Bilirubin metabolism: Applied physiology

Xia Wang, Jayanta Roy Chowdhury*, Namita Roy Chowdhury

Albert Einstein College of Medicine, New York, USA

Summary
Bilirubin is the breakdown product of the haem moiety of haemoglobin and other haemoproteins. Because of internal hydrogen bonding, bilirubin is water-insoluble and requires enzyme-mediated glucuronidation in the liver for biliary excretion. In normal circumstances, plasma bilirubin is mostly unconjugated and is tightly bound to circulating albumin. It is taken up by hepatocytes by facilitated diffusion, stored in hepatocytes bound to glutathione-S-transferases and conjugated to glucuronides by microsomal UGT1A1. Bilirubin glucuronides are actively transported into the bile canaliculi by the ATP-utilizing pump MRP2. Bilirubin is degraded in the intestine by bacteria into urobilinogens, which are partly excreted in the urine. Increased production, reduced uptake and low glucuronidation capacity can increase plasma unconjugated bilirubin levels. In cases of inherited or acquired deficiencies of bilirubin storage or excretion, both conjugated and unconjugated bilirubin accumulate in the plasma. Conjugated bilirubin is less tightly bound to albumin and is excreted in the urine. The capacities of the various steps of bilirubin throughput are finely balanced, and the expression of the gene products mediating these steps is coordinated by nuclear receptors.
© 2005 Elsevier Ltd. All rights reserved.

Practice points

- In normal circumstances, plasma bilirubin is mostly unconjugated (∼96%)
- The presence of a higher percentage of conjugated bilirubin suggests liver disease or inherited errors of bilirubin excretion. However, the 'direct-reacting' fraction in clinical tests slightly overestimates the conjugated fraction of bilirubin
- Unconjugated bilirubin is not excreted in urine in the absence of proteinuria. Therefore, the excre-
tion of conjugated bilirubin in the urine indicates the presence of an increased amount of conjugated bilirubin in the plasma
- When conjugated bilirubin accumulates in the plasma over a long time, a fraction of the pigment may bind irreversibly to albumin, generating a complex that is not excreted in the bile or urine. Thus, after surgical correction of biliary obstruction, direct-reacting hyperbilirubinaemia may linger for several weeks

Research directions

- Bilirubin is toxic to cells when its molar concentration in the plasma exceeds that of albumin. Mild

*Corresponding author. Tel.: +1 718 430 2265;
fax: +1 718 430 8975.
E-mail address: chowdhur@aecom.yu.edu (J.R. Chowdhury).

- hyperbilirubinaemia may be cytoprotective by virtue of its antioxidative effect. Further study is needed to determine types of cancer or other diseases mild hyperbilirubinaemia, as seen in Gilbert syndrome, may have a protective role
- Protein(s) that mediate the facilitated diffusion of bilirubin at the sinusoidal surface of the hepatocytes have not been conclusively identified

Introduction

Approximately 4 mg/kg body weight of bilirubin is produced daily from haem-containing proteins from erythroid and non-erythroid sources. Haemoglobin, released by the breakdown of senescent red blood cells, is the major erythroid source, but there is a significant contribution from free haem and haemoglobin that is produced but not incorporated into mature red cells (ineffective erythropoiesis). Approximately 20% of the total daily bilirubin production is normally contributed by other haemoproteins, primarily in the liver, such as cytochromes, catalase, peroxidase and tryptophan pyrrolase. Bilirubin is potentially toxic but is normally rendered harmless by tight binding to albumin and rapid conjugation and excretion by the liver. Bilirubin encephalopathy (kernicterus) is seen in severe cases of exaggerated neonatal jaundice and in patients with very high levels of unconjugated hyperbilirubinaemia owing to inherited disorders of bilirubin glucuronidation.[1]

Early and late-labelled peaks of bilirubin

Following the intravenous administration of the radiolabelled porphyrin precursors glycine or δ-aminolevulinic acid, the radioactivity is incorporated into bilirubin in two temporal peaks. The 'early labelled peak', derived mainly from liver enzymes and free haem, appears within 72 h. This peak is enhanced in 'ineffective erythropoiesis', for example congenital dyserythropoietic anaemias, megaloblastic anaemias, iron-deficiency anaemia, erythropoietic porphyria and lead poisoning. A late-labelled peak appears at approximately 110 days in humans and 50 days in rats, and is derived mainly from the haemoglobin of senescent erythrocytes. In haemolytic conditions, in which the lifespan of erythrocytes is shortened, this peak appears earlier.[2]

Enzymatic mechanism of bilirubin formation

Haem is a tetrapyrrole, the four pyrrole rings being connected by methane bridges. The four bridges are not equivalent because the side chains are asymmetrically distributed (Fig. 1). Haem is cleaved specifically at the α-methene bridge by a reaction catalysed by microsomal haem oxygenases, resulting in the formation of biliverdin and 1 mole of CO, and the release of an iron molecule. The reaction consumes three molecules of oxygen and requires a reducing agent, such as NADPH. The α-methene-bridge carbon is eliminated as CO, and the iron molecule is released.[3]
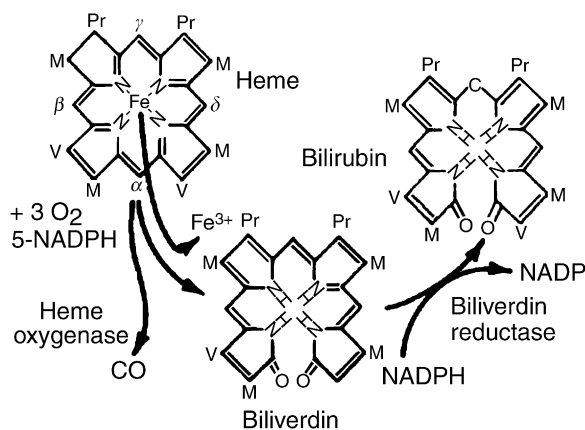

Figure 1 Enzymatic mechanism of bilirubin formation. The haem ring opens at the α-carbon bridge by the action of microsomal haem oxygenases, forming the green pigment biliverdin. Biliverdin is subsequently reduced to bilirubin by cytosolic biliverdin reductases.

There are three known isoforms of haem oxygenase (HO). The ubiquitous isoform HO-1 is inducible by haem and stress. HO-2 is a constitutive protein present mainly in the brain and the testis. HO-3 has a very low catalytic activity and may function mainly as a haem-binding protein. Subsequently, biliverdin is reduced to bilirubin by the action of cytosolic biliverdin reductase. The vasodilatory effect of CO regulates the vascular tone in the liver, heart and other organs during stress. The other products of haem breakdown, namely biliverdin and bilirubin, are potent antioxidants, which may protect tissues under oxidative stress (see below).

Since haem breakdown is by far the most important source of endogenous CO production, bilirubin formation can be quantified from CO exhaled in the breath. At steady state, bilirubin formation equals haem breakdown, which in turn equals haem synthesis. Breath CO excretion increases in haemolytic states. A small fraction of the CO may be formed by intestinal bacteria. Bilirubin production can be temporarily inhibited by administering dead-end inhibitors of haem oxygenase, such as tin-mesoporphyrin. In neonates, a single injection of tin-mesoporphyrin reduced serum bilirubin levels by 76% and prevented severe hyperbilirubinaemia in all recipients.[4]

Internal hydrogen bonding

Despite the presence of several polar groups, such as the propionic acid side-chains and the amino groups, bilirubin is insoluble in water. This apparent paradox is explained by internal hydrogen bonds between the propionic acid carboxyls and the contralateral amino and lactam groups (Fig. 2).[5] In nature, the hydrogen bonds are disrupted by glucuronidation of the propionic acid carboxyls. As a result, conjugated bilirubin is water-soluble and readily excretable in bile. The hydrogen bonds of unconjugated bilirubin bury the central methane bridge that connects the two dipyrrolic halves. Because of this, unconjugated bilirubin reacts very slowly with diazo reagents. In conjugated bilirubin, the central bridge is accessible to diazo reagents, so that the
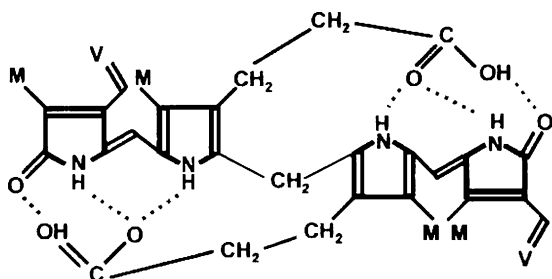
Figure 2 Internal hydrogen bonding of bilirubin IXα. Engagement of all the polar groups by internal hydrogen bonds makes bilirubin water-insoluble. The central CH_2 bridge is 'buried' and protected by the hydrogen bonds, so that unconjugated bilirubin reacts with diazo reagents only in the presence of accelerators ('indirect' van den Bergh reaction). Glucuronidation disrupts the hydrogen bonds, whereby conjugated bilirubin reacts immediately with diazo reagents ('direct' van den Bergh reaction).

reaction occurs rapidly ('direct' van den Bergh reaction). Total bilirubin can be measured by disrupting the hydrogen bonds by adding accelerators. The difference between total bilirubin and the direct-reacting fraction represents unconjugated bilirubin. Since 5–10% of unconjugated bilirubin gives a direct van den Bergh reaction, the direct-reacting fraction slightly overestimates conjugated bilirubin.

Exposure of the skin to light changes the geometric configuration of bilirubin, disrupting the internal hydrogen bonds and resulting in the excretion of unconjugated bilirubin in bile.[6] This is thought to be the main mechanism of reduction of serum bilirubin level by phototherapy, which is used in neonatal jaundice and in patients with Crigler–Najjar syndrome.

Bilirubin in serum, bile and urine[7]

About 96% of the bilirubin in normal plasma is unconjugated, although diazo-based clinical analytical methods slightly overestimate the conjugated fraction (see above). During haemolysis, the total serum bilirubin concentration increases, but the percentage of conjugated bilirubin tends to remain the same. In contrast, in inherited disorders associated with a deficiency of bilirubin glucuronidation, there is a further reduction in the proportion of the conjugated fraction. In biliary obstruction, hepatocellular injury or intrahepatic cholestasis, both conjugated and unconjugated bilirubin accumulate in the plasma, resulting in a marked increase in the proportion of conjugated bilirubin.

A tight binding of unconjugated bilirubin to albumin prevents its excretion in the urine, except in cases of albuminuria. Conjugated bilirubin binds to albumin less tightly, and the unbound fraction is excreted in the urine. Thus, bilirubinuria usually implies the accumulation of conjugated bilirubin in the urine.

Bilirubin diglucuronide constitutes about 80% of the bile pigments excreted in normal human bile. The proportion of bilirubin monoglucuronide increases in the presence of a reduced conjugating capacity of the liver, as in Crigler–Najjar syndrome type 2 and Gilbert syndrome.

Toxicity of bilirubin

Free unconjugated bilirubin exhibits a wide range of toxicity to many cell types, particularly neuronal cells. All known toxic effects of bilirubin are abrogated by binding to albumin. Cerebral toxicity (kernicterus) from bilirubin occurs when the molar ratio between bilirubin and albumin exceeds 1.0. Bilirubin toxicity is usually seen during exaggerated neonatal hyperbilirubinaemia and in patients with Crigler–Najjar syndrome at all ages. In neonates, serum unconjugated bilirubin levels above 340 μmol/l (20 mg/dl) are generally considered dangerous. Kernicterus can, however, occur at lower levels in the presence of sulphonamides, radiographic contrast media, coumarins and anti-inflammatory drugs that displace bilirubin from its albumin-binding sites, thereby increasing the level of unbound bilirubin. The immaturity of the blood–brain barrier in neonates has traditionally been implicated as a cause of susceptibility to kernicterus, but lower bilirubin clearance from the brain may play an important role.

Possible beneficial effects of bilirubin

Since bilirubin is a strong antioxidant, mild hyperbilirubinaemia may have a protective effect against ischemic cardiovascular disease and cancer. In a recent study on a large population, the odds ratios for a history of colorectal cancer were reported to be reduced to 0.295 in men and 0.186 in women per 1 mg/dl increment in serum bilirubin levels.[8] An inverse relationship between serum bilirubin levels and cancer mortality has also been reported. Such negative associations do not, however, conclusively establish a cause-and-effect relationship because of the presence of many potentially confounding variables.

Hepatic disposition of bilirubin

Plasma transport and hepatic uptake

Albumin-binding keeps bilirubin in solution, neutralises its toxic effects and transports the pigment from its site of production to the liver. The binding of bilirubin to albumin is usually reversible, but during prolonged conjugated hyperbilirubinaemia, a fraction of the conjugated bilirubin becomes irreversibly bound to albumin.[9] This fraction, termed δ-bilirubin, gives a direct van den Bergh reaction and is not excreted in the bile or urine. It therefore persists in the serum for a long time, reflecting the long half-life of albumin. The molar concentration of albumin (500–700 μmol/l) normally exceeds that of bilirubin (3–17 μmol/l). In cases of severe hyperbilirubinaemia, particularly in the presence of hypoalbuminaemia, the molar ratio of unconjugated bilirubin to albumin may exceed 1, resulting in kernicterus. As discussed above, drugs that displace bilirubin from albumin increase the unbound bilirubin concentration, increasing the risk of kernicterus in jaundiced infants.

Bilirubin dissociates from albumin at the sinusoidal surface of the hepatocytes, being taken up by facilitated diffusion. The transport requires inorganic anions, such as Cl^- and Cl^-/HCO_3^- exchange, and is non-energy-consuming.

A sinusoidal membrane organic anion transport protein, oatp-2, was reported to facilitate bilirubin uptake, although its physiological significance remains debatable. Inside the hepatocyte, bilirubin binds to cytosolic glutathione-S-transferases initially termed ligandins). Binding to glutathione-S-transferases keeps unconjugated bilirubin soluble in the cytosol of hepatocytes and increases the net uptake of bilirubin by reducing its efflux from the cell.[1]

UGT1A1-catalysed glucuronidation

Conversion to glucuronides is essential for the efficient biliary excretion of bilirubin. Bilirubin glucuronidation is catalysed by a specific isoform of uridinediphosphoglucuronate glucuronosyltransferase, termed UGT1A1. UGT1A1 is expressed from the UGT1A locus that expresses eight other UGT isoforms. The *UGT1A1* gene contains four consecutive exons (exons 2–5) at the 3′ end that are used in several other UGT isoforms. The amino-terminal half, which imparts it specificity for bilirubin, is encoded by a single unique exon.[10] Hepatic UGT1A1 activity is very low at birth and matures during the first 10 days of life. During intrauterine life, unconjugated fetal bilirubin is transferred to the maternal plasma by the placenta. UGT1A1 is induced by treatment with phenobarbital, diazepam, phenytoin, spironolactone and peroxisome proliferating agents (e.g. fibrates).

Since UGT1A1 is the only UGT isoform that significantly contributes to the glucuronidation of bilirubin, a reduced activity of this isoform results in various grades of unconjugated hyperbilirubinaemia. Delayed development of UGT1A1 is the most important cause of neonatal unconjugated hyperbilirubinaemia. This delayed development can be exaggerated because of some ill-defined factors in the maternal serum, leading to Lucey–Driscoll syndrome, which may cause a prolongation of severe hyperbilirubinaemia for several weeks and may even cause kernicterus.

A mild form of unconjugated hyperbilirubinaemia (bilirubin levels ranging from normal to 85 μmol/l), termed Gilbert syndrome, is found in up to 5% of Caucasian, black and South Asian populations. This condition is associated with a promoter variation (insertion of a TA residue in the TATA element) of UGT1A1.[11] Although 9% of Caucasian and black populations are homozygous for this genotype, all these subjects do not exhibit clinical hyperbilirubinaemia.

More severe unconjugated hyperbilirubinaemia is found with mutations or short deletions within the five exons that constitute the UGT1A1 mRNA. A complete loss of UGT1A1 activity resulting from these rare genetic lesions causes Crigler–Najjar syndrome type 1 (serum bilirubin levels of 250–650 μmol/l).[1,12] Crigler–Najjar syndrome type 1 is associated with kernicterus unless vigorously treated with phototherapy, and eventually requires liver transplantation. A partial deficiency of UGT1A1 activity arising from the substitution of single amino acids causes Crigler–Najjar syndrome type 2 (serum bilirubin levels of 130–255 μmol/l), in which kernicterus is rare and serum bilirubin levels are usually reduced by at least 25% upon treatment with UGT1A1-inducing agents, such as phenobarbitone.[13,14]

Canalicular excretion of conjugated bilirubin

Conjugated bilirubin is excreted into the bile canaliculus against a concentration gradient by an energy-consuming process. The energy is derived by ATP-hydrolysis by a canalicular membrane protein, belonging to the ATP-binding cassette (ABC) family, termed ABCC2 (also known as the multidrug resistance-related protein-2 (MRP2). This export pump is involved in the canalicular secretion of many other organic anions, particularly those which are conjugated with glucuronic acid or glutathione.[15] Most bile acids do not, however, use this pathway for excretion. Genetic lesions of ABCC2 cause the rare disorder Dubin–Johnson syndrome, in which both conjugated and unconjugated bilirubin accumulate in the plasma. Consistent with the defective excretion of many other non-bile-acid organic anions, there is accumulation of a black pigment.[2,15] A genetically unrelated disorder, Rotor syndrome, is caused by reduced hepatic storage capacity, resulting in mixed conjugated and unconjugated hyperbilirubinaemia but no pigment accumulation in the liver.[16]

The bile salt export pump, which is required for normal bile flow, and MDR-3, which transports phospholipids from the inner leaflet of the canalicular membrane to the outer leaflet, are also important in bilirubin secretion into the bile. During cholestasis, the accumulation of both conjugated and unconjugated bilirubin in the hepatocytes may lead to an upregulation of one or more other MRP molecules (e.g. MRP-3, MRP-4), which actively transport both conjugated and unconjugated bilirubin from the hepatocytes back into the plasma. This may explain the accumulation of both forms of bilirubin in the plasma in biliary obstruction or intrahepatic cholestasis.[3,15] (Fig. 3)

Fate of bilirubin in the gastrointestinal tract

Conjugated bilirubin is not reabsorbed from the intestine, but the small amount of unconjugated bilirubin that appears in the bile is partially reabsorbed. Cows' milk inhibits bilirubin reabsorption, but maternal milk does so less efficiently. This may be one reason for the higher serum bilirubin levels found in breast-fed compared with formula-fed infants. Intestinal bacteria degrades bilirubin into urobilinogen, most of which is absorbed from the intestine and undergoes enterohepatic recirculation.[17] A minor
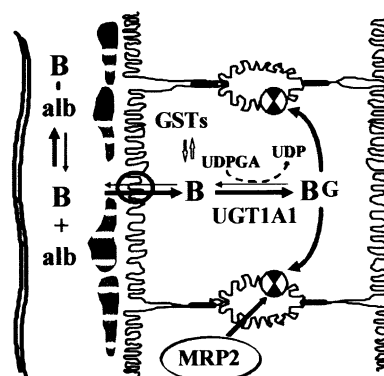

Figure 3 Bilirubin throughput by the hepatocyte.

fraction is then excreted in the urine. Urobilin, the oxidation product of urobilinogen, contributes to the colour of normal urine and stool. During severe cholestasis (e.g. the early phases of hepatitis A or B) or near-complete biliary obstruction (e.g. in carcinoma of the pancreas), bilirubin excretion in bile is markedly reduced, and the resulting lack of formation of urobilinogen causes the pale, so-called 'clay-coloured' stool.

Renal bilirubin elimination

As mentioned above, conjugated bilirubin is excreted in the urine. The kidney becomes the predominant route of excretion of bilirubin in severe cholestasis. Therefore, the coexistence of cholestasis and renal failure results in the highest serum bilirubin levels.

Acknowledgements

References

1. Roy Chowdhury J, Wolkoff AW, Roy Chowdhury N, Arias IM. Hereditary jaundice and disorders of bilirubin metabolism. In: Scriver CR, Boudet AL, Sly WS, Valle D, editors. *The metabolic and molecular bases of inherited disease*. 8th ed. New York: McGraw-Hill; 2001. p. 3063–101.

2. Roy Chowdhury N, Wang X, Roy Chowdhury J. Bile pigment metabolism and its disorders. In: Rimoin DL, Connor JM, Pyeritz RE, Korf BR, editors. Principles and practice of medical genetics, 5th ed., London: Churchill Livingstone, in press.

3. Roy Chowdhury N, Arias IM, Wolkoff AW, Roy Chowdhury J. Disorders of bilirubin metabolism. In: Arias IM, Jakoby WB, Schachter D, Shafritz DA, editors. *The liver: biology and pathobiology*. 3rd ed. New York: Raven Press; 2001.

4. Drummond GS, Kappas A. Chemoprevention of severe neonatal hyperbilirubinaemia. *Semin Perinatol* 2004;**28**:365–8.

5. Bonnett R, Davis E, Hursthouse MB. Structure of bilirubin. *Nature* 1976;**262**:327–8.

6. Itho S, Onishi S. Kinetic study of the photochemical changes of (ZZ)-bilirubin IX bound to human serum albumin. Demonstration of (EZ)-bilirubin IX as an intermediate in photochemical changes from (ZZ)-bilirubin IX to (EZ)-cyclobilirubin IX. *Biochem J* 1985;**226**:251–8.

7. Roy Chowdhury J, Roy Chowdhury N, Jansen PLM. Bilirubin metabolism and its disorders. In: Zakim D, Boyer T, editors. *Hepatology: a textbook of liver disease*. 4th ed. London: Saunders; 2003. p. 233–69.

8. Zucker SD, Horn PS, Serman KE. Serum bilirubin levels in the US population: gender effect and inverse correlation with colorectal cancer. *Hepatology* 2004;**40**:827–35.

9. Lauff JJ, Kasper ME, Ambros RT. Quantitative liquid chromatographic estimation of bilirubin species in pathological serum. *Clin Chem* 1983;**29**:800–5.

10. Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT, et al. A novel complex locus UGT1 encodes human bilirubin, phenol and other UDP-glucuronosyltransferase isozymes with identical carboxy termini. *J Biol Chem* 1992;**267**:3257–61.

11. Bosma PJ, Roy Chowdhury J, Bakker C, et al. Sequence abnormality in the promoter region results in reduced expression of bilirubin-UDP-glucuronosyltransferase-1 in Gilbert syndrome. *N Eng J Med* 1995;**333**:1171–5.

12. Bosma PJ, Roy Chowdhury N, Goldhoorn BG, Hofker MH, Oude Elferink RPJ, Jansen PLM, et al. Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler–Najjar syndrome, type I. *Hepatology* 1992;**15**:941–7.

13. Seppen J, Bosma P, Roy Chowdhury J, Roy Chowdhury N, Jansen PLM, Oude Elferink R. Discrimination between Crigler–Najjar syndrome type I and II by expression of mutant bilirubin-UDP-glucuronosyltransferase. *J Clin Invest* 1994;**94**:2385–91.

14. Arias IM, Gartner LM, Cohen M, Benezzer J, Levi AJ. Chronic nonhemolytic unconjugated hyperbilirubinaemia with glucuronosyltransferase deficiency: clinical, biochemical, pharmacologic, and genetic evidence for heterogeneity. *Am J Med* 1969;**47**:395.

15. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. *Annu Rev Biochem* 2002;**71**:537–92.

16. Wolkoff AW, Wolpert E, Pascasio FN, Arias IM. Rotor's syndrome: a distinct inheritable pathophysiologic entity. *Am J Med* 1976;**60**:173.

17. Watson CJ. The urobilinoids: milestones in their history and some recent developments. In: Berk PD, Berlin NI, editors. *Bile pigments: chemistry and physiology*. Washington, DC: US Government Printing Office; 1977. p. 469–82.