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Calculated *In Vivo* Free Bilirubin Levels in the Central Nervous System of Gunn Rat Pups

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ABSTRACT: *In vitro* studies suggest a free bilirubin (B_F) concentration in the range of 71–770 nmol/L can induce neurotoxicity. *In vivo* data regarding central nervous system (CNS) B_F levels have not been determined. We calculated *in vivo* CNS B_F levels in Gunn rat pups (15–19 d old; heterozygous nonjaundiced Gunn rats (J/j) and homozygous jaundiced Gunn rats (j/j); saline or sulfadimethoxine treated) based on 1) total brain bilirubin (TBB) content, 2) brain albumin level, 3) CNS bilirubin binding capacity attributable to brain albumin determined using an ultrafiltration technique, and 4) published Gunn rat albumin-bilirubin binding constants (*k*). Gunn rat brain bilirubin binding capacity was $\sim 22 \times 10^{-3}$ μmol/g, of which two thirds was accounted for by brain albumin. Using a Gunn rat pup *in vivo*, *k* of 9.2 L/μmol, calculated CNS B_F levels ranged from 72 to 112 nmol/L [95% confidence interval (CI)] in saline and from 59 to 156 nmol/L (95% CI) in sulfadimethoxine-treated J/j pups. These animals demonstrated no neurobehavioral abnormalities and normal cerebellar weight. Calculated CNS B_F levels were severalfold higher ($p < 0.001$) in saline (95% CI: 556–1110 nmol/L) and sulfadimethoxine-treated (95% CI: 3461–8985 nmol/L) j/j pups; the former evidenced reduced cerebellar weight; the latter both reduced cerebellar weight and acute neurobehavioral abnormalities. We conclude that calculated CNS B_F values in j/j pups are substantially higher than those in J/j animals. Given the absence of CNS abnormalities in J/j pups, the presence of such in j/j animals, and the CNS B_F levels in these groups, we speculate that the CNS B_F neurotoxicity threshold *in vivo* is subsumed within the range (71–770 nmol/L) reported *in vitro*. **(*Pediatr Res* 60: 44–49, 2006)**

The free or unbound fraction of unconjugated bilirubin (B_F) is believed to be neurotoxic (1,2). The threshold at which B_F produces changes in cellular function culminating in permanent cell injury and death has been a subject of study for some time. A recent review of *in vitro* investigations of cultured murine glial cells, astrocytes, and neurons suggests that a calculated B_F concentration in the range of 71–770 nmol/L encompasses the neurotoxic threshold (2), although higher thresholds have been reported in some studies (2). This broad *in vitro* B_F toxic range likely reflects differences in cell type, maturity, function, species of origin, and B_F exposure duration across studied lines (2).

Data regarding *in vivo* CNS B_F levels have not been reported. In the current study, we calculated *in vivo* CNS B_F levels in Gunn rat pups during peak hyperbilirubinemia (postnatal d 15–19) (3,4) to assess how calculated *in vivo* B_F levels compare with reported calculated *in vitro* B_F toxic thresholds. The Gunn rat (5) offers an intriguing model to explore this matter as this animal spontaneously develops newborn jaundice differing from others that rely on artificial means of inducing hyperbilirubinemia (6). Neonatal hyperbilirubinemia in homozygous j/j Gunn rat pups results from a deficiency of the bilirubin conjugating enzyme uridine-diphosphate-glucuronosyl transferase 1A1 (UDP-GT1A1) (7), homologous to human patients with Crigler Najjar type I syndrome and analogous to the relative deficiency of UDP-GT1A1 activity seen in human neonates during the first several days of life. Heterozygous J/j pups have reduced UDP-GT1A1 levels but do not develop neonatal hyperbilirubinemia and thus serve as an appropriate control.

We studied both J/j heterozygous and j/j homozygous genotypes in the presence and absence of sulfadimethoxine treatment 24 h before sacrifice. CNS B_F was calculated based on measures of 1) TBB content, 2) brain albumin levels, 3) published rat albumin-bilirubin binding constants, and 4) an estimate of total CNS bilirubin binding capacity attributable to brain albumin as measured using an ultrafiltration technique. We were interested in both 1) J/j heterozygous pups as these animals show normal neurobehavior and postnatal cerebellar weights whether treated with sulfadimethoxine or not, *i.e.* evidence a phenotype inconsistent with permanent bilirubin-induced brain injury, and 2) j/j homozygous pups as these animals show reduced postnatal cerebellar weight, and when treated with sulfadimethoxine, acute signs of hyperbilirubinemic encephalopathy and often death. We hypothesized that calculated *in vivo* CNS B_F levels in hyperbilirubinemic j/j pups would be significantly higher than those of their J/j counterparts and approximate or exceed the predicted B_F neurotoxic range reported *in vitro* (2), whereas calculated *in*

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Abbreviations: B_F, free bilirubin; **J/j,** heterozygous nonjaundiced Gunn rat; **j/j,** homozygous jaundiced Gunn rat; ***k*,** albumin-bilirubin binding constant; **TBB,** total brain bilirubin; **UDP-GT1A1,** uridine-diphosphate-glucuronosyl transferase 1A1 isoenzyme

vivo CNS B_F levels in asymptomatic J/j animals would be less than the calculated *in vitro* B_F neurotoxic range (2).

METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee of the Magee-Womens Research Institute. Studies were performed on 15- to 19-d-old paired littermate heterozygote (J/j) ($n = 40$) and homozygote (j/j) ($n = 40$) Gunn rats matched for gender and weight. This postnatal age was selected based on previous reports (3,4) and our own preliminary data that peak serum bilirubin in j/j Gunn rat pups occurs in this postnatal age range. Litters were the product of homozygous j/j male matings with heterozygous J/j females. ^{3}H-bilirubin (specific activity 580 mCi/mmole; 2.21×10^6 dpm/μg) prepared by sodium boro-[^{3}H]-hydride reduction of biliverdin (8) was supplied by Moravek (Brea, CA). On high-performance liquid chromatography of this material (5u Hypersil-BDS C-18, 2×150 mm; 6.4 vol% isopropanol in 0.05 mol/L KH_2PO_4; flow rate 0.1 mL/min), all radioactivity coeluted as a sharp peak with authentic bilirubin (Sigma Chemical Co.-Aldrich); 0.2 mCi of lyophilized ^{3}H-bilirubin was diluted in 0.1 N NaOH to give a 2-mmol/L stock solution that was stored at −20°C in the dark; fresh stock solutions were used, made at intervals of every 2 wk or less.

Twenty-four hours before sacrifice, pups of a given genotype (J/j or j/j) were injected with either sulfadimethoxine (200 mg/kg, i.p., $n = 20$) or equal volume of saline ($n = 20$). The former increases bilirubin displacement from albumin (4,9,10), and enhances brain bilirubin content. Pups were killed with a pentobarbital sodium overdose (50 mg/kg, i.p.), the descending aorta clamped, and the CNS vasculature flushed *in situ* with saline through the left ventricle (15 mL over 2 min) and the brain rapidly removed. Dural venous sinus effluent demonstrated no detectable albumin or Hb at the completion of vasculature flushing. TBB content was determined by acid-chloroform microassay extraction followed by diazotization (16) and reported as μmol bilirubin per g brain wet weight. Care was taken to protect tissue samples from ambient light to reduce the photodecomposition of bilirubin. Brain albumin was determined using a dye-binding assay employing bromcresol green (12). TBB and brain albumin were determined on undiluted whole brain homogenate.

The total bilirubin binding capacity of the CNS and that fraction of bilirubin binding capacity attributable to CNS albumin were determined in diluted whole-brain homogenates of J/j Gunn rats as outlined in Figure 1. Non–sulfadimethoxine-treated J/j pups were used in this phase of the study because their baseline brain bilirubin content was minimal ($<1.6 \times 10^{-3}$ μmol/g). J/j whole-brain homogenate was diluted in Ringers plus 10 mmol/L *N*-2-hydroxyethylpiperazine-*N* ^{1}-2-ethanesulfonic acid, pH 7.4 (1:5) and divided into two equal portions. One portion, A, was used to determine TBB binding capacity, whereas the other portion, B, was used to determine brain bilirubin binding attributable only to albumin. To portion A brain homogenate, a saturating concentration of ^{3}H-bilirubin (13 μmol/L, 0.65 μCi) was added and mixed for 30 min in the dark. The 2 mmol/L ^{3}H-bilirubin stock solution was diluted 1:150 in 2 mL of brain homogenate. The portion A mixture was placed in a Centricon YM-3 centrifugal filter device (Millipore, molecular weight cutoff = 3000 Da) and centrifuged at $3000 \times g$ for 15 min twice, then again at $6000 \times g$ for 60 min to isolate unbound bilirubin (filtrate;<3000 Da) from bound bilirubin (retentate; ≥3000 Da). A new filter device was used each time and retentate counts reflected those of all three devices used including the filter itself. From the retentate fraction of A, albumin content was measured and the total radioactivity (total bilirubin binding capacity) determined.

The homogenate in portion B was subjected to identical centrifugal conditions as with portion A (but in the absence of ^{3}H-bilirubin), resulting in retentate and filtrate fractions. To the filtrate fraction of portion B was added pure adult rat albumin (A-2018, Sigma Chemical Co.), in an amount equal to the albumin measured in the portion A retentate, along with an identical supersaturating concentration of ^{3}H-bilirubin (13 μmol/L), and this mixture subjected to the same ultrafiltration protocol as with portion A. The resulting retentate portion (total bilirubin bound to albumin only) was counted for radioactivity. The proportion of CNS bilirubin binding capacity attributable to albumin was determined by dividing the radioactive counts in the rat albumin retentate fraction of portion B by the radioactive counts in the retentate from portion A. Knowing this value permits the calculation of a "corrected" total brain albumin term that is used in the determination of B_F in the equation below.

The "corrected" total brain albumin term reflects both the CNS bilirubin binding attributable to brain albumin and that attributable to non-albumin binding substances and is given as the brain albumin concentration divided by the proportion of total CNS bilirubin binding capacity attributable to albumin, *e.g.* 3.0 μmol/L ÷ 0.67 = 4.48 μmol/L). This "corrected" brain albumin value together with the rat albumin-bilirubin binding constant permits the calculation of CNS B_F levels. More specifically, CNS B_F was calculated using the published *in vivo* albumin-bilirubin binding k mean (9.2 L/μmol) and range (2.4 and 33.6 L/μmol) values from Gunn rat pups (16 ± 0.5 d old) (13) in the following equation:

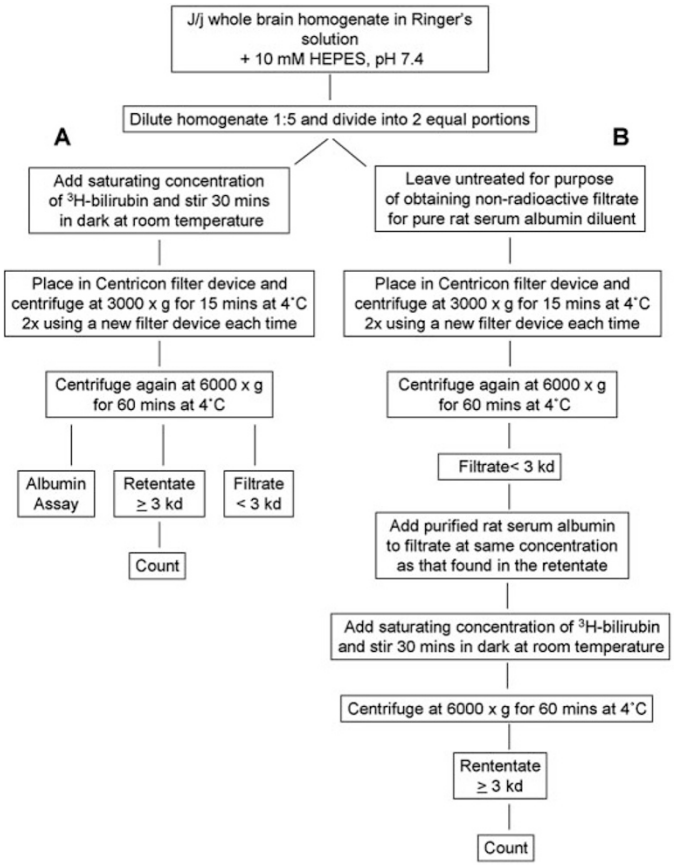

Figure 1. Schema demonstrating sequential steps in the determination of 1) TBB binding capacity (*A*); 2) total brain albumin content (A), and 3) bilirubin binding attributable to albumin content quantified in A (*B*). See Methods section for additional details.

$$\frac{TBB - B_F}{A} = \frac{B_F \times k_1}{1 + (B_F \times k_1)} + \frac{B_F \times k_2}{1 + (B_F \times k_2)}$$

This equation assumes independent binding of UCB to two sites on albumin (2,14): k_1 and k_2 are the binding constants for the first and second sites, respectively, with k_1 given by the binding constant values defined above (13) and k_2 equal to $k_1/15$ (2,14). Reported *in vitro* k values of purified adult rat serum albumin (15,16), either closely approximate or are encompassed by the range of k values observed in Gunn rat pups *in vivo* (13). A is the "corrected" brain albumin concentration defined above, and B_F is the CNS unbound bilirubin fraction.

Assessments for overt signs of neurologic dysfunction including ataxia, lethargy, failure of locomotion, and feeding difficulty were performed on all animals (17); the evaluator was not blinded to genotype as j/j animals are jaundiced, but was blinded to treatment assignment (sulfadimethoxine or saline). Cerebellar weights were obtained for all animals at sacrifice.

All data including CNS B_F levels were analyzed as a function of genotype (j/j *versus* J/j) and treatment (sulfadimethoxine *versus* saline) using analysis of variance (ANOVA) (Minitab Release 14, State College, PA) (18). In the event of a significant ANOVA, *post hoc* testing using Tukey's method was performed (18). Data are reported as a mean ± SD.

RESULTS

TBB contents are shown in Figure 2 and demonstrate significantly higher levels in j/j pups compared with their J/j counterparts ($p < 0.001$) as well as higher levels in sulfadi-

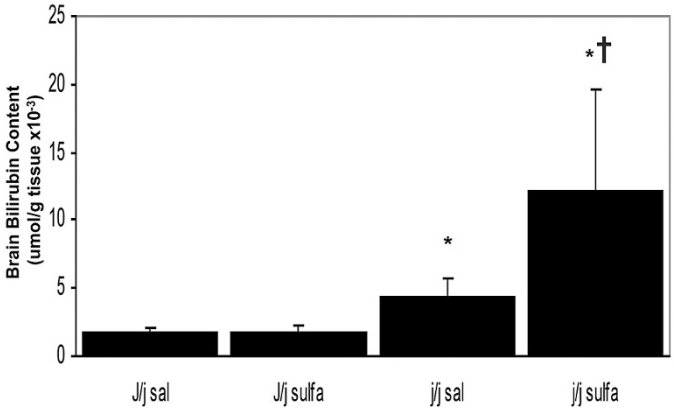

Figure 2. TBB content [expressed in μmol/g tissue ($\times 10^{-3}$)] is shown as a function of genotype and treatment condition [J/j saline (sal) ($n = 20$); J/j sulfadimethoxine (sulfa) ($n = 20$); j/j saline ($n = 20$); j/j sulfadimethoxine ($n = 20$)]. Homozygous hyperbilirubinemic j/j pups TBB content was significantly greater ($p < 0.001$) than that of their J/j counterparts and that of sulfadimethoxine-treated j/j greater than j/j saline-treated pups ($p < 0.01$). *$p < 0.001$ *vs* J/j pups;†$p < 0.01$ *vs* j/j saline.

methoxine-treated j/j *versus* saline-treated j/j animals ($p < 0.01$). Brain albumin content was 3.0 ± 1.1 μmol/L ($2.0 \pm 0.7 \times 10^{-3}$ μmol/g) and did not differ between J/j and j/j pups. TBB binding capacity was $22.1 \pm 3.5 \times 10^{-3}$ μmol/g and that fraction attributable to albumin was $14.8 \pm 2.4 \times 10^{-3}$ μmol/g, accounting for $67 \pm 13\%$ or approximately two thirds of the total.

Calculated CNS B_F levels from saline and sulfadimethoxine-injected heterozygous J/j pups are shown on the left in Figure 3 as a function of the reported mean and range of *in vivo* Gunn rat pup albumin-bilirubin binding constants (13).

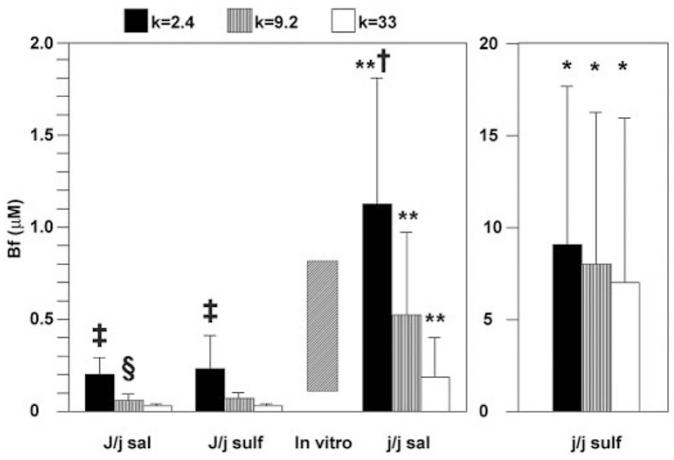

Figure 3. Calculated CNS B_F (expressed in μmol/L) is shown as a function of genotype: J/j heterozygous pups (*left*); j/j homozygous pups (saline treated) (*left*) and (sulfadimethoxine treated) (*right*), treatment condition [saline (sal) *vs* sulfadimethoxine (sulfa)] and k (L/μmol). Three different Gunn rat k were used to represent the range [2.4 L/μmol (*solid bars*); 33 L/μmol (*open bars*)], and mean [9.2 L/μmol (*vertically hatched bars*)] of reported *in vivo* values (13). The calculated *in vitro* B_F neurotoxic threshold range (71–770 nmol/L) (2) is plotted (*angled hatched bar*). *$p < 0.01$ *vs* j/j saline and $p < 0.001$ *vs* J/j sulfa, J/j saline; **$p < 0.01$ *vs* J/j saline, J/j sulfa; †$p < 0.05$ *vs* j/j saline $k = 9.2$ and 33 L/μmol; ‡$p < 0.05$ *vs* J/j $k = 9.2$ and 33 L/μmol saline and sulfa; §$p < 0.05$ *vs* J/j $k = 33$ L/μmol saline. Note the different scales on the ordinate between *left* (0–2 μmol/L) and *right* (0–20 μmol/L) panels; n of each group is same as in Figure 2.

CNS B_F did not differ between saline- and sulfadimethoxine-treated J/j pups at a given k value ($p \geq 0.56$), albeit calculated CNS B_F in absolute terms was lowest at $k = 33$ μmol/L, intermediate at $k = 9.2$ L/μmol, and highest at $k = 2.4$ L/μmol (ANOVA, $p < 0.05$). None of these animals evidenced any neurobehavioral abnormalities, and there was no difference in cerebellar weights between saline- and sulfadimethoxine-treated J/j animals ($p = 0.97$; Fig. 4).

Calculated CNS B_F levels from saline and sulfadimethoxine injected homozygous hyperbilirubinemic j/j pups are shown in Figure 3 as a function of the reported mean and range of *in vivo* Gunn rat pup k values (13). The calculated CNS B_F in homozygous j/j pups was significantly higher than in saline- and sulfadimethoxine-treated heterozygous J/j animals ($p < 0.001$); moreover, sulfadimethoxine-treated homozygous j/j pups B_F levels were significantly greater than those seen in their saline-treated homozygous j/j counterparts ($p < 0.001$). Calculated CNS B_F in absolute terms was lowest at $k = 33$ L/μmol; intermediate at $k = 9.2$ L/μmol; and highest at $k = 2.4$ L/μmol (ANOVA, $p < 0.05$) in saline-treated j/j pups. No differences in calculated CNS B_F were observed across the three k values in sulfadimethoxine-treated j/j pups, likely reflecting the appreciably greater CNS bilirubin levels in these animals, levels that consistently exceeded saturation of brain bilirubin binding capacity. Although saline-treated j/j pups did not show overt neurobehavioral abnormalities, they did demonstrate significantly lower cerebellar weights than J/j animals ($p < 0.05$; Fig. 4). Sulfadimethoxine-injected j/j pups demonstrated both 1) significantly lower cerebellar weights than J/j animals ($p < 0.05$; Fig. 4) and 2) acute signs of ataxia and lethargy in the 24 h after drug administration accompanied by feeding difficulties and failure of locomotion.

Figure 3 also demonstrates how calculated *in vivo* CNS B_F levels from each study group across all three k values (13) compare with the *in vitro* calculated B_F range reported to encompass the neurotoxic threshold (2). Notably, the greatest portion of the *in vitro* B_F neurotoxic threshold range is positioned between those levels observed in asymptomatic J/j pups (saline and sulfadimethoxine treated) and that seen in their symptomatic j/j pup counterparts (saline and sulfadimethoxine

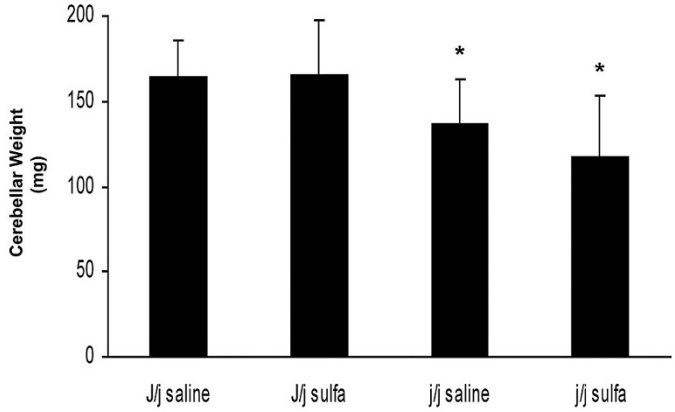

Figure 4. Cerebellar weight (expressed in mg) at sacrifice in Gunn rat pups as a function of genotype (J/j *vs* j/j) and treatment group [saline (sal) *vs* sulfadimethoxine (sulf)]. *$p < 0.05$ *vs* J/j pups; n in each group is same as in Figure 2.

treated). Even calculated *in vivo* CNS B_F values based on the *k* value of 33 L/μmol (the lowest calculated CNS B_F levels observed) in hyperbilirubinemic homozygous j/j pups lie well within [j/j saline: 221–537 nmol/L (95% CI)] or exceed [j/j sulfadimethoxine: 2744–8387 nmol/L (95% CI)] the neurotoxic range reported *in vitro* (71–770 nmol/L) (2); in contrast, nonjaundiced heterozygous J/j pup levels clearly fall below the neurotoxic range [J/j saline: 21–33 nmol/L (95% CI); J/j sulfadimethoxine: 16–51 nmol/L (95% CI)].

DISCUSSION

We conclude that 1) Gunn rat TBB binding capacity approximates 22×10^{-3} μmol/g, of which about two thirds was accounted for by brain albumin alone, and 2) calculated *in vivo* B_F levels in hyperbilirubinemic j/j Gunn rat pups were substantially higher than those of their J/j counterparts (four- to 18-fold greater in saline-treated j/j animals) and even more in sulfadimethoxine-treated j/j pups, where levels consistently exceeded calculated B_F levels postulated to be neurotoxic *in vitro* (2). Notably, at any given *k*, there was a large gap between the upper limit of the calculated CNS B_F 95% CI range in J/j pups (*e.g.* 156 nmol/L in sulfadimethoxine-treated J/j pups at $k = 9.2$ L/μmol) and the lower limit seen in the saline treated j/j pups (556 nmol/L at $k = 9.2$ L/μmol). Interestingly, it is within that *in vivo* B_F interval (51–221 nmol/L at $k = 33$ L/μmol; 156–556 nmol/L at $k = 9.2$ L/μmol; and 436–1199 nmol/L at $k = 2.4$ L/μmol) that either the threshold or greater part of the calculated toxic B_F range has been reported *in vitro* (2). Indeed, calculated *in vivo* B_F levels in both saline- and sulfadimethoxine-treated j/j pups were within or exceeded the proposed *in vitro* B_F neurotoxic range (2), regardless of which *k* value was used in the calculation; and these hyperbilirubinemic j/j animals showed low postnatal cerebellar weights and, in the case of sulfadimethoxine-treated j/j pups, also acute neurobehavioral abnormalities consistent with neurotoxic injury in the 24 h after sulfadimethoxine injection.

Not surprisingly, the markedly higher calculated *in vivo* B_F levels in sulfadimethoxine-treated j/j pups [~2700–10,000 nmol/L (95% CI)] were associated with the most evident CNS injury, these animals being the only ones across study groups to demonstrate overt neurobehavioral abnormalities, frequently severe, before sacrifice. Consistent with these observations is a previous report comparing the neuropathology of sulfadimethoxine-treated j/j with nontreated j/j Gunn rat pups (17). Two different neuropathologic patterns were observed: 1) sulfadimethoxine-treated j/j Gunn rat pups evidenced extensive neuropathology including acute necrotic lesions and neuronal cytolysis in the hippocampal cortex, inferior colliculi, globus pallidus, and pontomedullary tegmentum, whereas 2) nontreated j/j pups showed a paucity of abnormalities except for extensive neuronal degeneration of Purkinje cells in the cerebellar roof nuclei (17).

In contrast, calculated *in vivo* B_F levels in heterozygous J/j pups were less than or only partially overlapped the lower half of calculated B_F levels reported to be neurotoxic *in vitro* (2). These animals demonstrated no overt neurobehavioral abnormalities (short or long term), or impaired postnatal cerebellar weight whether saline or sulfadimethoxine treated. The preserved cerebellar weights in the current cohort of J/j animals are consistent with those of previous work demonstrating normal cerebellar volumes in saline and sulfadimethoxine-treated J/j pups to 30 d of postnatal life (9) and a lack of neuropathologic abnormalities by light or electron microscopy in formalin-fixed J/j brain tissue sections (17). We did not assess brainstem auditory evoked potentials, a more sensitive index of bilirubin-induced neuronal dysfunction and/or injury, in the current study. However, previous reports demonstrate an absence of notable brainstem auditory pathway abnormalities in nonjaundiced J/j pups whether saline or sulfadimethoxine treated (19–21). These findings taken together suggest that the calculated *in vivo* B_F levels observed in J/j pups were not neurotoxic, at least in terms of generating overt acute or long-standing injury, whereas those seen in j/j pups definitively were.

The calculated *in vivo* CNS B_F should be representative of B_F within the extracellular fluid compartment, the relevant *in vivo* analogue of the extracellular medium in which the comparable *in vitro* studies of B_F toxicity thresholds were performed (2), as B_F within the CNS should equilibrate among the intracellular and extracellular compartments as well as with bilirubin bound to CNS tissue and CNS albumin. Given the absence of CNS abnormalities in J/j pups, the presence of such in j/j animals, and the observed CNS B_F levels in these groups, we speculate that the CNS B_F neurotoxicity threshold *in vivo* is subsumed within the range (71–770 nmol/L) reported *in vitro* (2).

This is the first study to calculate *in vivo* B_F levels in the CNS of Gunn rat pups. Critical to this effort was a determination of the total CNS bilirubin-binding capacity and that portion attributable to brain albumin that was performed using an ultrafiltration technique. This process, based on centrifugal ultrafiltration is widely used to concentrate and purify proteins (22). We chose a molecular weight cutoff of 3000 Da to fractionate protein-membrane complexes (≥3000 Da), including albumin, from filtrate. Indeed, we did not detect albumin or any proteins in this filtrate fraction (data not shown). The rationale for using ultrafiltration was as follows. In portion A of the methodologic schema (Fig. 1), the ≥3000-Da fraction of brain homogenate–^{3}H-bilirubin mixture provided both a measure of TBB binding capacity and brain albumin content. In portion B, the filtrate provided a protein-free milieu that otherwise approximates that seen *in vivo*. To this portion B filtrate, albumin in an amount equal to that found in the portion A retentate and ^{3}H-bilirubin were added and subjected to ultrafiltration. Radioactive counts from the resultant retentate quantified the CNS bilirubin-binding capacity attributable to albumin alone. The ratio of the portion B to portion A counts equals that fraction of TBB binding capacity attributable to albumin. The measured Gunn rat pup brain albumin concentration divided by the proportion of total CNS bilirubin binding capacity attributable to albumin generates a "corrected" albumin term in the B_F calculation, a term that reflects both the CNS bilirubin binding attributable to brain albumin and that attributable to non-albumin binding substances. This term in combination with published Gunn rat *k* values (13) and

measurements of brain bilirubin content permitted the calculation of *in vivo* B_F.

Regarding the ultrafiltration protocol itself, the study conditions for portions A and B were identical with respect to dilution and temperature; therefore, the use of the portion B-to-portion A ratio to determine that fraction of total CNS bilirubin binding capacity attributable to albumin should cancel in part if not in full potential errors due to these factors. Of necessity, portion B was devoid of nonalbumin proteins to ascertain that fraction of TBB binding due to albumin alone; we have assumed that the effect of temperature is the same in portions A and B despite their different total protein concentrations. Moreover, the serial nature and cumulative centrifugal forces of the ultrafiltration protocol mirror those previously reported to remove weakly bound impurities (23) and therefore should have removed potential less well bound and filterable ^{3}H-degradation products (23). It also follows from the use of the portion B-to-portion A ratio that small amounts of polar impurities in the radiolabeled bilirubin, if present and not fully removed by ultrafiltration, would be canceled and not interfere with this calculation.

We are not aware of previous attempts to determine the total bilirubin binding capacity of Gunn rat CNS or that fraction attributable to CNS albumin. Indeed there have been only a few studies on CNS albumin in rat pups (24–27). These investigations, nevertheless, lend credence to the current study, demonstrating 1) the presence of endogenous albumin in developing and juvenile rat brain (24–27); 2) CNS albumin levels consistent with those observed [~3 μmol/L (190 μg/g)] (26,27); and 3) comparable brain albumin concentrations between J/j and j/j pups (26). Interestingly, similar brain albumin levels have also been reported in another mammalian species in the newborn period, namely, piglets (28).

Previous studies have reported TBB content in Gunn rat pups, but such investigations are also surprisingly few (15,29,30). The brain bilirubin content observed in our 15- to 19-d old j/j Gunn rat pups not treated with sulfadimethoxine ($5.24 \pm 1.57 \times 10^{-3}$ μmol/g = 3.06 ± 0.92 μg/g) is consistent with previous reports. Takahashi *et al.* (15) observed brain bilirubin contents of 2.8 ± 0.2 μg/g in 15-d-old jaundiced Gunn rats; Sawasaki *et al.* (29) reported brain bilirubin levels of 2.5 ± 0.6 μg/g in 16-d-old j/j pups; and Sato and Semba (30) noted cerebellar brain bilirubin contents of 2.75 ± 1.15 μg/g.

We used rat serum *k* values previously reported in the literature to calculate *in vivo* CNS B_F. Moreover, because the current data were collected in Gunn rat pups, we confined our calculations to *k* values reported in this animal model and age group (13). These published Gunn *k* values were generated *in vivo* using the modified peroxidase-diazo method by Ahlfors (31). Importantly, reported *in vitro k* values for purified adult rat serum albumin (15,16), either closely approximate or are encompassed by the range of *k* values observed in Gunn rat pups *in vivo* (13). The reported Gunn rat pup *in vivo k* values encompass a broad range (2.4–33.6 L/μmol) yet are characterized by a mean of 9.2 L/μmol and an SD of 7.9 (13). We reported calculated *in vivo* CNS B_F levels using *k* values from both ends of the *in vivo*–derived *k* value spectrum, in addition to the mean, to provide a comprehensive data set.

The effects of a given B_F level on CNS function may be determined in part by the duration of that exposure. Indeed, clinical data suggest that bilirubin-induced neurotoxicity is both a function of peak bilirubin and duration (32,33). One of the issues left unanswered by the current data set is at what combination of B_F level and exposure duration neurotoxicity may ensue. The absence of overt toxicity or impaired cerebellar growth in both the saline- and sulfadimethoxine-treated J/j Gunn rats, however, suggests that levels seen in these animals, even of protracted duration, are not likely to cause toxicity. Indeed, the pattern of hyperbilirubinemia seen in Gunn rat pups is typically that of a sustained plateau of several days while at the serum bilirubin peak (3,4,29), adding credence to this speculation.

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