

Experimental Bilirubin Encephalopathy: Importance of Total Bilirubin, Protein Binding, and Blood-Brain Barrier

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ABSTRACT. The cause of bilirubin encephalopathy has been variously ascribed to elevated total serum bilirubin concentration, high free bilirubin levels (or impaired albumin binding), and disruption of the blood-brain barrier. An experimental rat model for acute bilirubin encephalopathy was developed in which these three factors could be varied independently. Osmotic opening of the blood-brain barrier in the right hemisphere was produced by infusing a hypertonic arabinose solution into the right carotid artery. The total bilirubin level and bilirubin binding state were varied by adjusting the amount of bilirubin infused intravenously and/or by infusing human serum albumin. Brain electrical activity (EEG) served as an indicator of developing encephalopathy. Neither staining nor EEG changes occurred if the blood-brain barrier remained intact. Bilirubin staining without EEG evidence of encephalopathy sometimes occurred when the blood-brain barrier was open. Discriminant analysis showed that EEG changes were best predicted by the degree of blood-brain barrier opening (as indicated by brain bilirubin content) and by the quality of serum bilirubin binding. Serum total bilirubin concentration was not an important discriminator of encephalopathy. (*Pediatr Res* 20: 789-792, 1986)

Abbreviations

HSA, human serum albumin
BSA, bovine serum albumin
BBB, blood-brain barrier

Thirty-five years after the indictment of bilirubin as the cause of kernicterus (1), our understanding of the pathogenesis of bilirubin encephalopathy remains incomplete. Total bilirubin concentration (2), free bilirubin concentration (3, 4), and reduced serum bilirubin binding (5-7) have been implicated as critical elements in the pathogenesis of this condition, but fail to explain fully the variable response of infants to hyperbilirubinemia. More recently, disruption of the BBB has been proposed to be the cause of bilirubin toxicity (8).

Infusion of a hypertonic solution of urea or arabinose into a carotid artery of a rat will temporarily open the BBB in the ipsilateral hemisphere (9, 10). Large proteins such as albumin may then gain access to the interstitial space of brain. If Evans Blue dye is bound to the albumin, the ipsilateral hemisphere will be stained blue (10). If bilirubin is bound to the albumin, the brain will turn yellow, as reported by Levine *et al.* (8). Levine *et*

al. (8) suggested that kernicterus and bilirubin toxicity are caused by the albumin-bilirubin complex crossing a disrupted BBB and argued that the level of free bilirubin or nature of bilirubin binding may not be important in the development of bilirubin encephalopathy. However, Levine *et al.* (8) did not provide histological, physiological, or behavioral evidence of bilirubin neurotoxicity to support this view.

There is, on the other hand, abundant *in vitro* evidence that albumin-bound bilirubin is not toxic (11-15) and that the concentration of free bilirubin acid may be responsible for cell injury (4, 16). Recently, we examined the possibility that yellow staining may not be harmful if it represents the transport of albumin bound bilirubin across a disrupted BBB. To test this hypothesis, we utilized Levine's rat model, and, in addition, cerebrocortical electrical activity was recorded as an indicator of acute bilirubin toxicity. The initial studies (17) indicated that 1) neither brain staining nor EEG changes could be produced in young adult rats with intact BBB who were exposed to severe hyperbilirubinemia (serum bilirubin 42-74 mg/dl) for 60 min; 2) following osmotic opening of the BBB, the affected hemisphere was stained yellow following bilirubin infusion; 3) yellow staining was not always associated with encephalopathy as judged by EEG changes; 4) rats infused with human serum albumin, which binds bilirubin more tightly than does rat serum, required a higher total bilirubin level before EEG changes were observed. These data strongly suggested that while osmotic opening of the BBB might facilitate the equilibrium of bilirubin between albumin and brain cells, encephalopathy would only occur when the free bilirubin is sufficiently elevated to drive toxic levels of bilirubin onto functional cell membranes. This study examines further the relationship between serum total bilirubin concentration, barrier opening, serum bilirubin binding state, and the development of encephalopathy.

METHODS

Twenty-seven young male Wistar rats, 300-350 g, were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneal). The calvarium was exposed and cleaned of periosteum. Stainless steel screw electrodes were placed through burr holes drilled anteriorly and posteriorly 3-4 mm on each side of the midline, with a ground electrode overlying the frontal sinus. Bipolar anterior to posterior recordings were made from the right and left electrode pairs on a Grass Model 7D Polygraph. Temperature was monitored by a rectal probe. A tracheostomy was performed to ensure a patent airway. Catheters were placed in a femoral artery for blood sampling and in a femoral vein for infusion of bilirubin and albumin. A PE10 catheter was placed in the right external carotid artery with the tip lying near the branch of the internal carotid artery for infusion of hypertonic arabinose solution.

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In order to independently vary the quality of binding and bilirubin levels, rats were primed with either 1.5 g/kg HSA (25% HSA, Cutter Laboratories) (12 rats) or an equivalent volume of saline (15 rats). Ten minutes later, 0.8–1.0 ml of a saturated arabinose solution was infused into the carotid artery over 30 s. The infusion was terminated when a decrease in amplitude in the EEG appeared, indicating opening of the barrier (Fig. 1). The volume of arabinose administered was less than that reported by Levine *et al.* (8) since marked opening of the BBB invariably produced a prolonged cessation of electrical activity over the ipsilateral hemisphere. Two min after the arabinose infusion or when cortical electrical activity recovered, a bilirubin solution was infused with a Harvard Pump via the femoral vein. Fifteen mg bilirubin (Sigma) was dissolved in 2.5 ml of 0.1 N NaOH/isotonic saline (final pH ca 9.5) and administered at a rate of 1.71 mg bilirubin/min (0.29 ml/min). Infusion of alkaline bilirubin had a negligible influence on blood pH. The room was darkened during the bilirubin infusion to minimize photoisomerization of the pigment. The infusion was continued for 4 to 8 min in order to establish a range of maximum total bilirubin concentration. The infusion was stopped immediately if EEG changes appeared in order to identify threshold conditions for bilirubin levels and binding state.

Blood was sampled immediately after termination of the bilirubin infusion for measurement of total bilirubin and assessment of serum binding. A second sample was obtained 5 min later following which 7 mg of Evans Blue (10 mg/ml saline) was infused through the femoral vein. Two min later the animal was sacrificed by infusion of 8 molar KCl. A thoracotomy was performed immediately and icteric plasma and red cells were removed by perfusing the animal with 120–150 ml isotonic saline through a catheter placed in the left ventricle. The brain was removed and a single coronal section was made at the level of

the anterior thalamus to examine staining of deep structures. The brain was then divided into brainstem/cerebellum, right and left hemispheres, and frozen at -10°C until analyzed.

After weighing each brain segment and homogenizing in 2 N acetic acid, the bilirubin was extracted with chloroform/methanol and partitioned into the chloroform phase by adding water (18). The bilirubin concentration was determined by measuring the absorptivity at 452 nm. Two extractions were performed with each brain, and the total brain bilirubin extracted was expressed in μg bilirubin/g wet weight. Bilirubin content of brain was presumed to primarily represent interstitial bilirubin-albumin and, therefore, reflect the degree of BBB disruption (8).

In a preliminary study (17) serum binding was measured using the peroxidase method. Results were believed to be unreliable due to the high intrinsic oxidation rate in many sera and because human and rat albumin have different albumin-bilirubin dissociation rates. In this study, serum binding was evaluated by a spectrophotometric method which measures the ability of BSA to compete with rat serum for binding bilirubin (19). Twenty-five μl serum were added to 1.0 ml Sorensen's buffer, pH 7.4, and the total bilirubin concentration was determined from the absorptivity at 450 nm. Extinction coefficients for bilirubin bound to rat serum, rat/HSA serum, and BSA were determined at various wave lengths. Nonicteric rat serum had negligible absorptivity at the wave lengths measured. BSA has a higher molar absorptivity at longer wavelengths than either rat or human serum albumin, with the maximal difference occurring at 485 nm. After determining the serum bilirubin concentration, the change in absorption at 485 nm was measured after adding 20 μl BSA solution (3 g/dl) to the cuvette. The relative concentrations of BSA-bilirubin and rat serum bilirubin were then determined. In a model system, where bilirubin is distributed between primary binding sites of BSA and rat or human albumin, titration of icteric serum with BSA will theoretically yield a complete description of serum binding. In these experiments, where the bilirubin/albumin ratio exceeded 1.0, only semiquantitative data could be obtained. BSA served as a nonsaturable alternative receptor similar to Sephadex G-25. The binding index was defined as the percent total serum bilirubin transferred to BSA ($100 \times \text{BSA-bilirubin}/\text{total serum bilirubin}$). High values of binding index reflect weak serum binding and a high free bilirubin concentration.

EEG changes following or during bilirubin infusion were classified as none (normal), a decrease in amplitude of 20–60% over one or both hemisphere (moderate) (Fig. 1D'), or a decrease in amplitude of 80–100% over both hemispheres (severe) (Fig. 1D). In some animals, EEG changes progressed for 2–4 min after bilirubin infusion was stopped. The EEG was scored according to the lowest amplitude observed.

Stepwise discriminant analysis (20) was performed to analyze the extent to which total bilirubin concentration, bilirubin binding index, and brain bilirubin concentration in each hemisphere contributed to the development of EEG abnormalities. The discriminatory procedure first selected that variable which was the best single discriminator of the three EEG groups. Additional variables were selected stepwise until the addition of another variable did not improve significantly the ability to discriminate between the three groups. Significance of differences in means in the three groups was assessed using the Newman-Keuls multiple range test for unequal sample sizes (21).

RESULTS

In five rats, we were unsuccessful in opening the BBB, and none of these animals developed staining or EEG changes. Of the remaining 22 animals (nine receiving HSA), seven maintained normal cortical electrical activity during and following the bilirubin infusion, five developed some decrease in amplitude over one or both hemispheres, and 10 developed flattening or near flattening of the EEG over both hemispheres. Typical

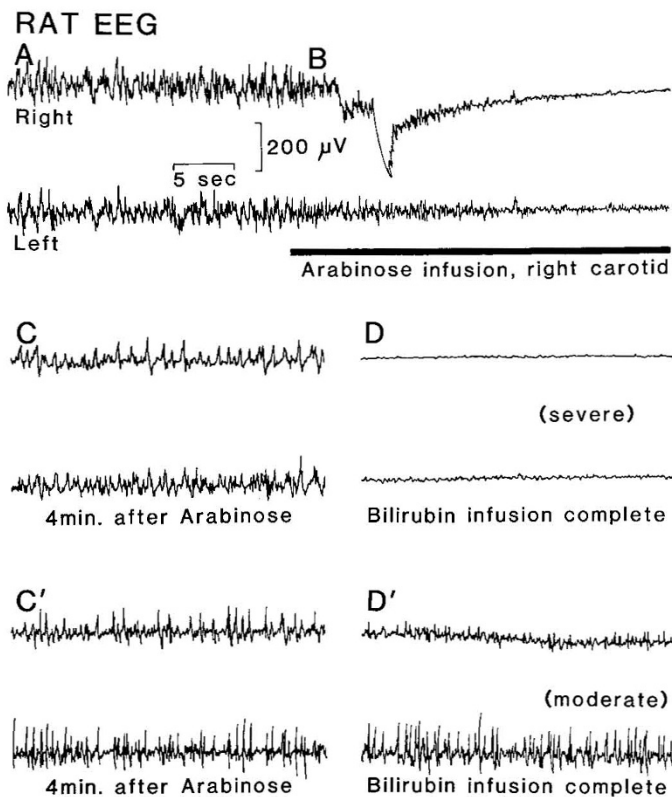


Fig. 1. Typical electroencephalographic changes. (A–D) EEG changes in a single rat before (A), during (B), and 4 min following (C) infusion of saturated arabinose solution in the right carotid artery. (D) Subsequent severe changes in EEG following bilirubin infusion. (C'–D') EEG recording in a rat demonstrating moderate changes induced by bilirubin.

responses are illustrated in Figure 1. In contrast to previous experience (17), infusion of HSA did not uniformly protect rats from EEG changes. However, the four HSA infused animals who developed moderate (3) or severe (1) encephalopathy had either poor serum binding despite the HSA infusion (more than 40% bilirubin bound to BSA) or deep staining of the right cerebral hemisphere.

Qualitative confirmation that the deeply stained right hemisphere was due to the movement of albumin-bilirubin across an open BBB was obtained by infusing Evans Blue dye intravenously 5 min after the infusion of bilirubin was terminated. Evans Blue binds to albumin without displacing bilirubin and will move into the interstitium if the barrier is permeable to proteins. When the BBB was opened, the dye produced a green color (bilirubin plus Evans Blue) in the right hemisphere and a small portion of the left frontal lobe which receives its blood supply from the common anterior cerebral artery. Separate blue or yellow stained areas were not observed. Staining was usually greatest in the temporal lobes and often extended into the thalamus and sometimes hypothalamic structures. The dorsum of the brain was variably stained.

The mean total bilirubin concentration was similar in all three groups (Table 1). The bilirubin content of the right hemisphere, reflecting the degree of BBB opening, was higher in rats with severe encephalopathy than in normal rats. Although the mean binding index appeared to be higher in the severely affected group, the difference did not reach statistical significance. There was no statistical correlation between bilirubin content in either right or left hemisphere and binding state. Most of the left hemisphere bilirubin content was presumed, therefore, to represent some cross-over effect of arabinose on the BBB and not movement of free bilirubin across an intact BBB.

Comparison of mean values (Table 1) can be deceptive when there is interaction between independent variables in producing an event. In this study, only the difference between "severe" and "normal" mean right hemispheric bilirubin content reached statistical significance. Possible interaction of bilirubin binding and right hemispheric barrier opening in the development of encephalopathy is suggested by Figure 2 which plots affected and nonaffected animals as a function of binding *versus* brain bilirubin concentration. When the free bilirubin was relatively low, *i.e.* when less bilirubin was transferred to BSA, encephalopathy occurred only when the barrier was very permeable to the albumin-bilirubin complex. When the serum binding was poor, encephalopathy developed at a much lower brain bilirubin-albumin concentration. Using a similar plot (Fig. 3), total bilirubin concentration did not appear to improve the predictive value of brain bilirubin alone.

Stepwise discriminant analysis confirmed that differences in

Table 1. Bilirubin values observed in relation to encephalopathy (\pm SD)

EEG Change	Normal	Moderate	Severe
<i>n</i>	7	5	10
Bilirubin (mg/dl)	37.7 (\pm 6.0)	40.0 (\pm 5.7)	35.9 (\pm 14.0)
Binding index (% extracted)	24.8 (\pm 21.8)	25.6 (\pm 13.4)	51.5 (\pm 28.8)
Right brain (μ g/g)	17.9 (\pm 6.4)	23.4 (\pm 4.8)	30.8* (\pm 9.8)
Left brain (μ g/g)	10.6 (\pm 1.7)	12.2 (\pm 4.6)	14.3 (\pm 4.5)

* Severe *versus* normal $p < 0.025$; remaining mean differences are not significant ($p > 0.05$, Neumann Keuls test).

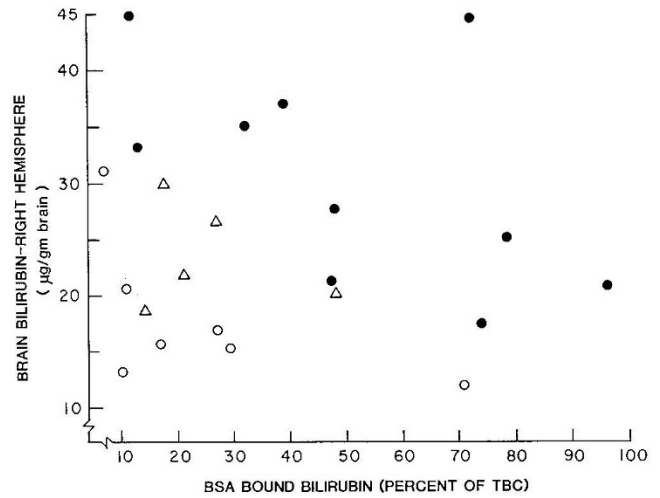


Fig. 2. Occurrence of EEG changes in relation to BBB opening in the right hemisphere and serum binding. With weaker binding (increasing free bilirubin) a higher percentage of total bilirubin is bound to BSA. EEG changes: \circ none, Δ moderate, \bullet severe.

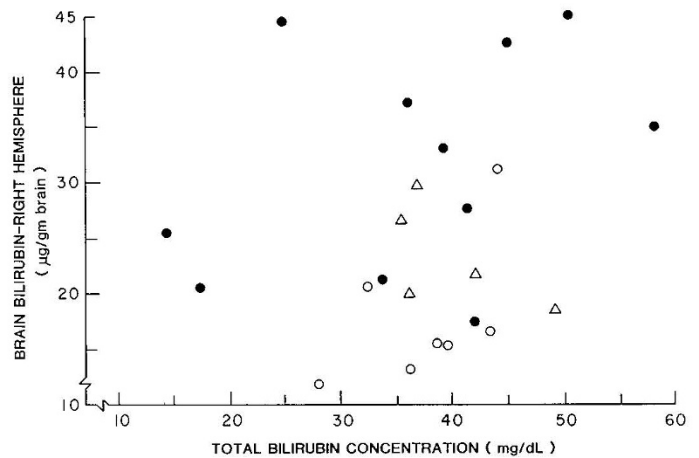


Fig. 3. Occurrence of EEG changes in relation to BBB opening in the right hemisphere and total bilirubin concentration. EEG changes: \circ none, Δ moderate, \bullet severe.

Table 2. Discriminant analysis: prediction of encephalopathy*

Actual group	<i>n</i>	Predicted group membership		
		Normal	Moderate	Severe
Normal	7	6	1	0
Moderate	5	1	4	0
Severe	10	0	2	8

* Discriminant analysis: EEG was best predicted by two discriminators: binding index and right hemisphere bilirubin concentration.

EEG findings could best be discriminated by two variables, the concentration of bilirubin in the right hemisphere and binding index. Total bilirubin concentration and left hemispheric bilirubin were not useful discriminating variables. The two discriminating factors classified 18/22 rats correctly, and in no case was a normal EEG misclassified as severe encephalopathy (Table 2).

DISCUSSION

We have confirmed that osmotic opening of the BBB produces yellow staining of the brain in icteric rats. However, yellow

staining occurred in some animals without EEG evidence of bilirubin toxicity. The risk for encephalopathy depended on both the degree of barrier disruption as defined by brain bilirubin content and the serum binding status. In these short-term experiments we could not produce toxicity without first disrupting the BBB.

The concentration of bilirubin-albumin, *i.e.* total bilirubin concentration, did not discriminate which animals would develop encephalopathy. Thus, these data do not support the hypothesis that albumin bound bilirubin crossing the BBB is toxic to the brain (8). However, disruption of the BBB permits leakage of the albumin-bilirubin complex into the interstitium of brain, bathing the neuropil in icteric serum and exposing neurons directly to the same free bilirubin concentration existing in serum. Under these conditions albumin provides a large and relatively static reservoir of bilirubin which will equilibrate with alternative binding sites on albumin or cellular membranes. If the free bilirubin level is sufficiently high and the reservoir sufficiently large, the amount of bilirubin bound to membranes will impair neuronal function resulting in abnormal electrical activity.

This study was designed to establish principles regarding the relative importance of total bilirubin levels, binding, and BBB opening with respect to neurotoxicity and not to imitate the neurophysiological state of the newborn. One must be cautious, therefore, in making direct extrapolations from these animal experiments to human kernicterus. Although the basal ganglia were usually involved, the staining of brain in these animals was diffuse, involving cortical gray matter as well as deeper structures. These studies permit the possibility that variations of BBB permeability to albumin may explain variations in susceptibility of human newborns to bilirubin staining and toxicity, but they provide no direct evidence that the BBB is altered in infants who develop kernicterus.

Two additional factors that might be important in explaining the variability of the newborn's response to bilirubinemia were not addressed in this study, *i.e.* differences in the transport of free bilirubin across the BBB (22) and variations in cell response to a given bilirubin load. Variations in susceptibility of target neurons, *e.g.* due to chronic or acute ischemia, have been implicated in the pathogenesis of kernicterus. In this study, infusion of hypertonic arabinose produced a transient change in EEG indicating at least a transient alteration in brain function (23). It is possible, therefore, that disrupting the BBB or exposing neural tissue to arabinose altered brain metabolism (24, 25), increasing neuronal susceptibility to bilirubin.

This experimental model supports the proposal that yellow staining cannot be equated with bilirubin toxicity and provides a possible explanation for the neuropathological observations of Turkel *et al.* (26). In many sick premature infants with yellow staining, they found spongy changes in brain consistent with interstitial edema (or interstitial bilirubin-albumin), but few changes in neurons that could be attributed to bilirubin. The presence of yellow "staining" in some rat brains without evidence of encephalopathy reinforces the need to define bilirubin encephalopathy on the basis of behavioral (27), physiological (28), neurological (29), or histopathological criteria (30) and not on gross neuropathological findings.

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