Proc. Indian natn. Sci. Acad. **B48** No. 2 pp. 163–175 (1982)

SUNDER LAL HORA MEDAL LECTURE 1981
Bilirubin—A Model for the Study of an Endogenous Toxic Chemical

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(Delivered on 1 January 1982)

"...Ideally the goal of medicine has always been to help man function successfully in his environment—whether he is hunting the mammoth, toiling for his daily bread or attempting to reach the moon."

—Rene Dubos
in Man Adapting 1965

I am deeply conscious of the signal honour conferred upon me by the Council of Indian National Science Academy in considering me worthy of receiving the Sunder Lal Hora Medal in Life Sciences.

Dr Sunder Lal Hora, a distinguished Fellow of the Academy, was one of the doyens, who laid the foundation for the science of Fisheries and Aquatic Biology in our country. An important component of the universal food web, fish is now-a-days the centre of many exciting studies. Many of these are oriented towards unravelling the role of fish as indicators of pollution of our inland and marine water resources. I have had not much to do in my research career with fish biology and as such I do not have the competence to talk about it with authority. There are, however, some aspects of fish biochemistry, particularly, their microsomal system which have fascinated me in recent years. This system in the fish appears to be very sensitive to changes in the ambient temperature of the aquatic medium. Again for a behavioural toxicologist, fish offer an exciting research tool for studying the mechanism of chemotactic signalling and through that the impact of environmental chemicals on behaviour.

I propose in this lecture to present an overview of our work on bilirubin, the common bile pigment, the villain of piece of jaundice. Before I do so, let me introduce the subject of toxic chemicals.

The stuff we are is made of chemicals. We are sustained by chemicals. Our continuity is the result of an information transfer mediated by chemicals. Functional aspects of all living systems including the activity of our brain, our behaviour, reproduction, etc. are controlled

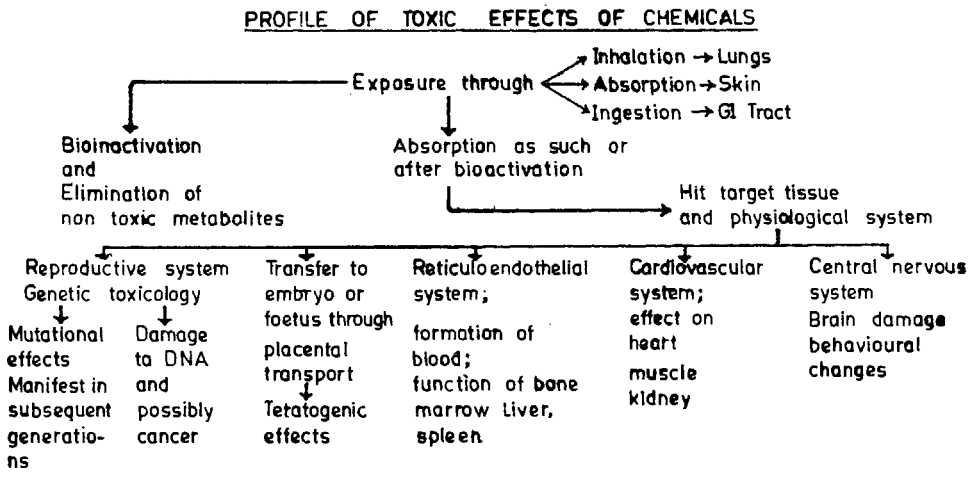


Figure 1 Health effects to toxic chemicals

by a variety of chemicals. The over bearing nature of this control is manifest as homeostasis. Furthermore, the associated equilibrium of biochemical reactions maintains the overall balance between constant synthesis and continuous degradation, repair process and rapport with the environment. It is well recognised that many chemicals can under certain circumstance modulate physiological equilibrium both constructively and destructively. The broad effects elicited by chemicals with potential to be toxic are summarised in figure 1. The theme of my presentation today is to stress the universality of the concept that all chemicals, depending upon dose, are potentially toxic and that homeostasis is the outcome of the efforts of the organism to counter the stress imposed by environmental factors of which chemicals are important constituents.

Bilirubin and Bile Pigments

The relationship between the bile pigment bilirubin and the chemical molecule pyrole was established in 1934 by Otto Hans Fischer and his colleagues. Extensive studies have since been conducted to elucidate the mechanism of formation, transport and disposal of bilirubin. What is bilirubin? It is a yellow pigment and is one of the end-products of the normal destructive process of recycling of red blood cells and other haeme containing proteins. The yellow tint acquired by the skin and the eyes in diseases connected with the liver has been known since very ancient times. Hence the significance of estimation of serum levels of bilirubin in the diagnosis of jaundice and related liver disorders. It is estimated that 15–24g of haemoglobin are turned over every twenty-four hours of human life. A schematic diagram of the breakdown of red blood cells is given in figure 2. The globin or protein moeity is returned to the general pool to be recycled by the cellular machinery of the body. The pigment moeity haem is made up of four porphyrin rings with iron in the centre fixed to the molecule by a coordination link.

The first step in the breakdown of haem is presumably the removal of iron by a process of sequestration involving

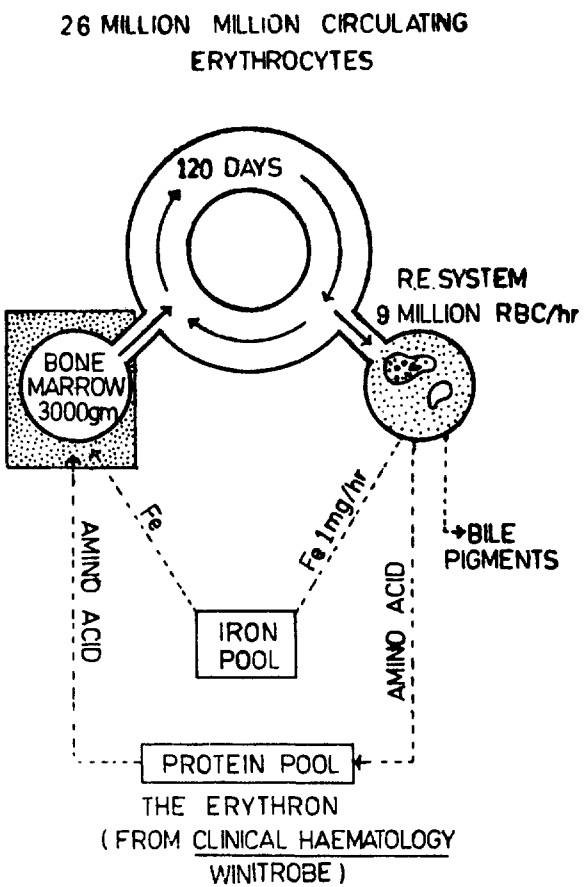


Figure 2 Breakdown of haemoglobin in the body

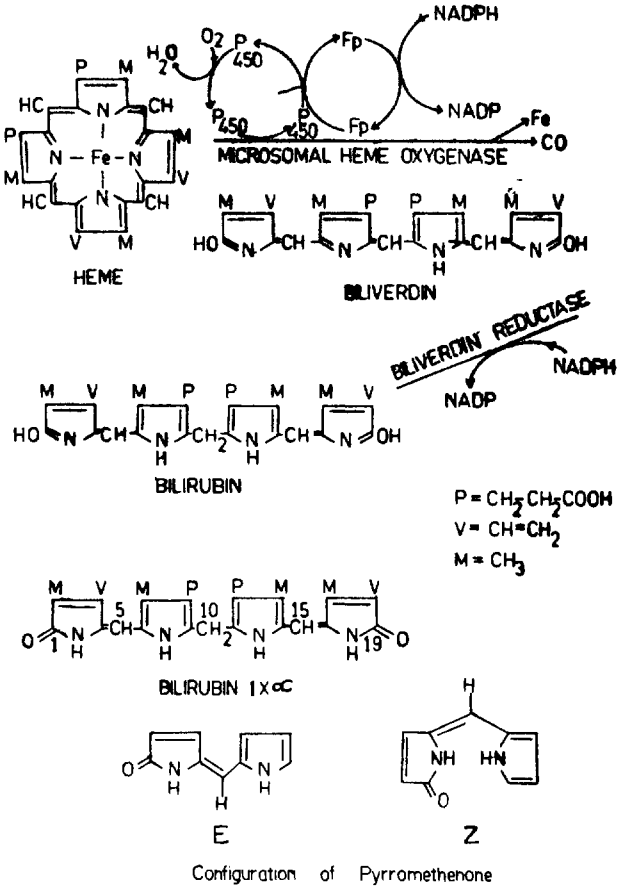
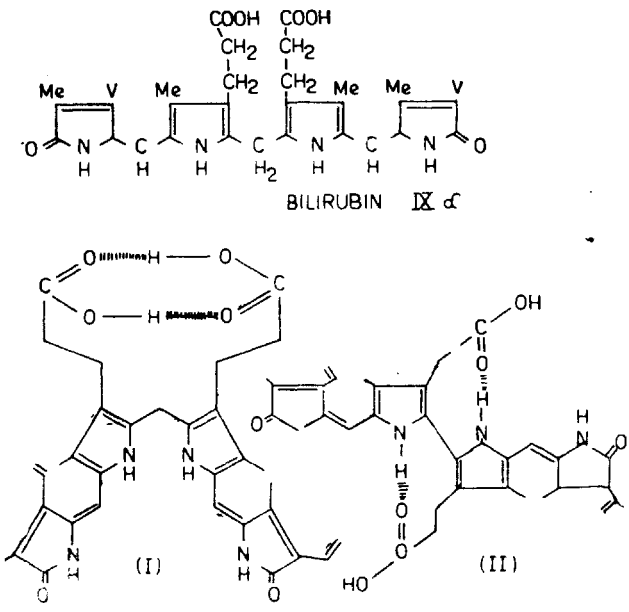


Figure 3 Biodegradation of heam



Stabilization of bilirubin by intramolecular hydrogen bonding

Figure 4 Linear tetra pyrole structure of bilirubin

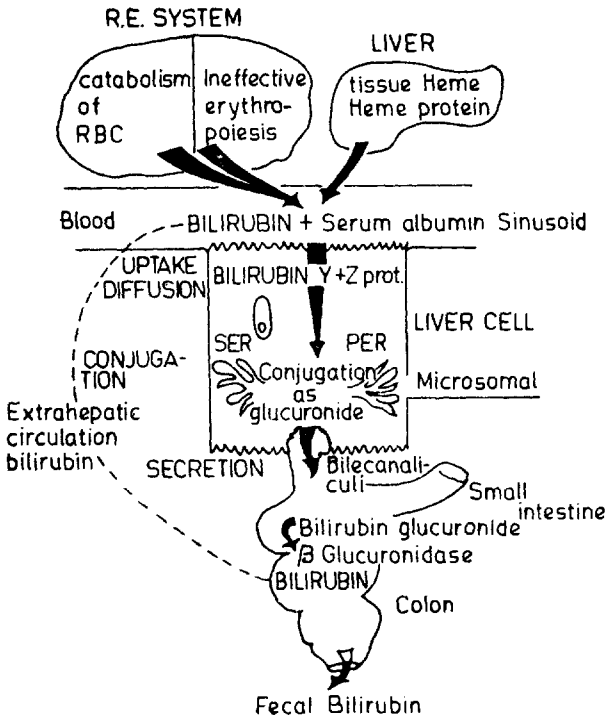
an enzyme haem oxygenase (figure 3). The ring structure is then transformed into a linear form (figure 4).

Increase in serum levels of bilirubin beyond 5–7 mg% does not constitute any serious threat but when it exceeds 7–10 mg%, serious consequences such as bile obstruction or irreversible brain damage can occur. The post-natal human baby is very susceptible to brain damage resulting from the toxic action of bilirubin on brain. The disease is known as "Kernicterus" and is often fatal. Very little is known about the mechanism of toxicity of bilirubin on human brain. It is, however, believed that post-natal Kernicterus accounts for a high percentage of mentally detarded children.

Neonatal Hyperbilirubinemia

In general, jaundice is the result of the over production of bilirubin or resurgication of conjugated bilirubin by liver or the retention of unconjugated bilirubin. Free bilirubin is relatively insoluble in water. Thus at physiological *p*H, less than 2 μ moles can be dissolved in one litre of water. Free bilirubin can bind itself tightly to albumin to form relatively soluble complexes. The transport of bilirubin from circulation to liver is, however, mediated mostly by the lipoprotein fraction of serum. The bilirubin bound thus to lipoproteins is taken to parenchymal cells of liver by facilitated transport across the liver membrane. Within minutes of its arrival in the liver more than 60% of bilirubin can be recovered in the supernatant fraction of liver homogenates in the form of a complex called bilirubin diglucuronide. The conjugation of bilirubin to glururoric acid is mediated by the microsomal enzyme UDP glucuronyl transferase. The soluble glucuronide passes into the bile canalculi and then to the bile helped by the active secretory system of the biliary tract. The fate of bilirubin in the body is shown in figure 5. The conjugation reaction leading to the formation of water soluble glucuronides is illustrated in figure 6.

What is the origin of the jaundice of the new born? Placental blood is degraded at a very rapid rate in the new born resulting in a high body load of bilirubin. This obviously leads to a situation where the cells setting out on their journey of development have to encounter a very highly toxic chemical generated by endogenous metabolic activity. The liver cells of the neonatal, in contrast to the adult, are not equipped with the enzyme machinery for conjugating bilirubin. The red blood cell forming ability of the new born is also yet to reach its peak level. In some rare genetic disorders and blood



Stages in the formation transport and excretion of bilirubin

Figure 5 Fate of bilirubin in the body

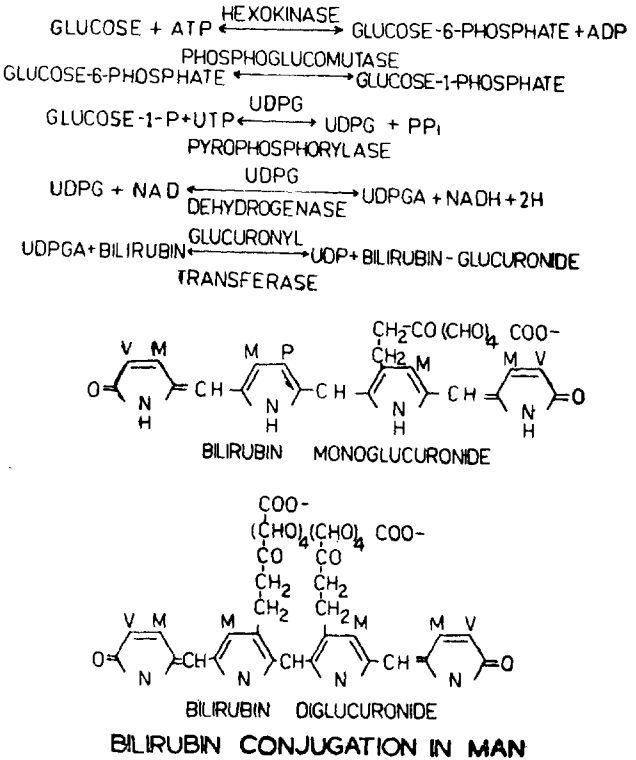


Figure 6 Conjugation of bilirubin by glucuronic acid

group incompatibility such as Rh factor inheritance, the neonatal life is called upon, in addition, to face other challenges for its survival.

It is here that the neonatal organism utilizes its own skin for defence against the toxicity of bilirubin. The skin and sclarae develop a deep yellow tinge due to accumulation of bilirubin. Visible light acts upon this and not only bleaches the skin but also degrades bilirubin to products which can now be excreted directly by kidneys by-passing the liver.

Experimental studies conducted by us jointly with the Pediatrics Department of K.G.'s Medical College, Lucknow, have shown that:

(a) nearly two thousand jaundiced neonates with serum bilirubin levels from 5–12 mg% responded favourably to controlled phototherapy. The management of jaundiced neonates by phototherapy was only marginally dependent on the conventional and the more/difficult exchange transfusion method (figure 7),

(b) mammalian skin including that of man provides a matrix for the spread of free bilirubin over a large area to facilitate interaction with light. The magnitude of plasma bilirubin decrease is correlated with the extent and depth of dermal ecterus in neonates,

(c) collagen, an important constituent of the skin, offers multiple sites for binding of bilirubin. Photo-oxidation of bilirubin bound to skin results in the formation of water soluble metabolites which can be disposed off by the renal route of clearance, and

(d) a number of catabolites could be recovered from urine of neonates exposed to light as against controls. See figure 8 for the profile of urinary metabolites of bilirubin.

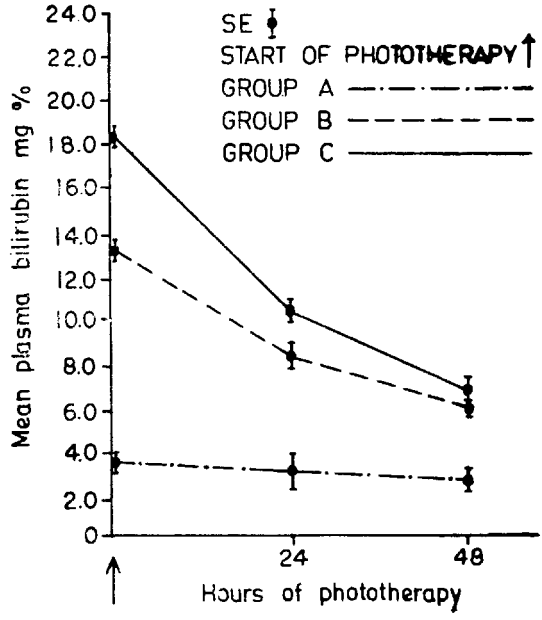


Figure 7 Phototherapy of neonatal jaundice

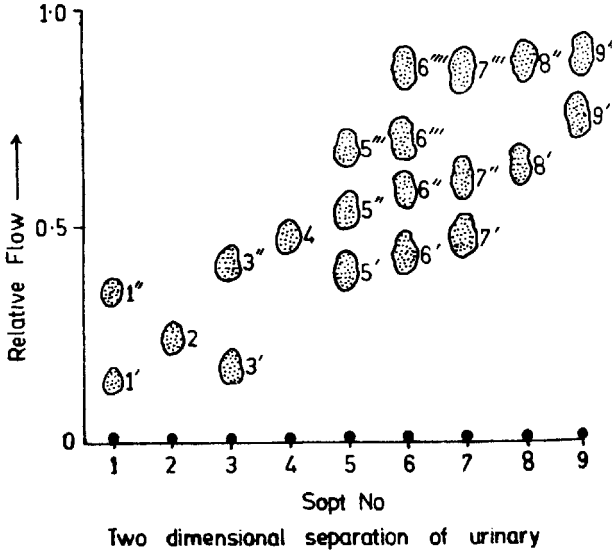


Figure 8 Profile of urinary metabolites of bilirubin

Mechanism of Photo-oxidation

Photo-oxidation of bilirubin involves photo-oxygenation, a reaction in which bilirubin acts as a photosensitizer for its own destruction. On the evidence that:

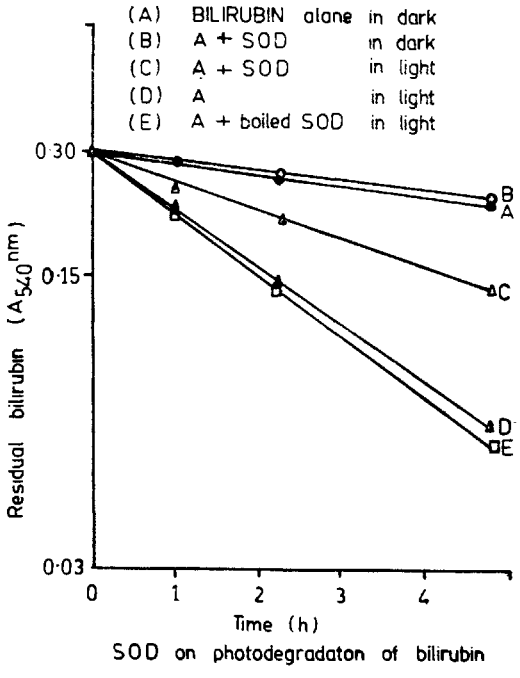
(i) photodecomposition of bilirubin is stimulated by singlet oxygen sensitizers like dyes,

(ii) singlet oxygen quenchers like β-carotene inhibit photodecomposition, and

(iii) trappers of singlet oxygen like 3:5-dimethyl furan suppress photodecomposition,

it is currently believed that singlet oxygen, a high energy form of molecular oxygen, is the mediator of photo-oxygenation. Studies conducted by Ravi Kaul and Hari Kaul in our laboratory have shown that superoxide anion may also be involved in the process. Please see figure 9 for xanthine and xanthine oxidase stimulation of photocatabolism of bilirubin. By inclusion of xanthine-xanthine oxidase as generators of superoxide radical, the dissociation constant of bilirubin is increased by a factor of one. At the same time exogenously added superoxide dismutase completely abolishes the photo-oxidation stimulated by xanthine-xanthine oxidase.

When there is a deficiency of UDP-glucuronyl transferase, jaundiced neonates excrete polar metabolites which resemble the degradation products obtained by in vitro photo-decomposition. See figure 10 for urinary catabolites and decrement in plasma bilirubin by phototherapy. Since there is an abundant supply of superoxide anion from the



SOD on photodegradaton of bilirubin

Figure 9 Role of xanthine-xanthine oxidase in photodecomposition of bilirubin

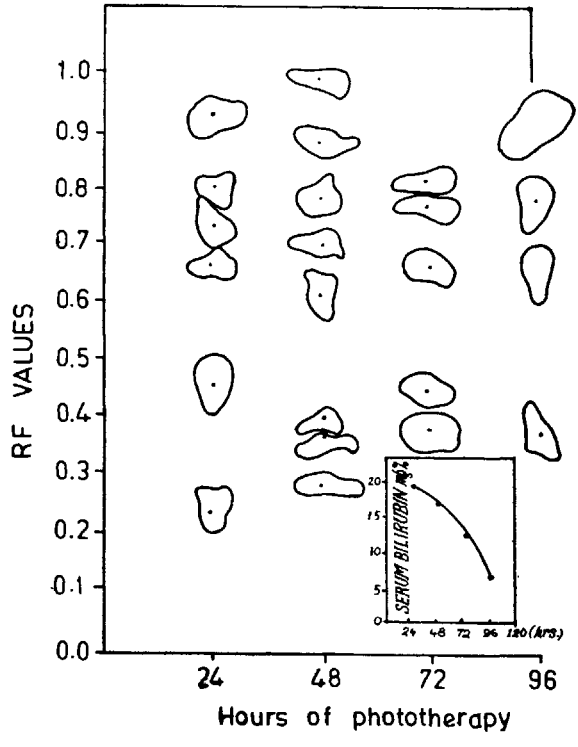


Figure 10 Urinary photocatabolites and decrement of plasma bilirubin

operation of xanthine-xanthine oxidase system in the body or from the oxygen haemoglobin exchange reaction, it is likely that there is a built-in mechanism in the body for direct biodegradation and detoxication of bilirubin. Further support for this view has come from the observation that plasma half-life of bilirubin in protein malnourished rats is extended substantially because of the well-known functional deficiency of xanthine-oxidase in protein malnutrition. See figure 11 and 12 for effect of half life of bilirubin by xanthine in experimental hyperbilirubinaemia in protein under-nourished rats.

Parturition associated hyperbaric oxygen toxicity is manifest in neonates as accelerated haeme catabolism leading to increased plasma concentration of free bilirubin. Partial hyperbilirubinemia not leading to kernicterus may be thus a natural defence mechanism for encountering hyperbaric stress associated oxygen toxicity. Challenged thus the organism uses free bilirubin to sequester the superoxide and singlet oxygen which otherwise may be detrimental to its survival.

Interaction of Bilirubin with Red Blood Cell

Results of studies conducted by Kapoor have indicated that bilirubin mediated

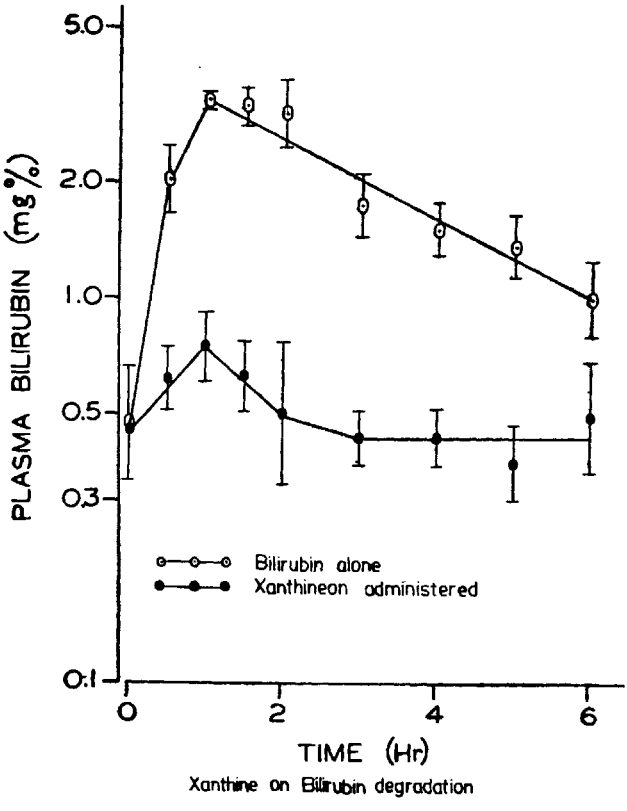


Figure 11 Role of xanthine in decreasing half life of bilirubin

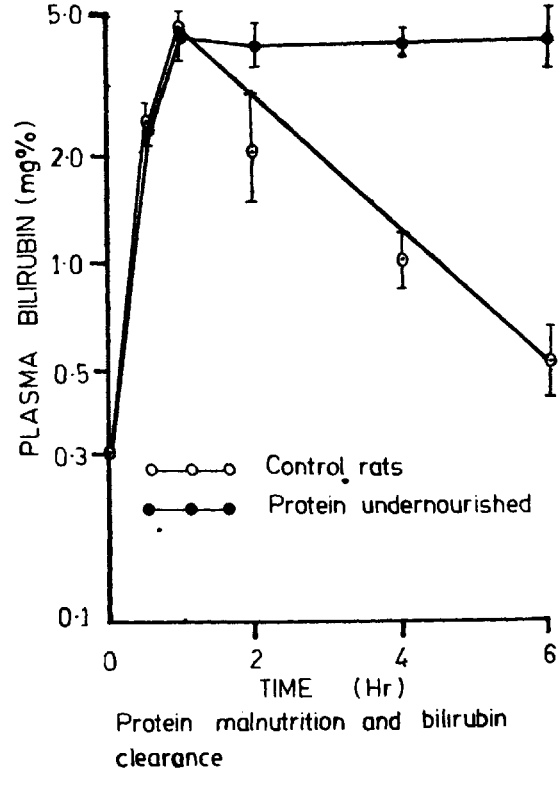


Figure 12 Protein deficiency and half life of bilirubin

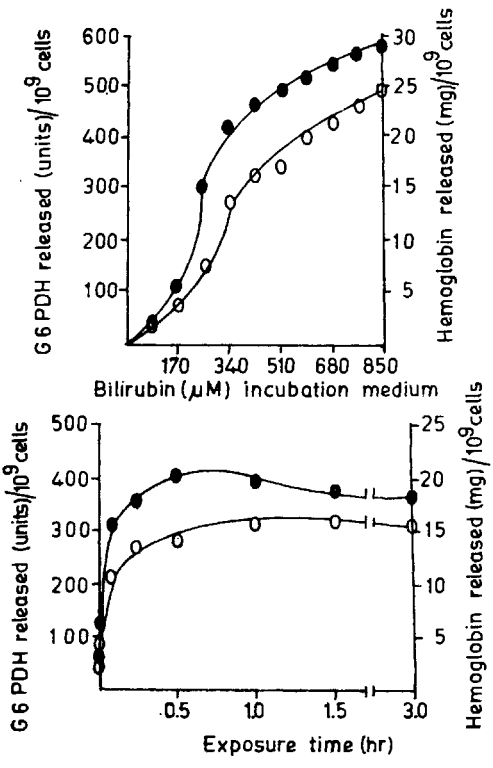


Figure 13 Haemolytic activity of bilirubin

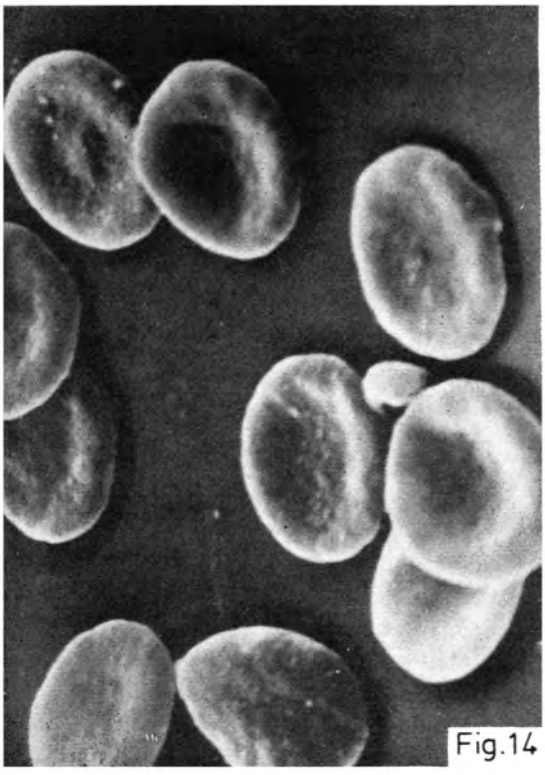
haemolysis is associated with binding of bilirubin to membrane localized receptors. See figure 13 for effects of in vitro haemolysis induced by bilirubin. We have followed the nature of in vitro bilirubin red cell membrane interaction by the invaluable method of scanning electron microscopy. Bilirubin interacts with the outer half of the bilayer couple of RBC membrane thereby increasing its surface relative to the inner half. As a result one sees the appearance of crenated RBC. Addition of albumin to the system reverses the effect. Jaundiced neonates show a large number of crenated erythrocytes. After phototherapy the number of such morphologically modified cells is substantially reduced. See figures 14–16 for scanning electron micrographs of normal RBC, RBC of jaundiced neonates and RBC of jaundiced neonates subjected to phototherapy. In contrast to free bilirubin, the photocatabolites of bilirubin or metabolites of bilirubin recovered from the urine of jaundiced neonates subjected to phototherapy were without any surface effect in RBC and were thus non-hemolytic.

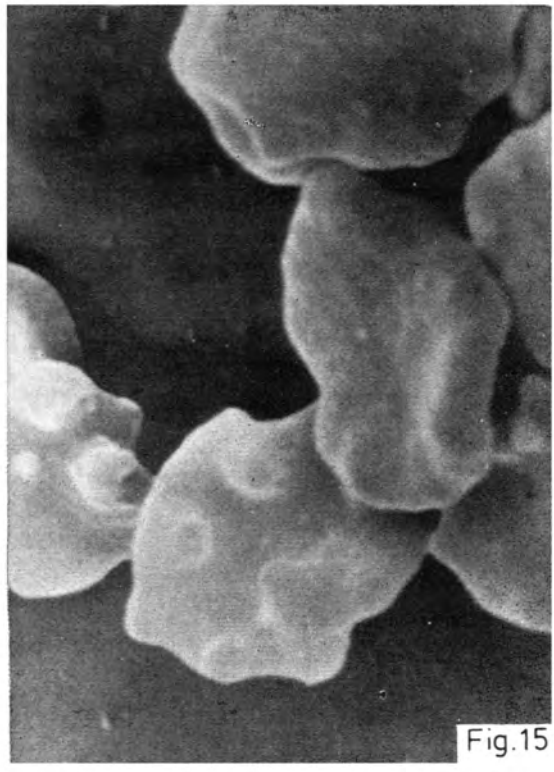
Towards a Model for Hyperbilirubinaemia

Neonatal jaundice or physiological of the new-born has been recognised since ancient times. Bilirubin has been identified as the cytotoxic agent which by interacting with cells of the target organ triggers various metabolic disturbances. Over-production of bilirubin, deficiency of uptake and disposal by liver or interference with the above processes by as yet unidentified maternal influence leading to increased intestinal absorption are considered to be some of the contributory factors. In addition to the immaturity of metabolic processes, pathological conditions such as *erythroblastosis fetalis*, maternofetal blood group incompatibility, hypoxia, acidosis, etc. are influence of the aetiology of hyperbilirubinaemia.

Attempts have been made in our laboratory to explore biochemical mechanisms for the pathological conditions associated with the neurotoxicity of bilirubin. Although fundamental cellular processes such as macro-molecular biosynthesis and oxidative phosphorylation are known to be affected by bilirubin, chemical steps that lead to damage of the brain have eluded our comprehension. It is also of urgency to know whether the damage inflicted by bilirubin in prenatal life is reversible or irreversible.

We have seen how studies with human red blood cells can throw some light on





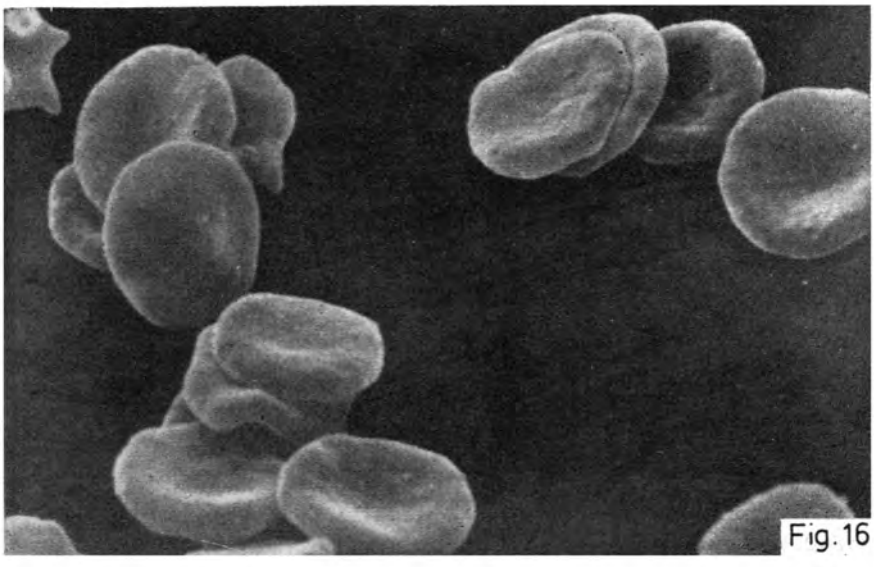


Figure 14 Scanning electromicrographs of RBC of a normal neonate
Figure 15 Scanning electromicrographs of RBC of a jaundiced neonate
Figure 16 Scanning electronmicrograph of RBC of a jaundiced neonate after phototherapy

the membrane mediated cascade of cellular reactions. Using an entirely different approach, Hari Kaul has succeeded in developing both transient and persistent hyperbilirubinaemia in neonatal rats by direct exposure of the animals to bilirubin (figure 17 & 18). Animals suffering from transient hyperbilirubinaemia show depressed levels of Na^+ K^+ ATPase and elevated levels of HCO^-_3 ATPase in brain. In contrast, both the enzymes are inhibited in liver. Thus inhibition of Na^+ Ka^+ ATPase may be one of the primary events leading to cytotoxicity of the pigment (figure 19).

Animals rendered hyperbilirubinaemic exhibited disturbances in the metabolism of L-tyrosine. A direct interaction of bilirubin with L-tyrosine amino transferase was also demonstrated, the enzyme activity being stimulated in brain and inhibited in liver. Using purified homogeneous preparations of the enzyme from brain and liver, the differential action of bilirubin was shown to be due to competition with pyridoxal phosphate in the liver enzyme and stimulation of the rate of enzyme-substrate complex formation of the brain enzyme. Besides, the binding of bilirubin to the enzyme produces, perturbances in the structural conformation of the latter as evident from quenching of fluorescence (figure 20).

Conclusion

Neonatal jaundice or the physiological jaundice of the new-born appears to be the manifestation of adaptation of the new-born to hyperbaric stress of postnatal environment. The growing human embryo leads a partially anaerobic life in the aquatic mileu of the uterus. Parturition exposes the young one to the sudden onslaught of hyperbaric oxygen external environment.

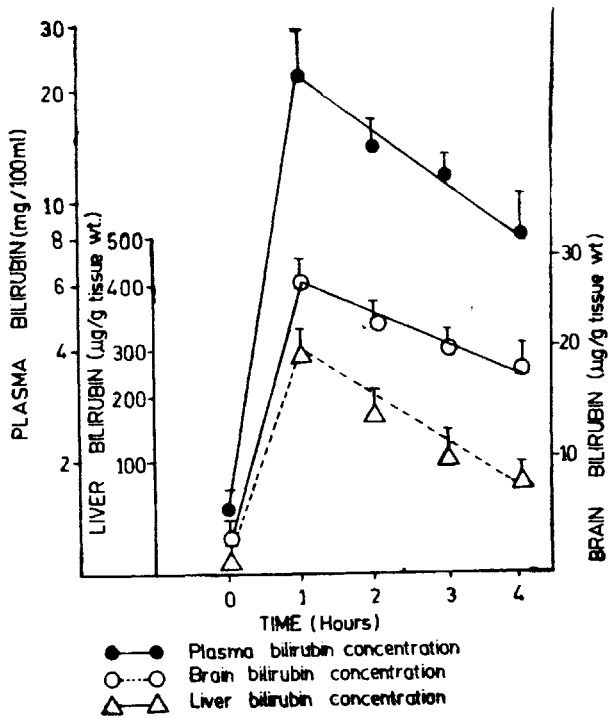


Figure 17 Transient experimental hyperbilirubinaemia

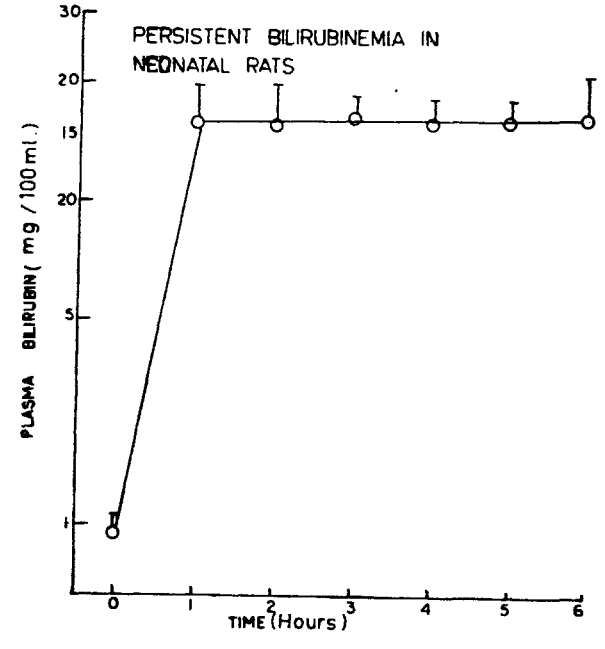


Figure 18 Persistent experimental hyperbilirubinaemia

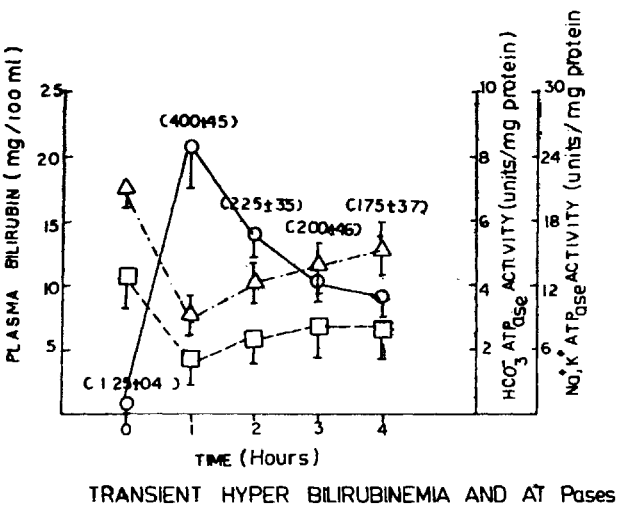


Figure 19 Modulation of ATPase in experimental hyperbilirubinaemia

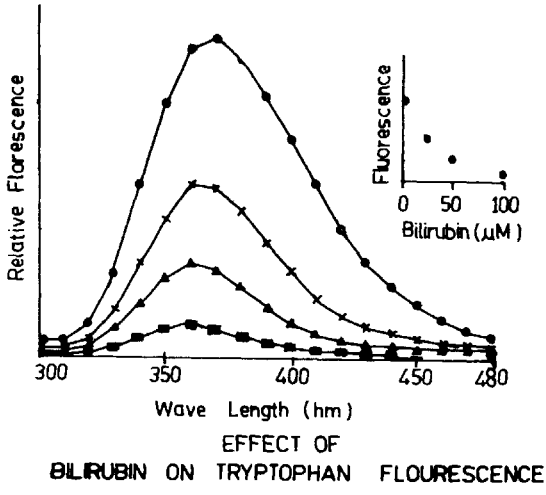


Figure 20 Quenching of fluorescence of aromatic amino acid by bilirubin

How does the child meet the situation? Since most of new-born babies go through the transition safely there must be a battery of protective biochemical reaction. One such system as evident from our studies, appears to be the induced haem oxygenase activity which leads to a rapid breakdown of maternal red blood cell to bilirubin. Bilirubin now acts as an internal scavenger of free radicals such as superoxide or singlet oxygen generated in the hyperbaric state. By its ability to bind to collagen, bilirubin spreads on to the skin and acts as its own photosensitizer and triggers the suicidal act of photodegradation or photodexication.

Since the major phase II biotransforming ability of liver, viz., conjugation, is still in a state of under-development in the critical period of post-natal life the skin provides the matrix for photodecomposition of bilirubin to non-toxic water soluble metabolites which are readily eliminated. The interaction of bilirubin with the membraneous barrier of the red blood cell may be a primary process in the induction of hemolytic anaemia. Changes in the activity of enzymes mediating transport of ions such as Na^+K^+ or HCO^-_2 are induced by circulating plasma free bilirubin. The pigment by its ability to bind to serum albumin also gains entry into brain crossing the blood brain barrier where conditions like acidosis prevails. The entry of bilirubin into brain can lead to irreversible damage. If, in contrast, the transport system is oriented towards spread on the skin and its subsequent removal by phototherapy or alternatively towards the liver for its removal by conjugate system stimulated by simple drugs such as barbiturate, the precious neonatal life can be saved from death.

In the elegant manner in which neonatal life deals with bilirubin, a toxic product of its own metabolism, there is a message to us when we consider the currently vexing problem of exposure to

toxic chemicals in our living environment. Co-existence with a toxic situation is rendered possibly by an inevitable process of biochemical adaptation. From the primitive amoeba to the highly evolved human, there is evidence of this adaptation at different phase of life. Discovery of the underlying cellular processes and the substaining molecular reactions is a challenge for biochemists.

Acknowledgements

My heart opens out in gratitude to the late Dr P C Bajpai, the former Professor of Pediatrics, K. G.'s Medical College, Lucknow, who was mainly responsible for stimulating my interest in neonatal jaundice. I am deeply indebted to my most devoted students Chiranjiv Lal Kapoor, Ravi Kaul and Hari Krishen Kaul, who obtained their Ph.D. working on different aspects of the problem. My special thanks are due to my former student and colleague Dr Maharaj Kishen Sahib for extremely valuable suggestions and various forms of help in conducting some of the studies and to Dr V K Bajpai and Dr A C Shipstone for help in taking scanning electron micrographs.

References

Bajpai P C, Misra P K, Das V K, Tripathy T K and Kapoor C L 1970 Evaluation of phototherapy in ABO hemolytic disease of the new born; *Indian J. med. Res.* **61** 1658

——, ——, ——, Singh D, Kapoor C L and Krishna Murthi C R 1973 Phototherapy in hyperbilirubinaemia of children; *Indian J. med. Res.* **64** 577

——, Tripathy T K, Singh D and Kapoor C L 1973 Phototherapy in Rh incompatibility hyperbilirubinaemia; *Indian J. med. Res.* **61** 221

——, Srivastava K L, Singh D, Krishna Murti C R and Kapoor C L 1976 Urinary catabolites of bilirubin in jaundiced neonates; *Indian J. med. Res.* **64** 529

Kapoor C L, Bajpai P C and Krishna Murti C R 1972 Toxic effect of bilirubin on human red blood cell and its reversal by RBC lipids; *Indian. J. med. Res.* **69** 918

——, Krishna Murti C R and Bajpai P C 1973 Uptake and release of bilirubin by skin; *Biochem. J.* **136** 35

——, —— and —— 1974 Role of human skin in the photodecomposition of bilirubin; *Biochem. J.* **142** 567

——, —— and ——1973 Interaction of bilirubin with human skin; *New England. J. med. USA* **288** 583

——1975 Biochemistry of bilirubin—An overview; *J. Sci. Industr. Res.* **37** 474

Kaul H K 1981 Chemical studies on the cytotoxicity of bilirubin, Ph.D. Thesis, Avadh University, Faizabad, U.P.

Kaul R, Kaul H K, Bajpai P C and Krishna Murti C R 1979 Evidence for the possible involvement of the superoride radical in the photodegradation of bilirubin; *J. Biosci.* **1** 377

——, —— and Krishna Murti C R 1980 An alternate pathway for bilirubin catabolism *FFBS Letters* **111** 290

——, Misra P K, Kaul H K and Bajpai P C 1980 Non-toxicity of bilirubin photocatabolites; *Indian J. med. Res.* **72** 381

——, Bajpai V K, Shipstone A C, Kaul H K and Krishna Murti C R 1981 Bilirubin induced erythrocyte membrane cytotoxicity; *Exptl. Mol. Pathol* **34** 1326

——, 1979 Chemical studies on photocatabolism of bilirubin, Ph.D. Thesis, Avadh University Faizabad, U.P.