

- 27 Vega RB, Huss JM, Kelly DP (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor  $\alpha$  in the transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20, 1868–1876.
- 28 Lehman JJ, Barger PM, Kovacs A *et al.* (2000) Peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest* 106, 847–856.
- 29 Zong H, Ren JM, Young LH *et al.* (2002) AMP kinase is required for mitochondrial biogenesis in response to chronic energy deprivation. *Proc Natl Acad Sci USA* 25, 15983–15987.
- 30 McGarry JD, Foster DW (1980) Regulation of hepatic fatty acid oxidation and ketone body production. *Annu Rev Biochem* 49, 395–420.
- 31 Browning JD, Horton JD (2004) Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 114, 147–152.
- 32 Yamashita H, Takenoshita M, Sakurai M *et al.* (2001) A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc Natl Acad Sci USA* 98, 9116–9121.
- 33 Lizuka K, Bruick RK, Liang G *et al.* (2004) Deficiency of carbohydrate response element binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc Natl Acad Sci USA* 101, 7281–7286.
- 34 Pessayre D, Fromenty B (2005) NASH: a mitochondrial disease. *J Hepatol* 42, 928–940.
- 35 Brady LJ, Brady PS, Rosmos DR (1985) Elevated hepatic mitochondrial and peroxisomal oxidative capacities in fed and starved adult obese (ob/ob) mice. *Biochem J* 231, 439–444.
- 36 Cook J, Gamble MSV (1987) Regulation of carnitine palmitoyl transferase by insulin results in decreased activity and decreased apparent  $K_i$  values for malonyl CoA. *J Biol Chem* 262, 2050–2055.
- 37 Chavin KD, Yang SQ, Lin HZ *et al.* (1999) Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *J Biol Chem* 274, 5692–5700.
- 38 St-Pierre J, Buckingham JA, Roebuck SJ *et al.* (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277, 44784–44790.
- 39 Kowaltowski AJ, Vercesi AE (2001) Reactive oxygen generation by mitochondria. In: Lemasters JJ, Nieminen AL (eds) *Mitochondria in Pathogenesis*. New York: Kluwer Academic/Plenum Publishers, pp. 281–300.
- 40 Walden WE (2002) From bacteria to mitochondria: aconitase yields surprises. *Proc Natl Acad Sci USA* 99, 4138–4140.
- 41 Demeilliers C, Maisonneuve C, Grodet A *et al.* (2002) Impaired adaptive resynthesis and prolonged depletion of hepatic mitochondrial DNA after repeated alcohol binges in mice. *Gastroenterology* 123, 1278–1290.
- 42 Elmore SP, Qian T, Grissom SF *et al.* (2001) The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 15, 2286–2287.
- 43 De Grey ADNJ (2002)  $\text{HO}_2\cdot$ : the forgotten radical. *DNA Cell Biol* 21, 251–257.
- 44 Wallace MA, Liou LL, Martins J *et al.* (2004) Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase. *J Biol Chem* 279, 32055–32062.
- 45 Szczesny B, Hazra TK, Papaconstantinou J *et al.* (2003) Age-dependent deficiency in import of mitochondrial DNA glycosylases required for repair of oxidatively damaged bases. *Proc Natl Acad Sci USA* 100, 10670–10675.
- 46 Cortopassi GA, Shibata D, Soong NW *et al.* (1992) A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. *Proc Natl Acad Sci USA* 89, 7370–7374.
- 47 Wang Y, Michikawa Y, Mallidis C *et al.* (2001) Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication. *Proc Natl Acad Sci USA* 98, 4022–4027.

## 2.3.5 Bilirubin metabolism

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### Bilirubin in medical history

Bilirubin has attracted the attention of physicians since antiquity. Its chemistry, metabolism and disposal have been studied systematically during the last two centuries as a model for hepatic disposal of biologically important organic anions of limited aqueous solubility [1]. The discovery of several inherited disorders of bilirubin metabolism and excretion during the twentieth century has led to renewed interest in inherited diseases associated with jaundice, some of which continue to pose a therapeutic challenge, providing impetus for further research. While physicians are mainly concerned with the toxic effect of bilirubin and its importance as a liver function test, the antioxidant property of bilirubin may provide a physiological defence against oxidative injury.

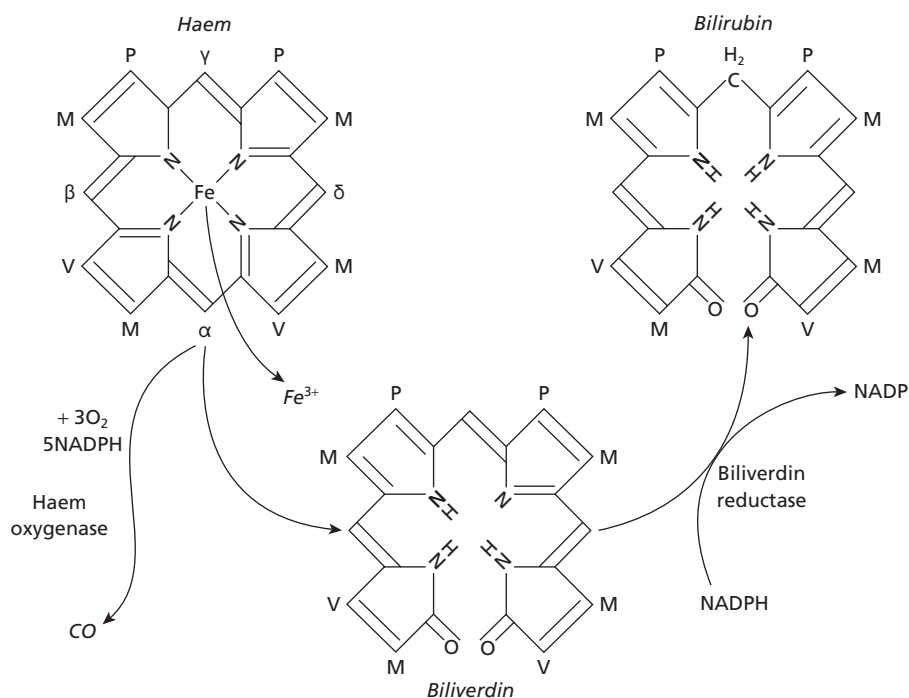
### Formation of bilirubin

#### Sources of bilirubin

Bilirubin is the breakdown product of the haem moiety of haemoglobin, other haemoproteins, such as cytochromes, catalase, peroxidase and tryptophan pyrrolase, and a small pool of free haem. In humans, 250–400 mg of bilirubin is produced daily, of which approximately 20% is produced from non-haemoglobin sources [2]. Following the injection of radio-labelled porphyrin precursors (glycine or  $\delta$ -aminolaevulinic acid), an ‘early-labelled peak’ of bilirubin (ELP) is excreted in bile within 72 h [3]. The initial component of ELP is derived mainly from hepatic haemoproteins. This is followed by a slower component, derived from both erythroid and non-erythroid sources, which becomes prominent in conditions associated with ‘ineffective erythropoiesis’, e.g. congenital dyserythropoietic anaemias, megaloblastic anaemias, iron-deficiency anaemia, erythropoietic porphyria and lead poisoning [4], and in accelerated erythropoiesis [5]. The ‘late-labelled peak’ appears at approximately 110 days in humans and coincides with the half-life of erythrocytes.

#### Enzymatic mechanism of bilirubin formation

The microsomal haem oxygenase (HO) enzymes catalyse the oxidation of haem (Fig. 1). Three molecules of  $\text{O}_2$  are consumed



**Fig. 1** Enzyme-catalysed degradation of haem. Haem degradation begins by haem oxygenase-catalysed oxidation of the  $\alpha$ -bridge carbon of haem, which is converted to CO, leading to opening of the tetrapyrrole ring and release of the iron molecule. The resulting biliverdin molecule is subsequently reduced to bilirubin by cytosolic biliverdin reductase.

in this reaction and a reducing agent, such as nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), is needed. The  $\alpha$ -methene bridge carbon is eliminated as CO and the iron molecule is released [6]. Of the three forms of HO, HO-1 is ubiquitous and inducible by haem [8] and stress [7]; HO-2 is a constitutive protein, expressed mainly in the brain and the testis. The catalytic activity of HO-3 is low, and this protein may function mainly as a haem binding protein. CO produced by HO activity has a vasodilatory effect and regulates the vascular tone in the liver, heart and other organs during stress. Similarly, biliverdin and its product bilirubin are potent antioxidants, which may protect tissues under oxidative stress [7,9] (see below).

Biliverdin is reduced to bilirubin by the action of cytosolic biliverdin reductases, which require NADH or NADPH for activity [10]. As discussed later, bilirubin requires energy-consuming metabolic steps for excretion in bile. Thus, the physiological advantage of its formation is not clear. The strong antioxidant activity of bilirubin may be particularly important during the neonatal period, when other antioxidants are scarce in body fluids.

### Measurement of bilirubin production

Bilirubin production can be quantified from the turnover of intravenously administered radioisotopically labelled bilirubin. Plasma bilirubin clearance is proportional to the reciprocal of the area under the radiobilirubin disappearance curve [11]. Bilirubin removal is calculated as the product of plasma bilirubin concentration and clearance. At a steady state of plasma bilirubin concentration, bilirubin removal equals bilirubin

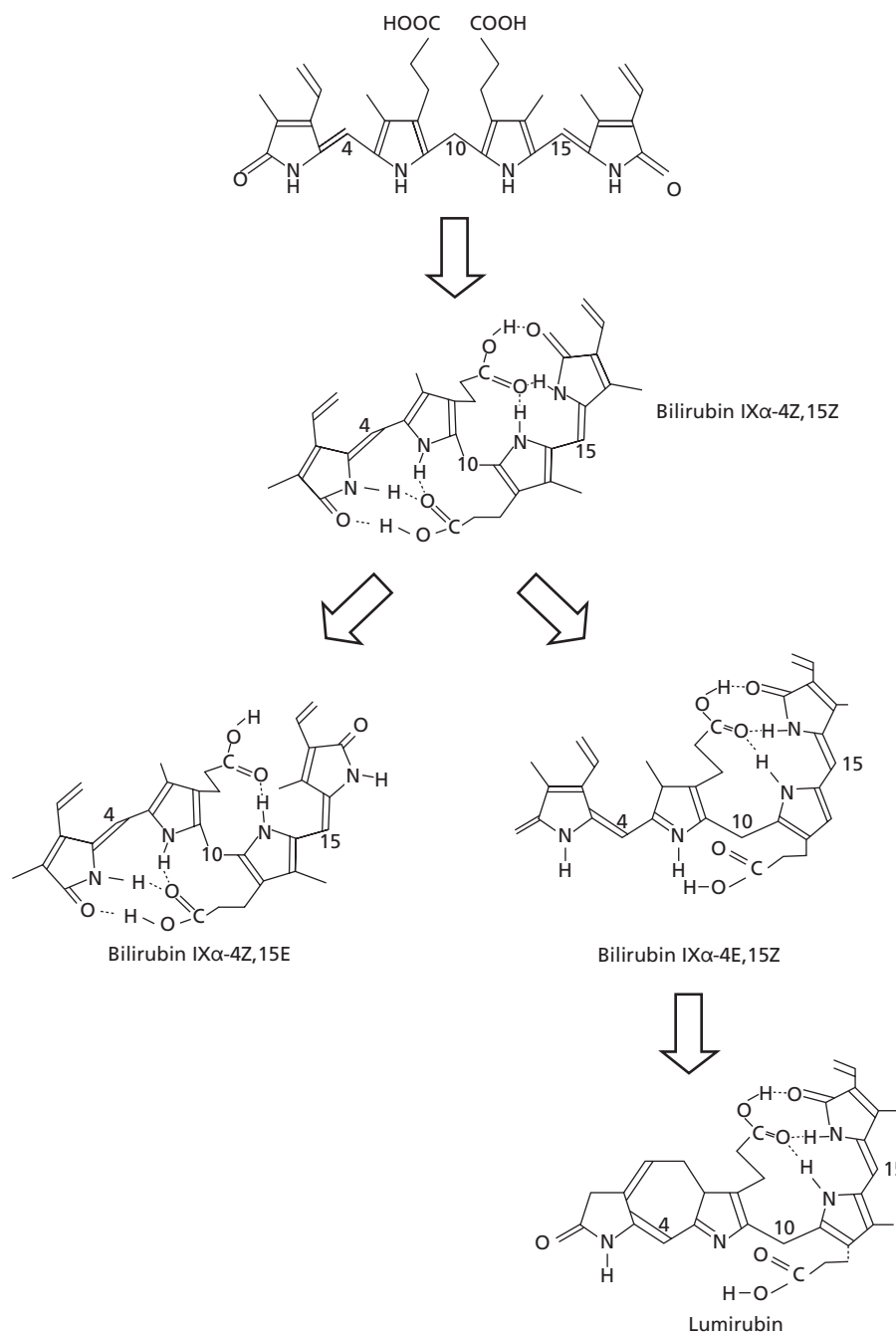
production. More conveniently, bilirubin formation can be quantified from CO, which is generated in equimolar amounts with bilirubin. Following rebreathing in a closed system, CO production is calculated from the CO concentration in the rebreathing mask and/or the increment in blood carboxyhaemoglobin saturation [12]. A small fraction of the CO may be formed by intestinal bacteria, which can be a significant source of CO in intestinal bacterial overgrowth syndromes [13].

### Inhibition of bilirubin production

Substances, such as tin-protoporphyrin and tin-mesoporphyrin, that bind irreversibly to HO, but are not broken down, serve as 'dead-end' inhibitors of the enzyme and reduce bilirubin production [14]. Injection of tin-mesoporphyrin lowers serum bilirubin levels by 76% in neonates [15].

### Chemical characteristics of bilirubin

The tetrapyrrole structure of bilirubin IX $\alpha$  (1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid [17]) was solved by Fischer and Plieninger [18]. X-ray crystallography has revealed that the propionic acid side-chains of bilirubin form hydrogen bonds with the pyrrolic and lactam sites on the opposite half of the molecule, giving rise to a distorted 'ridge tile' structure [19] (Fig. 2). Formation of hydrogen bonds requires the interpyrrolic bridges at the 5 and 15 position of bilirubin to be in *trans* or 'Z' configuration, whereby bilirubin is termed bilirubin IX $\alpha$ -ZZ. Engagement of all polar groups (two propionic acid carboxyls, four NH groups and two lactam oxygens) of bilirubin by the hydrogen bonds makes the molecule insoluble



**Fig. 2** Internal hydrogen bonding and photoisomerization of bilirubin. The carboxylic acid moiety of the propionic acid side-chains of bilirubin form internal hydrogen bonds with contralateral NH groups and the lactam oxygen, thereby engaging all polar groups of the molecule and making it insoluble in water. Upon exposure to light, configurational changes (Z to E) occur at the C4 and C15 interpyrrolic bridges, disrupting the hydrogen bonds. The bilirubin IX $\alpha$ -4E,15Z configurational isomer can be cyclized forming the so-called lumirubin. These configurational and geometric isomers are more polar than the hydrogen-bonded bilirubin IX $\alpha$ -4Z,15Z and are excreted in bile without requiring glucuronidation.

in water, necessitating chemical modification for excretion in bile. Disruption of the hydrogen bonds is accomplished *in vivo* by enzyme-catalysed esterification of the propionic acid carboxyl groups with a glycosyl moiety, mainly glucuronic acid (*vide infra*).

The hydrogen bonds 'bury' the central methane bridge, so that the unconjugated bilirubin reacts very slowly with diazo reagents, whereas bilirubin glucuronides, which lack hydrogen bonds, react rapidly ('direct' van den Bergh reaction). The addition of 'accelerators' such as methanol, ethanol, 6 M urea or

dimethyl sulphoxide to plasma disrupts the hydrogen bonds of bilirubin, so that both conjugated and unconjugated bilirubin react rapidly with diazo reagents ('total' van den Bergh reaction).

Bilirubin glucuronides in normal bile are 1-O-acyl conjugates linked to the propionic acid carboxyl of bilirubin in a  $\beta$ -D-ester linkage, which is hydrolysable by  $\beta$ -glucuronidase. However, during cholestasis, the migration of the 1-O-acyl bond from the C1 position to the C2, C3 or C4 position results in the generation of  $\beta$ -glucuronidase-resistant pigments [20], which are detectable in serum and bile by chromatographic analysis [21].

In cases of prolonged accumulation of conjugated bilirubin in plasma, as in cases of cholestasis or Dubin–Johnson syndrome, the pigment may become covalently bound to albumin [22]. This irreversibly protein-bound form, often termed delta-bilirubin, is included in the ‘direct’ fraction of bilirubin and is not eliminated in the bile or urine, which results in delayed clearance even after biliary obstruction or cholestasis is resolved.

### Effect of light

The main absorption band of unconjugated bilirubin IX $\alpha$  is at 450–474 nm in most organic solvents. Upon exposure to light, the ‘Z’ (*trans*) configuration of the 5 and/or 15 carbon bridges of bilirubin switches to the ‘E’ (*cis*) configuration. The resulting configurational isomers, ZE, EZ or EE, lack internal hydrogen bonds, are more polar than bilirubin IX $\alpha$ -ZZ and can be excreted in bile without conjugation [23]. The non-hydrogen-bonded molecule can be stabilized slowly by cyclization of the vinyl substituent in the endovinyl half of bilirubin IX $\alpha$ -EZ with the methyl substituent on the internal pyrrole ring, forming the stable structural isomer, E-cyclobilirubin. Because of its stability, this molecule is quantitatively important during phototherapy for neonatal jaundice [24]. Light and oxygen can also degrade a fraction of the bilirubin molecules into colourless fragments and biliverdin [25].

### Quantification of bilirubin

Bile pigments can be quantified as native or derivatized tetrapyrroles, or after conversion to azoderivatives. Conversion to azodipyrroles by reaction with diazo reagents is the most common method of measuring serum bilirubin levels in clinical laboratories. Electrophilic attack on the central bridge splits bilirubin into two diazotized azodipyrrole molecules. As discussed above, conjugated bilirubin reacts rapidly (‘direct’ fraction), while total bilirubin is determined after adding an accelerator. Unconjugated bilirubin is calculated by subtracting the direct fraction from total bilirubin. As 10–15% of unconjugated bilirubin may give a ‘direct’ diazo reaction, this method slightly overestimates conjugated bilirubin.

Bilirubin and its conjugates in serum or bile can be quantified more accurately as intact bilirubin tetrapyrroles by high-pressure liquid chromatography [26–28]. Bilirubin mono- and diconjugates can be converted to methyl esters by alkaline methanolysis prior to separation [29] but, because the sugar groups are cleaved off, this method does not permit identification of specific conjugates.

For repeated bilirubin measurements in jaundiced infants, as an extension of clinical evaluation of jaundice, bilirubin levels can be assessed by measurement of the intensity of yellow discoloration of the skin using a special reflectance photometer [30]. Two slide tests (Ektachem) are available for determination of total bilirubin and the unconjugated, conjugated and irreversibly protein-bound fractions.

### Bilirubin toxicity

Unconjugated bilirubin is toxic to many cell types, intracellular organelles and physiological processes. Bilirubin inhibits DNA synthesis [31] and ATPase activity of brain mitochondria [32], and uncouples oxidative phosphorylation. It has been reported to inhibit Ca<sup>2+</sup>-activated, phospholipid-dependent protein kinase C activity and cAMP-dependent protein kinase activity [33]. Which of these toxic effects is the predominant cause of bilirubin encephalopathy remains unclear at this time. Clinically, toxic effects of bilirubin, particularly on the brain, are seen in neonates and patients with severe inherited deficiency of bilirubin conjugation. Yellow discoloration of the hippocampus, basal ganglia and nuclei of the cerebellum and brain stem, found in infants with acute bilirubin encephalopathy, is termed kernicterus. Such discoloration is not found in patients with chronic encephalopathy, in whom focal necrosis of neurons and glia is seen [34].

As all toxic effects of bilirubin are abrogated by tight binding to albumin, cerebral toxicity is usually seen when there is a molar excess of bilirubin over albumin in plasma. At serum-unconjugated bilirubin concentrations over 20 mg/dL, newborn babies are at risk of kernicterus. However, kernicterus can occur at lower concentrations in the presence of substances such as sulphonamides, radiographic contrast dyes and coumarin, which inhibit albumin–bilirubin binding by competitive or allosteric displacement [35,36]. Although immaturity of the blood–brain barrier in neonates has been implicated in the increased susceptibility of neonates to kernicterus, evidence to support this concept is insufficient. Normally, bilirubin entering the brain is cleared rapidly, but the pigment may bind to damaged and oedematous brain inhibiting its clearance, thereby increasing the susceptibility to bilirubin encephalopathy [37].

### Potential beneficial effects of products of haem breakdown

Although clinicians are mainly concerned with the importance of bilirubin levels as a marker of liver disease and with the toxic effects of the pigment, biliverdin and bilirubin may exert some beneficial effects by virtue of their strong antioxidant properties. This may be relevant during the newborn period, when the level of other natural antioxidants is low. Bilirubin, which is toxic to neuronal cells at high concentrations, has been reported to have cytoprotective activity at lower concentrations. An inverse relationship between serum bilirubin levels and risk of ischaemic coronary artery disease has been observed [38], although whether such a protective effect extends to subjects with Gilbert syndrome is questionable [39]. Study of a large number of subjects in the United States has shown that the odds ratio for colorectal cancer is reduced to 0.295 in men and 0.186 in women per 1 mg/dL increment in serum bilirubin levels [40]. Similarly, a previous large study showed an inverse relationship between serum bilirubin levels and cancer mortality in a Belgian

population [41]. However, such associations do not conclusively prove a causative role for bilirubin, because possible confounding variables may exist.

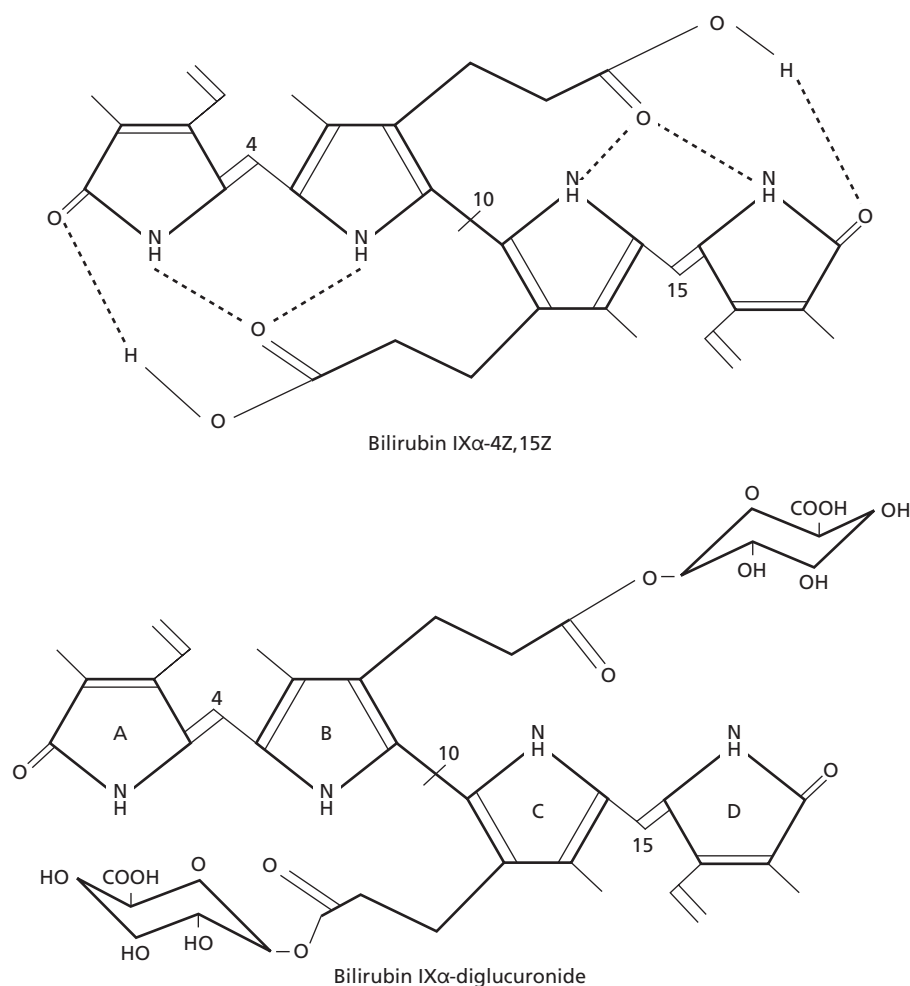
### Bilirubin in body fluids

About 4% of bilirubin in normal plasma is conjugated, but the clinical diazo-based methods overexpress this fraction (see above). In haemolytic jaundice, there is a proportional increase in 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. Haem in subcutaneous haematomas 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 conju-

gated bilirubin, which is less strongly bound to albumin, appears in urine. Bilirubin is present in normal human bile predominantly as diglucuronide, with bilirubin monoglucuronide and unconjugated bilirubin accounting for less than 10% and 1–4% of the pigments respectively. In the presence of reduced bilirubin glucuronidating capacity of the liver, as in Gilbert syndrome and Crigler–Najjar syndrome type 2 (see Chapter 16.6), the proportion of bilirubin monoglucuronide increases to 30% or above. In addition to the glucuronides, small amounts of glucosyl, xylosyl and mixed conjugates of bilirubin are found in human bile.

### Disposition of bilirubin

Disposition of bilirubin by hepatocytes comprises several specific steps, including transport of bilirubin to hepatocytes from sites of production, uptake by and storage within hepatocytes, enzyme-catalysed conjugation with glucuronic acid, active transport into the bile canaliculus and degradation in the intestinal tract. These steps are summarized in Figure 3 and discussed briefly below.



**Fig. 3** Glucuronidation disrupts internal hydrogen bonding of bilirubin. Glucuronidation of the propionic acid carboxyl groups results in disruption of the internal hydrogen bonds, making the molecule more polar and secretible in bile. Disruption of hydrogen bonding exposes the central CHH bridge to diazo reagents, whereby bilirubin glucuronides give the direct van den Bergh reaction.

## Transport in plasma

Unconjugated bilirubin circulates in plasma bound tightly but reversibly to albumin, which prevents its excretion in urine, except during albuminuria. Albumin binding keeps bilirubin in solution and abrogates its toxic effects. Conjugated bilirubin is bound less tightly to albumin, and the unbound fraction is excreted in the urine. As mentioned above, during prolonged conjugated hyperbilirubinaemia, a fraction of conjugated bilirubin 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 [22]. A small unbound fraction of unconjugated bilirubin is thought to be responsible for its toxicity [42]. Albumin has one high-affinity primary binding site for bilirubin. Additional sites are occupied when bilirubin is in molar excess. Normal plasma concentration of albumin (500–700  $\mu\text{mol/L}$ ) exceeds that of bilirubin (3–17  $\mu\text{mol/L}$ ). However, during exaggerated neonatal jaundice and in patients with Crigler–Najjar syndrome, the molar concentration of unconjugated bilirubin may exceed that of albumin. Hypoalbuminaemia resulting from inflammatory states, chronic malnutrition or liver disease may precipitate bilirubin toxicity. Sulphonamides, anti-inflammatory drugs, cholecystographic contrast media, fusidic acid, azapropazone, sodium caprylate and N-acetyl tryptophan displace bilirubin from albumin and increase the risk of kernicterus in jaundiced infants [43]. Binding of short-chain fatty acids to albumin causes conformational changes, decreasing bilirubin binding. Because of its pathophysiological importance, various methods have been devised to measure the unbound fraction of bilirubin and the reserve albumin binding capacity. These include ultrafiltration, ultracentrifugation, gel chromatography, affinity chromatography on albumin agarose polymers, dialysis and electrophoresis. Rapid degradation of unbound bilirubin by  $\text{H}_2\text{O}_2$  and horseradish peroxidase has been used to distinguish it from the bound fraction.

## Uptake by hepatocytes

At the sinusoidal surface of the hepatocyte (Fig. 4), bilirubin dissociates from albumin and is taken up by the hepatocyte by facilitated diffusion that requires inorganic anions, such as  $\text{Cl}^-$ . The protein(s) involved in sinusoidal bilirubin uptake have not been identified. A member of the organic anion transport protein family, termed OATP2 (also termed SLC21A6), has been proposed as the sinusoidal bilirubin transporter [44], but its importance in bilirubin transport has been questioned [45].

## Storage within the liver cell

After entering the hepatocyte, bilirubin binds to the major cytosolic proteins, glutathione-S-transferases (GSTs, formerly designated ligandin or Y-protein). The GST proteins, which

constitute 5% of the liver cytosol, bind various drugs, hormones, organic anions [46], a cortisol metabolite [47] and azo-dye carcinogens [48]. Bilirubin is a ligand for GSTs, but not a substrate for glutathione transfer. Binding to GSTs reduces the efflux of bilirubin from hepatocytes, thereby increasing its net uptake (Fig. 4). GST binding inhibits non-specific diffusion of bilirubin into various subcellular compartments, thereby preventing specific organellar toxicity, such as inhibition of mitochondrial respiration by bilirubin that is seen *in vitro* [49].

## Conjugation of bilirubin

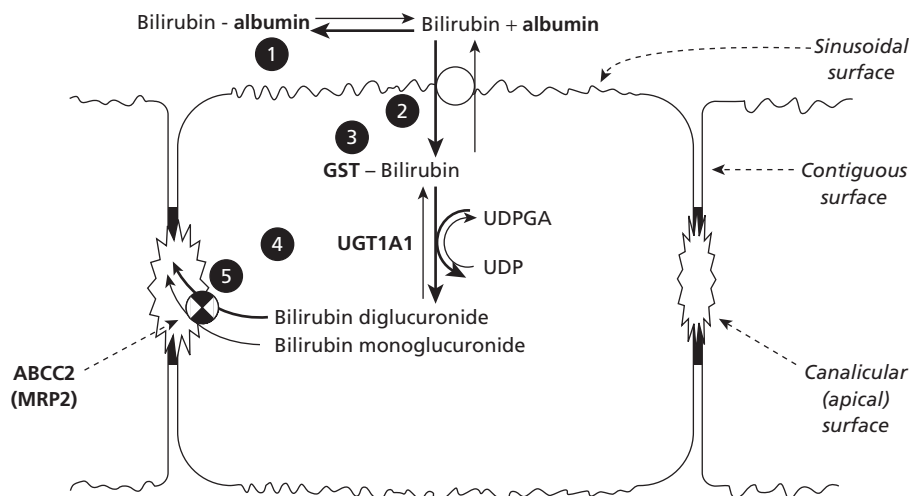
Conversion of unconjugated bilirubin to bilirubin diglucuronide or monoglucuronide by esterification of both or one of the propionic acid carboxyl groups is critical for efficient biliary excretion of bilirubin (Fig. 4).

### Bilirubin uridine diphosphoglucuronate glucuronosyltransferase

Bilirubin is one of the many endogenous and exogenous substrates whose conjugation with glucuronic acid is mediated by one or more isoform of uridine diphosphoglucuronate glucuronosyltransferase (UGTs). UGTs are enzymes concentrated in the endoplasmic reticulum (ER) and nuclear envelope of many cell types [50]. They catalyse the transfer of the glucuronic acid moiety of UDP-glucuronic acid to the aglycone substrates, forming polar and usually less bioreactive products. Bilirubin glucuronidation is catalysed predominantly by a single UGT isoform, UGT1A1 [51]. The UGT superfamily of genes comprises two major families, UGT1 and UGT2. Nine isoforms within the UGT1A subfamily are expressed from a series of exons clustered in a unique manner on chromosome 2 at the 2q37 region [61]. Four consecutive exons (exons 2–5) located at the 3' end of the UGT1A locus are used in nine different mRNAs. These encode the identical carboxy-terminal domains of these UGT isoforms, which contain the UDP-glucuronic acid binding site. Upstream of these four common region exons is a series of unique exons, each preceded by a separate promoter. Only one of these exons is utilized in a specific UGT mRNA. The unique exon encodes the variable N-terminal domain of the nine different UGT isoforms that impart aglycone specificity to the individual isoforms. Depending on which promoter is used, transcripts of various lengths are generated. In all cases, the unique exon, located at the 5' end of the transcript, is spliced to exon 2, and the intervening sequence is spliced out. The genes are named according to the unique first exon. Thus, UGT1A1 utilizes the unique exon 1A1, UGT1A6 utilizes exon 1A6, etc. [53].

The presence of a separate promoter upstream from each unique region exon permits differential regulation of individual UGT isoforms during development and in response to inducing agents. UGT1A1 is expressed after birth [54] and is induced by phenobarbital and clofibrate [55]. Delayed expression of UGT1A1 is a major cause of neonatal hyperbilirubinaemia in primates. Treatment of rats with triiodothyronine markedly

**Fig. 4** Bilirubin throughput by hepatocytes. Bilirubin is transported from sites of production to hepatic sinusoids bound to albumin (1). At the sinusoidal surface of hepatocytes, bilirubin dissociates from albumin and enters hepatocytes by facilitated diffusion (2). Binding to cytosolic glutathione-S-transferases (GSTs) increases net uptake of bilirubin by inhibiting its efflux (3). Bilirubin is converted to mono- and diglucuronide by the action of UGT1A1, which catalyses the transfer of the glucuronic acid moiety from UDP-glucuronic acid (UDPGA) to bilirubin (4). Bilirubin glucuronides are actively transported into bile against a concentration gradient by the ATP-utilizing pump ABCC2 (also termed MRP2) (5).



reduces UGT activity towards bilirubin, whereas the activity towards 4-nitrophenol is increased [56].

In humans, the expression of UGT1A1 is limited to hepatocytes and, to a lesser extent, the proximal small intestine. UGTs are integral to ER membranes. In addition to the enzyme content, UGT1A1 activity is affected by the lipids of the ER membrane. UGT activity in native microsomal vesicles is latent [57], probably because the ER membranes pose a barrier to the polar sugar donor UDP-glucuronic acid or as a result of the constraint of the enzyme by the membranes. Based on hydrophobicity analysis, the major portion of mature UGT molecules, including the UDP-glucuronic acid and the aglycone binding sites, is thought to be located within the ER cisternae. There is a single 17-amino-acid membrane-spanning segment and a 26-amino-acid cytoplasmic tail at the carboxy-terminal end of the molecule. Full enzyme activity is manifested *in vitro* by treatment of the microsomes with membrane-permeabilizing agents, such as digitonin or alamethacin. UDP-N-acetylglucosamine (UDP-glucNac) stimulates the internalization of UDP-glucuronic acid into intact microsomal vesicles and is thought to be the natural activator of UGTs within hepatocytes. UGT1A1 forms homodimers within the ER membrane, which may be required for its full catalytic activity [58]. In addition, it may interact with other UGT isoforms, as well as other proteins of the ER.

### Canalicular excretion of conjugated bilirubin

Conjugated bilirubin undergoes unidirectional transport into the bile against a concentration gradient, so that bilirubin concentration in the bile can be as high as 150-fold that in the hepatocyte. The electrochemical gradient of  $-35$  mV, generated by the sodium pump, may help in the canalicular transport but, by itself, is too small to account for this large concentration gradient. The energy for the uphill transport of bilirubin and many other non-bile salt organic anions is derived from adenosine

triphosphate (ATP) hydrolysis by the canalicular ATP-binding cassette protein, ABCC2 [also termed the MDR-related protein 2 (MRP2) or the multispecific organic anion transporter, MOAT]. ABCC2 pumps glutathione-, glucuronic acid- or sulphate-conjugated compounds across the canalicular membrane [59,60]. Canalicular transport of organic anions is unidirectional from the cytoplasm of the hepatocyte into the bile. Canalicular transport may be assisted by the membrane potential, but the contribution of membrane potential in organic anion transport has not been quantified. Mutant animals that lack ATP-dependent canalicular transport of non-bile acid organic anions retain normal activity with respect to potential-driven canalicular transport of non-bile acid organic anions, including bilirubin glucuronides [60]. The ATP-dependent canalicular organic anion transport is mediated by a canalicular membrane protein, termed canalicular multispecific organic anion transporter (cMOAT) or MRP2 [61].

Maximal bilirubin secretory capacity ( $T_{max}$ ) into the bile canaliculus depends on bile flow, which has bile salt-dependent and non-bile salt-dependent components. Bile acids increase the trafficking of vesicles containing MRP2 and the bile salt export pump (BSEP) from the Golgi apparatus to the apical domain of hepatocyte plasma membranes, thereby increasing the concentration of the transporters in the canalicular membrane [61].

### Fate of bilirubin in the gastrointestinal tract

Although conjugated bilirubin is not substantially absorbed from the intestines, a fraction of the small amount of unconjugated bilirubin that is excreted in bile is absorbed and undergoes enterohepatic circulation. In situations in which increased amounts of unconjugated bilirubin are excreted in bile, such as e.g. during phototherapy for neonatal jaundice or Crigler-Najjar syndrome, absorption of unconjugated bilirubin from the intestine may be clinically significant [62]. In these cases,

interruption of bilirubin reabsorption by ingestion of various substances, including calcium salts, can enhance the effect of phototherapy [63].

Degradation of bilirubin by intestinal bacteria generates urobilinogen and related products [64]. A major portion of the urobilinogen reabsorbed from the intestine is excreted in bile, but a small fraction is excreted in urine. Urobilinogen is colourless; its oxidation product, urobilin, contributes to the colour of normal urine and stool. During severe intrahepatic cholestasis or complete obstruction of the bile duct, urobilinogen and urobilin are absent in urine and stool, resulting in pale (so-called clay-coloured) stool. In liver disease and states of increased bilirubin production, urinary urobilinogen excretion is increased.

### Alternative routes of bilirubin elimination

In the absence of bilirubin glucuronidation, a fraction of bilirubin is excreted as hydroxylated products [65], probably by the action of microsomal P450s [66] and mitochondrial bilirubin oxidase in liver [67] and other tissues.

During intrahepatic or extrahepatic cholestasis, conjugated bilirubin accumulates in plasma. In total biliary obstruction, renal excretion becomes the major pathway of bilirubin excretion [68]. Renal excretion of conjugated bilirubin depends on glomerular filtration of the non-protein-bound fraction of conjugated bilirubin.

### References

- Chen TS, Chen PS (1984) *Understanding the Liver. A History*. Westport, CT: Greenwood Press, p. 99.
- London IM, West R, Shemin D *et al.* (1950) On the origin of bile pigment in normal man. *J Biol Chem* 184, 351–358.
- Schwartz S, Johnson JA, Stephenson BD *et al.* (1971) Erythropoietic defects in protoporphyria: a study of factors involved in labelling of porphyrins and bile pigments from ALA-<sup>3</sup>H and glycine-<sup>14</sup>C. *J Lab Clin Med* 78, 411–434.
- Robinson SH (1977) Origins of the early-labeled peak. In: Berk PD, Berlin NI (eds) *Bile Pigments: Chemistry and Physiology*. Washington, DC: US Government Printing Office, pp. 175–188.
- Come SE, Shohet SB, Robinson SH (1974) Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythroid stimulation or iron deficiency anemia. *Blood* 44, 817–830.
- Tenhunen R, Marver HS, Schmid R (1969) Microsomal heme oxygenase: characterization of the enzyme. *J Biol Chem* 244, 6388–6394.
- Elbirt KK, Bonkovsky HL (1999) Heme oxygenase: recent advances in understanding its regulation and role. *Proc Assoc Am Phys* 111, 438–447.
- Ishizawa S, Yoshida T, Kikuchi G (1983) Induction of heme oxygenase in rat liver. *J Biol Chem* 258, 4220–4225.
- Hayashi S, Takamiya R, Yamaguchi T *et al.* (1999) Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. *Circ Res* 85, 663–671.
- Tenhunen R, Ross ME, Marver HS *et al.* (1970) Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. *Biochemistry* 9, 298–303.
- Jones EA, Bloomer JR, Berk PD *et al.* (1977) Quantitation of hepatic bilirubin synthesis in man. In: Berk PD, Berlin NI (eds) *Bile Pigments: Chemistry and Physiology*. Washington, DC: US Government Printing Office, pp. 189–205.
- Berk PD, Rodkey FL, Blaschke TF *et al.* (1974) Comparison of plasma bilirubin turnover and carbon monoxide production in man. *J Lab Clin Med* 83, 29–37.
- Westlake DW, Roxburgh JM, Talbot G (1961) Microbial production of carbon monoxide from flavinoids. *Nature* 189, 510–511.
- Kappas A, Drummond GS, Henschke C *et al.* (1995) Direct comparison of tin-mesoporphyrin, an inhibitor of bilirubin production, and phototherapy in controlling hyperbilirubinemia in term and near-term newborns. *Pediatrics* 95 (4), 468–474.
- Valaes T, Petmezaki S, Henschke C *et al.* (1994) Control of jaundice in preterm newborns by an inhibitor of bilirubin production: studies with tin-mesoporphyrin. *Pediatrics* 93 (1), 1–11.
- Berk PD, Jones EA, Howe RB *et al.* (1980) Disorders of bilirubin metabolism. In: Bondy PK, Rosenberg LE (eds) *Metabolic Control and Disease*, 8th edn. Philadelphia: Saunders, p. 1009.
- Grandchamp B, Bissel DM, Licko V *et al.* (1981) Formation and disposition of newly synthesized heme in adult rat hepatocytes in primary cultures. *J Biol Chem* 256, 11677–11683.
- Fischer H, Plieninger H (1942) Synthese des biliverdins (uteroverdins) und bilirubins der biliverdine XIII, und III, sowie der Vinulneoxanthosaure. *Hoppe Seyler Z Physiol Chem* 274, 231.
- Bonnett R, Davis E, Hursthouse MB (1976) Structure of bilirubin. *Nature* 262 (5566), 327–328.
- Compernelle F, Van Hees GP, Blanckaert N *et al.* (1978) Glucuronic acid conjugates of bilirubin-IX $\alpha$  in normal bile compared with post-obstructive bile. Transformation of the 1-O-acylglucuronide into 2-, 3-, and 4-O-acylglucuronides. *Biochem J* 171, 185–201.
- Jansen PL (1981)  $\beta$ -Glucuronidase-resistant bilirubin glucuronides in cholestatic liver disease – determination of bilirubin metabolites in serum by means of high-pressure liquid chromatography. *Clin Chim Acta* 110, 309–317.
- Lauff JJ, Kasper ME, Ambros RT (1983) Quantitative liquid chromatographic estimation of bilirubin species in pathological serum. *Clin Chem* 29, 800–805.
- McDonagh AF, Palma LA, Lightner DA (1982) 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* 104, 6867.
- Itho S, Onishi S (1985) 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* 226, 251–258.
- McDonagh AF (1975) Thermal and photochemical reactions of bilirubin IX. *Ann NY Acad Sci* 244, 553–569.
- Onishi S, Itho S, Kawade N *et al.* (1980) An accurate and sensitive analysis by high pressure liquid chromatography of conjugated and unconjugated bilirubin IX $\alpha$  and in various biological fluids. *Biochem J* 185, 281–284.
- Spivak W, Carey MC (1985) Reverse-phase h.p.l.c. separation, quantification and preparation of bilirubin and its conjugates from native bile. *Biochem J* 225, 787–805.

- 28 Roy Chowdhury J, Roy Chowdhury N (1982) Quantitation of bilirubin and its conjugates by high pressure liquid chromatography. *Falk Hepatol* 11, 1649–1650.
- 29 Blanckaert N, Kabra PM, Farina FA *et al.* (1980) Measurement of bilirubin and its mono- and diconjugates in human serum by alkaline methanolysis and high performance liquid chromatography. *J Lab Clin Med* 96, 198–212.
- 30 Schumacher RE, Thornberry JM, Gutcher GR (1985) Transcutaneous bilirubinometry: a comparison of old and new methods. *Pediatrics* 76 (1), 10–14.
- 31 Schiff D, Chan G, Poznasky MJ (1985) Bilirubin toxicity in neural cell lines N115 and NBR10A. *Pediatr Res* 19 (9), 908–911.
- 32 Mustafa MG, Cowger ML, King TE (1969) Effects of bilirubin on mitochondrial reactions. *J Biol Chem* 244 (23), 6403–6414.
- 33 Sano K, Nakamura H, Tamotsu M (1985) Mode of inhibitory action of bilirubin on protein kinase C. *Pediatr Res* 19 (6), 587–590.
- 34 Vaughan VC, Allen FC, Diamond LK (1950) Erythroblastosis fetalis. IV. Further observations on kernicterus. *Pediatrics* 6, 706.
- 35 Gourley GR (1997) Bilirubin metabolism and kernicterus. *Adv Pediatr* 44, 173–229.
- 36 Odell GB (1973) Influence of binding on the toxicity of bilirubin. *Ann NY Acad Sci* 226, 225–237.
- 37 Lee K-S, Gartner LM (1983) Management of unconjugated hyperbilirubinemia in the newborn. *Semin Liver Dis* 3 (1), 52–64.
- 38 Breimer LH, Wannamethee G, Ebrahim S *et al.* (1995) Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clin Chem* 41, 1504–1508.
- 39 Bosma PJ, van der Meer, IM, Bakker CT *et al.* (2003) UGT1A1\*28 allele and coronary heart disease: the Rotterdam Study. *Clin Chem* 49, 1180–1181.
- 40 Zucker SD, Horn PS, Serman KE (2004) Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer. *Hepatology* 40, 827–835.
- 41 Temme EHM, Zhang J, Schouten EG *et al.* (2001) Serum bilirubin and 10-year mortality risk in a Belgian population. *Cancer Causes Control* 12, 887–894.
- 42 Bowen WR, Porter E, Waters WF (1959) The protective action of albumin in bilirubin toxicity in new born puppies. *Am J Dis Child* 98, 568–573.
- 43 Brodersen R (1986) Aqueous solubility, albumin binding and tissue distribution of bilirubin. In: Ostrow JD (ed.) *Bile Pigments and Jaundice*. New York: Marcel Dekker, pp. 157–181.
- 44 Cui Y, Konig J, Leier I *et al.* (2000) Hepatic uptake of bilirubin and its conjugates by the human organic anion-transporting polypeptide 2 (symbol SLC21A6). *J Biol Chem* 276, 9626–9630.
- 45 Wang P, Kim RB, Roy-Chowdhury J *et al.* (2003) Organic anion transport protein SLC21A6 (OATP2) is not sufficient for bilirubin transport. *J Biol Chem* 278 (23), 20695–20696.
- 46 Levi AJ, Gatmaitan Z, Arias IM (1969) Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein, and other anions. *J Clin Invest* 48, 2156–2167.
- 47 Morey KS, Litwack G (1969) Isolation and properties of cortisol metabolite binding proteins of rat liver cytosol. *Biochemistry* 8, 4813–4821.
- 48 Ketterer B, Ross-Mansell P, Whitehead JK (1967) The isolation of carcinogen-binding protein from livers of rats given 4-dimethylaminoazobenzene. *Biochem J* 103, 316–324.
- 49 Kamisaka K, Gatmaitan Z, Moore CL *et al.* (1975) Ligandin reverses bilirubin inhibition of liver mitochondrial respiration in vitro. *Pediatr Res* 9 (12), 903–905.
- 50 Roy Chowdhury J, Novikoff PM, Roy Chowdhury N *et al.* (1985) Distribution of uridinediphosphoglucuronate glucuronosyl transferase in rat tissues. *Proc Natl Acad Sci USA* 82, 2990–2994.
- 51 Bosma PJ, Seppen J, Goldhoorn B *et al.* (1994) Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* 269 (27), 17960–17964.
- 52 Ritter JK, Chen F, Sheen YY *et al.* (1992) A novel complex locus UGT1 encodes human bilirubin, phenol and other UDP-glucuronosyltransferase isozymes with identical carboxy termini. *J Biol Chem* 267 (5), 3257–3261.
- 53 Mackenzie PI, Owens IS, Burchell B *et al.* (1997) The UDP glucosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7 (4), 255–269.
- 54 Wishart GJ (1978) Functional heterogeneity of UDP-glucuronosyl transferase as indicated by its differential development and inducibility by glucocorticoids. *Biochem J* 174 (2), 485–489.
- 55 Roy Chowdhury J, Roy Chowdhury N, Mosconi AD *et al.* (1983) Differential regulation by triiodothyronine of substrate-specific uridinediphosphoglucuronate glucuronyl transferases in rat liver. *Biochim Biophys Acta* 761 (1), 58–65.
- 56 Lilienblum W, Walli AK, Bock KW (1982) Differential induction of rat liver microsomal UDP-glucuronosyltransferase activities by various inducing agents. *Biochem Pharmacol* 31 (6), 907–913.
- 57 Bossuyt X, Blanckaert N (1997) Carrier-mediated transport of uridine diphosphoglucuronic acid across the endoplasmic reticulum membrane is a prerequisite for UDP-glucuronosyltransferase activity in rat liver. *Biochem J* 323, 645–648.
- 58 Ghosh SS, Sappal BS, Ganjam VK *et al.* (2001) Homodimerization of human bilirubin-uridine-diphosphoglucuronate glucuronosyltransferase-1 (UGT1A1) and its functional implications. *J Biol Chem* 276, 42108–42115.
- 59 Ishikaowa T, Muller M, Klunemann C *et al.* (1990) ATP-dependent primary active transport of cysteinyl leukotrienes transport system for glutathione S-conjugates. *J Biol Chem* 265 (31), 19279–19286.
- 60 Nishida T, Gatmaitan Z, Roy-Chowdhury J *et al.* (1992) Two distinct mechanisms for bilirubin glucuronide transport by rat bile canalicular membrane vesicles. *J Clin Invest* 90 (5), 2130–2135.
- 61 Gatmaitan ZC, Nies AT, Arias IM (1997) Regulation and translocation of ATP-dependent apical membrane proteins in rat liver. *Am J Physiol* 272, G1041–G1049.
- 62 Brodersen R, Herman LS (1963) Intestinal reabsorption of unconjugated bilirubin. A possible contributing factor in neonatal jaundice. *Lancet* 1, 1242.
- 63 Van der Veere CN, Jansen PL, Sinaasappel M *et al.* (1997) Oral calcium phosphate: a new therapy for Crigler–Najjar disease? *Gastroenterology* 112, 455–462.
- 64 Watson CJ (1977) 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, pp. 469–482.
- 65 Berry CS, Zarembo JE, Ostrow JD (1972) Evidence for conversion of bilirubin to dihydroxyl derivatives in the Gunn rat. *Biochem Biophys Res Commun* 49 (5), 1366–1375.
- 66 Kapitunlik J, Ostrow JD (1978) Stimulation of bilirubin catabolism in jaundiced Gunn rats by an inducer of microsomal mixed function mono oxygenases. *Proc Natl Acad Sci USA* 75 (2), 682–685.

- 67 Cardenas-Vazquez R, Yokosuka O *et al.* (1986) Enzymic oxidation of unconjugated bilirubin by rat liver. *Biochem J* 236 (3), 625–633.
- 68 Cameron JL, Filler RM, Iber FL *et al.* (1966) Metabolism and excretion of  $^{14}\text{C}$ -labeled bilirubin in children with biliary atresia. *N Engl J Med* 274 (5), 231–236.

### 2.3.6 Metabolism of bile acids

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#### Introduction

Bile acids are synthesized in the liver from cholesterol; they are secreted in bile and stored in the gallbladder. After a meal, the gallbladder contracts, and stored bile is transferred to the duodenum and via the jejunum to the ileum. This movement is stimulated by intestinal propulsion. In the ileum, 90–95% of bile salts are reabsorbed and returned to the liver. The remainder is lost to the colon, where primary bile salts are transformed by bacterial metabolism into secondary bile salts. Some of the secondary bile salts are also reabsorbed, and the rest is removed with the faeces. Primary and secondary bile salts return to the liver via the portal circulation. In the liver, bile salts are taken up into hepatocytes, thereby completing the enterohepatic cycle.

Bile acids serve a number of functions: (i) they are the main solutes in bile and, as such, they are important for the generation of the so-called bile salt-dependent bile flow; (ii) bile salts are indispensable for the secretion of cholesterol and phospholipids from the liver; (iii) in bile, bile salts form mixed micelles that keep fat-soluble organic compounds in solution, including fat-soluble vitamins; (iv) in the intestine, bile salts promote the dissolution and hydrolysis of triglycerides by pancreatic enzymes; (v) bile salts act as signalling molecules in the regulation of enzymes and transporters of drug and intermediary metabolism.

The adult human liver produces about 500 mg of bile acids per day [1,2]. About three times this amount represents the total bile acid pool size that cycles through the enterohepatic circulation [2]. Bile acids complete an enterohepatic cycle about eight times per day. Enterohepatic cycling represents an efficient system for reuse of active components. Enterohepatic cycling not only serves to reclaim bile acids, but it also enables bile acids to act as messengers that carry signals from intestine to liver. Thus, they regulate their own synthesis and transport rates. Bile acids are also able to repress hepatic fatty acid and triglyceride synthesis [3,4].

#### Biosynthesis and metabolic defects

At least 16 different enzymes are involved in the biosynthesis of bile salts [1,5,6]. Most of these enzymes are active in the neutral (or classic) and acidic (or alternative) pathways, the two main

routes for the conversion of cholesterol to the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) (Fig. 1). The neutral pathway starts with the hydroxylation of the sterol nucleus of cholesterol by  $7\alpha$ -hydroxylase (CYP7A1) in the endoplasmic reticulum. CYP7A1 is regarded as the rate-limiting enzyme in bile acid biosynthesis, exemplified by the fact that mice deficient for Cyp7a1 have a 75% reduced bile acid pool size causing vitamin deficiencies, lipid malabsorption and liver failure [7–9]. The acidic pathway starts with the hydroxylation of the cholesterol side-chain by sterol 27-hydroxylase (CYP27). The CYP27 product, 5-cholesten- $3\beta$ -27-diol, is not a substrate for CYP7A1, but is hydroxylated at the C7 position by an alternative P450 enzyme, CYP7B1. From here on, the neutral and acidic pathways largely overlap. Double hydroxylated CDCA and triple hydroxylated CA are the principal bile acids. Their ratio depends on the activity of sterol 12 $\alpha$ -hydroxylase (CYP8B1). Bile acid synthesis is completed in hepatocyte peroxisomes, where bile acid coenzyme A:amino acid N-acyltransferase (BAAT) conjugates either taurine or glycine to CA or CDCA. At least 95% of the bile acid pool is generated through these two pathways. Extensive intracellular transport of bile acid intermediates occurs between various organelles. Transport in and out of these organelles may be mediated by transport proteins, but these have not been characterized in detail yet.

Bile acid synthesis defects (BASD) are rare genetic disorders that are the underlying cause of approximately 2% of persistent cholestasis in infants (see also Chapter 16.10, Genetic cholestatic diseases). BASDs are recognized by the absence or reduction of normal primary bile salts in serum and/or urine. Instead, non-typical bile acids and sterols are often detected in the body fluids of these patients. These can be identified by fast atom bombardment ionization–mass spectrometry (FAB-MS) and gas chromatography–mass spectrometry (GC-MS). Disease-causing mutations have been identified in 9 out of the 16 bile acid biosynthesis enzymes (Table 1). Cholestasis is a common clinical presentation of these diseases. The associated liver diseases may vary from mild to life-threatening but, in many cases, can be managed by replacement of deficient primary bile salts. This not only leads to restoration of normal bile function, but also induces feedback inhibition on the production of toxic bile acid intermediates.

Patients with CYP7A1 deficiency have a markedly reduced bile acid synthesis rate [10]. Symptoms include hyperlipidaemia, premature vascular disease and gallstones. A mutation in the CYP7A gene that results in truncation of the enzyme has been detected in these patients. Only one case of CYP7B1 deficiency has been reported to date [11]. This child produced no primary bile acids, and serum concentrations of the toxic 27 $\alpha$ -hydroxy cholesterol were increased. A mutation was identified in the CYP7B1 gene that truncates and inactivates the enzyme. In addition, it was found that expression of CYP7A, at both the mRNA and activity level, was absent. Bile acid treatment was ineffective, suggesting that the biosynthesis of toxic 27 $\alpha$ -hydroxy cholesterol cannot be suppressed.