blood brain barrier, and the integrity of the neural cells.

The mechanism of bilirubin encephalopathy has been extensively studied over the past decade. However, the primary target, the toxic manifestation and the nature of the interaction between bilirubin and neural cells remains unclear. Studies conducted on neural and on non-neural cells and tissues demonstrate that bilirubin may impair a large number of cellular functions. However, the use of bilirubin concentrations higher than those usually encountered in clinical situations, and the use of varying albumin concentrations may account for the multiplicity of effects and inconclusive results.

The use of bilirubin without the addition of albumin or at high bilirubin-to-albumin molar ratios causes rapid aggregation and precipitation, auto-oxidation, and decomposition of the pigment, as well as photoisomerization of the natural occurring

bilirubin IX-α isomer. Since bilirubin may be poisonous to cells, clearly it is important to establish appropriate experimental conditions under which bilirubin is maintained in solution throughout the time the cells are exposed to bilirubin.

The purpose of the work herein described was to establish the appropriate experimental conditions for studies related to bilirubin and its cellular interaction and to define the following:

1) The interaction between bilirubin and the neural cell.
2) The target and mechanism of bilirubin toxic effects.

In the following sections a number of subjects will be reviewed:

1) Neonatal jaundice.
2) Bilirubin metabolism, structure, and binding properties.
3) Bilirubin toxicity.
4) Kernicterus and bilirubin encephalopathy.
5) Studies on bilirubin toxic effects.

2. Neonatal Jaundice

Hyperbilirubinemia is a common occurrence during the neonatal period. Clinical hyperbilirubinemia is defined as a serum bilirubin concentration that exceeds 26 μmol/L, and is common to most newborn infants during their first week of life. In 10 to 15% of all normal-term babies, hyperbilirubinemia becomes sufficiently high to be visible as jaundice [1]. Although the majority of jaundiced full-term babies appear completely healthy, standard textbooks of newborn medicine mandate diagnostic investigation to rule out pathologic causes of jaundice in those infants whose serum bilirubin concentrations exceed a level of 170 to 220 μ mol/L [2,3]. The incidence of serum bilirubin concentrations above 220 μmol/L ranges from 4.5% to 20% during the first week of life [4,5]. Although the presence of hyperbilirubinemia engenders some concern, 56% of infants whose serum bilirubin concentrations exceed the above levels show no cause for the jaundice [6].

There are many causes for neonatal hyperbilirubinemia unique to the fetus and the newborn. During the last stages of fetal life, removal of erythrocytes provides an increasing load of hemoglobin for catabolism. This results in an increase in bilirubin production [7]. The normal newborn produces more than double the bilirubin production of 3.6 mg/kg/day observed in the adult. Moreover, no rate-limiting step in hemoglobin catabolism and unconjugated bilirubin formation is recognized in the mammalian fetus [8,9,10,11].

The disposal mechanism for bilirubin in the fetus involves two pathways. The vast majority of unconjugated bilirubin is cleared via the placental circulation into the maternal circulation, where it is disposed of by the maternal liver [8,10]. The second pathway involves excretion by the fetal liver. This pathway is limited due to several factors. Foremost among these is a marked deficiency in hepatic uridine diphosphate glucuronyltransferase, noted in human as well as other mammalian fetuses [7]. As a result, the conjugating capacity of fetal liver is almost undetectable. Other factors associated with decreased hepatic clearance of bilirubin in the fetus are reduced hepatic blood flow and low levels of bilirubin binding proteins [9,11]. However, as a result of the different disposal processes, unconjugated hyperbilirubinemia is rarely evident at birth, even in severe cases of hemolytic anemia in the fetus.

The newborn infant, like the fetus, has several impairments in bilirubin metabolism and transport. These include increased bilirubin production [7], deficiency of hepatic bilirubin binding proteins and decreased glucuronyltransferase activity [12,13], as well as increased enterohepatic circulation of bilirubin [14]. Taken together, these factors usually result in the occurrence of increased concentrations of serum unconjugated bilirubin during the first days of life. Clinically, this is usually defined as "physiologic jaundice of the newborn" [15]. Yet, in certain groups of infants this phenomenon is exaggerated and the jaundice becomes pathological. A variety of conditions may result in unconjugated

hyperbilirubinemia : hemolytic disorders, polycythemia, increased extravasation of blood, increased enterohepatic circulation of bilirubin, defects in bilirubin metabolism, breast feeding, inherited metabolic disorders and prematurity [2,3].

There are two functionally distinct periods in physiologic jaundice of the newborn . The first is observed during the first 5 days of life and is characterized, in the full term infant, by a rapid rise in serum unconjugated bilirubin concentration to a peak of 100-120 μmol/L on the third day of life, and a rapid decline until the fifth day. In the premature infant, the peak value is higher and does not occur until the fifth to seventh day of life. The second period of physiologic jaundice is characterized by a relatively stable serum unconjugated bilirubin level of about 35 μmol/L that lasts until the end of the second week, in term infants, or for more than a month in preterm infants. After the second stage, serum unconjugated bilirubin concentrations decline to levels observed in normal adults [3,15,16,17,18,19].

Many studies of serum bilirubin concentrations in normal-term and in premature babies have provided guidelines for the diagnosis of "physiologic" and pathologic jaundice [4]. Pathologic jaundice is suspected whenever the following criteria are present:

1) Clinical jaundice in the first 24 hours of life.
2) Total serum bilirubin concentration increasing by more than 85 μmol/L per day.
3) Total serum bilirubin concentration exceeding 220 μmol/L in term infant and 255 μmol/L in prematures.

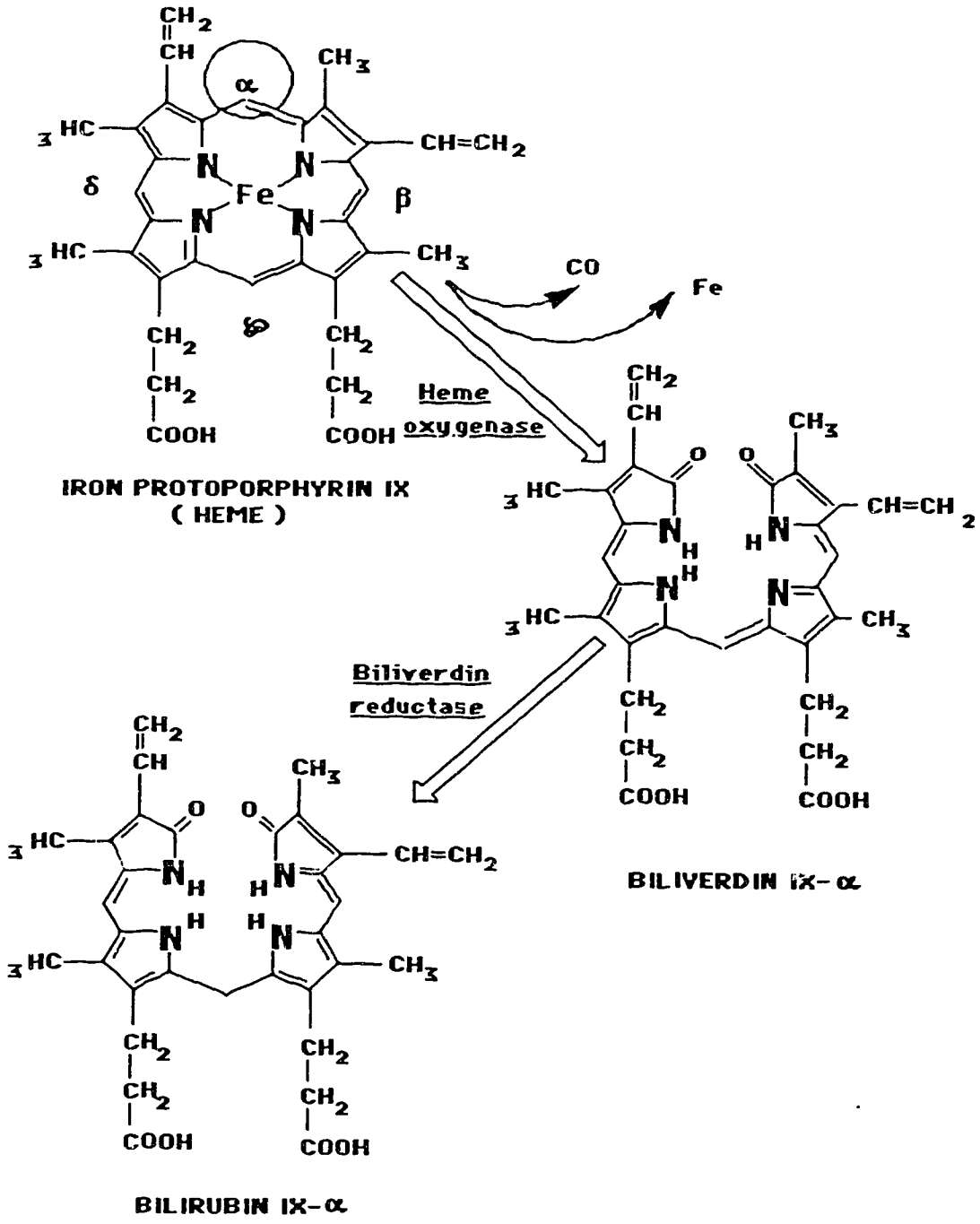
4) Direct serum bilirubin levels over 25-34 μmol/L.
5) Clinical jaundice lasting more than a week in a term baby, or two weeks in a premature infant [3].

3. Bilirubin Metabolism

Bilirubin is formed by the catabolism of different heme proteins including hemoglobin, myoglobin and heme-containing enzymes such as cytochromes, catalases and pyrolases [20]. Hemoglobin is the principal source of bile pigment in mammals, accounting for approximately 80% of the daily bilirubin production [21].

The metabolic pathway of heme catabolism has been clarified to a considerable extent [22]. Heme (Plate 1) is catabolized by a microsomal heme oxygenase localized primarily in the reticuloendothelial system [23], in tissue macrophages, and in the intestinal brush border membranes [24]. Inside the microsome, the porphyrin iron - located within the cyclic tetrapyrrole - is reduced, and an oxygen radicale is generated. Radicale attack and subsequent oxidation of the carbon atom at the α-methene carbon position, break the porphyrin ring. As a result, biliverdin IX-α is formed with loss of the iron atom and release of carbon monoxide. In mammals, biliverdin undergoes further reduction to bilirubin IX-α (Plate 2) [23]. The conversion is catalyzed by biliverdin reductase located in the cytosol [23].

Plate 1. Enzymatic oxidation of heme.



Bilirubin is a waste product and has no apparent function. Although the concentration of bilirubin in the serum is generally low, its concentration in the bile is significantly higher [20]. Five steps are involved in the transport of bilirubin from its sites of formation to the intestinal tract:

1) Transport in the plasma firmly bound to albumin [25].
2) Carrier-mediated transfer of bilirubin into the hepatocyte and binding to acceptor proteins located in the cytosol [26,27].
3) Hepatic conjugation that renders the pigment polar and water soluble [28].
4) Excretion of conjugated bilirubin into the bile [28]
5) Transport and elimination in the intestine [29,30].

Once inside the liver cell, bilirubin is transported to the smooth endoplasmic reticulum where the insoluble pigment is conjugated, thus converted into a water-soluble monoglucuronide pigment [29]. The final step in bilirubin metabolism, within the hepatocyte, is a second glucuronidation which takes place in the cytosol by a plasma membrane-bound enzyme [29]. Bilirubin mono- and diglucuronide are then excreted into the bile. When conjugated bilirubin reaches the sterile newborn intestine, the normal reduction of bilirubin to fecal stercobilinogen does not occur. Instead, a large proportion of the bilirubin is hydrolyzed by β-glucuronidase located in the brush border of the small intestine [29,30]. The resultant unconjugated bilirubin is reabsorbed in the gut and taken up by the portal system to start the disposal process

again [29,30], giving the so called enterohepatic circulation of bilirubin.

Disorders of bilirubin metabolism affect human beings from birth. The detrimental effects appear to arise chiefly from the virtual insolubility and instability of the pigment in aqueous solution at physiologic pH. Several bilirubin IX-α polar groups, namely, two carboxyl, two lactam, and two pyrrol groups render the substance soluble in water (Plate 2). The actual insolubility is explained by intra-molecular hydrogen bonding. In the hydrogen-bonded molecule (Plate 3), the hydrophilic polar COOH and NH groups are intimately associated and unavailable for interaction with polar groups in the environment. The insolubility of bilirubin-acid, with its two protonated carboxyl groups (Fig. 4), is considered the basis for its neurotoxicity. Understanding the conditions of bilirubin-acid formation is important for understanding the mechanism of its toxicity [31,32,33].

Bilirubin forms a saturated aqueous solution containing a very low concentration of the acid and a higher concentration of the dianion (Plate 4 & 5) [31,32]. Due to negative charges, the dianion is present in equilibrium with its dimer. The degree of dimerization is independent of pH, since hydrogen ions are not involved. However, with increasing hydrogen ion concentration some of the dimers and dianions take up protons from the medium, forming acid anions with fewer negative charges. The decrease in electric repulsion is followed by formation of large aggregates. During this aggregation the solution usually remains clear and

Plate 2: Bilirubin IX- α

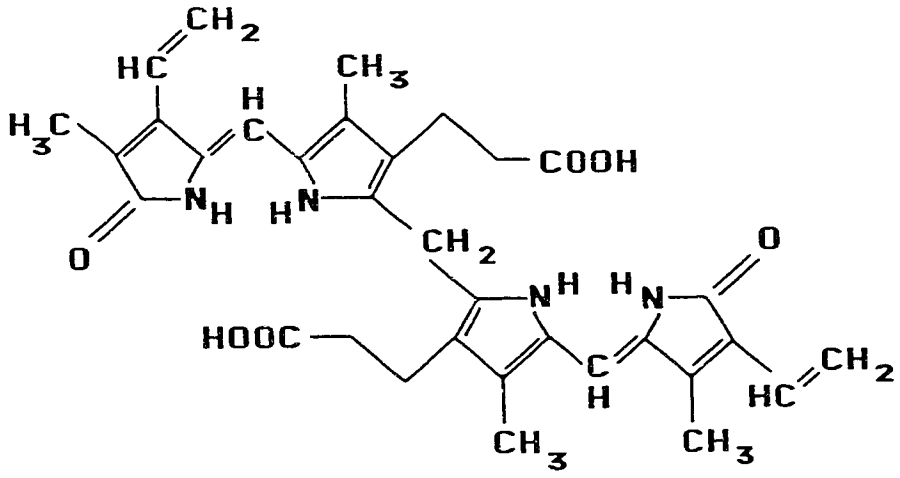


Plate 3: Bilirubin IX-α acid, intramolecularly hydrogen bonded.

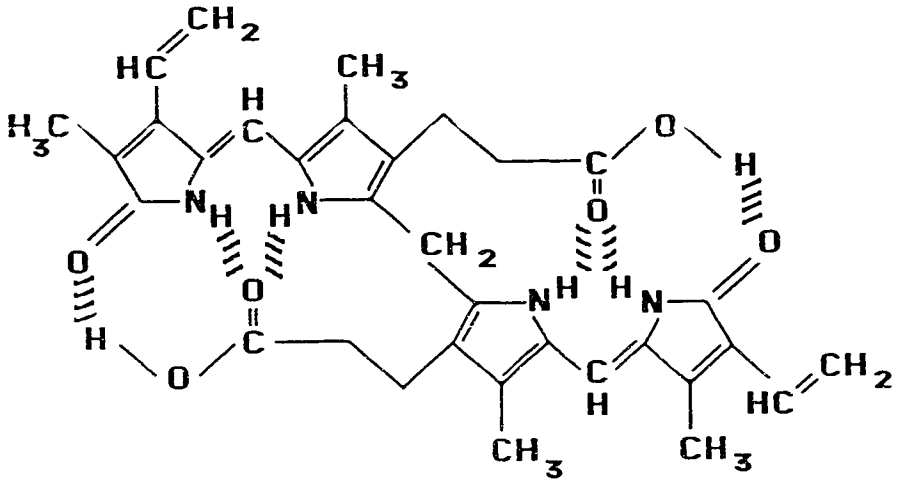


Plate 4: Bilirubin IX-α acid.

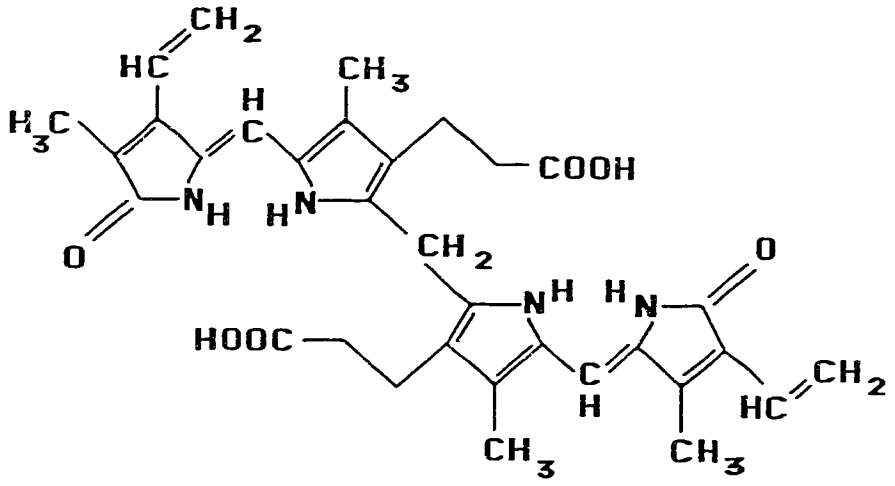
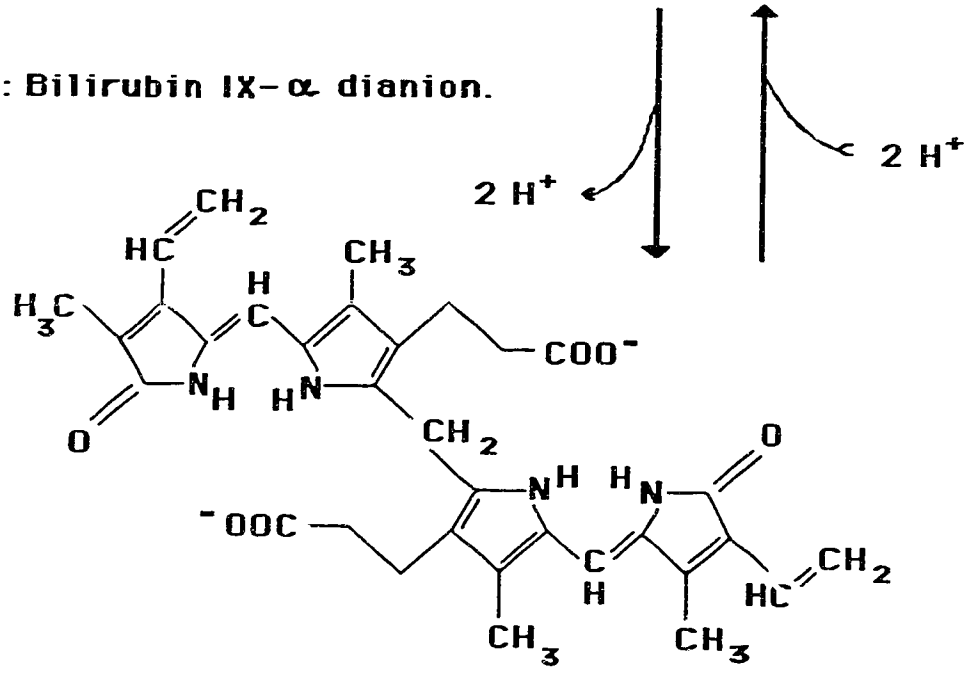


Plate 5: Bilirubin IX-α dianion.



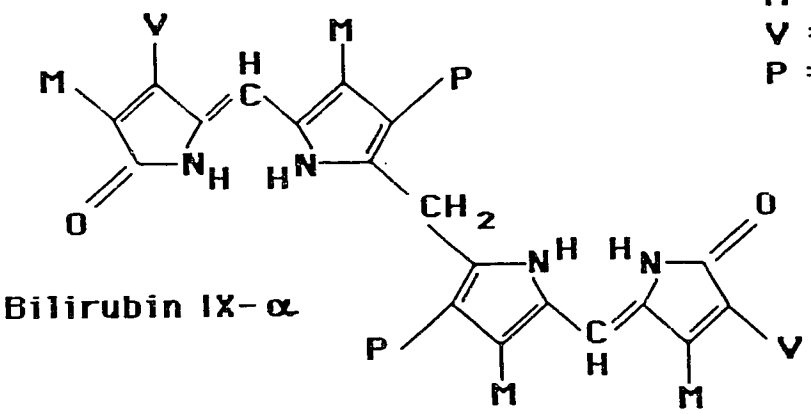
bright yellow when observed by the naked eye. The presence of strong light scattering indicates that a colloid suspension, and not a genuine bilirubin solution, is present [31,32,33,34].

When colloid formation is expected but fails to take place, the bilirubin solution is said to be in a supersaturated state. Under acidic pH conditions, supersaturation with extensive aggregation and precipitation of the insoluble protonated acid will occur [33]. Phospholipids accelerate the aggregation of bilirubin at acidic pH conditions as well as co-precipitation of bilirubin and phospholipids [34]. When a neutralized supersaturated solution of bilirubin is mixed with a suspension of erythrocyte membranes or mitochondria, and with liposomes or phospholipids ***in vitro*** , a process of binding and aggregation of bilirubin starts immediately and proceeds rapidly [33]. The end result is similar to colloid aggregates with the aggregates remaining attached to the membranes [34]. Since the same cellular structures are present in intact cells, it is reasonable to assume that the same process will occur ***in vivo.***

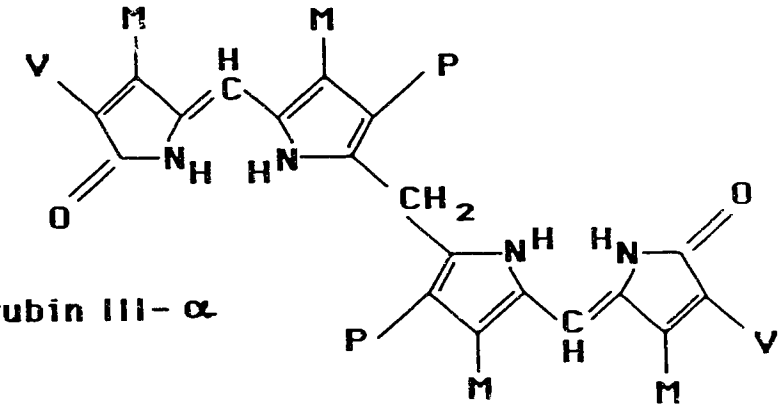
Besides its insolubility and tendency to aggregate and form colloids, bilirubin is unstable in solution and tends to auto-oxidize and decompose. Hydrogen-bonded bilirubin, dissolved in oxygenated alkaline aqueous solution, is unstable and may undergo rearrangement and auto-oxidation [35]. Furthermore, over a pH range from 7.4 to 12 [36] or in the presence of acid [37], bilirubin IX-α is cleaved at the central methylene bridge with subsequent rearrangement of the separate units to give a mixture of bilirubin III-α, and bilirubin XIII-α in addition to the natural IX-α isomer (Plate 6) [36,37].

Plate 6: Bilirubin isomers.

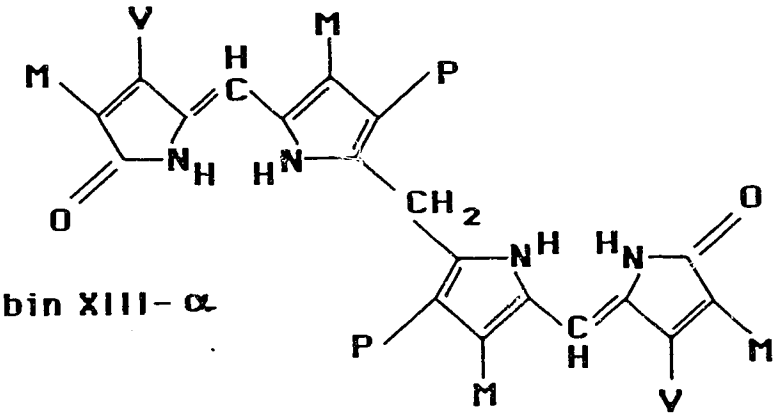
M = CH_3
V = CH=CH
P = CH_2CH_2COOH



Bilirubin IX-α



Bilirubin III-α



Bilirubin XIII-α

4. Bilirubin Binding to Albumin

In order to prevent bilirubin precipitation and toxicity, it is necessary to bind the pigment to a carrier. Serum albumin serves as a universal carrier, reversibly binding a large number of substances including bilirubin [32,38,39]. The importance of the interaction between bilirubin and albumin was demonstrated by several investigators. Bowen et al, demonstrated the protective role of albumin against unconjugated bilirubin injected into puppies [40]. Mustafa et al, found that one mole of albumin binds one mole of bilirubin and detoxifies it [41]. Odell [42] and Silverman et al [43] described increased bilirubin toxicity as a result of a dissociation of the pigment from its albumin binding site, caused by the use of different drugs.

In the blood, unconjugated bilirubin dianion is bound to a high affinity binding site on albumin with smaller amounts located at one or two lower affinity sites [44]. The binding process is fast, occurring in a matter of milliseconds [45], and is pH-independent within a pH range between 7 and 9.

Nevertheless, the distribution of bilirubin, ***in vivo***, between serum albumin and tissues is highly sensitive to pH changes, where acidosis favors a shift of the pigment from albumin to fat [46]. The shift is readily explained by a change in the solubility of the unbound pigment, formation of aggregates with lipoid membranes and lipids, and a shift in the bilirubin-albumin binding equilibrium [46]. Formation of the bilirubin-albumin complex is

reversible and is associated with protection of the pigment from degradation by various processes - e.g. photochemical degradation, isomerization, auto-oxidation and enzymatic reduction [47,48,49].

The same type of binding takes place ***in vitro*** , when a solution of albumin, at a slightly alkaline pH condition, is mixed with a solution of bilirubin dissolved in sodium hydroxide. Another type of binding occurs if a solution of albumin, ***in vitro*** , is mixed with a molar excess of bilirubin at pH 7.4 or below. Under such conditions a slow process of association takes place whereby large aggregates, consisting of large numbers of albumin and bilirubin acid molecules, are formed. This process results in co-crystallization of albumin and bilirubin with little bilirubin left in the solution [50]. Binding of bilirubin acid to albumin is pH-dependent since a high number of hydrogen ions are involved. Increasing the pH conditions of the solution towards an alkaline pH disintegrates most of the aggregates, and an equilibrium of binding of the anion is re-established [32,50].

5. Bilirubin Binding to Other Proteins

Although the only plasma protein with strong affinity for bilirubin is albumin, bilirubin can also bind to other blood components [51]. Binding to non-albumin proteins in the serum is important only when bilirubin concentrations exceed those of albumin and when the available primary binding sites on albumin are saturated. Bilirubin can bind to proteins such as serum β-lipoproteins and α-globulin, but when bilirubin is present in

the serum, the pigment distribution is always in favor of albumin [51,52,53,54].

Of physiological importance is bilirubin binding to proteins located in the cytosol, especially in hepatocytes. These proteins probably function as carriers for bilirubin within the cell and facilitate the uptake of the pigment [13]. Whether the binding is necessary for protection of the cell content against the pigment is unknown .

Other cellular and tissue components such as erythrocyte membrane [55,56,57], pulmonary hyaline membrane [58,59], mitochondria from heart and brain cells [60,61], glycolipids [62] , lipids and phospholipids [61,63,64] have been shown to bind bilirubin. Binding of the pigment to non-albumin proteins and other cellular components is of lesser importance and negligible when albumin exceeds bilirubin molar concentrations.

6. Bilirubin Interaction With Lipids .

The interaction of bilirubin with lipids has been studied by several investigators. Mustafa and King [61] suggested that bilirubin, in supersaturated solutions, is capable of binding to a variety of native membrane lipids as inferred by spectral changes. The changes observed were rapidly reversed by washing the liposomes with albumin, suggesting a loose binding of bilirubin to lipids. Weil and Menkes [62] have demonstrated that bilirubin interacts with gangliosides *in vitro* . In another study [63] bilirubin, at physiologic pH conditions, quenched a fluorescent

probe located within the lipid bilayer of membranes. Talafant [64] has found different binding qualities between the pigment and different phospholipids.

Of major importance is the knowledge of the interaction between bilirubin and the lipid bilayer. Eriksen et al [34] and Cestaro et al [65] demonstrated that bilirubin may be incorporated within the hydrophobic hydrocarbon domains of the bilayers, but migrate to the surface as equilibrium is achieved. On the other hand, Tipping et al [66] and Hayward et al [67], in a more recent study, were able to demonstrate that bilirubin is capable of passive diffusion across the lipid bilayer into liposomes. However, since no complete extraction of bilirubin from the liposomes was demonstrated, an interaction between the lipid bilayer and the pigment cannot be excluded.

The properties of bilirubin with regard to its effect on monolayers were demonstrated in two studies . In 1939 Stenhagen and Rideal [68] explored the interaction between bilirubin and various lipids and proteins. The results obtained suggest an interaction of the carboxyl groups of the pigment with the primary amide groups of lipids and proteins. Another series of monolayer experiments was carried out by Notter et al [69], exploring the effect of bilirubin on dynamic surface tension forces. Under acidic pH conditions, bilirubin-acid intercalates with the phospholipid acyl-fatty acid chains. At higher pH values, the more soluble bilirubin interacts with water away from the hydrophobic core of the lipid bilayer. Overall, it was shown that under acidic pH

conditions bilirubin is a highly surface-active material at the interface, and is capable of influencing the spreading behavior of membrane lipids [69].

7. Bilirubin Interaction With Membranes

The interaction of bilirubin with the central nervous system should consist of three steps: a) the entry of bilirubin into the brain from blood, b) the binding of bilirubin to the neural cell surface with or without subsequent internalization, and c) the interaction of bilirubin with plasma membrane, leading to alteration of membrane properties, or with intracellular targets.

Studying the interaction of bilirubin with synaptosomal plasma membrane, Vazquez et al [70] proposed a three step model for the interaction : 1) a rapid initial complex formation between anionic forms of bilirubin and the polar lipid head groups on the membrane surface, 2) a slow inclusion of bilirubin into the hydrophobic core of the lipid bilayers, and 3) the formation of bilirubin acid aggregates, by the remaining bilirubin molecules, on the surface of the plasma membrane [70]. While Vazquez demonstrated a multi-step interaction between bilirubin and the synaptosomal membrane, Leonard et al [71] suggested a different model for interaction. According to their results the interaction of bilirubin with model or biological membranes depends on the sizes of the free volumes, located within the membrane. These pools of free volumes varied according to the lipid composition and the presence or absence of proteins in the membranes. Bilirubin

appears to interact with neither the polar nor the apolar regions of the membrane but to partition with the free spaces in the apolar region of the lipid bilayer [71].

8. Bilirubin Toxicity

Despite the extensive knowledge of the chemical and biochemical properties of bilirubin, the question whether bilirubin is poisonous or only potentially toxic to the living organism has not been completely elucidated. While adults produce up to 250 mg of bilirubin daily without any harm and large doses have been injected intravenously into adults [72] and newborn babies [73] with no apparent ill effects, hyperbilirubinemia in newborn infants [44] and newborn rats [74] may cause bilirubin encephalopathy. Furthermore, studies in experimental animals indicate that unconjugated hyperbilirubinemia impairs liver mitochondrial function [41]. In contrast, no toxic hepatic effects have been seen in humans or Gunn rats suffering from prolonged unconjugated hyperbilirubinemia due to hepatic glucuronyltransferase deficiency [75,76].

A variety of pathologic conditions may result in severe or prolonged jaundice characterized by increased serum concentration of unconjugated bilirubin [77]. In several studies, bilirubin has been shown to be poisonous to neural and non-neural cells and tissues both ***in vitro*** and ***in vivo*** [78,79,80]. Bilirubin toxicity usually manifests as central nervous system damage which occurs almost exclusively during the early neonatal period [77]. Passage of

unconjugated bilirubin from the intravascular space - across the blood brain barrier - into the brain is thought to be the cause of kernicterus and bilirubin encephalopathy (see below).

9. Kernicterus and Bilirubin Encephalopathy

In 1903 Schmorl [81] coined the term kernicterus to describe the characteristic yellow staining of subcortical nuclei of the brain, that was commonly observed in jaundiced infants who died from severe erythroblastosis fetalis. The term was selected specifically to differentiate it from a more diffuse yellow staining of periventricular tissues and hemisphere surfaces, a condition considered secondary to passive diffusion of bilirubin following tissue necrosis [83]. Kernicterus, originally used as a pathologic term, is now associated with a particular clinical picture which varies from subtle neurologic changes such as high tone deafness to more extreme forms of severe choreoathetosis, mental retardation and, in some cases, to immediate death of the infant [82,83,84,85,86]. Moreover, in infants who survive the acute stages of hyperbilirubinemia but subsequently die, the staining may no longer be present, yet the basal ganglia display microscopic evidence of cell injury, neuronal loss and glial replacement [87,88,89]. Bilirubin encephalopathy is a more appropriate term to describe the clinical picture associated with the diffuse staining of the brain, the neuronal damage and the neurological picture associated with hyperbilirubinemia.

It is generally accepted that unconjugated bilirubin deposited in the brain is responsible for the yellow staining and the neurologic dysfunction characterizing bilirubin encephalopathy. To be toxic to the nervous system, unconjugated bilirubin has to cross the blood-brain barrier and specifically interact with vulnerable neural cells. The blood-brain barrier is a complex structure consisting of tight junctions cementing brain capillary endothelial cells plus adjoining foot processes of astroglial cells. Soon after contact with the astrocytes, continuous tight junctions seal the endothelial cells together and polar molecules no longer readily enter the brain by simple diffusion. Essential molecules such as glucose, organic acids and amino acids, therefore, require specific transporters to mediate their passage into the brain. Functionally, the blood-brain barrier comprises a series of carriers and transport mechanisms for various substances [90]. Permeation of the blood-brain barrier may result from changes in the anatomy and/or the function of its constituents.

The blood-brain barrier of the neonate is immature and thus may be more permeable [91]. Whether immaturity and increased permeability are responsible for the passage of free bilirubin into the neonatal brain is not clear. Many different factors, besides immaturity of the blood-brain barrier, account for the development of kernicterus. Among them are relative hypoalbuminemia, hypoxia, acidosis, hyperosmolarity, hypothermia, sepsis and drugs competing for bilirubin binding sites on albumin [92,93,94]. Endothelial cells of brain capillaries, as other cells, are

susceptible to injury by toxins and other abnormal metabolic conditions. Most evidence supports a passage of free bilirubin across the blood-brain barrier but a transfer of the albumin-bilirubin complex has not been excluded [95]. In normal infants, the restrictive nature of the blood-brain barrier is very well preserved despite immaturity of the endothelial cells composing the barrier. In infants with an intact blood-brain barrier, bilirubin will leave the blood to enter the brain only when the pigment is uncoupled from albumin and other plasma proteins. However, if brain endothelial cells are damaged, the altered barrier will then permit bilirubin, uncoupled from or complexed with albumin, to enter and damage the brain cells [95].

Another factor, the selective affinity of bilirubin for specific brain sites, complicates the picture of bilirubin encephalopathy. The vulnerability of specific brain areas to bilirubin toxic effects may be patterned by the blood flow to the brain [96,97,98] or affected by the different bilirubin binding affinities to various brain phospholipids [62,63,71].

Brodersen has suggested the possible existence of a bilirubin oxidase enzyme within the neural cells, which might play a role in protecting the cells by oxidizing the unbound bilirubin [99]. The presence of such an enzyme remains speculative.

Thus, protection of the newborn's brain from bilirubin may be attributed to a number of factors :

1) The interaction of bilirubin with albumin and /or different phospholipids [37,38,62,63,100].
2) The integrity of the blood brain barrier and of the brain cell membrane [95,98,101,102,103,].
3) The possible presence of a bilirubin oxidase enzyme [99].

The classical form of bilirubin encephalopathy , which was generally observed in term infants with hemolytic diseases, is virtually unknown today. This is a result of an improved and aggressive therapy directed at controlling hyperbilirubinemia with phototherapy, exchange transfusion, and prenatal management of the mother and fetus [104,105].

Unfortunately, kernicterus is still being observed at autopsies [105]. Small premature babies are the population at greatest risk for the development of bilirubin encephalopathy. In these infants, kernicterus has been found at bilirubin levels that are considered to be within the normal and "safe" range for the mature newborn [106,107,108]. Several potentiating factors that affect albumin binding of bilirubin or enhance tissue uptake of bilirubin have been suggested. Among these are low birth weight, hypothermia, asphyxia, acidosis, hypoalbuminemia, sepsis, meningitis and the use of drugs that displace bilirubin from its albumin binding sites [109,110]. To date, there is no proof for a direct relationship between the potentiating factors and the presence or absence of kernicterus and bilirubin encephalopathy [110,111,112]. The question as to what is affecting the newborn infant, still remains open. Is hyperbilirubinemia per se toxic, or is hyperbilirubinemia

an associated factor with the compounding effect of the other risk factors [113] ? Despite the uncertainty, measures have been taken to reduce the risk of bilirubin encephalopathy by adjusting the critical bilirubin concentrations to birth weight, gestational age and clinical situations at which medical intervention is indicated [114].

10. Studies on Bilirubin Toxicity

That bilirubin might be toxic to neural cells stems from the clinical association between the neurological picture and hyperbilirubinemia. However, despite a fairly detailed understanding of the chemistry and biochemistry of bilirubin there have been very few studies designed to define the interaction between bilirubin and the central nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions [78]. Specific kinetic studies carried out in hepatocytes [115,116,117,118,119,120] and human erythrocytes [121], have suggested the existence of saturable bilirubin binding sites. In other studies, the effects of pH and albumin on bilirubin binding to endothelial cells [122], fibroblasts [48,123], and isolated mitochondria [124] have been demonstrated. Our understanding of the interaction between bilirubin and neural cells is based on studies in which either the brain was exposed to bilirubin through opening of the blood brain barrier [95,98], or brain slices were exposed directly to bilirubin [125]. Both approaches present a relatively crude assessment of this interaction. To have a clear understanding of the mechanism of

bilirubin toxicity to the neural cell, knowledge of the interaction between bilirubin and the cell is critical.

Results of several studies indicate that bilirubin interferes with various cell functions [78,79,80]. Bilirubin toxicity to non-neural cells has been investigated extensively over the past years in fibroblasts [126,127,128,129], hepatocytes [130,131], erythrocytes [132,133,134,135], leukocytes [136,137], platelets [138] and Ehrlich ascites cells [139,140]. Toxic manifestations of bilirubin were demonstrated by non specific effects on cell viability and growth [126,127,128,129], cell morphology [135], and cell behavior [137,138]. More specific effects were observed when ATP synthesis [127] and membrane enzymes [133,134,139,140] were investigated.

Studies conducted on neural tissue demonstrated that bilirubin may impair a large number of cell functions such as changes in energy metabolism [41,141,142], alteration in the physical structure and function of cell membranes [61,62,63,64,65], changes in key intracellular enzymes [143,144,145,146,147], inhibition of both DNA [148,149] and protein synthesis [150,151,152,153], changes in carbohydrate metabolism [154,155] and modulation of neurotransmitter synthesis [156] and release [157]. Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, Gunn rats which suffer from hereditary unconjugated hyperbilirubinemia, served as a model [145,148, 150,151,152,153,155]. In the other, brain cells from normally developed animals were used

[144,146,147,154,155,157]. There is a major difference between the two. The use of the Gunn rat as a model for bilirubin encephalopathy is based on the assumption that the damage seen is primarily due to bilirubin. Although extensive damage to the nervous system in the Gunn rat can be attributed to bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded [158,159].

Bilirubin toxicity of the central nervous system is thought to occur in two stages : 1) an early reversible stage, sometimes referred to as subclinical and transient bilirubin-induced neurotoxicity, and 2) a later stage initiated when the sequelae become irreversible [80,160,]. Clinical studies in hyperbilirubinemic neonates have shown reversibility of the acute toxic bilirubin-induced changes in auditory nerve and brainstem responses [161,162,163]. Cowger demonstrated that bilirubin toxicity in an L-929 cell line was reversible with the addition of albumin [127]. Recently, Hansen et al demonstrated a similar phenomenon in hippocampal slices [157], and Wennberg provided evidence for the reversibility of bilirubin toxicity and mitochondrial uptake of bilirubin in erythrocytes [164]. On the other hand, working in a cell free system, Sano et al demonstrated that bilirubin inhibition of protein kinase C activity is irreversible [147].

A major concern when experimenting with a bilirubin-to-albumin molar ratio that exceeds one, is the instability of bilirubin

leading to the formation of bilirubin aggregates and co-aggregates of bilirubin and albumin [33,37,38,165]. Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been fully addressed in experiments dealing with bilirubin toxicity ***in vitro***. The frequent use of non-physiological bilirubin concentrations in ***in vitro*** studies, the addition of varying albumin concentrations with alteration of bilirubin-to-albumin molar ratios, and variations in the cells investigated, are among the major reasons for inconclusive results.

To date, few studies have been carried out in cultured neural cells. The question as to whether bilirubin is indeed toxic to the brain cell or whether the yellow staining of the brain is a coincidental finding has been raised. Schiff et al [149], reported recently that bilirubin toxicity in N-115, a murine neuroblastoma cell line, was dependent on bilirubin concentration, bilirubin to albumin molar ratio and time of exposure to bilirubin.

The present work will define the specific ***in vitro*** conditions under which bilirubin, when added to cells in media, is stable and remains so during the entire experiment. Working under these conditions and using N-115, a murine neuroblastoma cell line in culture, the present studies will attempt to characterize the following:

1) The interaction between bilirubin and the cell.
2) The target and the mechanism of bilirubin toxicity at the cellular level.

3) The possible reversibility of the toxic effects.

4) The delayed bilirubin effects after short-term bilirubin exposure during which no evidence of toxicity is manifested.

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CHAPTER 2

Publication No. 1:

Bilirubin - Neural Cell Interaction: Characterization of Initial Cell Surface Binding Leading to Toxicity in the Neuroblastoma Cell Line N-115.

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Introduction

Hyperbilirubinemia and bilirubin encephalopathy are well known occurrences in the newborn period [1,2]. It has been suggested that the protection of the newborn's brain to bilirubin toxicity may be due to a number of different factors. These include: a) the interaction of bilirubin with albumin and/or different phospholipids [3-9] and b) the integrity of the blood-brain-barrier and the brain cell membrane [10-14]. The fact that bilirubin might be toxic to neural cells stems from the clinical association of the neurologic picture that has emerged and the associated hyperbilirubinemia [15].

In spite of a fairly detailed understanding of the chemistry and biochemistry of bilirubin, there have been very few studies designed to define the interaction of bilirubin with the nervous system. The mechanism by which bilirubin enters the cell has been studied in many non-neural cells and subcellular fractions. Specific binding and kinetic studies carried out on hepatocytes [16-21] and human erythrocytes [22] have suggested the existence of saturable bilirubin binding sites. Other studies have demonstrated the effect of pH and albumin on the binding of bilirubin to L-929 cells [4], endothelial cells [23], fibroblasts [24] and isolated mitochondria [25].

The interaction of bilirubin with the central nervous system should consist of three steps, (i) the entry of bilirubin into the brain from the blood, (ii) the binding of bilirubin to neural cell surface with or without a subsequent internalization, and (iii) the interaction of bilirubin with intracellular targets (in the case of

internalization) or the alteration of plasma membrane properties leading to the toxic effect. There are two different views as to the mechanism of bilirubin entry into the brain. Some studies suggest that though bilirubin exists as a complex with albumin in the blood, only free bilirubin crosses the blood-brain-barrier (free bilirubin hypothesis) whereas other studies suggest that under certain conditions such as hyperosmolality, the blood-brain-barrier will be opened and bilirubin enters the brain as a bilirubin-albumin complex [10,11]. Once bilirubin enters the brain, the toxic effects will be determined by the interaction of bilirubin with the individual neurons.

Different approaches have been made to study the interaction of bilirubin with neural cells. There are studies that exposed either the whole brain [10,11] or brain slices to bilirubin [26]. These studies give a relatively crude assessment of the interaction because the exposure as well as the washing after the exposure will not be complete in a tissue and data are expressed in terms of total bilirubin uptake per gram of brain tissue. Another approach has been to characterize the interaction using membrane fractions and lipids of nervous tissue including components like sphingomyelin and gangliosides [9,27,28]. But these systems are far removed from the actual physiological situation with respect to the target as well as the form of bilirubin solution used. These studies employ supersaturated solutions of bilirubin, whereas in plasma, bilirubin is believed to be present predominantly as a complex with albumin. A better approach to the problem is to use a neural cell line under

the normal conditions of tissue culture in the presence of albumin as a model system. Such studies are almost lacking in the literature.

The present study examines the nature of the interaction of bilirubin with the murine neuroblastoma cell line N-115. The cells were exposed to bilirubin at different concentrations and different bilirubin to albumin molar ratios (B/A). The cellular uptake of bilirubin was characterized in terms of the kinetics, apparent equilibration (limiting values) and the effect of pH and temperature on the equlibration. The results indicate that the "free" form of bilirubin is the reactive species, and it interacts with the plasma membrane through a multistep binding process.

Materials and Methods

Materials. All reagents were of analytical grade and were purchased from Sigma Chemical Co. (USA). Bilirubin purity was verified by high performance liquid chromatography (HPLC), as indicated below, and was found to contain 92% IX-α isomer, 4.8% XIII-α isomer, and 2.8% III-α isomer. No other bile pigments were detected. Since all measurements of bilirubin extracted from cells were performed on HPLC, no further purification was carried out. [^{3}H]-bilirubin was prepared by ***in vivo*** labelling in rats using δ-amino [3,5(N)-^{3}H] levulinic acid (New England Nuclear) as the precursor [8]. [^{3}H]-bilirubin was purified from the bile as described by McDonagh [3], and was found to contain more than 98% bilirubin IX-α by HPLC (absorption at 454 nm), with specific activity of 1710 CPM/nmole bilirubin.

HSA (fraction V, Essentially Fatty Acid Free) obtained from Sigma Chemical Co. (St. Louis, MO), Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline (PBS) and fetal calf serum (FCS) were obtained from GIBCO (Canada). Solvents used were of HPLC grade (JT Baker Chemical Co.).

Bilirubin treatment of cells. The murine neuroblastoma cell line N-115 was seeded at a density of 3 x 10^6 cells/plate in 10 cm culture dishes (Falcon) and grown in standard DMEM plus 10% FCS, pH 7.4 at 37°C in a 5% CO_2 humidified atmosphere for 10-12 hours. The media was then removed, the cells washed twice with sterile PBS, and reincubated in 10 mL of protein-free media [29] containing human serum albumin plus 25 mM N-2-hydroxy-ethylpiperazine-N-2 ethanesulfonic acid (HEPES) to maintain a pH of 7.4 for another 12 hours, before the experiments with bilirubin were started. The albumin concentration was varied in different experiments to meet the required final B/A ratios. Three or four culture dishes were used in each experimental condition. These dishes were seeded with cells as above, with bilirubin being added to two or three of them. The remaining dish contained experimental media plus bilirubin, but no cells - a measure of non-specific binding of bilirubin to the plate.

A stock solution of bilirubin was made by dissolving 2 mg bilirubin in 1 mL of N_2-purged 0.1 N NaOH. Bilirubin was added to the culture media to achieve the appropriate experimental conditions, followed immediately by the addition of an amount of 0.1 N HCl equivalent to the amount of NaOH added to restore the pH of the

culture media to 7.4. Under the experimental conditions bilirubin-albumin mixtures were found to be stable when measured spectrophotometrically for a minimum of three hours and is reported elsewhere [30].

All procedures involving addition, incubation and extraction of bilirubin were carried out in a dimly lit room to avoid bilirubin photodegradation.

Measurement of bilirubin uptake by cells. At the end of the incubation period, the media was removed and saved for pH measurement. The cells were washed four times with ice cold PBS and then dislodged from the plate with a rubber policeman in 1.5 mL PBS and transferred into an Eppendorf Test tube. The cell suspension was then vortexed and 0.1 mL aliquots were taken for DNA analysis [31] and cell viability as measured by the nigrosin exclusion technique [32]. The remainder was spun down in a microfuge (Eppendorf) for 5 minutes and the supernatant removed. Bilirubin was extracted from the pellet by adding 0.9 mL of methanol:chloroform (1:2, v/v) followed by sonication for 10 minutes, and centrifugation for 10 minutes in an Eppendorf microfuge. The supernatant was dried under N_2 and kept at -20°C until HPLC analysis was performed [33].

Bilirubin extracts from the cells were analyzed by reverse-phase HPLC (Beckman Altex Ultrasphere IP, 5 µm, C-18, 25 x 0.46 cm column with Beckman Altex IP precolumn 4.5 x 0.46 cm) using 0.1 M di-n-octylamine acetate in methanol, pH 7.7, as eluant with a flow

rate of 1.0 mL/min - and the detector set at 454 nm [33]. Quantitation of peak areas was performed on a Gilson Data Master reporting integrator, using preweighed bilirubin (Sigma, Lot No. 25F-0584) as external standard.

The experiments with [^{3}H] bilirubin were also performed as described above except that instead of extracting cell-bound bilirubin with organic solvents and analysis by HPLC, the cells were suspended in 0.1 mL of 0.2 N NaOH and neutralized with 0.1 mL of 0.2 N HCl. The radioactivity was measured by liquid scintillation counting after adding 15 mL of aqueous counting scintillant (Amersham).

Results

In this study, uptake is defined as the total amount of bilirubin associated with the cells including both surface bound and internalized bilirubin. The results are the mean of the net uptake (total minus non-specific) of the two or three experimental dishes. The non-specific uptake was always less than 1.7% of the total uptake. If not mentioned otherwise, the bilirubin concentration refers to the total (input) concentration. The term "free bilirubin" is used to denote the bilirubin remaining after saturating the high affinity primary binding sites of albumin and as such include both "free bilirubin" in solution and the bilirubin loosely bound to albumin. The bilirubin-albumin solution were found to be stable for the time periods used in this study [31] and the isomeric composition of the bilirubin extracted from the cells was found to

be the same as the input bilirubin within error limits (1-2% increase in photoisomers).

Fig. 1 gives the time course of bilirubin uptake by N-115 cells when the cells are incubated with 100 μM bilirubin at different B/A ratios. The bilirubin uptake at a B/A ratio of 3 in 10 min is 80 pmole/μg DNA and plateaus at 125 pmole/μg DNA in 40 min. , whereas at a B/A ratio of 0.8 the rate is much slower and levels achieved are much less, < 5 pmole/μg DNA in 90 minutes. Thus, there is a sharp increase in the initial rate as well as the extent of uptake with increasing B/A ratio even though the input (total) concentration of bilirubin is held constant. The results support the idea that the "free" rather than the albumin-bound form of bilirubin is responsible for toxicity. Since the stoichiometry of albumin-bilirubin is 1:1 the concentration of "free bilirubin" will increase drastically as the B/A ratio increases from 0.8 to 3.0.

The effect of varying the bilirubin concentration on the initial rate of uptake of bilirubin by the neuroblastoma cell is given in Fig. 2. At a B/A ratio of 3, increasing the bilirubin concentration from 12.5 μM to 100 μM shows no evidence of saturation. A similar result was obtained at a B/A ratio of 1.5 with concentrations ranging up to 250 μM bilirubin. The apparent absence of saturation kinetics in either case likely rules out the possibility of carrier-mediated transport across the plasma membrane implicated in the uptake of bilirubin by hepatocytes [17-22]. The concentration of "free bilirubin" can also be varied by varying the B/A ratio at a constant total bilirubin concentration. The initial uptakes under

these conditions are plotted in two different forms in Fig. 3. As expected, the initial rate decreases rapidly with increasing albumin concentration almost linearly (probably) up to B/A = 2 and then very slowly (Fig. 3A). The same data plotted as a function of "free bilirubin" concentration calculated from the bilirubin-albumin stability constant of $3.2 \times 10^7 M^{-1}$ [4] is given in Fig. 3B, and shows no saturation up to 80 μM of "free bilirubin". (The curve suggests the possibility of saturation at higher bilirubin concentrations and a possible explanation for this is that at high B/A ratios the free bilirubin concentration is so high that it might form small aggregates, the reactivity of which might be less than that of the monomeric form. The results in Figs. 2 and 3 along with the known binding of bilirubin to lipids such as sphingomyelin and gangliosides with the affinity in the range of 10^5 - 10^6 M^{-1} [9,27,28] argue against the notion of a bilirubin carrier in N-115 cells.

The apparent equilibrium uptake (limiting values in Fig. 1) as a function of bilirubin concentration at B/A ratios of 1.5 and 3 are shown in Fig. 4. The curves are neither linear, expected for passive diffusion, nor hyperbolic, expected for a normal receptor-ligand system. The curves are parabolic (or rather part of a sigmoidal curve) suggestive of cooperative binding of bilirubin to the cells (At B/A = 1.5, a reasonable linear fitting can be done as shown by the solid line. However, there is considerable deviation from a linear extrapolation of early points as shown by the dotted lines). The uptake of [^{3}H] bilirubin by N-115 cells given in Table 1, also agree

with the non linear behavior seen in Fig. 4 effectively rulling out a diffusion mechanism .

To further characterize the binding we tested reversibility of binding by trying to extract cell-bound bilirubin with fresh albumin. Extraction was performed after incubating the cells with bilirubin for different time intervals and the results are given in Table 2. The uptake is partially reversible and the fraction reversible (extracted) decreases with an increasing period of incubation of cells with bilirubin. This indicates that the binding cannot be described by a simple receptor ligand system. The effect of temperature on bilirubin binding is given in Table 3. The temperature insensitivity of bilirubin uptake at B/A ratio of 1.5 suggests a specific binding to the cell because non-specific binding is expected to increase with increasing temperature due to increased concentration of "free bilirubin" in equilibrium with albumin at higher temperatures [3]. The difference in behavior at B/A ratios 1.5 and 3 could be a reflection of a complex binding process.

The effect of pH on bilirubin uptake by N-115 cells at a B/A ratio of 1.5 is given in Fig. 5. The uptake increases rapidly with decreasing pH - almost a 10 fold increase in uptake as the pH of the medium is lowered by 1 unit from pH 8.0 to pH 7.0. Changes in pH are reported to affect bilirubin deposition in the brain, erythrocytes and mitochondria [13,14,16,26,34]. One of the factors likely to contribute to this pH effect is the increased concentration of "free bilirubin" resulting from the decreased affinity of bilirubin for albumin with decreasing pH. Decreasing the pH from 7.4 to 7 leads to

a 4 fold increase in bilirubin uptake by N-115 cells whereas the expected change in "free bilirubin" concentration is negligible (16.729 μM at pH 7.4 and 16.738 μM at pH 7 calculated from binding constants of $3.2 \times 10^7\ M^{-1}$ at pH 7.4 and $2.8 \times 10^7\ M^{-1}$ at pH 7 [4]) suggesting that factors other than "free bilirubin" concentration may be responsible.

Discussion

The mechanism of bilirubin toxicity to the nervous system has been the subject of numerous investigations over the last few decades, yet the area is dominated by speculation rather than concrete ideas. This is mainly due to the peculiar properties of the bilirubin molecule. The molecule is neither hydrophilic nor hydrophobic, as indicated by its very poor solubility in aqueous media at neutral pH and poor to moderate solubility in organic solvents [6]. This has given rise to considerable limitation in experimentation as well as the interpretation of experimental data. It has also led to the use of a variety of model systems consisting of bilirubin solutions of varying kinds from supersaturated solutions at alkaline pH to bilirubin-albumin mixtures of different ratios and a range of targets from pure lipids and proteins to the whole brain. Though these studies have provided valuable information on different aspects of bilirubin action, a complete picture is still lacking. An important piece of information missing is the nature of bilirubin interaction with the plasma membrane. Studies with purified proteins and subcellular fractions have shown that bilirubin at micromolar concentrations can affect the activity of many

enzymes of cytosolic, mitochondrial and microsomal origin [2]. The relevance of these findings in relation to bilirubin toxicity ***in vivo*** requires an understanding of whether bilirubin can cross the plasma membrane and if so, what intracellular concentrations can be achieved under clinically relevant conditions. An integrated approach consisting of the quantification of bilirubin uptake and the measurement of consequent changes in some biochemical parameters of toxicity in the same system is desirable. Using a neural cell line we have shown recently that bilirubin affects mitochondrial function, protein synthesis and DNA synthesis in intact N-115 cells and the toxicity is determined by the concentration of bilirubin, B/A ratio and the period of exposure [35,36]. The complementary studies on the cellular uptake of bilirubin are presented here. In the clinical situation it is assumed that a B/A of less than one is safe, as the majority of bilirubin is bound to the primary "tight" binding site of the albumin molecule. In order to assess bilirubin interaction with the cell, we have used a B/A greater than 1 which would make available free bilirubin and/or loosely bound bilirubin [36].

The results in Figs. 1-4 clearly indicate that the uptake of bilirubin by N-115 cells increases with increasing period of exposure, B/A ratio and bilirubin concentration at a given B/A ratio consistent with our earlier results on the measurements of toxicity parameters under the same experimental conditions [35,36]. While this suggests that bilirubin enters the cell, the data presented here are not consistent with a simple transport mechanism. The data can be explained in terms of a multistep binding with the plasma

membrane similar to that proposed for the interaction of bilirubin with rat brain synaptosomal plasma membrane vesicles [28]. According to this model the interaction occurs in three steps: (i) bilirubin binding to the polar head group region of the membrane, (ii) insertion of the surface-bound bilirubin into the hydrophobic core of the membrane, and (iii) membrane induced aggregation of bound bilirubin on the surface of the membrane.

The unusual rate curve for bilirubin uptake at B/A = 1.5 (Fig. 1) could be a reflection of the multistep binding process. The effect is seen at bilirubin concentrations of 50 and 100 μM. Similar rate curves have been reported for the interaction of bilirubin with synaptosomal plasma membrane vesicles and liposomes made of lipids and proteins extracted from these vesicles [28]. The very low concentration of free bilirubin at B/A = 0.8 and a much faster uptake due to a high concentration of "free bilirubin" at B/A = 3 might explain the apparent normal behavior under these conditions. A multistep binding mechanism is also supported by the concentration-dependence of limiting uptake given in Fig. 4 and Table 1. The parabolic or probably sigmoidal curve is indicative of a cooperative process reflecting the aggregation of bilirubin on the membrane at high concentrations. The partial reversibility of bilirubin uptake, as assessed by the extraction with albumin (Table 2) also favors a multistep mechanisms. The bilirubin displaced from N-115 cells by albumin mainly represents the bilirubin bound to the cell surface (polar head groups), the initial step, because the fraction reversed decreases with increasing period of exposure. The

remaining non-extractable portion need not be irreversible in the thermodynamic sense because the dissociation of bilirubin aggregates and the desorption of bilirubin from the hydrophobic core of the membrane could be very slow processes as in the case of some lipids. The half-life for the desorption of membrane components such as phospholipids and glycolipids is in the order of days [37,38]. The difference in the effects of temperature on uptake at B/A ratios of 1.5 and 3 (Table 3) could be a further reflection of a multistep mechanism. At 50 μM bilirubin and B/A = 1.5, the "free bilirubin" concentration will be low so that the cell-bilirubin interaction is likely to be dominated by the initial step(s) whereas at 100 μM bilirubin and B/A = 3 the aggregation step is likely to be dominant. The step(s) following the initial binding is entropy driven as suggested by the increase in uptake with increasing temperature at B/A = 3. The most probable explanation for this is the penetration of bilirubin into the hydrophobic interior of the bilayer causing a disordering of acyl chains (increasing the fluidity). A recent study has suggested that the entropy gain may be due to the partitioning of bilirubin into free spaces in the bilayer [39]. The increased uptake with decreasing pH (Fig. 5) is also suggestive of hydrophobic interaction. As the pH is decreased the concentration of bilirubin monoanion will increase at the expense of bilirubin dianion and because of the reduced charge on the monoanionic form, penetration into the hydrophobic interior of the membrane will be favoured. A multistep binding mechanism including an aggregation of bilirubin on the surface has been suggested earlier for the interaction of

bilirubin with lipid vesicles and erythrocyte ghosts [22,27,28 40,41].

It is difficult to conclude from the present data on the question whether bilirubin crosses the plasma membrane and reaches intracellular targets. Some of the possibilities to be considered follow. Bilirubin may be confined to the plasma membrane and elicit the intracellular response by membrane-mediated transduction of information. Another possibility is that a fraction of the (plasma) membrane-bound bilirubin is transported into the cytosol by partitioning into a cytosolic carrier molecule. The ability of albumin to extract partially the cell-bound bilirubin (Table 2) and our earlier finding that bilirubin trapped in lipid vesicles can be extracted with albumin [42] support the idea. However, the presence of such carrier molecules for bilirubin has not yet been demonstrated in the nervous system though proteins such as Z-protein, glutathione-S-transferase and ligandin have been implicated to have such a role in the liver [20,43]. Finally, the possibility that bilirubin in the plasma membrane reaches intracellular membranes through membrane recycling or aqueous diffusion of the monomer as proposed for phospholipids and cholesterol [37,44] should also be considered. Experiments including subcellular fractionation of bilirubin-treated cells are in progress to obtain further insight into the mechanism of bilirubin transport across the plasma membrane.

Table 2-1. [^{3}H] bilirubin uptake by N-115 cells at 37°C. Cells were incubated with the indicated concentrations of bilirubin containing a constant amount of [^{3}H] bilirubin (28,000 CPM/dish) for the indicated periods and the cell-bound radioactivity was measured. The values are given as Mean ± S.E. of three dishes of cells.

B/A	Conc. of Bilirubin (μM)	Period of Incubation (min.)	[^{3}H]bilirubin Uptake (CPM/μg DNA)
1.5	5	5	7.86 ± 0.59
1.5	150	5	12.32 ± 1.26
1.5	5	60	12.78 ± 0.28
1.5	150	60	49.66 ± 1.12
3.0	5	60	19.46 ± 2.91
3.0	50	60	58.61 ± 1.80
3.0	75	60	68.58 ± 1.62
3.0	100	60	67.84 ± 2.03

Table 2-2. Reversibility of bilirubin uptake by N-115 cells at 37°C. For each case, 6 dishes of cells were treated with 100 μM bilirubin (B/A=3) at 37°C for the indicated period. Three dishes were subjected to uptake measurements by HPLC as usual (Control). To the remaining three dishes after a bilirubin washout, 33 μM of HSA was added and incubated at 37°C for 30 min. and then bilirubin remaining bound to the cell was measured as usual and this represented the uptake after extraction with albumin (Residual). The difference between control and residual uptakes gives the bilirubin extracted with albumin which represents the readily reversible portion of uptake. All uptake values are Mean ± S.E. from three dishes.

Period of Bilirubin Treatment	Bilirubin Uptake (pmole/μg DNA)		Bilirubin Extracted with Albumin	
(min.)	Control	Residual	pmole/μg DNA	% Control
3	26.8 ± 1.0	11.3 ± 1.6	15.5	57.8
10	80.5 ± 3.1	48.0 ± 1.8	32.5	40.4
20	72.5 ± 5.2	43.3 ± 4.7	29.2	40.3
40	124.6 ± 5.9	104.6 ± 3.4	20.0	16.1
60	132.8 ± 8.7	93.0 ± 4.6	39.8	30.0

Table 2-3. Effect of temperature on bilirubin uptake by N-115 cells.The cells were maintained at the indicated temperature for 2 hours and pH was maintained at 7.4 by adding appropriate amounts of 40 mM HEPES to the media. Bilirubin (100 µM at a B/A=3 and 50 µM at a B/A=1.5) was then added to the cells and incubated at the respective temperature for an additional 60 min. (B/A= 3) and 90 min. (B/A=1.5). Cell-bound bilirubin was extracted and measured by HPLC. The values at B/A= 3 are Mean ± S.E from three dishes while the values at B/A=1.5 are means from two dishes. The values in parenthesis give the pH of the medium at the end of incubation with bilirubin.

Temperature °C	Bilirubin Uptake (pmole / µg DNA) B/A = 1.5	B/A = 3
4	15.4 (7.68)	30.7 ± 1.8 (7.84)
15	9.2 (7.70)	52.1 ± 10.4 (7
25	15.9 (7.75)	59.6 ± 4.4 (7.98)
37	14.4 (7.70)	94.0 ± 10.7 (7.88)

Figure 2-1. Time course for the uptake of bilirubin by N-115 cells at 37 C. Cells were incubated with bilirubin for different time intervals at 37 C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells. 100 μM bilirubin at B/A=0.8 (x), B/A=1.5 (o), and B/A=3 ( ), 50 μM bilirubin at B/A=1.5 (Δ). The curves for B/A=0.8 & 3 are drawn as rectangular hyperbolas, whereas the curves for B/A=1.5 are drawn as smoothed interpolations because the fitting to rectangular hyperbola results in a straight line and the deviations are considerable.

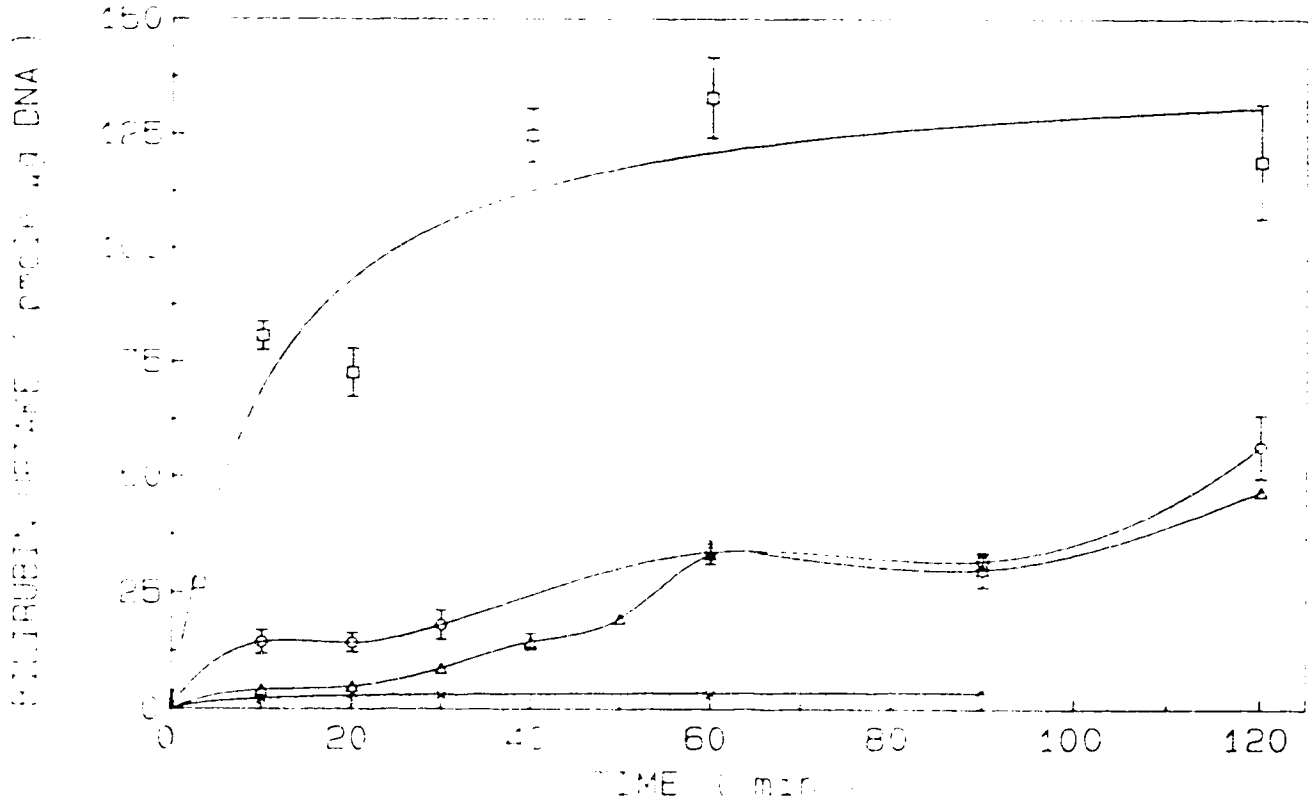


Figure 2-2. Initial rate of uptake of bilirubin by N-115 cells as a function of bilirubin concentration at constant B/A ratio. Cells were incubated with indicated concentrations of bilirubin for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=3 (Δ), and the mean of duplicates (which differ by <18%) for B/A=1.5 (□).

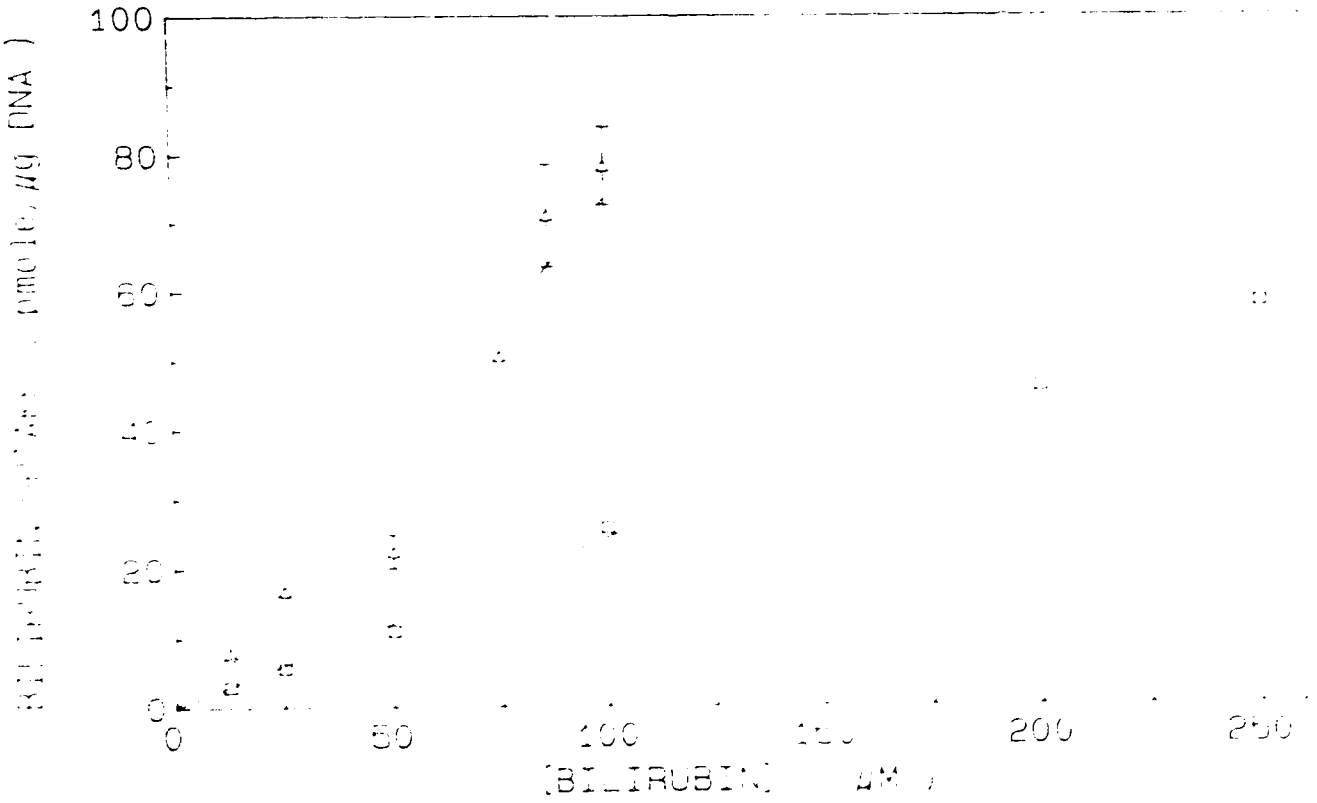


Figure 2-3. Initial rate of uptake of bilirubin by N-115 cells as a function of B/A ratio at a constant concentration of bilirubin. Cells were incubated with 100 μM bilirubin (and varying albumin concentration) for 10 min. at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Cellular uptake of bilirubin is plotted as a function of albumin concentration (A) and as a function of free bilirubin concentration (B). Concentration of free bilirubin was calculated assuming a bilirubin-albumin binding constant of 3.2 x 10^7 M^{-1} [4]. Each point represents the Mean ± S.E. from three dishes of cells.

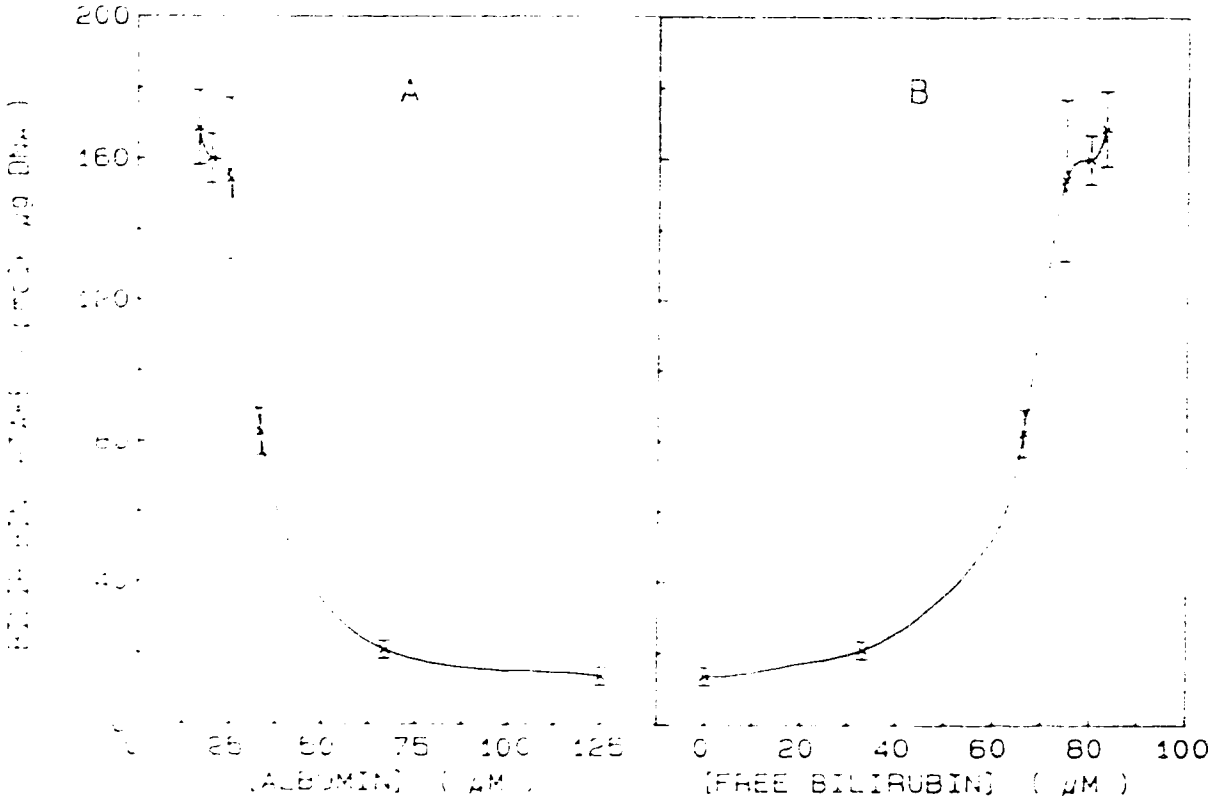
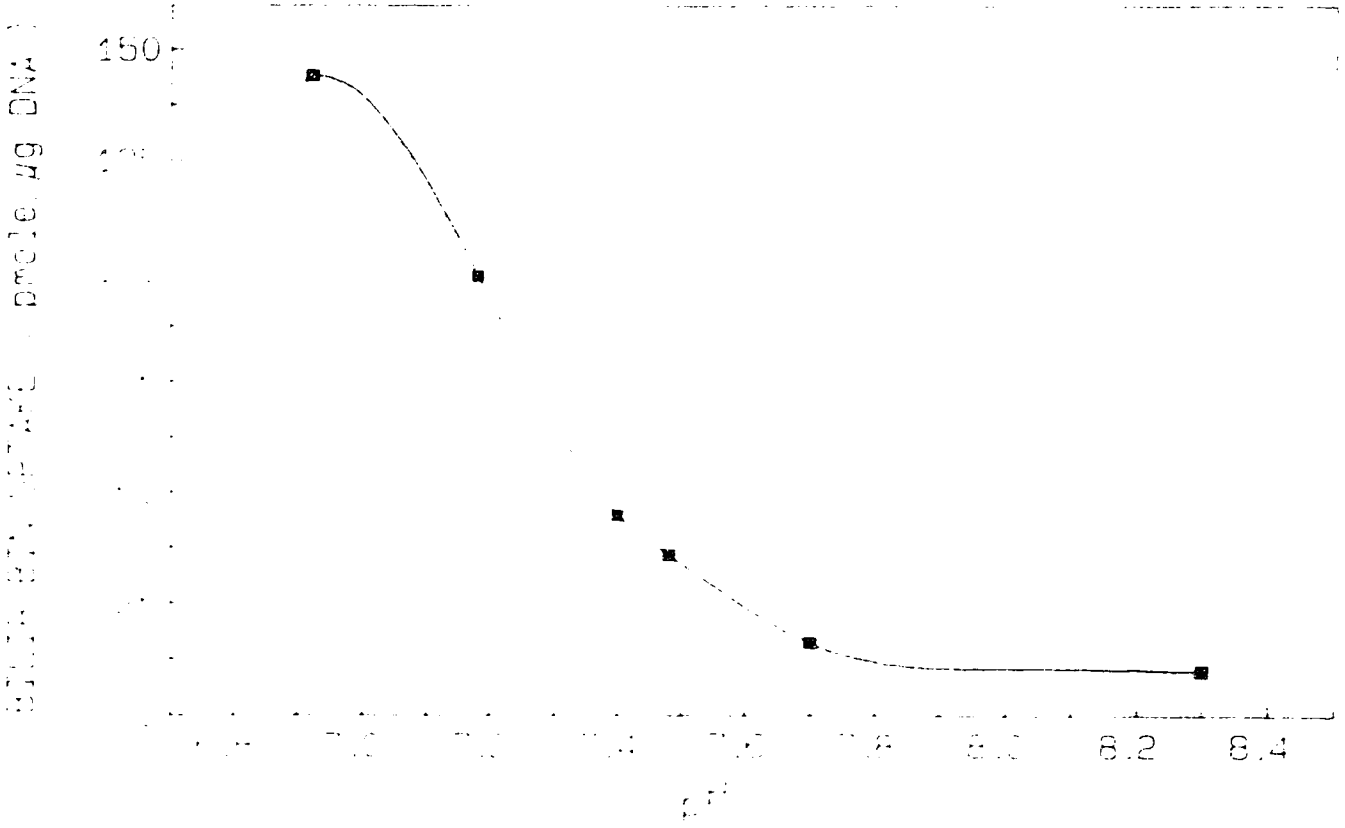


Figure 2-4. Limiting (apparent equilibrium) uptake of bilirubin by N-115 cells as a function of bilirubin concentration. Cells were incubated with indicated concentrations of bilirubin for 2 hours at 37°C and the cell-bound bilirubin was extracted and measured by HPLC. Each point represents the Mean ± S.E. from three dishes of cells for B/A=1.5 (x) and the mean of duplicates (which differ by < 14%) for B/A=3 (○). The dotted lines are linear extrapolations from points with bilirubin concentrations < 50 μM.

Figure 2-5. Effect of pH on bilirubin uptake by N-115 cells. Cells were grown as usual, the media was aseptically removed, 100-200 µL of sterile 1N HCl or 1N NaOH was added to achieve the desired pH and the media was gently poured back into the culture dish. Cells were incubated for 1 hour at 37°C and then 50 µM bilirubin at B/A=1.5 was added. After an additional 90 min. of incubation, cell-bound bilirubin was extracted and measured by HPLC. Each point represents the mean of duplicates (which differ by < 13%).



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CHAPTER 3

Publication No. 2:

Bilirubin Toxicity in a Neuroblastoma Cell Line N-115: I. Effects on Na^+ K^+ ATPase, [^{3}H]-Thymidine Uptake, L-[^{35}S]-Methionine Incorporation, and Mitochondrial Function.

A version of this chapter has been published :

Amit Y, Chan G, Fedunec S, Poznansky MJ,

and Schiff D.

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Introduction

The mechanism of bilirubin encephalopathy has been studied extensively over the past decade. Data has been obtained from tissue examinations using light and electron microscopy [1,2], ***in vitro*** assessment of bilirubin toxicity in different neural and non-neural tissues [3] and, more recently, ***in vivo*** studies of different brain cell functions in Gunn rats suffering from hereditary bilirubin encephalopathy [4].

The major biochemical defect underlying bilirubin encephalopathy has yet to be determined. Studies conducted on neural tissue demonstrated that bilirubin can impair a large number of cellular functions. Among them are: changes in energy metabolism [4,5,6], impairment of various membrane functions and intracellular key enzymes such as Na^+K^+ ATPase, glutamate decarboxylase, lactate dehydrogenase, protein kinase, to name a few [7-11]; alteration in the physical and functional state of the cell membrane [12-16], inhibition of both DNA [17,18] and protein synthesis [19-22], changes in carbohydrate metabolism [23,24], and modulation of neurotransmitter synthesis [25]. However, variation in different cellular functions, the use of concentration of bilirubin higher than usually encountered in the clinical situation, and the use of varying albumin concentrations, thus altering the bilirubin-to-albumin molar ratio (B/A), may account for the multiplicity of effects and inconclusive results. The use of bilirubin without added albumin, or the use of high bilirubin concentrations at high B/A causes rapid aggregation of bilirubin [26-29]. Once aggregates are formed,

changes in free bilirubin concentration occur, and hence may give rise to variable toxicity as the free form of bilirubin seems to be the reactive form.

As bilirubin can interfere with a number of cellular functions, it is clearly important to establish which functions are damaged first in a particular cell population. In the present study, using stable bilirubin-human serum albumin (HSA) mixtures at B/A of 0.8 and 1.5, the effect of bilirubin on $^{42}K^+$ influx, [^{3}H]thymidine uptake, L-[^{35}S]methionine uptake, and mitochondrial function in the neuroblastoma cell line N-115 in culture are investigated.

Materials and Methods

Chemicals . All reagents are analytic grade chemicals and include bilirubin (Lot #13F0846), HSA (fraction V, Essentially Fatty Acid Free), 3-(4,5 dimethylthiazol-y-yl)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma Chemical Co. (St. Louis, MO), Dulbecco's modified Eagle medium (DMEM), and FCS obtained from Grand Island Biological Co. [^{3}H]thymidine (sp act, 15.1 Ci/mmol) and L-[^{35}S]methionine (sp act, 1129 Ci/mmol) were purchased from Dupont, Mississauga, Ontario, Canada. $^{42}K^+$ was produced by irradiation of K_2CO_3 at the Slow Poke reactor, University of Alberta. The sp act was 0.36 mCi/mmol at the end of the radiation, and the experiment was carried out within 4 hr.

Preparation and stability of bilirubin-HSA mixtures. A stock solution of HSA (mol wt, 68,000) was prepared as a 50 μM solution in 50 mM Tris buffer (pH 7.4) and sterilized by filtration.

Immediately before the experiments, a stock solution of 10 mM bilirubin in 0.1 N NaOH was prepared. The composition was verified by high pressure liquid chromatography to contain 92% of the IX-α moiety, the rest being XIII-α and III-α. No other bile pigments were detected. Thus the bilirubin preparation was used without further purification. Then 5.85 mg bilirubin was dissolved in 0.5 mL 0.1 N NaOH. Once in solution, 0.5 mL of diluent at pH 7.8 containing 0.45% NaCl and 0.45% Na_2CO_3 was added. The bilirubin albumin solutions were mixed in various volumes to achieve the desired B/A.

MTT was made up as a 5 mg/mL solution in PBS (pH 7.4) and was filtered to sterilize. The yellow solution was sterile for several weeks when stored in the dark at 4°C. Just before use, one part MTT was mixed with nine parts of protein-free medium.

To determine the stability of bilirubin-HSA mixtures in 50 mM Tris buffer (pH 7.4) the freshly prepared bilirubin stock solution was centrifuged for 5 min at 10,000 x *g* to remove the undissolved material. The stability of bilirubin-HSA mixtures, using 5 μM HSA and bilirubin concentrations ranging from 4.5-36 μM, giving B/A from 0.9-7.2, was measured by spectrophotometry [28]. The mixtures of different B/A were made up in 6 cm tissue culture dishes and diluted to a final volume of 10 mL with Tris buffer, and incubated at 37°C in the dark. At different time intervals (0, 1, 2, and 24 hr), 1 mL of solution was removed into an Eppendorf centrifuge tube, centrifuged at 10,000 x *g* for 5 min, and absorbance at 460 nm measured. All experiments were carried out in a dimly lit room to avoid bilirubin photodegradation. As DMEM contains amino acids,

minerals, and vitamins, bilirubin stability may differ in the medium than in Tris buffer. Hence these experiments were repeated with DMEM. (DMEM also contains a dye, methyl red, which has an absorption maximum of 550 nm at pH 7.4. Interference of culture medium in bilirubin absorption was compensated for by using DMEM as the blank when measurements were made.)

N-115 cells. N-115 cells were seeded on 35-mm culture dishes and allowed to grow in standard DMEM with 10% FCS at 37°C in a 5% CO_2 humidified atmosphere for 12 hr. Then the medium was removed by suction and replaced with 1 mL protein-free media [30] plus HSA for another 12 hr, before the experiment with bilirubin was set up. Bilirubin was prepared as described before and added to the experimental media to make final bilirubin concentrations of 75, 100, or 125 μM and B/A of 0.8 and 1.5. The addition of bilirubin was immediately followed by an amount of 0.1 N HCl equivalent to the amount of NaOH to restore the pH to 7.4. Control cells were seeded and grown as above with HSA added. NaOH (0.1 N) and 0.1 N HCl were added to the media, with no bilirubin, in the same volume as in the bilirubin-treated cells.

$^{42}K^+$ influx. To assess the effect of bilirubin on Na^+K^+ ATPase activity, the following series of experiments were carried out. Cells were seeded at a density of 4-5 x 10^5/plate and prepared as described above. Bilirubin in concentrations of 75 μM and 100 μM and B/A 0.8 and 1.5 was added to the test cells and incubated for 2 and 4 hr. To determine the bilirubin effect, if any, on passive or active transport of K^+, another set of similar experiments was done

in which the cells were incubated in medium containing 0.5 mM ouabain for 10 min before the addition of $^{42}K^+$. At 1 hr before the end of the incubation period, 2 mM $^{42}K^+$ was added. At the end of the 60 min incubation period, the culture dishes were placed on ice, the medium was removed, and the cells washed five times with ice-cold PBS [31]. Then the cells were harvested in 0.5 mL PBS, scraped off into an Eppendorf test tube, and counted in a Beckman Gamma Counter (Beckman Instruments, Fullerton, CA) for 1 min. The background counts were always less than 1% of the total and were subtracted from the total counts.

$^{42}K^+$ influx. and [^{3}H]thymidine uptake. To compare the effect of bilirubin on $^{42}K^+$ influx and [^{3}H]thymidine uptake, the cells were grown as before and exposed to 125 μM bilirubin with B/A of 0.8 and 1.5 for 2, 4, and 6 hr. At 1 hr before the end of the incubation period, the cells were pulse labelled with $^{42}K^+$ and handled as described above. At the time of the addition of $^{42}K^+$, cells were also pulse labelled with [^{3}H]thymidine, 2 μCi/dish for 60 min to assess thymidine uptake by the cells. Uptake was assessed as reported previously [18].

MTT assay. The MTT assay has been used effectively for assessment of cell viability [32]. It assesses the ability of the mitochondria to cleave the dye to form a dark blue formazan. To achieve this, the cells were seeded and grown as described above and exposed to 100 μM bilirubin, B/A ratio of 1.5 for 0, 1, 2, 3, and 4 hr. MTT was prepared and sterilized as described before [18, 32]. At 60 min before the end of the exposure to bilirubin, 100 μl of the MTT

was added. At the end of the incubation period, the cleaved dye, seen as blue crystals within the cells, was dissolved in 1mL of isopropanol HCl (0.04 N) by agitation with repeated pipetting until a blue solution was obtained. The absorbance of the individual cultures was then read in a diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) with a test wavelength of 570 nm and a reference wavelength of 630 nm. The difference in absorbance is a direct measure of mitochondrial function and cell viability [32].

L-[^{35}S]methionine incorporation into protein. N-115 cells were seeded and grown as before and exposed to 100 μM bilirubin, at B/A ratio of 1.5, for 0, 1, 2, 3, and 4 hr. At 1 hr before the end of the incubation period, the cells were pulse labelled with 10 μL of 1/100 dilution of a stock solution of L-[^{35}S]methionine for 60 min. At the end of the incubation, the medium was removed into an Eppendorf test tube, the cells were suspended in 0.5 mL PBS and scraped off into another Eppendorf test tube. Proteins in the medium and in the cells were precipitated with 1 mL of 10% trichloroacetic acid solution. After centrifugation at 10,000 x *g* for 5 min, the supernatant was removed and the pellet redissolved in 1 N NaOH. Half of the pellet was measured for radioactivity by liquid scintillation counting using ACS (Amersham Corp., Arlington Heights, IL) as scintillant and the other half used for protein estimation [33].

Results

Bilirubin stability. Figure 1 demonstrates the stability of 35 μM bilirubin in 50 mM Tris buffer solution at different B/A in the range of 0.5 to 8 at 37°C. In the albumin free state, virtually all of the bilirubin precipitated out of the solution immediately. At a B/A of 1 or less, the absorbance of the bilirubin solution remained unchanged over a 24 hr period. The bilirubin solution became less stable as the B/A increased and declined to 60% of the initial level at a B/A of 8. The same pattern was seen when DMEM was used instead of Tris buffer.

$^{42}K^+$ influx. The effect of bilirubin on $^{42}K^+$ influx is given in Table 1. The total $^{42}K^+$ influx is inhibited by bilirubin only at a concentration of 100 μM and B/A 1.5, and this became manifest only after 4 hr of exposure. In this time frame, the portion affected is only the ouabain inhibitable or active (Na^+K^+ ATPase) component. No effect was seen on the ouabain resistant, or passive influx component.

$^{42}K^+$ influx. and [^{3}H]thymidine uptake. The effect of exposure to 125 μM bilirubin, at B/A of 1.5, on $^{42}K^+$ influx and [^{3}H]thymidine uptake, is depicted in Figure 2. Uptake of [^{3}H]thymidine was decreased by 40% of control values within 4 hr of exposure. $^{42}K^+$ influx was affected only after 4 hr of exposure, and to a lesser degree than the effect on [^{3}H]thymidine uptake.

MTT assay. The mitochondrial function measured as the difference in absorbance at 560 and 630 nm in the MTT assay is

given in table 2. The results show a decrease in the ability of the bilirubin treated cells to cleave the MTT dye shortly after the exposure. Although a 43% reduction in activity was seen after 2 hr of exposure to bilirubin, the most pronounced effect on viability (63% reduction of activity) was seen after 4 hr exposure.

L-[^{35}S]methionine incorporation into protein. At the end of 2, 3, and 4 hr exposure of the cells to 100-μM bilirubin, B/A of 1.5, there was a significant decrease in L-[^{35}S]methionine incorporation into protein compared to control (Fig. 3). The decrease in incorporation into protein was noticed in proteins extracted from cells and media.

Discussion

The mechanism of bilirubin toxicity to the central nervous system has been debated extensively over the past years. The difficulties in analyzing the results and the inability to point to a primary bilirubin target stems from variation in experimental designs, the use of different animal models and difficulties in correlating the chemical, biochemical, and clinical knowledge of the bilirubin molecule in ***in vitro*** and ***in vivo*** experiments.

Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, the hyperbilirubinemic Gunn rat served as the model; in the other, brain cells from normally developed animals were used. The difference between the two is a major one. The use of the Gunn rat as a model for bilirubin encephalopathy is based on the assumption that the

damage seen is primarily due to bilirubin. Studies in the Gunn rat have shown that bilirubin is indeed toxic to the mitochondria [4], causes changes in membrane morphology [2], affects glycolytic [23,24] and other cellular enzymes [9], modulates neurotransmitter synthesis [25] and may inhibit protein [19-22] and DNA [17] synthesis. Though extensive damage to the nervous system in the Gunn rat can be attributed to bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded [34,35].

Exposure of neural cells to bilirubin for a limited time period may not properly reflect the more prolonged influx of bilirubin encountered in the clinical situation. Yet the input from many studies points to damaging effects. Bilirubin was shown to impair mitochondrial reactions [5,6,18], and to inhibit various cellular enzymes either directly [7,8,10,11] or via alterations in the membranes [12-16]. Despite this, no specific target has been singled out as the primary one for bilirubin toxicity.

A major concern when experimenting with a B/A that exceeds one, is the instability of the bilirubin solution leading to formation of bilirubin aggregates and coaggregates of bilirubin and albumin [26-29]. Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been addressed in experiments dealing with bilirubin toxicity ***in vitro*** [24, 37-41]. In previously reported data, when either free bilirubin or bilirubin in excess of albumin was used, the toxic effect appears to be an instant one, within minutes

of exposure [4-7,10,15]. One often used solution to the bilirubin stability problem is raising the pH of the buffer to 8.2 or higher [16,42,43]. This proved to be an impractical approach when experimenting with live cells. When complexed with HSA, bilirubin in solution is stable at different concentrations [27]. In carrying out the studies herein described, attention is given to the stability of bilirubin in DMEM solutions. Under these conditions, where bilirubin is maintained in solution, toxicity is slow in occurring and is dependent on both the amount of free bilirubin and the time of exposure. Direct interaction of bilirubin with the purified enzymes, as opposed to interaction with the whole cell, may well be the reason for the time difference, but one cannot exclude the possibility that toxicity was delayed or did not occur as a result of bilirubin instability in solution.

In the present investigation, we have shown that bilirubin, at concentrations of 35-125 μM and B/A of 1.5, is stable over a 24-hr period in the medium (DMEM) for the neuroblastoma cell line N-115. When applying this approach to cell studies, it was noticed that at 50 μM bilirubin and B/A ≤ 0.8, bilirubin binding by the N-115 cell in monolayer culture was negligible. However at B/A 1.5, where loss of bilirubin after 24 hr was less than 10%, cellular uptake of bilirubin in 2 hr was found to be 110 ng/μg DNA [18].

In the studies herein reported, the effects of bilirubin on four vital cellular functions mitochondrial activity, protein synthesis, DNA synthesis, and ion transport were evident. A significant reduction in mitochondrial activity is seen within 2 hr of exposure.

This effect seems to occur early and is more pronounced than the effect seen on [^{3}H]thymidine uptake, L-[^{35}S]methionine incorporation into protein or $^{42}K^{+}$ influx. The bilirubin effect on $^{42}K^{+}$ influx is ouabain sensitive, and is a reflection of the effect of bilirubin on $Na^{+}K^{+}$ ATPase activity. In all these instances the effects seem to be dependent on the B/A, bilirubin concentration, and the duration of exposure. From these studies, it is not possible to single out conclusively the primary target for bilirubin toxicity, although the data suggest an earlier and more pronounced effect occurring with mitochondrial function. The effect on L-[^{35}S]methionine incorporation into protein seems to develop later in the course of the bilirubin exposure and is less pronounced then that seen with [^{3}H]thymidine uptake. It is difficult from these experiments to determine whether these two observations are a direct result of bilirubin toxicity or secondary effects arising from the initial effect of bilirubin toxicity on mitochondrial function.

It is therefore concluded that in studies involving cells in culture, where it becomes important to know that the amount of bilirubin utilized remains stable and in solution, the optimal B/A to use is < 2. In this fashion, reproducibility of conditions related to bilirubin toxicity of the cells can be achieved. Using this approach, it has been demonstrated that bilirubin affects mitochondrial function, [^{3}H]thymidine uptake, L-[^{35}S]methionine incorporation into protein and $Na^{+}K^{+}$ ATPase activity of the N-115 cell. As mitochondrial dysfunction precedes the other three effects and as ATP is required for protein and DNA synthesis, as well as for K^{+}

transport, these results point to the possibility that mitochondria may be the primary target of bilirubin toxicity.

Table 3-1. Effect of bilirubin treatment of N-115 cells on $^{42}K^+$ influx *

B/A ratio	Bilirubin (μM)	$^{42}K^+$ influx (cpm/μg DNA/hr) 2 hr bilirubin exposure: Ouabain resistant	$^{42}K^+$ influx (cpm/μg DNA/hr) 2 hr bilirubin exposure: Ouabain inhibitable	$^{42}K^+$ influx (cpm/μg DNA/hr) 4 hr bilirubin exposure: Ouabain resistant	$^{42}K^+$ influx (cpm/μg DNA/hr) 4 hr bilirubin exposure: Ouabain inhibitable
	Control	38 ± 3	210 ± 4.3	45 ± 6	202 ± 2.5
0.8	75	36 ± 5	207 ± 4.5	44.9 ± 3	208 ± 4.7
	Control	44 ± 3	214 ± 5.4	52 ± 0.5	207 ± 6.9
0.8	100	42 ± 5	208 ± 5.7	40 ± 0.3	207 ± 3.5
	Control	37 ± 2	166 ± 4.8	44 ± 4	166 ± 3.4
1.5	75	31 ± 6	167 ± 3.5	40 ± 4	123 ± 4.6 †
	Control	41 ± 4	169 ± 3.1	42 ± 3	163 ± 4.5
1.5	100	36 ± 5	160 ± 3.0	37 ± 3	130 ± 1.3 ††

* Values given are mean ± SD from three measurement x 2

† $p = 0.00001$

†† $p = 0.0003$

Table 3-2. MTT assay for viability of control and bilirubin-treated cells*

Duration of exposure (hr)	Differences in absorbance (A560-A630)		
	Control cells	bilirubin-treated cells	% Control
0	0.2548 ± 0.0122¶	0.2502 ± 0.0045	100
1	0.2318 ± 0.0062	0.2071 ± 0.0096	87
2	0.2220 ± 0.0172	0.1203 ± 0.0072	57
3	0.2066 ± 0.0046	0.1203 ± 0.0072	60
4	0.2153 ± 0.0014	0.0799 ± 0.0133	37

* Cells were treated with 100 μM bilirubin, B/A 1.5. An equivalent amount of HSA was added to control cells.

¶ Mean ± SD of two triplicate analyses.

Figure 3-1. The solubility of 35 μM bilirubin (Br) in 50 mM Tris buffer at B/A of 0.5 (1), 1.0 (2), 2.0 (3), 4.0 (4) and 8.0 (5). Curve (6) represents 35 μM bilirubin in Tris buffer in the absence of albumin. Absorbances were measured at 0, 0.5, 1, 3, and 24 hr. In the absence of albumin, bilirubin precipitates out of solution within 30 min., whereas at B/A up to 3, up to 10% bilirubin is lost by 24 hr. Similar results were obtained for DMEM.

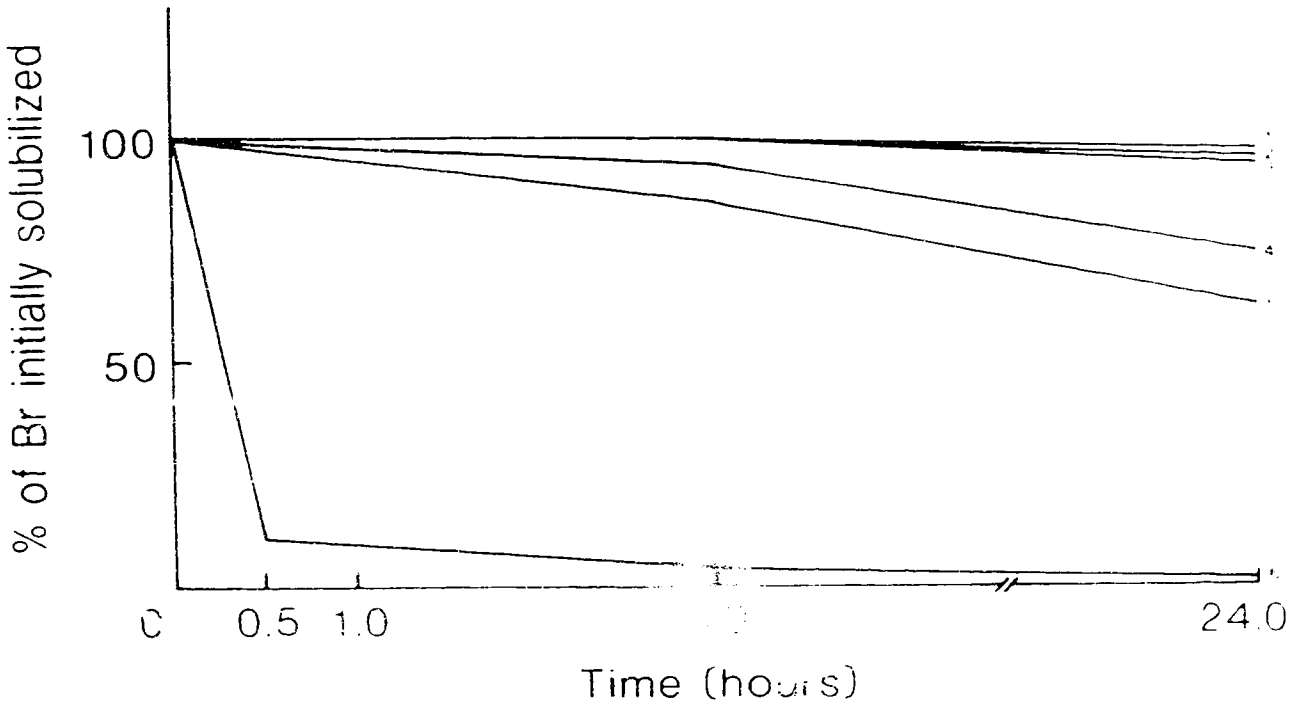


Figure 3-2. The effect of 125 μM bilirubin on [^{3}H]thymidine uptake (a) and $^{42}K^+$ influx by N-115 cells. Data expressed as CPM/μg DNA accumulated over 1 hr period. Control - (▭) , B/A = 0.8 - (▨), and B/A = 1.5 - (■).

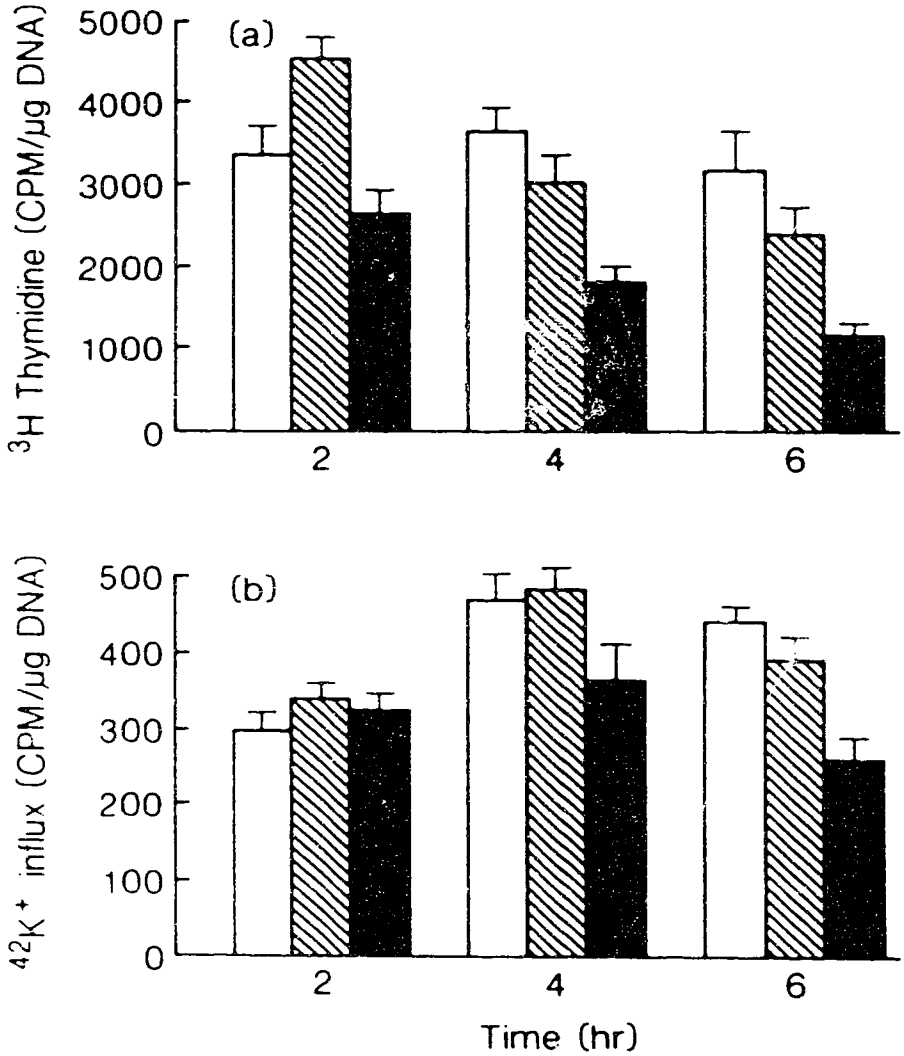
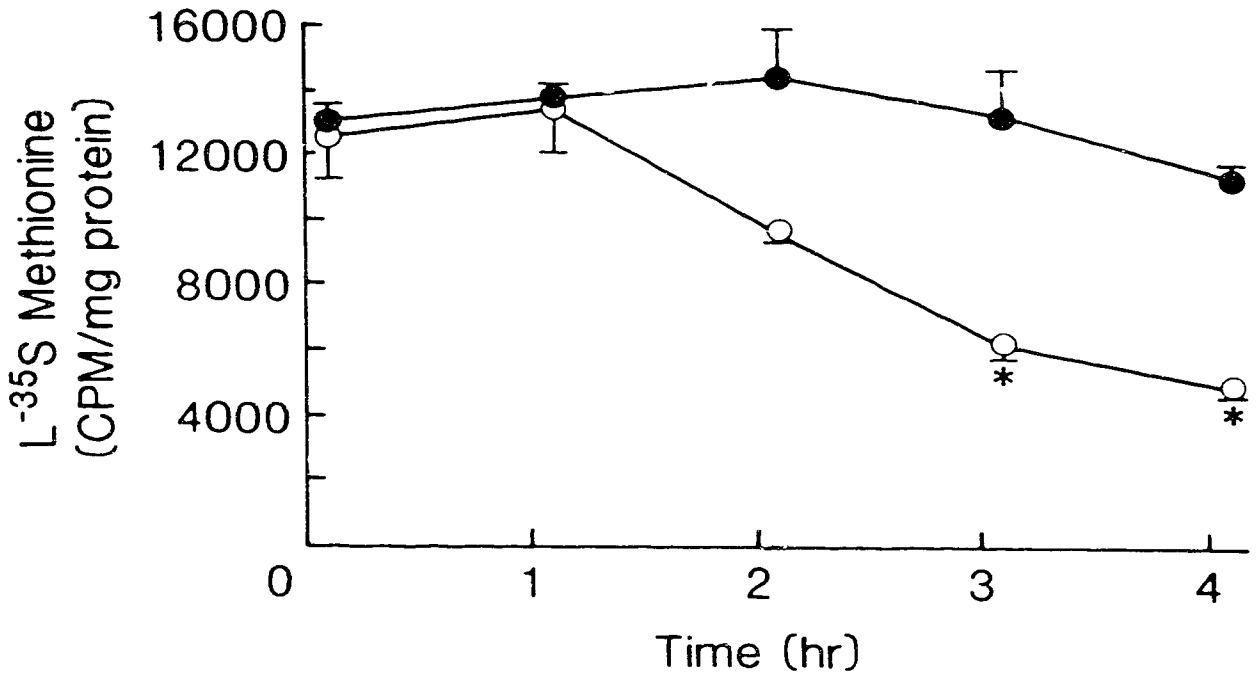


Figure 3-3. The effect of 100 μ M bilirubin on L-[^{35}S]methionine uptake by N-115 cells at B/A = 1.5 (o) compared to control (•). Data is expressed as CPM/mg protein accumulated over 1 hr period.



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CHAPTER 4

Publication No. 3:

Bilirubin Toxicity in a Neuroblastoma Cell Line N-115: II. Delayed Effects and Recovery

A version of this chapter has been published :

Amit Y, Poznansky MJ, and Schiff D.

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Introduction

Clinical studies have recently suggested that in the presence of hyperbilirubinemia the newborn infant will demonstrate abnormal evoked brain stem potentials, an indication of the early stages of bilirubin encephalopathy. Upon resolution of the hyperbilirubinemia, either spontaneously or after exchange transfusion, these abnormalities disappear. This suggests a reversibility of the early stages of bilirubin encephalopathy [1-3]. The role of albumin binding [4,5], the integrity of the blood brain barrier [6], and the possible presence of a bilirubin oxidase enzyme [7] have been suggested as possible mechanisms in this phenomenon. Cowger [8] demonstrated that bilirubin toxicity in a tissue culture system has shown a reversibility with the addition of albumin. More recently, Hansen et al [9] demonstrated a similar phenomenon in Hippocampal slices, and Wennberg [10] has shown a reversibility phenomenon in red blood cell and mitochondrial uptake of bilirubin.

Laboratory studies have demonstrated that bilirubin can affect a host of different cellular functions [11]. Recent studies from our laboratory have shown that bilirubin toxicity in the neuroblastoma cell line N-115 was dependent on bilirubin concentration, bilirubin-to-albumin molar ratio (B/A), and time of exposure [12]. The effect on mitochondrial function, [^{3}H]thymidine uptake and L-[^{35}S]methionine uptake become manifest after 2 hr of bilirubin exposure [12]. As these vital cellular functions are affected by a short-term exposure to bilirubin, the N-115 cell line

offers a good model system to study the early stages of bilirubin toxicity. The present study examines the reversibility of bilirubin toxicity in the early stages with respect to cellular function, as well as the possible delayed effects of a short-term exposure to three cellular functions, wherein no bilirubin toxicity was manifest. Contrary to the clinical situation, the present results indicate an irreversibility even in the early stages of bilirubin toxicity to the neural cell line.

Materials and Methods

Chemicals. All reagents were analytical grade chemicals and include bilirubin (Lot #13F0846), human serum albumin (HSA, fraction V, Essentially Fatty Acid Free), and 3-(4,5 dimethylthiazol-y-yl)-2,5-diphenyl tetrazolium bromide (MTT) purchased from Sigma Chemical Co., St. Louis, MO. Bilirubin purity was verified as previously described [12]. Dulbecco's modified Eagle medium (DMEM), Dulbecco's phosphate buffer saline (PBS), and fetal calf serum (FCS) were obtained from Grand Island Biological Co. [^{3}H]thymidine (sp act, 15.1 Ci/mmol) and L-[^{35}S]methionine (sp act, 1129 Ci/mmol) were purchased from Dupont, Mississauga, Ontario, Canada.

N-115 cells. Cells of the murine neuroblastoma cell line N-115 were seeded at a concentration of 5-8 x 10^{5} cells/plate on 35mm culture dishes (Falcon Labware, Oxnard, CA) and grown in standard DMEM plus 10% FCS, pH 7.4, at 37°C, in a 5% CO_2 humidified atmosphere for 12 hr. The medium was then removed,

the cells washed twice with sterile PBS and reincubated in 1 mL of protein-free medium (PFM) [12], plus HSA for another 12 hr before setting up the experiments with bilirubin. The HSA and bilirubin concentrations were varied in different experiments to meet the required final B/A.

Experimental conditions. All procedures involving bilirubin were carried out in a dimly lit room. Stock solutions of bilirubin in 0.1 N NaOH and HSA in PBS were prepared as described previously [12]. Bilirubin was added to the culture medium to achieve the appropriate bilirubin concentration and B/A. An equimolar amount of 0.1 N HCl was added to restore the pH of the media to 7.4. Control cells were grown as above, and 0.1 N NaOH and 0.1 N HCl were added to the media in the same volume as in the medium of the bilirubin-treated cells.

At the end of the exposure to bilirubin, the medium was gently removed, and the cells were washed twice with sterile PBS and reincubated in PFM plus HSA. Each of the studies outlined below were carried out in triplicate analyses.

Toxicity was assessed at appropriate intervals as follows :

1) To assess cell viability and mitochondrial function, MTT was prepared and sterilized as described before [12]. Aliquots of 100 μL were added to the medium and incubated for 60 min. Then the cleaved dye was dissolved in 1 mL isopropanol-HCl (0.04N), by agitation with repeated pipetting, until a blue solution was obtained. The absorbance of the individual culture dish was then

read in a diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) with a test wavelength of 570 nm and a reference wavelength of 630 nm [13]. Only live cell will cleave the dye to give an increase in absorbance at 570 nm. The difference in absorbance at 570 and 630 nm is a direct measure of mitochondrial function and cell viability .

2) [^{3}H]thymidine and L-[^{35}S]methionine uptake: At 1 hr before the end of the reincubation period, the cells were pulse labelled with either [^{3}H]thymidine (2 μCi/plate) or L-[^{35}S]methionine (25 μCi/plate) for 60 min. The medium was removed, the cells were washed twice with PBS, and dislodged from the plate and suspended in 0.5 mL PBS in an Eppendorf test tube. The cell suspension was then vortexed, and aliquots were taken for DNA [14] or protein [15] estimation and for measuring the radioactivity by liquid scintillation counting using ACS (Amersham Corp., Arlington Heights, IL) as scintillant. The radioactivity related to L-[^{35}S]methionine uptake was measured in the cellular protein fraction precipitated with 1 mL 10% trichloroacetic acid.

To determine whether cells exposed to bilirubin, without evidence of toxicity, continue to function normally after a bilirubin washout and whether cells that already demonstrate bilirubin toxicity can recover their function once removed from bilirubin, the following experiments were carried out:

1) Cells were exposed to 100 μM bilirubin, B/A 1.5 for 0.5, 1, 2, 3, and 4 hr. The cells were washed free of bilirubin and

reincubated in fresh PFM containing 66 μM HSA. Mitochondrial function (MTT assay), and [^{3}H]thymidine uptake were then assessed at 2, 8, and 24 hr after the bilirubin washout. L-[^{35}S]methionine uptake (TCA precipitable) was assessed in a similar fashion, but only after 1 and 2 hr bilirubin exposure.

2) To determine whether varying bilirubin concentration modifies the responses, cells were incubated with bilirubin concentrations of 25, 50, 75, and 100 μM, B/A 1.5, for 1 and 2 hr. The cells were then washed free of bilirubin and reincubated in fresh PFM with appropriate concentrations of HSA. [^{3}H]thymidine uptake was assessed at 2, 8, and 24 hr after the bilirubin washout.

3) To assess the role different B/A may have on these cells and their ability to recover from bilirubin toxicity, the following experiment was carried out. Cells were exposed to bilirubin concentrations ranging from 50 to 200 μM, with 100 μM HSA, yielding B/A of 0.5, 1, 1.5, and 2. After 2 hr exposure to bilirubin, the cells were washed free of bilirubin and reincubated in fresh PFM containing 100 μM HSA. [^{3}H]thymidine uptake, L-[^{35}S]methionine uptake, and MTT assay were carried out at 2 and 24 hr after the bilirubin washout.

Results

The effect of bilirubin (100 μM, B/A 1.5) exposure for different durations on [^{3}H]thymidine uptake and mitochondrial function are given in Figure 1 and Table 1. Cells exposed to bilirubin for 0.5 and 1 hr show no effect on [^{3}H]thymidine uptake

and MTT assay. The cells appear to be functioning normally 8 hr after the bilirubin washout. However, at 24 hr there is a significant reduction in these cellular functions. In cells exposed to bilirubin for 2 hours or longer, significant reductions in these functions are seen at 2 hr after the bilirubin washout, and this effect is progressive with time. At 24 hr, the cell viability is well below 20%. Similar results are obtained when toxicity is assessed by L-[^{35}S]methionine uptake as seen in Figure 2.

The effect of varying bilirubin concentration on [^{3}H]thymidine uptake is demonstrated in Figure 3. After exposure to bilirubin of 25, 50, 75, and 100 μM, B/A 1.5, the cells do not demonstrate any toxic effect for the first 8 hr after the bilirubin washout. After 24 hr, there is a significant decrease in [^{3}H]thymidine uptake, which is more pronounced at 100 μM bilirubin; 70% compared to 25 % at 25 μM bilirubin. After a 2 hr exposure to bilirubin, the suppression of [^{3}H]thymidine uptake becomes apparent at 2 hr after the bilirubin washout at concentrations above 50 μM bilirubin. At 8 hr and 24 hr, [^{3}H]thymidine uptake is reduced at all concentrations of bilirubin, and this is more pronounced at the higher bilirubin concentrations (Fig 3).

The effect of varying B/A on bilirubin toxicity and the inability of the cells to regain normal function after this exposure is demonstrated in Figure 4. After a 4 hr exposure of the cells to medium containing constant HSA and variable bilirubin concentrations, the 2 hr post bilirubin washout period is

associated with mitochondrial dysfunction after incubating the cells with solutions of B/A greater than 1. No signs of toxicity are evident at B/A 0.5. A similar effect is also seen with [^{3}H]thymidine and L-[^{35}S]methionine uptakes at B/A greater than one. After 24 hr the damaged cells demonstrate a continuing reduction in all cell functions tested with no signs of recovery. These effects are more pronounced at the higher B/A.

Discussion

It is well established that bilirubin is toxic to neural cells; however, the mechanism and pathogenesis of its toxicity remains unclear [6,8]. As preventive measures are taken very early during the course of neonatal hyperbilirubinemia, the number of cases with irreversible bilirubin encephalopathy are now of rare occurrence. However, clinical studies using auditory brainstem evoked responses in hyperbilirubinemic neonates have shown reversibility of the acute toxic effects of bilirubin. Once the hyperbilirubinemia has subsided, the abnormal responses were seen to normalize [1-3]. A similar phenomenon has been suggested in laboratory studies on different cell systems [8-10,16].

The removal of tissue-bound bilirubin is thought to be a process comprising either changes in B/A, enzymatic oxidation of bilirubin, or clearance of bilirubin from otherwise undamaged brain tissue to the blood. By virtue of the albumin's affinity for bilirubin the use of an albumin infusion during hyperbilirubinemia and/or

exchange transfusion has been proposed as a means of protecting the infant's brain from the pigment and/or indeed removing the bilirubin from brain tissue [4,17,18]. Brodersen [7] has suggested the possibility of a bilirubin oxidase enzyme within the neural cell, which might play a role in protecting the cell by oxidizing the unbound pigment. Bilirubin is capable of free diffusion across the lipid bilayer [19] and can effectively cross the cell membrane and move back into the circulation [6].

What makes some bilirubin toxic effects reversible and others irreversible is unclear. Although the auditory brain stem evoked responses still needs further refinement before a definitive conclusion can be made [20], in most of the laboratory studies, the toxic effects have been observed under bilirubin concentrations and B/A not usually encountered in the clinical situation [6].

In the studies reported here, where stable bilirubin solutions were used [21], it was possible to demonstrate that bilirubin is indeed toxic to various cellular functions. Once toxicity appeared, this was irreversible, despite reincubation of the cells with fresh medium and HSA in the absence of bilirubin. Moreover, after a short term exposure of 60 min, during which toxicity was not manifest, bilirubin-induced toxicity appeared later on. This can be used as an argument against the presence of a bilirubin oxidase enzyme system in the N-115 cell line. The presence of this enzyme in neural cells is speculative and to date has not been characterized. The fact that in this study the cells do not recover from bilirubin exposure and toxicity may be a reflection of the duration of the

exposure and the higher B/A used. In the study recently reported by Wennberg [10], the duration of bilirubin exposure was 15 min and B/A did not exceed 1.3. Under these conditions, he was able to remove bilirubin taken up by the red blood cell. The red blood cell is a known bilirubin-carrying agent and is a useful model for assessing bilirubin transport and bilirubin binding to tissues; as such, it may not necessarily reflect the mechanism for bilirubin toxicity in neural intracellular organelles. Nonetheless in both cell systems and in the clinical situation, the amount of free bilirubin available and the duration of exposure would appear to be among the critical factors leading to the irreversible stage of bilirubin toxicity. The amount of free bilirubin present in a system is dependent on the albumin concentration. The binding and buffering capacity of plasma albumin to bilirubin begins to break down rapidly as the B/A approaches unity [22]. Moreover, albumin binding is transitory and reversible with the bound and the free bilirubin molecules undergoing rapid exchange [23]. These dynamic changes could account for the toxicity seen in cells exposed to solutions of $B/A \geq 1$ and for the permanent effect bilirubin has on purified proteins [16].

It is concluded that under appropriate conditions of bilirubin concentration, B/A, and time of exposure, the bilirubin toxicity in the N-115 cell is a progressive and irreversible process. The critical safe time before the development of toxicity has not been defined for this cell line, and as yet not for the clinical situation. This has significant implications to our understanding of the

pathogenesis of bilirubin encephalopathy, and subsequent clinical management of the hyperbilirubinemic infant and prevention of bilirubin encephalopathy.

Table 4-1. Effect of bilirubin exposure time (A- 0.5 and 1 hr, B- 2, 3, and 4 hr) on cell viability and mitochondrial function and recovery potential 2, 8, and 24 hr after the cells are washed free of bilirubin. samples assayed in triplicate, and expressed as Mean ± S.D

A

Reincubation	Control		0.5 hr		1 hr	
(hr)	DNA*	MTT†	DNA	MTT	DNA	MTT
2	15.63 ±0.06	0.1735 ±0.0082	14.55 ±0.34	0.1261 ±0.0072	13.99 ±0.30	0.1331 ±0.0071
8	14.03 ±1.64	0.2185 ±0.0091	15.50 ±1.64	0.1734 ±0.0351	12.75 ±0.43	0.1989 ±0.0094
24	22.99 ±1.93	0.1567 ±0.0006	22.32 ±2.96	0.0618 ±0.0114	19.77 ±2.88	0.0422 ±0.0117

B

Re-incubation	Control		2 hr		3 hr		4 hr	
(hr)	DNA*	MTT†	DNA	MTT	DNA	MTT	DNA	MTT
2	9.48 ±0.81	0.1090 ±0.0103	9.34 ±1.12	0.0535 ±0.0044	9.21 ±0.76	0.0468 ±0.0082	8.28 ±1.35	0.0509 ±0.0027
8	18.81 ±0.34	0.1382 ±0.0091	18.23 ±2.51	0.0342 ±0.0059	15.66 ±3.56	0.0338 ±0.0096	17.53 ±2.27	0.0102 ±0.0021
24	22.17 ±0.81	0.0863 ±0.0145	19.79 ±1.82	0.0035 ±0.0061	19.17 ±1.82	0.0011 ±0.0019	17.27 ±1.05	0.0017 ±0.0030

* Expressed as µg/plate.
† Expressed as ΔABS (570-630) nm.

Figure 4-1. The effect of reincubation of cells in fresh bilirubin-free medium (after bilirubin removal) on the MTT assay (A) and [^{3}H]thymidine uptake (B) of N-115 cells initially exposed to 100 μM bilirubin, B/A 1.5 for 0.5 hr (•); 1 hr (o); 2 hr (▪); 3 hr (□); and 4 hr (Δ).

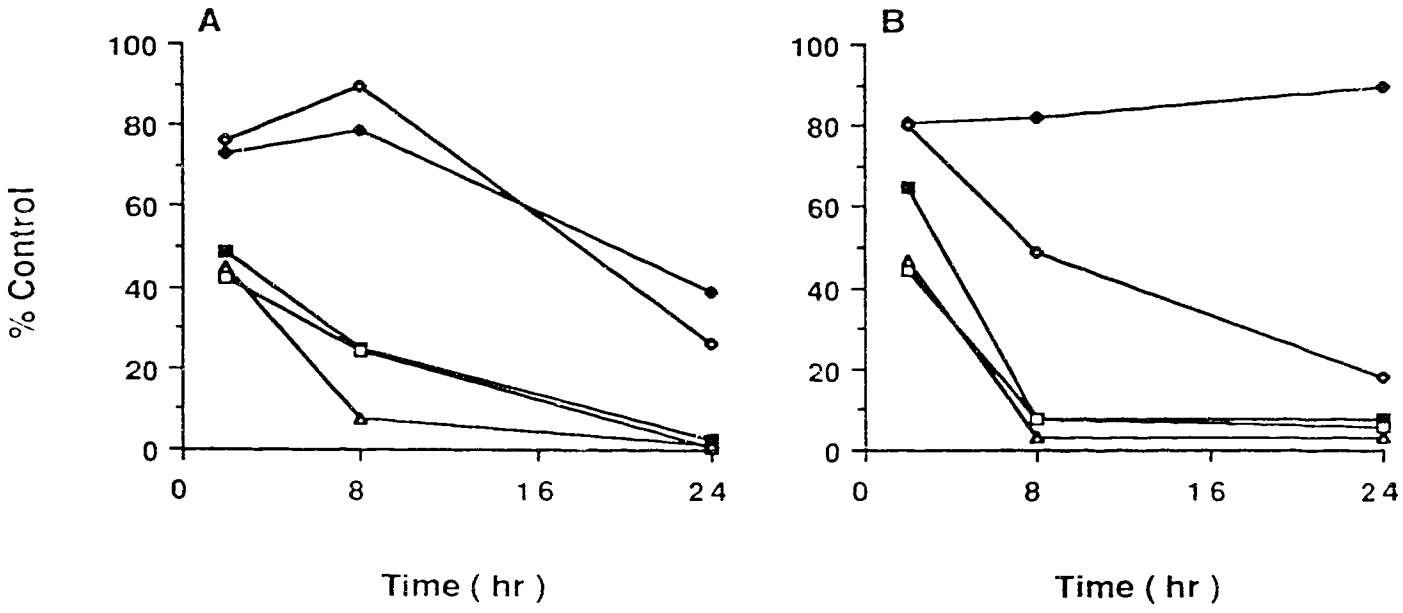


Figure 4-2. The effect of reincubation of cells in fresh bilirubin-free medium (after bilirubin removal) on L-[^{35}S]methionine uptake by N-115 cells initially exposed to 100 μM bilirubin, B/A 1.5 for 1 hr (•) and 2 hr (o).

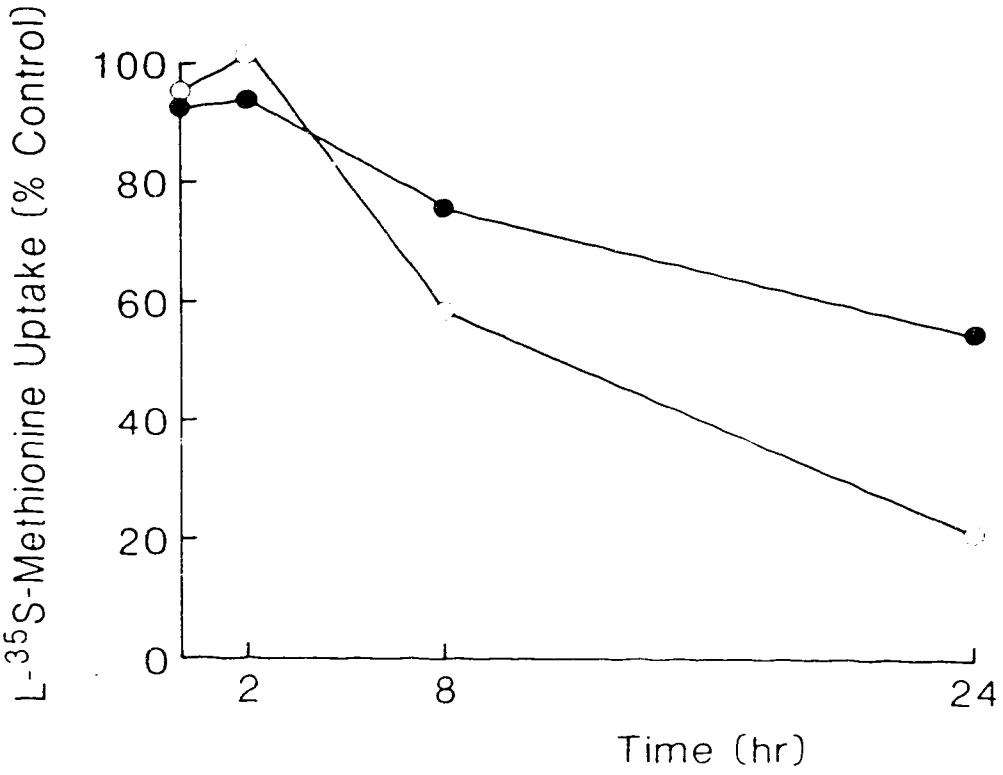


Figure 4-3. The effect of reincubation of cells in fresh bilirubin-free medium on [^{3}H]thymidine uptake. Cells were exposed to 25 μM bilirubin (•),50 μM bilirubin (o), 75 μM bilirubin (◆), and 100 μM bilirubin (◊), B/A 1.5, for 1 hr (A) and 2 hr (B).

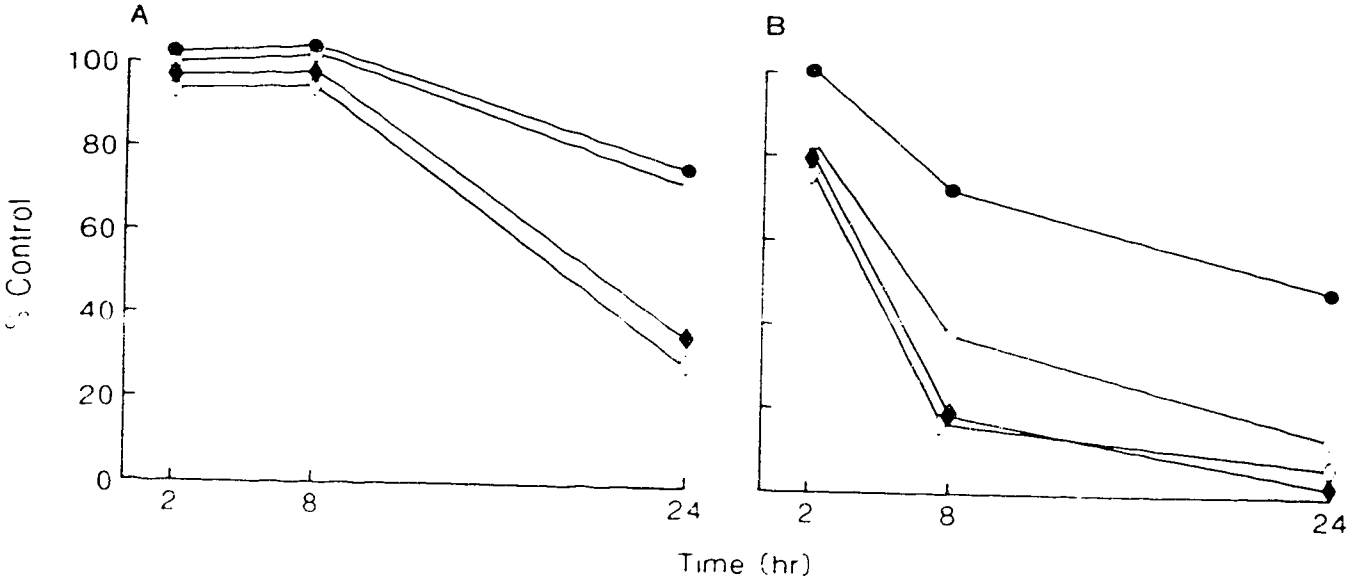
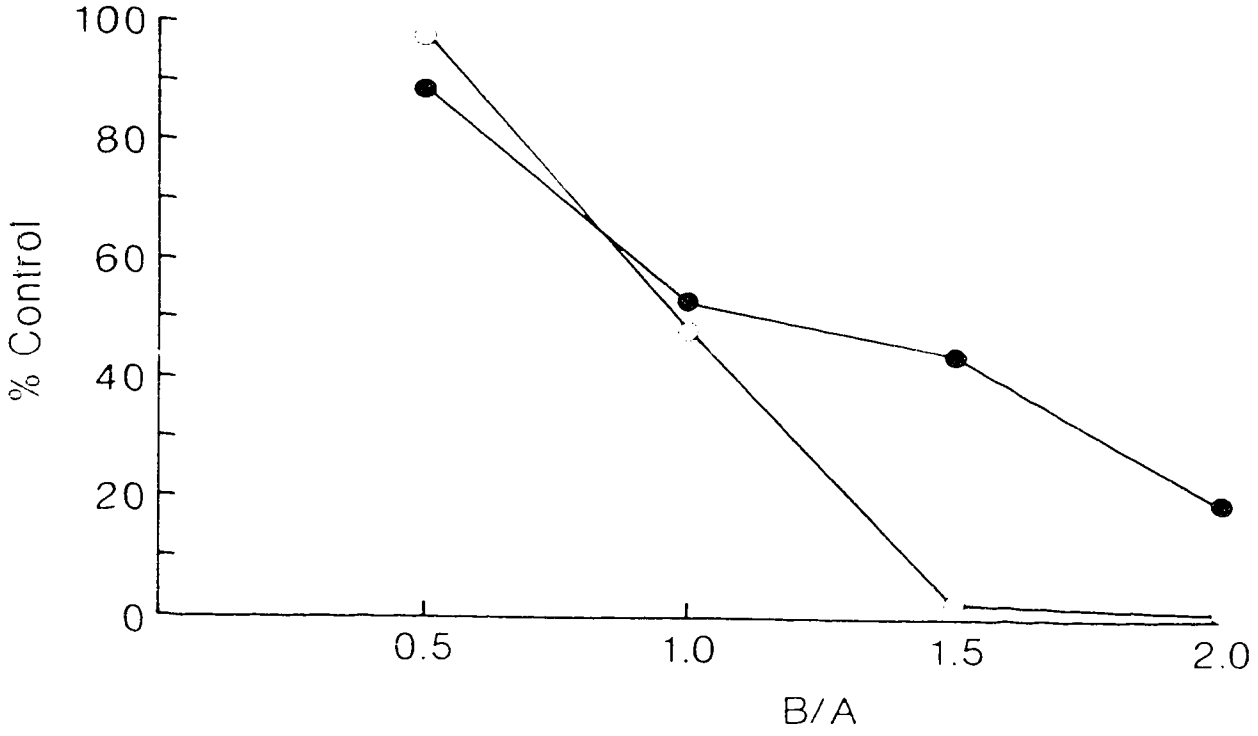


Figure 4-4. The recovery effect of bilirubin washout at 2 hr (•) and 24 hr (o) by N-115 cells on mitochondrial function using the MTT assay after 2 hr exposure to bilirubin at concentrations ranging from 50 μM to 200 μM and a fixed HSA concentration of 100 μM, thus yielding B/A 0.5, 1, 1.5, and 2.



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CHAPTER 5

Publication No. 4:

Effect of Bilirubin on Adenosine Nucleotide Level in the Neuroblastoma Cell Line N-115.

Introduction

The mechanism of bilirubin encephalopathy has been studied extensively over the past decade. Yet, the major biochemical defect underlying bilirubin toxicity to the nervous system has not been completely elucidated. Studies conducted on neural and non-neural cells and tissues demonstrate that bilirubin may impair a large number of cellular functions [1]. Among these are changes in energy metabolism and specifically the inhibition or uncoupling of oxidative phosphorylation in mitochondria [2-7]. However, there are considerable variations in the results reported. The use of bilirubin concentrations higher than usually encountered in clinical situations, and the use of varying albumin concentrations may account for the variability in resuits. The use of high bilirubin concentration without added albumin or at high bilirubin-to-albumin (B/A) molar ratios causes rapid aggregation and precipitation of bilirubin, and hence may give rise to variable toxicity [8-10].

In the present study, using a stable bilirubin-albumin mixture at B/A ratio of 1.5, the effect of bilirubin on energy metabolism in intact cells is investigated by measuring the adenosine nucleotide levels in the neuroblastoma cell line N-115 in culture.

Materials and Methods.

The conditions for growing N-115 cells and the preparation of bilirubin are as previously described [11]. Bilirubin is added to

the experimental media to make the final bilirubin concentration 100 μM and B/A ratio of 1.5. The addition of bilirubin is immediately followed by an amount of 0.1 N HCl equivalent to the amount of NaOH to restore the pH to 7.4. Control cell cultures contain the same concentration of albumin. 0.1 N HCl and 0.1 N NaOH are added to the media, with no bilirubin, in the same volumes as in the bilirubin treated cells. Another set of plates are seeded, grown and treated as described and are assessed in the presence of 20μM carbonyl cyanide-*p*-trifluoro-methoxyphenylhydrazone (FCCP), a potent uncoupler of oxidative phosphorylation, and hence functions as a positive control.

Nucleotide levels - Cells are exposed to 100μM bilirubin, at B/A molar ratio of 1.5, for 1 and 4 hours. At the end of the incubation periods the media is removed and the cells washed with 5 mL of 0.3 M sucrose to remove residual medium. The cells are then resuspended in 1 mL phosphate buffered saline. Aliquots are taken for DNA [12] and protein estimations [13]. Nucleotide levels in the cells are measured by high performance liquid chromatography (HPLC) analysis of the acid-soluble nucleotides extracted with 4% perchloric acid. To prevent interference with the chromatographic assay the acids are neutralized with an Alamine 336/ Freon-TF solution [14].

Results

Nucleotide levels - The effect of bilirubin on the adenine nucleotide levels of N-115 cells is given in Table 1.

Exposure of N-115 cells to 20 μM FCCP, a potent inhibitor of oxidative phosphorylation, results in a significant decrease in ATP level, compared to that of control cells. ADP level is decreased slightly while both AMP and c-AMP levels in the cells are increased (Table 1, Fig. 1). The same general trends are seen in the nucleotide levels after 4 hours exposure to 100μM bilirubin at B/A molar ratio of 1.5. ATP and ADP levels in the bilirubin treated cells decreased by 43% and 35% , respectively, while c-AMP level increased by 200% compared to control. Unlike the effect of FCCP, cells exposed to bilirubin demonstrate a decrease in AMP level. One hour exposure to bilirubin resulted in 1.5-3 fold increase in c-AMP, AMP and ADP levels with no effect on ATP level.

Adenylate Energy Charge - Oxidative phosphorylation supplies the major portion of ATP required for many vital cellular functions. Compounds which interfere with this process are probably lethal to the cell. From the formula : 2[ATP] + [ADP] / 2([ATP]+[ADP]+[AMP]+[c-AMP]) one can determine the adenylate energy charge of the cell, a measure of the possible high-energy phosphate bonds that are present in the adenine nucleotides. In a situation where a toxin affects oxidative phosphorylation ADP is not converted to ATP and, hence, reduces the cell adenylate energy charge [15]. When the adenylate charges in the different experimental groups are calculated (Table 2), FCCP treated cells showed a 66% decrease in adenylate charge from control cells, and cells exposed to

bilirubin also resulted in a decrease of adenylate charge, but to a lesser extent.

Discussion

In vitro studies of the effect of bilirubin on energy metabolism previously conducted on various neural and non neural cells and subcellular fractions, indicate that bilirubin has a powerful uncoupling effect on oxidative phosphorylation [2-7]. A major concern, when experimenting with a B/A molar ratio that exceeds one, is the instability of the bilirubin solution leading to formation of bilirubin aggregates and coaggregates of bilirubin and albumin [8-10]. Once aggregates are formed, changes in free bilirubin concentration occur, giving rise to experimental variability. This problem has not been fully addressed in experiments dealing with bilirubin toxicity ***in vitro*** [2-7]. In those experiments, when either free bilirubin or bilirubin in excess of albumin was used, the toxic effect appears to be an instant one, occurring within minutes of exposure [2,4,5,6]. Studies from our laboratory [11], have shown that bilirubin, at a concentration of 100 μM and B/A ratio of 1.5, is stable over a 24 hour period. Under these conditions bilirubin toxicity occurs at a slower rate and this is also evident from the present study (Table 1 and Fig. 1). Direct interaction of bilirubin with the purified respiratory enzymes [2-6], as opposed to interaction with the whole cell, may well be the reason for the rate difference, but one can not exclude the possibility that

toxicity is enhanced as a result of bilirubin instability in solution.

Bilirubin has been shown to impair mitochondrial functions [2-6], and to inhibit a large number of cellular enzymes [1]. Despite this, no specific target has been singled out as the primary one for bilirubin toxicity. Recently, we have demonstrated that bilirubin affects mitochondrial function earlier than other affected cell functions [11]. These results point to the possibility that mitochondria may be the primary target of bilirubin toxicity. While the effects on mitochondrial function are early in appearance, the effect on ATP levels is evident only after a longer exposure to bilirubin (Fig. 2). Moreover, while specific inhibition of oxidative phosphorylation results in a marked decrease in the cell adenylate energy charge, the effect of bilirubin is a moderate one (Table 2). FCCP is a weak lipophilic acid which is permeable across lipid bilayers in either protonated or deprotonated form [16]. By shuttling across the membrane it can catalyse the proton conductance of the membrane. In so doing the proton circuit is short-circuited allowing the generator of the proton electrochemical potential to be uncoupled from the ATP synthetase. The end result is a decrease in ATP synthesis [16]. Bilirubin may affect ATP synthesis in the same manner. Previous studies have demonstrated that bilirubin interacts with the lipid bilayer [17,18] and is capable of free diffusion across it [19].

Dissociated from albumin, bilirubin in its free dianionic form may function as a proton translocator affecting ATP synthesis.

The differences in toxicity observed after 1 and 4 hours exposure to bilirubin are also of interest. Previous investigations have demonstrated that bilirubin toxicity is dependent on bilirubin concentrations, bilirubin-to-albumin molar ratios and the length of exposure [20]. Exposure of N-115 cells to bilirubin for 1 hour is associated with an overall increase in adenine nucleotide levels. The same trend was observed when other cell and mitochondrial activities were measured [2,3,6]. Whether these findings reflect the changes associated with the changes in the culture media following the addition of bilirubin or the promoting effects of short term exposure to bilirubin has yet to be determined.

It is also possible that bilirubin affects ATP levels non-specifically, and that the oxidative phosphorylation reactions may be a secondary effect of bilirubin action on the cell. Further studies are needed to delineate the mechanism and primary target of bilirubin toxicity to the nervous system.

Table 5-1. Effect of bilirubin cell treatment on adenine nucleotide levels.

Exposure Time	Cell treatment	c - AMP*	AMP*	ADP*	ATP*
1 Hour	FCCP	26.1 ± 0.7¶	69.8 ± 3.9	25.7 ± 2.9	12.2 ± 1.3
	Control	12.4 ± 1.0	41.2 ±10.5	32.1 ± 9.5	73.6 ±13.9
	Bilirubin	21.6 ± 5.9	46.1 ± 3.4	31.4 ± 5.2	51.1 ± 8.8
4 Hours	Control	7.7 ± 0.6	25.3 ± 1.3	21.0 ± 2.1	49.5 ± 2.6
	Bilirubin	19.1 ± 1.2	32.6 ± 1.8	19.6 ± 1.3	40.7 ± 5.7
	Control	11.2 ± 1.6	38.5 ± 4.4	30.3 ± 4.4	71.0 ±15.4

* Expressed as pmole / µg DNA

¶ Values given are mean ± SD from three measurements.

Table 5-2. Effect of bilirubin treatment of N-115 cells on adenylate energy charge.*

Exposure Time	Cell treatment	Adenylate energy charge	% Control
	FCCP	0.19 ± 0.01¶	33.2
	Control	0.56 ± 0.01	100.0
1 Hour	Bilirubin	0.44 ± 0.02	76.6
	Control	0.58 ± 0.01	100.0
4 Hours	Bilirubin	0.45 ± 0.02	79.1
	Control	0.57 ± 0.02	100.0

* Adenylate energy charge = 2[ATP]+[ADP]/2([ATP]+[ADP]+[AMP]+[cAMP]).

¶ Calculated value of three measurements expressed as mean ± SD.

Fig 5-1: The effect of 100μM bilirubin, at B/A molar ratio of 1.5, and 20 μM FCCP on adenine nucleotide levels in N-115 cells, compared to control. cAMP ( ), AMP ( ), ADP ( ), ATP ( ).

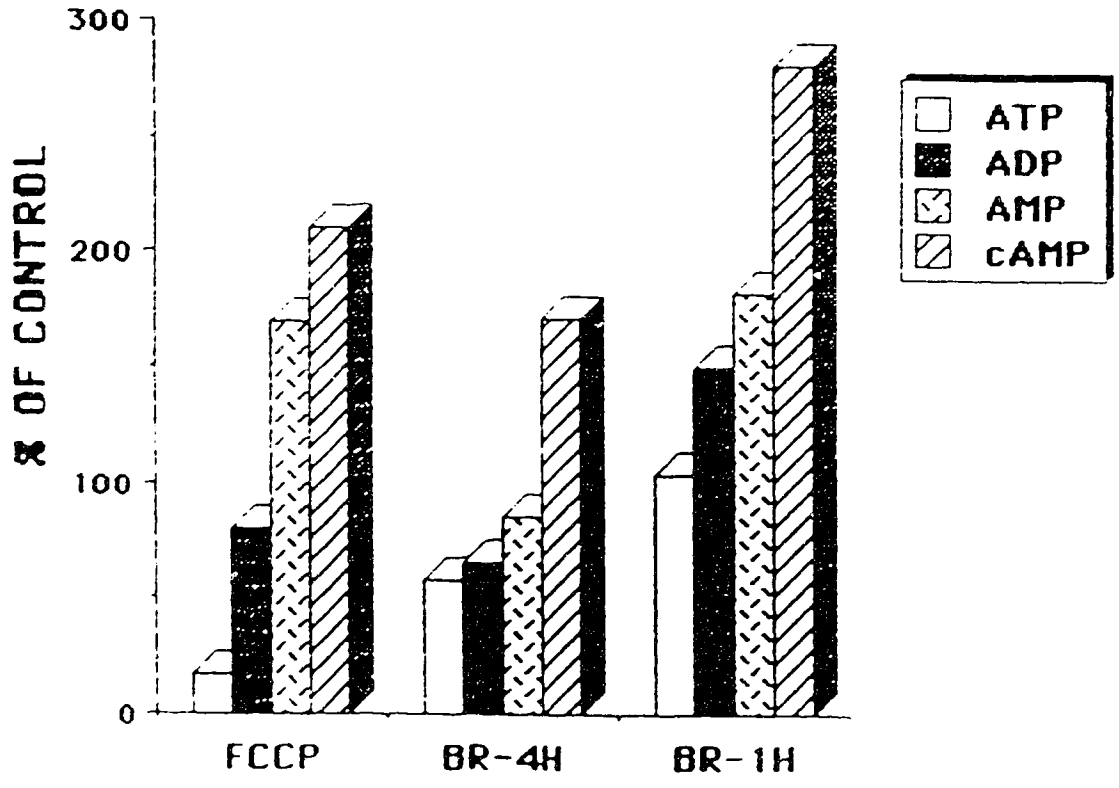
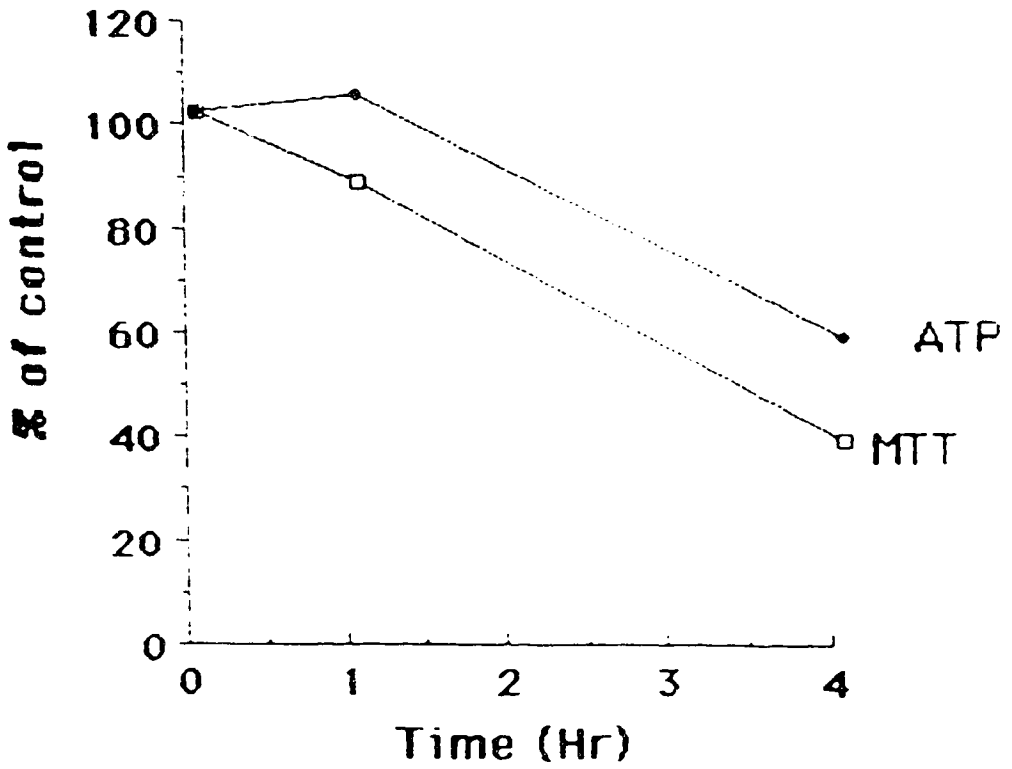


Figure 5-2: The effect of bilirubin on mitochondrial function assessed by the MTT assay (Ó) and ATP levels (♦) in N-115 cells.



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CHAPTER 6

GENERAL DISCUSSION

Discussion

Hyperbilirubinemia and bilirubin encephalopathy are well known occurrences in the newborn period. The mechanism of bilirubin toxicity to the central nervous system has been the subject of numerous investigations over the past decade [1,2]. Yet, the major biochemical defect underlying bilirubin encephalopathy has not been completely elucidated. The difficulties in analyzing the results and the inability to point to a primary target of bilirubin toxicity stems from variation in experimental design, the use of different animal models and different cell systems [2], and difficulties in correlating the chemical, biochemical and clinical knowledge of the bilirubin molecule in ***in vitro*** and ***in vivo*** experiments [3].

A major concern when experimenting with bilirubin is the solubility and stability of the pigment [3-6]. The use of bilirubin without albumin, or the use of high bilirubin concentrations at bilirubin-to-albumin (B/A) molar ratios that exceed one, causes the formation of bilirubin aggregates and coaggregates of bilirubin and albumin [3-6]. Once aggregates are formed, changes in free bilirubin concentration occur, and these changes can give rise to experimental variability. This problem has not been fully addressed in experiments dealing with bilirubin toxicity ***in vitro*** [7-12]. Thus, when experimenting with bilirubin it is important to ascertain that the pigment utilized remains fully stable and in solution.

The mechanism by which bilirubin enters the cell has been studied in several non-neural cells and subcellular fractions thereof [11,13-22]. Different approaches have been taken to study the interaction of the pigment with neural cells [23-26], these approaches include bilirubin exposure to the whole brain [23,24] or to brain slices [25], and exposure to membrane fractions and lipids extracted from various neural cells [26-29].

Most of the work done on bilirubin toxicity in neural tissues can be divided into two major groups. In one group, the hyperbilirubinemic Gunn rat served as a model [7,30-39]; in the other, brain cells from normally developed animals were used [27,40-49]. Though extensive damage to the nervous system of the Gunn rat can be correlated with bilirubin, a genetically determined bilirubin-independent abnormality in these animals cannot be excluded [50,51]. In addition, the results obtained from studies with purified proteins and subcellular fractions might have no relevance to bilirubin toxicity ***in vivo*** , as the question whether bilirubin crosses the plasma membrane has not been completely resolved. Obviously, an approach consisting of the quantification of bilirubin uptake and the measurement of consequent changes in cell behavior, in the same system, will be ideal. Thus, in studying the mechanism of bilirubin toxicity the use of a neural cell line and the presence of appropriate albumin concentrations, is advantageous.

Using N-115 cells, a murine neuroblastoma cell line, the purpose of the work presented herein was as follows:

a) to define the specific ***in vitro*** conditions under which bilirubin, when added to cell culture media ,is stable and remains so during the entire experiment.

b) to characterize the bilirubin-cell interaction, specifically:
   1) the interaction between bilirubin and the cell.
   2) the target of bilirubin toxicity at the cellular level.
   3) the reversibility of the toxic effect.
   4) the delayed bilirubin-toxicity.

Bilirubin stability . In the studies reported herein, we have shown that bilirubin in tissue culture media, at concentrations of 35-125 μM and B/A molar ratios up to 2, is stable over a 24 hour period [publication no. 2].

Bilirubin interaction with the cell. The results obtained in the present work clearly show that the binding of bilirubin to the cells is directly associated with the length of exposure, B/A molar ratio, bilirubin concentration at a given B/A ratio, temperature, and pH conditions of the media [publication no. 1]. In addition, the results indicate that the free form of bilirubin, rather than the albumin-bound form, is the reactive species, and it interacts with the plasma membrane through a multistep binding process. Moreover, under the same experimental conditions, the

binding parameters [publication no.1] are consistent with our results on measurements of toxicity parameters (see below and publications no. 2,3,4).

The binding data presented here [publication no.1] indicate a multistep binding process, similar to that recently proposed for the interaction of bilirubin with synaptosomal plasma membrane vesicles derived from rat brain [28]. It is not in accord with a simple transport mechanism such as passive diffusion [52] and carrier-mediated transport [11,16-18]. These binding steps are: 1) bilirubin binding to the polar head group region of the membrane, 2) insertion of the surface-bound bilirubin into the hydrophobic core of the membrane, and 3) membrane-induced aggregation of bound bilirubin on the surface of the membrane.

From the data presented here [publication no. 1] it is not possible to draw any conclusion regarding the question whether bilirubin crosses the plasma membrane and reaches intracellular targets. However, the correlation between cellular binding of bilirubin and cellular toxicity (see below) suggests that bilirubin probably enters the intracellular compartment.

Bilirubin toxicity. In the present study, using stable bilirubin-albumin mixtures at B/A molar ratios of 0.8 and 1.5, the effect of bilirubin on four vital cellular functions is shown. Specifically these are mitochondrial functions, [^{3}H]thymidine uptake, L-[^{35}S]methionine incorporation into protein and Na^{+}/K^{+} ATPase activity [publications no. 2,4]. In all these instances, the

toxic effects seem to be dependent again on B/A molar ratio, bilirubin concentration, and the duration of exposure.

Bilirubin may be confined to the plasma membrane and may elicit toxicity by perturbing membrane-mediated transduction of information. Another possibility is that a fraction of the membrane-bound bilirubin is transported into the cytosol allowing for the direct interaction between bilirubin and intracellular targets. This may be the result of either partitioning into a cytosolic carrier molecule, as demonstrated in the liver [16,53], or through membrane recycling, or aqueous diffusion of the pigment as proposed for phospholipids and cholesterol [54]. Our binding studies reveal a complex interaction at the level of the plasma membrane [publication no. 1]. Evidently, further studies, including subcellular fractionation of bilirubin-treated cells, are needed in order to obtain additional insight into the possible mechanism of bilirubin transport across the plasma membrane, the possible direct effects on intracellular targets, and to identify the primary target for bilirubin toxicity conclusively .

Reversibility and delayed bilirubin toxicity. Clinical studies using auditory brainstem evoked responses in hyperbilirubinemic neonates have shown reversibility of the acute toxic effects of bilirubin. Once the hyperbilirubinemia has subsided the abnormal responses seemed to normalize [55-57]. A similar phenomenon has been suggested in laboratory studies on different cell systems [58-61]. The removal of tissue-bound bilirubin is thought to be a process comprising either changes in B/A molar

ratio [62-64], enzymatic oxidation of bilirubin [64] and/or clearance of bilirubin from otherwise undamaged brain tissue to the blood [30,65]. In the studies reported herein we demonstrate that in N-115 cells, once toxicity appeared it was irreversible. Moreover, toxicity also appeared long after removal of the bilirubin containing media, following a short term exposure to bilirubin (during which no toxicity was manifest) [publication no. 3].

Binding interaction and toxicity. The multistep model suggested for the bilirubin interaction with synaptosomal plasma membrane vesicles [28] is in accord with the results obtained from our studies on bilirubin interaction with N-115 cells [publication no. 1], bilirubin toxicity [publication no. 2,4], delayed effects and recovery [publication no. 3]. Binding of bilirubin to the cell surface or aggregation of the pigment on the surface or within the lipid bilayer may elicit toxicity by alterations of cell membrane properties. Bilirubin binding to the cell is partially reversible by the addition of albumin. Bilirubin removed from the cells by albumin probably represents the bilirubin bound to the cell surface, since the fraction removed decreases with the increasing of the period of exposure. Since binding is time dependent, the longer the exposure the higher the amount of bilirubin irreversibly bound to the cell surface or penetrating the hydrophobic core of the membrane [publication no.1]. This results in a progressive and irreversible toxicity [publication no. 2,3,4]. With shortened periods of exposure, when no toxicity is demonstrated, some of the bilirubin molecules aggregate on the cell surface and/or within the

lipid bilayer leading to delayed toxic effects [publication no. 3]. Whether bilirubin is transported across the plasma membrane and affects intracellular targets directly is yet unknown.

In summary, we have shown that, under appropriate conditions of bilirubin concentration, bilirubin-to-albumin molar ratio, and time of exposure, the binding interaction between bilirubin and the plasma membrane is complex. Bilirubin affects various cellular functions in N-115 cells, and its toxicity is progressive and irreversible.

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