REVIEW ARTICLE

Bilirubin in clinical practice: a review

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Abstract
Bilirubin is an endogenous compound that can be toxic under certain conditions but, on the other hand, mild unconjugated hyperbilirubinaemia might protect against cardiovascular diseases and tumour development. Serum bilirubin levels are often enhanced under a variety of clinical conditions. These are discussed and the mechanisms are outlined.

Bilirubin is an endogenous compound that can be toxic (1), especially in neonates. However, it has recently been recognized that unconjugated bilirubin (UCB) exerts a strong anti-oxidant activity, and that mild hyperbilirubinaemia might have positive health effects. Bilirubin is the ultimate breakdown product of haemoglobin and serves as a diagnostic marker of liver and blood disorders. It has a complex metabolism, which is important in relation to several processes involved in drug metabolism.
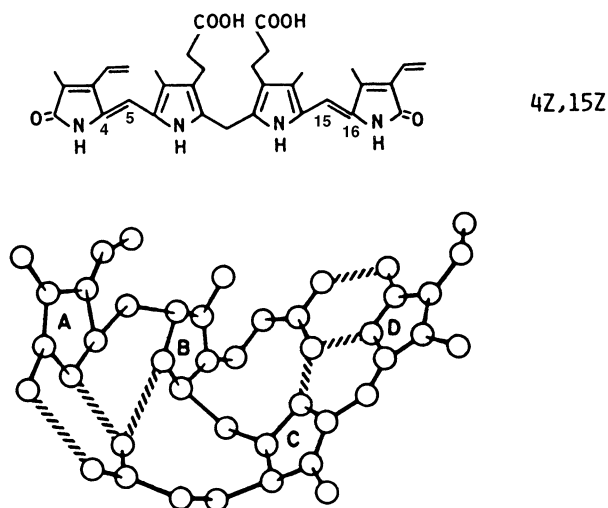
Bilirubin: chemical structure and formation

At first glance, bilirubin appears to be a simple molecule. However, the UCB IXα 4Z,15Z molecule, the major compound in mammals, has a peculiar stereo-chemical structure (Fig. 1). Indeed, all hydrophilic groups are involved in strong hydrogen bonds, and this turns the molecule into a closed molecule with a ridge-tile conformation (2, 3). These hydrogen bonds render UCB hydrophobic and they also shield

the central $-CH_2-$, which thus becomes inaccessible for the diazo-reagent (see further). Depending on the pH of the plasma, bile or urine, UCB can be present as uncharged diacid, as a monoanion or as a dianion (3). The uncharged diacid is by far the dominant species at low and physiological pH ($> 80\%$) but the ionized fractions become more important in an alkaline milieu, because the pK'a values have been determined to be 8.12 and 8.44, respectively, for the first and for the second anion (3).

Bilirubin is formed from haem by opening of the haem ring at the α carbon bridge. This cleavage is catalysed by the enzyme haem-oxygenase, and results in liberation of iron, and in the formation of carbonmonoxide and biliverdin IXα (Fig. 2). The latter is reduced by a cytosolic enzyme biliverdin-reductase to bilirubin IXα. The haem-oxygenase can temporarily be inhibited by mesoporphyrins, and this suppression results in a decreased UCB production as was shown in neonates (4). Cleavage at non-α sites is possible; it is probably non-enzymic and occurs

only to a minor extent. This results in the formation of other isomers; some can be detected in body fluids, although always in small amounts or under special conditions. The IXβ isomer is present in neonatal urine and in meconium (5), whereas the IXβ and IXγ isomers have been detected in Gunn rat bile (6). Because intramolecular hydrogen bonds cannot be formed in these isomers, they are more hydrophilic, and appear in urine or bile as an unconjugated pigment. Till now, IXδ has not been demonstrated in mammals. Phototherapy, used in the treatment of neonatal jaundice or in Crigler–Najjar disease, leads to the formation of another group of more hydrophilic derivatives of the natural UCB IXα, such as the 4E,15Z and the 4Z,15E and 4E,15E photoisomers, which can be excreted in bile without conjugation (3, 7–9) (Fig. 3).
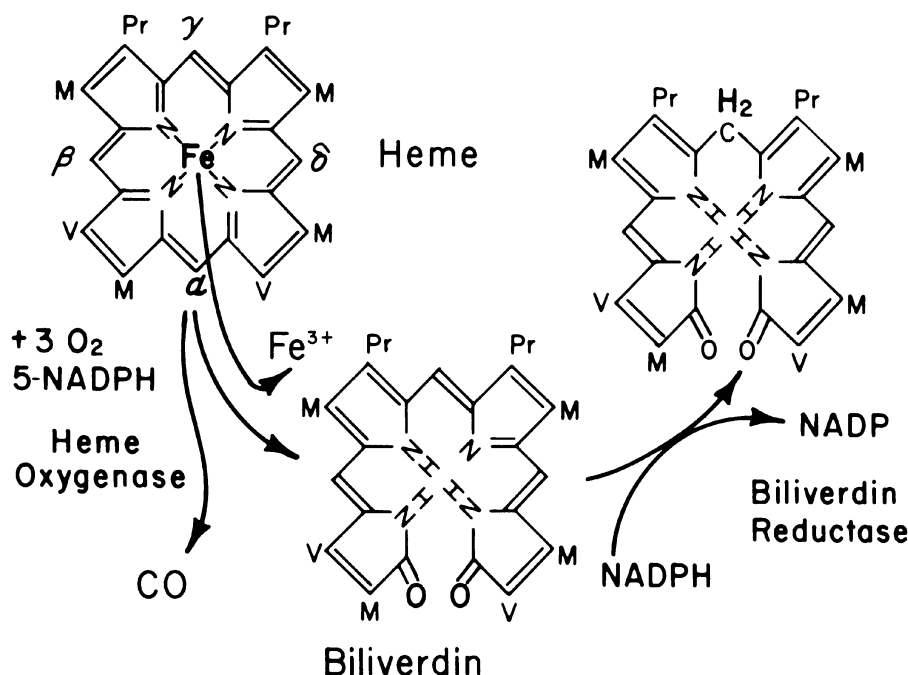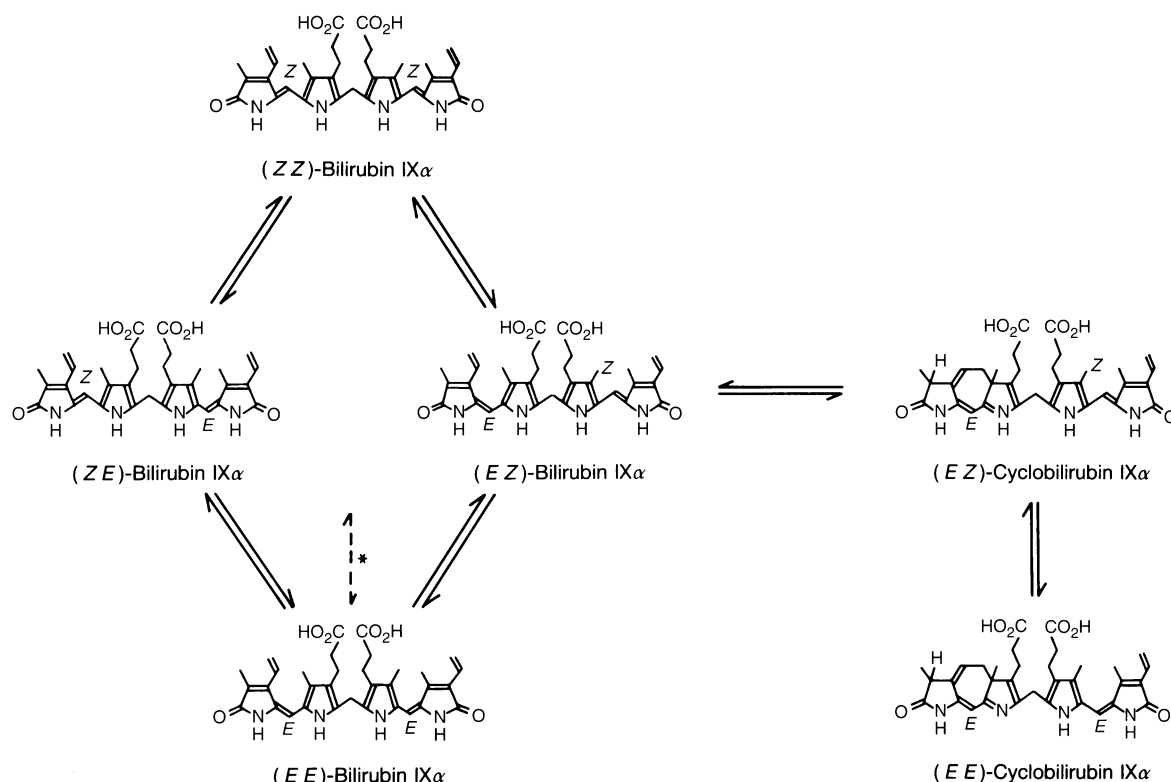
Bilirubin metabolism under normal conditions

Bilirubin derives from haem present in haemoglobin and is released during breakdown of senescent erythrocytes, whereas approximately 20% of the daily production is derived from haem proteins such as the cytochrome P 450 isoenzymes, myoglobin, etc. It is formed in the monocytic macrophages of the spleen and bone marrow and in hepatic Kupffer cells, and is released in plasma. Per 24 h 3.8 mg/kg or approximately 250–300 mg bilirubin is formed in a normal adult (10). More is formed in the neonate.

Because UCB is extremely poorly soluble in water, it is present in plasma strongly bound to albumin. The dissociation constant for the first albumin-binding site is $K_d = 7 \times 10^7 \, M^{-1}$ (11). Recent studies by Ostrow and collaborators, and reviewed in Ostrow et al. (3), determined the aqueous solubility of UCB IXα ZZ to be 70 nM in the non-ionized diacid form, which

Fig. 1. Chemical structure of the naturally occurring unconjugated bilirubin IXα 4Z,15 Z.

Fig. 2. Formation of bilirubin.

**Fig. 3.** Structure of isomers formed during phototherapy. From Fevery *et al.* (9) with permission.

is by far the most prominent species present in blood at physiological pH. The mono-anion is present at approximately 17% and the dianion is minimal (3).

Entry into the hepatocyte appears to be partly passive (12, 13) and partly mediated by organic anion transporter proteins (OATP 1B1 has the highest binding affinity) (13–15). The role played by OATPs has not yet been clarified quantitatively (16). In the hepatocytic cytosol, UCB is mostly bound to glutathione-*S*-transferase A (ligandin), and a small part is bound to the fatty acid-binding protein (3). As in serum, this binding keeps the free fraction (which is potentially toxic) low.

Bilirubin is conjugated in hepatocytic microsomes in an ester linkage (17) with sugar moieties donated by uridine diphosphate (UDP) sugars. The discovery of glucuronide conjugation of bilirubin was one of the milestones towards understanding bilirubin metabolism and was made almost simultaneously by three groups (18–20). The conjugation is catalysed by UDP-glucuronyltransferase (UDP-GT), an enzyme encoded for by the UGT1A1 gene (21). Both ligandin and UDP-GT appear to be tightly regulated by the nuclear constitutive androstane receptor (CAR) (22). In humans, conjugation occurs mainly with glucuronic acid, but glucose and xylose conjugates are also present in normal bile. The latter are more abundant in cats, dogs and rodents (23). One or two sugar moieties are coupled to the –COOH of the propionic acid side chain(s) of UCB in an ester linkage, resulting in monoconjugated or diconjugated bilirubin respectively. The esterification disrupts the intramolecular hydrogen bonds, thereby opening the molecule and rendering the conjugated bilirubins (CB) more water-soluble or amphipathic, allowing excretion in the bile. Conjugation also decreases the binding to albumin or to intracellular proteins 5–10-fold, and prevents intestinal re-absorption, because hydrophilic agents do not easily pass the intestinal wall. In addition, the central –CH_2– now becomes available for direct attack by the diazo-reagent.

The bilirubin conjugates formed in the hepatocytes are excreted in bile against a concentration gradient and mediated by the canalicular membrane transporter multidrug resistance-related protein 2 (MRP2) also termed ABC-C2, belonging to the adenosine triphosphate (ATP)-binding cassette family (24). The conjugates are incorporated into mixed micelles (with bile acids, phospholipids and cholesterol) and pass with the
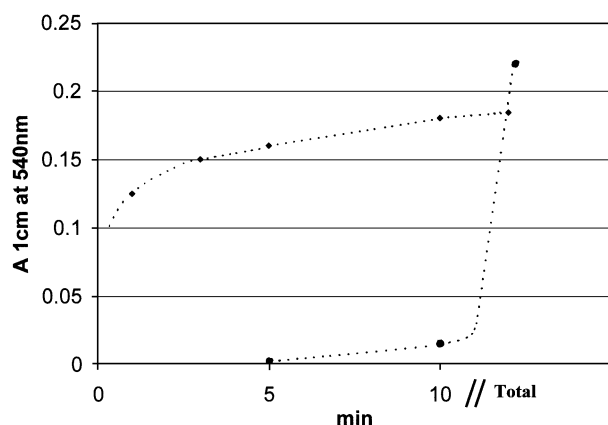
Fig. 4. Direct and total diazoreaction of bilirubindiglucuronide (triangles) and UCB (dots) added to human serum albumin with p-diazobenzenesulphonic acid at pH 2.6 and a nitrite concentration of 0.5%; means of three determinations are given (27).

bile into the intestine, where reductive breakdown into urobilinogens occurs by intestinal or bacterial enzymes. A minor part undergoes deconjugation mainly by bacterial enzymes, and the ensuing UCB can undergo intestinal re-absorption, in contrast to CB.

Bilirubin determination

Bilirubin has a yellow colour with, for the unconjugated molecule, a typical spectrographical peak at 450 nm (25). Bilirubins are very sensitive to oxidation and to light; therefore, serum samples should be protected from direct light and be analysed as soon as possible. For the study of biliary or urinary bile pigments, more stringent precautions are necessary because these fluids normally do not contain albumin to protect the bilirubins. Handling should only be carried out under subdued or red light and 1–5 mM ascorbate has to be added as an anti-oxidant (26).

Unconjugated bilirubin can be extracted from serum by chloroform in an acidic milieu and measured spectrophotometrically but in general the diazo-reaction is most often used. In the diazo-reaction, conjugated bilirubins are split to form dipyrrolic azopigments (so-called direct Hymans–Van den Berg reaction). In case of UCB, an accelerator substance such as urea, ethanol, dimethyl sulphoxide, etc. is needed to first disrupt the hydrogen bonds, rendering the central $-CH_2-$ available for coupling with the diazo-reagent in the so-called 'indirect reaction'. The azo-pigments formed have a typical purple colour with a spectrographical peak at 540 nm (26). The diazo-reaction is not entirely specific for differential quantification of unconjugated and conjugated bilir-

ubin, because UCB also shows some reaction (approximately 2.8%) without an accelerator and because the conjugated bilirubins have not yet reacted totally (approximately only 93%) within 10 min (Fig. 4), but the method using a total and a 10-min direct reaction is the best available approach (28–30). The most accurate and sensitive method to discriminate unconjugated from conjugated bilirubin is based on the formation of methyl derivatives in an alkaline milieu (31), because such alkaline methanolysis is not possible with UCB. The derivatives can be separated by thin-layer or more conveniently by high-pressure liquid chromatography (32).

Disturbed bilirubin metabolism (Table 1)

In clinical laboratories, serum total (TB) and direct-reacting (DB) bilirubin levels are usually determined. Disorders have accordingly been classified as unconjugated hyperbilirubinaemia when the ratio DB/TB is below 20–30%, whereas conjugated hyperbilirubinaemia is characterized by a ratio DB/TB > 70% and the mixed type with values in between (29).

Enhanced bilirubin production

The formation of bilirubin can be enhanced due to an abnormally high peripheral breakdown of haemoglobin, termed haemolysis (Table 2), or due to dyserythopoiesis (33). Dyserythopoiesis or inefficient erythropoiesis is a rather rare cause of enhanced bilirubin production, caused by an arrest in one of the phases of the mitosis, resulting in immature erythroid cells being present in the bone marrow and in the circulation. These immature or abnormal cells undergo rapid destruction, which leads to UCB formation. Several mutations have been detected recently. Dyserythopoiesis is also present in thalassaemia and in some acquired disorders such as Vit B 12 or folate deficiency, myelodysplasia, aplastic anaemia, etc.

Haemolysis (34) is a far more frequent cause of unconjugated hyperbilirubinaemia. Because erythrocyte synthesis in the bone marrow can be activated 6–8-fold, anaemia is often not present when red blood cells undergo accelerated destruction, and yet unconjugated hyperbilirubinaemia can be evident in chronic haemolysis. A large spectrum of disorders can give rise to haemolysis (Table 2). This becomes apparent from an enhanced reticulocute count, increased plasma UCB, lactate dehydrogenase (LDH), iron, decreased free haptoglobin and possible alterations in red cell morphology as seen in blood smears. On clinical examination, splenomegaly may be present, and the chronic hyperbilirubinaemia may induce pigment gall

Table 1. Hyperbilirubinaemia

Normal metabolism	Disorders	Hyperbilirubinaemia
1. Production (250–300 mg/day) from ●Erythrocyte haemoglobin degradation ●Breakdown of myoglobin, cytochromes ●Haem synthesis in the bone marrow	Haemolysis Dyserythropoiesis	Unconjugated
2. Transport in plasma bound to albumin	Competitive binding by salicylates, some fatty acids, Long acting sulphonamides	
3. Uptake in hepatocytes Membrane transit (via OATP?) Binding to ligandins and FABP	Inhibition by indinavir, cyclosporin A, rifamycin, etc. Neonatal immaturity of ligandin Mutant Southdown sheep Rotor syndrome??	
4. Conjugation in microsomes	Neonatal immaturity Crigler–Najjar diseases Gilbert syndrome Inhibition by novobiocin, atazanavir, amitriptyline, ketoconazole, etc. Escape form conjugation due to shunting (cirrhosis, TIPS)	
5. Biliary secretion ●Bile canaliculus ●Bile ducts	Neonatal immaturity Defect in MRP2: Dubin–Johnson syndrome MDR3/PFIC3: cholestasis of pregnancy Mutant Corriedale sheep Mutant TR − rat (Groningen, Japan) Hepatitis, cirrhosis PBC, PSC, mechanical obstruction	Conjugated
6. Intestinal fate ●Enzymic deconjugation ●Bacterial reduction to urobilinogens ●Faecal elimination	Neonatal absence of bacteria	

FABP, fatty acid-binding protein; MDR3, multidrug resistance 3; MRP2, multidrug resistance-related protein 2; OATP, organic anion transporter proteins; PBC, primary biliary cirrhosis; PFIC3, progressive familial intrahepatic cholestasis type 3; PSC, primary sclerosing cholangitis; TIPS, transjugular intrahepatic portosystemic shunt.

stone formation. The level of unconjugated hyperbilirubinaemia in haemolytic diseases, such as the more common spherocytosis and thalassaemia, also depends on the quite frequent association with Gilbert's syndrome (GS) (35, 36).

Disturbed conjugation

Bilirubin can only be eliminated efficiently out of the body following conjugation. Decreased conjugation rates will thus lead to unconjugated hyperbilirubinaemia.

The enzyme responsible for the conjugation, bilirubin UDP-GT, is immature at birth. This results in the so-called 'physiological jaundice of neonates', with peak bilirubin levels at day 3–4. Formation of UDP-GT is encoded by the UTG1A gene on chromosome 2. In the 51#8242 region of the UGT1A gene, a large set of unique first exons with individual proximal promoter elements are arranged in a tandem array upstream of four common exons. Each first exon

encodes a different substrate-specific N-terminal part of the protein and is spliced to the four common exons that encode the C-terminal part of the protein that binds the common substrate, UDP-glucuronic acid. In this way, a large set of isoforms are created, of which the UDGT1A1 is the bilirubin-conjugating isoform (21). Mutations in exons lead to Crigler–Najjar disease (37–41) and in Japanese individuals seemingly also to GS (41). A mutation upstream giving rise to an enlarged 5′ promoter TATA box, i.e. A (TA)7 instead of the normal A(TA)6, leads to decreased transcription; the reduced amount of enzyme formed is responsible for GS in Caucasian, black and South-Asian individuals (42). In GS, GT activity is approximately 30% of normal values, resulting in serum UCB levels of 1–3 mg/dl (37–121 μM). In addition to external factors, serum bilirubin levels in GS will also depend on whether the person is homozygous or heterozygous for the A(TA)7 variant (43–45).

The Crigler–Najjar type 1 disease is characterized by complete absence of enzyme activity with ensuing very

Table 2. Causes of haemolysis

1. Hereditary diseases
    1. Inherited haemolytic disorders
        (a) Membrane defects: spherocytosis, elliptocytosis
        (b) Stomatocytosis
        (c) Acanthocytosis
        (d) Echinocytes
        (c) Target cells: congenital LCAT deficiency
    2. Hereditary enzyme deficiency
        (a) Glucose and phosphate deficiency, GSH synthase deficiency, etc.
        (b) Disorders of glycolysis: pyruvate kinase deficiency, etc.
        (c) Disorders of erythrocyte nucleotide metabolism
    3. Congenital haemoglobinopathies: sickle cell disease, thalassaemia syndromes, etc.
2. Acquired disorders
    (a) Immunohaemolysis: transfusion reaction, autoimmune haemolysis, drugs behaving as haptens, etc.
    (b) Trauma and microangiopathy: prosthetic heart valves, haemolytic uremic syndrome, DIC, TTP, long-distance runners, etc.
    (c) Infections such as malaria, clostridia, bartonella, etc.
    (d) Chemical and toxic agents: snake venoms, copper, lead, dapsone, nitrites, aniline dyes, etc.
    (e) Membrane defects: paroxysmal nightly haemoglobinuria (PNH), spur cells, etc.
    (f) Hypophosphataemia

DIC, disseminated intravascular coagulation; GSH, glutathione; LCAT, lysolecithin cholesterol acyl transferase; TTP, thrombotic thrombocyto-paenic purpura.

high UCB levels in blood. This may lead to mental disturbances, called Kernicterus, because of deposition of UCB in brain tissue. Phototherapy can transform the UCB IXα 4Z,15Z into water-soluble photoisomers (Fig. 3), which can be excreted in bile and urine (1, 3, 7, 8, 46–48), and this therapy can maintain the UCB IXα 4Z,15Z at acceptable levels to protect Crigler–Najjar children till liver transplantation can be performed (49). In Crigler–Najjar type 2 disease, other mutations lead to the formation of an enzyme with markedly decreased conjugating activity (39, 40). In the latter syndrome, enzyme inducers such as phenobarbital can enhance the GT activity, allowing to maintain serum UCB levels around 10 mg/dl without side effects. The enzyme is also absent in the Gunn rat, a mutant strain of the Wistar R/A rat, which represents an animal model for Crigler–Najjar type 1 disease (50, 51).

The activity of the conjugating enzyme is also influenced by a variety of post-translational conditions, such as:

1. *Age*: The enzyme activity slowly increases after birth (52).

2. *Gender*: In serum of normal individuals, UCB is lower in females in the reproductive age than in males (52–56). This difference might be due to the effects of oestro-progestogens and of testosterone on the conjugation rate, because testosterone down-regulates UDP-GT, whereas the combination of oestro-progestogens enhances enzyme activity (52). The effect of testosterone might, however, explain the fact that GS is often detected in males around puberty, but there is no real gender preference for GS if one compares the enhanced UCB levels with the normal values taking age and gender into account.

3. *Microsomal enzyme-inducing agents, such as phenobarbital, spironolactone, gluthetimide, rifampicin, etc.*: They will enhance enzyme activity and will decrease serum bilirubin levels in Crigler–Najjar type 2 and in GS (53). Inhibiting agents are the antiretroviral protease inhibitor atazanavir, amitriptyline, ketoconazole, etc. (54).

4. *Thyroid hormones*: UDP-GT is decreased in rats with hyperthyroidism and increased in hypothyroid animals (55).

The conjugation rate is rate limiting for the overall bilirubin elimination out of the body in normal situations, because bilirubin can only be disposed off efficiently following conjugation. As such, the maximal biliary secretion rate, a measure of the hepatic elimination, was shown to depend on the conjugation rate, as documented under different experimental conditions (56). When the bilirubin production rate is enhanced as is the case in haemolysis, the relationship between conjugation and elimination rate, and consequently the serum UCB levels, remains identical but is situated at a higher level (57).

Decreased biliary secretion

Bile results from (i) a hepatocytic bile acid-independent secretion, with glutathione and Na^+ excretion, (ii) a hepatocytic bile acid-dependent secretion, whereby the osmotic flow is generated by bile acid formation and secretion, and (iii) a bile ductular secretion mainly consisting of Na^+ and HCO_3^-, stimulated by secretin and cholecystokinin, with involvement of the chloride channel CFTR gene ('cystic fibrosis'). Most of the solutes will be delivered in bile via mediation of special protein transporters or 'export pumps'. At the sinusoidal pole of the hepatocyte, unconjugated bile salts and part of UCB are taken up from plasma via the 'organic anion transporter proteins' (OATPs), and unconjugated and conjugated bile salts by the 'Na^+-dependent taurocholate cotransporter', whereas the transmembraneous potential

difference and the sodium gradient is sustained by a Na^+K^+ ATPase. At the canalicular site, several export pumps are active, and the biliary canaliculus behaves as an active contractile pump (58, 59) because of the action of microfilaments (which can be inhibited by administration of phalloidin or cytochalasin B) and of microtubules (inhibitable by e.g. colchicine, vinblastin, etc.). Inhibition of the contractile elements by these drugs leads to cholestasis (60).

A timely overview of the transport proteins [ATP-binding cassettes (ABC)] involved is given by Pauli-Magnus et al. (61, 62) and by Geier et al. (63). The most important transport proteins are given in Table 3. Cholestasis or bilirubinostasis can thus be because of either a congenital deficiency or absence of a given transporter or acquired suppression by toxins or diseases of the transporters (61–63) and/or of contractile elements (60) or because of decreased energy supply. Alterations at the cholangiocyte level can also produce cholestasis. Genetic disorders of cholangiocytes include cystic fibrosis and the Alagille syndrome; acquired disorders include primary biliary cirrhosis, primary sclerosing cholangitis, vanishing bile duct diseases, etc. (63).

During chronic cholestasis, the presence of *biliproteins* in plasma has been demonstrated. Acute biliary obstruction is characterized by a rapid short-lasting increase of alanine aminotransferase (ALT) (which is often missed because the patient presents later in time). This temporary increase in ALT is followed by an increase of serum-conjugated bilirubin and some days later by enhanced serum alkaline phosphatase (ALP) levels, because elevation of the latter enzymes requires new production by the cholestatic liver (64). Following relief of a mechanical biliary obstruction by endoscopy or by surgery, a rapid disappearance of itching and of the serum bile acids is noted but the jaundice disappears only slowly. It was also noticed that the urine had become clear already despite the fact that the jaundice still persisted. Investigations have shown that these discrepancies are due to the presence of 'biliproteins' or 'covalently albumin-bound bilirubin conjugates' in blood. These pigments consist of bilirubin conjugates in which one glucuronide side chain was replaced chemically by an albumin molecule (65, 66). This non-enzymic exchange between the glucuronide moiety and albumin occurs during stagnation of the bile. It can be compared with the chemical formation of glycosylated haemoglobin (HbA1C) in diabetes, whereby a glucose moiety becomes bound to haemoglobin. These albumin conjugates are diazo-positive, have a large molecular weight (because of the albumin attachment) and therefore cannot undergo ultrafiltration in the kidney. They thus do not appear in the urine. These biliproteins are catabolized in plasma when their albumin part undergoes proteolysis. They thus have a plasma half-life of 17 days, similar to that of natural albumin (66).

In contrast, the normal bilirubin conjugates are water soluble and appear in the urine. They mainly undergo glomerular filtration, but tubular re-absorption and secretion also occurs (67–69). However, because they are also bound to albumin (although far less strong than UCB), the ultrafiltrable fraction is only 0.5%. The renal bilirubin clearance is thus only approximately 0.5 ml/min or 0.5–1% of the normal glomerular filtration rate (68). This explains the low efficacy of haemo-dialysis in eliminating bilirubin conjugates. It can also be calculated that a serum total bilirubin concentration above 40 mg/dl points to the presence of either renal insufficiency (leading to decreased urinary output) or of bilirubin overproduction (haemolysis) in addition to the cholestasis (70).

Table 3. Most important transport proteins (61–63)

1. At the sinusoidal membrane
   - Organic anion transporter proteins (OATPs especially OATP1B1)
   - Na-taurocholate cotransporter protein (NTCP). This transporter is e.g. decreased by endotoxins and cytokines, which results in sepsis-induced cholestasis
2. At the canalicular membrane: export pumps
   Genetic mutations of some of these pumps give rise to the progressive familial intrahepatic cholestasis (PFIC) syndromes
   - BSEP or bile salt export pump or ABC B11, inhibited by cyclosporine, rifampycine, etc. and being defective in PFIC type 2 and in some patients with cholestasis of pregnancy
   - MDR 1 (multidrug resistance protein 1): utilized by organic compounds such as xenobiotics, cytotoxines, etc.
   - MRP 2 (multidrug resistance-assoc protein 2): mediating the secretion of bilirubin and bile salt glucuronides, defective in Dubin–Johnson syndrome
   - MDR 3 (multi drug resistance protein 3): a phosphatidylcholine flippase, defective in PFIC 3 and in several patients with cholestasis of pregnancy
   - ABC G5/G8: a cholesterol flippase, mutated in PFIC 1

Conjugated bilirubins are the dominant bile pigments in the urine of jaundiced patients, but very small amounts of UCB IXα may be present, probably resulting from tubular secretion (69). However, it should be noticed that the ratio UCB:albumin in these experiments was extraordinarily high (20:1). Furthermore, some deconjugation of CB is difficult to exclude. Bilirubin UDP-GT was demonstrated in rat and dog kidney (but not yet in the human kidney) (24) and in rat intestine (71), and transplantation of a Wistar rat kidney or intestine into a Gunn rat led to a significant reduction of plasma UCB levels (71, 72).

Intestinal breakdown of conjugated bilirubins

Bilirubin conjugates reach the intestinal lumen via the bile. In the intestine, deconjugation can take place. It is mainly carried out by intestinal enzymes. Further reductive alterations leads to the formation of several urobilinogen species (73). These reductions are mainly catalysed by bacterial enzymes and to a minimal part by intestinal enzymes. When deconjugation prevails, sizeable amounts of UCB are formed, and this pigment can undergo intestinal re-absorption ('enterohepatic recirculation'). Such an absorption can lead to enhanced serum UCB levels. In neonates, the bacterial flora is not yet developed, and reductive formation of urobilinogens will be negligible. Deconjugation will thus prevail and this adds to the enhanced serum bilirubin levels observed in neonatal jaundice (74).

Bile salts incorporate bilirubins in micelles and protect them from deconjugation. Normally, 95% of bile salts are re-absorbed in the terminal ileum, but not so in ileal disorders such as Crohn's disease or in patients with right-sided ileo-colectomy. In these patients, part of the bile salts escape re-absorption and appear in the colonic lumen. They keep UCB in solution, protected from bacterial reductive alterations, and this promotes UCB absorption and enterohepatic recirculation. The re-absorbed UCB augments the bilirubin content in serum, and following hepatic uptake and conjugation, also that of gall bladder bile. Because of the disease of the terminal ileum, subnormal amounts of bile salts are re-absorbed and secreted in bile after enterohepatic recirculation. The lower biliary bile salt content of the gallbladder decreases the solubility of the higher bilirubin content, and this can result in the formation of bilirubin gall stones (75).

How to differentiate hyperbilirubinaemia

In serum of normal individuals, the concentration of UCB is lower in females than that in males (52, 76–79),

and averaged 0.52 ± 0.003 mg/dl in women and 0.72 ± 0.004 mg/dl in men in a USA population study of 176 million individuals (79). Normal serum contains $96.4 \pm 2.0\%$ UCB, $1.8 \pm 2.0\%$ monoglucuronide and $1.9 \pm 2.0\%$ diglucuronide (78). The concentration of UCB is enhanced in haemolysis, but the relative proportions of UCB and CB remain identical to values of normal individuals (80) whereas in GS both the concentration and the percentage of UCB is enhanced, the latter attains 99% and the monoglucuronide is increased to 67% of the conjugated bilirubins (48). In normal human bile, UCB is $1.5 \pm 1.3\%$ of the total pigment, with $16.1 \pm 3.8\%$ monoconjugates and $80.8 \pm 3.9\%$ diconjugates. In GS, UCB and monoconjugates are enhanced till 3.2 ± 2.4 and $33.5 \pm 7.2\%$, respectively, whereas in haemolysis the percentages of the various pigments remain similar to those of normal individuals (81).

In the clinical context, the diazo-reaction is most often used and the determination of TB and DB will allow defining the hyperbilirubinaemia as:

1. *Unconjugated hyperbilirubinaemia*: DB/TB < 20–30%. In this condition, one has to consider:
- *Haemolysis*: Characterized by a high reticulocyte count, low free haptoglobin, high serum iron and LDH. Erythrocyte abnormalities may be recognized in blood smears. Splenomegaly is often detectable.
- *Dyserythropoiesis (acquired or more rarely congenital)*: A relatively low reticulocyte count, low free haptoglobin, low serum cholesterol (because it is utilized in the accelerated synthesis of red blood cell precursors), high serum iron and LDH (from the destruction of abnormal red cells) are present.
- *Gilbert's syndrome (or very rarely Crigler–Najjar type 2 disease)*: Increased UCB, but all other tests are normal. This can be documented by the demonstration of a mutated UGT1A1 gene (enhanced TATA box 6/7 or 7/7 instead of 6/6 in Caucasians, or mutated exons).

Table 4. Postoperative jaundice

1. Exacerbation of pre-existing liver disease
2. Toxic hepatitis or cholestasis due to anesthetic and other drugs used
3. Partial biliary obstruction
4. Post-transfusion hepatitis: before 1990, this was very frequent and mostly due to HCV infection from transfused blood
5. Ischaemic liver injury
6. Small for size liver syndrome
7. Benign postoperative jaundice

HCV, hepatitis C virus.

2. *Conjugated hyperbilirubinaemia*: *DB/TB > 70%*. This is because of cholestasis or the rare Dubin Johnson or Rotor syndrome.

3. *Mixed hyperbilirubinaemia*: DB/TB = 30–60%.

This condition is characterized by an increase in serum unconjugated and conjugated bilirubins. It can be seen in combined disorders leading to both enhanced production and decreased secretion rates, but also when UCB escapes the hepatic conjugation because of bypassing of the hepatocytes. Such shunting occurs when large intrahepatic or extrahepatic shunts (varices, splenorenal, etc.) are present either spontaneously in some patients with cirrhosis, or following placement of a transjugular intrahepatic portosystemic shunt or a surgical shunt. Shunting results in unconjugated or in mixed hyperbilirubinaemia, because most of the UCB is formed outside the liver and part of it will not reach the conjugating hepatocytes. The shunting will also lead to enhanced serum bile acids and ammonia, because these compounds will also escape hepatocytic metabolization.

Another example of combined disorders is present in the 'overloading syndrome' occurring sometimes in the postoperative situation. In general, 'postoperative jaundice' (82) can be due to several causes as given in Table 4. It is seen in the small for size liver syndrome following a partial liver resection, whereby the remaining liver might be too small to deal with a normal bilirubin production rate. This will lead to a temporary jaundice until the remaining liver regains compensatory hypertrophy. The jaundice is usually combined with shortage of clotting factors and with an elevated blood ammonia level. In older patients, it may take several weeks before the liver assumes its normal size. 'Benign postoperative jaundice' is another syndrome with mixed hyperbilirubinaemia. The jaundice is seen within 2–4 days after the operation. It occurs mostly in older, hypoxic, hypotensive or critically ill patients, who have undergone prolonged operations and have received blood transfusions. Transaminases remain below 100 IU/L, ALP is normal or only slightly increased and a mixed hyperbilirubinaemia is present (with CB being more increased than UCB). The jaundice is due to a combination of (i) bilirubin overproduction (because 10% of packed red cells haemolyse within 24 h and 0.5 L of transfused packed cells will thus result in an extra production of 250 mg bilirubin, doubling the normal daily production rate), (ii) decreased biliary secretion, because of inflammatory cytokines (which suppress the Na taurocholate cotransporter uptake protein), drugs, hypoxia and cardiac decompensation and (iii) renal dysfunction, which is often associated and will result in decreased renal elimination of bilirubin conjugates.

Mixed hyperbilirubinaemia can also be seen in alcoholic patients, when a decreased biliary secretion (because of the liver disease) is combined with overproduction of bilirubin due to haemolysis. Such a haemolysis can result from a decreased gluthathione content of the erythrocytes or from a decreased red cell membrane fluidity owing to high triglycerides (Zieve syndrome) or to the presence of echinocytes (a subtype of 'spur cells') (83). The latter have a high free cholesterol to phospholipid ratio in their membrane (normal red cells < 1.0, normal platelets < 0.4). This high free cholesterol results from the inability to esterify cholesterol because of a markedly decreased lysolecithin cholesterol acyl transferase (LCAT). This enzyme is formed in the liver, and can be markedly decreased in end-stage cirrhosis (81). An example is given by the following patient presenting with a TB of 6.6 mg/dl (or 112 µM), a DB of 2.1 mg/dl (or 35 µM), a low haemoglobin (9.6 g/dl), a high mean corpuscular volume and a very low haptoglobin (< 0.20 g/L), with 12% echinocytes in a peripheral blood smear, an active blood-forming bone marrow and a low serum cholesterol: 104 mg/dl (> 160), but a high free cholesterol (e.g. 57%), as a result of the low LCAT.

Additional aspects of disturbed bilirubin metabolism

Thyroid disorders and cardiac decompensation

Mild changes in serum aminotransferase levels and in bilirubin concentrations are frequent in thyroid diseases, but they often pass unnoticed. On rare occasions, clinical jaundice may be present with serum bilirubin levels as high as 19 mg/dl (84). Both mild unconjugated hyperbilirubinaemia as well as cholestasis and conjugated hyperbilirubinaemia can be seen.

In cardiac decompensation, mild unconjugated hyperbilirubinaemia may result from diminished uptake by the hepatocyte because of reduced flow, whereas a mild increase in conjugates can be present because of anoxic suppression of the biliary secretory mechanisms (85, 86).

Neonatal hyperbilirubinaemia

The so-called 'physiological jaundice of the neonate' is a complex phenomenon and results from a combination of the following:
- the larger haemoglobin mass of the neonate compared with the adult, leading to an increased bilirubin production;

● a lower plasma albumin level, which may decrease transport to the liver;

● a lower conjugation rate because of a low UDP-glucuronide content and immaturity of the conjugating enzyme UDP-GT;

● an immature biliary secretory apparatus; and

● the absence of bacterial flora resulting in a decreased reductive bilirubin breakdown, and in enhanced deconjugation of bilirubin di- or monoglucuronide to UCB with enhanced enterohepatic circulation.

Neonatal hyperbilirubinaemia can be a very serious condition, because UCB can become potentially toxic, especially in neonates, when the free or unbound UCB is enhanced. Especially, brain tissue is sensitive to the toxic effects of UCB, and this can lead to kernicterus with impairment of auditory, motor or mental functioning. Bilirubin-induced neurotoxicity has been encountered when serum UCB levels are above 20 mg/dl (340 μM), but it can occur at lower levels. As mentioned above, UCB is extensively bound to albumin and this binding keeps UCB in the plasma. However, when the molar ratios of UCB to albumin increase, the non-albumin bound or free UCB increases and this compound enters the cells and exerts toxicity. Its concentration can increase with high serum UCB levels, but also when the albumin concentration is low or when other compounds displace UCB from its binding to albumin. Such a displacement has been documented by sulphonamides, contrast media, anti-inflammatory drugs, etc. (47, 87). The free UCB concentration is very difficult to measure exactly, but the modified peroxidase method appears to be a clinically reliable method (1, 3, 47, 88). Neurotoxicity might also occur when UCB is not efficiently cleared by brain tissue itself because of low expression or activity of export carrier proteins, such as MRP1 and possibly multidrug resistance protein 1 or OATPs (47).

Gilbert's syndrome

Approximately 6–10% of the population has enhanced serum UCB levels (77), when the gender difference is taken into account. Serum and biliary UCB, and the bilirubin mono- to diglucuronide ratio are increased in GS because of a decreased bilirubin UDP-GT activity, which is approximately 30% of the normal enzyme activity. The lower amount of enzyme is the result of mutations of the UGT1A1 gene. In Caucasians, black and South-Asian populations, a longer A(TA)7 box is found in the promoter region instead of the normal A(TA)6, this mutated gene is termed UGT1A1*28 and is evenly present in male as in female

individuals (40). In Japan, GS seems to be characterized by a mutation in the coding region (41). Recent studies documented that this mutated UGT1A1*28 is frequently associated with mutations in UGT1A6 (89) and in other UGTs (UGT1A3 and UGT1A7 polymorphism), leading to a haplotype of four genetic variants (54). UGT1A6 is involved in the glucuronidation of 4-nitrophenol, 4-methylumbelliferone, etc. and UGT1A7 in the glucuronidation of irinotecan and of atazanavir. It is not yet clear whether such combined polymorphisms of UGTs in GS might exert a negative effect on the metabolization of other drugs or environmental toxic substances. In addition to decreased UDP-GT, several individuals with GS have a reduced hepatic uptake of UCB and ICG (44, 90). It is not yet clear whether this is due to a lower expression of OATPs. Serum bilirubin levels in GS will thus depend on the presence of a homozygous or a heterozygous mutation of the UGT1A1, of an additional reduced hepatic uptake, on hormonal influences (e.g. sex and thyroid hormones), on inhibiting or enzyme-stimulating medication, on fasting and on possibly associated haemolysis (35, 36).

The higher serum UCB levels appear to be advantageous because UCB is a strong anti-oxidant and inhibits lipid peroxidation (91). Population studies documented a reduced incidence of cardiovascular problems (92, 93), of carcinoma in general (94) and of colorectal carcinoma specifically (79) in individuals with higher serum UCB.

A disadvantage of the mutated UGT1A1 has been documented recently, because both irinotecan and indinavir are glucuronidated by the same GT as bilirubin. Irinotecan (Campto®, Pfizer Co) is a camptothecin analogue, a prodrug and requires bioactivation to the active 7-ethyl-10-hydroxycamptothecin (SN-38), which is a strong DNA topo-isomerase-1 inhibitor. SN-38 is detoxified to SN-38-glucuronide by UDP-GT (UGT1A1 genotype). Patients with GS will glucuronidate SN-38 more slowly and as such will have higher blood levels of the active SN-38. This results in more severe neutropaenia and diarrhoea following intake of irinotecan, in parallel with their bilirubin levels (95–98). Indinavir used in the therapy against the human immunodeficiency virus is detoxified more slowly in GS patients, and this can lead to haemolytic jaundice. Indinavir also inhibits the uptake transporter OATP 1B1 and this might additionally enhance the unconjugated hyperbilirubinaemia (15). Atazanavir, another antiretroviral protease inhibitor, is an inhibitor of bilirubin UDP-GT and is itself metabolized by the GT encoded by UGT1A7, which is often mutated in association with UGT1A1 (54). As a result

of both mutations being present in GS, atazanavir is more slowly catabolized and thus exerts an inhibition of bilirubin UDP-GT. This dual mechanism will lead to a marked hyperbilirubinaemia (54).

Gilbert's syndrome is characterized by an enhanced fasting hyperbilirubinaemia (99). Fasting for 24–48 h enhances serum bilirubin levels also in normal individuals because fasting results in an augmented haem-oxygenase activity, which leads to an increased production of bilirubin (100, 101). The absence of enteral feeding leads to a decreased intestinal motility and this may result in enhanced deconjugation by the bacterial flora with a greater intestinal re-absorption of UCB, adding to the serum UCB level (102, 103). In the case of GS, the lower UDP-GT will augment this UCB because of the decreased hepatic conjugation. Fasting hyperbilirubinaemia is normalized by enteral but not by intravenous administration of calories (99). Similarly, higher serum bilirubin levels have been observed in patients with GS and long-term parenteral nutrition (104) or associated achalasia (105) or in neonates with hypertrophical pyloric stenosis (106). Higher UCB levels in GS are also seen during fever (which induces mild haemolysis) and in general when GS is combined with a low-grade haemolysis. In one study, individuals with GS and haemolysis had levels of 3.9 ± 1.1 vs 2.6 ± 0.9 mg/dl in haemolysis alone and 2.2 mg/dl in GS alone (35).

Conclusion

Bilirubin is an interesting molecule, with special physico-chemical properties. Its complex metabolism is frequently disturbed. The conjugation is rate limiting under normal conditions and determines serum UCB and biliary excretion. Biliary secretion is the most susceptible step and is most easily disturbed, leading to conjugated hyperbilirubinaemia. The uptake mechanism needs more investigation.

Acknowledgements

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The reader interested in additional chemical or biochemical aspects is referred to the excellent book edited by J Donald Ostrow: Bile Pigments and Jaundice. Marcel Dekker Inc., New York & Basel, 1986.

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