Delta Bilirubin in Serum of Pediatric Patients: Correlations with Age and Disease

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At least four bilirubin fractions can be separated and identified by liquid chromatography, the least understood being the "delta" fraction (B₃), which apparently is covalently bound to albumin. To learn more about the incidence and significance of B₃, we assayed serum from 539 infants and children, both by the chromatographic method and the routine colorimetric Jendrassik–Grob method. The proportion of B₃ appeared to correlate with both age and disease course. For infants younger than 28 days B₃ generally was <2% of total bilirubin; for hyperbilirubinemic older infants and children the median B₃ value was 35%. High B₃ (>50% of total bilirubin) in newborns was associated with intra- and extra-hepatic cholestasis, biliary cirrhosis, biliary atresia, and hepatitis. Among older infants and children, a proportion of low B₃ (<10%) was found in hemolytic anemias, sepsis, shock, and other non-hepatic jaundice. In several cases, when low B₃ was accompanied by increased conjugated bilirubin, the prognosis was very poor. Delayed clearance of B₃ from the circulation together with its reactivity in direct diazo methods may interfere with interpretation of values for conjugated bilirubin as measured by classical methods.

Additional Keyphrases: liver disease - pediatric chemistry - neonates - prognostic sign - relation to traditional tests

A recently developed procedure for fractionation of bilirubin by "high-performance" liquid chromatography does not require complete deproteinization of the sample (1). This procedure reveals four species of bilirubin in serum. These appear to correspond to those reported earlier by Kuenzle et al. (2), which they designated, in order of elution from the chromatographic column, as α, unconjugated bilirubin; β, monoconjugated bilirubin; γ, diconjugated bilirubin; and δ, a protein-bound species. Historically, bilirubin species were classified according to reactivity in the diazo method as "indirect-reacting" or unconjugated bilirubin (B₂) and "direct-reacting," which was believed to be conjugated bilirubin (B₄). The existence of a protein-bound bilirubin species eluting as the δ fraction (B₃), which also reacts directly in diazo methods, was not widely recognized. Little is known about the nature or significance of B₃. It appears to be covalently linked to albumin through an amide bond (3).

Quantification of bilirubin by the total and direct-reacting methods is well documented as a useful tool in pediatric medicine for classifying neonatal jaundice. The etiology of hyperbilirubinemia in infants and children is often unique relative to "adult" disease states. Neonates comprise an interesting population in that bilirubin conjugation and excretion processes are in an early stage of development. Improvements in specialized care of the neonate have resulted in increased survival of premature infants as well as those with congenital abnormalities. Thus particular attention is now devoted to the immature or compromised liver of the infant. Treatments such as the use of total parenteral nutrition may result in hepatic complications, including cholestasis and inflammation, the etiology of which is poorly understood (4). The incidence and role of B₃ in these patients, and its potential diagnostic significance, were of particular interest to us.

We evaluated the bilirubin composition of 539 specimens from 347 different patients with hyperbilirubinemia: 242 from an acute-care referral facility and 105 from the well-baby nursery of a community hospital. Demographic and diagnostic information were obtained whenever possible. The accumulated data were evaluated relative to (a) age dependency and distribution of B₃ in the neonatal period, (b) presence of B₃ in various disease conditions, (c) bilirubin fractionation of sequential specimens from selected patients, and (d) the effect of the B₃ fraction on the measurement and interpretation of "direct" bilirubin.

Materials and Methods

Serum specimens submitted to the laboratory for bilirubin determination were frozen at −70°C within 3 h of collection. The blood had been sampled by routine venipuncture or skin puncture. In general, we selected patients for the study on the basis of visible icterus and increased total bilirubin in the serum as determined colorimetrically. Specimens were processed and analyzed in batches, weekly, with care to minimize exposure to light. Values for the bilirubin fractions were satisfactorily stable for samples so stored.

Liquid-chromatographic separation of bilirubin species was done as described by Lauff et al. (5), a procedure that does not require that sera be totally deproteinized before injection into the chromatograph. In brief, serum samples were treated with an excess of sodium sulfate solution to remove proteins larger than albumin. The filtered, diluted supernates were injected onto a supplier-prepared column

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containing Lichrosorb RP-8 (Brownlee Lab, Santa Clara, CA 95050). Separation required a 24-min elution with a linear-gradient eluent. The initial mobile phase consisted of phosphate buffer (50 mmol/L, pH 2) containing 50 mL of methoxyethanol per liter and a secondary mixture of methoxyethanol/isopropanol (5/95 by vol). Bilirubin fractions were detected by their absorbance at 436 nm and quantified from the relative peak areas and the total bilirubin concentration. Values of the monoconjugated (β) and diconjugated (γ) bilirubin fractions were generally combined and expressed as B₃ (conjugated bilirubin).

Total bilirubin was quantitated by a Jendrassik–Grodzio method adapted to a centrifugal analyzer (5). Samples were analyzed in duplicate when sample volume was adequate.

Results

Typical patterns for different disease states are shown in Figure 1a–d. These are grouped in the categories discussed below.

Neonates. Jaundiced neonates in the first two postnatal weeks tended to have a small proportion of bilirubin in the delta form. Some particular cases are represented in Figure 1a. The percentage of B₃ was highest in an infant infected with cytomegalovirus. At three days of age, 19% of the total bilirubin was B₃. Infants with no indication of hepatobiliary involvement had negligible concentrations of B₃ in their serum.

When we evaluated the proportion of B₃ in relation to age for infants younger than 90 days, the relation shown in Figure 2 was observed. In the case of the acute-care hospital, 168 hyperbilirubinemic neonates younger than 14 days had a median B₃ of 2% (range 0 to 19%). In infants older than 40 days, B₃ had shifted to approximately 35% (range 0 to 75%; n = 46)—comparable with the B₃ distribution found in hyperbilirubinemic adults (6). A distinct shift to the higher values is seen during the 20– to 40-day period.

Neonates from the community hospital (n = 105) had a median B₃ of 0.6% (range 0 to 12%). Figure 3 illustrates the

Fig. 2. Correlation of the percentage of B₃ in hyperbilirubinemia with age of the patient. Specimens submitted to the acute care hospital laboratory were selected based on increased bilirubin concentration, without conscious regard for the diagnosis or other clinical data.

Fig. 3. Distribution of B₃ in neonates less than 14 days old. (A) Patients from an acute care referral hospital. Median B₃ was 2%; n = 117. 95% were ≤12% B₃. (B) Patients from the well baby nursery of a community hospital. Median B₃ was 0.8%; n = 123. 95% were ≤5% B₃.
distributions of values for percent $B_3$ in neonates from these two hospitals.

We retrospectively reviewed medical records for patients with high $B_3$ (>50% of total bilirubin) and low $B_3$ (<20%). In many instances, multiple diagnoses or inconclusive diagnosis made such categorization difficult. Thus we grouped the observations into broad categories reflecting confirmed primary diagnosis, as follows.

**High $B_3$**. Figure 1b illustrates a representative selection of the 15 cases with $B_3$ ranging from 52 to 88% of total bilirubin. Six patients had cholestatic jaundice associated with administration of parenteral nutrition. Four patients were diagnosed as having biliary atresia and were at various stages of recovery. The remaining five patients had hepatic failure associated with confirmed hepatitis (type A or non A–non B).

**Low $B_3$ and low $B_0$**. A low percentage of $B_0$, as well as low $B_3$, was observed with jaundice associated with hemolytic or hemorrhagic events, including hemolytic anemia, trauma such as surgery or burns, and shock, as well as physiological jaundice of the newborn. Six cases are represented in Figure 1c. For 30 patients with diagnoses of sickle cell disease, the mean percentage of $B_3$ was 4% (range 0 to 14.5%).

**Low $B_3$ and high $B_0$**. A potentially significant observation may be the poor prognosis in cases where the percentage of $B_3$ was low but that of conjugated bilirubin was high ($B_0 > 47$%). Six of eight infants with this pattern died within a few days of sampling. This group included patients with diagnoses of hepatic failure associated with acute-phase leukemia, septic shock, $\alpha_1$-antitrypsin deficiency, and kidney transplant failure (Figure 1d).

The proportion of $B_3$ also depended on the stage of the disease. We followed the cases of several patients with obstructive jaundice. During recovery from obstruction, $B_3$ tended to return more quickly to normal than did total bilirubin but the percentage of $B_3$ increased steadily. A typical case is shown in Figure 4. These observations are consistent with a slower rate of clearance for the albumin-linked $B_3$.

**Discussion**

Recent developments in the resolution and determination of bilirubin conjugates and isomers have paved the way for better understanding of the mechanisms of bilirubin formation and elimination, as well as diagnostic implications of the various fractions in serum (6, 7). The $B_3$ fraction is of particular interest because of its novel covalent association with albumin.

The percentage of $B_3$ in the jaundiced neonates sampled in this study was low relative to hyperbilirubinemic adults. The apparent relation between the proportion of $B_3$ and the patient's age may reflect the state of maturation of the biochemical mechanism which produces $B_3$. The increased proportion of $B_3$ observed after three weeks of age lags behind the average maturation of the glucuronyl transferase system. It is tempting to infer that some mechanism for manufacturing $B_3$ is "turned on" at about three weeks. Alternatively, the presence of $B_3$ may depend on particular pathological states that increase in prevalence after the first three weeks of age.

Disease correlations in this study support and expand the earlier work of Kuenzle et al. (8). Although jaundice is rarely a result of a single pathophysiologic mechanism, several categorizations can be made.

Patients diagnosed with obstructive hepatobiliary diseases tend to have a substantial proportion of $B_3$, which tends to increase during recovery as the total bilirubin concentration decreases. The observation probably reflects slower clearance of $B_3$ from the circulation. Because it reacts in the direct diaz procedure (6), $B_3$ lingering in the circulation would continue to cause abnormal results in the traditional Jendrassik–Grof or Evelyn–Malloy measurement of direct-reacting bilirubin. These procedures are commonly used as an indicator of recovery from obstruction, and persistently increased $B_3$ after clearance of $B_0$ would diminish the sensitivity of direct-bilirubin results.

In the acute stage of hepatobiliary disease, $B_3$ and $B_0$ always increased concurrently. On the other hand, diseases characterized by hemolytic or hemorrhagic episodes were associated with low concentrations of $B_3$. The observations that $B_3$ does not accompany unconjugated hyperbilirubinemia and that $B_0$ is also present whenever $B_3$ is increased in the acute stage of disease suggest that $B_0$ may be a precursor to $B_3$. An animal model, in which hepatic insult could be controlled, could provide the means to study the conditions of formation of $B_3$ as well as the significance of its presence or absence.

In several of our cases increased $B_0$ was accompanied by a low percentage of $B_3$. The survival rate among these patients was low. Although the sample was too small for us to draw any conclusions, the observation may have significance in terms of the time required for $B_3$ to form after the onset of disease.

The addition of $B_3$ to the list of bilirubin species and of enzymes traditionally monitored in cases of jaundice may provide additional information about the clinical state of the patient. In particular, $B_3$ may aid in the determination of acute vs chronic stages of hepatobiliary disease. Consideration of $B_3$ separately from $B_0$ may enhance the correlation between increased $B_3$ and stages of cholestasis; failure to form $B_3$ may be indicative of the prognosis for hepatic recovery. Additional biochemical, physiologic, and clinical studies are needed that address these questions.

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**References**


**Fig. 4. Bilirubin fractions in a patient recovering from obstructive jaundice**

Concentrations in sequential serum specimens were determined as described in the text. TBIL, \( \bullet \); $B_3$, \( \circ \); $B_0$, \( \triangle \); $B_2$, \( \circ \); $B_1$, \( \bullet \); $B_0$, \( \circ \); $B_3$, \( \bullet \); $B_2$, \( \circ \).


