1 **MOLECULAR PHYSIOLOGY AND PATHOPHYSIOLOGY OF BILIRUBIN HANDLING BY THE BLOOD, LIVER,**
2 **INTESTINE, AND BRAIN IN THE NEWBORN**

3 THOR W.R. HANSEN¹, RONALD J. WONG², DAVID K. STEVENSON²

4 ¹*Division of Paediatric and Adolescent Medicine, Institute of Clinical Medicine, Faculty of Medicine,*
5 *University of Oslo, Norway*

6 ²*Department of Pediatrics, Stanford University School of Medicine, Stanford CA, USA*

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80 Bilirubin is the end-product of heme catabolism formed during a process that involves oxidation-
81 reduction reactions and conserves iron body stores. Unconjugated hyperbilirubinemia is common in
82 newborn infants, but rare later in life. The basic physiology of bilirubin metabolism, such as
83 production, transport, and excretion, has been well described. However, in the neonate numerous
84 variables related to nutrition, ethnicity, and genetic variants at several metabolic steps may be
85 superimposed on the normal physiologic hyperbilirubinemia that occurs in the first week of life and
86 results in bilirubin levels that may be toxic to the brain. Bilirubin exists in several isomeric forms that
87 differ in their polarities and is considered a physiologically important antioxidant. Here we review the
88 chemistry of the bilirubin molecule and its metabolism in the body with a particular focus on the
89 processes that impact the newborn infant, and how differences relative to older children and adults
90 contribute to the risk of developing both acute and long-term, neurologic sequelae in the newborn
91 infant. The final section deals with the interplay between the brain and bilirubin, and its entry,
92 clearance, and accumulation. We conclude with a discussion of the current state of knowledge
93 regarding the mechanism(s) of bilirubin neurotoxicity.

94 I. INTRODUCTION

95 Among the many transitional processes that take place in newborn infants, jaundice is arguably the
96 most visible, and also the most common cause for diagnostic and therapeutic intervention during the
97 first days of life (306, 329, 440, 491, 627). Neonatal jaundice (NNJ) is caused by the accumulation of
98 unconjugated bilirubin (UCB) in blood and tissues. The normal physiology of bilirubin production,
99 transport, and excretion has been well described (69-71, 710). However, the neonatal period is in
100 many respects unique in regard to bilirubin metabolism, as very significant elevations of UCB
101 concentrations in serum occur only exceptionally after this age. Because the actions of bilirubin
102 present something akin to a '*Janus face*', being not only a physiologically important antioxidant; but
103 also, a toxin, particularly in the brain, it is important to understand the factors that distinguish the
104 physiology and pathophysiology of NNJ from that in the more mature organism (445). Although NNJ
105 has been described in medical literature for centuries, the recognition that severely jaundiced infants
106 are at risk for neurotoxicity is more recent. In 1904, the German pathologist Georg Schmorl described
107 the bilirubin-staining pattern and neuropathological findings in brains from infants who had died
108 with severe jaundice and coined the term '*kernicterus*' (German for '*jaundice of the basal ganglia*')
109 (588). Descriptions of infants who survived severe NNJ with neurologic sequelae soon followed (44,
110 262).

111 Here we review our current understanding of bilirubin chemistry and
112 physiology/pathophysiology with particular reference to NNJ. Many phenomena which may appear
113 less interesting for the mature organism turn out to be important for an infant with significant NNJ.
114 Thus, bilirubin structure, solubility, and isomerization are all important in the pathophysiology of
115 kernicterus as well as in the therapies we employ to treat jaundiced infants. The balance between
116 the dual roles of bilirubin as an antioxidant and toxin is imperfectly understood (445). Because
117 bilirubin production is a potential target for therapeutic intervention, a more detailed understanding
118 of its molecular processes is needed (221, 487, 610, 713). The genetics of hepatic processing and
119 excretion of bilirubin as well as the molecular mechanisms of intestinal handling, may hold the keys

to predicting an infant's risk for developing significant NNJ (420, 689, 692, 732). There are many theories that attempt to explain the mechanisms for bilirubin entry into and processing by the brain, the differential sensitivity to bilirubin neurotoxicity both on the individual and cellular levels, and the 'basic mechanism of bilirubin neurotoxicity', if indeed there is only one (305). Neuroprotection has in recent years been developed for asphyxia-related brain damage in the newborn and may be a promising area for NNJ research. Drug treatment has been promising both in vitro as well as in vivo animal experiments, but is held back by concerns for toxicity (168, 233, 418, 557). Theoretically, the polar bilirubin photoisomers should be less toxic and perhaps also cross the blood-brain barrier (BBB) less easily than the predominant IX α (Z,Z) isomer, but experimental support is needed (304). Thus, the challenges involved in NNJ research remain a fertile field for the inquisitive mind.

II. BILIRUBIN IN THE BODY (FIGURE 1)

A. Bilirubin Chemistry

Bilirubin is formed in the reticuloendothelial system through the catabolism of heme. Hemoglobin (Hb) is the main source (80%–85%), but other heme-containing molecules (myoglobin, cytochrome, peroxidase, catalase) also contribute (70, 522). In hemolytic anemias erythropoiesis increases several-fold, and an even higher proportion of heme is derived from senescing red blood cells (RBCs) (71). Liver production of heme was estimated to contribute 13%–23% to the body's total production (84). The relative fraction of non-Hb heme may increase in conditions such as porphyria, protoporphyria, and lead poisoning (71).

In healthy humans, bilirubin production was estimated at 3.5–4.0 mg/kg body weight (BW)/day (69, 353), but in newborn infants it is twice as high – 8.5 $\pm$ 2.3 mg/kg BW/day (444). Increased bilirubin levels or 'hyperbilirubinemia' in the body are measured as total serum or plasma bilirubin (TSB) by spectrophotometry or co-oximetry, or in skin by transcutaneous bilirubinometry (TcB). Once bilirubin concentrations exceed certain levels, it can be visually detected as 'jaundice'. In humans, jaundice predominantly develops during the first week of life (transitional period) in 60%–80% of healthy newborn infants when hepatic bilirubin metabolism mechanisms are not fully mature

(75). The risk for bilirubin neurotoxicity explains why treatment to reduce TSB levels is so important in neonatal medicine (491). After the first 2 weeks of life, jaundice can sometimes be due to hepatic or bile duct disease, which causes accumulation of *conjugated* bilirubin (glucuronic acid-bound bilirubin). Rare inherited variants of bilirubin excretion and conjugation, such as Gilbert syndrome, Crigler-Najjar syndrome types I and II, Rotor syndrome, Dubin-Johnson syndrome, Aagenæs syndrome, and several other rare inherited and/or metabolic disorders, may cause jaundice after the newborn period.

1. *Bilirubin structure*

The chemical structure of bilirubin was defined in 1937 by Fischer and Orth (215) as a tetrapyrrol with a close relationship to Hb and its successful synthesis was reported in 1942 (FIGURE 2A) (216). In 1976, X-ray crystallography showed that the structure was a bis-lactam (87) (FIGURE 2B), confirmed by ¹⁵N nuclear magnetic resonance (NMR) spectroscopy studies in the 1980s (204, 279, 339). The bilirubin structure described by Fischer and Plieninger (216, 415) (FIGURE 2A) did not account for its stereochemistry(415). For many years, the structure of bilirubin was not consistently described and varied between the correct 4Z,15Z and 4E,15E configurations (415). However, many studies have since confirmed that bilirubin mainly occurs as the 4Z,15Z isomer (87, 88, 494). As will be discussed later, this may be important for the mechanism of bilirubin entry into the brain and its neurotoxicity. The 3-dimensional structure of bilirubin was shown by X-ray crystallography to be a 'ridge-tile' conformation (FIGURES 2C and 2D) (87, 88, 493, 494).

2. *Bilirubin solubility*

Bilirubin (4Z,15Z) appears to have very low solubility in aqueous media, ranging from 7–100 nM at a pH of 7.4 and temperature of 37°C (117, 270). After the neonatal period, TSB levels in non-jaundiced subjects range from 2–25 µmol/L (0.1–1.5 mg/dL), depending on the analytical method used (410, <http://ehandbok.ous-hf.no/document/105055>). In jaundiced infants, concentrations may be >300 µmol/L (17.5 mg/dL) and are vastly in excess of bilirubin solubility in aqueous, protein-free media. Thus, stability during the transport of bilirubin (4Z,15Z) in blood must involve additional factors.

These will be discussed in detail below (Section IV). Solubility and stability are challenges in studies of neonatal bilirubin pathophysiology. Some claim that the bilirubin concentrations used in many bilirubin toxicity experiments are in non-physiologic ranges (524). Others argue that concentrations of bilirubin in in vitro toxicity experiments should reflect the actual concentrations found in the brains of infants with kernicterus, as well as the brains of experimental animals with jaundice induced by bilirubin infusions or with genetic hyperbilirubinemia (101, 130, 153, 168).

3. Bilirubin isomers

Bilirubin (4Z,15Z) is the dominant isomer in the circulation, but other structural (constitutional) isomers as well as stereoisomers (conformational or configurational) may be present and differ in their aqueous solubility. The differences between these isomers may be of both physiological and clinical interest (70). Bilirubin first appears in human fetal bile (at about 14 weeks' gestational age [GA]) as the IX β isomer (85). At 20 weeks' GA, IX α comprises 6%, IX β 87%, IX γ 0.5%, and IX δ 6% of the bilirubin found in fetal bile (722). By 28 weeks' GA, the relative amount of bilirubin-IX α has increased to about 50% of total bilirubin (722), and by 30 weeks' GA, bilirubin-IX α replaces IX β as the main isomer (85). The IX β , IX γ , and IX δ isomers are excreted directly into bile (82) because they cannot form the intramolecular hydrogen bonds as does the IX α isomer, and thus behave as polar molecules (81, 82, 416, 467). The high proportion of bilirubin-IX β in early fetal bile does not necessarily prove that it is the major isomer produced by the fetus (462). Unlike the polar IX β isomer, the lipophilic IX α isomer may be excreted from the fetus through the placental barrier (49, 468). However, this may not be compatible with the increased proportion of bilirubin-IX α in fetal bile during pregnancy (85). The physiological details and significance of these observations require further study.

Bilirubin can assume different conformations since the two single carbon-carbon bonds that connect the two dipyrinones to the central methylene group can rotate (415). Further rotations may occur at the single carbon-carbon bonds inside the dipyrinones. Thus, four diastereomers of bilirubin can be formed: 4Z,15Z, 4E,15Z, 4Z,15E, and 4E,15E, of which only one, 4Z,15Z, represents the naturally occurring isomer (FIGURE 3). The practical importance of the *E*-isomers, which are

formed when the *Z,Z* isomer is exposed to light, will be described in more detail in the discussion of bilirubin photoisomers (II.C.2 and VII.G.10). Of note, some textbooks and research articles do not depict the *Z,Z* geometric isomer of bilirubin correctly (182).

4. *Heme degradation*

Theoretically, the heme ring can be opened at the 5(α)-, 10(β)-, 15(γ)-, and 20(δ)-methene bridges yielding bilirubin-IX α , -IX β , -IX γ and -IX δ , respectively (722). Opening at the 5(α) carbon is catalyzed by the rate-limiting enzyme heme oxygenase (HO), which is present in high concentrations in the fetal liver (1, 436). Non-enzymatic opening of the protoporphyrin-IX ring may also occur at other bridges than the 5(α)-carbon (70). However, the first bilirubin isomer found in bile during human fetal development is IX β (85), suggesting that cleavage of heme at other than the 5(α)-methene bridge does occur in mammalian organisms, but the role of enzymes in this process needs further study. Although the precise mechanism of heme cleavage to non- α isomers is unclear, Yamaguchi et al. (720) suggested that oxidative degradation of Hb heme by activated oxygen species in RBCs may be involved. Others have speculated that fetal Hb may play a role (601).

5. *Biliverdin and biliverdin reductase (BVR)*

Biliverdin is the first product of heme cleavage and is rapidly metabolized to bilirubin through the action of biliverdin-IX α reductase (BVR). Activity of BVR is high both in liver and spleen tissues (402, 614), particularly in the reticulo-macrophages (697). Human BVR has been purified and sequenced (439, 721) and appears to exist as four isoforms (720). Thus, isozymes I and II correspond to BVR-IX β (MW 21,000), while isozymes III and IV correspond to BVR-IX α (MW 34,000). All 4 isozymes can use NADH or NADPH as electron donors, but based on K_m values, NADPH is assumed to be the physiological electron donor (720). Biliverdin-IX β , -IX γ , and -IX δ are all substrates for isozymes I and II, while isozymes III and IV prefer biliverdin-IX α . CysteinyI residues are essential for the enzymatic activity of BVR-IX α , but not for the β -reductase (721). Thus, the biliverdin-IX α and -IX β reductases are different in their enzymatic action and are probably also phylogenetically distinct. This is supported by the finding that the BVR-IX α gene is localized to chromosome 19 (579), while the BVR-IX β gene is

localized to chromosome 7 (537). Biliverdin-IX β reductase appears to be identical to flavin reductase (601).

The activity of the biliverdin-IX β reductase isozymes relative to that of the α -reductases is considerably higher in the fetal than in adult liver (720), which may explain the preponderance of bilirubin-IX β in fetal bile (85, 722). Reducing biliverdin to bilirubin-IX β , a polar molecule, enables its secretion into bile and fetal intestine without the need for conjugation (85). This may help the fetus avoid accumulating potentially toxic levels of non-polar tetrapyrroles (540) (FIGURE 1).

BVR has been found in the cell membrane, cytoplasm, endoplasmic reticulum, mitochondria, and nucleus (697). It can translocate between compartments through processes that are regulated through nitrosylation, lipid modification, and phosphorylation (695, 696). It is induced by its substrate biliverdin as well as in response to oxidative stress (437). Activation of BVR through phosphorylation has been shown to be important for the reduction of biliverdin to bilirubin (530, 580), suggesting the possibility that bilirubin can inhibit its own production through a negative feedback loop. Thus, bilirubin inhibits the phosphorylation of a number of proteins/peptides in vitro (292, 296, 297). Indeed, given the central role of protein phosphorylation in the regulation of many cellular processes, inhibition of protein phosphorylation has been proposed as a candidate for the role in the '*basic mechanism of bilirubin toxicity*' (296). A study of hippocampal specimens from patients with Alzheimer's disease and others with milder cognitive impairment recently found that while levels of BVR- α were increased, the phosphorylation of its serine, threonine, and tyrosine residues were reduced, accompanied by decreased reductase activity (55). Furthermore, in the same brain specimens, BVR- α was shown to undergo post-translational oxidative and nitrosative modifications in the hippocampus, but not in the cerebellum (56). Concomitantly, inducible nitric oxide synthase (iNOS) was significantly upregulated in the hippocampus, but not in the cerebellum. Whether these new insights into the antioxidative and metabolic changes described in neurodegenerative disorders could have implications to our understanding of the pathophysiology of bilirubin toxicity in the newborn brain, will need further study. The ability of atorvastatin to modulate

250 BVR- α protein levels, phosphorylation, and activity in some brain regions in a canine model of
 251 preclinical Alzheimer's disease, further suggests the need to explore these mechanisms in the
 252 newborn/immature brain (58).

253 Evidence is accumulating regarding the role of BVR as more than an enzyme involved in the
 254 conversion of biliverdin to bilirubin (57, 697). It appears that BVR possesses pleiotropic functions,
 255 such as cellular signaling and the regulation of gene expression via mitogen-activated protein (MAP)
 256 kinase pathways in cancer and diabetes (239, 240, 357). In the rat brain, BVR levels increased 4-fold
 257 from day of life 1 to adulthood (199). The expression of BVR in brain regions (cortex, substantia nigra,
 258 hippocampus, cerebellum) changes with age, but not in the same pattern. This ontologic change in
 259 BVR activity in brain may modulate HO enzyme activity (199). BVR has serine/ threonine/tyrosine
 260 kinase activity and may have a role in the insulin signaling pathway, acting as a kinase for serine
 261 phosphorylation of insulin receptor substrate 1 (404). Another important role for BVR is transporting
 262 heme to the nucleus to regulate HO-1 gene expression (651). Finally, BVR-IX α may be involved in the
 263 regulation of inflammatory pathways (695), but whether this has any implications for the newborn
 264 infant has not been studied to date.

265 Biliverdin was recently shown to be a potent inhibitor of NF- κ B (239) and NFAT and was
 266 shown to enhance tolerance for cardiac allografts in mice (723). Biliverdin can trigger Ca²⁺/CaMKK
 267 signaling, resulting in phosphorylation of eNOS, which increases NO production in macrophages (696).
 268 S-nitrosylation of BVR occurs at the same time. Other effects attributed to biliverdin include
 269 significant antioxidant activity in brain microsomes (446), reduced mortality in experimental
 270 pancreatitis (509), and anti-inflammatory and antioxidative effects that involve reduced tumor
 271 necrosis factor- α (TNF- α), iNOS as well as other markers of oxidative stress (381). However, caution
 272 should be taken in interpreting these findings as many of the apparent effects of biliverdin have also
 273 been observed with bilirubin (696).

274 **B. Bilirubin As An Antioxidant**

275 The toxic potential of bilirubin is well documented (19, 106, 160, 282); however, biliverdin is polar,

might easily be excreted, and appears to be non-toxic (162). Further metabolism to bilirubin requires energy, a cofactor (NADPH), and an enzyme (BVR). As this process has been conserved phylogenetically, it seems likely that it serves a biological advantage (465).

During the second half of the 20th century, evidence increasingly suggested that bilirubin might be an antioxidant since HO is induced by oxidative stress (235). Bilirubin was shown to protect against oxidation of fatty acids and vitamin A and to be an effective scavenger of oxygen-free radicals (63, 631). Furthermore, bilirubin is an effective antioxidant (as potent as α -tocopherol) against lipid peroxidation (630, 632). Individuals with Gilbert syndrome have low circulating lipid concentrations, which could in part be due to their high TSB levels and hence high bilirubin antioxidant activity (128).

The normal range of TSB levels for healthy adult humans contributes an appreciable part of blood antioxidant capacity, and even more so in the newborn period when TSB levels are much higher (64). Intracellular and tissue concentrations of bilirubin are only 0.1%–1% of serum levels (280, 595), but even nanomolar concentrations of bilirubin may protect brain cells from the toxic effects of a substantial molar increase of hydrogen peroxide (185, 186). On the other hand, oxidant stress may also play a role in bilirubin neurotoxicity. In neuroblastoma cells (SH-SY5Y) exposed to 140 nM bilirubin in vitro, several antioxidant response genes are activated, in part through the Nrf2 pathway (546). It has been suggested that the high intracellular antioxidant activity of bilirubin is related to BVR recycling of biliverdin to bilirubin (595), but the experimental paradigm has been criticized (432, 464).

In some conditions where oxygen-free radicals may play a role, evidence has suggested that bilirubin may be protective (104, 128, 422, 594). However, the evidence may not yet be conclusive for cardiovascular disease. Thus, some studies are limited by having included males only (104, 594). In studies where women were included, positive effects of higher TSB values were only found in males (183, 412). Others failed to find any protective effects of higher-than-average TSB, or the effect was very limited (194, 388, 389, 625, 640). On the other hand, a recent review concluded that a number of studies have found low TSB concentrations (<10 mM) to be a predictor of current or

future risk for cardiovascular disease risk (387). A Belgian study investigated the association between TSB levels and cardiovascular and cancer mortality in 5460 men and 4843 women (640). In men with 'high' [$> 10 \mu\text{mol/L}$ (0.6 mg/dL)] versus 'low' [$\leq 3.4 \mu\text{mol/L}$ (0.1 mg/dL)] TSB levels, the adjusted relative risk (RR) for cancer mortality was 0.42 [95% CI: 0.26–0.68]. The associations between TSB levels and cancer mortality in women had the same direction but were not statistically significant.

In newborn infants, there is also some evidence that elevations of TSB levels may be associated with outcomes in diseases thought to involve oxidative stress. In infants with illnesses associated with increased oxygen-free-radical production (e.g., respiratory distress, circulatory failure, proven sepsis, aspiration syndromes, and asphyxia), the mean rise in TSB was significantly lower in the sick infants than in the control group (65). This seemed compatible with a hypothesis that bilirubin is consumed in vivo during oxidative stress. The same was found in preterm infants with necrotizing enterocolitis, bronchopulmonary dysplasia, intraventricular hemorrhage, and retinopathy of prematurity (ROP) (314). The results of studies, which have investigated if bilirubin might protect against ROP, have not been consistent. Several studies failed to find a protective effect (94, 177, 207, 229, 321, 480). Although others appear to have confirmed a protective association (315, 727), none of these studies were prospective, and in some cases the numbers of patients included were quite small. In the largest study to date, in which ROP was only one of many outcomes addressed, there was no difference in the occurrence of severe ROP between infants who received aggressive phototherapy, and thus had lower TSB levels, and those who received so-called conservative phototherapy, in whom TSB levels were significantly higher (489). In a recent case-control study of the association between breast milk nutrition, hyperbilirubinemia, and ROP, peak TSB levels were lower in ROP cases than in controls [mean 123 *versus* 135 $\mu\text{mol/L}$ (7.2 *versus* 7.9 mg/dL); $p = 0.045$], suggesting that bilirubin consumption was occurring in an oxygen-free radical disease (356). A negative association was found between the highest TSB levels and risk for ROP, but this association was not statistically significant [odds ratio (OR) = 0.82 per 17 $\mu\text{mol/L}$ (1 mg/dL) change in bilirubin; $p = 0.06$]. Thus, this question may not as yet have been adequately addressed.

328 In summary, the results of studies on the putative protective effects of bilirubin against
 329 oxygen-free radical diseases are conflicting. The greatest oxidative stress in newborn infants
 330 probably takes place during the first minutes and hours of life. However, in preterm infants, oxidative
 331 stress can persist for days and even weeks as intermittent hyperoxemia may continue to occur. Thus,
 332 we need more studies investigating the possible antioxidant effects of bilirubin in newborn infants,
 333 which might have great impact on treatment strategies for NNJ where the very vulnerable, extremely
 334 premature infants are likely to be most affected (654).

335 It has also been suggested that bilirubin may be a scavenger for NO (447, 448, 481). When
 336 exposed to peroxynitrate in plasma, the major oxidation product of bilirubin was biliverdin, possibly
 337 related to bilirubin binding to albumin (447, 481). Bilirubin may scavenge secondary oxidants through
 338 hydrogen donation (481). Bilirubin may also counteract nitrosative reactions both extra- and
 339 intracellularly (447, 448). Binding of NO to bilirubin causes formation of an N-nitroso derivative –
 340 bilirubin-NO, proposed as a new biomarker for oxidative/nitrosative stress (59). Another fascinating
 341 perspective on the yin-yang properties of bilirubin was revealed by the discovery that in cultured rat
 342 adrenal pheochromocytoma and cerebellar granule cells in the presence of neurotrophins, bilirubin
 343 promoted cell death by interfering with growth factor signaling (449). However, in the absence of
 344 neurotrophins, bilirubin appeared to be neuroprotective. The processes involved both NO (through
 345 activation of an NO-dependent cascade leading to activation of extracellular signal-regulated kinases
 346 (ERKs) above the baseline and to partial protection from cell death) and inhibition of phosphorylation
 347 of downstream effectors (Akt/protein kinase B and ERK1/2). A hypothesis was formulated that the
 348 degree of bilirubin toxicity vs neuroprotection in the brain may depend on the relative presence and
 349 activity of local neurotropic factors (449).

350 C. Bilirubin As A Toxin

351 Christian Georg Schmorl, the German pathologist who coined the term '*kernicterus*', noticed that in
 352 kernicteric brains, some neurons were more heavily stained than others, while glia were distinctly
 353 less stained than neurons (588). He speculated that the uptake/binding of bilirubin might cause cell

354 death. Soon after this, seizures were described in infants later shown in postmortem examinations to
355 have developed kernicterus (68, 197).

356 In early animal experiments, infusions of 100 mg/kg of bilirubin IV over a 2-hr period resulted
357 in the death of all experimental animals within 3–6 hrs. Mean TSB levels were 625 $\mu\text{mol/L}$ (36.5
358 mg/dL) and bilirubin was found in all organs, with the lowest concentration (8.5 nmol/g [0.5 mg/100
359 g]) in the brain (93). Some years earlier, very similar values had been found in the non-nuclear parts
360 of brains from 4 infants who had died with kernicterus (153) (153). However, the basal ganglia of
361 those brains contained 34 nmol/g (2 mg/100 g) bilirubin, with bilirubin concentrations in smaller,
362 more heavily-pigmented patches estimated to be 5–10 times greater (153). In later animal
363 experiments, total brain bilirubin values corresponded well to the data from human kernicteric
364 brains (281, 283, 293, 296, 300), but the high bilirubin concentrations found in the basal ganglia of
365 human infants were not approximated in animal experiments, being much less than the levels
366 estimated in the more heavily pigmented patches of the ganglia (153, 281, 283, 293, 296, 300).

367 Signs of bilirubin toxicity during extreme hyperbilirubinemia in animals have been both
368 general (diarrhea, hemorrhages, renal damage, death) and neurologic (seizures, involuntary
369 movements, head retraction) (517, 575, 576). Selective staining of the nuclear region of the brain, a
370 hallmark of kernicterus in humans, was not consistently observed in animal models (281, 283, 288,
371 293, 300), but have been described in newborn kittens with extreme hyperbilirubinemia, who then
372 also exhibited general signs of toxicity (575, 576). Albumin infusions done concurrently with bilirubin
373 significantly reduced toxicity in such models (93, 575).

374 Renal bilirubin toxicity has been shown both in animals and humans (115, 196, 234, 338, 517).
375 Findings have included impaired glomerular filtration rates, decreased concentrating ability,
376 increased sodium loss, decreased phenol red excretion, and enzymuria. Tissue concentrations have
377 shown a notable difference in that bilirubin concentrations in the renal papillae of jaundiced rats
378 were 100 times greater than in the cortex (517). The changes in renal function observed in jaundiced

infants may need to be considered in terms of drug dosing, particularly with respect to aminoglycosides that are commonly used in newborn medicine.

Bilirubin toxicity has been shown in several different human and non-human cells, such as hepatoma cells, fibroblasts, L-929 cells, RBCs, and others (102, 108, 113, 160, 161, 366, 373, 414, 459, 483, 510, 544, 552, 566, 643). In this section, we will address mainly those studies performed in cells that were not derived from brain tissue. Findings in these studies were quite wide-ranging, and included inhibition of growth, cell death, decreased intracellular ATP content, decreased synthesis of protein and DNA, increased membrane permeability, reduced membrane potential, membrane crenation in RBCs, membrane fusion events, altered membrane phospholipid content and distribution, inhibition of oxidative phosphorylation, oxidative stress, and inhibition of alanine uptake and bilirubin conjugation. Certain effects on membranes could be reversed by phototherapy, and toxicity was not apparent on exposure to bilirubin photoisomers (373). At physiologic concentrations, bilirubin, in the presence of bovine serum albumin (BSA), protected RBCs against oxidative stress, but significant cytotoxicity was observed at very high bilirubin concentrations ($\geq 510 \mu\text{mol/L}$ (30 mg/dL)) and a bilirubin:albumin molar ratio (BAMR) > 1.0 (483). Similarly, in adult human RBCs, exposure to low bilirubin concentrations was protective against hypotonic hemolysis and crenation, while high bilirubin concentrations induced hemolysis that was followed by membrane disruption (108). Apparently, in older RBCs and at high bilirubin concentrations, bilirubin enters deeper into the cell membrane bilayer, creating an unstable situation with aggregation of bilirubin acid leading to hemolysis and cell death (108). The extent of RBC shape changes as well as membrane phospholipid perturbations is increased with increased BAMR, suggesting a role for free or unbound bilirubin (UB), i.e. bilirubin not bound to the primary binding site on albumin (112, 114). Acidosis increases bilirubin toxicity (109), suggesting that cytotoxicity involves increased binding of bilirubin in its acid form to RBCs under such conditions (99, 551).

Several toxic effects of bilirubin have also been shown in in vitro experiments not involving intact cells or tissues. Thus, exposure of homogenized brain tissue to bilirubin concentrations > 350 –

450 $\mu\text{mol/L}$ (20–25 mg/dL) reduced cellular respiration by approximately 25% (171). However, this
 456 could be reversed/limited by oxidizing the bilirubin by adding methylene blue or cytochrome c. A
 457 similar study compared oxygen consumption in homogenized brain tissue from adult vs 2-day-old
 458 rats, and found a 22% reduction in adult brain vs a 67% reduction in newborn brain homogenates
 459 (694). In rat liver mitochondria, bilirubin at a concentration of 300 $\mu\text{mol/L}$ (17.5 mg/dL) uncoupled
 460 oxidative phosphorylation and was accompanied by the release of respiratory cofactors such as
 461 cytochrome c and diphosphopyridine nucleotide, leading to decreased respiratory activity (196). In
 462 mitochondria from primary rat neurons and astrocytes exposed to bilirubin, membrane permeability
 463 to cytochrome c was increased, probably in part due to involvement of the permeability pore. These
 464 effects could be prevented by ursodeoxycholate (UDCA) and tauroursodeoxycholate (566).

465 1. *Bilirubin effects on enzyme activity*

466 Many studies have examined the effects of bilirubin on enzyme function. An early review identified
 467 studies of 25 separate enzymes and four pathways, of which three were in vivo and the remainder in
 468 vitro, with the majority showing inhibitory effects of bilirubin (369). Only two studies noted a
 469 stimulatory effect of bilirubin for ATPase and glycogenesis, and for 7 enzymes no effect was noted.
 470 The sources of the enzymes studied included heart, liver, and brain from different species as well as
 471 L-929 and Ehrlich cells (369).

472 Other enzymes or pathways where bilirubin exerts inhibitory effects include protein kinase
 473 (156) and protein kinase C (582), mitochondrial ATPase (265), brain Na^+/K^+ -ATPase (370), lipolysis in
 474 rat lipocytes (607), purified respiratory enzymes from bovine heart (161), secretory phospholipase
 475 A(2) enzyme activities from several species (341), equine and human alcohol dehydrogenase (217),
 476 plus malate dehydrogenase and aspartate aminotransferase from mammalian mitochondrial malate-
 477 aspartate shuttle (472). Conversely, mitochondrial cytosolic glycerol-3-phosphate dehydrogenase
 478 from the malate-aspartate shuttle was not appreciably affected (472), and glucose oxidation in rat
 479 lipocytes was stimulated (607). Research into the mechanisms of bilirubin interaction with enzymes
 480 seems to have waned towards the end of the 20th century, with the exception of a single study using

circular dichroism spectroscopy to investigate bilirubin-enzyme interactions (739). Thus, a unifying theory remains elusive. Although protein phosphorylation appears to play a role in the regulation of some of these enzymes, and thus might hypothetically be connected to the widespread inhibition by bilirubin of phosphorylation reactions (296), this remains speculative at present.

2. Toxicity of bilirubin conjugates and isomers

Bilirubin, when conjugated with one or two molecules of glucuronic acid, becomes water soluble and appears to be non-toxic. Similarly, numerous studies have shown that when albumin is present in equimolar or greater ratios relative to bilirubin, toxicity is greatly diminished, probably reflecting the very low equilibrium concentration of UB that is present (132).

The photoisomers of bilirubin are polar, thus more water soluble than IX α (Z,Z), the isomer which normally dominates in humans. Therefore, it has been suggested that they may be less toxic than IX α (Z,Z) (373, 464). Unfortunately, the data on photoisomer toxicity are conflicting (304). Several interesting studies have been performed, but the experimental designs have flaws that limit their interpretation.

Several studies in cultured cells co-exposed to bilirubin and phototherapy lights have shown increased toxicity (150, 572, 608), which appears contrary to a hypothesis of photoisomers being less toxic. A possible explanation might be that phototherapy impacts antioxidant defense systems to cause oxidative stress (48). Also, specifics of irradiation conditions may influence oxidative stress (572). Fluorescent light in the 420 to 500 nm band wavelength caused DNA breaks, sister chromatid exchanges, and cell death in Chinese hamster cells (608). Therefore, concurrent in vitro exposure to bilirubin solutions and light may mask possible toxicity of bilirubin photoisomers.

Some in vitro studies do suggest that bilirubin photoisomers are less toxic (125, 151, 152, 570), but these findings must also be considered with caution. Experimental conditions were variable and/or inadequately described, including irradiances and other conditions pertaining to light, as well as composition and quantity of bilirubin isomers. When the first in vitro study was published in 1965, showing less toxicity of bilirubin photoproducts, photoisomers had not yet been discovered (125).

However, the mitochondria were exposed to very high bilirubin concentrations, and both an unstable solution and formation of photo-oxidative products probably limit the strength of the conclusions. Similar limitations apply to other early studies (609).

Several methodological questions challenge the interpretation of results from cell culture studies of bilirubin photoisomer toxicity. Some researchers have failed to find photoisomer formation when bilirubin was bound to cells, while photoisomers were found in irradiated bilirubin/albumin mixtures (149). However, in a previous study from the same group, cell toxicity was apparently reduced, irrespective of whether the bilirubin solution had been pre-irradiated or irradiated with cells and bilirubin present concurrently, suggesting that cells needed to be present to form photoisomers (152). The type of albumin (or possibly other proteins) present in the solutions may change the dynamics of photoisomerization. Thus, conversion of *E,Z*-bilirubin to the cyclobilirubin isomer occurs significantly faster in the presence of human serum albumin (HSA) than of non-human albumins (333). In one study, toxic effects were observed in calf serum, but not during incubation with human serum (152). Such discrepancies may, hypothetically, relate to bilirubin binding to albumin or other proteins, as well as to cell surface membranes (304).

Finally, the type of cells used in cell culture studies may further complicate attempts to understand mechanisms. For example, in mouse lymphoma cells, native *Z,Z*-bilirubin was reported to have limited cytotoxicity; whereas, the photoproducts formed after green light irradiation evinced less cytotoxicity than those resulting from blue-light irradiation (570).

Notably, in a recent well-controlled study where a SH-SY5Y human neuroblastoma cell line was exposed to bilirubin-IX α (*Z,Z*), or lumirubin, or a mixture of bilirubin-IX α (*Z,E/E,Z*) isomers, all carefully prepared and purified, the photoisomers did not affect cell viability, while the cells exposed to the IX α (*Z,Z*) isomer exhibited significant loss of viability that increased with time (344). Thus, although our understanding of the biology and toxicity of bilirubin photoisomers is still inadequate, this recent study clearly supports the hypothesis of less toxicity of the polar bilirubin isomers (373, 464).

Devising an appropriate in vivo model to test hypotheses related to relative toxicity and BBB passage

483 of photoisomers has been challenging and remains elusive.

484 **D. Other Functions/Roles**

485 *1. Drug displacement by bilirubin*

486 The importance of competition for albumin binding sites was discovered serendipitously in 1956, but
487 the underlying mechanism was not recognized (37). Infants who had received a sulfonamide had
488 significantly higher mortality rates and incidence of kernicterus than those who had received a
489 tetracycline (37). Using chemical and biochemical techniques, it was then shown that both organic
490 anions and some drugs (salicylate and sulfisoxazole) could dissociate bilirubin from its albumin
491 binding (514, 515). UB then can pass through semipermeable membranes and enter the brain. Many
492 drugs can displace bilirubin from its albumin binding, and testing of drugs used in neonates for their
493 bilirubin-displacing characteristics is standard of care (123).

494 However, the potential ability of bilirubin to increase the free concentration of drugs
495 competing for the same binding site(s) and thus cause toxic drug levels, has received much less
496 attention. This is particularly germane because serum concentrations of UCB are higher in the
497 neonatal period than at any other time of life, with the exception of patients with Crigler-Najjar
498 syndrome. Also, many drugs used in neonatal medicine have not been subjected to the rigorous
499 testing normally applied in the adult population.

500 Fetal and neonatal plasma in general have significantly lower drug binding capacity than
501 adults, and hyperbilirubinemia further decreases binding capacity, a finding not fully explained by
502 lower protein levels (193, 545). Unbound diphenylhydantoin levels were significantly higher in
503 plasma from umbilical cords vs adults and correlated significantly with TSB levels (384). In patients
504 with liver disease, the free fraction of diphenylhydantoin increased by 50% relative to healthy
505 individuals and was associated with changes in TSB levels (320). Albumin binding can profoundly
506 influence drug activity and marked reductions in binding may occur in hyperbilirubinemia (660).
507 Indeed, the binding relationship is such that drug displacement by bilirubin can be used to calculate
508 displacement of bilirubin by the same drug (124).

Regrettably, during the last two decades, little attention has been given to the possibility of drug displacement by bilirubin and the risks involved in unpredictable drug levels in sick infants with jaundice. Although increased free drug levels associated with hyperbilirubinemia in the newborn may carry risks of unwanted and unrecognized side effects or even toxicity, there may also be circumstances in which higher free drug levels may be desirable (560). In the complex care of seriously ill newborn infants, ignorance of such factors should probably no longer be accepted.

2. Bilirubin interactions with the immune system and inflammatory/infectious mechanisms

Clinical experience suggested that NNJ is associated with infection, and increase the risk of developing kernicterus (170, 539, 741). Such risk was thought to be associated with the lower serum albumin and lower reserve albumin binding capacity for bilirubin observed in infants suspected of having infection (192). However, in a recent prospective nationwide study of phototherapy in Norwegian NICUs, significantly fewer infants with a diagnosis of infection had NNJ needing phototherapy than infants without infection (491). On the other hand, an increased risk for kernicterus spectrum disorder (KSD) in infected infants who did develop NNJ was recently substantiated in a large case series from Egypt (225). Our understanding of the interplay between bilirubin and infection was further challenged by a report that bilirubin in a physiologically relevant concentration was able to reduce the replication of both human herpes simplex virus type1 and enterovirus EV71 in vitro (583). The authors of this report speculated that the mechanisms involved in the antiviral activity of bilirubin within the cell or at the interface blood/tissue could be related either to the stimulation of intracellular pro-survival pathways, such as the MAPK system, or the production of microbicidal molecules such as NO (583). However, they recognized that the absence of any evidence that conditions like Gilbert syndrome confer protection against infections must be considered.

Infants who were jaundiced as newborns may produce less antibodies following routine vaccination against diphtheria, tetanus, and measles, an effect which persists after jaundice has resolved – indeed antibody titers remained depressed as long as a year later (342). It is not clear

whether UCB depresses antibody production or whether hyperbilirubinemia in some other way modulates the development of the immune system.

Bilirubin inhibits several *in vivo* as well as *in vitro* expressions of cellular immunity, including cell migration, adhesion, proliferation, and infiltration (269, 342, 377, 479, 598, 644) and has been reported to promote *de novo* generation of T-regulatory cells in a mouse model (562). Bilirubin also interferes with both non-specific and specific immunity (667). Such effects might be due to inhibition of receptor activity/expression on the cell surface membrane. In a mouse islet cell transplant model, bilirubin suppressed the release of 'damage-associated molecular patterns' (DAMPs) as well as inflammatory cytokines and chemokines, and had tolerogenic effects on macrophages (3). The possible role of immune processes relative to bilirubin toxicity in the brain will be discussed elsewhere (107).

The anti-inflammatory properties of bilirubin include modulation of inflammation through regulation of HO activity (672, 709). Inhibition of the NF- κ B activation pathway has been described and appears also to involve biliverdin and BVR (697). However, in animal models, bilirubin did not influence NF- κ B or p38 MAP kinase, but rather it was suggested that bilirubin may be cytoprotective by inhibiting iNOS expression and stimulating local PGE₂ production (686). When congenitally-jaundiced rats were exposed to endotoxin, they had lower iNOS expression and were more resistant to hypotension or death than non-jaundiced controls (395). On the other hand, the cytotoxicity of bilirubin in mouse fibroblasts *in vitro* was increased by endotoxin and TNF- α (504). A full discussion of the possible/putative interactions between bilirubin and the immune system would exceed the limits of the present review, but a few selected effects are discussed below.

Both UCB and monoconjugated bilirubin evinced a dose-dependent inhibition of the classical pathway of the complement cascade *in vitro* at low micromolar concentrations, but the C1 step was most inhibited (47). Bilirubin interferes both with C1q-IgM and -IgG interactions, thus potentially explaining how bilirubin inhibits C1-mediated hemolysis (47). In a rat model, UCB also inhibited complement-mediated hemolysis *in vivo* (46). Bilirubin (and biliverdin) complement inhibition also

appears to protect tissues against inflammatory damage (500).

A molecular model posits that binding of UCB to C1q involves an electrostatic interaction between the negative charges present on the lactam oxygen atoms of bilirubin and the positively-charged arginine residues in the β chain of C1q (61). Accordingly, the anti-complement properties of bilirubin may ameliorate damage in diseases involving complement-mediated cell injury. However, considering the role of complement in mammalian infection defenses, hyperbilirubinemia may also increase susceptibility to infection. Complement deficiencies, e.g., in chronic liver disease or newborn infants, may be cases in point (61).

Several studies have demonstrated a negative relationship between levels of TSB and C-reactive protein (325, 672, 729). Thus, bilirubin along with biliverdin and BVR may play roles in the modulation, amelioration, and perhaps even the pathogenesis of chronic inflammatory and autoimmune conditions (184, 668, 677, 734). A hypothetical explanation for the many apparent effects of bilirubin on the immune system has been proposed (342). Thus, UCB was shown to cause widespread inhibition of protein kinases (296) and interacts with catalytic domains of various kinases, including PKC and I κ B kinase. In this way, downstream signaling cascades are interrupted and proinflammatory signaling intercepted. However, this interesting hypothesis requires confirmation, and many issues related to immunology, infection, or inflammatory processes and bilirubin metabolism require further research.

III. THE PRODUCTION OF BILIRUBIN IN THE BODY

A. Heme Catabolism and Its Regulation

As mentioned above, HO, the rate-limiting enzyme in the heme degradation pathway, produces equal amounts of iron (Fe^{2+}), carbon monoxide (CO), and biliverdin, which then is rapidly reduced by BVR to bilirubin (642) (FIGURE 1). CO then binds to Hb in circulating RBCs as carboxyhemoglobin (COHb), which then subsequently is released in exchange for inhaled oxygen to form oxyhemoglobin (HbO_2) with CO being exhaled in the breath. Because of this stoichiometry, the rate of CO production (or excretion) as measured as COHb (680, 681), total body CO excretion (VeCO) (626), or end-tidal

breath CO (ETCO) (617) can serve as an index of the rate of bilirubin production. Several studies have shown that COHb and ETCO, when corrected for inhaled CO, and VeCO levels correlate well in preterm and term neonates and have been used to identify those infants with increased rates of bilirubin production, particularly those undergoing hemolysis (77, 627). In the first week of life or *'transitional period'*, bilirubin production rates in newborns are increased due to a high turnover of RBCs (629, 682). Under normal steady-state conditions, the predominant source (> 86%) of endogenous CO production arises from the degradation of heme, which is primarily from senescing RBCs and the catabolism of other hemoproteins (e.g., myoglobin, catalases, cytochromes, peroxidases, NOS, and sGC). The remaining (< 14%) CO is derived from processes such as lipid peroxidation (683) or photo-oxidation (679).

HO is found in all cells (except in mature RBCs, which lack nuclei), with the highest activity in the newborn liver, adult spleen, placenta, and erythropoietic tissue (306). There are three functional isoforms: HO-1, HO-2, and HO-3. Of these, HO-1 is the inducible isoform, while HO-2 (435, 436) and the putative HO-3 are constitutive (461). The exact role of HO-3 however is not well described, although has been reported to be catalytically inactive (461). The enzymatic activity of HO can be inhibited by a class of synthetic heme derivatives called metalloporphyrins, which have been proposed and studied for many years as potential therapeutics for the treatment of newborn hyperbilirubinemia (78, 224, 367, 368, 438, 451, 547, 628, 657, 659). Their potency and various properties vary based on their central metal and ring side chains, yet no particular chemical structural feature has been readily identified that facilitates the prediction of which metalloporphyrins might be the most effective (589, 684, 713). Taken together, a favorable HO chemotherapeutic should include a biocompatible central metal, sufficient potency, negligible degradation, minimal photoreactivity, no effect on other enzymes (i.e., NOS, sGC), optimal duration of action, minimally upregulation of HO-1, be orally absorbable, and selectively inhibit the inducible HO-1 (221, 589, 684, 713). Almost all metalloporphyrins studied to date appear to be non-selective HO-1 inhibitors (684, 713). However, the development of the non-porphyrin imidazole-dioxalone

613 derivatives have been shown to be selective inhibitors for HO-1, although they have not been used in
 614 any human studies to date (163, 379, 487, 674).

615 1. Genetic variants in bilirubin production

616 An infant's genetic predisposition can affect their bilirubin production rate and subsequently impact
 617 their risk for developing bilirubin-induced neurologic dysfunction or BIND (349, 712). Although this
 618 concept is not novel, genetic mutations (e.g., glucose-6-phosphate dehydrogenase [G6PD] deficiency,
 619 pyruvate kinase deficiency, hereditary spherocytosis) are known clinical risk factors for
 620 hyperbilirubinemia, which can lead to hemolysis and hence increase bilirubin production. Genetic
 621 mutations or polymorphisms in the bilirubin conjugating enzyme, uridine 5'-diphospho-
 622 glucuronosyltransferase (UGT1A1) (60, 361, 453), singly or co-expressed with mutations in organic
 623 anion transporter genes [solute carrier organic anion transporter (SLCO)1B1] (13, 420), can decrease
 624 the hepatic uptake and conjugation of bilirubin and thereby lead to hyperbilirubinemia. Because TSB
 625 levels in circulation ultimately reflect the 'net' balance between bilirubin production and its
 626 elimination, mutations in those genes affecting one (or both) process will affect an infant's risk for
 627 developing severe hyperbilirubinemia, with the co-expression of genetic mutations and
 628 polymorphisms in either process may further exacerbate this risk.

629 Polymorphisms of the HO-1 gene promoter region have been recognized (200). The number
 630 of (GT)_n repeats or expansions in the promoter have been found to affect HO-1 expression, with
 631 short lengths (≤ 26) being associated with normal to high HO-1 expression; while long lengths (> 26)
 632 are associated with low HO-1 expression (145, 200, 316, 719). Individuals possessing long (GT)_n
 633 repeat lengths have been found to have higher incidences of vascular diseases, and among pregnant
 634 women, idiopathic recurrent miscarriages (178), intrauterine growth restriction (54), and pre-
 635 eclampsia (354). Because the expression of HO-1 can be upregulated by heme, any condition that
 636 leads to an increase in heme levels (such as hemolysis from any cause), might lead to a higher
 637 bilirubin production rate and thus a greater risk for developing hyperbilirubinemia in infants with
 638 short (GT)_n repeat lengths (363). A number of studies have been performed in a number of races and

ethnicities; however, these studies have not conclusively shown that (GT)_n repeat lengths directly correlate with the risk for developing hyperbilirubinemia nor BIND. A relationship seems to exist in the Japanese (371), Turkish (95), Taiwanese (700), Chinese (737), Indian (647), and Caucasian (328, 363) populations, but it is not so clear in African-American populations (592, 593). This difference in the impact of (GT)_n repeat length may lie in the underlying ancestral genetics in African-Americans that long repeat lengths as well as a tri-modal distribution of allele lengths are more common (599, 685), which evolved as an adaptation conferring resistance to malaria (227, 638) and sickle cell disease (241). Therefore, bilirubin elimination disorders may play a larger part in the risk for developing hyperbilirubinemia in African-American populations. It seems more probable that the net effect of the combined contributions of the genetic variations in bilirubin production rates and hepatic bilirubin uptake and conjugating capacities as well as the bilirubin binding capacity (BBC) determine an infant's overall tissue bilirubin burden, and hence his/her risk profile of developing pathologic hyperbilirubinemia.

B. The Effect of Hemolysis

In the newborn, there is a normal '*imbalance*' of increased bilirubin production and decreased elimination, which is in a dynamic equilibrium such that TSB levels do not rise to hazardous levels unless there are unrecognized causes (362). Because all newborns have an impaired hepatic bilirubin conjugating capacity after birth due to the low expression of UGT1A1, any condition that causes an increased bilirubin production rate leads to severe hyperbilirubinemia. These conditions include immune or non-immune causes of hemolysis. If left uncontrolled and undetected, hyperbilirubinemia may reach dangerously high levels, which can then cause bilirubin neurotoxicity presenting as acute bilirubin encephalopathy (ABE) and if chronic, KSD (349, 712). Therefore, identification of these high-risk infants with hemolysis is critical to the prevention of BIND.

Bilirubin production decreases; whereas bilirubin elimination increases as a function of postnatal age in days. Thus, a deviation in this normal pattern of transitional hyperbilirubinemia reflects the likelihood of developing significant hyperbilirubinemia as defined by the Bhutani hour-

specific bilirubin nomogram (TSB > 95th percentile prior to age 7 days) (76, 77). By using this nomogram, an infant's hour-specific TSB level can be categorized into defined risk zones (low, low-intermediate, high-intermediate, or high) based on percentiles in order to guide interventions and follow-up. Hazardous TSB levels or bilirubin thresholds reflect an exaggerated imbalance between increased bilirubin production and impaired elimination (362) that are more likely to overwhelm BBC (394) and increase the risk of neurotoxicity (and the development of KSD). Infants with high bilirubin production rates are most at risk because their rate of TSB rise (> 0.2 mg/dL/hr) can overwhelm the natural protective capacity in a matter of hours rather than days unlike those infants with delayed bilirubin elimination. Thus, known clinical causes of hemolytic hyperbilirubinemia have been extensively studied such as Rh disease, Coombs-positive ABO incompatibilities, bacterial sepsis, etc. On the other hand, covert, or unrecognized hemolysis [such as Coombs-negative ABO incompatibilities, G6PD deficiency, and inborn RBC disorders] may account for a substantial number of cases of idiopathic KSD.

1. Disorders associated with increased bilirubin production

Immune hemolytic disorders are due to a variety of known causes, such as isoimmunization (Rh disease, ABO incompatibility, and other immunoglobulin-mediated hemolytic diseases); RBC enzyme deficiencies (G6PD deficiency, hexokinase or pyruvate kinase deficiency, and others); and RBC membrane defects (hereditary spherocytosis, elliptocytosis) (718) as well as extravascular causes, such as cephalohematoma or closed-space bleeding. Non-immune hemolytic disorders could be due to genetic mutations of RBC enzymes such as G6PD, which is common in infants of African or Mediterranean ethnicity (72, 708) or pyruvate kinase deficiency, another common neonatal RBC enzymopathy. Other causes are genetic disorders of the RBC membrane such as congenital hereditary spherocytosis, which is the most common inherited hemolytic disease among those of Northern European descent, and the condition is probably significantly underdiagnosed as a cause of NNJ (148). Other RBC membrane disorders that are less common are hereditary poikilocytosis and elliptocytosis, and are primarily associated with severe anemia in the newborn and less with

hyperbilirubinemia (497). Except for α -thalassemia, hemoglobinopathies are not a cause of severe anemia in the neonatal period (497).

IV. BILIRUBIN BINDING AND TRANSPORT IN BLOOD

Bilirubin is transported in plasma bound to albumin with a binding affinity at the primary binding site of 10^7 to 10^8 per mole (118, 306). A secondary binding site also exists, but has a much lower affinity (117, 118). Albumin contains three domains (313, 634), with each domain containing two subdomains. The high-affinity binding site for bilirubin is believed to be localized to Site I on Subdomain 2A, where a lysine residue appears to be involved in this binding (336, 383, 482, 541). However, crystallographic analysis of the binding of bilirubin IX α (4Z,15E) to HSA showed that it is bound to an L-shaped pocket in Subdomain IB, and indirect data seemed to show that the IX α (4Z,15Z) could also bind to this site (742). Another photoisomer, lumirubin, also seems to bind at or near Subdomain 1B, but with a much lower binding affinity to albumin than IX α (4Z,15Z) (344). Although the binding sites for IX α (4Z,15Z) and lumirubin appear different, they are not independent (344). Data obtained by circular dichroism have suggested that while the high-affinity bilirubin binding site on HSA is located in Subdomain IIA, low-affinity binding sites may be found both in Subdomains IB and IIIA (242). Because of albumin's high affinity for bilirubin, normal circulating levels of UB are present in nanomolar concentrations, even in infants with significant hyperbilirubinemia (134, 337). However, when the BBC is exceeded, UB concentrations can increase significantly (118, 337). Although BBC increases with postnatal age, in sick newborns and those having endogenous or exogenous bilirubin displacers, it becomes reduced (67, 80, 100, 191). In addition to albumin, bilirubin can also bind to other proteins (e.g. α -fetoprotein and ligandin) as well as to lipoproteins, and to erythrocytes (83, 98, 99, 636).

An infant's risk for neurologic injury arising from hyperbilirubinemia is dependent upon the absolute TSB level, BBC, and any underlying clinical condition(s) or exposure(s) that can alter the binding affinity of albumin for bilirubin (5, 134, 394). By taking into account UB, TSB, BBC, and the equilibrium dissociation constant (K_D) of bilirubin binding to albumin, the ability to assess a neonate's

risk of developing BIND can be improved and better than the reliance on any single parameter (6). BBC and K_D reflects how much (i.e., BBC) and how tightly (i.e., K_D) plasma binds bilirubin at a given TSB and/or UB level (5, 6). For example, an infant with 'poor' binding (low BBC) and a high bilirubin production rate (high ETCOc or rapid TSB rate of rise) will have more bilirubin move into tissues at a given TSB, and will thus be at a relatively higher risk of developing BIND (as bilirubin binding is exceeded) than one with 'good' BBC at the same TSB level (as bilirubin binding is available). A number of recent studies (23, 25, 394) and reviews (6, 23) have addressed the importance of incorporating bilirubin binding parameters into the evaluation of an infant's risk for developing BIND, especially in the context of ongoing hemolysis.

Several compounds (such as benzyl alcohol (455), sulfisoxazole (37), ibuprofen (618), free fatty acids (267, 622), ceftriaxone (214, 257) to name a few) and conditions (such as infection/sepsis, hypothermia, acidosis, and asphyxia) (486) have been shown to displace bilirubin from albumin and increase UB levels. However, UB levels are not routinely measured clinically in the US, but only in the research setting. This is mainly because the methodology is currently time-consuming and laborious and not well-suited for a routine clinical laboratory assay.

V. BILIRUBIN IN THE LIVER

A. Hepatocellular Uptake and Intracellular Processing

Before entering liver cells, circulating bilirubin must dissociate from albumin. This is accomplished by two mechanisms: by carrier-mediated or 'passive' diffusion and by organic anion transporter proteins (OATP). Once in the cytoplasm, bilirubin can bind to two major intracellular transport proteins: ligandin (glutathione-S-transferase A) or B-ligandin (Y protein), or Z protein (at a low affinity) (711). There are high ($K_a = 5 \times 10^7$ per mole) and low ($K_a = 3 \times 10^5$ per mole) affinity binding sites on ligandin (74). However, competitive inhibition of the enzymatic activity of ligandin occurs at the low affinity site (74). Neonates are relatively deficient in ligandin, thus affecting (decreasing) their ability to retain bilirubin within hepatocytes, which may result in bilirubin re-entering the circulation. The concentration of ligandin can be increased pharmacologically, such as by the administration of

phenobarbital (711). Evidence has alluded to the possibility that polymorphisms of the glutathione S-transferase gene GSTM1 may affect ligandin function, showing that individuals without GSTM1 may have higher TSB levels in the neonatal period (498). Lysine may be involved in bilirubin binding both to albumin and ligandin (138, 158, 261, 335, 336, 738) and may modulate susceptibility to bilirubin toxicity (297).

In addition, studies have shown that the immaturity of the neonatal hepatic bilirubin conjugation system during the first 3 or 4 days of life is the primary contributor to the development of unconjugated hyperbilirubinemia rather than just a reduction of bilirubin uptake, which may play the primary role during the second week of life as bilirubin conjugation reaches normal adult levels (620).

B. Bilirubin Conjugation

Bilirubin is water-insoluble and needs to be conjugated so that it can be excreted with bile. It binds (conjugated) to glucuronic acid in a reaction catalyzed by UGT (EC 2.4.1.17), which is located in the endoplasmic reticulum of hepatocytes (249). In the newborn, monoconjugates are predominantly formed. Diconjugates are created at the membrane level by a tetrameric form of UGT. The activity of UGT in the fetus is very low (only 0.1% at 16–32 weeks' GA) and increases to ~1% of adult values by term (374), but increases to adult levels by 4–8 weeks of age. Because treating pregnant women with phenobarbital can increase the bilirubin conjugating ability in neonates (554), such therapy has been used both before and after birth to prevent and/or treat NNJ (658). The use of dexamethasone (398), and clofibrate (421) can also increase UGT activity.

Conjugated forms of bile pigments have been isolated in the liver of fetuses with Rh incompatibility (175). Bilirubin IX α glycosyl conjugates appear in fetal bile at 20 weeks' GA, while monoglucuronides appear 2–3 weeks later (85). Monoconjugates of bilirubin IX α are predominant in the fetal bile at around 30 weeks' GA. Monoglucuronides constitute the major pigment at term when conjugation with glucuronic acid begins (85), constituting < 2% of total bile pigment in serum (495), while diconjugates comprise 20% of the total conjugated fraction (495). In adults, bile is comprised of

mostly bilirubin-IX diglucuronide (80%) with the remainder being primarily monoglucuronide (18%) (213). Kawade and Onishi (374) have reported that in premature infants, hepatic UGT activity is accelerated.

The final step in conjugation occurs at the hepatocyte cell membrane (343) with excretion of conjugated bilirubin into bile being an active process and the rate-limiting step (43, 502). In fact, infants with the Dubin-Johnson syndrome have a defect in the gene encoding the hepatocyte bilirubin conjugate export pump (MRP2) (334, 376). Barbiturates can be used to stimulate bile flow affecting the transport process (249).

1. Genetic variants in bilirubin conjugation

There are over 50 mutations and polymorphisms in the UGT1A1 gene, which are associated with severe unconjugated hyperbilirubinemia (355, 655, 689). These mutations are autosomal recessive, but different heterozygous mutations can be co-inherited and manifest as clinical diseases with distinctive clinical phenotypes and also with variable patterns of unconjugated hyperbilirubinemia. Their presentations and severities are based on how they affect the rates of bilirubin production (particularly in combination with increased hemolysis) (364) and/or excretion as well as nutritional status and intercurrent illness. Thus, any observed interindividual variations in the progression and severity of NNJ may have an underlying genetic cause and thus should be further investigated, warranting the need to develop a genetic panel to identify these infants at high risk for developing BIND.

a. Crigler-Najjar syndrome type I

Crigler-Najjar syndrome type I is a rare autosomal recessive disorder where there is an absence of the UGT1A1 gene. Several nonsense mutations that affect synthesis or cause deletions in key amino acid sequences of UGT1A1 have been reported in infants with Crigler-Najjar syndrome type I (355, 655). Severe, prolonged unconjugated hyperbilirubinemia [at TSB levels of 340–765 $\mu\text{mol/L}$ (20–45 mg/dL)] is apparent in infancy and continues throughout life such that life-long phototherapy, gene replacement, or orthotopic liver transplantation is required to prevent bilirubin neurotoxicity.

b. Crigler-Najjar syndrome type II

Patients with Crigler-Najjar syndrome type II have a partial UGT1A1 activity and thus conjugating defect, which is caused by numerous single-site missense or insertion mutations (355). Bilirubin conjugates may be formed, but only in small quantities, thus severe unconjugated hyperbilirubinemia [with peak TSB levels of 100–340 $\mu\text{mol/L}$ (6–20 mg/dL)] may still occur in the newborn period and may even persist into adulthood. Phenobarbital administration has been shown to induce UGT enzyme synthesis or activity.

c. Gilbert syndrome

Gilbert syndrome presents as a mild form of unconjugated hyperbilirubinemia. The most common mutation is an insertion of two extra bases (TA) in the TATAA portion of the 5' promoter region of the UGT1A1 gene, resulting in a sequence of A (TA)₇ TAA in place of the normal A (TA)₆ TAA (355, 689). It has a high carrier rate in some ethnic groups. Polymorphisms with 5 to 8 TA repeat sequences have been reported (73, 364).

Infants with Gilbert syndrome or who are both heterozygotes for the Gilbert promoter and possess mutations of UGT1A1 have an increased risk for developing severe hyperbilirubinemia. In addition, infants who also have a hemolytic disease, such as G6PD deficiency, hereditary spherocytosis, or ABO incompatibility, also have an increased risk (454). As with individuals with Crigler-Najjar syndrome type II, increasing UGT enzyme synthesis and activity can be achieved by phenobarbital administration.

2. Genetic variants in transporter proteins

Some infants with hypertrophic pyloric stenosis present with NNJ that may be related to a Gilbert-type variant. Polymorphisms in the OATP-2 gene have resulted in an increased (3-fold) risk for developing severe NNJ, and in those co-expressing a variant UGT1A1 gene mutation this risk further increases 22-fold (323, 689). Polymorphisms in the ligandin gene may also contribute to higher TSB levels in some infants.

820 C. Bilirubin Excretion

821 NNJ due to an elevation of conjugated bilirubin levels is suggestive of the presence of a defect or an
 822 insufficient bile secretion mechanism, biliary flow, or both, and is always pathologic (36, 365, 666). It
 823 is frequently associated with increases in other bile components (i.e., bile salts, phospholipids).
 824 'Cholestasis' is the term used to describe these disorders associated with a reduction in bile flow (36,
 825 365, 666). The increase in conjugated bilirubin levels may result from primary defects in bile
 826 transport or excretion in the liver, or secondarily to defects in bile duct function and/or structure.
 827 Treatment, if possible, is primarily focused on the underlying disease process or processes, since
 828 sequelae are specific to the diseases causing cholestasis (365). More detailed descriptions of this
 829 process and related conditions go beyond the scope of this review and have been well-described in a
 830 number of textbooks and elsewhere.

831 In brief, under normal conditions, bile secretion involves transporting conjugated bilirubin
 832 through the hepatocyte cell membrane by canalicular contraction and microvillous motility, which
 833 produces intrahepatic bile flow (146). Between hepatocytes are 'tight junctions,' which provide a
 834 barrier that efficiently prevents the bile from entering the space of Disse or vascular compartments.
 835 Thus, any hepatocellular injury that impairs bile excretion or affects the integrity of tight junctions,
 836 can result in cholestasis. Mechanical bile flow obstruction, biochemical pathway dysfunction or
 837 defects in bile secretion, as well as bacterial or viral infections can cause intrahepatic diseases.
 838 Ultimately hepatocellular damage will result from any chronic abnormality in bile flow, such as in the
 839 'bile plug syndrome' or the 'inspissated bile syndrome'; choledocholithiasis (most common in
 840 neonates presenting with severe intrauterine hemolysis or in those receiving total parenteral
 841 nutrition); cysts in the biliary tree (congenital hepatic fibrosis, Caroli disease); or the presence of
 842 tumors (primary hepatoblastoma and metastatic neuroblastoma) or masses (enlarged periductal
 843 lymph nodes, distended bowel) (365).

844 With respect to the newborn infant, cholestasis is also associated with an increase in bilirubin
 845 production caused by hemolysis, which is caused by lipid profile changes in the circulation leading to

rheologic changes in RBC membranes (581). In addition, cholestasis is also associated with the use of hyperalimentation, which is the most common cause in preterm infants, and probably related to inappropriate mixtures and combinations of amino acids in these preparations (16, 36, 533, 641).

VI. BILIRUBIN IN THE INTESTINES

A. Excretion into the Intestine

Bilirubin enters the intestines in bile through the common bile duct, primarily in its conjugated form, only a very small fraction is unconjugated. Bile from term infants contains mainly bilirubin-IX α monoconjugates (85). Experimental evidence from Gunn rats also suggests that bilirubin can be transported from blood to the intestinal lumen directly through the bowel wall (382, 406). Higher amounts of UCB are found in the feces of Gunn rats and Crigler-Najjar patients than in control subjects (382). Such transmural transfer is likely to be the result of equilibration of UB in blood with bilirubin in the intestinal lumen. Indeed, treatment of Gunn rats with Orlistat[®] has been shown to reduce TSB levels, probably due to capture of bilirubin by intestinal fats following transmucosal clearance of UB (268, 410). Transmucosal clearance of bilirubin is also supported by the observation that oral calcium phosphate reduces TSB levels both in Gunn rats and in Crigler-Najjar patients (662, 663).

B. Role/Function of Bilirubin in Intestines

UCB, but not conjugated bilirubin or biliverdin, inhibits the activity of digestive proteases, e.g., trypsin and chymotrypsin, which causes mucosal damage as well as reducing the expression of tight junction molecules (548). Interestingly, UCB binds to the catalytic site of α -chymotrypsin (739). In a rat bile duct ligation model, the absence of bile was followed by significant increases in gut trypsin and chymotrypsin as well as mucosal injury manifested by atrophy and edema of villi, dilatation of lacteal canals, and increased intestinal permeability (736). In the same model, UCB supplied to the gut reduced the histological and biochemical evidence of damage to the gut barrier. Thus, UCB may be an endogenous serine protease inhibitor in the bowel (736).

The anti-inflammatory potential of bilirubin may also be active in intestines. In a mouse model (male C57BL/6 mice), inflammatory colitis was induced by dextran sodium sulfate (DSS) (740). Mice treated with DSS and concurrent bilirubin (intraperitoneal injections of 30 mg/kg BW) had less weight loss, lower serum nitrate levels as well as lower disease severity (shown by histopathological analyses) than control animals. The authors concluded that bilirubin prevented DSS-induced colitis by inhibiting the migration of leukocytes across the vascular endothelium and by suppressing iNOS expression (740). Anti-inflammatory effects of UCB were also observed in rats with colitis induced by trinitrobenzenesulfonic acid (736). Oxidative stress in intestinal mucosa and the possible role of bilirubin in that setting was studied by administering *Escherichia coli* lipopolysaccharide (LPS) IP to male Wistar rats and harvesting intestinal mucosa at intervals (526). Septic oxidative stress rapidly induced HO-1 in intestinal mucosa followed by production of bilirubin, suggesting a possible role for bilirubin as an antioxidant through scavenging of oxygen-free radicals. The peaks in mucosal concentration of bilirubin and its oxidation products occurred at the same point in time, thus bilirubin reacts quickly to increasing oxygen-free radical levels. The small intestinal mucosa seems to participate actively in response to sepsis, and the antioxidant effects of bilirubin may play a role in local mucosal defenses (526).

C. Re-Uptake/Enterohepatic Circulation of Bilirubin (FIGURE 1)

1. Breast milk jaundice

Bilirubin diglucuronide is not reabsorbed in significant quantities from the intestines, while the monoglucuronide may be absorbed, albeit in rats only one fifth is absorbed and re-excreted (407, 408, 561). However, UCB may be reabsorbed and may contribute to hyperbilirubinemia in neonates (122, 407, 408).

Two groups of full-term infants were formula-fed, and an intervention group was given agar to stabilize bilirubin in the bowel and prevent its bacterial conversion (543). In agar-fed infants TSB concentrations did not increase after the 13th hr of life, while TSB only peaked on the 4th day of life in control infants. Fecal bilirubin excretion remained higher in the agar-fed infants throughout the 6

days of the study (543). Thus, reabsorption of bilirubin from the intestine may be an important contributor to physiologic jaundice in the neonate, as previously also suggested by others (122).

Factor(s) in breast milk may contribute to or enhance intestinal reabsorption of bilirubin (18). This phenomenon is often referred to as 'breast milk jaundice', and must be distinguished from 'breastfeeding jaundice', in which insufficient breast milk causes delays in the transit of intestinal contents (228). Thus, breastfeeding is likely to be the most important contributor to the increased incidence of significant NNJ, increased postnatal weight loss, and decreased urine and stool outputs observed in infants who are exclusively breastfed compared with those receiving partial or complete formula nutrition (142).

The mechanism(s) underlying breast milk jaundice remain under investigation, with several proposed candidate explanations. While NNJ in the majority of infants is a transient phenomenon in which TSB values peak at around 2–4 days of life (441), in about 1/5 to 1/3 of exclusively breastfed infants, unconjugated hyperbilirubinemia, manifesting as visually apparent jaundice [typically requiring TSB > 85 $\mu\text{mol/L}$ (5 mg/dL) for the eye to perceive], may persist for more than 4 weeks (141, 440, 645) and in some for up to 3 months (228).

Several studies have shown that breast milk in itself may contribute to the 'breast milk jaundice' phenomenon (246, 248, 249). In healthy, term, vaginally-delivered infants randomized to receive either breast milk or one of two formulas (a casein hydrolysate product and a standard whey-predominant formula) ad libitum, the jaundice index (measured by TcB) was lowest in the casein hydrolysate group and highest in the breast milk group (249). In 4 groups of breastfed infants, the control group received breast milk only, while the study groups, in addition to breast milk, received six 5-mL aliquots daily of either L-aspartic acid, enzymatically hydrolyzed casein, or whey/casein protein (ratio 60/40) (248) (248). All intervention groups had significantly lower TcB levels than the control group (75.8%, 69.6%, and 69.2%, respectively, of the control mean [146 $\mu\text{mol/L}$ (8.5 mg/dL)]) at peak bilirubin on day 4. L-aspartic acid and hydrolyzed casein were chosen for the intervention because of their known ability to inhibit β -glucuronidase, with L-aspartic acid being the principal β -

glucuronidase inhibitor in the casein hydrolysate (246, 247, 385), however no such effects have been described for the whey/casein preparation. Lower TcB levels in the L-aspartic acid and casein hydrolysate groups were thought to result from β -glucuronidase inhibition and increased fecal excretion of bilirubin, while a different mechanism may explain the reasonably equivalent results in the whey/casein group (248). Another possible interpretation, not suggested by the authors, is that the lowering of bilirubin levels in all three intervention groups may be due to an unknown mechanism and have nothing to do with β -glucuronidase. Thus, in a study of breast milk β -glucuronidase levels on the 4th and 15th days of life in 3 groups of neonates with either physiologic jaundice, early breastfeeding jaundice, or late breast-milk jaundice, no differences were found in breast milk β -glucuronidase levels between the groups (728). In a humanized UGT1A1*28 mouse model feeding of human breast milk resulted in severe hyperbilirubinemia, while mice fed formula did not exhibit this phenomenon (222). Of note, the human breast milk used for the study came from a single donor, and the authors do not specify whether the donor's baby had breast milk jaundice. However, this breast milk apparently suppressed intestinal I κ B kinase α and β , leading to inactivation of nuclear factor- κ B and loss of expression of intestinal UGT1A1. Formula feeding was associated with induction of both intestinal UGT1A1, as well as Cyp3a11 and Cyp2b10 gene expression, the latter was induced >200-fold (222). Thus, it seems possible that the beneficial effect of formula feeding in cases of prolonged and/or excessive breast milk jaundice may be due to induction of intestinal UGT1A1.

Epidermal growth factor (EGF) in human milk may play a role in fetal and/or postnatal intestinal growth and development (251). Higher concentrations of EGF in serum as well as in breast milk were found in infants with prolonged jaundice (TSB above 171 μ mol/L (10.0 mg/dL) after the 3rd week of life) compared to infants without jaundice (386). Concentrations of EGF in breast milk correlated significantly with TSB and EGF levels. The mechanisms of jaundice relative to EGF are not known, but inhibition of gastric motility, increased reabsorption of bilirubin, and activation of bilirubin transport may be possible explanations (386).

A genetic contribution to breast milk jaundice has also been suggested (10, 11). Thus, the allele frequency of the missense mutation G-to A at nucleotide 211 in the UGT1A1 gene, causing an amino acid change of glycine to arginine at codon 71 (Gly71Arg) (which manifests clinically as Gilbert syndrome in older children and adults), was significantly higher in Japanese newborns with hyperbilirubinemia than in healthy controls (10). Neonates with this mutation had a gene dose-dependent increase of TSB levels on days 2–4, the time around which NNJ typically peaks, and the frequency of the same mutation was significantly higher in neonates who needed phototherapy than in infants who did not need treatment (11). This was confirmed in 170 Japanese infants with breast milk jaundice in whom more than half were homozygous for the Gly71Arg mutation (UGT1A1*6 allele) (452). In these infants, TSB levels were significantly higher than in infants with other genotypes. However, breast milk jaundice was observed in almost 50% of infants who did not have the UGT1A1*6 allele, and must be due to other causes (573). In 240 term breastfed Chinese infants followed prospectively to investigate potential risk factors for significant hyperbilirubinemia, only predischarge TSB levels on the third day and the variant UGT1A1 gene at nucleotide 211 (Gly71Arg) were predictors of significant hyperbilirubinemia (140). In addition, in the same ethnic group male breastfed infants with the Gly71Arg variant had a higher risk than females for prolonged hyperbilirubinemia (141). Ethnic variations in gene allele frequency likely explain some of the variable results from different groups. Thus, a Gilbert syndrome genotype (TA7/7), which did not involve the Gly71Arg mutation seen in Japanese, Chinese and Korean infants, appeared to be related to prolonged unconjugated hyperbilirubinemia in European breastfed term neonates (485).

In an early study of breast milk jaundice, pregnane-3 (α) 20 (β)-diol was identified in the breast milk of 7 infants with prolonged jaundice and shown to competitively inhibit UGT1A1 activity in vitro (42). However, later studies had not been able to confirm these findings (496, 549). This issue was recently revisited with newer genetic techniques by analyzing the inhibitory effect of pregnanediol on the transcriptional and enzyme activities of UGT1A1 (525). In the presence of 100- μ M pregnanediol, bilirubin glucuronidation by G71R-UGT1A1 was reduced to 51% of wild-type levels.

This suggests that pregnanediol may contribute to breast milk jaundice, but perhaps limited to carriers of G71R. The mechanisms underlying breast milk jaundice are likely to be multifactorial, and more studies are needed to elucidate this important phenomenon.

2. *Effects of perturbed intestinal transit*

Interrupted or delayed transit of intestinal contents may increase enterohepatic circulation of bilirubin. Examples are found in infants with intestinal atresia, those who are not fed orally because they are gravely ill, and those who have difficulty establishing lactation and have insufficient enteral nutrition (86, 529). Inadequate intake of calories has been implicated in the causation of NNJ (584). Because of the high proportion of monoconjugated bilirubin in the newborn, deconjugation in the proximal intestine will produce relatively more UCB, which is more easily reabsorbed (669). Establishment of enteral nutrition will reduce the enterohepatic circulation, and the same can be achieved by increasing the frequency of feeding (172) and also by giving agar or bilirubin oxidase orally (352, 516). The use of oral agar or bilirubin oxidase here is 'off-label'. Apparently intestinal UGT1A1 can be induced by intake of calories in the form of glucose supplementation (38).

D. **Metabolism of Bilirubin in the Intestine**

In transit through the intestines, bilirubin may be metabolized through processes that may be related both to intestinal contents and to bowel tissue per se. In an early analysis of bilirubin and its conjugates in feces from newborn infants during the first 1–2 weeks of life, the bilirubin found was mostly unconjugated and concentrations were higher than in blood plasma (122). It was speculated that the low degree of bilirubin conjugation present after intestinal passage was due to the activity of β -glucuronidase, which was quantitated and studied, concluding that the enzyme likely came from the bowel itself and not from microbes (122). Epithelial cells in the villi of the jejunum have UGT activity, which show a progressive increase in concentration from the crypt to the villar tip (147). The contribution of bilirubin glucuronidation in the proximal small intestine to overall intestinal bilirubin metabolism in normal physiology remains unknown, but 2 animal studies suggest that this function may be relevant (473, 637). When Gunn rats, who are congenitally deficient in UGT activity, were

transplanted orthotopically with small bowel from normal Wistar rats, both TSB and UCB levels dropped rapidly and in a sustained fashion (473). The reduction occurred more slowly and was not well sustained in animals who received heterotopic transplants. Gunn-to-Gunn transplants did not evince reductions in bilirubin levels. These findings have been confirmed by others (637). Apparently, the transplanted bowel contains UGT activity and enhances the metabolism and clearance of bilirubin.

Evidence supporting a role for intestinal UGT1A1 in bilirubin metabolism was strengthened in a study of UGT1A1 expression in humanized *Ugt1*^{-/-} mice, which included the human UGT1 locus and encoded all 9-UGT1A genes (223). During the first 2 weeks of life TSB levels in the pups increased in both *Tg(UGT1A1*1)Ugt1*^{-/-} and *Tg(UGT1A1*28)Ugt1*^{-/-} mice and in some pups exceeded 255 µmol/L (15.0 mg/dL). However, during the 3rd week of life, TSB levels fell quickly to adult levels (223). This rapid reduction in TSB levels did not reflect increased hepatic UGT1A1 activity, but UGT1A1 gene expression and protein in the small intestine increased significantly during this period, concordant with changes in TSB. Thus, at least in humanized UGT1A1 mice, glucuronidation of bilirubin by UGT1A1 in the intestine may play a role in bilirubin clearance in the neonatal period (223). Further studies using the same genetic model showed that while formula feeding will induce UGT1A1 activity and reduce hyperbilirubinemia, breast milk will inhibit UGT1A1 and augment jaundice (222). Furthermore, deletion of the intestinal nuclear receptor co-repressor 1 (NCoR1) was recently shown to almost abolish hyperbilirubinemia in newborn hUGT1 mice, and control of NCoR1 function/depression was linked to the function of the inhibitor of NF-κB kinase subunit β (IKKβ) (144). NCoR1 played a significant role in repressing intestinal developmental maturation. It is intriguing that the function and regulation of NCoR1 appears dependent on phosphorylation events (347, 490). Bilirubin has previously been shown to inhibit phosphorylation of several proteins/peptides (292, 296, 297, 305) although its interaction with NCoR1 appears not to have been studied specifically. Thus, several processes in bowel homeostasis appear to be linked, contributing to control of the developmental repression of UGT1A1 and to the modulation of hyperbilirubinemia (144).

Intestinal bilirubin metabolism is also influenced by the gut microbiota. However, as the gut of the newborn infant is sterile, the role of microbes in bilirubin metabolism is likely to be very limited in the first few days of life. In apparent confirmation of this, urobilinoids were found in the stools of 57% of newborn human infants on the 5th day of life, but not earlier (670). At this early time point urobilinoid production was low and unlikely to contribute much to bilirubin removal. UCB in feces increased from 169 nmol/g on the 2nd day of life to a peak of 2,204 on day 5, but by 6 weeks decreased 15-fold as the colonic microbiota was established and urobilinoid production approached adult levels (670). Thus, the newborn intestinal microbiota favors deconjugation of UCB over urobilinoid production, leading to increased enterohepatic circulation of UCB. Microbiologic analyses of the neonatal stools were done in some of the cases, and the activity of isolated bacteria as far as ability to metabolize bilirubin was studied in vitro. Two strains of *Clostridium perfringens* and *difficile* were both able to reduce bilirubin to urobilinoids (670), apparently different from the two strains of microbes previously shown to convert bilirubin to urobilinoids – *C. ramosum* (477) and *Bacteroides fragilis* (201).

Gunn rats received either clindamycin/neomycin or co-trimoxazole orally for 4 days, resulting in the disappearance of fecal urobilinoids and a significant increase in TSB levels in the clindamycin/neomycin-treated rats, while co-trimoxazole had no effect on either level (673). When the intestines were re-colonized with *C. perfringens*, which has been shown to reduce bilirubin, urobilinoids reappeared in the feces and TSB decreased significantly, although less impressively than the increase which had followed sterilization of the gut. In comparison, re-colonization with *C. pasteurianum*, which does not reduce bilirubin, had negligible effects (673). Thus, metabolism of bilirubin by the intestinal microbiota significantly influences overall bilirubin metabolism.

A strain of *C. perfringens* from neonatal stools was incubated under anaerobic conditions with both native and synthetic bile pigments, which were then separated by thin layer chromatography and analyzed by spectrophotometry, spectrofluorometry, and mass spectrometry (671). Several bilirubin reduction products were found, representing different urobilinogen species,

but the reduction process apparently does not proceed to stercobilinogen. Bilirubin diglucuronide was not reduced to urobilinoid conjugates, suggesting that hydrolysis of glucuronides must take place before reduction of the double bond (671). For this reason, UCB may be reduced more rapidly.

The above data suggest that during the first few days of life bilirubin is metabolized in the intestines primarily through deconjugation by enterocytes, thereby contributing to NNJ (212, 670). Further breakdown to colorless urobilinoids depends on the establishment of the intestinal microbial flora (671), and thus will come later (212, 646). These latter processes may take place mainly in the distal ileum and colon (646). Infants who are formula-fed appear to excrete urobilinoids earlier than infants who are breastfed, and it has been speculated that that this may be due to the effects of formula on the establishment of intestinal flora (730).

E. Fecal Excretion

The content of bilirubin in meconium represents about 5 to 10 times the daily production (249). Of this, approximately 50% is unconjugated and may be reabsorbed. In the meconium that is passed first, bilirubin-IX β is the predominant bile pigment. It decreases during the first weeks of life, though more slowly in preterm infants. However, along with zinc coproporphyrin, it can be considered a biochemical marker of meconium (49, 50).

Delayed meconium passage has been thought to cause NNJ, but studies involving facilitation of meconium passage have yielded conflicting results. Infants who received rectal stimulation due to rectal temperature measurement passed yellow stools significantly earlier than those assigned to axillary temperature control, and at about 3d of age TSB levels in the rectally-stimulated group were on average 17 $\mu\text{mol/L}$ (1.0 mg/dL) lower than in controls ($p = 0.042$) (159). However, in another study TSB levels during the first 72 hrs of life in infants randomized to receive a glycerin suppository every 4 hrs were no different from those in untreated controls, although meconium was evacuated earlier in the treatment group (698). Others have found similar results (51, 143).

Manna is a plant extract with purgative effects that was used to treat NNJ in the 18th and 19th centuries in Europe (307), and it is still being used for this indication in Iran and South East Asian

countries (205). A recent study compared infants on phototherapy with two other study groups (n=30 per group) as far as reduction of TSB levels and length of hospital stay – one intervention group received drops of manna as a laxative and the other group received glycerin suppositories in addition to phototherapy (205). TSB levels at 12, 24, and 48 hrs were significantly lower in both intervention groups than in controls, and length of hospital stay was shorter. However, a recent review of randomized controlled trials concluded that there was no evidence of effects of rectal purging on NNJ (621). Thus, the current evidence that suppositories, enemas, laxatives, or rectal stimulation are effective in decreasing TSB levels is equivocal. Therefore, until larger and appropriately designed and powered studies become available, routine clinical use cannot be considered evidence based.

Loose stools during phototherapy for NNJ were described in several early studies (126, 348, 427, 428). Several groups have tried to elucidate the mechanism(s) behind this phenomenon. Thus, a carmine test was performed on 3 groups of newborn infants: 1) healthy full-term newborns; 2) jaundiced full-term newborns who had received phototherapy for 24 hrs at the time of the test; and 3) non-jaundiced full-term newborns who also received phototherapy with carmine testing after 24 hrs (577). Significantly shortened intestinal transit time (from about 14 to 7 hrs) was observed *only* in the newborns that were jaundiced *and* were receiving phototherapy. The authors speculated that the photoisomers of bilirubin might be responsible for the diarrhea, and that reduced intestinal transit time might even be beneficial in reducing the enterohepatic circulation of bilirubin (577). Intestinal lactase activity in duodenal biopsies obtained with a hydraulic capsule was compared in 6 jaundiced newborn infants with diarrhea on phototherapy and 8 healthy controls (52). The infants were also subjected to lactose tolerance tests. Both results from the lactose tolerance tests and the biopsies were judged to confirm a diagnosis of lactase deficiency. A normal biopsy from a 2-year-old girl was exposed to bilirubin in vitro, and no lactase activity was found. Stools normalized after introduction of a lactose-free breast milk substitute, but recurred when breast milk was re-instituted in infants who were still on phototherapy (52). However, other groups have not been able to confirm any significant role for lactose intolerance in phototherapy-associated loose stools (127, 188, 189).

1105 A suggestion that diarrhea in infants under phototherapy for NNJ might be of the secretory
 1106 type seemed supported by findings from in vivo studies of perfused hamster small intestines, in
 1107 which perfusion with bilirubin solution caused secretion of sodium and water, while control animals
 1108 exhibited absorption of both (174, 706). Bile from Gunn rats receiving phototherapy had anti-
 1109 absorptive effects when perfused through the jejunum of Wistar rats, and UCB had a dose-
 1110 dependent secretory effect on transport of water and electrolytes in the same system (255). Other
 1111 studies had also shown UCB to be an intestinal secretagogue (255, 706). A rectal dialysis bag was
 1112 used to study water, sodium chloride, and potassium absorption and showed that absorption was
 1113 impaired in infants who received phototherapy, but impairment was transient and abated when
 1114 jaundice receded and phototherapy was discontinued (173). The authors concluded that the
 1115 observed anti-absorptive effects must be due to the combination of hyperbilirubinemia and
 1116 phototherapy and speculated that the large amounts of UCB present in bile was responsible (173).

1117 In summary, it seems clear that bilirubin has effects on the intestines during its passage from
 1118 duodenum to rectum. Some effects, such as antioxidant and anti-inflammatory, appear to model
 1119 those observed in other organ systems. Others, such as effects on absorption and secretion, may be
 1120 more unique to the intestinal environment. However, compared to what is known about the actions
 1121 and effects of bilirubin in other organ systems, our knowledge about bilirubin and the gut is clearly
 1122 still limited.

1123 **VII. BILIRUBIN IN THE BRAIN**

1124 **A. Clinical Picture of Kernicterus**

1125 Kernicterus is a pathoanatomic term and describes intense yellow coloring of the basal
 1126 ganglia while the background is pale yellow. In descending order of frequency stained areas include:
 1127 hippocampus, thalamus, hypothalamus, corpus striatum, medulla, olives, pons, and dentate nucleus
 1128 (4). Ultrastructural details include changes in membranes, calcium granules, and dense cytoplasmic
 1129 bodies, which probably represent degenerated mitochondria (602). Such changes were thought to be
 1130 irreversible and to correlate with the clinical picture of KSD in survivors. At the beginning of the 20th

century, cases were reported of infants who survived extreme jaundice, and who later exhibited neurologic sequelae with choreoathetosis (262). Subsequent case reports and case series contributed to the understanding that the clinical manifestations of KSD evolved over time (131). Hypertonicity, absent Moro reflex, opisthotonos, high-pitched cry, and poor feeding dominated the clinical picture during the first 2 to 3 months. Decreased muscle tone, hyperreflexia, and persistence of immature postural patterns was accompanied by significantly delayed motor development, which then became increasingly manifest during the first 2 years of life. Athetosis could be variably present, ranging from hardly noticeable to completely disabling, and with age of presentation ranging from the end of the second year of life until 8-9 years of age. Hearing loss of varying degree was noted in the majority. A fairly obligatory paresis of upward gaze appeared to distinguish KSD from other types of cerebral palsy. Few patients had developmental delay and/or intellectual deficits. KSD is described in similar terms even in the most recent discussions of this condition (678).

The chronic sequelae of bilirubin brain toxicity may be multifaceted, and involve both the clinical findings, changes that occur over time, and severity (603). The recent proposal to use the overarching term KSD reflects the idea that the effects of bilirubin on the brain can take different forms as far as both the type and severity of the insult (397). While the term ABE describes neurological symptoms which are caused by ongoing bilirubin exposure, in the case of KSD bilirubin exposure had happened in the past, and it is the long-term sequelae of that time-limited exposure which causes the signs and symptoms currently observed in the patient (397). In addition to KSD, modifier terms for severity can be used to describe the condition as mild, moderate, and severe. Further, subtype modifiers such as auditory, motor, and classical can be used to describe auditory-predominant, motor-predominant, and combined auditory and motor sequelae. Finally, the term '*subtle kernicterus*' can be applied to infants with neurodevelopmental disabilities that, having excluded other diagnoses, may perhaps be understood on the background of a history of extreme NNJ and/or ABE (397).

Although kernicterus in the strict sense affects the basal parts of the brain, bilirubin toxicity

may also affect cortical neurons, albeit the cortex appears less vulnerable than other areas (28, 106). However, the cortex is intimately connected to the basal ganglia via several circuits, which together play vital roles in cognitive functions (14, 271), which is why bilirubin-induced injury of certain basal ganglia-cortical circuits may lead to impulse and stimulus challenges seen e.g. in children with adult attention deficit/hyperactivity disorder (ADHD) (45). These circuits are also important for learning and memory functions such as acquisition of motor skills and learning modes: perceptual-motor, stimulus-response, and reward-based (92). Impairments of these functions may cause language impairment and clumsiness. Cerebellar toxicity is also well described in connection with hyperbilirubinemia (591), and it has been suggested that disruption of feedback loops between the cerebellum and cortex could contribute to autism spectrum disorders among survivors of NNJ (29, 31). In the hippocampus bilirubin inhibits arborization both of dendrites and axons (106). Several studies have also shown toxic effects of bilirubin on synaptic functions (139, 292, 311, 613). Bilirubin hippocampal toxicity in the newborn period may have adverse effects on synaptic plasticity and lead to memory deficits with negative effects on learning (28). Bilirubin toxicity in the auditory system, discussed elsewhere in this review, may also have negative consequences for language development.

On this background it is not surprising that several groups have described intellectual and neurobehavioral deficits in children and adults, thought to represent sequelae of NNJ (319, 429, 434, 506, 597). Thus, in a study of 18-year-old male Norwegian army conscripts, 55 of whom had a history of NNJ, 7 individuals who had been DAT-positive and had hyperbilirubinemia for >5 days were found to have significantly lower IQ scores than the national average (506). Similarly, in 17-year-old Israeli army recruits the risk for having an IQ score <85 was significantly higher in males, but not in females, with documented TSB levels in the neonatal period of >342 $\mu\text{mol/L}$ (20 mg/dL) (597). In a cohort study 128 term Finnish children who had experienced NNJ with TSB >340 $\mu\text{mol/L}$ (20 mg/dL) or had needed an exchange transfusion were followed up prospectively at 5, 9, 16, and 30 years of life and compared to 82 non-jaundiced controls (319). The odds of a child with NNJ having neurobehavioral symptoms at 9 years of age was significantly increased compared to controls (OR 4.68). The 45% of

NNJ subjects who had neurobehavioral issues, had significantly lower results on all cognitive function tests, and continued to have problems in adulthood, with lower academic achievement and lower ability to complete secondary and tertiary education (319). Persisting cognitive challenges affected both reading, writing, and mathematics. A registry-based follow-up study of 733826 Danish children showed that those with a recorded diagnostic code of NNJ (4.87% of the total cohort) had a 56%–88% increased risk of developing a psychological development disorder (434). The risk for autism spectrum disorders was significantly increased only for boys, and was present only for infants born in winter months, a finding the authors speculate could be due to more prolonged exposure to NNJ because of shorter daylight hours. However, prospective data from a 1-year study of phototherapy in Norwegian NICUs did not show evidence for increased need for phototherapy in the darkest months of the year (Mreihil K, personal communication). In contrast to these findings, in another Danish study, in which 463 army conscripts with a recorded diagnosis of NNJ were compared to 12,718 non-jaundiced conscripts, no association was found between level of hyperbilirubinemia and cognitive scores (190). These apparently contradictory findings suggest that studies based on registries may be vulnerable to precision in diagnostic coding. Thus, while in the latter study the population with a diagnostic code for NNJ constituted 3.51% of the total, in the former study 4.87% had been so coded.

Some studies suggest that even moderate degrees of NNJ may have long-term effects on behavior and neurodevelopmental outcomes (28, 429, 430, 604, 619). However, both patient selection and sensitivity *versus* specificity of the methods used for assessment must be carefully considered (429). The concept of minor neurological dysfunction (MND) has evolved, and it is possible that those with complex MND are of special interest as regards sequelae of moderate neonatal hyperbilirubinemia (266, 430). Thus, more studies will be necessary to delineate the details.

Present-day bilirubin researchers seem to agree that bilirubin in brain cells causes a primary toxic event, it is not just a dye or marker. In clinical experience tolerance for bilirubin brain toxicity varies between infants (20, 187, 276, 431, 443, 476). Some term infants may tolerate extremely high TSB levels [> 500 to $600 \mu\text{mol/L}$ (>29.2 to 35.1 mg/dL)] without suffering any long-term toxicity, while

others develop KSD with TSB just slightly above 350 $\mu\text{mol/L}$ (20.5 mg/dL). In preterm infants, this has happened at even lower TSB levels (310, 322, 484, 693). While in some cases increased vulnerability may be due to illness or immaturity, variable vulnerability is also seen in infants judged to be healthy and mature. More research is needed to elucidate the mechanisms behind such differences, which could be due to altered or disrupted bilirubin metabolism in the brain or to bilirubin passage through the BBB, e.g. through both genetic, intrinsic, and extrinsic modulation of membrane transporters.

Biological risk factors for ABE and KSD involve both those that impact production, transport/binding, and/or excretion of bilirubin, those that involve transfer across the BBB as well as BBB permeability, and probably also characteristics/vulnerability of brain cells as well as bilirubin processing by those cells. Further details of ABE and KSD biology and risk will be discussed below. On a larger scale, the risk of an infant with severe hyperbilirubinemia going on to develop ABE and KSD depends on the availability of healthcare services, delays in seeking care, and structural as well as organizational roadblocks within health care systems (15, 179, 521, 555, 656). A further discussion of these challenges is beyond the scope of this paper.

Below we will discuss the merits versus weaknesses of theories regarding the '*basic mechanism(s) of bilirubin neurotoxicity*', as well as research on bilirubin effects in, and interactions with, the brain. These studies have used a wide range of methods, from 'pure' in vitro, to cell cultures, ex vivo organotypic cultures, and animal models. This topic has recently been extensively reviewed, and will only be briefly outlined here (91, 460). Studies in cell cultures first tended to use immortalized cell lines, such as e.g. mouse or human neuroblastoma cells, human astrocytoma cells, and differentiated human NT2-N neurons (35, 151, 273-275, 344, 504, 505, 546, 570). Later, cell cultures obtained by primary culture techniques have increasingly been used, including both neurons and several types of glia (107). These cells have yielded many interesting data on differential sensitivity to bilirubin toxicity, as well as insight into possible pathways and mechanisms. Common challenges to all in vitro studies is bilirubin solubility and stability in solution, leading to discussions about 'physiologically relevant' bilirubin concentrations for such studies (464, 523). This has been

particularly challenging when studying the toxicity of bilirubin photoisomers (304). Also, cultures involving a single cell type will not be able to study the interplay between e.g. neurons and glia which appears to modulate bilirubin cell toxicity (107, 202, 203).

Organotypic cultures consist of slices of brain regions of interest, which for a time retain the complexity of the brain tissue of origin. Bilirubin toxicity has primarily been studied in hippocampal slices, both as far as synaptic function and plasticity as well as the interplay between glia and neurons (139, 164, 297, 613, 687). Different experimental setups and conditions have led to apparently contradictory results, potentially opening door to new discoveries through refinement of the paradigms (164, 166).

The classical in vivo model of kernicterus is the Gunn rat, a spontaneous mutant discovered in the 1930s (259). These rats have a complete deficiency of UGT1A1, leading to hyperbilirubinemia which peaks around 2 weeks of age, and with brain damage in the form of cerebellar hypoplasia (91, 460). Deaths from kernicterus and TSB levels corresponded reasonably well (351). Later on, crossbreeding to other strains resulted in two strains (ACI/N-j and RHA/N-j) with very different mortality rates, which thus far is unexplained (303). The Gunn rat has been extensively used as a model of bilirubin neurotoxicity, e.g. by ABRs which have yielded important information on the importance of UB (22, 24, 26, 27, 31, 260, 294, 390, 603, 714).

New methods for genome manipulation have been used to create constitutive and conditional knock-out, knock-in, and transgenic strains. These models have been reviewed quite recently (91). In brief, it has been possible to model the human Crigler-Najjar syndrome, and to study interactions between UGT1A and the pregnane X and constitutive androstane receptors by mating with null strains of the latter (see [Bortolussi and Muro 2019](#) for a review). UGT1A null mouse mutants exhibit more severe neurological damage than Gunn rats and eventually die, though the reasons for the observed differences in mortality between strains is as yet unexplained, as remains true of the Gunn ACI/N-j and RHA/N-j mutants mentioned above. Thus, further studies of these models appear to be of great interest.

B. The Role of the BBB

1. *Permeability and its modulation*

For bilirubin brain toxicity to occur, bilirubin must gain entry into brain. Bilirubin in the blood vs the brain is in an unstable equilibrium, influenced by several factors (FIGURE 4). Chemically speaking bilirubin is amphipathic, but it behaves in many respects as lipophilic, binding to and crossing phospholipid membranes (120, 293, 331). Although this characteristic is thought to enable UB to cross the intact BBB and gain access to the brain (281), it does so to a lesser extent than one would expect of a typical lipophilic molecule (332). Thus, the blood-brain gradient is high – in study animals with an intact BBB the brain bilirubin concentration is only 1% to 2% of serum concentrations (283). The characteristics of molecules that can cross the BBB in significant amounts include: 1) molecular weight <400 Daltons; 2) lipid solubility; and 3) not being a substrate for an efflux transporter (534). Bilirubin does not really satisfy any of these criteria: its molecular weight is 585, lipid solubility varies with the isomeric form, and it is a substrate for phosphoglycoprotein (P-gp) (690). This may explain why bilirubin entry into brain is limited.

Albumin-bound bilirubin probably does not cross an intact BBB (118, 300) (see Section IV). Nanomolar concentrations of UB are always present in plasma during significant neonatal hyperbilirubinemia. The “free bilirubin theory” posits that UB is the moiety which enters the brain and causes injury (702). The first clinical observations that supported this theory came from the use of drugs that turned out to compete with bilirubin for its albumin binding, thus increasing the UB concentration (37). Subsequently, many studies have provided both clinical and experimental evidence for how UB impacts the risk for kernicterus/KSD (7-9, 31, 137, 514, 515, 518, 733). According to the laws of equilibrium, UB in high serum concentrations will force more bilirubin into tissues, such as the brain (FIGURE 4). Increased concentrations of UB occur in the presence of altered albumin binding characteristics or exo-/endogenous binding competitors and increase bilirubin entry into brain (80, 100, 118, 135, 707). Many drugs act as binding competitors with bilirubin relative to

serum albumin (37, 455). Increased entry of bilirubin into brain following administration of bilirubin-displacing substances has also been documented in several animal studies (118, 281, 283, 293, 702).

The BBB in newborn animals appears to behave differently relative to bilirubin passage than it does in more mature subjects (399, 400, 409). It is possible that expression of P-gp, which appears to evolve with increasing maturity, may contribute to the apparent increase of bilirubin passage through the BBB in immature subjects (649). Albumin permeability may also be reduced with age, though not all published data are in agreement on this (263, 399, 519).

The function of biologic membranes may be impacted by bilirubin, leading to questions of whether BBB function could also be perturbed by bilirubin (35, 160, 459). The permeability of the BBB both to a dye and to bilirubin itself was increased by pre-exposure to bilirubin (256, 568). Glial cells, particularly astrocytes, play a fundamental role in the maintenance of the BBB (111). Therefore, toxicity of bilirubin to glial cells could also contribute to effects on BBB function (33, 635). UCB disturbs homeostasis in endothelial cells by inducing increased eNOS expression, which is followed by accumulation of nitrites, suggesting nitrosative stress (531). In the same experiments, brief exposure of brain endothelial cells to UCB first inhibited the release of IL-6, IL-8, and vascular endothelial growth factor (VEGF), but this was later followed by an increased release, first of IL-6, then of IL-8 and later on of VEGF. Thus, UCB may cause injury to endothelial cells, affecting important mediators that may increase inflammation in the brain and perhaps affect BBB integrity (111). The interaction between glial cells and bilirubin is discussed further below.

Several conditions that occur quite frequently in seriously ill newborn infant may affect BBB permeability (FIGURE 4). The hypercarbia that accompanies respiratory failure, the hyperosmolality seen in severe hyperglycemia, IV hyperalimentation, or renal failure, and the damage caused by asphyxia have all been shown to increase bilirubin entry into brain (129, 130, 281, 283, 300, 330, 704). This seems clearly relevant for management of jaundice in NICU infants. Hypercarbia increases brain blood flow, and most of the bilirubin enters brain as UB; while in hyperosmolality albumin also enters

the brain in significant amounts along with bilirubin (130, 300). Furthermore, in hypercarbia, the acute entry of bilirubin into brain is increased compared to control conditions (129, 283).

In vitro studies appear to show that albumin in equimolar concentrations blocks the toxic effects of bilirubin (97, 102, 160, 414). When the BBB is opened, bilirubin neurotoxicity increases (301, 330, 704). In the presence of an open BBB, both albumin-bound and UB enter the brain, but in the immature subject entry of albumin-bound bilirubin may occur even with an intact BBB (520). Total brain bilirubin content appears to be the best predictor of toxicity, while increased albumin binding of bilirubin may be protective (704).

2. Transport mechanisms

a. The role of 'flippases'

P-gp is an ATP-binding membrane transporter. Expression of such transporters has been observed both in normal and in diseased tissues and limit the entry of xenobiotics into cells (238). In a preliminary report UCB was reported to be a weak substrate for multi-drug resistance protein (MDR1) (245). Bilirubin has many properties in common with P-gp substrates, including hydrophobicity when unconjugated as well as certain structural elements that facilitate interaction with P-gp (596, 691). These elements involve electron donor groups with a spatial separation of about 2.5 ± 0.3 Å (691). In vitro verapamil, a P-gp inhibitor, inhibited [3 H]bilirubin transport through human Caco-2 monolayer cells, providing further support that bilirubin is a substrate for P-gp (433).

However, the interaction between bilirubin and P-gp may have more than one perspective – as bilirubin may inhibit P-gp function (345, 392, 690). Therefore, it was hypothesized that changes in P-gp expression/function could modulate bilirubin entry into brain. To test this hypothesis the entry of bilirubin into brain was first compared between P-gp knockout and wild-type mice, showing that brain bilirubin content was almost two-fold higher in knock-out mice (690). Then, drugs known to inhibit P-gp function were shown to significantly increase bilirubin entry into rat brain (219, 220, 277). Studies in postmortem brain tissue sections from human infants born at 22 to 42 weeks' GA showed P-gp to be expressed in a regionally and cell specific pattern which was also dependent on

maturation, findings clearly relevant to the putative role of P-gp in modulating bilirubin uptake into and/or extrusion from brain (167).

b. Other BBB molecules with relevance for brain bilirubin uptake and excretion

MRP1 (encoded by the ABCC1 gene) may also limit bilirubin access to cells (559). Cytotoxicity and intracellular accumulation of [³H]bilirubin were higher in fibroblasts from MRP1 knockout mice than in than in cells from wild-type controls (132). Bilirubin appeared to upregulate MRP1 in cultured astrocytes, thus reducing sensitivity to bilirubin toxicity (237). Given the important role of astrocytes in BBB function and the apparent ability of bilirubin exposure to increase both BBB and blood-cerebrospinal fluid (CSF) barrier permeability to bilirubin itself, the interplay between bilirubin, barrier elements, and transport proteins appears complex and clearly in need of further study (111, 568).

C. Brain Blood Flow

Hypercarbia leads to increased brain blood flow, which in experimental animals is followed by increased bilirubin entry into brain (129, 283, 300). There will always be a significant blood-to-brain bilirubin concentration gradient, thus when brain blood flow increases, each circulating bilirubin molecule will pass the BBB more often, consequently the opportunities to equilibrate with bilirubin in the brain will increase.

D. Excretion

1. The CSF "sink"

Yellow coloring of the CSF was noticed early on during autopsies of neonates with kernicterus (587, 588). When CSF bilirubin was analyzed in infants with 'physiologic' NNJ as well as due to isoimmunization, CSF bilirubin was predominantly unconjugated and bilirubin values were mostly 1%–3% of TSB, corresponding remarkably well to brain tissue data from later animal studies (281, 283, 623). A subsequent study showed that CSF bilirubin correlated with total protein levels in CSF. The relationship between CSF bilirubin concentrations and TSB was not linear, suggesting that there are individual variations in BBB permeability during the first days of life (624).

A scatterplot of the relationship between TSB and CSF bilirubin in 100 newborn infants, of whom 34 were normal term infants, 49 were normal preterm, and 17 had erythroblastosis due to Rh- or ABO-incompatibility, showed a correlation coefficient of 0.58 (501). While CSF bilirubin in the preterm infants was on average 4.4% of the TSB, with wide range of variation (from 1.7%–15.6%), in term infants CSF bilirubin was 3.0% of the TSB (ranging from 0.95%–11.9%). In older jaundiced control subjects, CSF bilirubin was 0.65% of TSB values. The higher CSF bilirubin:TSB ratio in the premature infants is likely to reflect increased permeability of the BBB for bilirubin, as also shown in animal studies (399, 400, 409).

Rabbit choroid plexus in vitro accumulates ^3H -bilirubin, suggesting that the choroid plexus may be involved in transporting bilirubin from the CSF to blood (340). An active mechanism for bilirubin transport out of the CSF also seemed compatible with studies of 45 newborn infants (475). CSF bilirubin correlated linearly with UB concentration in serum, but CSF bilirubin values were approximately 150 times greater than the concentrations of UB in serum, indicating a pronounced CSF-to-serum concentration gradient (474).

P-gp/ABCB1 and MRP1/ABCC are both expressed in the choroid plexus epithelium in the developing human CNS (167). It was suggested that the complementary patterns of P-gp/ABCB1 and BCRP/ABCG2 at the BBB with MRP1/ABCC1 at the blood-CSF barrier may limit CNS uptake and retention of drugs and toxins in neonates (167). However, P-gp/ABCB1 seems to be localized to the apical membrane of the choroid plexus and from that position might be expected to mediate transport of substrates from blood into the CSF. The implication of such P-gp/ABCB1 expression for bilirubin brain toxicity appears not to have been studied (405, 538).

Organic anion transporters, such as MRPs, have also been hypothesized to limit bilirubin entry into the CSF (523). In choroid plexus epithelial cells from Gunn rat pups exposed to bilirubin in vitro, MRP1 protein was down-regulated, a phenomenon also observed in choroid plexa isolated from homozygous (*jj*) Gunn rats when compared to their non-jaundiced (*Jj*) littermates (230). The authors speculated that down-regulation of Mrp1 protein at the blood-CSF barrier, which probably

results from a direct effect of bilirubin on epithelial cells, can perturb the MRP1-mediated neuroprotective functions of the blood-CSF barrier and possibly accentuate bilirubin neurotoxicity (230).

2. *The BBB*

Bilirubin is not static once it has entered the brain. The half-life has been estimated with different techniques yielding different results. This first study performed with unilateral hyperosmolar opening of the BBB induced by arabinose infusion into one carotid artery yielded a half-life of bilirubin in brain of 1.7 hrs and in blood 1.6 hrs (409). This technique opens the BBB only ipsilaterally and was thought to be reversible within 1 hr (409). However, later studies reduced the estimate of the time to reversibility of permeability changes to 10 min (550). Serum osmolality during or after arabinose infusion was not reported. Later work estimated a much lower bilirubin half-life in brain - 16–18 min during baseline conditions and approximately 38 min during general hyperosmolality induced by urea injection (283, 293). Serum osmolality was increased from the normal 290 to 395 mosm/L, which was sustained until sacrifice (283). As both UB and albumin-bound bilirubin enter the brain when the BBB is opened, with rapid closure the much larger bilirubin-albumin complex will probably be ‘trapped’ on the brain side of the BBB, while clearance of UB will be more rapid. This may explain the differences between the half-life data from earlier vs later studies (283, 293, 409). Time-limited unilateral opening of the BBB provides important experimental data, but sustained global opening is more likely to be representative of conditions in vivo. However, in both models, bilirubin retention in the brain is clearly more prolonged than during control conditions. It is not clear whether prolonged exposure increases toxicity, but limited experimental and clinical data do suggest that, in addition to the level of TSB, the duration of hyperbilirubinemia may impact the risk for neurologic sequelae (301, 578). In hypercarbia both acute brain entry of bilirubin and clearance are rapid (129, 283). Hypothetically, the more rapid clearance might be explained by increased opportunities for equilibration across the BBB pursuant to increased brain blood flow.

E. **Bilirubin Metabolism in the Brain**

Experimental studies showed that clearance of bilirubin from the brain could be more rapid than from blood, suggesting that additional mechanisms might contribute to bilirubin clearance from the brain (289, 293, 409). An enzyme on the inner mitochondrial membrane of brain cells as well as in other tissues capable of oxidizing bilirubin had been described (121) and verified by others (17, 280, 287, 288, 290, 295, 302, 303). The activity has enzyme characteristics, such as pH and temperature maxima for activity, and denaturability (288). Cytochrome P-450 2A5 may contribute to hepatic oxidation of bilirubin, but it is not known whether brain and hepatic metabolism are the same (2). The activity of the brain enzyme is lower in the immature organism and in neurons *versus* glia (290), observations that seem compatible with the clinical impression that infants are more vulnerable to bilirubin toxicity than older individuals, and the same applies to neurons *versus* glia. This enzyme possesses some of the characteristics of the cytochrome oxidases, but has not with certainty been identified as such (284, 308).

Oxidation of bilirubin will reduce the concentration of toxic bilirubin, but it is not known with certainty whether the oxidation products are more or less toxic than bilirubin itself. Genetic variability exists between different strains of Gunn rats, however the Gunn rat strain with higher specific bilirubin-oxidizing capacity was also the strain that exhibited significantly higher early spontaneous mortality (302). However, there could be reasons for these differences in mortality rates that have no connection with bilirubin.

Molecules with structures similar to bilirubin oxidation products are found in the CSF of patients with cerebral vasospasm following subarachnoid hemorrhage, and bilirubin oxidation products have similar effects on blood vessels both in vitro and in vivo (154). In an in vitro model of subarachnoid hemorrhage the production of bilirubin oxidation products was significantly enhanced when cytochrome oxidase was stimulated, but was attenuated by cyanide (425). In support of previous data from others the authors suggested that mitochondrial cytochrome oxidase could be a major source of bilirubin oxidation (291, 308, 425).

1440 In jaundiced Gun rat pups brain bilirubin content and expression of cytochrome P450 oxidase
1441 (CYP) mRNA were inversely related (232). Delayed induction of CYP enzymes was found in brain
1442 regions typically involved in kernicterus. The functional induction of Cyp1A1, 1A2, and 2A3 as well as
1443 the ability of membranes to oxidize bilirubin were also studied in a subcellular fraction containing
1444 microsomes, nuclear membranes, and mitochondrial membranes from cultured rat brain cortical and
1445 cerebellar astrocytes (226). Cyp1A1 could be induced by β -naphthoflavone in astrocytes from both
1446 cortex and cerebellum. However, this enzyme oxidized bilirubin only after uncoupling by 3, 4,3',4'-
1447 tetrachlorobiphenyl. On the other hand, Cyp1A2 was most active in bilirubin oxidation without
1448 uncoupling, but inducible only in cells from the brain cortex. Cyp2A3 could not be induced.
1449 Cytochrome P-450 2A5 may contribute to hepatic oxidation of bilirubin, but it is not known whether
1450 brain and hepatic metabolism are the same (2).

1451 Following the original discovery of a bilirubin-oxidizing activity in brain (121), two different
1452 groups have applied themselves to the study of this phenomenon (17, 226, 232, 284, 287, 288, 290,
1453 291, 302, 303, 308). Currently, it is not clear that the findings from these two groups can be
1454 reconciled in the sense that they study and describe the same enzyme activity, or whether in fact
1455 more than one enzyme could be involved in bilirubin oxidation in brain. Thus, more work is needed
1456 to clarify the implications of the apparent differences. One group has focused its studies on
1457 measuring bilirubin oxidation in brain mitochondrial membranes, though in recent exploratory work
1458 they also investigated microsomal membranes from mouse brain and did not find any evidence for
1459 bilirubin oxidation (308). The other group has primarily addressed the questions from the angle of
1460 specific CYPs, their induction, and the implications for bilirubin content in specific regions, but in
1461 parts of one study also used membranes from several subcellular organelles (226).

1462 CYPs in liver are found in microsomes and endoplasmic reticulum, while brain CYP activity is
1463 mostly found associated with the mitochondrial subcellular fraction (478). One of the groups
1464 mentioned above used a pure preparation of glial cells as their starting material (226, 232), while the
1465 other group performed subcellular fractionations with whole rat and mouse brains, yielding

mitochondria from both glia and neurons. However, in one study they compared the mixed mitochondrial membranes with membranes from a pure neuronal source (synaptosomes) and found that the bilirubin oxidation rate per milligram protein was significantly lower using the pure neuronal membranes, leading the authors to speculate that this might explain the greater vulnerability of neurons compared with glial cells to bilirubin toxicity (290). Another clue that different enzymes may be involved is that while one group needed NADPH to start their bilirubin oxidation reactions (226), the other group showed that in their assay, neither NAD, NADP, NADH, NADPH, GSH, nor GSSH had any effect on oxidation velocity (291). The reaction was cytochrome c dependent, and it could also be inhibited by clotrimazole and ketoconazole, both known to inhibit the cytochrome P450 oxidase group of enzymes (291). While data from the pure glial source also pointed to the cytochrome P450 oxidases, specifically Cyps 1A1 and 1A2 (226, 232), known inhibitors of these enzymes (omeprazole and fluvoxamine) were unable to inhibit the activity measured in mixed brain mitochondrial membranes (308). Recently CYP1A2 mRNA levels were found to increase with maturation in rat brain and liver microsomes, but microsomal fractions did not affect bilirubin or its metabolites, suggesting that physiologically this CYP may not have a role in bilirubin oxidation (411). Also, recent attempts to purify the mixed mitochondrial enzyme activity by salt fractionation showed peak activity in fractions around 205 mM NaCl (308). Parsing of proteomics data from these fractions yielded several candidate proteins in the cytochrome oxidase group. However, testing with antibodies currently available against these enzymes has as yet not resulted in a firm conclusion (308).

We do not as yet know what, if anything, metabolism of bilirubin in brain might mean for clinical practice, thus more studies are needed. It should be noted, however, that the term '*bilirubin oxidase*' does not identify this activity (288). Commercial '*bilirubin oxidase*' behaves differently from the mitochondrial enzyme discussed above (17, 121, 284, 287, 288, 291, 302, 303). There appears to be no evidence that '*bilirubin oxidase*' catalyzes bilirubin oxidation in brain (291).

F. Regional and subcellular localization

The question of how bilirubin localizes to the basal ganglia is likely to be important for a full

understanding of bilirubin brain toxicity, as the clinical syndrome of KSD in survivors of ABE in the newborn period involves movement disorders associated with basal ganglia dysfunction (262, 397). Extraction of bilirubin from the brains of four infants who died with severe jaundice found concentrations of about 35 nmol/g in the basal ganglia and 8 nmol/g in the rest of the brains. Much higher bilirubin concentrations were thought to be present in the most intensely stained regions of the nuclei, but could not be quantitated (153).

Many have tried to recreate this staining pattern in animal models, but success has been limited. Though regional differences in brain bilirubin concentration were found in piglets following a [³H]bilirubin infusion during conditions of hypercarbia or hyperosmolality (129, 130), such differences could have been due both to variations in bilirubin entry into or disappearance from the brain, or to redistribution, or to binding to tissue or cell elements. Significant differences were found in brain regional bilirubin concentrations in 15–19-day-old Gunn rat pups pretreated with sulfadimethoxine (133). The concentration of bilirubin in the cerebellum was 32 nmol/L; while the brainstem and cortex contained 18 and 8 nmol/L respectively. But while higher brain bilirubin concentrations were found in male pups, suggesting that sex could influence brain bilirubin uptake or clearance, the regional differences did not correspond to a typical kernicteric staining pattern, nor is it clear how they arose. Studies in Sprague-Dawley rats who received infusions of bilirubin-containing solutions stabilized with albumin and accompanied by manipulations of bilirubin binding, BBB opening, and brain blood flow, have not succeeded in mimicking the basal ganglia accumulation (281, 283, 293, 300). Furthermore, no inter-regional differences between bilirubin entry and clearance from brain were shown. Regional differences in brain bilirubin metabolism did not explain the kernicteric staining pattern (288).

In order for the classical theory of bilirubin inhibition of mitochondrial respiration to be supported, bilirubin concentrations in the mitochondria during relevant levels of hyperbilirubinemia must be high enough to cause toxicity (196). Only one study appears to have addressed this question. [³H]bilirubin was given as an IV bolus to rats and the brains of animals sacrificed after 10 and 30 min

were processed by subcellular fractionation (280). Absolute values for bilirubin could not be calculated, but bilirubin content related to protein concentration was much lower in mitochondria than in the cytoplasmic and membrane fractions.

G. Mechanism(s) of Bilirubin Neurotoxicity

1. Transient versus permanent effects

A discussion of the mechanisms of bilirubin-induced brain injury must include events associated with cell death. However, bilirubin also appears to have transient effects on the brain. Jaundiced neonates may be lethargic/drowsy, have reduced muscle tone, and difficulties with feeding (20, 198, 678). Auditory brainstem response (ABR) studies, both in humans and in animal models, have yielded more objective evidence of transiently altered neuronal function, although some have also found more permanent changes (22, 24, 243, 260, 294, 390, 603, 714). Both acute and chronic auditory neuropathy has been shown to be associated with levels of UB, but not TSB (22, 24, 26, 27, 31). An increased incidence of apneas in jaundiced premature infants compared to less jaundiced controls gradually disappears from the second week of life, and that is also associated with UB levels as well as appearing to follow changes in the ABR (24, 30). Such changes in neuronal function and behavior likely reflect bilirubin effects on the brain, and although these apparently transitory effects of bilirubin on the brain may be referred to as early phase ABE, clinicians probably would not use the term kernicterus/KSD about such reversible toxicity, nor is there a diagnostic code for this condition in the diagnostic coding systems (20, 62, 656, 715).

The signs of early phase ABE versus KSD could represent extreme ends of a continuum of toxicity. But one must also consider the possibility that separate and distinct mechanisms are involved in cell death versus milder, and perhaps transitory, perturbation of neuronal signaling (285). The term ‘*subtle kernicterus spectrum disorder*’ was part of the recent proposal for a revision of terminology and could possibly be applicable to the behavioral and neurodevelopmental effects mentioned above (397). Herein, we discuss bilirubin effects on brain in a wide sense, and also include processes that may not lead to cell death and lasting damage.

2. *Inhibition of cell respiration*

The first in vitro studies of bilirubin toxicity showed that bilirubin inhibited respiration in a rat brain homogenate (171). In rat liver mitochondria bilirubin [300 $\mu\text{mol/L}$ (17.5 mg/dL)] partially inhibited respiration and almost completely inhibited phosphorylation (196). Uncoupling of oxidative phosphorylation, causing energy failure, was for many years the main theory on the 'basic mechanism' of bilirubin toxicity (171, 196, 460, 523). Evidence for bilirubin toxicity to mitochondria has been found in several in vitro studies. Bilirubin perturbed the mitochondrial membrane leading to increased permeability, decreased potential, release of cytochrome c, and triggering of apoptosis (563-566). Mitochondrial toxicity was also suggested from in vivo observations. Ultrastructural changes were found in the brain mitochondria of Gunn rats (346, 590, 591). Significantly lowered phosphocreatine and ATP were found in the brains of rats with hyperbilirubinemia induced by IV infusion; although opening of the BBB with hyperosmolality was required to elicit such changes (705). Magnetic resonance spectroscopy in five infants with severe NNJ showed that one of the infants had an abnormally high lactate:N-acetyl aspartate ratio (252). Only this infant had abnormalities in the basal ganglia on MRI and, along with one other infant, at follow-up was neurologically abnormal. The increased ratio of lactate:N-acetyl aspartate could have been due to changes in mitochondrial function. A cartoon which shows some of the different effects and interactions of bilirubin with cells is shown in **Figure 5**.

However, other data are not compatible with mitochondria as the principal targets of in vivo bilirubin toxicity. Electron microscopy of Gunn rat brains appeared to show that changes in the mitochondria was most likely caused by prior changes in the cytoplasm, however this interpretation has been critiqued (460, 590). In cultured L-929 cells bilirubin effects, when compared to compounds known to uncouple oxidative phosphorylation and inhibit reduced NAD, were more likely on the cell membrane than on the respiratory chain (160). Newborn pigs with brain bilirubin levels comparable to those found in infants with kernicterus did not show changes in cerebral oxygen, glucose, and lactate metabolism, as would have been expected if mitochondrial function were disturbed (96).

Bilirubin perturbed neurotransmitter metabolism in permeabilized synaptosomes in vitro with high exogenous ATP present, thus loss of endogenous ATP is unlikely to have caused the observed effects (295). Of six infants with extreme jaundice, of whom four were neurologically abnormal at 1 year of age, none had demonstrated high brain lactate levels during the period of hyperbilirubinemia (511). Indeed, the reported findings seemed more likely to be due to changes in the sensitivity of the N-methyl-D-aspartate (NMDA) receptor.

When comparing neurons and astrocytes from young versus old rats, the immature cells were most vulnerable to bilirubin toxicity, albeit the mitochondria from the young animals were more resistant to toxicity than those from the older rats (567). Thus, mitochondrial injury may be neither the only nor the primary mechanism for bilirubin neurotoxicity. Also, although the experimental data is limited, it has not been shown that sufficient quantities of bilirubin get to mitochondria in vivo to disturb their function (280). However, more bilirubin was found in the mitochondria after hyperosmolar opening of the BBB, which may be noteworthy in light of the lower phosphocreatine and ATP found in the brains of rats with infusion-induced hyperbilirubinemia following hyperosmolar opening of the BBB, but not when the BBB was intact (705). However, bilirubin likely has more direct access to mitochondria in in vitro cell cultures than in the intact brain in vivo. Thus, whether in vitro observations are necessarily relevant to the quest for the mechanisms of bilirubin toxicity in the living organism is uncertain and must await further study.

3. *Membrane effects*

Evidence from several sources suggests that bilirubin interacts with biological membranes (35, 160, 459). In rats who were given [^3H]bilirubin IV, the bilirubin concentration (relative to protein) was higher in membranes than in other subcellular fractions (280). Autoradiography of brain slices exposed to [^3H]bilirubin in vitro showed that bilirubin bound most strongly to neurons, suggesting a stronger affinity for neuronal cell membranes (165).

Bilirubin interaction with membrane polar lipids may play a role in the mechanism of toxicity (119). In cell cultures containing bilirubin cytosolic enzymes leaked into the medium, suggesting

increased membrane permeability (160). Scanning electron microscopy of erythrocytes from jaundiced neonates showed crenation of the surface, suggesting that bilirubin interacted with the outer half of the erythrocyte plasma membrane bilayer complex (373). The membrane changes were reversed by phototherapy, an interesting observation in light of hypotheses that bilirubin photoisomers may be less able to cross the BBB and perhaps also are less toxic (304). Crenation of the RBC outer surface has been shown in the presence of bilirubin concentrations ranging from 1×10^7 to 1×10^{-5} mol/L (108). Bilirubin-induced depression of the synaptosome membrane potential was thought to involve changes in ion permeability (195). However, in synaptosomes permeabilized with streptolysin O (which excludes plasma membrane polarity), bilirubin inhibited Ca^{2+} -dependent neurotransmitter exocytosis and disrupted norepinephrine storage in vesicles at higher bilirubin concentrations (295).

Bilirubin apparently has high affinity for cell membrane phospholipids and may form complexes with these (116, 117, 403). Partitioning into membranes was increased when these contained proteins, but specific binding sites were not involved (403). Bilirubin acid may precipitate when acidosis is present, leading to irreversible aggregation (120, 701). Bilirubin aggregation and precipitation is a theory that still has support (523). However, binding of the monovalent anion of bilirubin acid to membranes may be reversible (703). The finding that bilirubin binding to liposomes and RBCs is reversible is in line with this speculation, as are clinical and experimental observations showing that the milder signs of bilirubin influence on the brain may be reversed (456, 508, 701, 703). However, both the model and the experimental conditions seem to influence the reversibility of membrane changes. For example, washing of erythrocytes with albumin did not completely reverse bilirubin-associated membrane toxicity (112).

Bilirubin may also interact with membrane-localized enzymes, pumps, or transporters, and it is possible that some of the observed effects of bilirubin on cell membranes could involve such elements. For example, bilirubin changed the temperature dependence of $\text{Na}^+ - \text{K}^+$ -ATPase to lower levels in young rats, whereas enzyme from adult rats was not affected, suggesting that the enzyme,

1622 bilirubin, and the surrounding membrane lipid environment may interact (370, 375). Similarly, the
1623 temperature dependence of NOS activity changed in the presence of bilirubin (648). Changes in NOS
1624 activity due to bilirubin were reduced by 7-nitroindazole, a specific inhibitor of neuronal NOS (535).
1625 $\text{Na}^+\text{-K}^+\text{-ATPase}$ and acetylcholinesterase activities were more strongly inhibited in young vs old rat
1626 brains following in vivo bolus administration of bilirubin (650). It was suggested that this could be
1627 due to differences in the lipid environments which surround the enzyme during membrane
1628 development. $\text{Mg}^{++}\text{-ATPase}$ was not similarly affected.

1629 Bilirubin inhibits the enzymes that transfer reducing equivalents across the inner
1630 mitochondrial membrane as well as vasopressin-stimulated water and Na^+ transport across toad
1631 bladder membranes (105, 472). Bilirubin also inhibits K^+ transport across cell membranes, but not
1632 reversibly (157). In murine hepatoma cells, bilirubin, possibly a ligand for the aryl hydrocarbon
1633 receptor, caused apoptosis and disruption of cell membrane integrity (600).

1634 The NMDA receptor ion-channel complex at the surface of cell membranes, including the
1635 synaptic membrane, is activated by glutamate, causes opening of an ion channel, and appears to be
1636 important during development (469, 633). In developing rat brain neurons, apoptosis could be
1637 reduced by MK-801 an NMDA blocker, and these observations were confirmed in human neurons
1638 (139, 253, 254, 274, 275). However, MK-801 did not protect primary cultures of rat hippocampal cells
1639 against bilirubin toxicity, nor did it prevent ABR abnormalities in jaundiced Gunn rat pups after
1640 injection of a displacer (606). In piglets given bilirubin IV, the affinity of the NMDA receptor for the
1641 blocking agent MK-801 was increased (318). When ABE was induced in homozygous Gunn rats by
1642 injection of a displacer, concurrent treatment with MK-801 reduced the effects (470). However, in a
1643 different experimental model, bilirubin was not found to interact with the NMDA receptor (687).
1644 When comparing the results of the latter two studies, significant differences in the experimental
1645 paradigms suggest that the apparent contradiction may be due to these differences rather than to
1646 bilirubin pathophysiology per se. Thus, one group examined sequelae in the form of histological
1647 tissue loss 5 days after an acute in vivo intervention in Gunn rats (470), while the other group studied

transverse hippocampal slices and Müller cells acutely in vitro by cell clamp technique (687).
Therefore, although some of the published data seem to support a role for NMDA-mediated
excitotoxicity in ABE, apparent contradictions will need to be reconciled (Figure 5).

Using voltage-clamp recordings from bushy cells in the ventral cochlear nucleus from rat
pups, it was shown that acute administration of bilirubin increased voltage-gated calcium channel
currents mediated by high voltage-activated P/Q-type calcium currents (413). This appeared to occur
via Ca^{2+} and calmodulin dependent mechanisms, causing excessive Ca^{2+} within the neurons. There
are more P/Q-subtype calcium channels in neonatal neurons than at more mature stages, thus
subtype-specific increase of P/Q-type Ca^{2+} currents may be involved the vulnerability of neurons to
bilirubin toxicity in auditory as well as other brain regions (413). A role for Ca^{2+} channels in bilirubin
neurotoxicity was also supported by studies of recombinant $\text{Ca}_v2.3 + \beta_3$ channel complexes and ex
vivo electroretinograms from wildtype and $\text{Ca}_v2.3$ -deficient mice (12). Thus, 10 μM UB produced
changes in the voltage-dependence of activation and prepulse inactivation. Also, exposure of mouse
retina to UCB suppressed responses of the inner retina from wildtype compared to $\text{Ca}_v2.3$ -deficient
mice, and recovery after washout was more complete, and occurred more rapidly in retinae that did
not have $\text{Ca}_v2.3$ channels (12).

4. Neurotransmitter metabolism

In studies of brain specimens from human kernicterus cases, both of ABE and in cases with chronic
sequelae, changes have been found in the expressions of neurotransmitters and neuropeptides as
well as calcium-binding proteins, suggesting that bilirubin neurotoxicity may impact these important
molecules and processes (264). Thus, in cases who had died with ABE during the newborn period, the
expression of tyrosine hydroxylase was reduced both in the putamen and in the globus pallidus, and
in the latter nucleus also in cases of chronic post-kernicteric bilirubin encephalopathy. The expression
of methionine–enkephalin was also reduced in the external segment of the globus pallidus, both in
cases of acute and chronic post-kernicteric BE. Finally, immunoreactivity for substance P was

significantly reduced in both internal and external segments in cases of chronic post-kernicteric BE, but only mildly affected in cases who had died in the newborn period (264).

In the experimental setting bilirubin reversibly inhibited both evoked potentials and synaptic activation in rat transverse hippocampal slices in vitro, which suggests an effect of bilirubin on neurotransmitter metabolism (297). Although this study may be critiqued because of the very high bilirubin concentrations used to elicit the reported changes, others have also found changes in stimulus-response in in vitro hippocampal slices, specifically in the Schaffer collateral CA1 synapses, and at bilirubin concentrations as low as 10 $\mu\text{mol/L}$ (139). Phosphorylation of synapsin I is an important step in synaptic neurotransmitter release. Bilirubin inhibits synapsin I phosphorylation, which also points to an effect of bilirubin on neurotransmitter cycling (292).

Reduced neurotransmitter uptake into synaptic vesicles may be due to an inhibitory interaction between bilirubin and transport proteins in vesicle membranes, causing decreased synaptic function in the jaundiced brain (574). But uptake of dopamine and glutamate were equally inhibited, which is interesting in light of the assumption that these are driven by different mechanisms (proton gradient and membrane potential, respectively). This suggests that bilirubin inhibition of neurotransmitter uptake may be mediated by an effect on the transmembrane domains of the transporter proteins, possibly at the protein/lipid interphase (574).

It was suggested that perturbations of membrane function by bilirubin trigger a cascade that leads to excitotoxicity and energy failure in mitochondria (688). However, this hypothesis may be contradicted by the observation that bilirubin inhibited both exocytotic release and synaptic vesicular storage of brain catecholamines in permeabilized synaptosomes (295). Given the characteristics of the model, these effects were clearly dependent neither on endogenous ATP nor on the membrane potential (295). Thus, bilirubin may have (at least) two distinct effects on transmitter release in presynaptic catecholaminergic terminals: *i*) by decreasing the efficiency of Ca^{2+} -dependent secretion; and *ii*) at higher bilirubin concentrations, disrupting vesicular norepinephrine storage. In intact neurons these effects would decrease the evoked release of the neurotransmitter. Furthermore, in in

vitro studies with purified kinases and peptides, in the absence of membranes and in the presence of excess ATP, widespread inhibitory effects of bilirubin on peptide-kinase interactions were still present (288).

Inhibitory effects of bilirubin were detectable in synaptosomes, both as far as the uptake of tyrosine (dopamine precursor), and formation of dopamine (34). Bilirubin was also shown to inhibit the direct uptake of dopamine into synaptosomes, but not release (513). Endogenous acetylcholine release was inhibited and the synaptosomal membrane was depolarized. However, inhibition of dopamine release by bilirubin was observed by other investigators using a different stimulus for release (136).

Bilirubin inhibited uptake of glutamate in rat cortical astrocytes in vitro (610). Release of glutamate and cell death followed bilirubin exposure of astrocytes, microglia, as well as neurons in culture, and immature cells were more vulnerable to loss of glutamate (202, 210, 244). L-carnitine protects neurons in culture from glutamate toxicity, and its presence significantly reduced bilirubin toxicity in cultured cerebellar granule cells, lending further credence to the putative role of glutamate and excitotoxicity in bilirubin cell toxicity (639).

5. *Enzyme induction*

As already discussed, bilirubin appears to inhibit a wide range of enzyme activities. However, bilirubin may apparently also induce some enzymes. Thus, in oligodendrocytes from newborn rats bilirubin induced NOS mRNA expression, leading to increased nitrite production (236). This was accompanied by apoptosis of the oligodendroglia, which was dependent on bilirubin concentration and time of exposure. Exposure of rat cerebellar granule neurons in vitro to bilirubin led to activation of p38 MAP kinase, followed by cell death (419). Pretreatment of the cells with a p38 MAP kinase inhibitor (SB 203580) significantly reduced bilirubin neurotoxicity. In contrast to the widespread inhibitory effects of bilirubin on protein phosphorylation, p38 MAP kinase phosphorylation was upregulated by bilirubin exposure, and seemed to be the trigger for bilirubin-induced neuronal death (419).

6. *Apoptosis and necrosis*

Neuronal loss is a key event in kernicterus and in the brain lesions of homozygous Gunn rats, in whom cerebellar Purkinje cells appear to be particularly vulnerable (155, 169, 272, 460, 532, 656, 678). Bilirubin caused apoptosis in rat cerebellar granule cells (417). Cell death could be blocked by inhibiting the synthesis of RNA and proteins, suggesting that de novo synthesis of RNA and protein may be necessary to initiate cell death. The sequence of events involved in cell death was examined in developing rat brain astrocytes and neurons (565, 566). Signs of impaired mitochondrial metabolism and membrane perturbations in the form of altered lipid polarity and fluidity, protein order, and redox status were observed before apoptosis became apparent. The effect on cell membranes, evinced by increased lipid polarity, was noted almost immediately. Neurons were around 30% more vulnerable than astrocytes (565). UDCA, a mitochondrial-membrane stabilizing agent, and cyclosporine A, an inhibitor of the permeability transition, prevented apoptosis which appeared to be triggered by mitochondrial depolarization and Bax translocation (563, 566). Cytochrome c was released from the intermembrane space of the mitochondria. This suggested that bilirubin interacted directly with mitochondria by influencing membrane lipid and protein properties, redox status, and cytochrome c content, and that induction of apoptosis by bilirubin might be mediated, at least in part, by physical changes in the mitochondrial membrane (563, 566).

The above studies were carried out in cells of animal origin. However, NT2-N neurons (a human neuron-like cell line) have also been used to assess the ability of bilirubin to cause apoptosis and/or necrosis and showed that induction of apoptosis, as opposed to necrosis, may depend on bilirubin concentration. Thus, high bilirubin concentrations (100 $\mu\text{mol/L}$) induced early necrosis while low-to-moderate concentrations (0.66–25 $\mu\text{mol/L}$) predominantly induced delayed apoptosis (273). Using a specific caspase-3 inhibitor (zDEVD.FMK), a general caspase inhibitor (zVAD.FMK), and an NMDA receptor antagonist (MK-801) bilirubin-induced cell death was shown to involve both NMDA receptor-mediated and caspase-mediated pathways (274). Synergistic protection was seen following concurrent inhibition of both pathways. While caspase inhibition did not positively impact cell

survival after short-term bilirubin exposure in these cells, the number of undamaged nuclei was significantly increased by NMDA blockade without effects on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, another measure of cell viability (275). A possible role for NMDA receptors has also been confirmed in rat transverse hippocampal slices in vitro (139).

7. Cell metabolism

Bilirubin inhibits protein synthesis; however, one recent study did not confirm this finding (35, 250, 261, 274). Inhibitory effects of bilirubin on carbohydrate metabolism were shown in older studies, but these findings do not appear to have been replicated (372, 542, 607). Bilirubin was shown to inhibit DNA synthesis, while strand breakage in DNA was increased when bilirubin exposure was combined with phototherapy (35, 571, 572, 586, 643). In a mouse model of NNJ (UGT1^{-/-}) DNA damage in the cerebellum was shown to occur in vivo (553). In vitro, SH-SY5Y cells which are derived from human neuroblastoma and often used to model neuronal function in vitro, evinced DNA damage when exposed to 140nM UB, as determined by Western blot and immunofluorescence analyses. If these cells were concomitantly exposed to N-acetyl-cysteine, a scavenger of free radicals, DNA damage was prevented. This was seen to support the concept that DNA damage was caused by bilirubin-induced oxidative stress. Exposure of the cells to bilirubin also activated the main DNA repair pathways through homologous recombination and non-homologous end joining (553).

Exposing SH-SY5Y cells to 140 nM UB increased intracellular oxygen-free radical levels and accumulation of Nrf2 protein, which regulates the expression of antioxidant proteins, in the nucleus (546). This resulted in increased expression of multiple antioxidant response gene mRNAs. Thus, in these cells the response against bilirubin-mediated oxidative stress involves activation of antioxidant defenses, partly through the Nrf2 pathway.

Exposure of PC12 cells (from rat pheochromocytoma) and rat cerebellar granule cells to bilirubin (0.5–10 μ M) significantly decreased nerve growth factor and brain-derived neurotrophic factor signaling to Akt and extracellular signal-regulated kinases, showing that bilirubin can interfere

with important pro-survival signaling pathways (449). This involved reduced phosphorylation, and thus reduced activation, of important downstream effectors, which could be partially reversed by a phosphatase inhibitor. The possible role of inhibition of peptide/protein phosphorylation as a basic mechanism of bilirubin toxicity is discussed further in Section G.13 – ‘A common mechanism?’.

8. *Infection and immunology*

Infection/sepsis has long been considered a risk factor for ABE/KSD, but published support was limited and equivocal (378, 539, 652). However, newer data provide stronger support for an association between sepsis and kernicterus (225, 380, 511, 699). Among 100 Pakistani infants with ABE, 52 had been diagnosed with (unspecified) sepsis (380). Among 288 Taiwanese infants with TSB > 342 $\mu\text{mol/L}$ (20 mg/dL) 15 developed ABE and/or KSD. The OR for adverse outcomes in infants with sepsis was 161.7 (95% CI: 11.7–2242.8), a higher OR than for any other potentially contributing factor (699). Among 249 newborn infants with TSB $\geq 427 \mu\text{mol/L}$ (25 mg/dL) admitted to Cairo University Hospital with ABE on admission ($n=44$) and/or neurological evidence of KSD at the time of death or discharge ($n = 35$), rigorously defined sepsis greatly increased the risk for bilirubin neurotoxicity (OR = 20.6) although the highest risk for ABE/KSD was found among infants with Rh-incompatibility (OR = 48.6) (225). Similarly, in a cohort of 21 infants with ABE from Nigeria, 15 had septicemia (512).

Infected infants tend to develop higher TSB levels, which may contribute to their increased risk of ABE/KSD (181, 527, 528). In endotoxemic rats, both total and UB were elevated, leading to increased net accumulation of bilirubin in brain (298). Infected human infants had lower total albumin concentrations and also a lower reserve albumin binding capacity for bilirubin as estimated by the MADDS method (192). It is a reasonable assumption that these infants also had higher UB concentrations.

Inflammatory cytokines may increase BBB permeability, which might then facilitate bilirubin passage into the brain (499). Changes in BBB permeability may be secondary to disruption of the barrier, involving modifications of tight junctions, endothelial damage, degradation of the glycocalyx, breakdown of the glia limitans, and changes in the astrocytes (665). However, changes in barrier

permeability may also be non-disruptive and involve membrane transporters, cytokines, prostaglandins, and cellular transmigration (665). The complexity of this system, and the possibility for both unintended as well as perhaps planned perturbation of such processes, may be illustrated by the finding that acetaminophen, a drug commonly used for pain relief in sick neonates, can cause upregulation of P-gp protein expression through the constitutive androstane receptor pathway (616). While the interactions of some of these processes/mechanisms with bilirubin have been discussed in this review and also reviewed by others, other areas have apparently not been studied with a specific focus on the BBB (231).

Endotoxin and TNF- α increased the cytotoxicity of bilirubin in mouse fibroblasts in vitro (504). When astrocytes were exposed to bilirubin in conditions that induced < 10% cell death, the release of TNF- α and IL-1 β was significantly increased (210). Young astrocytes in culture were more vulnerable to bilirubin-induced cell death than older cells and also showed greater inflammatory response (202). As in the fibroblast model (504), endotoxin increased bilirubin cytotoxicity in astrocytes (202). When exposed to bilirubin in vitro, microglia were activated and released high levels of TNF- α , IL-1 β , and IL-6, suggesting that bilirubin-induced cytokine production may increase neurotoxicity (244).

The response of the developing cerebellum to bilirubin toxicity was studied in UGT^{1-/-} mouse pups (675, 676). Notable findings were early activation of oxidative stress, endoplasmic reticulum (ER) stress, and inflammatory markers. TNF α and NFK β were important mediators of and inflammatory reaction, which led to apoptosis and eventually opening of the autophagy pathway. During this process M1 type microglia were activated (676). Using the same model these processes were later shown to be amenable to modification by minocycline treatment (675). Reduction of neurodegeneration, neuroinflammation, and apoptosis of cerebellar neurons translated into a dose-dependent reduction of lethality. Further, decreased M1 microglia activation was accompanied by a reduction in oxidative and ER stress markers in these cells. These data support the concepts that neurodegeneration and neuro-inflammation are important elements in bilirubin-induced neonatal lethality in this model.

1828 Recently an acute mouse model was developed using young (up to 22 days of age) CBA/Ca
1829 mice in which acute/transient hyperbilirubinemia and neurotoxicity were induced by intraperitoneal
1830 injection of bilirubin (up to 450 mg/kg) combined with sulfadimethoxine (300 mg/kg) (585). Clinical
1831 neurotoxicity was assessed with a behavioral score and ABR, and whole genome gene expression
1832 studies were carried out on brain tissue (cerebellum and auditory brainstem) and investigated
1833 further using immunoblotting. In vivo the mice showed impairment in behavior and the auditory
1834 threshold was raised. Whole genome gene expression analysis showed that ER stress and
1835 inflammation were important factors in bilirubin auditory neurotoxicity. Both known and novel anti-
1836 inflammatory drugs which interfere with NF- κ B and TNF α signaling were shown to protect the
1837 auditory pathway from bilirubin toxicity. The authors suggest that the rapid and reversible onset of
1838 bilirubin toxicity in this model may prove useful in screening potential therapeutic compounds,
1839 including anti-inflammatory drugs (585).

1840 Clearly the interaction between bilirubin and the immunologic cascade is quite complex.
1841 Bilirubin induced a rapid rise in the levels of TNF- α receptor 1 in astroglia, followed by activation of
1842 p38 MAP kinase and NF- κ B (209). Therefore, NF- κ B may play a role in astroglial response to bilirubin
1843 through inflammatory pathways, although the experimental evidence seems contradictory. Thus, in
1844 animal models no effect was found of bilirubin on NF- κ B or p38 MAP kinase. On the contrary –
1845 bilirubin appeared to exert a cytoprotective effect through inhibition of iNOS expression, as well as
1846 through stimulation of local PGE₂ production (686). Jaundiced rats were more resistant to endotoxin-
1847 induced hypotension or death compared to non-jaundiced controls and showed reduced expression
1848 of iNOS (395). Neural progenitor cells transplanted into the striatum of 20-day old homozygous vs
1849 heterozygous Gunn rat pups had a higher survival rate in the brains of jaundiced pups, suggesting
1850 that elevated brain bilirubin levels in jaundiced pups may protect the grafted cells, either by an
1851 antioxidant or immunosuppressive effect (724, 725). However, the authors did not discuss how we
1852 might reconcile the increased survival of transplanted cells in jaundiced brains with the fact that cells
1853 native to the same brain are lost due to bilirubin toxicity, thus this puzzle will need to be addressed

further. Also, endotoxemia or sepsis did not affect bilirubin metabolism in rat brain, though whether this is relevant for the putative connection between septicemia and ABE/KSD is as yet unknown (17).

It is also possible that innate immunity signaling may ameliorate bilirubin neurotoxicity. Very high TSB levels in *UGT1A1*28* mice caused systemic oxidative stress, as shown by a decreased ratio of glutathione/glutathione disulfide, and by activation of the NADPH oxidase complex and brain antioxidant response genes in the brain (731). Very high TSB levels led to inflammation in neurons, shown by activation of microglia and astrocytes. Apparently, the toll-like receptor 2 signaling pathway was key to the regulation of gliosis, pro-inflammatory mediators, and oxidative stress, and served as a protective mechanism in the presence of severe hyperbilirubinemia (731). This was shown by the significantly higher mortality rates in jaundiced *hUGT1A1*28/Tlr22/2* mice pups, who failed to activate glial cells, pro-inflammatory cytokines, and stress response genes (731).

As a further example of the yin-and-yang of bilirubin biology, there is also evidence that bilirubin may have anti-inflammatory activities in brain tissue. Thus, in rodent experimental autoimmune encephalomyelitis bilirubin, injected intraperitoneally to produce TSB levels of ~60 $\mu\text{mol/L}$ 30 min after injection, delayed the onset and alleviated the severity of the chronic form of the disease (423). Conversely, depleting endogenous bilirubin by treatment with zinc protoporphyrin exacerbated the disease. The authors suggest that the effect of bilirubin cannot have been due solely to its antioxidant effect as α -tocopherol, a compound with antioxidant effects similar to bilirubin, was much less effective than bilirubin in changing the course of the disease. They also investigated the effect of bilirubin (20 and 150 μM) on the proliferative responses of naive SJL/J-mouse CD4^+ T cells and protein lipid peptide (PLP)-specific CD4^+ T cells following stimulation with Con A, anti-CD3 mAb with or without anti-CD28 mAb, or PLP in vitro. At these concentrations, bilirubin inhibited CD4^+ T cell reactivity across a range of actions which included inhibition of costimulatory activities, suppression of immune transcription factor activation, and down-regulation of inducible MHC class II expression. The authors suggested that bilirubin actions were direct, and not through induction of immune deviation or regulatory T cells (423).

Thus, the evidence points to a role for immunology, inflammation, and/or infection in the pathophysiology of ABE/KSD, as well as to a possible modulatory effect of bilirubin on immune mechanisms. However, a number of questions still remain to be addressed and explored. Our discussion herein concerning the role of inflammation/infection in the genesis of bilirubin neurotoxicity is by necessity limited. For a more complete discussion of these aspects, the reader is referred to an extensive review by Brites (107), in whose laboratory much of the important work regarding these questions has been carried out.

9. *Differential sensitivity*

With very few exceptions, extreme unconjugated hyperbilirubinemia occurs in newborn infants. Therefore, descriptions of ABE and KSD are focused on this age group, giving the impression that bilirubin neurotoxicity is primarily related to increased vulnerability in the immature brain. Within this age group, however, some infants can suffer neurologic sequelae at moderately elevated TSB levels, while others seem to tolerate extreme hyperbilirubinemia without brain damage. Furthermore, preferential accumulation of bilirubin in e.g. basal ganglia suggests greater sensitivity in some brain regions and cell populations than in others.

However, brain damage due to bilirubin can also occur in more mature individuals, as shown by a few patients with Crigler-Najjar syndrome who escaped neurological sequelae for many years due to meticulous follow-up and continued phototherapy, only to suffer neurological damage during a medical or surgical emergency which caused significant increase of TSB (393, 653, 664, 678). Apparently, in these individuals, cerebellar sequelae were predominant, which is more unusual when ABE occurs in the newborn period.

In vitro, most cells appear vulnerable to bilirubin toxicity, but some more so than others. The age of the cells may modify their vulnerability, and different cellular processes vary in sensitivity. Glial cells appear to be more resistant to lethal bilirubin toxicity than neurons. For example, bilirubin toxicity was seen in mouse neuroblastoma cells in vitro, but not in rat astrocytoma cells (507). When these neuroblastoma cells had differentiated under exposure to PGE₁ and cAMP, they lost their

1906 sensitivity to bilirubin toxicity (507). Rat brain astrocytes in vitro tolerated higher bilirubin
 1907 concentrations than neurons before showing signs of injury as evinced by release of lactate
 1908 dehydrogenase, perhaps suggesting increased membrane leakage, necrosis, and apoptosis (611).
 1909 However, when cell function was measured through glutamate uptake and MTT reduction, a
 1910 measure of cell viability, astrocytes were more susceptible. MTT reduction has been regarded as a
 1911 sign of mitochondrial dysfunction, although that assumption has been questioned (424). Therefore
 1912 the authors suggested that bilirubin toxicity in neural cells might involve two distinct mechanisms: i)
 1913 a severe insult which causes cell death and is responsible for the irreversible damage primarily
 1914 observed in neurons, and ii) less pronounced effects that compromise only some cellular functions
 1915 and lead to reversible insults, perhaps more common in glial cells such as astrocytes (611).
 1916 Differences may also exist between classes of glia, as microglia appear more sensitive to bilirubin
 1917 toxicity than astrocytes (244). Thus, the pathogenesis of kernicterus may be complex and involve
 1918 more than one type of cell and one mechanism (611).

1919 When rat glial cells were grown in culture for 12 days, they became more resistant to
 1920 bilirubin effects than cells cultured for only 2 days, suggesting that immature cells may be more
 1921 vulnerable (33). Similar observations were made using different cells and techniques (202, 556). The
 1922 expression of P-gp in both mouse and rat brain increases with maturation, apparently with a
 1923 localization to the brain capillaries that suggests a function related to the BBB (457, 649). Increased
 1924 expression of MRPs in astrocytes may protect cells against bilirubin cytotoxicity, and exposure to
 1925 bilirubin may increase translocation of P-gp from the Golgi apparatus to the cell membrane (237).
 1926 Thus, lowered sensitivity to bilirubin toxicity with increasing age may perhaps be tied to increasing
 1927 expression of membrane transporters in BBB cells.

1928 Both cAMP and PGE₁ increase phosphorylation of membrane P-gp in human platelets (39, 40),
 1929 a noteworthy finding in light of the decreased sensitivity to bilirubin toxicity observed in
 1930 neuroblastoma cells exposed to these agents (507). MRP1 and P-gp are both expressed in cultured
 1931 rat astrocytes, and studies in humans show that drug treatment may lead to overexpression of P-gp

1932 and be associated with therapy resistance in epilepsy (66, 176, 426, 450). However, although both P-
 1933 gp and MRP1 may be expressed in neurons in experimental or refractory epilepsy, their expression in
 1934 neurons in the absence of such stimulation appears to be negligible to non-existent (317, 396, 615).
 1935 MRP5 has been found in pyramidal neurons from human brain samples, but the implications for
 1936 bilirubin neurotoxicity are unknown (90).

1937 With increasing BAMR, neuroblastoma and glioblastoma cells in vitro became more
 1938 vulnerable than fibroblasts and hepatocytes (505). A comparison of two neuronal cell lines (NBR10A
 1939 and N115) showed that the latter had more tolerance for bilirubin toxicity (586). Indeed,
 1940 pathoanatomical data from kernicterus in humans as well as from Gunn rats show that not all
 1941 neurons are damaged by bilirubin. Also, in the hippocampus of Gunn rats the density of parvalbumin-
 1942 positive cells is reduced both in the CA1 and CA3 regions as well as in total hippocampus compared
 1943 to Wistar controls, and the loss of these cells was found to correlate with TSB levels (312). In human
 1944 kernicterus autopsy cases the number of interneurons in the external segment of the globus pallidus,
 1945 which were immunoreactive to parvalbumin, was decreased mainly in cases of ABE as compared to
 1946 chronic/post-kernicteric brains (264). The reasons for such differences in sensitivity are not clear.

1947 Mitochondrial membranes from glial cells oxidize bilirubin at a greater rate than
 1948 mitochondrial membranes from a pure neuronal source (303). If such oxidation can be shown to be
 1949 protective, the different vulnerabilities to bilirubin toxicity might, perhaps in part, involve this
 1950 mechanism (226, 284). A similar speculation might be applied to the observation of increased
 1951 bilirubin oxidation by mitochondrial membranes from more mature brain cells (282). Further,
 1952 bilirubin may also have a greater binding affinity for neurons, as suggested by the observation of
 1953 [³H]bilirubin binding to hippocampal pyramidal and granular cells, as well as to Purkinje cells in rat
 1954 brain slices (165). However, at this point no explanation for this phenomenon has been suggested.

1955 Evidence suggests that bilirubin may perturb the interplay between neurons and glia, and as
 1956 activation and damage of glia appear to be important to the processes leading to neurodegeneration,
 1957 further studies to delineate these processes may be important steps in advancing our understanding

(106, 612, 613). A recently developed mouse model of kernicterus, which involves deletion of the Ugt1a1 gene and the Ugt1 locus in liver tissue from UAC mice, may become a useful tool enhance our understanding of these processes (53). In this model severe hyperbilirubinemia leads to clinical signs of ABE, including seizures, and kernicterus, and involves marked cerebellar hypoplasia accompanied by marked loss of Purkinje cells and reduced arborization of those remaining, reduction of myelination, and increased astrogliosis and microgliosis in the cerebellum, pons, and medulla oblongata (53).

Another fascinating perspective on the nuances of differential sensitivity to bilirubin toxicity was shown in a study of synaptic transmission in the medial nucleus of the rat trapezoid body in vitro (311). Increased latency and reduced amplitude evinced transmission failure following bilirubin exposure, which on more detailed examination was shown to be due to presynaptic damage, while postsynaptic characteristics were unaffected. Electron microscopy revealed loss of presynaptic calyceal terminals, while postsynaptic neurons were undamaged. When 7-nitroindazole, a nNOS antagonist, was given to the Gunn rats before administration of a displacer, the detrimental effects of bilirubin toxicity were prevented. Neurons from the medial nucleus of the trapezoid body have been shown to highly express nNOS, thus supporting the concept that NO may be implicated in bilirubin neurotoxicity (311).

Another study that addressed differential sensitivity examined the ototoxic potential of bilirubin and used organotypic cultures from rat pup cochlea and vestibula (726). These were exposed to bilirubin in a concentration range from 0–250 μ M for 24h. Auditory nerve fibers and vestibular nerve endings were most sensitive, evincing toxicity at bilirubin concentrations of 10-50 μ M. With increasing bilirubin concentration, a dose-dependent gradual shrinkage of spiral and vestibular ganglion neurons became evident together with condensation or fragmentation of nuclei. Only at bilirubin concentrations of 250 μ M did loss of cochlear and vestibular hair cells become evident (726). The clinical relevance of the bilirubin concentrations used at the highest end of the range may fairly be questioned in a setting intending to mimic the brain (524) (473). However, these

1984 data nevertheless align with others as far as indicating that further studies of the characteristics cells
 1985 such as these may expand our understanding of the differential toxicity of bilirubin.

1986 *10. Neuroprotection*

1987 Minocycline inhibits the activity of glial caspase 1 and iNOS, may reduce bilirubin toxicity in granule
 1988 cells from rat cerebellum in vitro, significantly reduces loss of Purkinje cells, and limits cerebellar
 1989 hypoplasia in homozygous Gunn rat pups (418). In a Gunn rat model of acute bilirubin neurotoxicity
 1990 (induced by injection of sulfadimethoxine and monitored by ABRs), minocycline 50 mg/kg injected IP
 1991 15 min prior to the displacer provided complete protection against the decreased waves II and III
 1992 amplitudes and increased interwave I–II and I–III intervals seen in the controls, but at lower doses
 1993 minocycline protection was only partial (233). Using the same rat model, but giving the minocycline
 1994 30–120 min *after* the displacer, complete neuroprotection was observed when minocycline was
 1995 dosed 30 min after the displacer, but was only partial when the dosing interval for minocycline was
 1996 extended to 120 min (557). Based on a hypothesis that oxidative stress might play a role in mediating
 1997 bilirubin neurotoxicity, minocycline, which is known to have antioxidant properties, was compared to
 1998 tauroursodeoxycholic acid and 12S-hydroxy-1,12-pyrazolinominocycline in Gunn rat pups given a
 1999 displacer at the time of peak postnatal hyperbilirubinemia (168). Bilirubin-induced neurological
 2000 dysfunction was recorded 24 hrs post-intervention with a rating scale that quantifies gait
 2001 abnormalities and dystonia. After sacrifice, cerebellar lipid peroxidation and protein oxidation were
 2002 measured. All the inhibitors reduced lipid peroxidation (but not protein oxidation) to control levels,
 2003 but only minocycline prevented neurological dysfunction (731). Thus, inhibition of lipid peroxidation
 2004 alone was not sufficient to prevent neurotoxicity, suggesting that the mechanism for minocycline
 2005 protection may not involve lipid peroxidation, or may involve additional mechanisms (731).

2006 Whether minocycline might prevent or ameliorate acute bilirubin neurotoxicity in human
 2007 infants has, as yet, not been studied. There appears to be at least two challenges as far as its use.
 2008 First, the timing of minocycline dosing appears to be critical as far as obtaining neuroprotection, but
 2009 the sentinel event to be used as a timing parameter is as yet undefined and needs to be determined.

2010 Second, minocycline is, along with other tetracyclines, considered contraindicated in infants as well
 2011 as during the first years of life due to risk of permanent dental enamel discoloration. Thus, a
 2012 substitute drug which mimics the molecular mechanism of neuroprotection against bilirubin toxicity,
 2013 but lacks the aforementioned side effect, would need to be developed.

2014 UDCA prevents apoptosis induced by several different factors, suggesting that different
 2015 apoptotic pathways may share a common mechanism. Bilirubin caused cell death through apoptosis
 2016 both in immature astrocytes and neurons (611). UDCA inhibited cell death in both cell types, and this
 2017 was not seen with other bile acids tested (611). Researchers also compared the ability of IL-10 and
 2018 glyoursodeoxycholic acid (GUDCA) to modulate cell responses to bilirubin (211). Only GUDCA
 2019 prevented bilirubin-induced cell death, inhibited suppression of IL-6, and also inhibited TNF- α - and IL-
 2020 1A-converting enzymes, as well as prevented maturation and release of these cytokines. Neither
 2021 reagent inhibited the extracellular accumulation of glutamate (211). Another study showed
 2022 glutamate and NO to be keys to the early and lasting deficits in neurite extension and ramification
 2023 induced by bilirubin (613). Both GUDCA and IL-10 prevented bilirubin inhibition of neurite extension
 2024 and ramification in vitro, but only GUDCA limited neuronal death and changes in the synapses. The
 2025 authors suggested that GUDCA might be used to prevent BE in at-risk neonates. However, no studies
 2026 appear to have been performed in vivo to explore this potential. The role of oxidative stress in
 2027 bilirubin-induced cell death was also studied in immature rat neurons incubated with 50- or 100- μ M
 2028 bilirubin in the presence of 100- μ M HSA, either alone or in combination with 100 μ M N(G)-nitro-L-
 2029 arginine methyl ester (NAME), an inhibitor of NOS, or with 50- μ M GUDCA (110). Bilirubin induced
 2030 protein oxidation and lipid peroxidation, events that correlated with the extent of cell death. Protein
 2031 oxidation, lipid peroxidation, and cell death were counteracted by NAME and largely prevented when
 2032 GUDCA was present (110). The authors speculated that these findings might point the way to a new
 2033 therapeutic approach for bilirubin-induced neurotoxicity.

2034 Organotypic rat brain cultures have been used to compare bilirubin toxicity in different brain
 2035 regions, as well as to study the potential for neuroprotection using different drugs (indomethacin

[anti-inflammatory], MgCl_2 [glutamate channel blocker], curcumin [antioxidant], or a cocktail of these three drugs (164). Minocycline was used as a control considered at present to be the 'gold standard' for protection against bilirubin neurotoxicity. Single drug treatment (indomethacin, curcumin, or MgCl_2) improved cell viability in all regions studied, while the three-drug cocktail almost completely prevented toxicity in the most affected area (hippocampus). These findings seem to indirectly support the roles both of inflammation, glutamate toxicity, and oxidant injury as mechanisms of bilirubin neurotoxicity, but also point the way towards the possibility of intervening with drug treatments in infants with signs of ABE. In support of this, anti-inflammatory compounds which interfere with NF- κ B and TNF α signaling were recently shown in a new, acute mouse model of ABE, to protect the auditory system against bilirubin toxicity (585). Thus, this avenue is in need of further exploration.

Hypothermia is neuroprotective following asphyxia, both in animals and in humans (258). The same was found for meningitis and traumatic brain injury (180, 661). Based on the effects of therapeutic hypothermia on neuronal metabolism and on the progression of events in brain injury a hypothesis of protection in bilirubin neurotoxicity may be postulated. Newborn pigs were divided into a hypothermic ($34.0^\circ\text{--}35.0^\circ\text{C}$) and a normothermic ($38.0^\circ\text{--}39.0^\circ\text{C}$) group, and hyperbilirubinemia was induced by infusion of bilirubin over a 4-hr period, followed by a displacer to increase bilirubin entry into brain. Decreased cerebral cortical cell membrane $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity and increased lipid peroxidation products evinced bilirubin neurotoxicity in the normothermic group, while such changes were significantly attenuated in the hypothermia group, which also showed less evidence of reduction in brain ATP. The changes in phosphocreatine and blood and brain lactate levels seen in the control group were also less pronounced with hypothermia (536). Recently, moderate hypothermia was also shown to protect against bilirubin-induced cell death in mouse neurons in vitro (391).

Thus, therapeutic hypothermia appears promising as a strategy to ward off bilirubin neurotoxicity, but no clinical studies have been published, and no ongoing clinical trials are recorded

in the registries we have accessed (Clinicaltrials.gov, Australian New Zealand Clinical Trials Registry, EU Clinical Trials Register, ISRCTN registry, Health Canada's Clinical Trials Database, ICTRP Search Portal). Also, no case reports could be found in PubMed or Medline. This suggests that recruitment or selection of suitable patients, either for a trial or for compassionate use, is challenging. Indeed, in the animal study discussed above, hypothermia was induced concurrently with bilirubin infusion, a scenario not applicable to clinical practice. Also, given the difficulty of selecting an entry point for testing this potential therapy and the rarity of ABE in most settings, extensive scientific scrutiny and discussion will be necessary before proceeding. Then, a setting in a low- or middle-income country, where ABE unfortunately continues to be reported with some regularity, may be most feasible.

The predominant bilirubin isomer in humans is bilirubin-IX α (Z,Z), found either in the charged dianion form, or as bilirubin acid. While the dianion is to some extent water soluble at neutral pH due to its eight hydrophilic groups, the acid form is nearly insoluble because of intramolecular hydrogen bonds (117, 218). Photoisomerization of bilirubin yields more polar forms of the molecule (442). While the lipophilic bilirubin-IX α (Z,Z) isomer is associated with toxicity, water-soluble isomers have been hypothesized to be nontoxic (442, 609, 643). Photoisomers have in some cell culture studies nevertheless increased bilirubin toxicity (150, 569, 572, 608). However, it is uncertain whether in vitro cell models are appropriate for testing a hypothesis relating to phototherapy and bilirubin neurotoxicity. Before embarking on further in vitro studies of bilirubin photoisomer toxicity it is necessary to ascertain whether photoisomers actually cross the BBB and gain access to brain cells. While a hypothesis that these polar isomers do *not* cross the BBB seems supported by our knowledge of the physicochemical characteristics of these molecules, no in vivo studies to support these speculations are on record (304, 466). Clinical evidence suggests that aggressive phototherapy, in some cases used in combination with other therapeutic tools such as exchange transfusion and IV immune globulin (IVIG), can reverse acute intermediate-to-advanced BE, but it is not clear whether decreased BBB penetration of bilirubin photoisomers plays any role in these fortuitous outcomes (286, 299, 309).

2088 11. Hemolysis

2089 Most therapeutic guidelines recommend more aggressive management of severe hemolytic NNJ, as
 2090 hemolysis is believed to increase the risk for bilirubin neurotoxicity (20, 77, 79, 358, 360).(77, 79)
 2091 G6PD deficiency has been a risk factor in cases of kernicterus reported in recent years (359). Among
 2092 infants admitted to Cairo University Children's Hospital during the year 2008, Rhesus incompatibility
 2093 with a hematocrit < 35% had an OR of 48.6 (95% CI: 14–168) for ABE on admission, assessed by
 2094 "BIND score", and/or death or bilirubin encephalopathy on discharge, based on residual neurological
 2095 abnormalities compatible with bilirubin sequelae (225, 349, 678). In this particular study, ABO
 2096 incompatibility with a hematocrit < 35% was *not* significantly associated with ABE or neurological
 2097 abnormalities at discharge (225), but others have concluded that both ABO and Rh incompatibility are
 2098 risk factors for KSD (329, 350).

2099 The mechanisms underlying increased risk for bilirubin brain toxicity in hemolysis are not
 2100 known. Hemolysis increases the Hb available for bilirubin production and leads to increased TSB
 2101 values. The important question is, however, whether hemolysis simply increases the risk of bilirubin
 2102 neurotoxicity by increasing TSB, or whether at any given value of TSB that risk is greater in the
 2103 presence of hemolysis than in its absence.

2104 In rats with hemolytic anemia induced by phenylhydrazine, bilirubin entry into and clearance
 2105 from brain after an IV bolus of bilirubin were the same as in control animals at equivalent TSB values
 2106 (288). In Gunn rats phenylhydrazine-induced anemia was followed by signs of ABE in the form of
 2107 decreased auditory brainstem evoked potential wave II and III amplitudes and increased I–II and I–III
 2108 interwave intervals, but these changes reflected TSB levels and not the anemia per se (558). The
 2109 immunological processes at play in infants with blood group incompatibility were not modeled by
 2110 these two studies. But neither is immunology involved in G6PD deficiency, a well-described risk
 2111 factor for kernicterus, nor in most other congenital hemolytic anemias (359). Whether other
 2112 products of hemolysis beyond bilirubin could contribute to bilirubin neurotoxicity, appears not to
 2113 have been studied. Thus, while hemolysis must be regarded as a risk factor for ABE and KSD, the

2114 underlying mechanism(s) are not clear and require further study (358).

2115 12. *Bilirubin binding*

2116 In the organism bilirubin binds to albumin in serum and to ligandin in hepatocytes. In both binding
 2117 sites lysine seems to be present (336, 716). The literature suggests that lysine may be a constituent
 2118 of the active sites of many of the reactions perturbed by bilirubin, including the ATP-binding
 2119 Subdomain II of the protein kinase family (278, 458). In an in vitro model peptide-kinase system,
 2120 lysine-containing peptides modulated the toxic effects of bilirubin (297). Thus, the possibility that
 2121 bilirubin-lysine binding may mediate and/or modulate bilirubin neurotoxicity appears worthy of
 2122 further investigation.

2123 The concentration of UB, the '*culprit*' in bilirubin neurotoxicity, is intimately tied to the
 2124 binding capacity of albumin for bilirubin, and although bilirubin can bind to other serum proteins as
 2125 well as erythrocytes, the primary albumin binding site is most important in this context. Bilirubin-
 2126 albumin binding is reversible, and the binding affinity depends both on albumin characteristics, such
 2127 as immaturity, and on other factors such as illness and binding competitors (21, 25). Known risk
 2128 factors for bilirubin neurotoxicity which are associated with reduced albumin binding include
 2129 prematurity, sepsis, acidosis, hypothermia, asphyxia, and many of the drugs and nutrients used to
 2130 treat sick newborn infants (21).

2131 13. *A common mechanism?*

2132 In light of the many seemingly different, mostly inhibitory, toxic effects of bilirubin, it seems
 2133 appropriate to consider whether they may have something in common. Protein-peptide
 2134 phosphorylation has been shown to regulate many cell processes (41, 324, 503). An overview of the
 2135 literature suggests that regulation by protein-peptide phosphorylation might be a common theme for
 2136 many of the processes affected by bilirubin. Only a few examples can be mentioned here, the first
 2137 being the observation that both the binding of cAMP to protein kinase and the phosphorylation of
 2138 histone were inhibited by bilirubin (156). In cell-free preparations from newborn rabbit brains
 2139 protein phosphorylation was inhibited when bilirubin had been administered IV before sacrifice to

produce brain bilirubin levels ranging from 14–51 nmol/g tissue (488). As noted above, these brain bilirubin levels are comparable to those observed both in experimental animals as well as human infants with kernicterus. Bilirubin inhibition could be partly reversed by aminophylline, suggesting that bilirubin effects on brain involve both synaptic transmission and nuclear activation through histone phosphorylation. Bilirubin also inhibited phosphorylation of endogenous proteins in fibroblasts (32).

In vitro, bilirubin inhibited phosphorylation of synapsin I, a protein that is preferentially localized to presynaptic vesicles (292). Phosphorylation of synapsin I promotes neurotransmitter release at the synaptic cleft, while dephosphorylated synapsin I inhibits such release. Bilirubin inhibited synaptic activation in transverse rat hippocampal slices, which might be due to a lower degree of synapsin I phosphorylation (297). Drowsiness in jaundiced infants, along with reduced brain stem auditory evoked responses, could also be due to reduced synapsin I phosphorylation (605). Many other protein-kinase interactions are also inhibited by bilirubin (296). Thus, a speculation that inhibition of protein-peptide phosphorylation might contribute to the effects of bilirubin in biological systems does not seem far-fetched. Hypothetically, lysine binding could be involved in these reactions.

As discussed elsewhere in this review, P-gp is a membrane pump that may limit the intracellular and intracerebral accumulation of bilirubin and is regulated through phosphorylation (103, 277, 326, 327, 345, 401, 690, 735). Both protein kinases A and C are implicated in P-gp phosphorylation, and the interaction between these kinases and other peptide-protein substrates is inhibited by bilirubin (296). Conceivably, inhibition of P-gp phosphorylation may have a role in bilirubin toxicity. For example, pre-exposure to bilirubin has been shown to increase the permeability of the BBB for bilirubin itself (568). Reduced P-gp activity has already been shown to increase bilirubin entry into brain (277, 690). Although this has not been studied specifically for the bilirubin P-gp interaction, another BBB transport protein, breast cancer resistance protein (BCRP/ABCG2), is co-localized with P-gp and shares substrates with the latter (206). In rats with hyperbilirubinemia, both

2166 BCRP expression and function at the BBB were downregulated (717). Bilirubin levels correlated
 2167 negatively with brain BCRP expression. These in vivo results were replicated in vitro in Madin-Darby
 2168 canine kidney cells expressing human BCRP (717). Thus, the role of bilirubin relative to regulation of
 2169 P-gp function at the BBB, possibly involving phosphorylation, appears to be worthy of further study.

2170 *14. A note of caution*

2171 Some years ago McDonagh expressed a cautionary note regarding the many in vitro studies of
 2172 bilirubin toxicity (464). He suggested that bilirubin might be a 'promiscuous inhibitor'. The term
 2173 'promiscuous inhibitor' was applied to drugs that showed strong in vitro activity against many
 2174 potential protein receptor targets, but failed to show 'drug-like' activity on further testing (208, 471).
 2175 Common to 'promiscuous inhibitors' were these characteristics: high hydrophobicity and molecular
 2176 flexibility, along with the ability to form microaggregates. Bilirubin shares these properties. The
 2177 hypothesis that bilirubin is a promiscuous inhibitor seems compatible with the findings that bilirubin
 2178 inhibits many enzymes in vitro yet seems not to have any effects on them in vivo (463). Therefore,
 2179 McDonagh pointed to the risk involved in "*deducing the biochemical pathways of kernicterus from*
 2180 *the numerous in-vitro studies*" (464).

2181 Bilirubin toxicity has been found in many biological reactions and systems. Whether these
 2182 are implicated in the effects of bilirubin observed in jaundiced infants is not clear. It follows that
 2183 there is no agreement on the basic mechanism for bilirubin neurotoxicity. Indeed, we have probably
 2184 shown that the mechanism(s) of kernicterus, ABE, and KSD continues to elude our understanding.
 2185 Not surprisingly, translating research data into clinical guidelines has been challenging, leading to
 2186 significant practice variability (89, 492). While current knowledge regarding bilirubin neurotoxicity
 2187 does provide at least some input into the practical management of NNJ, more work is needed to
 2188 understand the basic mechanisms of bilirubin neurotoxicity in the infant brain. Armed with such
 2189 information, we may be in a position to develop more robust clinical protocols to limit, and even
 2190 block, the acute effects of bilirubin on the newborn brain as well as its effects on long-term
 2191 neurodevelopmental outcomes.

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- 3955

FIGURE LEGENDS

FIGURE 1. Bilirubin metabolism in the body. ALB, albumin; B, bilirubin; BCRP, breast cancer resistance protein (also referred to as ATP-binding cassette super-family G member 2 [ABCG2]); BDG, bilirubin diglucuronide; BG, bilirubin glucuronides; BMG, bilirubin monoglucuronide; BSEP, bile salt export pump (also referred to as ATP-binding cassette, sub-family B member 11 [ABCB11] or sister of P-glycoprotein [sP-gp]); BT, proposed bilirubin transporter; BVR, biliverdin reductase; COHb, carboxyhemoglobin; ER, endoplasmic reticulum; GST, glutathione-S-transferase; Hb, hemoglobin; HO, heme oxygenase; NADP, nicotinamide adenine dinucleotide phosphate; MATE1, multidrug and toxin extrusion 1; MRP2 and 3, multidrug resistance–associated proteins 2 and 3 [also referred to as ‘ATP binding cassette subfamily C members 2 and 3’ (ABCC2, ABCC3)]; OATP1B1/B3, organic-anion-transporting proteins 1B1 and 1B3; O₂, oxygen; UB, unbound bilirubin; UCB, unconjugated bilirubin; UCB-A, albumin-bound bilirubin; UGT1A1, bilirubin-UDP-glucuronosyltransferase.

FIGURE 2. Bilirubin structure. **A.** Planar structure of bilirubin as first presented by Fischer and Plieninger (redrawn from reference 216); **B.** Bis-lactam structure of bilirubin according to Bonnett, Davies, and Hursthouse (redrawn from reference 87); **C.** Bilirubin ridge-tile conformation (courtesy of Professor Antony F. McDonagh, PhD [deceased]); **D.** Ridge-tile structure of bilirubin (from reference 415, with permission).

FIGURE 3. Bilirubin diastereomers. Linear drawings of the four diastereomers of bilirubin (from reference 415, with permission).

FIGURE 4. Bilirubin-brain interaction. A, albumin; ABR, auditory brainstem response; ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; B, bilirubin; B-A, albumin-bound bilirubin; BCRP, breast cancer resistance protein (also referred to as ATP-binding cassette super-family G member 2 [ABCG2]); BV, blood vessel; CSF, cerebral spinal fluid; CPEC, choroid plexus epithelial cells; MRP1, multidrug resistance–associated protein 1 [also referred to as ‘ATP binding cassette subfamily C member 1’ (ABCC1)]; P-gp,

3982 phosphoglycoprotein (also referred to as multidrug resistance protein 1 [MDR1] or ATP-
 3983 binding cassette sub-family B member 1 [ABCB1]); ST, stroma; TJ, tight junction.

3984 FIGURE 5. Cellular effects and interactions of bilirubin. IL-1 β , Interleukin 1 beta; NADPH,
 3985 dihydronicotinamide-adenine dinucleotide phosphate; NF- κ B, nuclear factor kappa-light-
 3986 chain-enhancer of activated B cells; NMDA, N-methyl-D-aspartate receptor; TNF α , tumor
 3987 necrosis factor alpha.

3988 **Call-out box**

3989 This is an updated review of the biochemical and molecular mechanisms involved in the
 3990 development of newborn jaundice. It focuses on those aspects that differentiate newborn jaundice
 3991 from those in the more mature organism, particularly on how newborns are at an increased risk of
 3992 brain toxicity that can result in life-long, devastating neurological sequelae.

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 3995 Correspondence to: Thor WR Hansen, Langmyrgrenda 45 B, 0861 Oslo, Norway
 3996 t.w.r.hansen@medisin.uio.no
 3997

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Figure 1. Bilirubin metabolism in the body.

Figure 2. Bilirubin structure

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