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Imbalance Between Production and Conjugation of Bilirubin: A Fundamental Concept in the Mechanism of Neonatal Jaundice

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ABSTRACT. *Objective.* The objective of this study was to evaluate the roles of production and conjugation of bilirubin, individually and in combination, in the mechanism of neonatal jaundice.

Methods. A cohort of healthy, term male newborns was sampled on the third day of life, coincident with routine metabolic screening, for blood carboxyhemoglobin determination, a reflection of heme catabolism, and for serum unconjugated and conjugated bilirubin fractions, reflecting bilirubin conjugation. The former was determined by gas chromatography, corrected for inspired CO (COHbc), and expressed as percentage of total hemoglobin. Serum bilirubin fractions were quantified by alkaline methanolysis and reverse phase high performance liquid chromatography. The sum of all bilirubin fractions comprised serum total bilirubin (STB). Total conjugated bilirubin (TCB) was comprised of the sum of the conjugated fractions and was expressed as percentage of STB (TCB[%]). A "bilirubin production/conjugation index" (COHbc/[TCB(%)] represented the combined roles of these modalities in the mechanism of bilirubinemia. Relationships between STB concentrations on the one hand, and COHbc values, TCB(%) proportions, and the production/conjugation index on the other, were determined by applying a best-fit regression analysis methodology.

Results. Mean (\pm standard deviation) STB concentration at the time of sampling was $114 \pm 48 \mu\text{mol/L}$ (range: 8–263 $\mu\text{mol/L}$). Mean COHbc value was $0.77 \pm 0.19\%$, and median (interquartile range) TCB(%) was 0.737 (0.465–1.260)%. COHbc values correlated directly with STB concentrations ($r = 0.38$; $s = 46.1$), and TCB(%) correlated inversely with STB ($r = 0.40$; $s = 45.8$). The production/conjugation index correlated positively with STB values ($r = 0.61$; $s = 45.8$), the r value for the index being higher than that of either COHbc or TCB(%), individually. The bilirubin production/conjugation index seemed to have a biphasic relationship to STB: STB values rose steeply in concert with increasing index values

in the lower range of the index, and subsequently plateaued in the higher range of the index.

Conclusions. Within the range of STB concentrations encountered, both increasing bilirubin production and diminishing bilirubin conjugation contributed to STB. The production/conjugation index confirmed that imbalance between production and conjugation of bilirubin plays an important role in the mechanism of neonatal bilirubinemia. *Pediatrics* 2002;110(4). URL: <http://www.pediatrics.org/cgi/content/full/110/4/e47>; alkaline methanolysis, bilirubin, bilirubin conjugation, carbon monoxide, carboxyhemoglobin, gas chromatography, hemolysis, high performance liquid chromatography, physiologic jaundice.

ABBREVIATIONS. STB, serum total bilirubin; CO, carbon monoxide; COHb, carboxyhemoglobin; COHbc, COHb corrected for inspired CO; UGT, uridine diphosphoglucuronate glucuronosyltransferase 1A1; G-6-PD, glucose-6-phosphate dehydrogenase; TCB, serum total conjugated bilirubin.

Jaundice is common during the first days of postnatal life and affects almost two thirds of human newborns. The mechanism of this bilirubinemia is multifactorial, as recently summarized, and comprises primarily processes contributing to increased bilirubin load, or diminished bilirubin clearance.^{1,2} The former may be the result of factors that augment bilirubin production and the enterohepatic circulation, whereas the latter is primarily the result of immature conjugative capacity, although impaired hepatic uptake or excretion may also play a part. It has been suggested that serum total bilirubin (STB) concentrations that remain within the physiologic range result from equilibrium between bilirubin production and elimination. In contrast, in some neonates, imbalance between these processes may occur, with bilirubin production being relatively higher than conjugation. This imbalance is thought to result in hyperbilirubinemia.³

Assessment of the role of hemolysis may be accomplished through assessment of endogenous carbon monoxide (CO) production by accurately measuring blood carboxyhemoglobin (COHb), or end tidal CO, both with correction for ambient CO. The principle involved is that equimolar quantities of CO and biliverdin, (the latter subsequently metabolized to bilirubin), are released for each molecule of heme catabolized through the action of the enzyme heme oxygenase. Elevated endogenous CO production increases in concert with increased bilirubin produc-

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tion, and frequently accompanies increased serum STB levels. Non- or minimally invasive techniques are available for this determination.⁴⁻⁷

Hepatic activity of the bilirubin conjugating enzyme, uridine diphosphoglucuronate glucuronosyltransferase 1A1 (UGT), is almost always diminished in the immediate postnatal period.^{8,9} It is to be expected, therefore, that diminished bilirubin conjugation should also contribute to the development of bilirubinemia. However, in contrast to measurements of bilirubin production, the lack of minimally invasive methods for assessment of neonatal bilirubin conjugation has limited the understanding of its role in the development of bilirubinemia. It is recognized that, even under physiologic conditions, some bilirubin conjugates efflux from the hepatocyte to the serum,¹⁰⁻¹³ and that accurately determined serum concentrations of conjugated bilirubin will reflect intrahepatocytic bilirubin.

We hypothesized that an important factor in the mechanism of jaundice in the first days of life would be increased bilirubin production relative to conjugation. This imbalance would be affected by either exacerbated hemolysis, diminished conjugation, or a combination of both. The aim of this study was to test the validity of this hypothesis through determination of the relative roles of increased bilirubin production as well as diminished conjugation activity. This was to be accomplished by measuring blood COHb corrected for inspired CO (COHbc) levels and serum unconjugated and conjugated bilirubin fraction concentrations in samples drawn simultaneously from a cohort of healthy neonates on the third day of life. This time was chosen so as to allow for sampling simultaneous with routine metabolic screening tests, so as to avoid a blood-taking procedure specially for the purpose of the study. Furthermore, very little serum conjugated bilirubin is detectable during the first day of life, whereas measurable quantities of serum conjugated bilirubin have been shown to be present by the third postnatal day.^{14,15}

METHODS

The study protocol was approved by the institutional review board of the Shaare Zedek Medical Center. The patient cohort consisted of healthy neonates delivered at ≥ 37 weeks' gestational age at the Shaare Zedek Medical Center. As glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is common in some subsections of the Israeli population and may be associated with differing rates of bilirubin metabolism,¹⁶ males only were included so as not to encounter female G-6-PD-deficient heterozygotes. Infants with direct Coombs' positive hemolytic conditions, G-6-PD deficiency, sepsis, maternal diabetes, extensive bruising, or Down syndrome were excluded. This cohort was previously studied in relation to UDP glucuronosyltransferase 1A1 promoter polymorphism, seen in Gilbert's syndrome,¹⁷ but no infant was excluded based on this genotype. All infants received routine clinical management during their nursery stay.

A sample of blood for the purpose of the study was drawn close to the beginning of the third day of life. For analysis of the unconjugated and conjugated bilirubin fractions, 0.5 mL of serum was separated, stored in the dark in a dark box at -70°C , and subsequently shipped on dry ice to the University of Padua, Italy. Whole blood for COHb determination (150 μL) was collected into custom-made capillary tubes containing heparin and saponin, as previously described^{18,19} and stored at -18°C . Before shipping, the samples were allowed to thaw and were transported on ice to Stanford University. Simultaneously with the blood collection a

sample of room air from the nursery in which the infant was being cared was collected and stored in a special container designed for this purpose until its CO concentration was determined (Bistable Gas Sampler, Chemical Projects Limited, Toronto, Ontario, Canada).

Laboratory Methods

COHb was determined at Stanford University by a gas chromatographic method, and its concentrations expressed as a percentage of total hemoglobin, which was quantitated by the cyanmethemoglobin method, as previously described.^{18,19} Using this method, the within-day and between-day coefficients of variation for reference blood samples are 3% and 8%, respectively.²⁰ The CO concentration of the room air specimens was determined at the Shaare Zedek Medical Center using a sensitive electrochemical CO analyzer supplied by Stanford University (Stanford, CA).²¹ Room air CO concentrations were used to correct measured COHb for COHbc by a previously derived formula ($\text{COHbc} = \text{measured COHb} - 0.17 \mu\text{L CO/L room air}$).²²

Quantitation of the unconjugated and conjugated bilirubin was performed at the University of Padua via alkaline methanolysis followed by reverse-phase high-performance liquid chromatography, according to the method of Muraca and Blanckaert.²³ For this method, the within-day coefficient of variation is 5% to 8%, and that for between-day is 6% to 13%.

G-6-PD Mediterranean mutation was determined at the Scripps Research Institute, La Jolla, California, by polymerase chain reaction followed by allele-specific oligonucleotide hybridization, courtesy of Ernest Beutler, MD.

Data Analysis

The sum of the measured concentrations of serum unconjugated, mono-, and diconjugated bilirubin was defined as STB, and that of the mono- and diconjugated bilirubin concentrations, the total conjugated bilirubin (TCB) value. The role of bilirubin conjugation in the pathogenesis of bilirubinemia was determined by calculating serum TCB as a percentage of the STB (TCB[%]) (See "Discussion"). The percentage value for each infant was individually calculated, and then the median and interquartile range of these calculations were computed. Parametric data are presented as mean \pm standard deviation, and nonparametric data as median (interquartile range).

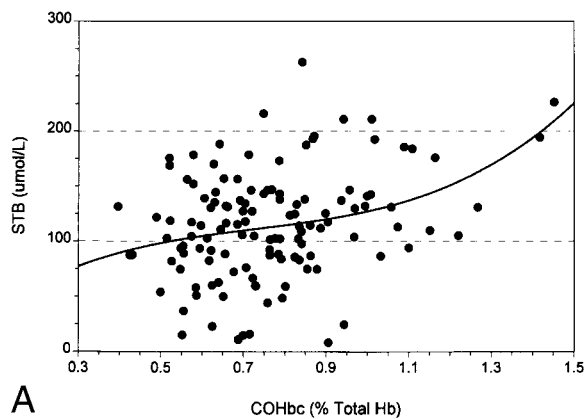
To assess the combined role of bilirubin production and conjugation in the pathogenesis of jaundice, an index representative of bilirubin production divided by bilirubin conjugation, (bilirubin "production/conjugation" index, $\text{COHbc}/[\text{TCB}(\%)]$) was used.¹⁷ Using this index, low bilirubin production rates along with efficient bilirubin conjugation should be expected to result in a low index, whereas high COHbc values relative to TCB(%) should result in a higher index.

Because of the nonparametric nature of the TCB data, and because visual inspection of the scattergrams between STB and the parameters studied, including the production/conjugation index, suggested that the relationship between these parameters may be curvilinear rather than linear (Figs 1 and 2), regression analysis was performed by applying a best-fit methodology, using a software program Curve Expert version 1.3, for Windows (Starkville, MS).

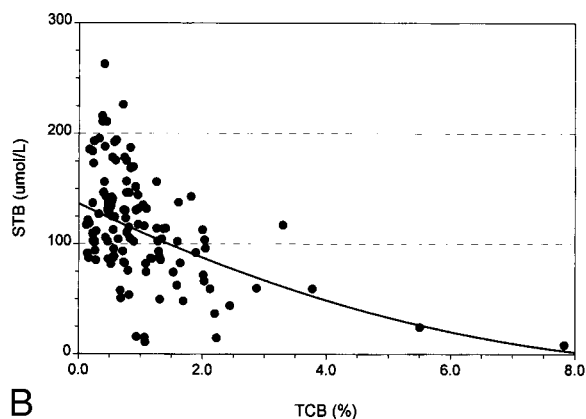
RESULTS

A total of 131 male infants was appropriate for inclusion in the study and had all measurements completed. Mean birth weight was 3336 ± 470 g, and gestational age 39.6 ± 1.2 weeks. Ninety-one percent were delivered vaginally, and 73% were exclusively breastfed. Mean age at the time of sampling was 52 ± 8 hours.

Measured values included blood COHbc (percentage of total hemoglobin), $0.77 \pm 0.19\%$ (mean \pm standard deviation); unconjugated serum bilirubin, $113 \pm 48 \mu\text{mol/L}$; monoconjugated bilirubin, 0.740 (0.140 – 1.130) (median, interquartile range); and diconjugated bilirubin, 0.00 (0.00 – 0.050) $\mu\text{mol/L}$. Cal-



A



B

Fig 1. A, Curvilinear regression analysis between STB values and carboxyhemoglobin corrected for ambient CO (COHbc). Increasing STB values correlated positively with COHbc ($r = 0.38$; $s = 46.1$. $y = 9.36 + 323.5x - 378.4x^2 + 172.5x^3$). B, Curvilinear regression analysis between STB values and TCB, expressed as percentage of STB (TCB[%]). Increasing STB values were inversely proportional to TCB(%) ratio ($r = 0.40$; $s = 45.8$. $y = 136.5 - 27.0x + 1.3x^2$).

culated values included STB, $114 \pm 47 \mu\text{mol/L}$ (range: $8\text{--}263 \mu\text{mol/L}$) (sum of unconjugated and conjugated bilirubin fractions); TCB, 0.810 ($0.475\text{--}1.260$) $\mu\text{mol/L}$ (sum of conjugated bilirubin fractions); and percentage of STB (TCB[%]), 0.737 ($0.465\text{--}1.260$)%.

Best-fit regression analysis was performed with STB as the dependent variable and COHbc and TCB(%) the independent variables. There was a positive correlation of COHbc to STB ($r = 0.38$; $s = 46.1$) and an inverse correlation of TCB(%) to STB ($r = 0.40$; $s = 45.8$; (Figs 1A and 1B, respectively). STB values and the bilirubin production/conjugation index also correlated in a curvilinear fashion ($r = 0.61$; $s = 39.1$). This correlation seems to be biphasic: for index values $< \sim 2$ (75th percentile), STB concentrations rose steeply in concert with increasing values for the index, as expected. However, for index values > 75 th percentile, additional increases were associated with a plateau effect for STB concentrations, although these STB values were generally higher than those associated with index values < 2 . It will be noted that the r value for the index was higher than that of individual r values for either COHbc or TCB(%)

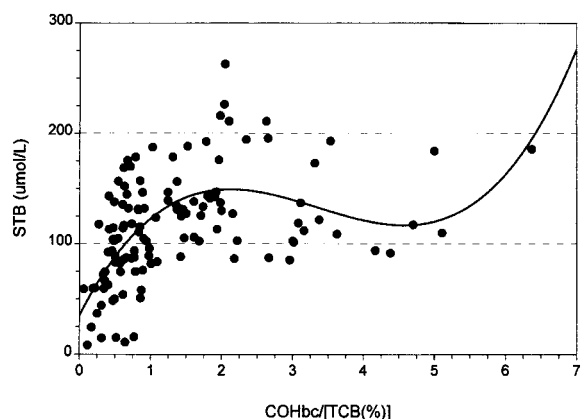


Fig 2. Curvilinear regression analysis between STB values and the combined effect of bilirubin production and conjugation, reflected by the bilirubin production/conjugation index COHbc/[TCB(%)]. Increasing values of STB correlated positively to this index ($r = 0.61$; $s = 39.1$. $y = 32.1 + 132.1x - 45.8x^2 + 4.6x^3$).

DISCUSSION

To our knowledge, this is the first time that the contributions of production and conjugation of bilirubin to STB, and the combined effects of these 2 processes, have been evaluated in the same neonatal cohort. In this study, we clearly demonstrate that over the entire range of STB concentrations observed in these otherwise normal and healthy neonates, STB values correlate with both increasing COHbc levels as well as diminishing TCB(%) values. Especially illustrative of this combination of factors is the bilirubin production/conjugation ratio, the correlation of which to STB was higher than that of individual r values for either COHbc or TCB(%). The combination of forces reflected by the production/conjugation index may be likened to the fluid level in a sink of water. The depth of water in the sink will be dependent on inflow from the faucet (analogous to bilirubin production) as well as outflow from the drain (analogous to elimination).³ Theoretically, increase in the production/conjugation index with resultant jaundice may result from minimally increased hemolysis combined with immature bilirubin conjugation, whereas conversely, excessively increased hemolysis in the presence of optimal conjugative ability may not affect the index and the development of jaundice. The relationship of the index to STB confirmed that imbalance between production and conjugation of bilirubin is an important concept in the mechanism of neonatal bilirubinemia.

The biphasic nature of the relationship of the production/conjugation index to STB is intriguing. In the majority of cases, which fell within the lower range of the index, small increases in index values were accompanied by large increases in STB concentrations. However, at index values of ~ 2 , equivalent to the 75th percentile, bilirubin levels seemed to plateau, and additional increases in the index were no longer associated with the steep increases in STB that were noted previously. Possible explanations for this plateau effect may include changes in albumin carrying capacity, or increased uptake into the hepatocyte, at higher rates of bilirubin production. Bilirubin

conjugation has been shown to be induced by increasing bilirubin load. It is also possible that at higher STB concentrations the gradient between bilirubin in the serum and tissues increases, with greater bilirubin efflux from the serum to the tissues. Any of these factors, individually or in combination, may contribute to modify a direct relationship between the index and actual STB concentrations. The study was not designed to evaluate the interaction between production and conjugation of bilirubin in infants with severe hyperbilirubinemia, although it does seem logical to presume that additional imbalance between these processes should lead to even higher STB concentrations than were encountered in our patients on the third day of life.

Relatively low r values in the correlations between STB concentrations and COHbc values and TCB(%) ratios indicate that factors additional to the above contribute to the genesis of STB. Some of these factors may include interindividual variations in bilirubin production or conjugation, or differing rates of hepatic uptake and excretion. In the neonate, immaturity of the bilirubin conjugating process, hour to hour changes in STB, which are common during the first days of life, and bilirubin resorption via the enterohepatic circulation, may further confound the relationship between bilirubin production and conjugation on the one hand, and actual serum bilirubin levels on the other. It is therefore perhaps not surprising that the serum bilirubin levels were not directly mathematically related to the bilirubin production rate or the conjugation rate, although it should be noted that the r value for the production/conjugation index was higher than that of the individual r values for either COHbc or TCB(%).

Measurement of serum conjugated bilirubin fractions have been used as a minimally invasive method of assessing bilirubin conjugation in neonates,^{15,24,25} children,^{26,27} and adults,^{12,28} alike. It is recognized that physiologically, a small fraction of bilirubin conjugates effluxes from the hepatocyte to the serum.¹⁰⁻¹³ This concept has been demonstrated in studies of serum bilirubin fractions in UGT-deficient rats, as well as normal rats who had been loaded with bilirubin.¹² Human serum bilirubin studies of individuals who had either Gilbert's syndrome or Crigler-Najjar syndrome, or alternatively, hemolytic conditions, have confirmed that there is bidirectional transfer of both unconjugated bilirubin as well as conjugated bilirubin fractions across the sinusoidal membrane.¹² Similarly, sera from patients with biopsy-proven liver disease have demonstrated increased serum conjugated bilirubin fractions.²⁹ Furthermore, in man bilirubin conjugation seems to occur only in the liver,^{11,12} and conjugated bilirubin compounds are not absorbed from the bowel. Therefore, as all the neonates included in this cohort were otherwise healthy, free of overt hemolysis as determined by direct Coombs' positivity or G-6-PD deficiency, and with no evidence of hepatic disease, we believe that this test represented an accurate reflection of bilirubin conjugation.

In this study, for data analysis, we used the ratio of serum TCB expressed as a percentage of the STB to

evaluate bilirubin conjugation and compare conjugated bilirubin fractions, rather than actual measured conjugated bilirubin concentrations. The rationale is that, in animals as well as in humans, bilirubin may modulate its own metabolism via regulation of UGT expression. As a result, in individuals with normal hepatic conjugation capacity, serum conjugated bilirubin fractions may fluctuate in parallel with changes in serum unconjugated bilirubin concentrations.^{12,30-34} Actual conjugated bilirubin fractions can be used for comparisons in situations where the total bilirubin is similar between groups being compared, but may artifactually give the impression of improved hepatic conjugative capacity in individuals who have higher STB values than others, or when comparing individuals with differing bilirubin conjugative ability. In individuals with diminished hepatic conjugation, the conjugated bilirubin fractions may not respond by rising in parallel to increasing bilirubin load. Thus, serum TCB values expressed as a percentage of STB, will facilitate comparison of bilirubin conjugation between subjects with varying total bilirubin values. It should be noted that for each infant, calculations were first individually performed, and then the mean or median value computed. As a result, the measured data in the results section may seem to be unrelated to the calculated ratios.

Although it might have been useful to study the neonates closer to the peak of their bilirubinemia, or in a developmental context at several points during early postnatal life, we could not do so as on the first day of life there is very little measurable serum conjugated bilirubin, and most of the infants were discharged on or shortly after the third day postnatal day. However, there is every reason to assume that the principle of imbalance between bilirubin production and conjugation in the mechanism of jaundice is operative both at earlier and later stages of neonatal life.

We are, however, confident that the study design did give an accurate representation of bilirubin conjugation in the neonatal period as Muraca et al¹⁵ have shown that TCB values, although variable on the first day of life, stabilize over the next 3 days. Furthermore, Rubaltelli et al³⁵ have shown that conjugated bilirubin profiles on the third and fifth day of life are similar between breastfed and formula-fed infants, and Stevenson et al³⁶ have demonstrated similar end tidal CO concentrations between these groups, so we were not concerned that pooling of nursing and bottle-fed infants would lead to any discrepancy in the interpretation of the results.

Unfortunately, neither of the components of the production/conjugation index are readily available for routine clinical use. The index does, however, shed light on the mechanism of neonatal jaundice and confirms the multifactorial cause of the condition. When evaluating a newborn with jaundice, both bilirubin production and elimination should be investigated. Although physiologic jaundice is usually a benign condition, because of infrequent cases in which severe hyperbilirubinemia may develop, the course of physiologic jaundice can not be regarded as

benign until it stabilizes. Additional studies will address the relationship between bilirubin production and conjugation in high-risk situations, such as G-6-PD deficiency and ABO blood group heterospecificity. In an era when we are witnessing a possible resurgence of kernicterus,³⁷ neonatal jaundice should be of concern to pediatricians and parents alike.

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