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The Lab's Role in Diagnosis of Neonatal Hyperbilirubinemia

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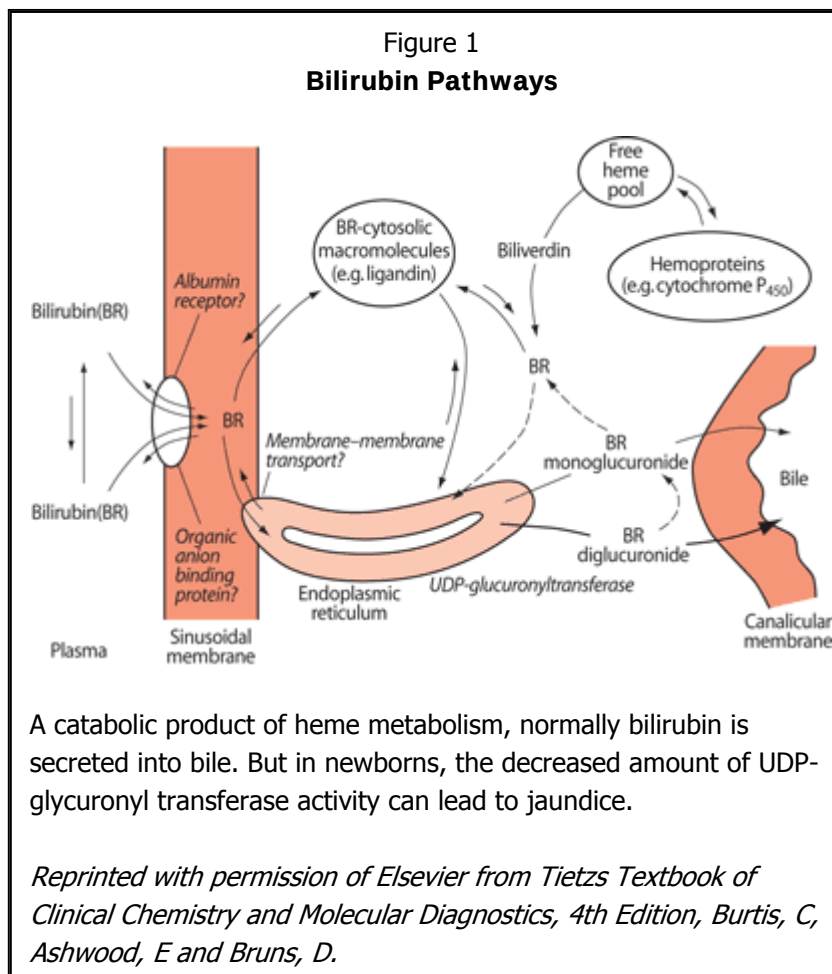
Hyperbilirubinemia is one of the most common problems encountered in term newborns. Neonatal jaundice typically occurs as a part of normal newborn physiology 3–5 days after birth and is characterized by yellow-pigmented skin and increased bilirubin concentrations in blood. Normally, this final product of heme degradation binds to serum albumin, is transported to the liver where it is conjugated with glucuronic acid, and eventually gets secreted in bile. Due to the relative lack of glucuronyl transferase activity in the first days after birth, however, bilirubin concentrations increase in most newborns. While most suffer no ill effects, if bilirubin levels do not normalize, infants can progress to severe bilirubin encephalopathy and kernicterus, a form of brain damage due to deposition of bilirubin in the basal ganglia and brainstem nuclei. Infants who survive may develop movement disorders, gaze abnormalities, and auditory abnormalities.

Because the effects of bilirubin toxicity are often devastating and irreversible, determining total bilirubin concentrations in newborns is essential for appropriate diagnosis and management of hyperbilirubinemia. Laboratory testing therefore plays an important role in management of affected infants. This article reviews bilirubin metabolism and describes lab methods for measuring different bilirubin fractions in serum.

Bilirubin Metabolism

A catabolic product of heme metabolism, up to 85% of bilirubin is derived from hemoglobin released

from senescent red blood cells, while a minor amount comes from ineffective development of red blood cells and other hemoproteins, such as myoglobin, cytochromes and peroxidases. Within the reticuloendothelial system, hemoxygenase catalyzes the degradation of heme, producing biliverdin and carbon monoxide. Reduction of the green-colored biliverdin by biliverdin reductase forms bilirubin, an orange-yellow pigment (Figure 1).



Unconjugated bilirubin is insoluble in water because at physiologic pH the highly polar carboxyl groups of propionic acid are hydrogen-bonded internally to the nitrogen atoms of the pyrrole rings. The six internal hydrogen bonds stabilize the Z-Z structure of unconjugated bilirubin and prevent its interaction with water. The other structural isomers, E-Z-bilirubin and Z-E-bilirubin formed by rupture of three hydrogen bonds, occur from rotation around the double bonds between the pyrrole rings of bilirubin. These more open structures are water soluble, allowing the molecules to be removed from circulation.

Unconjugated bilirubin is transported from the reticuloendothelial system to the surface of the liver. A specific carrier protein transports the conjugated molecule across the sinusoidal membrane where it binds to S-glutathione transferase B. It then travels to the microsomes where its internal hydrogen bonds are disrupted by conjugation with glucuronic acid. **Bilirubin uridine diphosphate glucuronyl transferase (UDPGT) catalyzes the covalent bonding of glucuronic acid and bilirubin and produces water-soluble bilirubin mono- and diglucuronide. It is this enzyme, which can be induced by phenobarbital, that is inactive at birth and requires a few days to be induced.**

The majority of conjugated bilirubin exists in the diconjugated form. Once conjugated, bilirubin is secreted into bile in a space located between liver cells, the bile canaliculus. From the canaliculi, the conjugated bilirubin travels to the common bile duct and combines with bile released from the gall bladder. A pump transporter and carrier proteins deliver the bile containing the conjugated bilirubin to the small and large intestines. Anaerobic microbes found in the intestine convert bilirubin into urobilinogens, which are then spontaneously oxidized to urobilins and excreted in the feces.

Normally, fetuses do not produce conjugated bilirubin. Any that is formed, however, is cleared by the placenta or excreted into the amniotic fluid. In fact, conjugated bilirubin in amniotic fluid typically signals a hemolytic condition. At birth, due to removal of the placenta, nearly all of the bilirubin in a newborn's blood is in the unconjugated form. Total bilirubin measurements in newborns therefore represent unconjugated bilirubin concentrations. Within 24–48 hours, however, monoconjugated bilirubin appears in the bile followed by the diconjugated form. As mentioned previously, lack of a fully functional conjugation system in newborns ultimately results in increased bilirubin concentrations, commonly observed as physiologic jaundice.

Development of Methods for Measuring Bilirubin

In 1916, van den Bergh and Muller were the first to use the diazo reaction to determine total bilirubin concentrations in serum. They discovered that diazotized sulfanilic acid added to serum from hepatitis patients yielded a red color immediately, but it produced little or no color when added to serum from jaundiced neonates. When alcohol, an accelerator or promoter of the diazo coupling reaction, was added to the jaundiced neonate serum, however, color development occurred more quickly. The researchers called the bilirubin present in adult sera "direct" for direct-reacting and that in neonatal sera "indirect" because it required an accelerator. Subsequently, alcohol was replaced by more efficient accelerators, such as carreine-benzoate, dyphilline-benzoate, and a variety of surface active agents, which presumably disrupt the internal hydrogen bonds of unconjugated bilirubin, thereby increasing its solubility in water and therefore its reactivity. The coupling reaction produces two isomeric azopigments, whose color, red at pH 2–7 and blue at alkaline pH, is measured spectrophotometrically.

Since then, researchers have developed many variations of the diazo method. The most extensively studied is the Doumas modification of the Jendrassik-Grof method. In this method, caffeine and sodium benzoate accelerate the reaction between the diazo reagent and unconjugated bilirubin. Researchers have postulated that caffeine, and perhaps benzoate, displace the unconjugated bilirubin from its albumin carrier and keep it in solution by hydrogen bonding. Because of its excellent precision and accuracy, the Clinical and Laboratory Standards Institute has deemed this method the reference method for measuring total bilirubin in serum (1). Clinical laboratories have adopted a variety of modified versions of this method, and manufacturers of clinical analyzers also use the reference method for establishing traceability to field bilirubin methods used in clinical labs.

Laboratory Methods for Measuring Bilirubin

Today, clinical laboratory methods measure total bilirubin and its various forms: unconjugated bilirubin (α); conjugated bilirubin (monoglucuronide or β -bilirubin and diglucuronide or γ -bilirubin); and delta-bilirubin (δ) (Table 1), which is formed by spontaneous reaction, in vivo and in vitro, between mono- or diglucuronide and serum albumin. The reaction involves the carboxyl group of propionic acid and the ϵ -amino group of a lysine moiety forming an amide covalent bond. δ -Bilirubin has a half-life of

approximately 19 days, consistent with that of albumin, and therefore remains in the blood long after the resolution of a hepatic obstruction or other inflammatory events.

Table 1				
Bilirubin Fractions Measured by Different Methods				
HPLC Peak	α	β	γ	δ
Bilirubin Fraction	Unconjugated	Monoglucuronide	Diglucuronide	Delta bilirubin
Traditional Diazo Methods	Total			
	Indirect (Total—Direct)	?	Direct	
Vitros Methods	Total			
	Unconjugated (Bu)	Conjugated (Bc)		Delta (Total—[Bu+Bc])
	Neonatal (Bu+Bc)			
	Direct (Total—Bu)			

Note: The reactivity of traditional diazo methods for direct bilirubin varies with each traditional diazo method (?).
Used with permission of Phil Bach, Primary Children's Medical Center, Salt Lake City, Utah.

Understanding the chemistry of the assay method is helpful for understanding the various forms of bilirubin being measured. When the accelerator is left out of the direct reaction, the mono- and diglucuronide (β - and γ -forms) and δ -bilirubin react more quickly, or directly, with the diazo reagent due to their solubility in water. The fraction of bilirubin that does not react with the diazo reagent in the direct reaction is primarily unconjugated bilirubin, commonly called "indirect bilirubin". It is important to note that **because both the indirect and direct fractions are in solution, the assay method must minimize the reactivity of the reagents with the indirect fraction or the results will be falsely elevated.** The difference between the total bilirubin and direct bilirubin values is known as indirect bilirubin or unconjugated bilirubin.

It is also important for laboratorians to understand that direct-reacting bilirubin, which is defined by the reactivity without an accelerator in the reaction, is not the same as conjugated bilirubin, which represents the sum of the two glucuronide forms. Direct bilirubin is the sum of the two glucuronides plus a variable amount of δ -bilirubin. **Laboratorians often use the terms indirect bilirubin and direct-reacting bilirubin interchangeably with unconjugated and conjugated bilirubin, respectively, but this is not correct and can lead to confusion.**

Most manufacturers' automated assays determine concentrations of total, direct-reacting, and indirect bilirubin, which is a calculated value. **Only Ortho Clinical Diagnostics has methods on its Vitros analyzers that provide total, unconjugated, and conjugated bilirubin results.** Understanding what the different assays measure is critical for clear communication with clinicians. For example, the **TBIL slide on the Vitros instruments measure all fractions of bilirubin by a diazo method, while the BuBc slide measures unconjugated (α -bilirubin or Bu) and the sum of monoglucuronide and diglucuronide bilirubin (β -bilirubin and γ -bilirubin, respectively, or Bc) by direct spectrophotometry. The Vitros neonatal method (NBIL or BuBc slide) is the sum of Bu and Bc; it does not include δ -bilirubin.**

To calibrate total and direct bilirubin methods, some instrument manufacturers use bovine serum, instead of human serum, enriched with unconjugated bilirubin and ditaurobilirubin. With the reference method and diazo methods on clinical analyzers, unconjugated bilirubin in human serum reacts completely; however, its reaction in bovine serum is incomplete and unpredictable (2). Therefore, it is virtually impossible to assign accurate bilirubin values to calibrators using bovine serum as a protein base. In human serum, researchers reported that ditaurobilirubin was underestimated with two out of

seven clinical analyzers tested, and in bovine serum with all analyzers, including the reference method. It is important to note that the calibrators of the two analyzers were made in bovine serum.

More Analytic Methods for Bilirubin

Like the direct spectrophotometric methods, blood-gas analyzers measure total bilirubin with an oximeter. An oximeter is essentially a spectrophotometer that uses various wavelengths for measuring different hemoglobin species and bilirubin. By comparing the sample spectrum to stored reference spectra, oximeters can determine total bilirubin concentrations in newborns.

Bilirubin levels also can be determined by high performance liquid chromatography (HPLC), but this method is used primarily by research labs. The origin of the terminology, α -, β -, γ -, and δ -bilirubin fractions stems from the elution times on a normal-phase column. For clinical analysis, however, HPLC is too laborious and increases turnaround time of bilirubin results. In addition, the method requires special technical expertise to set up and maintain.

Noninvasive Methods

Noninvasive methods are also available for measuring bilirubin in newborns. The transcutaneous method uses reflectance densitometry and is particularly attractive because it eliminates the trauma of a heel stick for the newborn, is suitable for point-of-care testing, and produces an instantaneous result without the risk of skin injury and infection for the newborn. The method also eliminates sharps-related injuries for staff who perform heel sticks. Consequently, such instruments are becoming more prevalent in newborn nurseries and physician office settings.

For the most part, bilirubin results from either transcutaneous devices or clinical lab instruments show good correlation up to 15 mg/dL bilirubin (3). At higher concentrations, a confirmatory measurement such as a serum total bilirubin is usually necessary. Transcutaneous measurements, however, are not interchangeable with serum total bilirubin determinations. They generally fall within 2–3 mg/dL of the serum bilirubin measurement. At this time, the effect of infants' inherent skin pigmentation on transcutaneous bilirubin measurements has not been well defined, and further studies are needed to determine its potential role in screening and managing infants with hyperbilirubinemia.

Clinical Indications of Hyperbilirubinemia

A few days after birth, newborns normally excrete bilirubin in bile, but elevated serum levels may indicate certain diseases. Too much bilirubin in blood may mean that too much is being produced, usually due to increased hemolysis, or that the liver is incapable of adequately removing bilirubin in a timely manner due to blockage of bile ducts or inherited problems with bilirubin metabolism, conjugation, and elimination.

Inherited Hyperbilirubinemia. Increased serum bilirubin concentrations may develop as a result of three causes: hemolytic disorders; inherited disorders of bilirubin metabolism; or jaundice of the newborn. In newborns, hemolytic disorders arise from incompatibility with the mother's Rhesus blood factor or ABO blood group, as well as glucose-6-phosphate dehydrogenase (G6PD) deficiency.

Inherited disorders resulting in increased unconjugated bilirubin levels include Gilbert's Syndrome and

Crigler-Najjar Syndrome. The former, a benign condition that affects about 4% of the population, is characterized by unconjugated bilirubin concentrations <3 mg/dL. Hepatic UDPGT, the enzyme that conjugates glucuronic acid to bilirubin, has decreased activity in these patients.

Unconjugated Hyperbilirubinemia of the Neonate. As described in the introduction, healthy, full-term newborns frequently have higher than normal unconjugated bilirubin concentrations (>5 mg/dL). Such increased levels cause a physiological jaundice of the skin. Unconjugated bilirubin accumulates in newborns for several reasons: 1) the conjugation system for the removal of bilirubin has yet to mature; 2) newborns' red blood cells have a decreased lifespan, which increases the bilirubin pool; 3) absorption of bilirubin is increased in the intestine due to β -glucuronidase, which hydrolyzes conjugated bilirubin into the unconjugated form and is passively reabsorbed into the bloodstream; and 4) breast milk provides a source of β -glucuronidase, as well as other inhibitors of conjugation, including pregnanediol and nonesterified fatty acids.

Conjugated Hyperbilirubinemia of the Neonate. Unlike hyperbilirubinemia caused by high levels of unconjugated bilirubin, which is common and benign in neonates, elevated levels of conjugated bilirubin are typically associated with disease. These newborns require follow up testing within 24 hours for proper diagnosis.

Extrahepatic biliary atresia, a condition in which the bile ducts are damaged and unable to send bile containing conjugated bilirubin to the intestinal tract, is one of the most common diseases associated with increased conjugated bilirubin. The drainage problem can be surgically repaired using the Kasai procedure so that the bile drainage is sent directly to the bowel.

Diagnosis and Management of Unconjugated Hyperbilirubinemia

Today, visual estimation of bilirubin levels that assess the degree of jaundice are considered prone to error, especially in heavily pigmented skin. Clearly, lab measurements of total serum bilirubin concentrations are the only accurate measurement of hyperbilirubinemia and can properly guide clinicians' diagnosis of hyperbilirubinemia in newborns. However, improvement in the currently used lab methods is still needed (5).

In 2004, the American Academy of Pediatrics (AAP) published a clinical practice guideline to assist physicians with diagnosis of hyperbilirubinemia (6). The Canadian Paediatric Society published a similar guideline in 2007 (7). Among the many recommendations in the AAP guideline, the recommends total serum bilirubin or transcutaneous measurements on every infant suspected of jaundice within the first 24 hours after birth or at a minimum before the newborn is discharged from the hospital.

The guidelines also recommend that bilirubin levels be interpreted according to the hour-specific, percentile-based nomogram (6). The hour-specific risk can have significantly different interpretations. For example, a total serum bilirubin of 10 mg/dL at 12 hours falls into a high-risk category, while at 48 hours this level falls into the low-intermediate risk zone. It is important to understand that the nomogram does not represent the normal course of hyperbilirubinemia. It predicts newborns' risk for hyperbilirubinemia based on a predischarge total serum bilirubin measurement. (8)

Contrary to AAP's recommendations, the U.S. Preventative Services Task Force concluded that there is insufficient evidence to recommend screening infants for hyperbilirubinemia (9). According to the

authors of the AAP guidelines, their recommendations are based on the best available evidence, consensus of expert opinion, and support from several independent reviewers. The authors also acknowledge that questions regarding the effectiveness of the guidelines in preventing kernicterus, as well as their effectiveness, are unknown (10).

Nevertheless, follow up testing of infants discharged with higher-than-normal bilirubin levels routinely occurs. *The decision to retest newborns is a clinical judgment determined by the zone in which the total serum bilirubin falls, the age of the infant, other known risk factors, and the expected course of hyperbilirubinemia.* Risk factors fall into one of three categories: major; minor; or decreased (Table 2).

Table 2 Risk Factors for Hyperbilirubinemia in Newborns		
Major Risk	Minor Risk	Decreased Risk
predischage bilirubin levels in the high-risk zone	predischage bilirubin levels in the high intermediate-risk zone	bilirubin levels in the low-risk zone
jaundice observed within 24 hours of birth	jaundice observed before discharge	
blood group or Rh factor incompatibility and other known hemolytic diseases		
gestational age of 35–36 weeks	gestational age 37–38 weeks	gestational age ≥ 41 weeks
phototherapy treatment of a sibling	previous sibling with jaundice	discharge from hospital after 72 hours
significant bruising		
exclusive breast feeding	maternal age ≥ 25 years of age	exclusive bottle feeding
East Asian race	male gender	black race
Source: AAP Guidelines, reference 6.		

Treatment of Hyperbilirubinemia

The most common treatment for acute hyperbilirubinemia is phototherapy. Based on bilirubin concentration and limited evidence, an age-specific nomogram in the AAP practice guidelines provides recommendations for intervention. Many different phototherapy units are available, as well as several different lamps emitting different wavelengths of light at different intensities. In general, light near the 450-nm wavelength is used. This wavelength of light disrupts the internal hydrogen bonding of unconjugated bilirubin and results in a number of different water-soluble isomers of bilirubin, allowing them to be cleared from circulation in the bile. Fortunately, phototherapy is considered a safe

procedure and will not only decrease bilirubin levels, but also prevent the newborn from undergoing an exchange blood transfusion. In rare cases, however, bilirubin levels are high enough to warrant this treatment.

The Laboratory and Hyperbilirubinemia

The role of the laboratory in the assessment of hyperbilirubinemia is to provide accurate bilirubin results. A number of methods are available on clinical analyzers, some of which need to be improved, as well as transcutaneous devices that are becoming more prevalent. More extensive studies on the correlation between transcutaneous devices and clinical laboratory analyzers need to be completed to determine the acceptability of the transcutaneous measurement within the context of screening for hyperbilirubinemia.

There are a number of causes for hyperbilirubinemia, both conjugated and unconjugated, including jaundice of the newborn and inherited disorders. In acute hyperbilirubinemia in term newborn infants, in which unconjugated bilirubin is the major fraction for consideration, the interpretation of bilirubin results is guided by a nomogram that uses the age of the infant in hours and a total serum bilirubin. Intervention also depends on an evaluation for major and minor risk factors and the normal course of hyperbilirubinemia. As laboratorians, we should be ready to assist clinicians in interpreting bilirubin results to help ensure that affected newborns receive appropriate treatment, thereby avoiding serious complications.

REFERENCES

1. Dumas BT, Kwok-Cheung PP, Perry BW, Jendrzczak, et al. Candidate reference method for determination of total bilirubin in serum: development and validation. *Clin Chem* 1985;31:1677–82.
2. Lo SF, Jendrzczak B, Dumas BT. Bovine serum–based bilirubin calibrators are inappropriate for some diazo methods. *Clin Chem* 2010 56: 869–872.
3. National Academy of Clinical Biochemistry. *Laboratory Medicine Practice Guidelines. Evidence-based practice for point-of-care testing*. AACC Press, 2007. Chapter 2. Transcutaneous bilirubin testing. Pages 5–12. <http://www.aacc.org/members/nacb/lmpg> (Accessed April, 2010).
4. Volpe, JJ. *Neurology of the newborn*, 4th ed, WB Saunders, Philadelphia, 2001, p. 521.
5. Lo SF, Dumas BT, Ashwood ER. Performance of bilirubin determinations in US laboratories—revisited. *Clin Chem* 2004;50:190–194.
6. American Academy of Pediatrics, Subcommittee on Hyperbilirubinemia. Clinical practice guideline: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics*. 2004;114:297–316.
7. Canadian Paediatric Society, Fetus and Newborn Committee. Guidelines for detection, management and prevention of hyperbilirubinemia in term and late preterm newborn infants (35 or more weeks' gestation). *Paediatr Child Health* 2007;12 (5):1B–12B.
8. Bhutani VK, Johnson L, Sivieri EM. Predictive ability of a predischARGE hour-specific serum bilirubin for subsequent significant hyperbilirubinemia in healthy term and near-term newborns. *Pediatrics*. 1999;103:6–14.
9. US Preventive Services Task Force. Screening of infants for hyperbilirubinemia to prevent chronic bilirubin encephalopathy: recommendation statement. *Pediatrics* 2009;124 (4):1172–1177.
10. Maisels MJ, Bhutani VK, Bogen D, Newman TB, et al. Management of hyperbilirubinemia in the newborn infant 35 weeks' gestation: an update with clarifications. *Pediatrics* 2009;124 (4):1193–1198.



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