Unbound (Free) Bilirubin: Improving the Paradigm for Evaluating Neonatal Jaundice

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BACKGROUND: The serum or plasma total bilirubin concentration ($B_T$) has long been the standard clinical laboratory test for evaluating neonatal jaundice, despite studies showing that $B_T$ correlates poorly with acute bilirubin encephalopathy (ABE) and its sequelae including death, classical kernicterus, or bilirubin-induced neurological dysfunction (BIND). The poor correlation between $B_T$ and ABE is commonly attributed to the confounding effects of comorbidities such as hemolytic diseases, prematurity, asphyxia, or infection. Mounting evidence suggests, however, that $B_T$ inherently performs poorly because it is the plasma non–protein-bound (unbound or free) bilirubin concentration ($B_f$), rather than $B_T$, that is more closely associated with central nervous system bilirubin concentrations and therefore ABE and its sequelae.

CONTENT: This article reviews (a) the complex relationship between serum or plasma bilirubin measurements and ABE, (b) the history underlying the limited use of $B_f$ in the clinical setting, (c) the peroxidase method for measuring $B_f$ and technical and other issues involved in adapting the measurement to routine clinical use, (d) clinical experience using $B_f$ in the management of newborn jaundice, and (e) the value of $B_f$ measurements in research investigating bilirubin pathochemistry.

SUMMARY: Increasing evidence from clinical studies, clinical experience, and basic research investigating bilirubin neurotoxicity supports efforts to incorporate $B_f$ expeditiously into the routine evaluation of newborn jaundice.

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Clinical Chemistry recently published a Citation Classic entitled “Unbound Bilirubin: A Better Predictor of Kernicterus?” (1). The article noted that preventing acute bilirubin encephalopathy (ABE) and its serious neurological sequelae in jaundiced newborns remains a serious and unresolved concern for clinicians. It went on to suggest that the serum or plasma non–protein-bound bilirubin concentration (free or unbound bilirubin, $B_f$) as measured by the much-cited peroxidase method (1, 2) may provide better guidance for therapy than the conventionally used total bilirubin concentration ($B_T$). The issue of $B_T$ vs $B_f$ was also raised in a recent prospective National Institute of Child Health and Human Development (NICHD) phototherapy study that found considerable overlap in $B_f$ values in very low birth weight infants with and without bilirubin-associated deafness, and those authors suggested that $B_f$ might have better differentiated the 2 groups (3).

Although evidence suggesting that $B_f$ would be superior to $B_T$ in the clinical management of jaundiced newborns first appeared in the 1950s (4), $B_f$ has languished for decades on the periphery of clinical practice as well as basic research investigating the pathochemistry of bilirubin neurotoxicity. Many clinicians, clinical chemists, and even researchers working in the field of bilirubin neurotoxicity are unfamiliar with $B_f$ measurement or its clinical application. Recent events have renewed interest in $B_f$ (5), and the intent of this review is to provide a framework for understanding how $B_f$ measurements might augment both the care of jaundiced newborns and our understanding of the pathochemistry of ABE.

Neonatal jaundice is caused by the retention of unconjugated bilirubin (UCB) during the normal, transient postnatal imbalance between UCB production and elimination. Although the condition is generally inconsequential except for the noticeable jaundice,
the 4Z,15Z UCB-Iα isomer produced (Fig. 1) is neurotoxic and in rare cases may reach concentrations sufficient to cause ABE, resulting in death or serious sequelae known collectively as kernicterus (athetoid cerebral palsy, auditory dysfunction, ocular movement disorders, and dental enamel dysplasia) (6). Bilirubin-induced neurological dysfunction (BIND) has recently been introduced to encompass both the classic and more subtle neurological impairments thought to be due to ABE (7). Without intervention with phototherapy or exchange transfusion, ABE occurs in roughly 1% of babies born prematurely (8) and in up to 15% of term newborns with hemolytic disorders (9). This is in stark contrast to the estimated nonintervention incidence of <1 per 30,000 in the 60% of healthy newborns who become clinically jaundiced (10).

It is important to note that B_{T} and B_{p} in samples from jaundiced newborns, as well as solutions prepared from commercial sources of 4Z,15Z UCB-Iα, usually contain varying amounts of presumably nontoxic bilirubin isomers that may also be measured by the laboratory tests employed to measure the neurotoxic 4Z,15Z UCB-XIα isomer (11–14). Those isomers include conjugated bilirubin, UCB-III and UCB-XIII (commercial UCB preparations), and UCB photoisomers (11–14). Nonetheless, the American Academy of Pediatrics recommends that B_{T} be used to guide jaundice therapy because conjugated bilirubin in the newborn is generally a tiny fraction of B_{T} and the neurotoxicity of the major photoisomer (4Z,15E UCB-Iα, Fig. 1) is unknown (6, 11–14). B_{T} and B_{p} should therefore be viewed as the best estimates currently available for the concentrations of total and non–protein-bound neurotoxic UCB isomers, respectively. Hereafter UCB will refer specifically to the neurotoxic isomers of UCB unless otherwise indicated.

It is well recognized that B_{T} has poor specificity (many false positives) as a predictor of ABE (6, 10, 15, 16), requiring large numbers of otherwise healthy jaundiced babies to undergo very costly, unnecessary intervention to prevent a very few cases of ABE (15, 16). Despite the considerable investment, ABE still occurs in jaundiced newborns, healthy or otherwise (17, 18). Nonetheless, proposals to modify current clinical practice cannot be considered without strong evidence that they may decrease unnecessary treatment or further reduce ABE (19). Measurement of B_{p} has the potential to do both.

**ABE, B_{T}, and B_{p}: The Rationale for Measuring B_{p} in Jaundiced Newborns**

The pathogenesis of ABE is intimately related to the factors governing UCB steady-state kinetics, in which B_{p} plays a critical role. We illustrate this using the UCB steady-state kinetics models reported for healthy adults (20) and a child with Crigler-Najjar I disease (21), which is an inherited absence of hepatic UCB conjugation resulting in severe, lifelong UCB retention characterized by marked jaundice, unconjugated hyperbilirubinemia, and increased risk of ABE (22).
Fig. 2A shows the 3-compartment model of UCB steady-state kinetics for healthy adults, as derived from the elegant studies of Berk et al. (20). Two important features to note are (a) the likelihood of ABE, i.e., the concentrations of UCB in and around the cells of the central nervous system (CNS), depends on the concentration of UCB in the nonhepatic extravascular compartment, not BT per se (23) and (b) under normal conditions, vascular UCB is nearly all bound to plasma proteins. Protein-bound UCB crosses capillary walls very slowly compared with unbound UCB and does not cross the blood–brain barrier or cell membranes at all (24–28).

The mass action relationship between $B_T$, $B_f$ the concentration of plasma bilirubin binding proteins (P), and the UCB–protein equilibrium association binding constant ($K_f$) is shown in Equation 1.

$$B_f = \frac{B_T - B_i}{K_f(P - B_T + B_i)}$$

For the purposes of this discussion, $K_f$ refers to the clinically relevant high-affinity UCB binding site (27), but it should be noted that UCB also binds to additional sites with much lower affinity constants (29).

Albumin, which is by far the major component of P, and $K_f$ are highly variable in the newborn, with CVs of about 15% and 40%, respectively (27, 30). Fig. 2B shows that whereas substantial changes in either P or $K_f$ at a given UCB production and elimination rate will greatly alter the direct correlation between $B_T$ and ABE (i.e., the correlation between $B_T$ and the nonhepatic extravascular UCB concentration), the correlation between $B_T$ and ABE remains unchanged. This complex and often paradoxical
The interplay between the UCB binding variables in Equation 1 and ABE is not accounted for when a single BT intervention threshold is applied across a diverse population of newborns.

Extrapolating the concepts illustrated in Fig. 2 to neonatal jaundice, the rate of vascular UCB increase and peak BT will be directly correlated with (a) the magnitude of the imbalance between UCB production and elimination and (b) the magnitudes of P and $K_f$.

Increases in the UCB production/elimination imbalance at constant P and $K_f$ will increase BT, $B_T$, and the risk of ABE. However, increases in P and $K_f$ at constant UCB production/elimination imbalance will increase BT but not BT or the risk of ABE (Fig. 2B). Variations in P and $K_f$ in the newborn undermine the ability of BT but not that of BT to predict ABE, and BT should inherently correlate better than BT with ABE in jaundiced newborns.

**Fig. 3.** (A), UCB steady state in a child with Crigler-Najjar disease (albumin = 604 μmol/L) [Schmid and Hammaker (21)]. Although BT is 50-fold greater vs Fig. 2A, extravascular UCB is 5-fold greater, suggesting decreasing extravascular UCB accumulation as the UCB load increases. (B), Steady state with sulfisoxazole occupying 25% of the UCB-binding sites. BT decreases, but BT and ABE risk are increased.
molysis, prematurity, asphyxia, infection) or extenuating CNS more susceptible to UCB neurotoxicity (e.g., he-...

The extravascular UCB resides in the CNS in animal models. The extravascular UCB reservoirs such as fatty tissue (31, 32), nonconjugating pathways for UCB catabolism (31, 32), and increasing saturation of non-CNS extravascular UCB reservoirs such as fatty tissue (<1% of the extravascular UCB resides in the CNS in animal models) (33).

Fig. 4 illustrates the clinical interplay of $B_T$, $B_f$, and ABE as the UCB load increases. Paradoxically, as $P$ becomes saturated with UCB, the incremental increase in $B_T$ per incremental increase in the UCB load decreases while the incremental increase in $B_f$, which is still a tiny fraction of $B_T$ (29), accelerates. This in turn accelerates the extravascular accumulation of UCB and the risk of ABE. Therefore, without a confirming $B_T$ measurement, an increased but stable $B_f$ in the clinical setting should not be (but often is) taken as reassurance that the risk of ABE is not increasing.

Despite the rationale and arguments presented above, the poor correlation between $B_T$ and ABE is customarily attributed to comorbidities that may make the CNS more susceptible to UCB neurotoxicity (e.g., hemolysis, prematurity, asphyxia, infection) or extenuating clinical circumstances (15, 19, 34). Even the well-documented association between illness and impaired plasma protein binding of ligands, including UCB, is rarely considered (35, 36). On closer examination, however, this tendency to marginalize the role of UCB binding in ABE results more from historical circumstances than a failure to appreciate the relationship between UCB binding and ABE (6, 15). Invoking UCB binding to explain the poor correlation between $B_T$ and ABE without a substantial amount of supporting clinical data is merely speculation, and there has been a remarkable paucity of UCB-binding data in the clinical literature.

Where’s the $B_T$? A Brief Clinical History of ABE, $B_T$, and $B_f$

In retrospect, $B_T$ remains entrenched at the cores of clinical algorithms designed to prevent ABE (6) primarily because $B_T$ measurements were not available until 20 years after UCB was clearly established as the cause of ABE. By then, other advances in newborn care had significantly altered the clinical outcomes of newborn jaundice and the perceived need for $B_T$ measurements.

In the early 1950s, ABE was a serious problem mainly for babies who had Rh or ABO hemolytic disease or who were born prematurely (8, 9). Treatment with the newly described exchange transfusion was risky but significantly lowered the incidence of ABE in both of these high-risk groups. The suggestion that the procedure be used at $B_T \geq 20$ mg/dL (342 μmol/L) for babies with hemolytic disease (9) was quickly applied to all jaundiced newborns.

The complex interplay between $B_T$, $B_f$, and ABE soon became apparent when premature babies given sulfisoxazole for infection prophylaxis developed ABE and died despite a “low” $B_T$ (23). It was soon discovered that sulfisoxazole competes with UCB for protein binding sites, producing a new UCB steady state with a lower $B_T$ but higher $B_f$ and extravascular UCB, as illustrated in Fig. 3B. The increased extravascular UCB often reached levels sufficient to cause ABE despite the low $B_T$ (23), a phenomenon that still haunts clinical practice (37, 38).

The sulfisoxazole experience alerted clinicians to the role of UCB binding in the pathogenesis of ABE, and methods for measuring UCB binding were pursued in hopes of better identifying babies truly needing a potentially lifesaving but also very risky exchange transfusion (4). Measuring $B_f$ directly proved elusive, however, and the early tests measuring plasma “satura-

Fig. 3A uses the UCB steady-state kinetics in an otherwise healthy child with Crigler-Najjar I disease (21) to illustrate that the relationships between $B_T$, $B_f$, and ABE change as UCB accumulates. Compared with Fig. 2A, $B_T$ in Fig. 3A is about 50 times greater (439 vs 8.3 μmol/L), but the nonvascular UCB is only about 5 times higher (492 vs 93 μmol of extravascular and hepatic UCB). This difference suggests that as UCB accumulates, an increasingly smaller fraction of the UCB load resides extravascularly. This phenomenon may result from increased activity of facilitated and active transport mechanisms for extravascular efflux of UCB (28), nonconjugating pathways for UCB catabolism (31, 32), and increasing saturation of non-CNS extravascular UCB reservoirs such as fatty tissue (<1% of the extravascular UCB resides in the CNS in animal models) (33).
Ironically, by the time the peroxidase method for measuring Bf finally arrived on the scene in 1974 (2), ABE had become a rare event in the newborn populations at greatest risk. Rhogam prophylaxis had nearly eliminated Rh hemolytic disease, and phototherapy to enhance UCB elimination substantially reduced the numbers of exchange transfusions needed to prevent ABE in premature babies. Furthermore, a prospective study of ill, premature newborns found neither Bf nor BT to be predictive of kernicterus at autopsy (41). Although this study was later shown to have serious technical shortcomings (42), the now-rare incidence of ABE made prospective determination of the Bf threshold for ABE problematic in any case. Phototherapy seemed a safe and effective method for maintaining Bf below the concentrations at which exchange transfusion would be needed in most babies, and it became increasingly difficult to see how measuring Bf would substantially alter clinical care (43).

With the issue of ABE in high-risk neonatal populations apparently resolved without Bf measurement, attention turned to a major problem with jaundice in the large group of healthy newborns. The a priori Bf ≥20 mg/dL exchange transfusion guideline, now whimsically christened vigiliphobia (fear of 20) (44), had morphed over the years into the clinical (and legal) cornerstone for jaundice intervention in healthy newborns. However, a Bf ≥20 mg/dL, unlike ABE, was a fairly common occurrence in healthy newborns despite the availability of phototherapy (34), and exchange transfusion with its considerable morbidity and mortality (45) was frequently called for to prevent a rare illness (10). It is not surprising that clinicians arbitrarily began treating most of these babies with phototherapy alone. Historical evidence seemed supportive of this untested practice and even suggested that jaundice in healthy babies was benign and being managed far too aggressively (46). Unfortunately, ABE suddenly appeared in previously healthy newborns in the mid-1980s when the increasingly laissez-faire approach to jaundice collided with early postnatal hospital discharge (47). A worldwide reexamination of the management of newborn jaundice ensued that largely focused on systematic issues such as patient monitoring and follow-up, and Bf was considered only in passing (6, 19, 34).

Evidence also emerged questioning the adequacy of the varied and experientially based Bf exchange transfusion guidelines used for premature newborns (30). In a prospective NICHD study done in the 1970s comparing phototherapy with exchange transfusion, there were 3 cases of autopsy proven kernicterus in the 216 babies with birth weights <1250 g, but only 1 had a Bf above the study exchange transfusion threshold of 10 mg/dL (171 μmol/L) (48). There were also reports of hearing deficits associated with peak Bf below accepted exchange transfusion guidelines (49), but more importantly, 2 observational studies now documented a significant association of ABE with Bf but not BT in sick, premature newborns (50, 51). It was the introduction of the auditory brainstem response (ABR) for assessing newborn hearing in the early 1980s, however, that resurrected the issue of Bf in the management of newborn jaundice in earnest.

The ABR provided for the first time a noninvasive and quantifiable outcome measure for assessing UCB neurotoxicity. UCB was quickly shown to induce ABR changes that could progress to permanent signal loss or be reversed by exchange transfusion (52, 53). There is now unequivocal evidence that UCB-induced ABR changes are predicted by Bf but not BT in both premature and term newborns (54–57). In addition, UCB injury in susceptible areas of the CNS can now be documented using newer imaging techniques in vivo such as MRI (37, 58), providing yet another new outcome measure for documenting UCB injury.

It once again seems warranted and feasible to test the hypothesis that Bf measurements will improve the clinical management of newborn jaundice by better identifying babies needing treatment and minimizing unnecessary intervention (5). The peroxidase test for measuring Bf is well suited for this purpose.

The Peroxidase Test for Measuring Bf

The horseradish peroxidase (HRP) methodology of Jacobsen and Wennberg (1, 2) is based on the observation that HRP catalyzes UCB oxidation by peroxide (typically hydrogen peroxide or ethyl hydrogen peroxide), but protein-bound UCB is protected and only Bf reacts (2, 31). The first-order rate constant for the reaction (Kp) is determined from the reaction velocity (rate of decrease in UCB peak light absorbance at 440 nm) in the absence of UCB-binding proteins (i.e., [UCB] = Bp Equation 2a) and then used to calculate Bf from the reaction velocity (rate of decrease in UCB light absorbance peak at 460 nm) when binding proteins are present (Equation 2b) (2).

\[
\frac{d[UCB]}{dt} = K_p[HPR][UCB] \tag{2a}
\]

\[
\frac{dB_f}{dt} = K_p[HPR]B_f \tag{2b}
\]

Integrated forms of the equations can also be used to obtain Kp and Bf (59), and Bf is calculated from the initial absorbance of the sample corrected for any hemoglobin interference (2). The test has been automated (60, 61), requires small sample volumes (<100 μL of serum or plasma), and is quantitative, rapid (2–3
min), and inexpensive. Because the method directly assay Bf, the lower-affinity UCB-binding sites will not obscure test endpoints as they do with the binding tests that attempt to measure serum or plasma UCB binding capacity (29, 39, 40).

A US Food and Drug Administration (FDA)-approved commercial spectrophotometer (UB-A1 Analyzer, Arrows Co Ltd) (62) provides automated readouts of Bf (mg/dL) and Bt (μg/dL). Bf is obtained after manually diluting the sample 41-fold with phosphate buffer, and a HRP reagent is added to obtain Bt. The reagents are stable at 8 °C for a month after reconstitution, and controls for Bt and Bf are provided. The CVs for Bt and Bf are about 2% and 5%, respectively. Although many clinical studies have been done with this device (11–14, 27, 51, 54–57, 61, 63, 64), it is not currently marketed in the US.

Several things interfere with the Bt measurement by the peroxidase test: (a) Hemoglobin is a weak peroxidase and has considerable absorbance at 460 nm. Moderate hemolysis (hemoglobin >5 g/dL in the undiluted sample (62)) adds peroxidase activity that can falsely increase Bf, and severe hemolysis overwhelms UCB light absorbance at 460 nm, falsely lowering Bf while falsely increasing Bt (2, 62). (b) Conjugated bilirubin is more readily oxidized than UCB and, at levels higher than about 1 mg/dL, will falsely increase Bf (13, 65). The test can be modified to avoid this problem (65). (c) Paraben preservatives falsely increase Bf by generating free radicals that rapidly oxidize UCB (66). (d) Bilirubin photoisomers appear to have little effect on Kt (Equation 1) in clinical studies (11, 12), although a recent in vitro study using nonneonatal samples suggests that photoisomers may interfere unpredictably with the peroxidase test (14). The peroxidase method reacts far more readily with 4Z,15Z UCB-IXe than with UCB photoisomers, and the 4Z,15Z UCB-IXe oxidation products have far less light absorbance than those of the photoisomers (11). (e) The pH and ionic composition (particularly chloride and phosphate ion) of the reaction medium may alter binding and the measured Bf (59, 63). (f) As with all enzyme-catalyzed reactions, Bf will vary with reaction temperature (62). (g) Substantial non–HRP-catalyzed UCB oxidation may occur depending on the type of peroxidase used (2, 62) and will falsely increase Bf unless a correction is applied (2). (h) The large sample dilution (typically 40-fold) can attenuate the effect of weak UCB binding competitors such as sulfisoxazole (Fig. 3B), causing underestimation of Bf (42).

Perhaps the most serious error made when using the peroxidase test is the failure to appreciate that the Bt determined by the peroxidase test is a steady-state Bt (Btst) (27), where

\[
B_{tst} = \frac{k_d(P - B_T + B_{tst})}{k_a(P - B_T + B_{tst}) + (K_{f[HPRP]})}
\]

and \(k_d\) and \(k_a\) are the UCB–protein dissociation and association rate constants, respectively (\(k_a/k_d = K_f\) in Equation 1). Btst is nearly equal to the equilibrium Bf only when \(k_a(P - B_T + B_{tst}) >> K_f[HPRP]\) and Equation 3 and Equation 1 are nearly identical. When this is not the case, Btst underestimates the equilibrium Bf, and the error worsens as the equilibrium Bf increases (27, 63). This error is easily avoided by measuring Bt at 2 or more HRP concentrations. When Btst is independent of the HRP concentration (Equation 3 vs. Equation 1), Btst is a reliable estimate of the equilibrium Bf at each HRP concentration. When Btst decreases with increasing HRP, however, the equilibrium Bf is equal to the inverse of the intercept of the plot of \(1/B_{tst}\) vs [HRP], as explained in detail elsewhere (27, 59, 61).

A pseudo-error in the method occurs when diluted samples are used because the apparent \(K_f\) for the albumin binding of UCB as well as many other ligands increases with sample dilution (61, 63, 67, 68). \(K_f\) increases steeply below albumin concentrations of about 100 μmol/L, most likely due to the dissociation of albumin oligomers that bind UCB less avidly than the albumin monomer (68). Bt measured at the standard 40-fold sample dilution traditionally used for the peroxidase test (2) is lower than but correlates directly and significantly with Bt measured at minimal sample dilution (61, 63). Furthermore, Bt measured in diluted samples has been superior to Bt in predicting ABE- or UCB-induced ABR changes (50, 51, 54–57). Nonetheless, because dilution alters both intrinsic albumin binding and may also attenuate the effects of weak UCB binding competitors, it would seem prudent to measure Bt at minimal sample dilution if possible.

The only clinical laboratory control for Bt is supplied with the commercial instrument described above. \(K_p\) may be determined directly using solutions of UCB (Equation 2A), but the instability of UCB solutions makes this approach problematic for clinical laboratories. UCB surrogates such as purpurogallin or the more stable biliverdin (69) could be employed to verify HRP activity, but specific Bt controls with a human albumin matrix to stabilize UCB are more desirable. Current Bt clinical laboratory controls may also prove useful in this regard (61), and it may be feasible to develop new Bt controls composed of UCB and human albumin solutions that are cross-referenced to Bt measured by other binding methods (68).

Intervention (reference) Bt thresholds for using phototherapy or exchange transfusion can only be approximated from available data at this time (30, 51, 54–57, 64, 70). P and \(K_f\) will need to be determined in larger
populations of newborns to determine the range of $B_f$ values occurring at the specific $B_T$ currently used to guide therapy (6, 30, 34). UCB-binding isotherms can readily be constructed for this purpose by titrating individual samples and umbilical cord blood with UCB and measuring $B_f$ over the range of $B_T$ observed clinically (2, 60). Because both $B_T$ and the $B_T$/albumin ratio guidelines for exchange transfusion decrease with gestation and birth weight (30), $B_f$ intervention thresholds should encompass these parameters as well.

Can $B_f$ Alter Clinical Practice? A Brief Summary of a Clinical Experience with the Peroxidase Test

The first author of this review (C.E. Ahlifors) has used the peroxidase test in the clinical management of newborn jaundice. $B_T$ and $B_f$ were measured at 2 HRP concentrations using a UB-A1 Analyzer in a CLIA-certified laboratory. The reference exchange transfusion $B_f$ threshold, extrapolated from existing literature and adjusted for birth weight, was 1.3 $\mu$g/dL per kg of birth weight (22 nmol/L per kg), with a maximum allowable $B_f$ of 4 $\mu$g/dL (66 nmol/L) (41, 50, 51, 64, 70). Given the limited clinical experience with the reference $B_f$, however, we restricted its use such that exchange transfusions were never administered or withheld on the basis of $B_f$ alone. Exchange transfusions were performed when the $B_T$ or the $B_T$/albumin ratio reached exchange transfusion criteria (6, 30) or symptoms suggestive of ABE were present (6).

$B_f$ was measured 294 times in 257 babies over 4 years (Table 1). Several publications resulted from this limited data set, including the first measurement of $B_f$ in a case of lethal kernicterus in the US (64) and repudiation of a published model for brain UCB uptake based on $B_T$ (27). Most importantly, we reported that $B_f$ but not $B_T$ was significantly associated with abnormal automated ABR hearing screens in term babies (56), and that $B_f$ and especially the $B_f/B_T$ ratio (a measure of binding affinity that is proportional to $1/K_f$), but not $B_T$, predicted abnormal automated ABR hearing screens, regardless of clinical circumstances (57).

The American Academy of Pediatrics guidelines recommend exchange transfusion at $B_T \geq 30$ mg/dL (6), and above this concentration about half of the babies with Rh hemolytic disease develop ABE (9). We encountered 4 babies with $B_T \geq 30$ mg/dL. One had lethal kernicterus ($B_T$ 7.63 $\mu$g/dL, 130 nmol/L) and died before exchange transfusion could be performed (64), and the other 3 received exchange transfusions. Only one of the 3 had a $B_f$ above the exchange transfusion threshold ($B_f$ 4.41 $\mu$g/dL, 75 nmol/L), and that baby was lethargic and failed an automated ABR hearing screen before exchange transfusion but then passed the ABR screen (64). The other 2 babies ($B_f$ 2.87 $\mu$g/dL and 2.80 $\mu$g/dL) had no obvious symptoms and passed the automated ABR before receiving exchange transfusions. One also had a normal MRI examination. Adding $B_f$, automated ABR, and MRI to our evaluation of these babies enhanced our assessments of the likelihood of ABE and the urgency with which treatment was needed in these babies.

The American Academy of Pediatrics guidelines consider $B_T$ between 25 and 30 mg/dL a gray zone where phototherapy should be provided but exchange transfusion is left to the discretion of the clinician (6). We readmitted 64 babies for jaundice, 13 of whom had $B_T$ between 25 and 30 mg/dL. All the $B_f$ values, which ranged from 1.23 to 2.99 $\mu$g/dL (21–51 nmol/L), were below the reference $B_f$ concentrations, and all 13 babies passed an automated ABR hearing screen on admission. This provided us considerable reassurance that phototherapy alone was sufficient for these babies.

An important but underappreciated tactic we often used was to measure $B_f$ early in the clinical course when babies were at increased risk of ABE (e.g., had hemolysis or were born prematurely). This allowed us to estimate the $B_T$ and $B_T$/albumin ratio at the reference $B_f$ for comparison with the recommended exchange transfusion $B_T$ and $B_T$/albumin ratio for babies with that condition (30). $K_f$ was calculated from the albumin concentration, $B_T$, and $B_f$ using Equation 1, and then reinserted into the equation along with the albumin and reference $B_f$ (22 nmol/L × birth weight in kg up to 66 nmol/L) to calculate the $B_T$ and the $B_T$/albumin ratio at the reference $B_f$. Comparing these with the recommended intervention $B_T$ and the $B_T$/albumin ratio allowed us to individualize our intervention strategies for each patient. The importance of applying this tactic as well as the general concepts outlined in this review to clinical practice and research are illustrated by comparing our clinical experience with premature newborns of <1 kg birth weight (Table 1) with that of the recent NICHD phototherapy study (3).

The NICHD phototherapy study admitted 984 babies into its conservative phototherapy group in which phototherapy and exchange transfusion were considered at $B_T$ thresholds of 8 and 10 mg/dL and 13 and 15 mg/dL for birth weights ≤0.750 kg and ≥0.751 kg, respectively. Peak $B_T$ averaged 9.8 mg/dL, and 13 babies reached $B_T$ exchange transfusion thresholds, with 3 receiving the procedure (0.3%) and 10 receiving augmented phototherapy according to study guidelines. Twenty-eight babies (3%) had severe hearing loss defined as requiring bilateral hearing aids, about 10 times the rate of congenital deafness in the newborn population (71). We measured UCB binding in 59 similar babies (Table 1), half of whom had binding measured before 48 h of age. Phototherapy was applied at the discretion of the attending physician and using our ex-
We performed 3 exchange transfusions (5%), and 3 additional babies did not pass their automated ABR hearing screen at hospital discharge. The binding and automated ABR data for these 6 babies are summarized in Table 2. The mean $K_f$ of the samples from these 6 babies (28 L/μmol) is significantly lower than that of the remaining samples (75 L/μmol, $P < 0.0001$). Only 1 baby (birth weight 0.840 g) reached the exchange transfusion threshold for the NICHD study ($B_T = 15.2$ mg/dL, $B_f = 0.90$ μg/dL), but $B_T$ had decreased substantially by the time the blood arrived for exchange transfusion and it was not performed. None

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<th>Albumin, μmol/L</th>
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<td>11.5</td>
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<td>347–664</td>
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<td>3.4–46.0</td>
<td>39–179</td>
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<td>≥2.5</td>
<td>136</td>
<td>124</td>
<td>5</td>
<td>336</td>
<td>559</td>
<td>0.61</td>
<td>21.5</td>
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<td>574</td>
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<td>91</td>
<td>86</td>
<td>0.18</td>
<td>16.1</td>
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<tr>
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<td>151–710</td>
<td>0.21–1.36</td>
<td>0.9–130</td>
<td>27–318</td>
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</table>
of our 50 surviving babies have required bilateral hearing aids.

As we have pointed out in this review, the increase in the UCB load required to increase the Bf from 8 to 10 mg/dL (the NICHD study phototherapy thresholds for 0.750 and 0.751 kg babies, respectively) can be substantial. Even worse, ABE may occur before reaching 10 mg/dL if P is approaching saturation at 8 mg/dL, as illustrated in Fig. 4. Measuring UCB binding early in the clinical course in the NICHD study could have helped identify patients for whom the study intervention criteria might have been inappropriate. In addition, UCB-binding measurements would have also been very informative in all 16 NICHD study babies that reached the BT exchange transfusion criteria, only 5 of whom received the procedure.

Comparing our clinical experience with conventional approaches to jaundice management clearly indicates that Bf measurements, especially when coupled with ABR and MRI imaging, can substantially improve clinical practice. Babies with very poor UCB binding can be identified who might otherwise undergo undetected and unsuspected ABE at BT considered too low for ABE to occur. Screening for jaundice at newborn discharge is now being considered, and screening with UCB binding to obtain Kf would be valuable. A higher Kf at increased hour-specific discharge Bf argues against excessive UCB production, whereas a lower Kf at nondescript discharge Bf identifies an increased risk for ABE at unexpectedly low Bf (6). The ultimate advantage of measuring UCB binding is individualization of jaundice management, where one Bf intervention criterion clearly can never fit all.

**Research Applications of Bf Measurements**

Measuring Bf in the media of cell culture models of bilirubin toxicity has been addressed only recently (59, 72). It has been now been demonstrated directly that in vitro cytotoxicity is accurately predicted by Bf but not Bf, irrespective of whether human or bovine serum albumin or fetal calf serum is used in the medium (59). Such measurements with cultured neurons and astrocytes may provide considerable insight into the concentrations of Bf in the cerebrospinal fluid (CSF) and extracellular fluid of the CNS that are likely to produce bilirubin neurotoxicity in vivo (73). In addition, Bf measurements may help evaluate the considerable variation in susceptibility of cell lines to bilirubin toxicity and the specific anatomical patterns of kernicterus that occur in vivo (74).

In vitro aspects of UCB binding may also be relevant in vivo. The enhanced effect of albumin dilution on UCB–albumin binding noted above may play an important role in the movement of UCB from blood to CSF across the choroid plexus. The albumin concentration in the CSF is about 2% that of the blood, and, consistent with the enhancement of albumin binding affinity with dilution noted above (67–69), the reported BT/albumin ratios in CSF are higher than the corresponding ratios in the blood (75). The higher affinity of the less concentrated albumin in the CSF would create a gradient favoring net diffusion of unbound UCB from plasma into the CSF.

**Summary and Conclusions**

There is now strong evidence that Bf measurements could significantly improve the clinical management of newborn jaundice by better detecting those babies needing treatment and by reducing unnecessary intervention. Bf measurements in basic and clinical research may provide further insight into the pathochemistry of ABE. We must continually remind ourselves that our goal in measuring vascular bilirubin levels is not to prevent hyperbilirubinemia per se, but to prevent bilirubin-induced brain injury. The emerging clinical evidence strongly supports the theoretical considerations indicating that measurement of Bf may contribute substantially to achieving this goal.

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**Table 2. Bilirubin binding variables (UB-A1 Analyzer, 1:41 sample dilution, 2 HRP concentrations) in babies born at <1 kg who received exchange transfusions or had an abnormal automated ABR hearing screening at hospital discharge.**

<table>
<thead>
<tr>
<th>Birth weight, kg</th>
<th>Exchange transfusion</th>
<th>Automated ABR at discharge</th>
<th>Bf, μmol/L (mg/dL)</th>
<th>Bf, nmol/L (μg/dL)</th>
<th>Albumin, μmol/L (g/dL)</th>
<th>Kf, L/μmol</th>
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<tr>
<td>0.823</td>
<td>Yes</td>
<td>Normal</td>
<td>111 (6.5)</td>
<td>21 (1.22)</td>
<td>211 (1.4)</td>
<td>53</td>
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<tr>
<td>0.580</td>
<td>Yes</td>
<td>Not done</td>
<td>166 (9.7)</td>
<td>42 (2.46)</td>
<td>498 (3.3)</td>
<td>12</td>
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<tr>
<td>0.622</td>
<td>Yes</td>
<td>Died</td>
<td>173 (10.1)</td>
<td>34 (1.99)</td>
<td>513 (3.5)</td>
<td>15</td>
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<tr>
<td>0.777</td>
<td>No</td>
<td>Abnormal</td>
<td>54.7 (3.2)</td>
<td>5.2 (0.31)</td>
<td>423 (2.8)</td>
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<tr>
<td>0.583</td>
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<td>Abnormal</td>
<td>66.7 (3.9)</td>
<td>8.2 (0.48)</td>
<td>544 (3.6)</td>
<td>17</td>
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<td>0.619</td>
<td>No</td>
<td>Abnormal</td>
<td>94.0 (5.5)</td>
<td>5.3 (0.34)</td>
<td>483 (3.2)</td>
<td>42</td>
</tr>
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</table>
References

41. Ritter DA, Kenny JD, Norton HJ, Rudolph AJ. A