

Simulation of Bilirubin Detoxification in the Newborn Using an Extracorporeal Bilirubin Oxidase Reactor

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ABSTRACT. Jaundice, which is characterized by an excessive accumulation of bilirubin in the blood and tissues, occurs in 13% of newborns. The common treatments for neonatal jaundice are phototherapy and blood exchange transfusion. A novel approach using an extracorporeal blood filter containing immobilized bilirubin oxidase was recently proposed to detoxify jaundiced blood, and a prototype device markedly reduced serum bilirubin in genetically jaundiced Gunn rats. The primary toxicologic effect in that study was a 20% reduction in red blood cell count. Using a compartmental model for bilirubin metabolism, a mathematical simulation of the extracorporeal treatment's ability to reduce serum bilirubin levels in jaundiced infants is presented. Using a 10-mL reactor volume containing immobilized bilirubin oxidase, the simulation predicts a 32 to 65% decrease in plasma bilirubin concentration over a 4-h treatment for a 2 kg preterm hyperbilirubinemic newborn. In addition, a new approach to altering support material has essentially eliminated red blood cell lysis *in vivo* using Gunn rats and *in vitro* using adult blood. (*Pediatr Res* 26: 452-457, 1989)

This involves the replacement of the infant's blood with bilirubin-free adult blood. This procedure presents several risks, including blood damage, fluid shifts, risk of transmission of infectious diseases, and possibly death (2).

Although these treatments have reduced the occurrence of bilirubin toxicity, the associated risks remain a serious problem. Lavin *et al.* (7) reported the use of a highly specific enzyme to remove bilirubin from the bloodstream. With this method, a small reactor containing a bilirubin detoxifying enzyme is placed in an extracorporeal circuit and the bilirubin detoxified blood is returned to the patient. A prototype device markedly reduced serum bilirubin concentrations *in vitro* using human umbilical-cord blood to which bilirubin was added, and *in vivo* using genetically jaundiced Gunn rats. The primary toxicologic effect observed in the previous studies was a 20% reduction in red blood cell count (7).

This report evaluates the efficacy of the extracorporeal treatment by providing a mathematical simulation of the reduction in plasma bilirubin in jaundiced newborns. Also, a new approach is presented that essentially eliminates red blood cell lysis *in vitro* with adult blood and *in vivo* with Gunn rats by altering the support material in the reactor.

Bilirubin originates in the body with the catabolism of the cyclic tetrapyrroles, the major pigment in bilirubin production being Hb (1). All human newborns accumulate bilirubin to levels greater than those in adults, and 20% accumulate enough to stain their skin, resulting in jaundice (2). Clinically elevated bilirubin levels have been associated with mental retardation, cerebral palsy, seizures, deafness, or death (2).

The two most common treatments for neonatal jaundice are phototherapy and exchange transfusion. In phototherapy, bilirubin is converted to more readily excreted photoisomers by exposing the infant to blue light. Phototherapy has been used extensively for plasma bilirubin concentrations up to 15 mg/dL, but it suffers from the inherent limitation that the total plasma bilirubin concentration can be lowered by only 15 to 20% within 48 h (3, 4). In addition, the possible long-term dangers of exposing the newborn to blue light have not been fully evaluated. DNA as well as several essential amino acids and vitamins have been shown to be light sensitive (5, 6).

For severe cases of jaundice (plasma bilirubin >15 mg/dL), it may be necessary for the infant to undergo exchange transfusion.

MATERIALS AND METHODS

Reagents. All reagents were analytical grade. Lyophilized bilirubin oxidase (EC.1.3.3.5) obtained from *Myrothecium verrucaria* MT-1 (Amano International Enzyme Co. Troy, VA.) had a specific activity of 2.27 U/mg. One unit of bilirubin oxidase is defined as the enzyme quantity which can oxidize 1 μ mol of bilirubin per min in a phosphate buffer (0.05 mol/L, pH 7.4) containing 20 μ mol/L bilirubin, 30 μ mol/L serum albumin, and 20 mmol/L sodium-cholate. Bilirubin was purchased from Porphyrin Products (Logan, UT) and was used without further purification. Tresyl-Chloride (2,2,2-trifluoroethanesulfonyl chloride) was from Fluka AG (Ronkonoma, NY). Nonactivated Biogel A 1.5 (8% agarose) beads were from Bio-Rad Laboratories (Rockaway, NY). Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Heparin (sodium injection, 10,000 USP U/mL) was from Elkins-Sinn, Inc. (Cherry Hill, NJ).

Assays. The enzyme protein concentration was determined by the method of Lowry *et al.* (8) (Sigma research kits and reagent no. P5656, Sigma Chemical Co., St Louis, MO) using BSA (Sigma) as a standard. Serum albumin concentrations were measured by the method of Doumas *et al.* (9) (Sigma diagnostic kit and reagent no. 631; Sigma) using BSA or rat serum albumin (Sigma) as standard. Total plasma bilirubin concentrations were

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determined using the method of Jendrassik and Grof (10) (Sigma diagnostic kit and reagent no. 605; Sigma).

Preparation of activated agarose beads. The agarose beads were screened using sieves of different mesh sizes (USA Standard Sieve Series, Newark Wire Cloth Co., Newark, NJ) and beads ranging from 180 to 250 μm in diameter were used. The volume of swollen beads was measured by centrifugation in a graduated tube at $1000 \times g$ for a period of 5 min.

The beads were activated by a sulfonyl chloride method using tresyl-chloride under anhydrous conditions as described by Nilsson and Mosbach (11). Using elemental sulfur analysis (12), the activation yield was 325 μmoles of tresylate groups per dry g of agarose.

Bilirubin oxidase immobilization and activity. Lyophilized bilirubin oxidase powder contained approximately 50% wt/wt protein. To remove the salts present in the crystalline enzyme, the powder was dissolved in sodium bicarbonate buffer (0.02 mol/L, pH 8.3) and the solution passed over a 25-mL Sephadex G-25 column preequilibrated with the buffer. The enzyme solution examined by SDS-PAGE, as described by Yang *et al.* (13) showed a single protein band indicating high enzyme purity. Immobilization of bilirubin oxidase onto tresyl-chloride activated agarose beads was performed as described by Comfort *et al.* (12). Briefly, 1 mL of enzyme solution at a concentration of 3 mg/mL was incubated at 22°C for 4 h in the presence of 1 mL of active beads. To desorb noncovalently bound enzyme molecules, the support was washed with several volumes of a PBS (0.5 mol/L NaCl, 0.07 mol/L phosphate, pH 7.0) as described by Mullon *et al.* (14). The amount of enzyme immobilized determined from the difference between the initial total protein and the amount recovered in the phosphate-saline wash was 1.7 mg/mL of beads. The enzymatic activity of the beads was determined at 37°C in bilirubin enriched plasma prepared as follows.

Plasma from freshly drawn adult blood heparinized at 10 U/mL was used. The albumin concentration measured by the method of Dumas *et al.* (9) was 620 $\mu\text{mol/L}$. A bilirubin solution was prepared by dissolving 5.0 mg of the pigment in a few drops of 0.1 N NaOH. The solution was protected from the light by aluminum foil and used within 15 min after preparation. The bilirubin enriched plasma solution was then prepared by adding the bilirubin solution to 19.5 mL of plasma. The pH of the enriched plasma was adjusted to 7.4 by adding a few drops of 0.02 N HCl. The enriched plasma contained 420 $\mu\text{mol/L}$ bilirubin and a bilirubin serum albumin molar ratio of 0.69.

The initial enzymatic activity of the beads was measured spectrophotometrically at 460 nm by following the disappearance of bilirubin in the bilirubin plasma mixture as described by Mullon *et al.* (15). Typically, the catalytic activity was 25 nmol/min/mL of beads.

Reactor. The reactor was made of agarose beads packed in a 1.5-cm diameter, 10-cm ht column (Biorad Econo-Column, Biorad Laboratories, Richman, CA). The column was maintained at 37°C using a 10-cm ht Water-Crest jacket (Thomas Scientific, Swedesboro, NJ) filled with flowing water. Blood was passed through the column using a peristaltic pump (LKB microperpex peristaltic pump, LKB, Sweden) and silicone tubing (Silastic, Dow-Corning, Midland, MI).

Hemocompatibility. In their *in vivo* and *in vitro* studies on bilirubin detoxification, Lavin *et al.* (7) reported no statistically significant changes in most blood components except a 20% decrease in red blood cell count in both experiments and controls. The reactor was made of commercially available agarose beads used in chromatography. These beads have a diameter ranging from 60 to 140 μm . If the beads are assumed perfectly spherical, it can be calculated that the smallest opening between adjacent beads would permit the passage of about 10 μm diameter spherical particles (16). This is approximately the diameter of red blood cells. Agarose beads of 180 to 250 μm in diameter were selected in our study because they have a larger theoretical

minimum space between beads that allows passage of about 30 μm diameter spheres (16). Using these larger diameter beads, red cell counts were monitored both *in vitro* and *in vivo*.

To simulate the potential *in vivo* use of the reactor, the *in vitro* studies were performed using a closed loop circuit. Freshly drawn adult human blood (140 mL) heparinized at 10 U/mL was placed in a reservoir and perfused through the reactor. The *in vivo* studies were performed according to Lavin *et al.* (7). All animal care was in accordance with the NIH guidelines and was approved by the Massachusetts Institute of Technology Committee on Animal Care. Briefly, homozygous male Gunn rats (Blue Spruce Farms, Altamont, NY) of 350 g body wt were used. After the rats were anesthetized using nembutol (Henry Schein Inc., Port Washington, NY; 4 mg of anesthetic per 100 g of body wt), the left carotid artery and jugular vein were cannulated using PE-50 polyethylene tubing (Clay Adams, Parsippany, NJ), and 2000 U of heparin were injected. The blood, pumped at 0.7 mL/min from the artery, was passed through the reactor containing 3 mL of beads and returned to the rat via the jugular vein. The apparatus was initially primed with about 4 mL of 0.9% saline solution containing 1000 U of heparin. All the rats regained full consciousness before the experiment began. Blood samples of 0.7 mL each were collected from the arterial shunt at the beginning and at the end of the 90-min study. All blood samples were drawn in vacutainer tubes containing EDTA (Becton Dickinson, Rockville, MD).

Complete blood cell counts were measured by Bioran Medical Laboratory (Cambridge, MA) using a coulter counter (Coulter Electronics, Hialeah, FL), and the cell counts were normalized by the count at the start of the experiment. Serum albumin and plasma bilirubin concentrations in Gunn rats were determined by the method of Dumas *et al.* (9) and the method of Jendrassik and Grof (10), respectively. Typically the serum albumin and bilirubin concentrations were 500 $\mu\text{mol/L}$ and 6 mg/dL, respectively.

Simulation of bilirubin detoxification. Most bilirubin circulates in the blood bound to serum albumin (17, 18). Stoichiometric bilirubin-serum albumin binding analysis and experimental measurements of unbound bilirubin showed that the unbound bilirubin concentration in plasma is small (3 to 30 nM) and is well approximated by the following expression (15, 19)

$$b = s/K_1(1.1 - s) \quad (1)$$

where b is the unbound bilirubin concentration, s is the molar ratio of the total bilirubin concentration divided by the serum albumin concentration, and K_1 is the bilirubin serum albumin stoichiometric equilibrium constant ($K_1 = 60 \mu\text{M}^{-1}$).

The mechanism of bilirubin toxicity *in vivo*, whereas poorly understood, probably stems from its low aqueous solubility [5 nM at pH 7.4 (17, 18)] which may lead to the transport and accumulation of unbound bilirubin in several tissues where it interferes with cell functions (19). From the plasma disappearance curve of intravenous injections of radiolabeled bilirubin in adults, a three-compartment model of bilirubin distribution within the body (Fig. 1) has been considered to be the most physiologically reasonable representation (20, 21). The three compartments are considered to represent plasma, hepatic, and extrahepatic-extravascular pools of unconjugated bilirubin. However, the presence of unconjugated bilirubin in meconium stools from both premature and term infants (19, 22) suggests that unconjugated bilirubin is present in the gut during neonatal jaundice. A fourth compartment was then added to represent the intestinal pool of unconjugated bilirubin (Fig. 1). Bilirubin production results mainly from the catabolism of Hb in the cells of the reticuloendothelial system which include macrophages and phagocytic endothelial cells lining blood sinuses in various organs (23). However, the bilirubin production rate for each organ is not known. Therefore, an overall bilirubin production function is commonly used (20, 21) and represented as a single

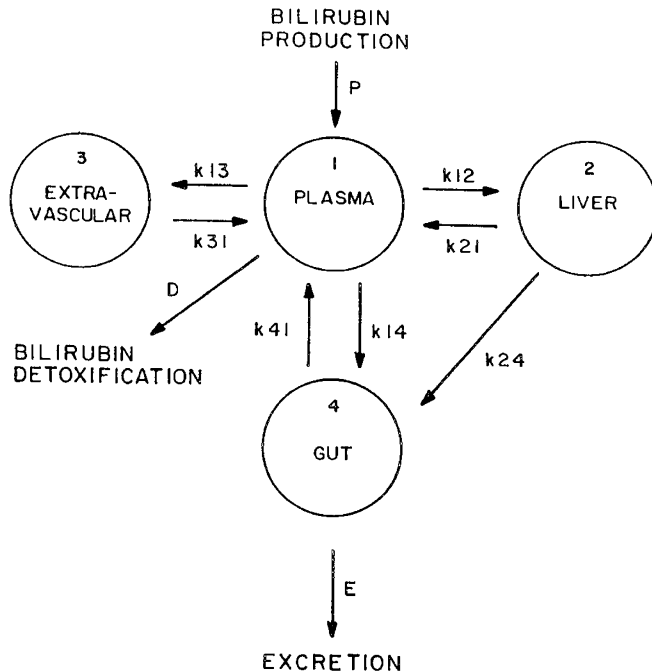


Fig. 1. Compartment model of bilirubin distribution within the body; plasma (1), hepatic (2), extrahepatic-extravascular (3), and intestinal (4) pools of unconjugated bilirubin.

input into the plasma pool (Fig. 1). Bilirubin released into the plasma by the reticuloendothelial cells circulates bound to serum albumin and is eliminated from the bloodstream by the hepatocytes which are represented in Figure 1 by the hepatic pool. Excretion of conjugated bilirubin by the hepatocytes is represented by the transport of bilirubin from the hepatic to the intestinal pool. In the gut, bacterial flora deconjugate and reduce the pigment to various compounds which are excreted in feces and urine. The formation of these compounds from unconjugated bilirubin is represented as a single arrow leaving the intestinal pool. The potential reabsorption of unconjugated bilirubin during its enterohepatic circulation is represented by a set of arrows between both intestinal and plasma pools. The several other arrows represent bilirubin transport between the different pools.

According to the kinetic study of Berk (21), bilirubin transport from one compartment to another may be represented by first order kinetics and the rate of change of bilirubin in a given pool by an ordinary differential equation. Thus, the bilirubin metabolism and the extracorporeal bilirubin detoxification (Fig. 1) may be modeled by a set of differential equations, as follows:

$$dC_1/dt = -(k_{12} + k_{13} + k_{14})C_1 + k_{21}C_2 + k_{31}C_3 + k_{41}C_4 + wP/V_1 - D/V_1 \quad (2)$$

$$dC_2/dt = -(k_{21} + k_{24})C_2 + k_{12}C_1 \quad (3)$$

$$dC_3/dt = -k_{31}C_3 + k_{13}C_1 \quad (4)$$

$$dC_4/dt = -k_{41}C_4 + k_{24}C_2 + k_{14}C_1 - wE/V_4 \quad (5)$$

with P , bilirubin production function; D , reactor detoxification function; C_i , bilirubin concentration in pool i (Fig. 1); w , body wt; V_1 , volume of the plasma pool; V_4 , volume of the intestinal pool; E , excretion function; k_{ij} , rate constants.

The intercompartmental rate constants k_{21} , k_{13} and k_{31} , k_{41} were taken as reported by Berk and coworkers (20, 21). The bilirubin production (P), hepatic uptake (k_{12}), and conjugation (k_{24}) rates were modeled according to Gartner *et al.* (24) and the bilirubin excretion rate from the intestinal pool according to Odell and coworkers (19, 22). These studies showed that during

the first days after birth the rates of bilirubin production, hepatic uptake, hepatic conjugation, and excretion vary exponentially with time. Accordingly, the several rates and rate constants were modeled as follows:

Bilirubin production

$$P = P_n \times P_{\text{birth}} \times e^{-k_1 t} \text{ for } t \leq t_p \quad (6)$$

with

$$k_1 = [\ln P_{\text{birth}}]/t_p \quad (7)$$

and

$$P = P_n \text{ for } t > t_p \quad (8)$$

where P_n is the normal adult bilirubin production and P_{birth} is a constant by which the normal adult bilirubin production was multiplied to conform with the production at birth. The parameter t_p is the time at which the rate P becomes the normal adult value.

Hepatic uptake

$$k_{12} = (k_{12})_n (1 - e^{-k_2 t}) \quad (9)$$

where $(k_{12})_n$ is the normal adult bilirubin hepatic uptake rate constant.

Hepatic conjugation

$$k_{24} = (k_{24})_n (1 - e^{-k_4 t}) \quad (10)$$

where $(k_{24})_n$ is the normal adult bilirubin conjugation rate constant.

Bilirubin excretion

$$E = E_n (1 - e^{-k_5 t}) \quad (11)$$

where E_n is the bilirubin excretion rate in infants of 6 d of age and older (19, 22).

The extracorporeal rate of bilirubin detoxification from the plasma pool or bilirubin detoxification function (D) may be represented by the bilirubin concentration change over the reactor multiplied by the flow rate.

$$D = Q(C_{\text{in}} - C_{\text{out}}) \quad (12)$$

The *in vitro* performance of a packed bed reactor containing immobilized bilirubin oxidase has been studied by Mullon *et al.* (15). The bilirubin concentration change over the reactor was represented as follows:

$$C_{\text{out}} - C_{\text{in}} = -(k E b_i V)/Q \quad (13)$$

where C_{out} , bilirubin concentration at the reactor outlet; C_{in} , bilirubin concentration at the reactor inlet; Q , flow rate; V , volume of beads; E , immobilized enzyme per volume of beads; b_i , unbound bilirubin concentration at the reactor inlet; k , constant of proportionality.

Equation 13 combined with equation 12 yields the bilirubin detoxification function:

$$D = R V = (k E b_i) V \quad (14)$$

The unbound unconjugated bilirubin concentration at the reactor inlet (b_i) is defined by the plasma bilirubin and albumin concentrations (equation 1), and by the presence of displacing substances competing at the bilirubin-serum albumin binding sites. Bilirubin oxidase catalyzes the oxidation of bilirubin to biliverdin which is a more soluble tetrapyrrole (1, 17). Biliverdin is then further oxidized to a purple pigment (25). The second oxidation step may yield products more soluble and more rapidly cleared from the plasma than biliverdin. However, both reaction products are bilirubin displacing substances (15, 26), and therefore, the unbound bilirubin concentration at the reactor inlet depends on the clearance rate of the products of the reaction from the plasma pool.

The metabolism and the clearance rate of the reaction products

from the plasma pool are unknown. To simulate the *in vivo* performance of the extracorporeal detoxification, two product clearance conditions were considered: 1) rapid clearance of both products; 2) slow clearance of both reaction products. The true *in vivo* product clearance should be within these conditions.

Rapid clearance of reaction products. The unbound bilirubin concentration at the reactor inlet is not affected by the presence of the reaction products. The inlet unbound concentration (b_i) is given by equation 1 and the detoxification function becomes:

$$D = (kEV/K_1)(C_1/1.1A - C_1) \quad (15)$$

where A is the serum albumin concentration.

Slow product clearance. The products of the reaction stay in the plasma pool during the treatment. According to the study by Mullon *et al.* (15) the *in vitro* unbound bilirubin concentration in the presence of the products of the reaction is well approximated by the following equation:

$$b = C_{10}/[K_1(1.1A - C_{10})] \quad (16)$$

where C_{10} is the plasma bilirubin concentration at the onset of the treatment. Combining equations 15 and 16 leads to,

$$D = kEvb = \text{constant} \quad (17)$$

Using the fourth order Runge-Kutta algorithm (27), equations 2 to 17 were solved numerically on a VAX 11/750 computer running DEC VMS operating system (Digital Equipment Corporation, Maynard, MA).

RESULTS AND DISCUSSION

Simulation of *in vivo* bilirubin detoxification. Hyperbilirubinemia in the neonate was simulated with the following production rate, excretion rate, and intercompartmental rate constants: $k_{21} = 0.4 \text{ h}^{-1}$, $k_{13} = 0.28 \text{ h}^{-1}$, $k_{31} = 0.1 \text{ h}^{-1}$, $k_{14} = 0.005 \text{ h}^{-1}$, $k_{41} = 0.4 \text{ h}^{-1}$, $(k_{12})_n = 1.0 \text{ h}^{-1}$, $(k_{24})_n = 0.67 \text{ h}^{-1}$, $P_{\text{birth}} = 2.0$, $P_n = 3.8 \text{ mg/kg/d}$, $E_n = 5 \text{ mg/kg/d}$, $V_1 = 40 \text{ mL/kg}$ of body wt, $V_4 = 80 \text{ mL/kg}$ of body wt, and the initial concentrations $C_1 = C_2 = C_3 = 1.0 \text{ mg/dL}$ and $C_4 = B_4/V_4$ where B_4 is the initial amount of unconjugated bilirubin in the intestinal pool ($B_4 = 250 \text{ mg}$ (19)). The intercompartmental rate constants were from Berk and coworkers (20, 21) and the bilirubin production factor (P_{birth}) from Valaes (28). The initial concentrations, the production rate and the excretion rate were from Odell and coworkers (19, 22).

The adjustable parameters k , k_2 , k_4 , and t_p were evaluated by comparison with the data reported by Gartner *et al.* (24) who studied bilirubin transport and metabolism in human newborn infants and in newborn rhesus monkeys. Figure 2 shows good agreement between the serum bilirubin concentrations reported

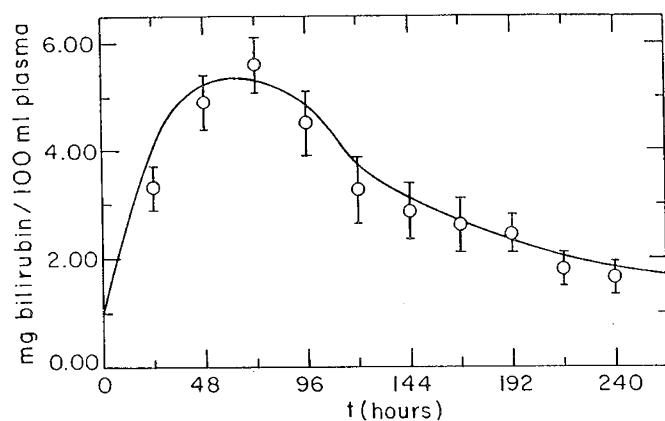


Fig. 2. Plasma bilirubin concentration as a function of time. The open circle and SEM represent the clinical data, of Gartner *et al.* (24) for human newborn infants ($n = 29$). The solid line represents plasma bilirubin concentrations predicted by the computer simulation.

by Gartner *et al.* (24) for human newborn infants and the serum bilirubin concentrations predicted by the model ($k = 0.005 \text{ h}^{-1}$, $k_2 = -0.045 \text{ h}^{-1}$, $k_4 = -0.0015 \text{ h}^{-1}$, and $t_p = 30 \text{ d}$).

Simulation of a 4-h treatment in a hyperbilirubinemic newborn infant is presented in Figure 3 for both rapid and slow clearance of the products of the reaction. The bilirubin production rate was chosen to be four times the normal bilirubin production in newborns. This rate of production is within the bilirubin production range for hemolytic jaundice (19, 28). The treatment was simulated for a 2-kg premature infant, a reactor containing 10 mL of agarose beads with a catalytic activity of 25 nmol/min/mL of bead, a blood flow rate of 3.0 mL/min through the reactor and a serum albumin concentration of 3.5 g/dL (19). The treatment was initiated as the plasma bilirubin concentration reached 17 mg/dL, reflecting the bilirubin plasma concentration at which blood exchange transfusion has been suggested for a 2.0-kg premature infant (2, 29). Figure 3 shows a significant decrease in plasma bilirubin concentration for both reaction product clearance conditions. The simulation of a 4-h treatment predicts a 32% decrease in plasma bilirubin concentration for rapid clearance of the reaction products and a 65% decrease for slow clearance of the reaction products. Higher reduction of plasma bilirubin with the slow clearance condition was expected because the products of the reaction compete for the bilirubin binding sites on the serum albumin molecules (15, 26) which leads to a higher unbound bilirubin concentration and a higher enzymatic rate of bilirubin degradation (equation 14). The true plasma clearance of the products of the reaction should be within the slow and fast clearance conditions and the reduction in plasma bilirubin concentration within the predicted percentages (32 to 65%). Such an efficacy of the device and associated decrease in plasma bilirubin might alleviate the need for blood transfusion.

In vivo and in vitro red cell hemolysis. In their *in vivo* and *in vitro* studies on bilirubin detoxification, Lavin *et al.* (7) reported no statistically significant change in platelet or white cell count but a 20% decrease in red blood cell count using immobilized bilirubin oxidase and agarose beads only. The present *in vivo* and *in vitro* studies were aimed at reducing or eliminating red blood cell hemolysis for future use of the reactor in humans. Agarose beads of 180 to 250 μm in diameter were selected because they have a higher theoretical minimum space between beads (30 μm) than the 60 to 140 μm beads used by Lavin *et al.* (7) (minimum space between beads 10 μm). Hemolysis may result from poor blood biocompatibility of the extracorporeal circuit associated with a direct mechanical effect on the red blood

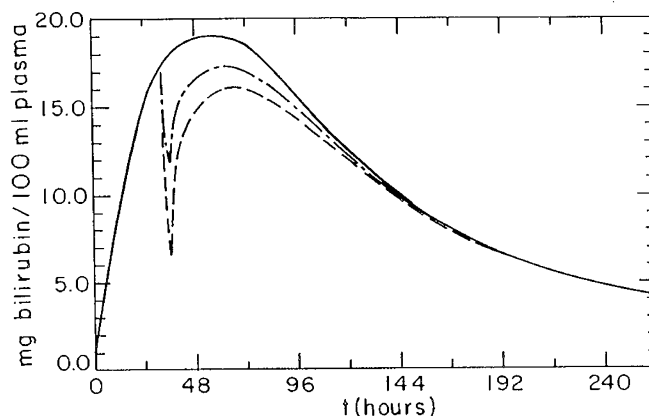


Fig. 3. Predicted plasma bilirubin concentration as a function of time in hyperbilirubinemic newborn infants. The bilirubin production was assumed eight times the normal adult bilirubin production. Predicted plasma bilirubin concentrations: (—) in the absence of extracorporeal treatment, (---) for a 4-h treatment and fast clearance of the products, (- -) for a 4-h treatment and slow clearance of the products.

cells through shear forces (16, 30). A higher theoretical space between beads lowers the mechanical effect through lower shear forces.

Tables 1 and 2 show the red blood cell recovery under *in vitro* and *in vivo* conditions. Four Gunn rats were treated for 90 min with a reactor containing 3.0 mL of agarose. The blood flow rate through the reactor was 0.7 mL/min and two of the four Gunn rats were treated with immobilized bilirubin oxidase (1.7 mg enzyme/mL agarose). Besides the four *in vivo* experiments, two *in vitro* experiments were conducted using a closed loop circuit. Freshly drawn human blood (140 mL) heparinized at 10 U/mL was pumped through 4.0 mL of agarose beads at a flow rate of 1.2 mL/min for a period of 2 h. In one of the two *in vitro* experiments, the blood was exposed to immobilized bilirubin oxidase (1.7 mg enzyme/mL agarose beads). For both *in vitro* and *in vivo* conditions, with and without bilirubin oxidase, the red blood cell count did not drop during the course of the experiments. Although the plasma bilirubin concentration stayed unchanged in the two rats treated with agarose beads only (experiments A and B), the plasma bilirubin concentration decreased by 15 and 20% in the two rats treated with a reactor containing bilirubin oxidase (experiments C and D). The observed decreases in plasma bilirubin concentration are in agreement with the data reported by Lavin *et al.* (7) who observed a 50% decrease in plasma bilirubin concentration in Gunn rats using a 8-mL reactor volume. Lavin *et al.* (7) used cyanogenbromide-activated agarose beads with the same amount of enzyme immobilized per volume of beads. However, the use of bilirubin oxidase immobilized onto tresyl-chloride-activated beads may be preferable because tresyl-chloride chemistry has been shown to yield more stable protein-support bonds (12).

CONCLUSION

A simulation of bilirubin distribution in the body and subsequent removal by extracorporeal bilirubin oxidase treatment was developed to provide additional understanding of the detoxification and to predict the treatment efficacy in hyperbilirubinemic neonates. Using a 10-mL reactor volume, the simulation predicts a lowering of 32 to 65% of the initial plasma bilirubin concentration (17 mg/dL) in a 2-kg preterm infant during a 4-h treatment. This drop in plasma bilirubin concentration might be

Table 1. *In vivo* red blood cell recovery: normalized red blood cell counts (final RBC/initial RBC)*

Experiment	Reactor vol (mL of beads)	Enzyme (mg/mL beads)	RBC (final/initial)
A	3	0	1.07
B	3	0	1.20
C	3	1.7	1.01
D	3	1.7	1.06

* Four Gunn rats with an initial plasma bilirubin concentration ranging from 5.5 to 6.5 mg/dL were treated for 90 min. The blood flow rate in the extracorporeal reactor was 0.7 mL/min. Upon completion of the treatment, the plasma bilirubin concentration was unchanged in experiments A and B. In experiments C and D, 15% and 20% decrease in plasma bilirubin concentration were observed, respectively.

Table 2. *In vitro* red blood cell recovery: normalized red blood cell counts (RBC/initial RBC)*

Experiment	Reactor vol (mL of beads)	Enzyme (mg/mL beads)	RBC ratios	
			t = 60 min	t = 120 min
E	4	0	0.92	0.90
F	4	1.7	1.00	0.98

* Heparinized human blood (140 mL, 10 U heparin/mL) was recycled through the reactor at a flow rate of 1.2 mL/min.

sufficient to alleviate the use of blood transfusion. Other investigators have reported the use of hemoperfusion over ion exchange and affinity chromatography resins for removal of bilirubin (31, 32) but due to the limited adsorption capacity of the resins, the recommended sizes of the columns ranged between 25 to 65% of the neonate's blood volume. The resins also retained other essential compounds such as cortisol and thyroxine (31, 32). Bilirubin oxidase catalyzes the oxidation of bilirubin to biliverdin and biliverdin to a purple pigment (25). Researchers studying the toxic effects of bilirubin and biliverdin on the metabolism of mammalian cells *in vitro* (33, 34) found that biliverdin is much less toxic than bilirubin. The purple pigment is still unknown and further work must be done to characterize its toxicity. However, it was shown that the products of the reaction (biliverdin, purple pigment) are noncytotoxic, nonmutagenic, and nonteratogenic (7). Although the bilirubin displacing property of the products of the reaction was considered in the enzymatic rate of bilirubin degradation (slow clearance and fast clearance conditions, equations 15 and 16), their accumulation in the blood stream may further affect the efficiency of the detoxification process. For instance, biliverdin (about 30% of the reaction products) may be reconverted to bilirubin by the cells of the reticuloendothelial system. The products may also interfere with the net movement of bilirubin from the tissues. The potential effect of the reaction products on the rate constants between bilirubin pools is not known and was not included in the model. However, the displacement of unconjugated bilirubin from several tissues would probably increase the unbound bilirubin concentration in the blood stream leading to a higher rate of bilirubin degradation by the bilirubin oxidase reactor (equation 14).

The simulation predicts the bilirubin concentration in the plasma as well as in the extravascular compartment where the toxic effect of bilirubin occurs. Unfortunately, data concerning bilirubin concentrations in the extravascular compartment are not available for comparison. Such data would provide additional information in estimating the adjustable parameters.

The absence of red blood cell lysis using agarose beads of 180 to 250 μ m in diameter instead of 60 to 140 μ m (7) represents a potentially safer use of the device. However, because neonatal red blood cells differ in their membrane properties from adult red blood cells, whether the degree of hemolysis would be similarly reduced still needs to be investigated. Additional studies should also be carried out with lower amounts of blood anticoagulant (heparin) to use this technique clinically.

In summary, this reactor may provide a new approach to the treatment of neonatal jaundice and serve as a model for the clinical application of immobilized enzymes for the detoxification of compounds in blood.

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