Abstract

Bilirubin is one of the degradation products of haeme. Bilirubin is hydrophobic because of internal hydrogen bonds and must be bound to albumin for transportation. It is converted into a water-soluble and excretable form in the liver by glucuronidation. Bilirubin concentration elevations (icterus) are used in the (differential) diagnosis of hepatopathies and biliary stasis disorders as well as increased haemolysis. Excessive elevations of the neurotoxic bilirubin in neonates must be diagnosed and treated. The antioxidant effects of bilirubin and its metabolites have been researched in recent times. Slight bilirubin concentration elevations are associated with a lower prevalence and risk of cardiovascular and neurodegenerative disorders, diabetes mellitus, overall mortality and (perhaps also) cancer. Further research into the molecular causes is necessary.

Keywords: bilirubin, hepatopathies, haemolysis, icterus, kernicterus, antioxidant, risk markers
hough BR contains hydrophilic groups, such as two propionic acid residues, it is sparingly soluble in water and is not excreted renally. Internal hydrogen bonds between the two propionic acids and the NH and lactam groups give rise to what is known as a folded ZZ configuration and BR gains hydrophobic/lipophilic properties, which can promote accumulation in the central nervous system at relatively high BR concentrations (> 15 mg/dl) (see kernicterus).

Blood transport of BR can take place only after binding to water-soluble albumin. In analytical processes with a diazo reagent, this combination is termed indirect unconjugated bilirubin (BRu). Albumin is dissociated at the surfaces of hepatocytes and the endothelia and the free BR is transported into the cell energy-dependently and with the aid of transport proteins and bound to specific cytosol proteins (y protein [ligandin] and z protein). Prior to excretion in the bile, glucuronidation of one or two propionic acids takes place in the endoplasmic reticulum by means of a specific UDP glucuronosyltransferase (UGT1A1 gene, see glossary) [6]. In diazo analysis, it is termed direct conjugated bilirubin (BRc). Under normal circumstances, up to 70–90% of BR is glucuronidated twice and the remainder once. Less than 1% is free BR, which is particularly important in the prevention of gallstones. The glucuronidated BRc is secreted in hepatic bile (bile A) by MRP-2 (multidrug resistance protein 2) transport proteins and multispecific organic anion transporters (MOAT) through the canalicular membranes of hepatocytes [7]. Every day, the liver excretes approximately 1 g of bilirubin in the bile, equivalent to 2–5 times the newly produced quantity. Besides minimal quantities of BRu, the hepatic bile contains only BRc, in concentrations of 42.8±16 mg/dl (723±289 µmol/l). Biliary bile contains only a vanishingly small concentration [8].

In the presence of prolonged glucuronidated BRc elevation, such as occurs in obstructive jaundice, albumin binding also takes place. This fraction is termed delta-bilirubin (BRδ) and is not detectable in the serum of healthy individuals. Due to albumin binding, BRδ has a longer half-life (~ 18 days) than other albumins. This means that, particularly during convalescence from cholestasis, BRδ can account for up to 90% of total bilirubin, which must be taken into account in the post-analytical interpretation of the disease process unless specific BRδ determination is performed.

Conjugated BR excreted in the bowel undergoes hardly any resorption. Only after cleavage of glucuronic acid by bacterial glucuronidases is BR converted to bile pigments by means of a redox reaction [1,2,9]. Hydrogenation of the two vinyl groups gives rise to mesobilirubin and further hydrogenation of methine groups to colourless mesobilirubinogen. Other products are today grouped together as urobilinogens: D-urobilinogen, i-mesobilinogen and stercobilinogen. Approximately 20% of these is reabsorbed, reaches the liver, and is excreted again in the bowel via enterohypoxic cycling. Only 2–5% reaches the blood (53±32 mg/l) and the urine (< 4 mg/dl). The colourless products are oxidised to urobilin, mesobilin and stercobilin (40–280 mg/dl) in the colon and/or urine. Together with other degradation products (tri- and dipyrroles, bilifuchsin, mesobilifuchsin) they are responsible for the colour of the stools and urine. Detection or determination (including of BR) takes place with various aldehyde or azo dye reactions, mostly using dipstick tests these days [1,2]. BR remains unchanged by colon sterilisation with antibiotics and can be converted into green biliverdin by atmospheric oxygen.

Approximately 250 mg of BR is produced every day: 1 g of haemoglobin gives 35 mg of BR; daily Hb turnover in an adult is 6.25 g (90 µmol), equivalent to 220 mg of BR. On top of this, a further 20–30 mg of BR is produced by the degradation of haeme proteins (myoglobin, cytochromes, haeme peroxidases) and 1–2 mg by ineffective erythropoiesis (termed shunt bilirubin).

**Indications**

- Differential diagnosis and monitoring of prehepatic, hepatic and posthepatic icterus
- Calculations for liver transplantation, such as the MELD or Child-Pugh score
- Dose adjustment in chemotherapy
- Monitoring of neonatal jaundice
- Monitoring of Rh and ABO incompatibilities by measurement of amniotic fluid BR and analysis in the Liley nomogram
- Genetic disorders of bilirubin catabolism

**Fig. 1: BVR mechanism**

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Pre-analytics

The examination material is serum or plasma. The samples must be stored in the dark because sunlight destroys ~ 50% of BR in one hour. In light-protected serum, bilirubin is stable for 1–2 d at room temperature and for 7 d at 3 – 8°C.

Prolonged fasting increases the concentration of BR (from 0.5 to 0.9 mg/dl after 48 h), much more so in Gilbert’s syndrome (1.5 to 3.4 mg/dl, which has sometimes been recommended as a test for diagnosis of the syndrome). BR elevations due to anabolic steroids, allopurinol, diuretics, indometacin, sulphonamides, and haemolytic serum and indoxyl derivatives in uraemia. BR can be lowered by the pill.

Analytics

• The diazo reagent described by Paul Ehrlich in 1883 was expanded into a direct and indirect diazo determination for bilirubin by A. A. H. van den Bergh and P. Müller in 1916 [10] and is usually used in the variant by L. Jendrassik and P. Gröf [11] and also proposed as a reference method. Total BR (BRc, BRu and BRδ) is calculated using a reagent (“accelerator”) to dissociate albumin (caffeine, methanol, etc.). Direct BR (BRc, BRδ and a minimal quantity of BRu) reacts immediately without a dissociation reagent. Indirect BR is an oper-and (total BR minus direct BR) and is meaningful only in haemolytic and genetic hyperbilirubinaemia when no BRδ is present. According to the guidelines of the German Federal Medical Association (‘Rill-BÄK’), the permissable relative deviation of the individual value from total BR is 13% at >2 to 30 mg/dl and 22% at 0.1 to <2 mg/dl. The individual biological deviation from total BR is ~ 25% and is thus larger than for many other parameters. The azo reaction must be performed at pH 4.75 for specific determination of BRc.

• DPD method: 2,5-dichlorophenyl diazonium salt in 0.1 mol/l HCl with Triton X-100 detergent

• Bilirubin oxidase converts BR to biliverdin and forms purple pigments with O2 at pH 10, only BRc is oxidised. Differences in relation to the azo method result from incomplete oxidation of conjugated BRc [12].

• With appropriate preparation, HPLC measures the individual fractions exactly; BRc, in particular, comes out lower than with the diazo method.

• Multilayer film technique with different cartridges

• Direct photometric measurement of neonatal samples without BRc at 460 nm (BR) and for differentiation against haemoglobin at 550 nm or measurement between 350 and 600 nm with differentiation above the linear absorption line (DA450)

• Urine test strips use diazonium salts for semiquantitatively analysable colour changes (for historical tests, see glossary)

Interpretation

Hyperbilirubinaemia, icterus (jaundice) [1,2,13]
The yellowing first becomes apparent in the sclera at a total BR of > 2 mg/dl (34.4 µmol/l). A level of 1.2 – 2.9 mg/dl is referred to as subicterus and from 3.0 mg/dl as icterus, which is stratified into 3 categories according to whether its onset takes place inside or outside the liver:

• Prehepatic icterus (haemolytic icterus) with predominantly indirect, unconjugated bilirubin (until albumin-binding capacity has been achieved). Increase in BR production due to haemolysis, blood transfusion complications, the breakdown of extravascular blood clots, myolysis and burns. Genetic disorders or increased haemolysis can also lead to prehepatic icterus: thalassaemia, sickle cell anaemia, pyruvate kinase deficiency and glucose-6-phosphate dehydrogenase deficiency, megaloblastic anaemia as well as malaria. The following are used to differentiate it from hepatic icterus: free haemoglobin ↑, haptoglobin ↓, reticuloocytes ↑, ALT n, GGT n as well as AST and LDH ↑↑ (from haemolysed red blood cells!), bile acids normal. In the urine, urobilinogen is greatly increased, non-water-soluble BRu is absent. This category includes neonatal jaundice, which is caused by a physiological increase in foetal red blood cell breakdown, and lasts for ~ 8 d in full-term infants and for ~ 14 d in pre-term infants. Due to the lack of bacterial colonisation of the bowel, urobilinogen is not detectable until the 5th day. Massively increased BR values (15 µmol/dl; 257 µmol/l) shortly after parturition (blood group Rh incompatibility, pre-term birth) create a risk of kernicterus (bilirubin encephalopathy) with lethargy, hypertonia or hypotonia, seizures, fever and death. The reasons for BR transport into the CNS are the minimal activity (1% at birth) of glucuronosyltransferase in the liver and a blood-brain barrier that is not functioning or has been damaged by hypoxia or hyperthermia in newborn infants. At BR concentrations of > 20 mg/dl (> 360 µmol/l) on the 3rd day blue light therapy (or sunbaths) can be used, breaking the hydrogen bonds in the non-water-soluble ZZ bilirubin. Transportation of lumobilirubin into the CNS is inhibited and biliary excretion is enhanced. Even more severe forms require an exchange transfusion.

• Hepatocellular icterus (intrahepatic icterus) is caused by a) impaired uptake of BR into the liver (viral hepatitis, steatosis, [primary] biliary cirrhosis, medicines); b) glucuronidation disorders (see also genetic disorders); c) impaired transportation of BRc from the hepatocyte via the canaliculi of the liver into the intrahepatic bile ducts (known as intrahepatic cholestasis). Conjugated BRc increases much more sharply than unconjugated BRu and to some extent reaches the blood, and thus also the urine, via the lymph. Urobilinogen is slightly elevated in the urine. Hepatocellular damage is indicated by elevations of hepatic enzymes and bile acids.

• Posthepatic cholestatic icterus (obstructive jaundice, obstructive icterus, canalisation icterus) is caused by disorders of bile drainage through the common bile duct into the duodenum: gallstones, tumours (initially pain-free; investigation is important!), biliary atresia in newborn infants, cholestasis in pregnancy, pancreatitis and other disorders. Direct BR is greatly increased, indirect BR is normal or slightly increased. In total biliary tract obstruction, the BR concentration increases daily by 2.8 mg/dl (48 µmol/l) and after 15 d reaches a steady state of ~ 20 mg/dl (344 µmol/l). In dark urine, urobilinogen is reduced or absent and at serum concentrations of BRc > 0.5 mg/dl (> 8.5 µmol/l) BR is slightly to greatly increased. The stools will be pale yellow in colour because no BR or other bile pigments are rea-
ching the stools. Of the hepatic enzymes, GGT and alkaline phosphatase in particular will be greatly increased (by 10 and 5 times, respectively), and to a lesser extent AST and ALT. Concurrently increased bile acid levels cause severe pruritus. During monitoring, it should be taken into consideration that the prolonged half-life of BR (50–60% of total BR) can simulate a prolongation of the obstruction phase when using the azo method. Imaging diagnostics are essential. In the presence of biliary tract infections, BR is converted into urobilinogen there and its blood and urine levels are increased.

Genetic causes of hyperbilirubinaemia [1,2,6]

Gilbert’s syndrome, Gilbert-Meulengracht’s syndrome, intermittent juvenile jaundice: A frequently autosomal dominant, chronic, intermittent form of hyperbilirubinaemia caused by disorders of BR uptake in the liver and by glucuronidation disorders [14]. The diagnosis is confirmed shortly after parturition or later in life, particularly in stress situations. In most cases it is characterised by scleral icterus, sometimes combined with weakness, vomiting and abdominal symptoms, but it has a good prognosis. The symptoms and BR concentrations (3.5–5.0 mg/dl; 60–85 µmol/l) are increased after fasting, stress, infections and alcohol. The prevalence is between 4% and 12% of the population (!) and therefore shifts the distribution curve upwards for the population as a whole. Men are affected more frequently than women (2:1 to 7:1). The activity of UDP glucuronosyltransferase (see glossary) is reduced to ~ 30%, meaning that mono-glucuronyl BR predominates. The genetic changes differ in ethnic groups and the phenotype is consistent. Gilbert’s syndrome patients have a reduced risk of arteriosclerosis, tumours and COPD (see below) with normal hepatic function.

Crigler-Najjar syndrome (CNS): The autosomal recessive disorders affect the UGT1A1 gene of UDP glucuronosyltransferase. With CNS I, little or no activity is present (loss of 13 base pairs in exon 2 or mutation with chain termination): BRuv level already of 20–35 mg/dl (342–600 µmol/l) at birth with the risk of kernicterus and early death. The weaker genetic variant CNS II has BR concentrations of < 20 mg/dl (< 342 µmol/l) and transferase activity of < 10% of the normal value. Phenobarbital can be tried as a transferase inducer. Icterus occurs in the first year of life in 50%.

Dubin-Johnson syndrome: An autosomal recessive BRc excretion disorder due to reduced MRP2 transporters. Female predominance: ingestion of oestrogens contraindicated. BRc accumulates in the blood: total BR up to 5 mg/dl, rarely up to 20 mg/dl due to stress, alcohol and menstruation; direct BR ~ 3 mg/dl (~ 50 µmol/l). Urinary excretion of coproporphyrin I and a brown/black liver are characteristic.

Rotor’s syndrome: Described in 1948 by A. B. Rotor et al. and initially equated with Dubin-Johnson syndrome, but separated in 1976 by A. W. Walhoff due to little or no coproporphyrin I. BRc concentrations of 3–10 mg/dl (51–100 µmol/l) with normal transaminases. Particularly common in the Philippines.

Idiopathic recurrent cholestasis, Summerskill-Walshe-Tygstrup syndrome with intermittent intrahepatic obstructive icterus in children and adolescents: Direct BR of up to 20 mg/dl (340 µmol/l), alkaline phosphatase elevated, sometimes associated with tiredness, pruritus and hepatomegaly.

Physiological significance of bilirubin and its degradation products

The antioxidant effects of BR on vitamin A and essential fatty acids [15] were first reported in 1954, followed 33 years later by its physio-
logical significance as an antioxidant in various chronic diseases [16]. BR offers greater protection than α-tocopherol against lipid oxidation and its efficacy in the tissues is similar to or better than glutathione (GSH), although BR accounts for only approximately one-tenth (20–50 nmol/l) of the GSH concentration in the cells. 10 nmol/l BR should provide protection against a 10,000 times higher hydrogen oxide concentration (the quantities probably depend on the experimental conditions). The mechanisms have not yet been fully elucidated but the connections are confirmed by significant correlations.

A significant inverse correlation between BR concentrations and overall mortality and/or specific diseases such as cardiovascular disease (CVD) and cancer has been found in various extensive epidemiological studies (and animal studies) [6,17–20]. The associations are stronger for men than for women (influence of sex hormones) but are applicable only to people without hepatic disease. Numerous studies have been performed in patients with Gilbert’s syndrome due to the raised BR concentrations: 24 deaths/10,000 person-years as compared to 50 deaths/10,000 person-years in people without Gilbert’s syndrome (see genetic causes). The antioxidant effects of BR in CVD have been studied extensively: it lowers LDL cholesterol, LDL cholesterol oxidation, triglycerides, proinflammatory cytokines, platelet activation and insulin resistance, but it increases HDL cholesterol. Individuals in the highest BR quartile (> 1.2 mg/dl; > 20.6 μmol/l) had a 41% lower risk than those in the lowest quartile (< 0.8 mg/dl; < 13.7 μmol/l) [21]. Gilbert’s syndrome patients have a 50% lower risk of peripheral arterial disease and stroke.

The mixing of terms is clear. The formation of pigment gallstones requires the recirculation of unconjugated BR in the presence of high biliary salt concentrations (cholic acids), which keep insulin sensitivity: HO-1 also plays a major role here, directly or indirectly: activity in the highest quartile was associated with an 8-fold increase in the risk of diabetes [18,22].

The association of BR with tumour prevalence is weaker than its association with CVD and is greatly influenced by patient gender. Gilbert’s syndrome patients have 4 times less risk of colon cancer than people without Gilbert’s syndrome. Whether BR inhibits genotoxic effects, such as due to trinitro-9-fluorenone, and has antimutagenic effects against aromatic and heterocyclic hydrocarbons and amines is also being discussed [23,24].

The antioxidant effect of BR is probably also significant for the central nervous system (CNS) because the CNS is very sensitive to reactive oxygen and nitrogen species (ROS and RNS) and possesses hardly any other antioxidant defence mechanisms. For example, Alzheimer’s disease is accompanied (or triggered?) by oxidative stress, inflammation, GSH loss, mitochondrial malfunction and apoptosis. The haemoxynagebiliverdin reductase system is up-regulated by oxidative stress [25] and increases BR concentrations in the cerebrospinal fluid [26]. Similar observations have been made in Parkinson’s disease and multiple sclerosis. Further study of the relevance of BR in CNS disease is needed as a matter of urgency [25].

The released iron reacts with H$_2$O$_2$ due to inflammation and induction of HO-1 to create hydroxyl radicals which form various oxidation products of bilirubin together with other ROS. With monoclonal antibodies, groups of Japanese researchers found several oxidised tripyrroles and dipyrroles without a diazo reaction and grouped them together as substances known as biopyrrins (bilirubin oxidative metabolites). Due to their good water solubility, they are rapidly excreted in the urine and are believed to be a measure of oxidative stress, such as following a cardiac infarction or after abdominal surgery [27]. Excretion was considerably higher in septic patients than in non-septic patients (21.6±2.5 versus 1.4±0.4 μmol/g creatinine) and there was a strong correlation with body temperature, CRP and leukocyte count [28].

Dihydro-monopyrroles are formed as bilirubin oxidation end-products (BOXes); they contain the known substituents of BR and are present in isomeric forms (BOX A and BOX B). In small quantities, it has been possible to produce BOXes from BR with H$_2$O$_2$ or cytochrome oxidase, and they are now also readily accessible synthetically. Using LC-ESI-MS/MS Z-BOX A was found in a level of 14.4±5.1 nmol/l and Z-BOX B in a level of 10.9±3.1 nmol in the serum, with half-lives of 11.8 and 25.1 min, respectively. BOXes are significant for delayed cerebral vasospasms following subarachnoid haemorrhage and for neuronal deficits due to changes in potassium channel activity [29].

**Glossary**

The ancient theory of the four humours differentiated between blood, phlegm, yellow bile and black bile. The term yellow bile is easy to understand from the colour of bilirubin. What was known as black bile is not easy to interpret these days, with associations often cited with the spleen, testicles and adrenal glands, but also with a sad disposition, such as melancholy (melas black; chole bile). According to Hippocrates of Kos, melancholy is an excess of burnt black bile that pours into the blood (“my bile is up”). A black colour is found in aged gallstones (calcium bilirubinate), blood clots and in isolated cases in the urine (urina nigra, e.g. in alkaptonuria), so a mixing of terms is clear. The formation of pigment gallstones requires the recirculation of unconjugated BR in the presence of high biliary salt concentrations (cholic acids), which keep the BR in solution and encourage passive resorption [30]. It should not be forgotten that cholesterol was first found by F. Poulletier de la Salle in gallstones in 1769 and was named “cholestérine” (sterein solid) in 1816 by M. E. Chevreul (1786–1889). The term cholera (rhein flow) was based on the false assumption that it was a form of “bile diarrhoea” or “bile diarrhoea with vomiting”.

Biliverdin: The water-soluble precursor of bilirubin which can be excreted rapidly. Why evolution favoured the sparingly soluble bilirubin is explained by the fact that only BR can penetrate the placenta and remove the haeme degradation product from the foetus. The green colour of biliverdin can occasionally be seen in haematomas. Like BR, it possesses antioxidant and antimutagenic properties. In
the animal kingdom, biliverdin is involved in the colour of eggs, hells, the blood of saltwater fish and the wings of moths and butterflies. Binding of biliverdin to bacterial phytochromes produces infra-red-emitting chromophores for in vivo imaging.

Biliverdin reductase: A zinc-binding pleiotropic enzyme. Isoform A is particularly present in reticulo-macrophages in the liver and spleen and reduces biliverdin (after dissociation from haeme oxygenase-1) to bilirubin with hydrogenated NADP. When reactive oxygen species convert the antioxidant BR back to bilirubin, it is reduced to BR again immediately. This redox cycle guarantees the antioxidant effect of BR and is important for cell preservation. A genetic switch-off of biliverdin reductase leads to increased ROS production and associated cell death. Secondly, biliverdin reductase induces biliverdin-producing haeme oxygenase-1 in response to oxidative stress. As a pleiotropic protein, bilirubin reductase activates glucose metabolism and cell proliferation via the insulin receptor cascade.

Haeme oxygenase (HO) [3]: The inducible isoform 1 (HO-1) is up-regulated by many stimuli: growth factors, lipid peroxides, NO, oxidative stress, biliverdin reductase and others. HO-1 acts together with its end-product carbon monoxide [4]: promotion of neovascularisation, particularly also in tumours which express large quantities of HO-1, and inhibition of inflammatory processes, new connective tissue formation and apoptosis. HO-1 and CO are needed during embryonal development for the formation of new blood vessels. The concentration of HO-1 in the placenta and CO in exhaled breath is reduced in pre-eclampsia.

Uridine-5-diphospho-glucuronosyltransferase (UGT) (synonym: UDP glucuronosyltransferase) catalyses the transfer of glucuronic acid to hydrophobic endogenous and exogenous metabolites and xenobiotics and increases their water solubility for biliary or renal excretion. From the two known gene families, the UGT1 gene codes for at least 6 different enzymes, with the UGT1A1 gene in turn possessing over 100 genetic variants (polymorphisms, mutations), some of which are associated with reduced or absent UGT activity: Gilbert’s syndrome often has a TA insertion in the TATA promoter region with a frequency of ~30% in Europeans and ~50% in black Americans. By contrast, glycine-to-arginine exchange is more common in Asians. These mutations can also lead to changes in the toxicity of chemotherapeutic agents (neutropenia and diarrhoea with irinotecan administration). Crigler-Najjar syndromes have other mutations of different severities (see text).

Hans Fischer [31]: chemist and doctor, 1881–1945. Awarded his post-doctoral lecturing qualification in 1912 with the topic “Urobilin und Bilirubin”. 1916–1918 Chair of Medical Chemistry in Innsbruck, 1918–1921 in Vienna and 1921–1945 Chair of Organic Chemistry at the Technical University of Munich. His main field of work was pyrrole pigments: porphyrins, urobilin, biliverdin, bilirubin and many others. In 1928 he synthesised haemnin, in 1942 bilirubin. Responsible for structural elucidation of chlorophyll. In 1930, was awarded the Nobel Prize for chemistry.

Historical bilirubin detection:

• Gmelin’s test: After urine is covered with the same volume of nitric acid, a green ring of biliverdin is produced. Leopold Gmelin (1788–1853) particularly studied gastric juice (hydrochloric acid identified for the first time) and bile fluid, in which he together with L. F. Tiedemann discovered cholesterol and taurine. He is best known for his multivolume “Handbuch der Chemie” (“Handbook of Chemistry”).

• Fouchet’s test (André Fouchet, born 1894): After protein precipitation, bilirubin is oxidised to biliverdin by iron(III) chloride; the reaction can also be carried out on paper impregnated with barium chloride (Harrison spot test).

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