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Bilirubin is the end product of degradation of the heme moiety of hemoproteins. Hemoglobin, derived from senescent erythrocytes, is the major source of bilirubin. Significant fractions are also derived from other hemoproteins of liver and other organs. Historically, hyperbilirubinemia has attracted the attention of clinicians as a marker of liver dysfunction. Subsequently, the studies of bilirubin chemistry, synthesis, transport, metabolism, distribution, and excretion have provided important insights into the transport, metabolism, and excretion of biologically important organic anions, particularly those with limited aqueous solubility.

Bilirubin is potentially toxic, but is normally rendered harmless by tight binding to albumin, and rapid detoxification and excretion by the liver. Patients with very high levels of unconjugated hyperbilirubinemia are at risk for bilirubin encephalopathy (kernicterus). Kernicterus is found in some cases of severe neonatal jaundice and in inherited disorders associated with severe unconjugated hyperbilirubinemia. This chapter provides a brief review of bilirubin metabolism and its inherited disorders.

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FORMATION OF BILIRUBIN

Sources of Bilirubin

The breakdown of hemoglobin, other hemoproteins, and free heme generates 250 to 400 mg of bilirubin daily in humans, approximately 80% of which is derived from the hemoglobin (1). Intravenously administered radiolabeled porphyrin precursors (glucose or δ-aminolevulinic acid) are incorporated into bile pigments in two peaks (2). The “early-labeled” peak appears within 72 hours. The initial component of this peak is derived mainly from hepatic hemoproteins such as cytochromes, catalase, peroxidase, and tryptophan pyrrolase, and a small, rapidly turning over pool of free heme. The slower phase of the early-labeled peak is derived from both erythroid and nonerythroid sources, and is enhanced in conditions associated with “ineffective erythropoiesis,” e.g., congenital dyserythropoietic anemias, megaloblastic anemias, iron-deficiency anemia, erythropoietic porphyria and lead poisoning (3), and in accelerated erythropoiesis (4). A “late-labeled” peak appears at approximately 110 days in humans and 50 days in rats, and represents the contribution from the hemoglobin of senescent erythrocytes.

Enzymatic Mechanism of Bilirubin Formation

Heme (ferroprotoporphyrin IX) (Fig. 20.1) is cleaved by selective oxidation of the α-methene bridge, catalyzed by microsomal heme oxygenase. This reaction requires three molecules of O₂ and a reducing agent, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), and results in the formation of the linear tetrapyrrole, biliverdin, and 1 mol of CO. The iron molecule is released (5). Three forms of heme oxygenase have been identified (6). Heme oxygenase 1 is ubiquitous and is a major inducible stress-related protein. Heme oxygenase 1 synthesis is upregulated by heme (7). In contrast, heme oxygenase 2 is a constitutive protein, expressed mainly in the brain and the testis. Heme oxygenase 3 has very low catalytic activity and may function mainly as a heme-binding protein. The products of heme oxygenase, biliverdin (which is subsequently reduced to bilirubin), and CO have significant physiologic effects. CO, a potent vasodilator, regulates the vascular tone in the liver and in other organs, such as the heart, under conditions of stress. Biliverdin and bilirubin are potent antioxidants, and may protect tissues under oxidative stress (6,8).

In most mammals, biliverdin is reduced to bilirubin by the action of biliverdin reductases. The physiologic advantage of this process is not clear, because bilirubin requires energy-consuming metabolic modification for excretion in bile. The stronger antioxidant activity of bilirubin may be particularly important during the neonatal period, when concentrations of other antioxidants are low in body fluids. Biliverdin reductases are cytosolic enzymes that require reduced nicotinamide adenine dinucleotide (NADH) or NADPH for activity (9).

Quantification of Bilirubin Production

At a steady-state condition of blood hemoglobin level, the rate of bilirubin production equals the rate of heme synthesis. Therefore, the heme synthesis rate can be estimated from the rate of bilirubin production. In humans, bilirubin production can be quantified from the turnover of intravenously administered radioisotopically labeled bilirubin. Plasma bilirubin clearance (the fraction of plasma from which bilirubin is irreversibly extracted) is proportional to the reciprocal of the area under the radiobilirubin disappearance curve (10). Bilirubin removal is calculated from the product of plasma bilirubin concentration and clearance. When plasma bilirubin concentrations remain constant, removal of bilirubin equals the amount of newly synthesized bilirubin entering the plasma pool. Alternatively, bilirubin formation can also be quantified from carbon monoxide production. Following rebreathing in a closed system, CO production is calculated from the CO concentration in the breathing chamber and/or the increment in blood carboxyhemoglobin saturation (11). CO production exceeds plasma bilirubin turnover by 12% to 18%, because a fraction of bilirubin produced in the liver is excreted into bile without appearing in serum. A small fraction of the CO may be formed by intestinal bacteria (12).
Inhibition of Bilirubin Production

Nonmetabolized “dead-end” inhibitors of heme oxygenase, such as tin-protoporphyrin or tin-mesoporphyrin, inhibit heme oxygenase activity (13). Injection of tin-mesoporphyrin in neonates reduces serum bilirubin levels by 76% (14).

CHEMISTRY OF BILIRUBIN

The systemic name of bilirubin IXα is 1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid (15, 16). The linear tetapyrrole structure of bilirubin was solved by Fischer and Plieninger (17). X-ray diffraction studies of crystalline bilirubin have revealed that the propionic acid side chains of bilirubin are internally hydrogen-bonded to the pyrrolic and lactam sites on the opposite half of the molecule (18). The molecule takes the form of a “ridge tile” in which the two dipyrrolic halves of the molecule lie in two different planes with an interplanar angle of 98 to 100 degrees (Fig. 20.2). The integrity of the hydrogen-bonded structure requires the interpyrrolic bridges at the 5 and 15 position of bilirubin to be in trans- or Z configuration. The hydrogen-bonded structure of bilirubin explains many of its physicochemical properties. As both the carboxylic groups, all four NH groups, and the two lactam oxygens are engaged by hydrogen bonding, bilirubin is insoluble in water. The hydrogen bonds “bury” the central methene bridge, so that the molecule reacts very slowly with diazo reagents. In vivo, the hydrogen bonds are disrupted by esterification of the propionic acid carboxyl group with glu- curonic acid (see below). Because of this disruption, conjugated bilirubin reacts rapidly with diazo reagents (“direct” van den Bergh reaction). Addition of methanol, ethanol, 6 M urea, or dimethyl sulfoxide to plasma disrupts the hydro- gen bonds of bilirubin, rendering the molecule water soluble and making the central methene bridge readily accessible, so that both conjugated and unconjugated bilirubin react rapidly with diazo reagents (“total” van den Bergh reaction) (19).

Absorption Spectra and Fluorescence

The main absorption band of unconjugated bilirubin IXα is at 450 to 474 nm in most organic solvents. Although pure bilirubin does not fluoresce, when dissolved in deter- gents, albumin solution, or alkaline methanol, an intense fluorescence is observed at 510 to 530 nm. Fluorescence determination has been utilized for rapid quantification of blood bilirubin concentrations and the unsaturated bilirubin-binding capacity of albumin (see below).

Effect of Light

The “Z” (trans) configuration of the 5 and/or 15 carbon bridges of bilirubin is changed to the “E” (cis) configuration upon exposure to light. The resulting ZE, EZ, or EE isomers lack internal hydrogen bonds, are more polar than bilirubin IXα-ZZ, and can be excreted in bile without conju- gation (20). The vinyl substituent in the endovinyl half of bilirubin IXα-EZ is slowly cyclized with the methyl sub- stituent on the internal pyrrole ring, forming the stable structural isomer, E-cyclobilirubin, which is quantitatively important during phototherapy for neonatal jaundice (21). In the presence of light and oxygen, a fraction of the biliru- bin molecules is also converted to colorless fragments (22). A small amount of biliverdin is also formed (22).

QUANTIFICATION OF BILIRUBIN IN BODY FLUIDS

Bile pigments are quantified as native or derivatized tetrapyroles, or after conversion to azodervatives. Total bilirubin can also be quantified indirectly by quantification of the intensity of yellow discoloration of the skin. Conversion to azodipyroles by reaction with diazo reagents is used commonly for determination of serum bilirubin levels for clinical purposes. Electrophilic attack on the central bridge splits bilirubin into two diazotized azodipyrole molecules.
As discussed above, conjugated bilirubin reacts rapidly in this system (direct fraction). In the presence of accelerators, both unconjugated and conjugated bilirubin react rapidly (total bilirubin). Unconjugated bilirubin (the “indirect” fraction) is calculated by subtracting the direct fraction from total bilirubin. As 10% to 15% of unconjugated bilirubin may give the direct diazo reaction, this method slightly overestimates conjugated bilirubin. The irreversibly albumin-bound fraction of serum bilirubin, which is formed in the serum of patients with prolonged conjugated hyperbilirubinemia, also exhibits direct diazo reaction (23). For more accurate quantification and for separating the different sugar conjugates, the intact bilirubin tetrapyrroles can be separated by thin-layer or high-performance liquid chromatography (24–26). Bilirubin mono- and diconjugates can be converted to methyl esters by alkaline methanolysis prior to separation (27), but because the sugar groups are cleaved off, this method does not permit identification of specific conjugates.

For repeated bilirubin measurements, particularly in jaundiced infants, special clinical methods have been devised. Measurement of the yellow color of the skin by analysis of reflected light provides a noninvasive approach for estimating serum bilirubin (28), which has been verified in a large study (29). Two slide tests are available for determination of total bilirubin, and the unconjugated, conjugated, and irreversibly protein-bound fractions. Fluorescence characteristics of bilirubin have been utilized for determining total bilirubin, albumin-bound bilirubin, and reserve bilirubin-binding capacity from as little as 0.1 mL of whole blood (30).

About 4% of bilirubin in normal plasma is conjugated, but the clinical diazo-based methods overexpress this fraction (see above). In hemolytic jaundice, there is a proportionate increase of plasma unconjugated and conjugated bilirubin. In contrast, in inherited disorders of bilirubin conjugation, the conjugated bilirubin is absent or reduced in proportion. In biliary obstruction or hepatocellular diseases, both conjugated and unconjugated bilirubin accumulate in plasma. Bilirubin is present in exudates and other albumin containing body fluids and binds to the elastic tissue of skin and sclera. Heme in subcutaneous hematomas is sequentially converted to biliverdin and bilirubin, resulting in a transition from green to yellow discoloration. Because of tight binding to albumin, unconjugated bilirubin is not excreted in urine in the absence of albuminuria, but conjugated bilirubin, which is less strongly bound to albumin, appears in urine. Bilirubin is present in normal human bile predominantly as diglucuronide, with unconjugated bilirubin accounting for only 1% to 4% of the pigments (see below).

TOXICITY OF BILIRUBIN

The toxic effect of bilirubin on the brain of neonates has been known since antiquity. Yellow discoloration of basal ganglia is termed kernicterus. Bilirubin exhibits a wide range of toxic effects in cell culture systems and in cell homogenates. Bilirubin inhibits DNA synthesis in a mouse neuroblastoma cell line (31), and uncouples oxidative phosphorylation and inhibits adenosine triphosphatase (ATPase) activity of brain mitochondria (32). In mutant rats (Gunn strain) with congenital nonhemolytic hyperbilirubinemia (see below), bilirubin inhibited RNA and protein synthesis, and carbohydrate metabolism in brain (33). In a cell-free system, bilirubin inhibited Ca2+-activated, phospholipid-dependent, protein kinase (protein kinase C) activity and 3',5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase activity (34), which may be relevant in the mechanism of its toxicity. Albumin binding inhibits toxic effects of bilirubin, both in vitro and in vivo.

At serum unconjugated bilirubin concentrations over 20 mg/dL, newborn babies are at risk of kernicterus. However, kernicterus can occur at lower concentrations (35). Serum albumin concentrations, pH, and substances that compete for albumin binding are important in the genesis of bilirubin encephalopathy (36). The immaturity of the blood–brain barrier in neonates is often held responsible for increased susceptibility of neonates to kernicterus. Tight junctions between capillary endothelial cells and foot processes of astroglial cells that restrict the exchange of water-soluble substances and proteins between blood and brain are the anatomic constituents of the blood–brain barrier (37). In addition, specific transport processes for ions, water, and nutrients from plasma to brain may provide a functional blood–brain barrier. However, there is little evidence to support the concept of immaturity of the blood–brain barrier in the neonate. The opening of the blood–brain barrier is expected to permit the entrance of albumin-bound bilirubin into the brain, which should not result in increased bilirubin toxicity. The entry of the non–albumin-bound (free) fraction of bilirubin into the brain is independent of the intactness of the blood–brain barrier. Damaged and edematous brain may bind bilirubin avidly, and therefore be unable to clear it rapidly, increasing the susceptibility to bilirubin encephalopathy (38).

DISPOSITION OF BILIRUBIN

Hepatocellular disposition of bilirubin requires several specific physiologic mechanisms, including transport to the hepatocytes from the major sites of production, efficient internalization into the hepatocyte, enzyme-catalyzed conjugation with glucuronic acid, active transport into the bile canalculus, and degradation in the intestinal tract. These steps are summarized in Fig. 20.3, and briefly discussed below.

Bilirubin Transport in Plasma

Bilirubin is tightly but reversibly bound to plasma albumin. Albumin binding keeps bilirubin in solution and transports the pigment to the liver. Unconjugated bilirubin is bound
tightly to albumin and, therefore, is not excreted in urine, except during albuminuria. Conjugated bilirubin is bound less tightly to albumin, and the unbound fraction is excreted in the urine. During prolonged conjugated hyperbilirubinemia, a fraction of the pigment becomes irreversibly bound to albumin. This fraction, termed delta-bilirubin, is not excreted in the bile or urine and disappears slowly, reflecting the long half-life of albumin (23).

Albumin binding protects against the toxic effects of bilirubin. A small unbound fraction of bilirubin is thought to be responsible for its toxicity (39). Normally, the molar concentration of albumin (500 to 700 μmol/L) exceeds that of bilirubin (3 to 17 μmol/L). However, during neonatal jaundice, in patients with Crigler–Najjar syndrome, the molar ratio of unconjugated bilirubin to albumin may exceed 1. Reduction of serum albumin levels during inflammatory states, chronic malnutrition, or liver diseases may accentuate bilirubin toxicity. Sulfonamides, antiinflammatory drugs, and cholecytographic contrast media displace bilirubin competitively from albumin and increase the risk of kernicterus in jaundiced infants (40). Binding of short chain fatty acids to albumin causes conformational changes, decreasing bilirubin binding.

Because of the pathophysiologic importance of the unbound fraction of unconjugated bilirubin, ultrafiltration, ultracentrifugation, gel chromatography, affinity chromatography on albumin agarose polymers, dialysis, and electrophoresis have been used to separate free from bound bilirubin. Unbound bilirubin is rapidly destroyed by treatment with H₂O₂ and horseradish peroxidase, as compared with bound bilirubin. Binding of bilirubin to albumin induces bilirubin fluorescence, and quenches the protein fluorescence. This phenomenon has been utilized for differentiating free from albumin-bound bilirubin.

**FIGURE 20.3.** Summary of hepatic metabolism of bilirubin. Bilirubin is strongly bound to albumin in the circulation (1). At the sinusoidal surface of the hepatocyte, this complex dissociates, and bilirubin enters hepatocytes by facilitated diffusion (2). This process is non-adenosine triphosphate (ATP)-dependent and bidirectional. Within the hepatocyte, bilirubin binds to a group of cytosolic proteins, mainly to glutathione-S-transferases (GSTs) (3). GST binding inhibits the efflux of bilirubin from the cell, thereby increasing the net uptake. A specific form of uridine diphosphoglucuronate glucuronyltransferase (UGT) (BUGT, also termed UGT1A1), located in the endoplasmic reticulum, catalyzes the transfer of the glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to bilirubin, forming bilirubin diglucuronide and monoglucuronide (4). Glucuronidation is necessary for efficient excretion of bilirubin in bile (5). Canalicular excretion of bilirubin and other organic anions (except most bile acids) is primarily an energy-dependent process, mediated by the ATP-utilizing transporter multidrug resistance-related protein (MRP2), also termed canalicular multispecific organic anion transporter (cMOAT).

**Bilirubin Uptake by the Hepatocytes**

At the sinusoidal surface of the hepatocyte (Fig. 20.3), bilirubin dissociates from albumin and is taken up by the hepatocyte by facilitated diffusion. The transport requires the presence of inorganic anions, such as Cl⁻. A family of organic anion transport proteins (oatp) has been identified. One oatp isoform, oatp-1, mediates Na⁺-independent taurocholate transport and is associated with HCO₃⁻ exchange (41). However, the role of oatp family of proteins in bilirubin transport has not been directly established (see Chapter 7).

**Hepatocellular Storage of Bilirubin**

Within the hepatocyte, bilirubin is kept in solution by binding to cytosolic proteins, which were originally designated Y and Z. The Y group of proteins, which constitute 5% of the
liver cytosol, binds various drugs, hormones, organic anions, a cortisol metabolite, and azo-dye carcinogens, and was termed "ligandin" (42). Subsequently, ligandin was found to be a family of proteins, identical to the α class of glutathione-S-transferases (GST) in the rat liver (43). There are corresponding proteins in human hepatocytes as well. Binding to GSTs increases the net uptake of bilirubin by reducing efflux from the hepatocyte (Fig. 20.3). GST binding may inhibit the toxicity of bilirubin by preventing its nonspecific diffusion into specific subcellular compartments. For example, binding to GSTs prevents the inhibition of mitochondrial respiration by bilirubin in vitro (44).

### Conjugation of Bilirubin

Conversion to bilirubin diglucuronide or monoglucuronide by esterification of both or one of the propionic acid carboxyl groups is critical for efficient excretion of bilirubin across the bile canalculus (Fig. 20.3). Bilirubin diglucuronide accounts for about 80% of pigments excreted in normal bile (24–26). Bilirubin monoglucuronide constitutes about 10% of the pigments, but in states of partial deficiency of hepatic bilirubin glucuronidation the proportion of bilirubin monoglucuronide increases (see Crigler–Najjar Syndrome Type 2 and Gilbert Syndrome, below). Smaller amounts of glucosyl and xylosyl conjugates are also found.

**Bilirubin-Uridine Diphosphoglucuronate Glucuronosyltransferase**

Glucuronidation of bilirubin is catalyzed by a specific isoform of uridine diphosphoglucuronate glucuronosyltransferase (UGT). UGTs comprise a family of enzymes that are integral components of the endoplasmic reticulum of various cell types (45). UGTs mediate the conversion of a wide variety of xenogenous and endogenous toxic metabolites to less bioreactive, polar compounds that are readily eliminated in bile or urine. Based on the degree of homology of the messenger RNA (mRNA) sequences, UGTs have been categorized into several families and subfamilies (46). Only one of these UGT isoforms, currently termed UGT1A1, contributes significantly to bilirubin glucuronidation (47). The gene that expresses bilirubin-UGT (UGT1A1) is termed UGT1A (48). The UGT1A locus contains four consecutive exons (exons 2 to 5) at the 3′ end that are used in all mRNAs expressed from this locus, and encode the identical upidine diphospho (UDP)-glucuronic acid-binding carboxy-terminal domain of the UGT isoforms (Fig. 20.4). Upstream to these four common-region exons is a series of unique exons, each preceded by a separate promoter, only one of which is utilized in specific UGT mRNAs. The unique exon encodes the variable aglycone-binding N-terminal domain of individual UGT isoforms. Depending on which promoter is used, transcripts of various lengths are generated. The unique exon, located at the 5′ end of the transcript, is spliced to exon 2, and the intervening sequence is spliced out. Within the UGT1A locus, genes encoding individual isoforms are named after the unique exon that is utilized in the specific mRNA. Bilirubin-UGT mRNA, which consists of the first unique region exon of the UGT1A locus (plus exons 2 to 5), is named UGT1A1 according to this terminology.

The presence of a separate promoter upstream to each unique region exon (Fig. 20.4) permits differential expression of individual UGT isoforms during development (49) and enzyme induction (50). UGT1A1 develops after birth (49) and is induced by phenobarbital and clofibrate (51). Treatment of rats with triiodothyronine markedly reduces UGT activity toward bilirubin, whereas the activity toward 4-nitrophenol is increased (50).

### Canalicular Excretion of Conjugated Bilirubin (See Chapters 24, 25, and 26)

Conjugated bilirubin is excreted across the bile canalculus against a concentration gradient, which can be as high as 150-fold. The energy for the uphill transport of bilirubin is provided by an adenosine triphosphate (ATP)-dependent system in the canalicular membranes that is specific for non–bile-acid organic anions, including bilirubin and other glucuronides, and glutathione conjugates (52,53). A canalicular membrane protein, termed canalicular multispecific organic anion transporter (cMOAT) or multidrug resistance-related protein (MRP2) (54), mediates the ATP-dependent canalicular organic anion transport.

### Fate of Bilirubin in the Gastrointestinal Tract

Conjugated bilirubin is not substantially absorbed from the gastrointestinal tract. When there is enhanced excretion of unconjugated bilirubin into the intestine, e.g., during phototherapy for neonatal jaundice or Crigler–Najjar syndrome, absorption of unconjugated bilirubin from the intestine may be clinically significant (55). Milk inhibits intestinal absorption of unconjugated bilirubin, but such inhibition is less with human milk than with infant milk formula. Intestinal bacteria degrade bilirubin into a series of urobilinogen and related products (56). Most of the urobilinogen reabsorbed from the intestine is excreted in bile, but a small fraction is excreted in urine. Absence of urobilinogen in stool and urine indicates complete obstruction of the bile duct. In liver disease and states of increased bilirubin production, urinary urobilinogen excretion is increased. Urobilinogen is colorless; its oxidation product, urobilin, contributes to the color of normal urine and stool.
Alternative Routes of Bilirubin Elimination

In the absence of bilirubin glucuronidation, a small fraction of bilirubin is excreted as hydroxylated products. Induction of a specific isoform of microsomal P-450s by administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in UGT1A1-deficient Gunn rats resulted in a sevenfold increase in the fractional turnover of bilirubin and reduction of the bilirubin pool (57). A mitochondrial bilirubin oxidase in liver (58) and other tissues may catalyze oxidative degradation of bilirubin.

During intrahepatic or extrahepatic cholestasis, the plasma conjugated bilirubin concentrations increase. In total biliary obstruction, renal excretion becomes the major pathway of bilirubin excretion. Renal excretion of conjugated bilirubin depends on glomerular filtration of the non–protein-bound fraction of conjugated bilirubin.

Antioxidant Property of Bilirubin

Although bilirubin has been thought of conventionally as a waste product with little utility, antioxidant activity of bilirubin may serve an important tissue protective role. Both unconjugated (59) and conjugated (60) bilirubin inhibit lipid peroxidation. The tissue cytoprotective role of heme oxygenase in various tissues may be mediated by biliverdin and bilirubin.

DISORDERS OF BILIRUBIN METABOLISM RESULTING IN UNCONJUGATED HYPERBILIRUBINEMIA

Hepatic transport of bilirubin involves four distinct but probably interrelated stages: (a) uptake from the circula-
Neonatal Jaundice

By adult standards, every newborn baby has increased serum bilirubin levels, and about half of all neonates become clinically jaundiced during the first 5 days of life. Serum bilirubin is predominantly unconjugated. Exaggeration of this “physiologic jaundice” exposes the baby to the risk of kernicterus (see Toxicity of Bilirubin, above). In 16% of newborns, maximal serum bilirubin concentrations equal or exceed 10 mg/dL, and in 5% serum bilirubin levels are above 15 mg/dL. In the normal full-term neonate, serum bilirubin peaks at approximately 72 hours and subsequently declines to normal adult levels in 7 to 10 days. Physiologic jaundice of the newborn results from a combination of increased bilirubin production and immaturity of the bilirubin disposal mechanisms of the liver. Bilirubin production rate is high in newborns, because of the increased early-labeled peak from erythroid and nonerythroid sources, and decreased erythrocyte half-life (61). Net hepatic bilirubin uptake is low in neonates because of low hepatocellular ligandin levels (62). Delayed closure of the ductus venosus may permit the bilirubin-rich portal blood to bypass the liver. Low caloric intake may also reduce hepatic bilirubin clearance. UGT activity toward bilirubin is low in the liver of the newborn and takes about 10 days to mature to adult levels (63). Deficiency of UGT activity may be prolonged and exaggerated in some inherited disorders due to inhibitory factor(s) in maternal milk or serum (see Gilbert syndrome, below). A variant TATAA element within the promoter region of UGT1A1 has been found to be associated with Gilbert syndrome (64) (see Gilbert syndrome, below). Presence of this variant promoter reduces the expression of bilirubin-UGT (UGT1A1) and may accentuate and prolong neonatal jaundice (65).

Plasma bilirubin concentrations tend to be higher in breast-fed infants than in formula-fed babies. The hyperbilirubinemia resolves on discontinuation of breast-feeding, and kernicterus occurs only rarely (66). Maternal milk jaundice is associated with an inhibitor of UGT activity in maternal milk but not maternal serum (67). Free fatty acids resulting from the digestion of fat by lipase secreted in the milk of some women are thought to inhibit hepatic bilirubin-UGT activity (68). Intestinal absorption of unconjugated bilirubin is high in neonates. Bilirubin absorption may be higher in breast-fed infants than in formula-fed babies.

Inhibitory factors present in the plasma of some mothers may delay the maturation of bilirubin-UGT (69). Peak serum bilirubin concentrations of 8.9 to 65 mg/dL are reached within 7 days. This condition, termed Lucey-Driscoll syndrome, is distinguished from maternal milk jaundice by earlier onset of hyperbilirubinemia, a more severe and protracted course, and occasional kernicterus.

In the great majority of cases, neonatal hyperbilirubinemia is innocuous. But vigilance is needed for the occasional case in which severe neonatal jaundice can expose the newborn to the risk of kernicterus. Although plasma bilirubin levels of 20 mg/dL or higher are considered dangerous, bilirubin encephalopathy may occur at lower concentrations (see Toxicity of Bilirubin, above). Phototherapy is the most common treatment used. In severe cases exchange transfusion is employed to reduce serum bilirubin levels rapidly. Inhibition of heme oxygenase activity by the administration of tin-mesoporphyrin at birth has been shown to prevent the development of significant levels of neonatal jaundice, thereby abrogating the need for phototherapy or exchange transfusion.

Bilirubin Overproduction

Bilirubin overproduction results in unconjugated hyperbilirubinemia, which rarely exceeds 3 to 4 mg/dL in the absence of hepatobiliary dysfunction. Bilirubin overproduction occurs commonly in hemolytic conditions and during resolution of large hematomas. Ineffective erythropoiesis occurs in thalassemia, pernicious anemia, and some rare hereditary anemias, termed congenital dyserythropoietic anemias (70). In addition to unconjugated bilirubin, a small amount of conjugated bilirubin may accumulate in the serum (~4% of total bilirubin), probably because of diffusion out of the hepatocyte. This does not necessarily indicate that the rate of bilirubin production has exceeded the hepatic excretory transport maximum for conjugated bilirubin.

Crigler–Najjar Syndrome Type 1

Crigler–Najjar syndrome type I is a rare disorder, characterized by severe lifelong nonhemolytic unconjugated hyperbilirubinemia (71) (Table 20.1). Hepatic bilirubin-UGT activity is absent or nearly so. Without treatment, the majority of patients used to die of kernicterus during the first 18 months of life. Exceptional patients survived beyond puberty, but succumbed to bilirubin encephalopathy in young adult life (72,73). With the routine use of phototherapy and intermittent plasmapheresis during crises, survival until puberty is usual, but the risk of bilirubin encephalopathy increases at this age (74). Orthotopic or auxiliary liver transplantation cures the disease.
Laboratory test results in Crigler–Najjar syndrome type 1 are normal except for the serum bilirubin levels, which are usually 20 to 50 mg/dL (340–850 μM), but may increase during intercurrent illness to as high as 50 mg/dL (75). Serum bilirubin is unconjugated and there is no bilirubinuria. There is no evidence of hemolysis. As the canalicular excretion process is normal, oral cholecystography visualizes the gallbladder. There is an increased incidence of pigment gallstones, probably because of increased concentrations of unconjugated bilirubin in bile, resulting from phototherapy. Liver histology is normal except for the presence of “pigment plugs” in bile canaliculi.

As UGT1A1 (bilirubin-UGT1) is the only isoform that contributes significantly to bilirubin metabolism (47), genetic lesions within the coding region of the UGT1A1 gene can abolish hepatic bilirubin glucuronidation, causing Crigler–Najjar syndrome type 1. Since the initial description of the molecular basis of Crigler–Najjar syndrome in 1992 (48,76) numerous mutations, deletions, and insertions in any of the five exons of UGT1A1 have been shown to cause the disease (77–89) (Table 20.2). In addition to exonic lesions, mutations of the splice donor or splice acceptor sites within the intronic sequences can cause inappropriate splicing of the transcript, resulting in the loss of UGT1A1 activity. These molecular lesions have been reviewed recently (90). As in all rare recessively inherited disorders, known or unknown consanguinity is common, but not always found (91). Crigler–Najjar syndrome is found in all races. As many different mutations can cause the disease, no single mutation is common in any race. An exception to this is found among the Amish and Mennonite communities of Pennsylvania (92), where the disease is relatively common and all patients have the same mutation, reflecting a strong founder effect. In patients with genetic lesions within the unique exon 1, only UGT1A1 activity is abnormal, but when the mutation affects one of the common region exons (exons 2 to 5), all UGT1A group of isoforms are expected to be abnormal.

The differential diagnosis includes Crigler–Najjar syndrome type 2, with or without coexisting hemolysis. Although serum bilirubin levels are relatively lower in Crigler–Najjar syndrome type 2, the ranges overlap in the two disorders. In most cases of Crigler–Najjar syndrome type 2, the serum bilirubin concentrations are reduced by more than 25% after phenobarbital administration (60 to 120 mg for 14 days), which differentiates it from Crigler–Najjar syndrome type 1 (75). Chromatographic analysis of pigments in bile collected from the duodenum through a perorally placed duodenal catheter or an upper gastrointestinal endoscope provides rapid differentiation of the two conditions. In

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**TABLE 20.1. CHARACTERISTICS OF INHERITED UNCONJUGATED HYPERBILIRUBINEMIA**

<table>
<thead>
<tr>
<th></th>
<th>Crigler–Najjar Syndrome Type 1</th>
<th>Crigler–Najjar Syndrome Type 2</th>
<th>Gilbert Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver function tests other than serum bilirubin and liver histology</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Serum bilirubin concentrations</td>
<td>20–50 mg/dL (340–850 μM)</td>
<td>7–20 mg/dL (120–340 μM)</td>
<td>Reduced proportion of bilirubin glucuronides</td>
</tr>
<tr>
<td>Pigments excreted in bile</td>
<td>Small amounts of unconjugated bilirubin and only traces of bilirubin glucuronides</td>
<td>Reduced proportion of bilirubin diglucuronide</td>
<td>Reduced proportion of bilirubin diglucuronide</td>
</tr>
<tr>
<td>Hepatic bilirubin-UGT activity</td>
<td>Virtually absent</td>
<td>Markedly reduced but detectable</td>
<td>Reduced to about 30% of normal</td>
</tr>
<tr>
<td>Effect of phenobarbital administration</td>
<td>No significant reduction of serum bilirubin levels</td>
<td>Reduction of serum bilirubin levels by &gt;25%</td>
<td>Normalization of serum bilirubin</td>
</tr>
<tr>
<td>Inheritance</td>
<td>Autosomal recessive</td>
<td>Autosomal recessive</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>Molecular basis</td>
<td>Genetic lesions within the coding region or at splice sites of UGT1A1</td>
<td>Point mutations within the coding region of UGT1A1</td>
<td>Insertion of a TA dinucleotide within the TATAA element of UGT1A1a</td>
</tr>
<tr>
<td>Prevalence</td>
<td>Rare (&lt;1:1,000,000)</td>
<td>Rare (&lt;1:1,000,000)</td>
<td>Phenotype in ~4% of the population; among Caucasians and Africans, ~9% are homozygous for the genotype (less common in Japan)</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Kernicterus, unless vigorously treated; currently, liver transplantation is the only curative treatment</td>
<td>Kernicterus is uncommon, but has been reported</td>
<td>No encephalopathy; increased intensity of neonatal jaundice; toxicity of some drugs may be increased</td>
</tr>
<tr>
<td>Animal model</td>
<td>Gunn rat</td>
<td>—</td>
<td>Bolivian subpopulation of squirrel monkeys</td>
</tr>
</tbody>
</table>

aSome mutations of the coding region of UGT1A1 may be associated with serum bilirubin levels that overlap with the range seen in Gilbert syndrome (Table 20.3). UGT, uridine-diphosphoglucuronate glucuronosyl-transferase.
Crigler–Najjar syndrome type 1, bilirubin glucuronides are virtually absent in bile, whereas significant amounts of bilirubin conjugates are found in Crigler–Najjar syndrome type 2, although the proportion of bilirubin diglucuronide is reduced (see below). Liver biopsy is not needed for diagnosis, unless a coexisting liver disease is suspected. If a biopsy is performed, UGT activity toward bilirubin is virtually undetectable. The diagnosis can be made also by genetic analysis of DNA extracted from blood, buccal scrapings, or other tissue. The five exons and the flanking intronic sequences are amplified by polymerase chain reaction and the nucleotide sequences are determined (76). If a previously uncharacterized mutation is found, the mutation can be generated in an expression plasmid by site-directed mutagenesis, and its effect can be determined after transfection of the plasmid into COS cells (47). Genetic analysis permits identification of heterozygous carriers and prenatal diagnosis based on chorionic villus sampling or amniocentesis (93).

Animal Model

A mutant strain of Wistar rats, termed Gunn rats (94,95), manifests nonhemolytic unconjugated hyperbilirubinemia due to a lack of bilirubin-UGT activity. The jaundice is inherited as an autosomal trait. The Gunn rat is the only experimental animal that develops kernicterus spontaneously. Much of the knowledge of cellular and biochemical mechanisms of bilirubin encephalopathy has been gained from studies on Gunn rats. The molecular basis of UGT1A1 deficiency in this strain is the deletion of a guanosine base in the common region exon 4. Consequently, in addition to UGT1A1, other enzymes of the UGT1A group are also abnormal.

Treatment

Treatment is aimed at reduction of serum bilirubin levels. Because there is hardly any residual bilirubin-UGT activity, enzyme-inducing agents, such as phenobarbital, are ineffective in Crigler–Najjar syndrome type 1 (75). Phototherapy is the routine treatment. An array of 140-W fluorescent lamps is used for 8 to 12 hours a day with the eyes shielded. After puberty, phototherapy becomes less effective because of skin thickening, pigmentation, and decreased surface area in relation to body mass. Phototherapy converts bilirubin IXα-ZZ into geometric and structural isomers that are

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**TABLE 20.2. GENETIC LESIONS OF UGT1A1 THAT ABOLISH BILIRUBIN–UGT ACTIVITY (CRIGLER–NAJJAR SYNDROME TYPE 1)**

<table>
<thead>
<tr>
<th>Site of Lesion</th>
<th>Nucleic Acid Alteration</th>
<th>Predicted Mutation of UGT1A1</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>Del C,T at nt 120,121, respectively</td>
<td>Truncated (frameshift)</td>
<td>Inactive</td>
<td>79</td>
</tr>
<tr>
<td>Exon 1</td>
<td>Ins 4 bp after codon 80</td>
<td>Truncation (frameshift)</td>
<td>Inactive</td>
<td>80</td>
</tr>
<tr>
<td>Exon 1</td>
<td>Ins T after codon 158/del codon 170</td>
<td>Truncated (frameshift)/del of phenylalanine</td>
<td>Inactive/inactive</td>
<td>81</td>
</tr>
<tr>
<td>Exon 1</td>
<td>Del of codon 170</td>
<td>Del phenylalanine</td>
<td>Inactive</td>
<td>81–83</td>
</tr>
<tr>
<td>Exon 1/exon 2</td>
<td>T529C/del nt 879–892</td>
<td>C177Truncated (frameshift)</td>
<td>Inactive/inactive</td>
<td>83</td>
</tr>
<tr>
<td>Exon 1</td>
<td>G826T</td>
<td>G276R</td>
<td>Inactive</td>
<td>83</td>
</tr>
<tr>
<td>Exon 1/exon 3</td>
<td>A835T/C1069T</td>
<td>B279Y/Q357X</td>
<td>ND/inactive</td>
<td>76,83,84</td>
</tr>
<tr>
<td>Exon 1</td>
<td>C840A</td>
<td>C280X</td>
<td>Inactive</td>
<td>85</td>
</tr>
<tr>
<td>Intron 1</td>
<td>Splice donor, G→C</td>
<td>Truncated</td>
<td>Inactive</td>
<td>86</td>
</tr>
<tr>
<td>Exon 2</td>
<td>Skipping of exon 2</td>
<td>Truncated</td>
<td>Inactive</td>
<td>83</td>
</tr>
<tr>
<td>Exon 2/exon 4</td>
<td>C872T/A1282G</td>
<td>A291V/K426E</td>
<td>Inactive</td>
<td>84</td>
</tr>
<tr>
<td>Exon 2</td>
<td>T880A and del 881–893</td>
<td>Truncated (frameshift)</td>
<td>Inactive</td>
<td>78,83</td>
</tr>
<tr>
<td>Exon 2</td>
<td>G923A</td>
<td>G308E</td>
<td>Inactive</td>
<td>84,87</td>
</tr>
<tr>
<td>Exon 2</td>
<td>C991T</td>
<td>Q331X</td>
<td>Inactive</td>
<td>77</td>
</tr>
<tr>
<td>Exon 3/exon 4</td>
<td>G1005A/G1102A</td>
<td>W335X/A368T</td>
<td>Inactive</td>
<td>84</td>
</tr>
<tr>
<td>Exon 3</td>
<td>nt: C1006T</td>
<td>R336W/N</td>
<td>Inactive</td>
<td>88</td>
</tr>
<tr>
<td>Exon 3</td>
<td>C1021T</td>
<td>R341X</td>
<td>Inactive</td>
<td>89</td>
</tr>
<tr>
<td>Exon 3</td>
<td>C1069T</td>
<td>Q357X</td>
<td>Inactive</td>
<td>76,83,84</td>
</tr>
<tr>
<td>Exon 3/exon 4</td>
<td>C1069T/G1201C</td>
<td>Q357X/A401P</td>
<td>Inactive</td>
<td>76,83,84</td>
</tr>
<tr>
<td>Exon 3</td>
<td>A1070G</td>
<td>Q357R</td>
<td>Inactive</td>
<td>84</td>
</tr>
<tr>
<td>Exon 3/exon 4</td>
<td>C1081T/G1159,1160GT</td>
<td>Q361X/P387R</td>
<td>Inactive/inactive</td>
<td>79</td>
</tr>
<tr>
<td>Intron 3/exon 1</td>
<td>Splice acceptor site, A→G/nt C145T</td>
<td>Truncated protein/Q49X</td>
<td>Inactive</td>
<td>86</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C1124T</td>
<td>S376F</td>
<td>Inactive</td>
<td>77,83,87</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C1143G</td>
<td>S381R</td>
<td>Inactive</td>
<td>84</td>
</tr>
<tr>
<td>Exon 4</td>
<td>CC1159,1160GT</td>
<td>P387R</td>
<td>Inactive</td>
<td>79</td>
</tr>
<tr>
<td>Exon 4</td>
<td>G1201C</td>
<td>A401P</td>
<td>Inactive</td>
<td>84</td>
</tr>
<tr>
<td>Exon 4/exon 5</td>
<td>G1201C/A1308T</td>
<td>A401P/K437X</td>
<td>Inactive</td>
<td>84</td>
</tr>
</tbody>
</table>

bp, base pair; nt, nucleotide; del, deletion; ins, insertion; N, normal; ND, not determined. Note: A slash separating two mutations indicates that the patient was a compound heterozygote for two different mutations.
excreted in bile without conjugation (see above). A portion of the unconjugated bilirubin excreted in bile is reabsorbed in the small intestine. Oral administration of agar, cholestyramine, or calcium salts inhibits bilirubin reabsorption, thereby slightly enhancing the effect of phototherapy. Plasmapheresis can be used to reduce serum bilirubin concentrations rapidly during crisis, although the effect is short-lived (96). Orthotopic or auxiliary liver transplantation is the only curative therapy available at this time (97). Because of the associated risk of liver transplantation and the need for lifelong immunosuppression, alternative experimental therapies are being explored. Hepatocyte transplantation by infusion into the portal vein through a percutaneously placed portal venous catheter has reduced the serum bilirubin level significantly in one patient (98,99), but the optimum number of cells that should be transplanted for this disease is not known clearly. Immunosuppression is needed for prevention of allograft rejection. Gene therapy methods using recombinant retrovirus, adenovirus, and SV40, as well as nonviral vectors are being explored in studies on Gunn rats. Recently, site-directed gene repair has been used to reduce serum bilirubin levels in Gunn rats. These methods have been reviewed recently (100) and are also described in Chapter 62.

**Crigler–Najjar Syndrome Type 2 (Arias Syndrome)**

In this variant of Crigler–Najjar syndrome, serum bilirubin concentrations usually range from 7 to 20 mg/dL, the prognosis is much less severe, and serum bilirubin levels are usually reduced by over 25% after administration of bilirubin-UGT inducing agents, such as phenobarbital (101). Serum bilirubin levels may be as high as 40 mg/dL during fasting (102) or intercurrent illness (103). The bile contains significant amounts of bilirubin glucuronides (Table 20.1). Bilirubin encephalopathy is unusual, but has been reported (102,103). In normal bile, over 90% of the conjugated bilirubin is bilirubin diglucuronide. In Crigler–Najjar syndrome type 2, the major pigment is bilirubin monoglucuronide (103,104). The liver has markedly reduced bilirubin-UGT activity (103).

Crigler–Najjar syndrome type 2 occurs in families (101). There is no sex predilection. The inheritance is autosomal recessive. As in Crigler–Najjar syndrome type 1, the disease is caused by mutations of one of the five exons that encode bilirubin-UGT (UGT1A1) (91). However, in Crigler–Najjar syndrome type 2, the genetic lesions are always point mutations that result in the substitution of a single amino acid that markedly reduces, but does not abolish, bilirubin-UGT activity (Table 20.3 (105–115)). In Table 20.3, we have classified all published mutations of the UGT1A1 coding region that result in incomplete deficiency of bilirubin glucuronidating activity as in Crigler–Najjar syndrome type 2. Although in most of these cases serum bilirubin concentrations are clearly consistent with Crigler–Najjar syndrome type 2, some point mutations, described in Japanese individuals, cause serum bilirubin levels that overlap with those seen in Gilbert syndrome (see Gilbert syndrome, below). Based on the serum bilirubin levels, some of the latter cases have been reported in the literature by some Japanese investigators as Gilbert syndrome (106–109,115). Such semantic difficulty in correlating genetic diagnosis with the nomenclature that had been developed before the discovery of molecular

**TABLE 20.3. MUTATIONS WITHIN THE CODING REGION OF UGT1A1 THAT REDUCE, BUT DO NOT ABOLISH BILIRUBIN–UGT ACTIVITY**

<table>
<thead>
<tr>
<th>Site of Mutation</th>
<th>Nucleic Acid Mutation</th>
<th>Predicted Amino Acid Substitution</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>T44G</td>
<td>L15R</td>
<td>Reduced activity</td>
<td>105</td>
</tr>
<tr>
<td>Exon 1</td>
<td>G211A</td>
<td>G71R</td>
<td>Reduced activity</td>
<td>106–108</td>
</tr>
<tr>
<td>Exon 1</td>
<td>G211A/N</td>
<td>G71R/N</td>
<td>Reduced activity</td>
<td>107,108</td>
</tr>
<tr>
<td>Exon 1/exon 5</td>
<td>Double homozygote for</td>
<td>G71R and Y486D</td>
<td>Reduced activity</td>
<td>109</td>
</tr>
<tr>
<td>Exon 1</td>
<td>T395C</td>
<td>L132P/N</td>
<td>Reduced activity</td>
<td>108</td>
</tr>
<tr>
<td>Exon 1</td>
<td>T524A</td>
<td>L175Q</td>
<td>Reduced activity</td>
<td>83</td>
</tr>
<tr>
<td>Exon 1/exon 2</td>
<td>T524A/del of nt 973</td>
<td>L175Q/truncated (frameshift)</td>
<td>Reduced activity; truncated—inactive</td>
<td>83</td>
</tr>
<tr>
<td>Exon 1</td>
<td>T625C</td>
<td>R209W</td>
<td>Reduced activity</td>
<td>83,111</td>
</tr>
<tr>
<td>Exon 1</td>
<td>C686A</td>
<td>P229Q/N</td>
<td>Reduced activity</td>
<td>107</td>
</tr>
<tr>
<td>Exon 2</td>
<td>T881C</td>
<td>i294T</td>
<td>Reduced activity</td>
<td>88</td>
</tr>
<tr>
<td>Exon 2</td>
<td>A992G</td>
<td>Q331R</td>
<td>Reduced activity</td>
<td>112</td>
</tr>
<tr>
<td>Exon 2</td>
<td>C991T</td>
<td>Q331X</td>
<td>Reduced activity</td>
<td>113</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C1095G</td>
<td>R367G</td>
<td>Reduced activity</td>
<td>107,108</td>
</tr>
<tr>
<td>Exon 5</td>
<td>A781G</td>
<td>Z464A</td>
<td>Reduced activity</td>
<td>114</td>
</tr>
<tr>
<td>Exon 5</td>
<td>T1456G</td>
<td>Y486D</td>
<td>Reduced activity</td>
<td>107,115</td>
</tr>
</tbody>
</table>

*These patients had serum bilirubin levels that overlap with the range seen in some patients with Gilbert syndrome, and have been reported in the literature as Gilbert syndrome.

UGT, uridine-diphosphoglucuronate glucuronosyl-transferase.
bases of these conditions is not unexpected. We propose that reduction of UGT1A1 activity, resulting from any structural mutation of UGT1A1, should be classified as Crigler–Najjar syndrome type 2, and that due to a promoter abnormality should be classified as Gilbert syndrome (see below).

**Gilbert Syndrome**

Gilbert syndrome, also known as “constitutional hepatic dysfunction” or “familial nonhemolytic jaundice” (116), is characterized by mild, chronic, and unconjugated hyperbilirubinemia (Table 20.1). Familial occurrence is common, but not always found. The syndrome is often diagnosed in young adults, usually males, who present with mild, predominantly unconjugated hyperbilirubinemia. Serum bilirubin levels may fluctuate from normal to 3 mg/dL, and increase during fasting or intercurrent illness. Occasional patients complain of fatigue and abdominal discomfort, which are probably manifestations of anxiety. Other than icterus, physical examination and routine laboratory tests are normal. Percutaneous liver biopsy, which is not required for diagnosis, when performed, shows normal liver histology, except for a nonspecific accumulation of lipofuscin pigment in the centrilobular zones. Hepatic bilirubin-UGT activity is reduced to approximately 30% of normal. A minority of patients exhibit reduced hepatic uptake of bilirubin and other organic anions (117). Whether such uptake defects are related pathophysiologically to Gilbert syndrome or are merely coincidental is unknown. A 48-hour fast exaggerates the unconjugated hyperbilirubinemia of Gilbert syndrome (118). Serum bilirubin levels also increase in normal individuals and in patients with liver diseases upon fasting. Therefore, the fasting test is of limited diagnostic value. Intravenous injection of nicotinic acid also increases serum bilirubin levels in Gilbert syndrome (119). However, it does not clearly separate patients with Gilbert syndrome from normal subjects or those with hepatobiliary disease. As splenectomy abolishes nicotinic acid-induced hyperbilirubinemia (119), the effect of nicotinic acid may be based on increased erythrocyte fragility and enhanced splenic heme oxygenase activity, leading to increased bilirubin formation.

**Molecular Mechanism**

The normal TATAA element within the promoter region upstream to exon 1 of UGT1A1 has the sequence A[TA]_{7}TAA. A variant TATAA box, which contains a longer dinucleotide repeat, A[TA]_{7}TAA, has been found to be associated with Gilbert syndrome (120). Subjects of Caucasian, black, or Asian Indian origin, who have a clinical diagnosis of Gilbert syndrome, have been found to be homozygous for the variant TATAA element, which reduces the expression of the structurally normal UGT1A1. This fits with the definition of autosomal-recessive type of inheritance. However, all subjects with this genotype do not exhibit abnormally high bilirubin levels, which also depend on other contributory factors, including the rate of bilirubin production. For example, Gilbert syndrome is diagnosed clinically much more commonly in males, although the variant promoter is equally distributed in both genders, probably because of a higher daily production of bilirubin in males. Approximately 9% of the general population in Europe and the United States are homozygous for the Gilbert type promoter (gene frequency 0.3). The incidence of this genotype may be lower in Japan. Some mutations in the structural region of UGT1A1 have been reported to result in levels of hyperbilirubinemia that are consistent with the diagnosis of Gilbert syndrome (106–109,115). These mutations are listed in Table 20.3 (see Crigler–Najjar Syndrome Type 2, above).

Because of the very high incidence of the Gilbert-type promoter, some heterozygous carriers of Crigler–Najjar syndromes type 1 or 2 mutations have the variant TATAA box on the structurally normal allele. The consequent reduction of expression of the only structurally normal allele can reduce the hepatic UGT1A1 activity to a level that may increase serum bilirubin levels to a range compatible with the clinical diagnosis of Crigler–Najjar syndrome type 2. This explains the frequent finding of intermediate levels of hyperbilirubinemia in the family members of patients with Crigler–Najjar syndrome types 1 and 2.

Although Gilbert syndrome is considered innocuous, the diagnosis is important to avoid confusion with other liver diseases and unnecessary investigations. Gilbert syndrome is diagnosed in individuals with mild unconjugated hyperbilirubinemia without evidence of hemolysis or elevation of liver enzymes. Although hemolysis is not a part of the syndrome, coexistent clinical or subclinical hemolysis may increase the bilirubin load, thereby exacerbating the hyperbilirubinemia and bringing the patient to the attention of the physician. When necessary, the diagnosis can be established by analysis of pigments in duodenal juice. The reduced hepatic bilirubin-UGT activity is reflected by a reduction of bilirubin diglucuronide to monoglucuronide ratio in bile (104). Normally, bilirubin monoglucuronide accounts for 10% or less of all forms of bilirubin excreted in bile. In Gilbert syndrome the percentage increases to 14% to 34%. Genetic analysis of DNA extracted from blood leukocytes or any other tissue can aid in the diagnosis.

**Animal Model**

The Bolivian population of squirrel monkeys (Saimiri sciureus) has a higher serum unconjugated bilirubin levels and a greater degree of increase upon fasting than does a closely related Brazilian population of the species (121). The Bolivian monkeys have slower plasma clearance of intravenously administered bilirubin, a lower level of hepatic bilirubin-UGT activity, and an increased bilirubin
monoglucuronide to diglucuronide ratio in bile. In these respects, the Bolivian squirrel monkeys are a model of human Gilbert syndrome. Fasting hyperbilirubinemia is rapidly reversed by oral or intravenous administration of carbohydrates, but not by lipid administration.

**DISORDERS OF BILIRUBIN METABOLISM THAT RESULT IN PREDOMINANTLY CONJUGATED HYPERBILIRUBINEMIA**

Conjugated bilirubin may accumulate in plasma because of “leakage” from the liver cells, as in hepatocellular diseases, such as hepatitis, or from disordered canalicular excretion or biliary obstruction. In all such cases, both conjugated and unconjugated bilirubin accumulate in plasma. Rapid advances in molecular genetic studies have revealed the mechanism of several disorders of the hepatocyte that can, directly or indirectly, lead to the accumulation of conjugated bilirubin in plasma. These include Dubin–Johnson syndrome, three types of progressive intrahepatic cholestasis, and benign recurrent intrahepatic cholestasis. Progressive familial intrahepatic cholestasis syndromes have been discussed in Chapter 26. The molecular basis of Rotor syndrome remains unknown. In addition to the hepatocellular excretory abnormities, developmental anomalies of bile ductules can cause cholestasis. The genetic mechanism of one of these disorders, Alagille syndrome, has been discovered.

**Dubin–Johnson Syndrome**

Dubin–Johnson syndrome is characterized by conjugated hyperbilirubinemia and black pigmentation of the liver, in the absence of other abnormalities of clinicocochemical tests for liver dysfunction, including serum alanine and aspartate aminotransferase, alkaline phosphatase, γ-glutamyltranspeptidase, and albumin levels (122–124) (Table 20.4). Dubin–Johnson syndrome is rare except in Jews of Middle Eastern origin, in whom the incidence is 1 in 1,300 (124). In Middle Eastern Jews, Dubin–Johnson syndrome is associated with clotting factor VII deficiency, but this linkage is not tight. Occasionally, patients complain of vague abdominal discomfort and some have hepatosplenomegaly. In most cases, however, the patients are asymptomatic.

Serum bile acid levels are normal and pruritus is absent (125). Serum bilirubin levels are usually between 2 and 5 mg/dL, but may be normal at times and may be as high as 20 to 25 mg/dL during intercurrent illness, use of oral contraceptives, and pregnancy (125). Fifty percent or more of total serum bilirubin is conjugated and bilirubinuria is frequently found. Continuous retention of bilirubin glucuronides in plasma results in the formation of irreversible adducts of bilirubin with plasma proteins, particularly albumin (δ-bilirubin), which is not excreted in urine or bile and gives a direct van der Bergh reaction.

**Organic Anion Excretion**

Canalicular excretion of many organic anions, other than bile acids, is defective in Dubin–Johnson syndrome. These anions include bilirubin, bromosulfophthalein (BSP), dibromosulfophthalein (DBSP), indocyanin green (ICG), and 125I-labeled rose Bengal. In most patients, following an intravenous injection of BSP there is normal initial plasma disappearance of the dye, so that the concentration is normal or mildly elevated 45 minutes after the injection. How-

**TABLE 20.4. INHERITED DISORDERS CAUSING RETENTION OF BOTH CONJUGATED AND UNCONJUGATED BILIRUBIN**

<table>
<thead>
<tr>
<th>Dubin–Johnson Syndrome</th>
<th>Rotor Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum bilirubin</td>
<td>Predominantly conjugated, usually 50–100 μM, occasionally as high as 340 μM</td>
</tr>
<tr>
<td>Routine liver function tests</td>
<td>Normal except for hyperbilirubinemia</td>
</tr>
<tr>
<td>Serum bile salt levels</td>
<td>Normal</td>
</tr>
<tr>
<td>Plasma bromsulfophthalein (BSP) retention</td>
<td>Normal at 45 min; secondary rise at 90 min</td>
</tr>
<tr>
<td>Plasma BSP clearance</td>
<td>Tmax is very low; storage is normal</td>
</tr>
<tr>
<td>Oral cholecystogram</td>
<td>Usually does not visualize the gallbladder</td>
</tr>
<tr>
<td>Urinary coproporphyrin excretion pattern</td>
<td>Total—normal; &gt;80% as coproporphyrin I</td>
</tr>
<tr>
<td>Appearance of liver</td>
<td>Grossly black</td>
</tr>
<tr>
<td>Histology of liver</td>
<td>Dark pigments, predominantly in centrilobular areas; otherwise normal</td>
</tr>
<tr>
<td>Mode of inheritance</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>Prevalence</td>
<td>Rare (except in Middle Eastern Jews: 1 in 1,300 births)</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Benign</td>
</tr>
<tr>
<td>Animal model</td>
<td>Mutant TR rats/mutant Corriedale sheep/golden lion Tamarin monkey</td>
</tr>
<tr>
<td></td>
<td>Normal, no increase in pigmentation</td>
</tr>
<tr>
<td></td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td></td>
<td>Rare</td>
</tr>
<tr>
<td></td>
<td>Benign</td>
</tr>
<tr>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>
ever, 90 minutes after the injection, there is a secondary rise because of the reflux of glutathione-conjugated BSP from the liver cell into the circulation prior to excretion by the hepatocytes (126,127). A similar secondary rise has been described following intravenous administration of unconjugated bilirubin. However, such secondary rise can also occur in other cholestatic disorders. Because of the organic anion excretion defect, oral cholecystographic contrast dyes do not visualize the gallbladder even after a double dose.

Hepatic Pigmentation

Macrosopically, the liver is black. Liver histology is normal except for the accumulation of a dense pigment, which is contained within lysosomes (128). Infusion of 3H-epinephrine in the Corriedale sheep (an animal model of Dubin–Johnson syndrome, see below) revealed reduced biliary excretion of radioactivity and incorporation of the isotope into the hepatic pigment, suggesting its relationship with melanin (129). When TR− rats (another model of Dubin–Johnson syndrome) are fed a diet enriched in aromatic amino acids (phenylalanine, tyrosine, and tryptophan), lysosomal pigmentation develops, probably because of impaired excretion of anionic metabolites of tyrosine, phenylalanine, and tryptophan, with subsequent oxidation, polymerization, and lysosomal accumulation (130). Electron spin resonance spectroscopy suggests that the pigment differs from authentic melanin, but could be composed of polymers of epinephrine metabolites. Computed tomography of the liver shows higher than normal attenuation values in Dubin–Johnson syndrome. Interestingly, the pigment is cleared during acute viral hepatitis and reaccumulates slowly after recovery (131).

Urinary Coproporphyrin Excretion

The total urinary coproporphyrin excretion is normal in Dubin–Johnson syndrome, but the ratio of coproporphyrin I to coproporphyrin III is greater (4:1), than that seen normally (1:3) (132). In obligate heterozygotes (i.e., unaffected parents and children of patients with Dubin–Johnson syndrome), total urinary coproporphyrin excretion is reduced by 40%, because of a 50% reduction in coproporphyrin III excretion (133). In heterozygote carriers, the proportion of coproporphyrin I in urine was intermediate between findings in controls and in patients with Dubin–Johnson syndrome. Based on these data, Dubin–Johnson syndrome is inherited as an autosomal-recessive characteristic. No other hepatobiliary disorder or porphyria has been described in which a combination of normal total urinary coproporphyrin excretion and a great predominance of coproporphyrin I is seen. Thus, in the presence of a consistent history and physical examination, urinary coproporphyrin excretion appears to be diagnostic of this disorder.

Molecular Mechanism

Organic anions, other than bile acids, such as conjugated bilirubin, and other glucuronide or glutathione conjugated substances, are transported across the bile canalicular membrane by an ATP-dependent energy-consuming process, mediated by MRP2 [also known as the canalicular multispecific organic anion transporter (cMOAT)] (Fig. 20.5) (see Chapters 24 and 25). TR− rats have a frame-shift mutation in the gene encoding MRP2 (134). The human MRP2 gene is located on chromosome 10q23-q24. A number of mutations causing Dubin–Johnson syndrome have been identified, a significant proportion of which are in the critical ATP-binding domain (135–139) (Table 20.5). A mutation at an intrinsic splicing donor site that results in abnormal splicing of the transcript has also been identified in a patient with Dubin–Johnson syndrome (139).

Animal Models

A mutant strain of the Corriedale sheep was found to have a metabolic defect similar to that in Dubin–Johnson syndrome. Biliary excretion of conjugated bilirubin, gluthathione-conjugated BSP, iopanoic acid, and ICG is decreased in this strain, whereas taurocholate transport is normal. The secretion of unconjugated BSP is unimpaired. As in patients with Dubin–Johnson syndrome, total urinary coproporphyrin excretion is normal with increased proportion of the isomer I. The most extensively studied animal model for Dubin–Johnson syndrome is the TR− rat (140). The organic anion excretion defect and the pattern of

![FIGURE 20.5. Four adenosine triphosphate–utilizing transport proteins concentrated in the bile canalicular membrane have been recognized to be important in canalicular transport. Multidrug resistance–related protein (MRP2) [also known as canalicular multispecific organic anion transporter (cMOAT)] mediates the transport of most non–bile-acid organic anions, including bilirubin glucuronides. Bile salt export pump (BSEP) [also known as sister of p-glycoprotein (SPPG)] is the major bile acid transporter. FIC1 translocates acidic phospholipids (such as phosphatidylethanolamine) from the outer to the inner leaf of the plasma membrane. MDR3 transports phospholipids from the inner to the outer leaflet of the bile canalicus. Genetic lesions of MRP2 cause Dubin–Johnson syndrome. Inherited abnormalities of the other three genes are associated with various intrahepatic cholestasis syndromes (see text). FIC, familial intrahepatic cholestasis; MDR, multi drug resistance.](image)
Coproporphyrin excretion in urine are similar to that in Dubin–Johnson syndrome. For organic anions, such as glutathione-conjugated leukotriene (LT)C4, the canalicular secretion defect is nearly complete, whereas for bilirubin glucuronides there is about 10% residual transport activity (141). The TR− rat and patients with Dubin–Johnson syndrome have normal canalicular excretion of bile salts, except those that have double-negative charges because of conjugation at the 3-OH position (142). The ATP-driven component of bilirubin glucuronide transport by canalicular plasma membrane vesicles is absent in the TR− rats, but the membrane potential-dependent mechanism provides the residual transport (53). A mutant strain of golden lion tamarins (Leontopithecus rosalia rosalia) with Dubin–Johnson-like syndrome has been described (143).

Rotor Syndrome

This disorder is characterized by accumulation of conjugated bilirubin in the plasma in the presence of normal liver function tests (144) (Table 20.4). In contrast to Dubin–Johnson syndrome, there is no increased pigmentation of the liver. Oral cholecystographic agents result in roentgenologic visualization of the gallbladder in Rotor syndrome. Unlike the findings in Dubin–Johnson syndrome, patients with Rotor syndrome exhibit marked retention of BSP at 45 minutes after injection, but biphasic plasma BSP peaks are not found and conjugated BSP does not appear in plasma. There is also marked plasma retention of intravenously administered unconjugated bilirubin and ICG.

Studies using a constant infusion of BSP indicate that while in Dubin–Johnson syndrome the transport maximum \( T_{\text{max}} \) is virtually zero and hepatic storage is normal, in Rotor syndrome the \( T_{\text{max}} \) is 50% of normal, but the hepatic storage is reduced by 75% to 90%. Thus, Dubin–Johnson syndrome represents a canalicular excretion disorder, whereas Rotor syndrome is a disorder of hepatic storage and may be identical with the so-called familial hepatic storage disease (145).

Urinary Coproporphyrin Excretion

Compared to normal, total urinary coproporphyrin excretion is increased by 250% to 500% and the proportion of coproporphyrin I in urine is approximately 65% of total (146). These results are similar to those seen in many other hepatobiliary disorders and distinguish this disorder from Dubin–Johnson syndrome. Rotor syndrome is inherited as an autosomal-recessive characteristic. Its molecular basis is unknown.

Progressive Familial Intrahepatic Cholestasis Syndromes

In contrast to Dubin–Johnson and Rotor syndromes, progressive familial intrahepatic cholestasis syndromes (PFICs) are autosomal-recessive disorders, associated with various degrees of cholestasis. In most cases PFICs cause progressive liver damage. An exception is benign recurrent intrahepatic cholestasis, which is an episodic and milder disorder. These conditions have been discussed in Chapter 26.

Alagille Syndrome

Several inherited disorders of bile duct development have been described. Of these, the Alagille syndrome was the first to be characterized at the molecular level. Alagille syndrome is characterized by the paucity or absence of small bile ducts, resulting in progressive intrahepatic cholestasis, and abnormalities of the eye, heart, and vertebrae. The disorder is inherited as an autosomal-dominant characteristic. The responsible gene, \( JAG1 \), has been mapped to chromosome 20p12 (147). \( JAG1 \) encodes an unidentified ligand that binds to the notch receptor, which is crucial for cell plate development in Drosophila and mammals. In rare cases, the gene is deleted. In other cases of Alagille syndrome, various point mutations in \( JAG1 \), each of which abolishes expression of the altered allele, have been described.

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### TABLE 20.5. MUTATIONS IDENTIFIED IN PATIENTS WITH DUBIN–JOHNSON SYNDROME TYPE

<table>
<thead>
<tr>
<th>Site of Mutation</th>
<th>Nucleic Acid Change</th>
<th>Predicted Change of Amino Acid in MRP2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 13</td>
<td>Del, nt 1669–1815</td>
<td>Truncated protein</td>
<td>138</td>
</tr>
<tr>
<td>Intron 15</td>
<td>Splice donor site, T→C</td>
<td>Truncated protein</td>
<td>139</td>
</tr>
<tr>
<td>Exon 18</td>
<td>C2302T</td>
<td>R368W</td>
<td>138</td>
</tr>
<tr>
<td>Exon 18</td>
<td>Del, nt 2272–2439</td>
<td>Truncated protein</td>
<td>138</td>
</tr>
<tr>
<td>Exon 23</td>
<td>C3196T</td>
<td>R1066X—truncated protein</td>
<td>137</td>
</tr>
<tr>
<td>Exon 31</td>
<td>Del, nt 4175–4180</td>
<td>Truncated protein</td>
<td>135</td>
</tr>
</tbody>
</table>

MRP, multidrug resistance-related protein.
REFERENCES


