

DISORDERS OF BILIRUBIN METABOLISM

NAMITA ROY CHOWDHURY
IRWIN M. ARIAS
ALLAN W. WOLKOFF
JAYANTA ROY CHOWDHURY

FORMATION OF BILIRUBIN 292

Sources of Bilirubin 292
 Enzymatic Mechanism of Bilirubin Formation 292
 Quantification of Bilirubin Production 292
 Inhibition of Bilirubin Production 293

CHEMISTRY OF BILIRUBIN 293

Absorption Spectra and Fluorescence 293
 Effect of Light 293

QUANTIFICATION OF BILIRUBIN IN BODY FLUIDS 293

TOXICITY OF BILIRUBIN 294

DISPOSITION OF BILIRUBIN 294

Bilirubin Transport in Plasma 294
 Bilirubin Uptake by the Hepatocytes 295
 Hepatocellular Storage of Bilirubin 295
 Conjugation of Bilirubin 296
 Canalicular Excretion of Conjugated Bilirubin 296

Fate of Bilirubin in the Gastrointestinal Tract 296
 Alternative Routes of Bilirubin Elimination 297
 Antioxidant Property of Bilirubin 297

DISORDERS OF BILIRUBIN METABOLISM RESULTING IN UNCONJUGATED HYPERBILIRUBINEMIA 297

Neonatal Jaundice 298
 Bilirubin Overproduction 298
 Crigler–Najjar Syndrome Type 1 298
 Crigler–Najjar Syndrome Type 2 (Arias Syndrome) 301
 Gilbert Syndrome 302

DISORDERS OF BILIRUBIN METABOLISM THAT RESULT IN PREDOMINANTLY CONJUGATED HYPERBILIRUBINEMIA 303

Dubin–Johnson Syndrome 303
 Rotor Syndrome 305
 Progressive Familial Intrahepatic Cholestasis Syndromes 305
 Alagille Syndrome 305

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 dys-

function. 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.

N. Roy Chowdhury: Department of Medicine and Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York 10461.

I. M. Arias: Departments of Physiology and Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111.

A. W. Wolkoff: Departments of Medicine and Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461-1602.

J. Roy Chowdhury: Department of Medicine and Molecular Genetics, Albert Einstein College of Medicine Liver Center; Department of Medicine, Jack D. Weiler Hospital of Albert Einstein College of Medicine, Bronx, New York 10461.

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 (glycine 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 O_2 and a reducing agent, such as reduced

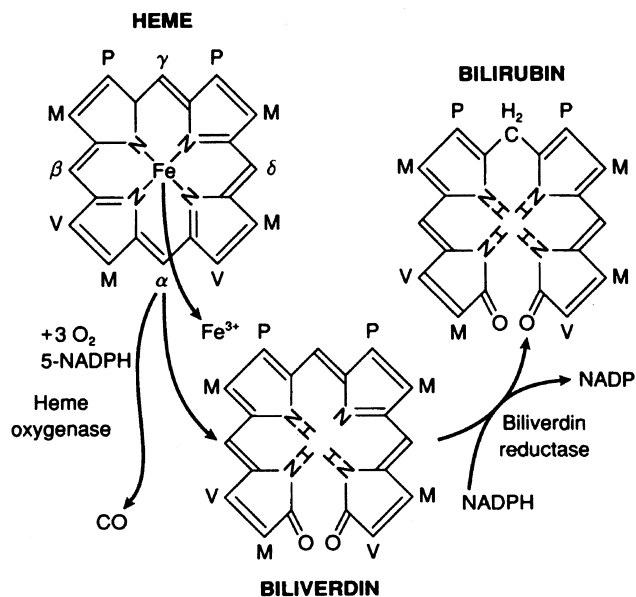


FIGURE 20.1. Mechanism of heme ring opening and subsequent reduction of biliverdin to bilirubin.

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 IX α is 1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid (15, 16). The linear tetrapyrrole structure of bilirubin was solved by Fischer and Plieninger (17). X-ray diffraction studies of crystalline bilirubin has 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

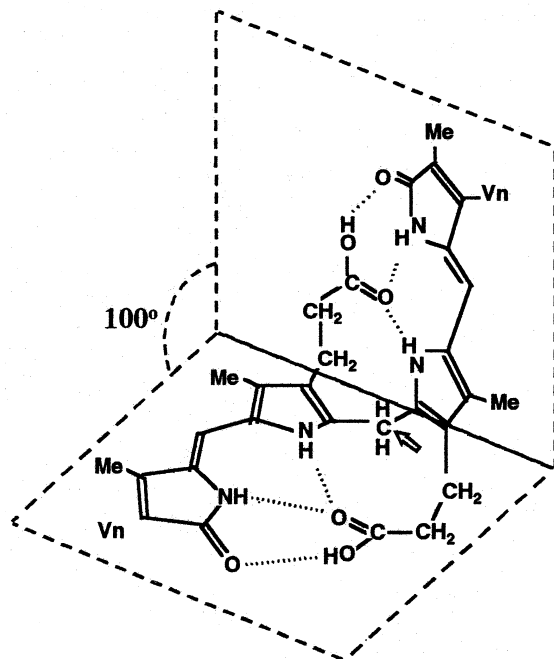


FIGURE 20.2. X-ray crystallographic structure of bilirubin showing a ridge-tile configuration caused by internal hydrogen bonding of the propionic acid carboxyls to the amino groups and the lactam oxygen of the pyrrolenone rings of the opposite half of the molecule. The bonds between the pyrrolenone rings A and B and C and D are in the *Z* (*trans*) configuration.

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 glucuronic 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 hydrogen 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 IX α is at 450 to 474 nm in most organic solvents. Although pure bilirubin does not fluoresce, when dissolved in detergents, 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 IX α -*ZZ*, and can be excreted in bile without conjugation (20). The vinyl substituent in the endovinyl half of bilirubin IX α -*EZ* is slowly cyclized with the methyl substituent 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 bilirubin 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 tetrapyrroles, or after conversion to azoderivatives. Total bilirubin can also be quantified indirectly by quantification of the intensity of yellow discoloration of the skin. Conversion to azodipyrroles 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 azodipyrrole 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 Ca^{2+} -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 pathogenesis 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 canaliculus, 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

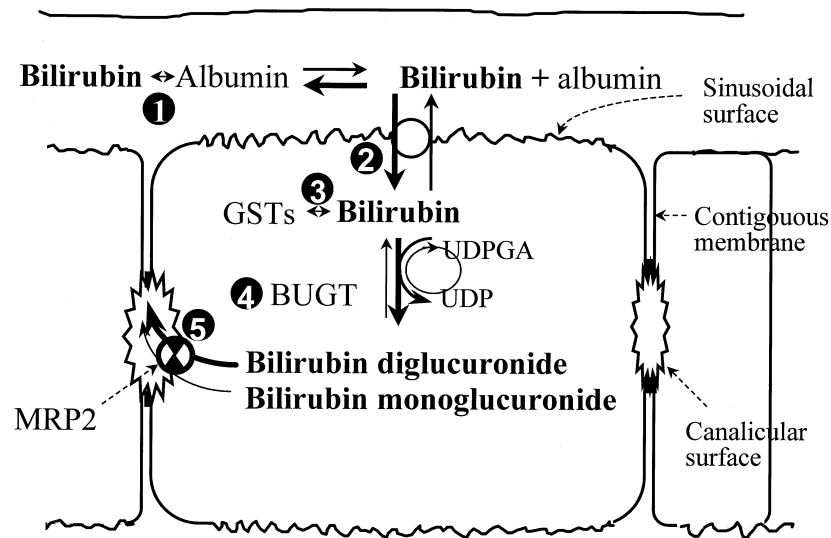


FIGURE 20.3. Summary of hepatic metabolism of bilirubin. Bilirubin is strongly bound to albumin in the circulation (1). At the sinusoidal surface of the hepatocyte, this complex dissociates, and bilirubin enters hepatocytes by facilitated diffusion (2). This process is non-adenosine triphosphate (ATP)-dependent 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 glucuronosyltransferase (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*).

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 $\mu\text{mol/L}$) exceeds that of bilirubin (3 to 17 $\mu\text{mol/L}$). 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 cholecystographic 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 chro-

matography on albumin agarose polymers, dialysis, and electrophoresis have been used to separate free from bound bilirubin. Unbound bilirubin is rapidly destroyed by treatment with H_2O_2 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.

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 HCO_3^- 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 canaliculus (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 exogenous 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 canaliculus 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.

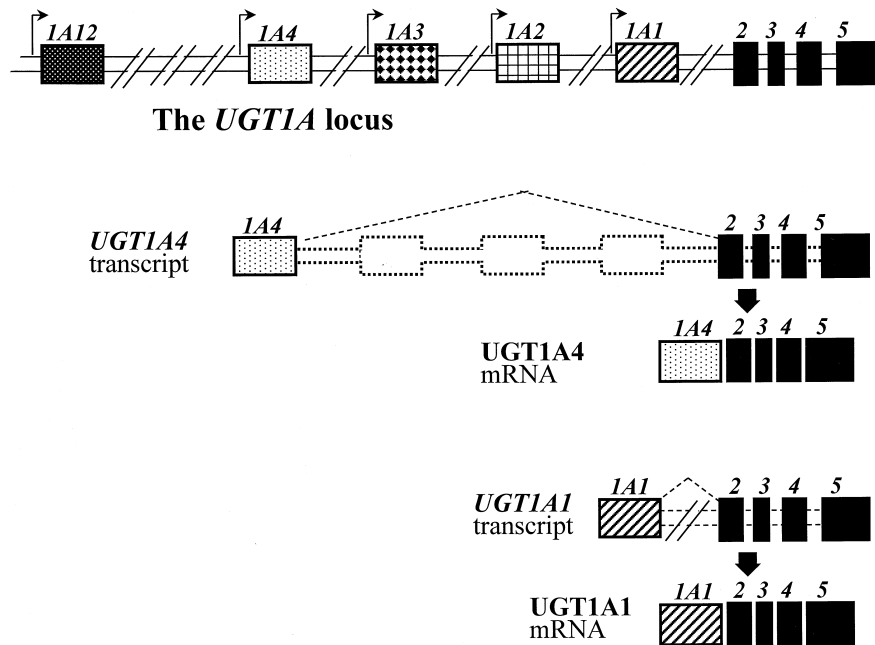


FIGURE 20.4. Schematic representation of the human *UGT1A* locus, located at 2q37. This locus comprises a number of genes of the *UGT1A* family that include bilirubin-UGT. Exons 2, 3, 4, and 5, located at the 3' end of *UGT1A*, encode the identical carboxyl terminal domains of all *UGT1A* isoforms expressed from this locus. Upstream to these "common region" exons are a series of "unique exons" (exons 1A1 through 1A12), each of which encodes the variable amino terminal domain of a different *UGT1A* isoform. Each unique region exon is preceded by a separate promoter region (arrows), permitting independent regulation of gene expression. Transcription may start from any of the promoters, producing transcripts of varying lengths, two examples of which are shown in the figure. The unique exon located at the 5' end of the primary transcript is spliced to the 5' end of exon 2, and other unique region exons present in the transcript (shown in dotted lines) are spliced out. Genes belonging to the *UGT1A* locus are named according to the unique exon utilized in the processed mRNA. Thus, when the transcription starts at exon 1A4 (**upper** transcript in the figure), the mRNA for *UGT1A4* is generated after splicing. This gene, which consists of exon 1A4 plus the common region exons 2 to 5, is termed *UGT1A4*, and the expressed enzyme is termed *UGT1A4*. Similarly, if the transcription starts at exon 1A1 (**lower** transcript in the figure), an mRNA consisting of exon 1A1 plus exons 2 to 5 is generated. According to the current system of terminology, this gene is named *UGT1A1*, and the expressed isoform is termed *UGT1A1*. *UGT1A1* is the only isoform that contributes significantly to bilirubin glucuronidation in humans. This structure predicts that disorders of bilirubin glucuronidation resulting from genetic lesions within exon 1A1 should affect UGT activity toward bilirubin, but other *UGT1A* isoforms should be normal. In contrast, mutations of exons 2 to 5 should affect all isoforms of the *UGT1A* family.

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-

tion; (b) intracellular binding or storage; (c) conjugation, largely with glucuronic acid; and (d) biliary excretion. Abnormalities in any of these processes may result in hyperbilirubinemia. Complex clinical disorders, such as hepatitis or cirrhosis, may affect multiple processes. In several inherited disorders, the transfer of bilirubin from blood to bile is disrupted at a specific step. Study of these disorders has permitted better understanding of bilirubin metabolism in health and disease. Each disorder is characterized by varied degrees of hyperbilirubinemia of the unconjugated or conjugated type.

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 neonates, 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 unconju-

gated 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.

TABLE 20.1. CHARACTERISTICS OF INHERITED UNCONJUGATED HYPERBILIRUBINEMIA

	Crigler–Najjar Syndrome Type 1	Crigler–Najjar Syndrome Type 2	Gilbert Syndrome
Liver function tests other than serum bilirubin and liver histology	Normal	Normal	Normal
Serum bilirubin concentrations	20–50 mg/dL (340–850 μ M)	7–20 mg/dL (120–340 μ M)	Normal to 5 mg/dL (<85 μ M)
Pigments excreted in bile	Small amounts of unconjugated bilirubin and only traces of bilirubin glucuronides	Reduced proportion of bilirubin diglucuronide	Reduced proportion of bilirubin diglucuronide
Hepatic bilirubin-UGT activity	Virtually absent	Markedly reduced but detectable	Reduced to about 30% of normal
Effect of phenobarbital administration	No significant reduction of serum bilirubin levels	Reduction of serum bilirubin levels by >25%	Normalization of serum bilirubin
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive
Molecular basis	Genetic lesions within the coding region or at splice sites of <i>UGT1A1</i>	Point mutations within the coding region of <i>UGT1A1</i>	Insertion of a TA dinucleotide within the TATAA element of <i>UGT1A1</i> ^a
Prevalence	Rare (<1:1,000,000)	Rare (<1:1,000,000)	Phenotype in ~4% of the population; among Caucasians and Africans, ~9% are homozygous for the genotype (less common in Japan)
Prognosis	Kernicterus, unless vigorously treated; currently, liver transplantation is the only curative treatment	Kernicterus is uncommon, but has been reported	No encephalopathy; increased intensity of neonatal jaundice; toxicity of some drugs may be increased
Animal model	Gunn rat	—	Bolivian subpopulation of squirrel monkeys

^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.

Laboratory test results in Crigler–Najjar syndrome type 1 are normal except for the serum bilirubin levels, which are usually 20 to 25 mg/dL, 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

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

Site of Lesion	Nucleic Acid Alteration	Predicted Mutation of <i>UGT1A1</i>	Activity	Reference
Exon 1	Del C,T at nt 120,121, respectively	Truncated (frameshift)	Inactive	79
Exon 1	Ins 4 bp after codon 80	Truncation (frameshift)	Inactive	80
Exon 1	Ins T after codon 158/del codon 170	Truncated (frameshift)/del of phenylalanine	Inactive/inactive	81
				82
Exon 1	Del of codon 170	Del phenylalanine	Inactive	81–83
Exon 1/exon 2	T529C/del nt 879–892	C177R/truncated (frameshift)	Inactive/inactive	83
Exon 1	G826T	G276R	Inactive	83
Exon 1/exon 3	A835T/C1069T	B279Y/Q357X	ND/inactive	76,83,84
Exon 1	C840A	C280X	Inactive	85
Intron 1	Splice donor, G→C	Truncated	Inactive	86
Exon 2	Skipping of exon 2	Truncated	Inactive	83
Exon 2/exon 4	C872T/A1282G	A291V/K426E	Inactive	84
Exon 2	T880A and del 881–893	Truncated (frameshift)	Inactive	78,83
Exon 2	G923A	G308E	Inactive	84,87
Exon 2	C991T	Q331X	Inactive	77
Exon 3/exon 4	G1005A/G1102A	W335X/A368T	Inactive	84
Exon 3	nt: C1006T	R336W/N	Inactive	88
Exon 3	C1021T	R341X	Inactive	89
Exon 3	C1069T	Q357X	Inactive	76,83,84
Exon 3/exon 4	C1069T/G1201C	Q357X/A401P	Inactive	76,83,84
Exon 3	A1070G	Q357R	Inactive	84
Exon 3/exon 4	C1081T/CC1159,1160GT	Q361X/P387R	Inactive/inactive	79
Intron 3/exon 1	Splice acceptor site, A→G/nt C145T	Truncated protein/Q49X	Inactive	86
Exon 4	C1124T	S376F	Inactive	77,83,87
Exon 4	C1143G	S381R	Inactive	84
Exon 4	CC1159,1160GT	P387R	Inactive	79
Exon 4	G1201C	A401P	Inactive	84
Exon 4/exon 5	G1201C/A1308T	A401P/K437X	Inactive	84

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.

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 IX α -ZZ into geometric and structural isomers that are

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 I, 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

Site of Mutation	Nucleic Acid Mutation	Predicted Amino Acid Substitution	Activity	Reference
Exon 1	T44G	L15R	Reduced activity	105
Exon 1	G211A ^a	G71R	Reduced activity	106–108
Exon 1	G211A/N	G71R/N	Reduced activity	107,108
Exon 1/exon 5	Double homozygote for G211A and T1456G	G71R and Y486D	Reduced activity ^a	109
Exon 1	T395C	L132P/N	Reduced activity ^a	108
Exon 1	T524A	L175Q	Reduced activity	83
Exon 1/exon 2	T524A/del of nt 973	L175Q/truncated (frameshift)	Reduced activity; truncated—inactive	83
Exon 1	T625C	R209W	Reduced activity	83,111
Exon 1	C686A	P229Q/N	Reduced activity ^a	107
Exon 2	T881C	I294T	Reduced activity	88
Exon 2	A992G	Q331R	Reduced activity	112
Exon 2	C991T	Q331X	Reduced activity ^a	113
Exon 4	C1099G	R367G	Reduced activity ^a	107,108
Exon 5	A1391C	Z464A	Reduced activity	114
Exon 5	T1456G	Y486D	Reduced activity	107,115

^aThese 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]₆TAA. A variant TATAA box, which contains a longer dinucleotide repeat, A[TA]₇TAA, 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 inher-

itance. 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 abnormalities, 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 clinicochemical 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 den 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 ^{125}I -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

	Dubin–Johnson Syndrome	Rotor Syndrome
Serum bilirubin	Predominantly conjugated, usually 50–85 μM , can be as high as 340 μM	Predominantly conjugated, usually 50–100 μM , occasionally as high as 340 μM
Routine liver function tests	Normal except for hyperbilirubinemia	Normal except for hyperbilirubinemia
Serum bile salt levels	Normal	Normal
Plasma bromosulfophthalein (BSP) retention	Normal at 45 min; secondary rise at 90 min	Elevated; but no secondary rise at 90 min
Plasma BSP clearance	T_{max} is very low; storage is normal	Both T_{max} and storage are reduced
Oral cholecystogram	Usually does not visualize the gallbladder	Usually visualizes the gallbladder
Urinary coproporphyrin excretion pattern	Total—normal; >80% as coproporphyrin I	Total—elevated; ~50–75% coproporphyrin I
Appearance of liver	Grossly black	Normal
Histology of liver	Dark pigments, predominantly in centrilobular areas; otherwise normal	Normal, no increase in pigmentation
Mode of inheritance	Autosomal recessive	Autosomal recessive
Prevalence	Rare (except in Middle Eastern Jews: 1 in 1,300 births)	Rare
Prognosis	Benign	Benign
Animal model	Mutant TR^- rats/mutant Corriedale sheep/golden lion Tamarin monkey	None

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

Macroscopically, 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 intronic splice 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, glutathione-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

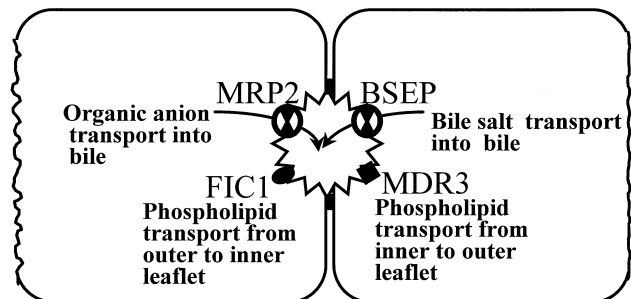


FIGURE 20.5. Four adenosine triphosphate-utilizing transport proteins concentrated in the bile canalicular membrane have been recognized to be important in canalicular transport. Multidrug resistance-related protein (*MRP2*) [also known as canalicular multispecific organic anion transporter (cMOAT)] 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 (*SPGP*)] is the major bile acid transporter. *FIC1* translocates acidic phospholipids (such as phosphatidylserine and phosphatidyl-ethanolamine) from the outer to the inner leaf of the plasma membrane. *MDR3* transports phospholipids from the inner to the outer leaflet of the bile canalculus. 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.

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

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

MRP, multidrug resistance-related protein.

coproporphyrin excretion in urine are similar to that in Dubin–Johnson syndrome. For organic anions, such as glutathione-conjugated leukotriene (LT)₄, 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 (*Leontopittheous 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_{\max}) is virtually zero and hepatic storage is normal, in Rotor syndrome the T_{\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.

ACKNOWLEDGMENTS

The authors thank Dr. Ajit Kadakol for assistance in preparation of this review. This work was supported in part by National Institutes of Health grants DK-46057, DK-39137, DK-41296, DK-23026, DK-35652, and DK-34926.

REFERENCES

- London IM, West R, Shemin D, et al. On the origin of bile pigment in normal man. *J Biol Chem* 1950;184:351.
- Schwartz S, Johnson JA, Stephenson BD, et al. Erythropoietic defects in protoporphyria: a study of factors involved in labeling of porphyrins and bile pigments from ALA-3H and glycine-14C. *J Lab Clin Med* 1971;78:411.
- Robinson SH. Origins of the early-labeled peak. In: Berk PD, Berlin NI, eds. *Bile pigments: chemistry and physiology*. Washington, DC: US Government Printing Office, 1977;175.
- Come SE, Shoher SB, Robinson SH. Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythroid stimulation or iron deficiency anemia. *Blood* 1974;44:817.
- Tenhunen R, Marver HS, Schmid R. Microsomal heme oxygenase: Characterization of the enzyme. *J Biol Chem* 1969;244:6388.
- Elbirt KK, Bonkovsky HL. Heme oxygenase: recent advances in understanding its regulation and role. *Proc Assoc Am Physicians* 1999;111:438.
- Ishizawa S, Yoshida T, Kikuchi G. Induction of heme oxygenase in rat liver. *J Biol Chem* 1983;258:4220.
- Hayashi S, Takamiya R, Yamaguchi T, et al. Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. *Circ Res* 1999;85:663.
- Tenhunen R, Ross ME, Marver HS, et al. Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. *Biochemistry* 1970;9:298.
- Jones EA, Bloomer JR, Berk PD, et al. Quantitation of hepatic bilirubin synthesis in man. In: Berk PD, Berlin NI, eds. *Bile pigments: chemistry and physiology*. Washington, DC: US Government Printing Office, 1977;189.
- Berk PD, Rodkey FL, Blaschke TF, et al. Comparison of plasma bilirubin turnover and carbon monoxide production in man. *J Lab Clin Med* 1974;83:29.
- Westlake DWS, Roxburgh JM, Talbot G. Microbial production of carbon monoxide from flavinoids. *Nature* 1961;189:510.
- Kappas A, Drummond GS. Direct comparison of tin-mesoporphyrin, an inhibitor of bilirubin production, and phototherapy in controlling hyperbilirubinemia in term and near-term newborns. *Pediatrics* 1995;95:468.
- Valaes T, Petmezaki S, Henschke C, et al. Control of jaundice in preterm newborns by an inhibitor of bilirubin production: studies with tin-mesoporphyrin. *Pediatrics* 1994;93:1.
- Berk PD, Jones EA, Howe RB, et al. Disorders of bilirubin metabolism. In: Bondy PK, Rosenberg LE, ed. *Metabolic control and disease*, 8th ed. Philadelphia: WB Saunders, 1980;1009.
- Grandchamp B, Bissel DM, Licko V, et al. Formation and disposition of newly synthesized heme in adult rat hepatocytes in primary cultures. *J Biol Chem* 1981;256:11677.
- Fischer H, Plieninger H. Synthese des biliverdins (uteroverdins) und bilirubins der biliverdine XIII, und III, sowie der Vinulneoxanthosaure. *Hoppe Seyler Z Physiol Chem* 1942;274:231.
- Bonnet RJ, Davis E, Hursthouse MB. Structure of bilirubin. *Nature* 1976;262:326.
- Kuenzle CC, Weibel MH, Pelloni RR. The reaction of bilirubin with diazomethane. *Biochem J* 1973;133:357.
- McDonagh AF, Palma LA, Lightner DA. Phototherapy for neonatal jaundice. Stereospecific and regiospecific photoisomerization of bilirubin bound to human serum albumin and NMR characterization of intramolecularly cyclized photoproducts. *J Am Chem Soc* 1982;104:6867.
- Itho S, Onishi S. Kinetic study of the photochemical changes of (ZZ)-bilirubin IX bound to human serum albumin. Demonstration of (EZ)-bilirubin IX as an intermediate in photochemical changes from (ZZ)-bilirubin IX to (EZ)-cyclobilirubin IX. *Biochem J* 1985;226:251.
- McDonagh AF. Thermal and photochemical reactions of bilirubin IX. *Ann NY Acad Sci* 1975;244:553.
- Lauff JJ, Kasper ME, Ambros RT. Quantitative liquid chromatographic estimation of bilirubin species in pathological serum. *Clin Chem* 1983;29:800.
- Onishi S, Itho S, Kawade N, et al. An accurate and sensitive analysis by high pressure liquid chromatography of conjugated and unconjugated bilirubin IX α and in various biological fluids. *Biochem J* 1980;185:281.
- Spivak W, Carey MC. Reverse-phase h.p.l.c. separation, quantification and preparation of bilirubin and its conjugates from native bile. *Biochem J* 1985;225:787.
- Roy Chowdhury J, Roy Chowdhury N. Quantitation of bilirubin and its conjugates by high pressure liquid chromatography. *Falk Hepatol* 1982;11:1649.
- Blanckaert N, Kabra PM, Farina FA, et al. Measurement of bilirubin and its mono- and diconjugates in human serum by alkaline methanolysis and high performance liquid chromatography. *J Lab Clin Med* 1980;96:198.
- Schumacher RE, Thornberry JM, Gutcher GR. Transcutaneous bilirubinometry: a comparison of old and new methods. *Pediatrics* 1985;76:10.
- Tayba R, Gribetz D, Gribetz I, et al. Non-invasive estimation of serum bilirubin. *Pediatrics* 1998;102:28.
- Brown AK, Eisinger J, Blumberg WE, et al. A rapid fluorometric method for determining bilirubin levels and binding in the blood of neonates: comparison with other methods. *Pediatrics* 1980;65:767.
- Schiff D, Chan G, Poznasky MJ. Bilirubin toxicity in neural cell lines N115 and NBR10A. *Pediatr Res* 1985;19:908.
- Mustafa MG, Cowger ML, Kind TE. Effects of bilirubin on mitochondrial reactions. *J Biol Chem* 1969;244:6403.
- Caroh R, Kashiwamata S, Niwa F. Studies on cellular toxicity of bilirubin: effect on the carbohydrate metabolism in the young rat brain. *Brain Res* 1975;83:81.
- Sano K, Nakamura H, Tamotsu M. Mode of inhibitory action of bilirubin on protein kinase C. *Pediatr Res* 1985;19:587.
- Gourley GR. Bilirubin metabolism and Kernicterus. *Adv Pediatr* 1997;44:173.
- Odell GB. Influence of binding on the toxicity of bilirubin. *Ann NY Acad Sci* 1973;226:225.
- Rappaport SI. *Blood-brain barrier in physiology and medicine*. New York: Raven Press, 1976.
- Lee K-S, Gartner LM. Management of unconjugated hyperbilirubinemia in the newborn. *Semin Liver Dis* 1983;3:52.
- Bowen WR, Porter E, Waters WF. The protective action of albumin in bilirubin toxicity in new born puppies. *Am J Dis Child* 1959;98:568.
- Harris RC, Lucey JF, MacLean JR. Kernicterus in premature infants associated with low concentrations of albumin in plasma. *Pediatrics* 1958;21:875.
- Satlin LM, Amin V, Wolkoff AW. Organic anion transporting polypeptide mediates organic anion/HCO₃⁻ exchange. *J Biol Chem* 1997;272:26340.
- Fleischner G, Robbins J, Arias IM. Immunological studies of Y protein: a major cytoplasmic organic anion binding protein in rat liver. *J Clin Invest* 1972;51:677.
- Rowe JD, Nieves E, Listowsky I. Subunit diversity and tissue distribution of human glutathione S-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem J* 1997;325:481.
- Kamisaka K, Gatmaitan Z, Moore CL, et al. Ligandin reverses

- bilirubin inhibition of liver mitochondrial respiration in vitro. *Pediatr Res* 1975;9:903.
45. Roy Chowdhury J, Novikoff PM, Roy Chowdhury N, et al. Distribution of uridinediphosphoglucuronate glucuronosyl transferase in rat tissues. *Proc Natl Acad Sci USA* 1985;82:2990.
 46. Mackenzie PI, Owens IS, Burchell B, et al. The UDP glucosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 1997;7:255.
 47. Bosma PJ, Seppen J, Goldhoorn B, et al. Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* 1994;269:17960.
 48. Ritter JK, Chen F, Sheen YY, et al. A novel complex locus UGT1 encodes human bilirubin, phenol and other UDP-glucuronosyltransferase isozymes with identical carboxy termini. *J Biol Chem* 1992;267:3257.
 49. Wishart GF. Functional heterogeneity of UDP-glucuronosyl transferase as indicated by its differential development and inducibility by glucocorticoids. *Biochem J* 1978;174:485.
 50. Roy Chowdhury J, Roy Chowdhury N, Moscioni AD, et al. Differential regulation by triiodothyronine of substrate-specific uridinediphosphoglucuronate glucuronosyl transferases in rat liver. *Biochim Biophys Acta* 1983;761:58.
 51. Lillienblum W, Walli AK, Bock KW. Differential induction of rat liver microsomal UDP-glucuronosyltransferase activities by various inducing agents. *Biochem Pharmacol* 1982;31:907.
 52. Ishikaowa T, Muller M, Klunemann C, et al. ATP-dependent primary active transport of cysteinyl leukotrienes transport system for glutathione S-conjugates. *J Biol Chem* 1990;265:19279.
 53. Nishida T, Gatmaitan Z, Roy Chowdhury J, et al. Two distinct mechanisms for bilirubin glucuronide transport by rat bile canalicular membrane vesicles. *J Clin Invest* 1992;90:2130.
 54. Cole SPC, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992;258:1650.
 55. Brodersen R, Herman LS. Intestinal reabsorption of unconjugated bilirubin. A possible contributing factor in neonatal jaundice. *Lancet* 1963;1:1242.
 56. Watson CJ. The urobilinoids: milestones in their history and some recent developments. In: Berk PD, Berlin NI, eds. *Bile pigments: chemistry and physiology*. Washington, DC: US Government Printing Office, 1977;469.
 57. Kapitulnik J, Ostrow JD. Stimulation of bilirubin catabolism in jaundiced Gunn rats by an inducer of microsomal mixed function mono oxygenases. *Proc Natl Acad Sci USA* 1978;75:682.
 58. Cardenas-Vazquez R, Yokosuka O, Billing BH. Enzymic oxidation of unconjugated bilirubin by rat liver. *Biochem J* 1986;236:625.
 59. Stocker R, Yamamoto Y, McDonagh AF, et al. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043.
 60. Stocker R, Peterhans E. Antioxidant properties of conjugated bilirubin and biliverdin; biologically relevant scavenging of hypochlorous acid. *Free Radic Res Commun* 1989;6:57.
 61. Vest M, Strelbel L, Hauensein D. The extent of "shunt" bilirubin and erythrocyte survival in the newborn infant measured by the administration of (15N) glycine. *Biochem J* 1965;95:11c.
 62. Levi AJ, Gatmaitan Z, Arias IM. Deficiency of hepatic organic anion-binding protein, impaired organic anion uptake by liver and "physiologic" jaundice in newborn monkeys. *N Engl J Med* 1970;283:1136.
 63. Brown AK, Zuelzer WW. Studies on the neonatal development of the glucuronide conjugating system. *J Clin Invest* 1958;37:332.
 64. Bosma PJ, Roy Chowdhury J, Bakker C, et al. A sequence abnormality in the promoter region results in reduced expression of bilirubin-UDP-glucuronosyltransferase-1 in Gilbert syndrome. *N Engl J Med* 1995;333:1171.
 65. Roy Chowdhury N, Deocharan B, Bejjanki HR, et al. The presence of a Gilbert-type promoter abnormality increases the level of neonatal hyperbilirubinemia. *Hepatology* 1997;26:370a.
 66. Maisels MJ, Newman TB. Kernicterus in otherwise healthy breast-fed term newborns. *Pediatrics* 1995;96:730.
 67. Arias IM, Gartner LM, Seifter S, et al. Prolonged neonatal unconjugated hyperbilirubinemia associated with breast feeding and a steroid, pregnane-3(alpha), 20(beta)-diol, in maternal milk that inhibits glucuronide formation in vitro. *J Clin Invest* 1964;43:2037.
 68. Foliot A, Ploussard JP, Housett E, et al. Breast milk jaundice: in vitro inhibition of rat liver bilirubin-uridine diphosphate glucuronyl transferase activity and Z protein-bromosulphophthalein binding by human breast milk. *Pediatr Res* 1976;10:594.
 69. Arias IM, Wolfson S, Lucey JE, et al. Transient familial neonatal hyperbilirubinemia. *J Clin Invest* 1965;44:1442.
 70. Robinson S, Vanier T, Desforges JE, et al. Jaundice in thalassemia minor: a consequence of ineffective erythropoiesis. *N Engl J Med* 1962;267:512.
 71. Crigler JE, Najjar VA. Congenital familial non-hemolytic jaundice with kernicterus. *Pediatrics* 1952;10:169.
 72. Childs B, Sidbury JB, Migeon CJ. Glucuronic acid conjugation by patients with familial non-hemolytic jaundice and their relatives. *Pediatrics* 1959;23:903.
 73. Berk PD, Martin JE, Blaschke TE, et al. Unconjugated hyperbilirubinemia: physiological evaluation and experimental approaches to therapy. *Ann Intern Med* 1975;82:552.
 74. Kapitulnik J, Kaufmann NA, Goitein K, et al. A pigment found in the Crigler-Najjar syndrome and its similarity to an ultra-filterable photo-derivative of bilirubin. *Clin Chim Acta* 1974;57:231.
 75. Arias IM, Gartner LM, Cohen M, et al. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronosyl transferase deficiency: clinical, biochemical, pharmacologic, and genetic evidence for heterogeneity. *Am J Med* 1969;47:395.
 76. Bosma PJ, Roy Chowdhury N, Goldhoorn BG, et al. Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I. *Hepatology* 1992;15:941.
 77. Bosma PJ, Roy Chowdhury J, Huang TJ, et al. Mechanism of inherited deficiencies of multiple UDP-glucuronosyltransferase isoforms in two patients with Crigler-Najjar syndrome, type I. *FASEB J* 1992;6:2859.
 78. Ritter JK, Yeatman MT, Ferreira P, et al. Identification of a genetic alteration in the code for bilirubin UDP-glucuronosyltransferase in the UGT1 gene complex of a Crigler-Najjar syndrome, type I. *J Clin Invest* 1992;90:150.
 79. Ciotti M, Obaray R, Martin M, et al. Genetic disease at the UGT1 locus associated with Crigler-Najjar syndrome type-1 disease, including a prenatal diagnosis. *Am J Med Genet* 1997;68:173.
 80. Clarke DJ, Moghrabi N, Monaghan G, et al. Genetic defects of UDP-glucuronosyltransferase-1 (UGT1) gene that cause familial non-hemolytic unconjugated hyperbilirubinemias. *Clin Chim Acta* 1997;266:63.
 81. Rosatelli MC, Meloni A, Faa V, et al. Molecular analysis of patients with Sardinian descent with Crigler-Najjar syndrome type 1. *J Med Genet* 1997;34:122.
 82. Ritter JK, Yeatman MT, Kaiser C, et al. Phenylalanine codon deletion at the UGT1 gene complex locus of a Crigler-Najjar type I patient generates a pH-sensitive bilirubin UDP-glucuronosyltransferase. *J Biol Chem* 1993;268:23573.
 83. Seppen J, Bosma PJ, Goldhoorn BG, et al. Discrimination

- between Crigler-Najjar type I and II by expression of mutant bilirubin uridine diphosphate-glucuronosyltransferase. *J Clin Invest* 1994;94:2385.
84. Labrune PH, Myara A, Hadchouel M, et al. Genetic heterogeneity of Crigler-Najjar syndrome type I; a study of 14 cases. *Hum Genet* 1994;94:693.
 85. Aono S, Yamada Y, Keino H, et al. A new type of defect in the gene for bilirubin uridine 5'-diphosphate-glucuronosyltransferase in a patient with Crigler-Najjar syndrome type I. *Pediatr Res* 1994;35:629.
 86. Gantla S, Bakker CTM, Deocharan B, et al. Splice site mutations: a novel genetic mechanism of Crigler-Najjar syndrome type I. *Am J Hum Genet* 1998;62:585.
 87. Erps LT, Ritter JK, Hersh JH, et al. Identification of the two single base substitutions in the UGT1 gene locus which abolish bilirubin uridine diphosphate glucuronosyltransferase activity in vitro. *J Clin Invest* 1994;93:564.
 88. Ciotti M, Chen F, Rubatelli FF, et al. Coding and a TATA Box mutation at the bilirubin UDP-glucuronosyl transferase gene cause Crigler-Najjar syndrome type 1 disease. *Biochem Biophys Acta* 1998;1407:40.
 89. Moghrabi N, Clarke DJ, Burchell B, et al. Cosegregation of intragenic markers with a novel mutation that cause Crigler-Najjar syndrome type I: implication in carrier detection and prenatal diagnosis. *Am J Hum Genet* 1993;53:722.
 90. Kadakol A, Ghosh SS, Sappal BS, et al. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert's syndrome: correlation of genotype to phenotype. *Hum Mutat* 2000;16:297.
 91. Jansen PLM, Bosma PJ, Roy-Chowdhury J. Molecular biology of bilirubin metabolism. In: Boyer JL, Ockner RK, ed. *Progress liver diseases*, vol 13. Philadelphia: WB Saunders, 1995;125.
 92. Deocharan B, Gantla S, Morton DH, et al. Interaction of a Crigler-Najjar syndrome type I mutation and a Gilbert type promoter defect results in two grades of hyperbilirubinemia in members of an Amish and a Mennonite kindred of Lancaster County, Pennsylvania. *Gastroenterology* 1997;112:1255A.
 93. Sengupta K, Gantla S, Bommineni VR, et al. Prenatal identification of Crigler-Najjar syndrome type I genotype by analysis of chorionic villus sample DNA. *Hepatology* 1994;20:320A.
 94. Gunn CH. Hereditary acholuric jaundice in a new mutant strain of rats. *J Hered* 1938;29:137.
 95. Schmid R, Axelrod J, Hammaker L, et al. Congenital jaundice in rats due to a defective glucuronide formation. *J Clin Invest* 1958;37:1123.
 96. Blaschke TF, Berk PD, Scharschmidt BF, et al. Crigler-Najjar syndrome. An unusual course with development of neurologic damage at age eighteen. *Pediatr Res* 1974;8:573.
 97. van der Veere CN, Sinaasappel M, McDonagh AF, et al. Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatology* 1996;24:311.
 98. Fox IJ, Roy Chowdhury J, Kaufman SS, et al. Treatment of Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998;338:1422.
 99. Roy Chowdhury J, Strom S, Fox IJ. Human hepatocyte transplantation: gene therapy and more? *Pediatrics* 1998;102:647.
 100. Ghosh SS, Takahashi M, Thummala NR, et al. Liver-directed gene therapy: promises, problems and prospects at the turn of the century. *J Hepatol* 2000;32:238.
 101. Arias IM. Chronic unconjugated hyperbilirubinemia without overt signs of hemolysis in adolescents and adults. *J Clin Invest* 1962;41:2233.
 102. Gollan JL, Huang SM, Billing B, et al. Prolonged survival in three brothers with severe type II Crigler-Najjar syndrome. Ultrastructural and metabolic studies. *Gastroenterology* 1975;68:1543.
 103. Gordon ER, Shaffer EA, Sass-Kortsak A. Bilirubin secretion and conjugation in the Crigler-Najjar syndrome type II. *Gastroenterology* 1976;70:761.
 104. Fevery J, Blanckaert N, Heirwegh KPM, et al. Unconjugated bilirubin and an increased proportion of bilirubin monoconjugates in the bile of patients with Gilbert's syndrome and Crigler-Najjar syndrome. *J Clin Invest* 1977;60:970.
 105. Seppen J, Steenken E, Lindhout D, et al. A mutation which disrupts the hydrophobic core of the signal peptide of bilirubin UDP-glucuronosyltransferase, an endoplasmic reticulum membrane protein, causes Crigler-Najjar type II. *FEBS Lett* 1996;390:294.
 106. Soeda Y, Yamamoto K, Adachi Y, et al. Predicted homozygous missense mutation in Gilbert syndrome. *Lancet* 1995;346:1494.
 107. Koiwai O, Nishizawa M, Hasada K, et al. Gilbert's syndrome is caused by a heterozygous missense mutation in the gene for bilirubin UDP-glucuronosyltransferase. *Hum Mol Genet* 1995;4:1183.
 108. Aono S, Adachi Y, Uyama E, et al. Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. *Lancet* 1995;345:958.
 109. Aono S, Yamada Y, Keino H, et al. Identification of a defect in the gene for bilirubin UDP-glucuronosyltransferase in a patient with Crigler-Najjar syndrome type II. *Biochem Biophys Res Commun* 1993;197:1239.
 110. Seppen J, Bosma PJ, Roy Chowdhury J, et al. Discrimination between Crigler-Najjar syndromes type I and II by expression of mutant bilirubin-UDP-glucuronosyltransferase. *J Clin Invest* 1994;94:2385.
 111. Bosma PJ, Golhoorn B, Oude Elferink RP, et al. A mutation in bilirubin uridine 5'-diphosphate glucuronosyltransferase isoforms 1 causing Crigler-Najjar syndrome type II. *Gastroenterology* 1993;105:216.
 112. Moghrabi N, Clarke DJ, Boxer M, et al. Identification of an A-to-G missense mutation in exon 2 of the UGT1 gene complex that causes Crigler-Najjar syndrome type 2. *Genomics* 1993;18:171.
 113. Koiwai O, Aono S, Adachi Y, et al. Crigler-Najjar syndrome type II is inherited both as a dominant and as a recessive trait. *Hum Mol Genet* 1996;5:645.
 114. Chalasani N, Roy Chowdhury N, Roy Chowdhury J, et al. Kernicterus in an adult who is heterozygous for Crigler-Najjar syndrome and homozygous for Gilbert type genetic defect. *Gastroenterology* 1997;112:2099.
 115. Maruo Y, Sato H, Yamano T, et al. Gilbert's syndrome caused by homozygous missense mutation (Tyr486Asp) of bilirubin-UDP glucuronosyl transferase. *J Pediatr* 1998;132:1045.
 116. Gilbert A, Lereboullet P. La cholestase simple familiale. *Semin Med* 1901;21:241.
 117. Berk PD, Bloomer JR, Howe RB, et al. Constitutional hepatic dysfunction (Gilbert's syndrome): a new definition based on kinetic studies with unconjugated radiobilirubin. *Am J Med* 1970;49:296.
 118. Felsler BF, Rickard D, Redeker AG. The reciprocal relation between caloric intake and the degree of hyperbilirubinemia in Gilbert's syndrome. *N Engl J Med* 1970;283:170.
 119. Fromke VL, Miller D. Constitutional hepatic dysfunction (CHD: Gilbert's disease): a review with special reference to a characteristic increase and prolongation of the hyperbilirubinemia in response to nicotinic acid. *Medicine (Baltimore)* 1972;51:451.
 120. Bosma PJ, Roy Chowdhury J, Bakker C, et al. A sequence abnormality in the promoter region results in reduced expression of bilirubin-UDP-glucuronosyltransferase-1 in Gilbert syndrome. *N Engl J Med* 1995;333:1171.

121. Portman OW, Roy Chowdhury J, Roy Chowdhury N, et al. A non-human primate model for Gilbert's syndrome. *Hepatology* 1984;4:175.
122. Dubin IN. Chronic idiopathic jaundice: a review of fifty cases. *Am J Med* 1958;23:268.
123. Sprinz H, Nelson RS. Persistent nonhemolytic hyperbilirubinemia associated with lipochrome-like pigment in liver cells: report of four cases. *Ann Intern Med* 1954;41:952.
124. Shani M, Seligsohn U, Gilon E, et al. Dubin-Johnson syndrome in Israel. I. Clinical, laboratory, and genetic aspects of 101 cases. *West J Med* 1970;39:549.
125. Cohen L, Lewis C, Arias IM. Pregnancy, oral contraceptives, and chronic familial jaundice with predominantly conjugated hyperbilirubinemia (Dubin-Johnson syndrome). *Gastroenterology* 1972;62:1182.
126. Erlinger S, Dhumeaux D, Desjeux JF, et al. Hepatic handling of unconjugated dyes in the Dubin-Johnson syndrome. *Gastroenterology* 1973;64:106.
127. Mandema E, De Fraiture WH, Neiweg HO, et al. Familial chronic idiopathic jaundice (Dubin-Sprinz disease), with a note on bromsulphalein metabolism in this disease. *Am J Med* 1960;28:42.
128. Ehrlick JC, Novikoff AB, Platt R, et al. Hepatocellular lipofuscin and the pigment of chronic idiopathic jaundice. *Bull NY Acad Med* 1960;36:488.
129. Arias IM, Bernstein L, Roffler R, et al. Black liver diseases in Corriedale sheep: metabolism of tritiated epinephrine and incorporation of isotope into the hepatic pigment in vivo. *J Clin Invest* 1965;44:1026.
130. Kitamura T, Alroy J, Gatmaitan Z, et al. Defective biliary excretion of epinephrine metabolites in mutant TR⁻ rats: relation to the pathogenesis of rat liver in Dubin-Johnson syndrome and Corriedale sheep with an analogous excretory defect. *Hepatology* 1992;15:1154.
131. Ware A, Eigenbrodt E, Naftalis J, et al. Dubin-Johnson syndrome and viral hepatitis. *Gastroenterology* 1974;67:560.
132. Koskelo P, Toivonen I, Adlercreutz H. Urinary coproporphyrin isomer distribution in Dubin-Johnson syndrome. *Clin Chem* 1967;13:1006.
133. Wolkoff AW, Cohen LE, Arias IM. Inheritance of the Dubin-Johnson syndrome. *N Engl J Med* 1973;288:113.
134. Paulusma CC, Bosma PJ, Zaman GJR, et al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* 1996;271:1126.
135. Tsujii H, Konig J, Rost D, et al. Exon-intron organization of the human multidrug resistance protein 2 (MRP2) gene mutated in Dubin-Johnson syndrome. *Gastroenterology* 1999;117:653.
136. Toh S, Wada M, Uchuimi T, et al. Genomic structure of the canalicular multispecific organic anion transporter gene (MRP2/cMOAT) and mutations in the ATP-binding cassetted region in Dubin-Johnson syndrome. *Am J Hum Genet* 1999;64:739.
137. Paulusma CC, Kool M, Bosma PJ, et al. A mutation in the human cMOAT gene cause the Dubin-Johnson syndrome. *Hepatology* 1997;25:1539.
138. Wada M, Toh S, Taniguchi K, et al. Mutations in the canalicular multispecific organic anion transporter gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome. *Hum Mol Genet* 1998;7:203.
139. Kajihara S, Hisatomi A, Mizuta T, et al. A splice site mutation in the human canalicular multispecific organic anion transporter (cMOAT) gene causes Dubin-Johnson syndrome. *Biochem Biophys Res Commun* 1998;253:454.
140. Oude Elferink RPJ, Meijer DKE, Kuipers F, et al. Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. *Biochim Biophys Acta* 1995;1241:215.
141. Jansen, PLM, van Klinken JW, van Gelder M, et al. Preserved organic anion transport in mutant TR rats with a hepatobiliary secretion defect. *Am J Physiol* 1993;265:G445.
142. Kobayashi K, Sogame Y, Hayashi K, et al. ATP stimulates the uptake of S-dinitrophenylglutathione by rat liver plasma membrane vesicles. *FEBS Lett* 1988;240:55.
143. Schulman FY, Montali RJ, Bush M, et al. Dubin-Johnson-like syndrome in Golden Lion Tamarins (*Leontopithecus rosalia rosalia*). *Vet Pathol* 1993;30:491.
144. Rotor AB, Manahan L, Florentin A. Familial nonhemolytic jaundice with direct van den Bergh reaction. *Acta Med Phil* 1948;5:37.
145. Dhumeaux D, Berthelot P. Chronic hyperbilirubinemia associated with hepatic uptake and storage impairment: a new syndrome resembling that of the mutant Southdown sheep. *Gastroenterology* 1975;69:988.
146. Wolkoff AW, Wolpert E, Pascasio FN, et al. Rotor's syndrome. A distinct inheritable pathophysiologic entity. *Am J Med* 1976;60:173.
147. Oda T, Elkaholoun AG, Meltzer PS, et al. Identification and cloning of the human homolog (Jag1) of the rat jagged 1 gene from the Alagille syndrome critical region at 20p12. *Genomics* 1997;43:376.

