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

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Hansen TWR, Wong RJ, Stevenson DK. Molecular Physiology and Pathophysiology of Bilirubin Handling by the Blood, Liver, Intestine, and Brain in the Newborn. *Physiol Rev* XX: XX, 2020. – Bilirubin is the end-product of heme catabolism formed during a process that involves oxidation-reduction reactions and conserves iron body stores. Unconjugated hyperbilirubinemia is common in newborn infants, but rare later in life. The basic physiology of bilirubin metabolism, such as production, transport, and excretion, has been well described. However, in the neonate numerous variables related to nutrition, ethnicity, and genetic variants at several metabolic steps may be superimposed on the normal physiologic hyperbilirubinemia that occurs in the first week of life and results in bilirubin levels that may be toxic to the brain. Bilirubin exists in several isomeric forms that differ in their polarities and is considered a physiologically important antioxidant. Here we review the chemistry of the bilirubin molecule and its metabolism in the body with a particular focus on the processes that impact the newborn infant, and how differences relative to older children and adults contribute to the risk of developing both acute and long-term, neurologic sequelae in the newborn infant. The final section deals with the interplay between the brain and bilirubin, and its entry, clearance, and accumulation. We conclude with a discussion of the current state of knowledge regarding the mechanism(s) of bilirubin neurotoxicity.

I. INTRODUCTION

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

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

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

130 **II. BILIRUBIN IN THE BODY (FIGURE 1)**

131 **A. Bilirubin Chemistry**

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

139 In healthy humans, bilirubin production was estimated at 3.5–4.0 mg/kg body weight
140 (BW)/day (69, 353), but in newborn infants it is twice as high – 8.5±2.3 mg/kg BW/day (444).
141 Increased bilirubin levels or '*hyperbilirubinemia*' in the body are measured as total serum or plasma
142 bilirubin (TSB) by spectrophotometry or co-oximetry, or in skin by transcutaneous bilirubinometry
143 (TcB). Once bilirubin concentrations exceed certain levels, it can be visually detected as '*jaundice*'. In
144 humans, jaundice predominantly develops during the first week of life (transitional period) in 60%–
145 80% of healthy newborn infants when hepatic bilirubin metabolism mechanisms are not fully mature

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

153 *1. Bilirubin structure*

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

165 *2. Bilirubin solubility*

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

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

178 *3. Bilirubin isomers*

179 Bilirubin (4*Z,*15*Z*) is the dominant isomer in the circulation, but other structural (constitutional)
180 isomers as well as stereoisomers (conformational or configurational) may be present and differ in
181 their aqueous solubility. The differences between these isomers may be of both physiological and
182 clinical interest (70). Bilirubin first appears in human fetal bile (at about 14 weeks' gestational age
183 [GA]) as the IXβ isomer (85). At 20 weeks' GA, IXα comprises 6%, IXβ 87%, IXγ 0.5%, and IXδ 6% of the
184 bilirubin found in fetal bile (722). By 28 weeks' GA, the relative amount of bilirubin-IXα has increased
185 to about 50% of total bilirubin (722), and by 30 weeks' GA, bilirubin-IXα replaces IXβ as the main
186 isomer (85). The IXβ, IXγ, and IXδ isomers are excreted directly into bile (82) because they cannot
187 form the intramolecular hydrogen bonds as does the IXα isomer, and thus behave as polar molecules
188 (81, 82, 416, 467). The high proportion of bilirubin-IXβ in early fetal bile does not necessarily prove
189 that it is the major isomer produced by the fetus (462). Unlike the polar IXβ isomer, the lipophilic IXα
190 isomer may be excreted from the fetus through the placental barrier (49, 468). However, this may
191 not be compatible with the increased proportion of bilirubin-IXα in fetal bile during pregnancy (85).
192 The physiological details and significance of these observations require further study.

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

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

201 *4. Heme degradation*

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

212 *5. Biliverdin and biliverdin reductase (BVR)*

213 Biliverdin is the first product of heme cleavage and is rapidly metabolized to bilirubin through the
214 action of biliverdin-IXα reductase (BVR). Activity of BVR is high both in liver and spleen tissues (402,
215 614), particularly in the reticulo-macrophages (697). Human BVR has been purified and sequenced
216 (439, 721) and appears to exist as four isoforms (720). Thus, isozymes I and II correspond to BVR-IXβ
217 (MW 21,000), while isozymes III and IV correspond to BVR-IXα (MW 34,000). All 4 isozymes can use
218 NADH or NADPH as electron donors, but based on Km values, NADPH is assumed to be the
219 physiological electron donor (720). Biliverdin-IXβ, -IXγ, and -IXγ are all substrates for isozymes I and II,
220 while isozymes III and IV prefer biliverdin-IXα. Cysteinyl residues are essential for the enzymatic
221 activity of BVR-IXα, but not for the β-reductase (721). Thus, the biliverdin-IXα and -IXβ reductases are
222 different in their enzymatic action and are probably also phylogenetically distinct. This is supported
223 by the finding that the BVR-IXα gene is localized to chromosome 19 (579), while the BVR-IXβ gene is

224 localized to chromosome 7 (537). Biliverdin-IXβ reductase appears to be identical to flavin reductase
225 (601).

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

231 BVR has been found in the cell membrane, cytoplasm, endoplasmic reticulum, mitochondria,
232 and nucleus (697). It can translocate between compartments through processes that are regulated
233 through nitrosylation, lipid modification, and phosphorylation (695, 696). It is induced by its
234 substrate biliverdin as well as in response to oxidative stress (437). Activation of BVR through
235 phosphorylation has been shown to be important for the reduction of biliverdin to bilirubin (530,
236 580), suggesting the possibility that bilirubin can inhibit its own production through a negative
237 feedback loop. Thus, bilirubin inhibits the phosphorylation of a number of proteins/peptides in vitro
238 (292, 296, 297). Indeed, given the central role of protein phosphorylation in the regulation of many
239 cellular processes, inhibition of protein phosphorylation has been proposed as a candidate for the
240 role in the '*basic mechanism of bilirubin toxicity'* (296). A study of hippocampal specimens from
241 patients with Alzheimer's disease and others with milder cognitive impairment recently found that
242 while levels of BVR-α were increased, the phosphorylation of its serine, threonine, and tyrosine
243 residues were reduced, accompanied by decreased reductase activity (55). Furthermore, in the same
244 brain specimens, BVR-α was shown to undergo post-translational oxidative and nitrosative
245 modifications in the hippocampus, but not in the cerebellum (56). Concomitantly, inducible nitric
246 oxide synthase (iNOS) was significantly upregulated in the hippocampus, but not in the cerebellum.
247 Whether these new insights into the antioxidative and metabolic changes described in
248 neurodegenerative disorders could have implications to our understanding of the pathophysiology of
249 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-IXα 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-κB (239) and NFAT and was
266 shown to enhance tolerance for cardiac allografts in mice (723). Biliverdin can trigger Ca^{2+}/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,

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

279 During the second half of the 20th century, evidence increasingly suggested that bilirubin
280 might be an antioxidant since HO is induced by oxidative stress (235). Bilirubin was shown to protect
281 against oxidation of fatty acids and vitamin A and to be an effective scavenger of oxygen-free radicals
282 (63, 631). Furthermore, bilirubin is an effective antioxidant (as potent as α-tocopherol) against lipid
283 peroxidation (630, 632). Individuals with Gilbert syndrome have low circulating lipid concentrations,
284 which could in part be due to their high TSB levels and hence high bilirubin antioxidant activity (128).

285 The normal range of TSB levels for healthy adult humans contributes an appreciable part of
286 blood antioxidant capacity, and even more so in the newborn period when TSB levels are much
287 higher (64). Intracellular and tissue concentrations of bilirubin are only 0.1%–1% of serum levels (280,
288 595), but even nanomolar concentrations of bilirubin may protect brain cells from the toxic effects of
289 a substantial molar increase of hydrogen peroxide (185, 186). On the other hand, oxidant stress may
290 also play a role in bilirubin neurotoxicity. In neuroblastoma cells (SH-SY5Y) exposed to 140 nM
291 bilirubin in vitro, several antioxidant response genes are activated, in part through the Nrf2 pathway
292 (546). It has been suggested that the high intracellular antioxidant activity of bilirubin is related to
293 BVR recycling of biliverdin to bilirubin (595), but the experimental paradigm has been criticized (432,
294 464).

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

302 future risk for cardiovascular disease risk (387). A Belgian study investigated the association between
303 TSB levels and cardiovascular and cancer mortality in 5460 men and 4843 women (640). In men with
304 *'high'* [> 10 µmol/L (0.6 mg/dL)] versus *'low'* [≤ 3.4 µmol/L (0.1 mg/dL)] TSB levels, the adjusted
305 relative risk (RR) for cancer mortality was 0.42 [95% CI: 0.26–0.68]. The associations between TSB
306 levels and cancer mortality in women had the same direction but were not statistically significant.
307 In newborn infants, there is also some evidence that elevations of TSB levels may be
308 associated with outcomes in diseases thought to involve oxidative stress. In infants with illnesses
309 associated with increased oxygen-free-radical production (e.g., respiratory distress, circulatory failure,
310 proven sepsis, aspiration syndromes, and asphyxia), the mean rise in TSB was significantly lower in
311 the sick infants than in the control group (65). This seemed compatible with a hypothesis that
312 bilirubin is consumed in vivo during oxidative stress. The same was found in preterm infants with
313 necrotizing enterocolitis, bronchopulmonary dysplasia, intraventricular hemorrhage, and retinopathy
314 of prematurity (ROP) (314). The results of studies, which have investigated if bilirubin might protect
315 against ROP, have not been consistent. Several studies failed to find a protective effect (94, 177, 207,
316 229, 321, 480). Although others appear to have confirmed a protective association (315, 727), none
317 of these studies were prospective, and in some cases the numbers of patients included were quite
318 small. In the largest study to date, in which ROP was only one of many outcomes addressed, there
319 was no difference in the occurrence of severe ROP between infants who received aggressive
320 phototherapy, and thus had lower TSB levels, and those who received so-called conservative
321 phototherapy, in whom TSB levels were significantly higher (489). In a recent case-control study of
322 the association between breast milk nutrition, hyperbilirubinemia, and ROP, peak TSB levels were
323 lower in ROP cases than in controls [mean 123 *versus* 135 µmol/L (7.2 versus 7.9 mg/dL); p = 0.045],
324 suggesting that bilirubin consumption was occurring in an oxygen-free radical disease (356). A
325 negative association was found between the highest TSB levels and risk for ROP, but this association
326 was not statistically significant [odds ratio (OR) = 0.82 per 17 µmol/L (1 mg/dL) change in bilirubin; p
327 = 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 neurotropins, bilirubin
343 promoted cell death by interfering with growth factor signaling (449). However, in the absence of
344 neurotropins, 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 µmol/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

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

381 Bilirubin toxicity has been shown in several different human and non-human cells, such as
382 hepatoma cells, fibroblasts, L-929 cells, RBCs, and others (102, 108, 113, 160, 161, 366, 373, 414, 459,
383 483, 510, 544, 552, 566, 643). In this section, we will address mainly those studies performed in cells
384 that were not derived from brain tissue. Findings in these studies were quite wide-ranging, and
385 included inhibition of growth, cell death, decreased intracellular ATP content, decreased synthesis of
386 protein and DNA, increased membrane permeability, reduced membrane potential, membrane
387 crenation in RBCs, membrane fusion events, altered membrane phospholipid content and
388 distribution, inhibition of oxidative phosphorylation, oxidative stress, and inhibition of alanine uptake
389 and bilirubin conjugation. Certain effects on membranes could be reversed by phototherapy, and
390 toxicity was not apparent on exposure to bilirubin photoisomers (373). At physiologic concentrations,
391 bilirubin, in the presence of bovine serum albumin (BSA), protected RBCs against oxidative stress, but
392 significant cytotoxicity was observed at very high bilirubin concentrations (≥ 510 µmol/L (30 mg/dL))
393 and a bilirubin:albumin molar ratio (BAMR) > 1.0 (483). Similarly, in adult human RBCs, exposure to
394 low bilirubin concentrations was protective against hypotonic hemolysis and crenation, while high
395 bilirubin concentrations induced hemolysis that was followed by membrane disruption (108).
396 Apparently, in older RBCs and at high bilirubin concentrations, bilirubin enters deeper into the cell
397 membrane bilayer, creating an unstable situation with aggregation of bilirubin acid leading to
398 hemolysis and cell death (108). The extent of RBC shape changes as well as membrane phospholipid
399 perturbations is increased with increased BAMR, suggesting a role for free or unbound bilirubin (UB),
400 i.e. bilirubin not bound to the primary binding site on albumin (112, 114). Acidosis increases bilirubin
401 toxicity (109), suggesting that cytotoxicity involves increased binding of bilirubin in its acid form to
402 RBCs under such conditions (99, 551).

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

405 450 µmol/L (20–25 mg/dL) reduced cellular respiration by approximately 25% (171). However, this
406 could be reversed/limited by oxidizing the bilirubin by adding methylene blue or cytochrome c. A
407 similar study compared oxygen consumption in homogenized brain tissue from adult vs 2-day-old
408 rats, and found a 22% reduction in adult brain vs a 67% reduction in newborn brain homogenates
409 (694). In rat liver mitochondria, bilirubin at a concentration of 300 µmol/L (17.5 mg/dL) uncoupled
410 oxidative phosphorylation and was accompanied by the release of respiratory cofactors such as
411 cytochrome c and diphosphopyridine nucleotide, leading to decreased respiratory activity (196). In
412 mitochondria from primary rat neurons and astrocytes exposed to bilirubin, membrane permeability
413 to cytochrome c was increased, probably in part due to involvement of the permeability pore. These
414 effects could be prevented by ursodeoxycholate (UDCA) and tauroursodeoxycholate (566).

415 *1. Bilirubin effects on enzyme activity*

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

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

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

435 *2. Toxicity of bilirubin conjugates and isomers*

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

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

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

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

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

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

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

476 Notably, in a recent well-controlled study where a SH-SY5Y human neuroblastoma cell line was
477 exposed to bilirubin-IXα(*Z,Z*), or lumirubin, or a mixture of bilirubin-IXα(*Z,E/E,Z*) isomers, all carefully
478 prepared and purified, the photoisomers did not affect cell viability, while the cells exposed to the
479 IXα(*Z,Z*) isomer exhibited significant loss of viability that increased with time (344). Thus, although
480 our understanding of the biology and toxicity of bilirubin photoisomers is still inadequate, this recent
481 study clearly supports the hypothesis of less toxicity of the polar bilirubin isomers (373, 464).
482 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).

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

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

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

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

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

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

546 The anti-inflammatory properties of bilirubin include modulation of inflammation through
547 regulation of HO activity (672, 709). Inhibition of the NF-κB activation pathway has been described
548 and appears also to involve biliverdin and BVR (697). However, in animal models, bilirubin did not
549 influence NF-κB or p38 MAP kinase, but rather it was suggested that bilirubin may be cytoprotective
550 by inhibiting iNOS expression and stimulating local PGE_2 production (686). When congenitally-
551 jaundiced rats were exposed to endotoxin, they had lower iNOS expression and were more resistant
552 to hypotension or death than non-jaundiced controls (395). On the other hand, the cytotoxicity of
553 bilirubin in mouse fibroblasts in vitro was increased by endotoxin and TNF-α (504). A full discussion
554 of the possible/putative interactions between bilirubin and the immune system would exceed the
555 limits of the present review, but a few selected effects are discussed below.

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

561 appears to protect tissues against inflammatory damage (500).

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

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

579 **III. THE PRODUCTION OF BILIRUBIN IN THE BODY**

580 **A. Heme Catabolism and Its Regulation**

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

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

597 HO is found in all cells (except in mature RBCs, which lack nuclei), with the highest activity in
598 the newborn liver, adult spleen, placenta, and erythropoietic tissue (306). There are three functional
599 isoforms: HO-1, HO-2, and HO-3. Of these, HO-1 is the inducible isoform, while HO-2 (435, 436) and
600 the putative HO-3 are constitutive (461). The exact role of HO-3 however is not well described,
601 although has been reported to be catalytically inactive (461). The enzymatic activity of HO can be
602 inhibited by a class of synthetic heme derivatives called metalloporphyrins, which have been
603 proposed and studied for many years as potential therapeutics for the treatment of newborn
604 hyperbilirubinemia (78, 224, 367, 368, 438, 451, 547, 628, 657, 659). Their potency and various
605 properties vary based on their central metal and ring side chains, yet no particular chemical
606 structural feature has been readily identified that facilitates the prediction of which
607 metalloporphyrins might be the most effective (589, 684, 713). Taken together, a favorable HO
608 chemotherapeutic should include a biocompatible central metal, sufficient potency, negligible
609 degradation, minimal photoreactivity, no effect on other enzymes (i.e., NOS, sGC), optimal duration
610 of action, minimally upregulation of HO-1, be orally absorbable, and selectively inhibit the inducible
611 HO-1 (221, 589, 684, 713). Almost all metalloporphyrins studied to date appear to be non-selective
612 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

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

652 **B. The Effect of Hemolysis**

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

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

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

678 *1. Disorders associated with increased bilirubin production*

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

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

693 **IV. BILIRUBIN BINDING AND TRANSPORT IN BLOOD**

694 Bilirubin is transported in plasma bound to albumin with a binding affinity at the primary binding site
695 of 10^7 to 10^8 per mole (118, 306). A secondary binding site also exists, but has a much lower affinity
696 (117, 118). Albumin contains three domains (313, 634), with each domain containing two
697 subdomains. The high-affinity binding site for bilirubin is believed to be localized to Site I on
698 Subdomain 2A, where a lysine residue appears to be involved in this binding (336, 383, 482, 541).
699 However, crystallographic analysis of the binding of bilirubin IXα (4*Z,*15*E*) to HSA showed that it is
700 bound to an L-shaped pocket in Subdomain IB, and indirect data seemed to show that the IXα
701 (4*Z,*15*Z*) could also bind to this site (742). Another photoisomer, lumirubin, also seems to bind at or
702 near Subdomain 1B, but with a much lower binding affinity to albumin than IXα (4*Z,*15*Z*) (344).
703 Although the binding sites for IXα (4*Z,*15*Z*) and lumirubin appear different, they are not independent
704 (344). Data obtained by circular dichroism have suggested that while the high-affinity bilirubin
705 binding site on HSA is located in Subdomain IIA, low-affinity binding sites may be found both in
706 Subdomains IB and IIIA (242). Because of albumin's high affinity for bilirubin, normal circulating levels
707 of UB are present in nanomolar concentrations, even in infants with significant hyperbilirubinemia
708 (134, 337). However, when the BBC is exceeded, UB concentrations can increase significantly (118,
709 337). Although BBC increases with postnatal age, in sick newborns and those having endogenous or
710 exogenous bilirubin displacers, it becomes reduced (67, 80, 100, 191). In addition to albumin,
711 bilirubin can also bind to other proteins (e.g. α-fetoprotein and ligandin) as well as to lipoproteins,
712 and to erythrocytes (83, 98, 99, 636).

713 An infant's risk for neurologic injury arising from hyperbilirubinemia is dependent upon the
714 absolute TSB level, BBC, and any underlying clinical condition(s) or exposure(s) that can alter the
715 binding affinity of albumin for bilirubin (5, 134, 394). By taking into account UB, TSB, BBC, and the
716 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

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

748 In addition, studies have shown that the immaturity of the neonatal hepatic bilirubin
749 conjugation system during the first 3 or 4 days of life is the primary contributor to the development
750 of unconjugated hyperbilirubinemia rather than just a reduction of bilirubin uptake, which may play
751 the primary role during the second week of life as bilirubin conjugation reaches normal adult levels
752 (620).

753 **B. Bilirubin Conjugation**

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

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

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

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

777 *1. Genetic variants in bilirubin conjugation*

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

788 *a. Crigler-Najjar syndrome type I*

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

795 *b. Crigler-Najjar syndrome type II*

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

802 *c. Gilbert syndrome*

803 Gilbert syndrome presents as a mild form of unconjugated hyperbilirubinemia. The most common
804 mutation is an insertion of two extra bases (TA) in the TATAA portion of the 5' promoter region of the
805 UGT1A1 gene, resulting in a sequence of A $(TA)_7$ TAA in place of the normal A $(TA)_6$ TAA (355, 689). It
806 has a high carrier rate in some ethnic groups. Polymorphisms with 5 to 8 TA repeat sequences have
807 been reported (73, 364).

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

814 2. *Genetic variants in transporter proteins*

815 Some infants with hypertrophic pyloric stenosis present with NNJ that may be related to a
816 Gilbert-type variant. Polymorphisms in the OATP-2 gene have resulted in an increased (3-fold) risk for
817 developing severe NNJ, and in those co-expressing a variant UGT1A1 gene mutation this risk further
818 increases 22-fold (323, 689). Polymorphisms in the ligandin gene may also contribute to higher TSB
819 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

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

849 VI. BILIRUBIN IN THE INTESTINES

850 A. Excretion into the Intestine

851 Bilirubin enters the intestines in bile through the common bile duct, primarily in its conjugated form,
852 only a very small fraction is unconjugated. Bile from term infants contains mainly bilirubin-IXα
853 monoconjugates (85). Experimental evidence from Gunn rats also suggests that bilirubin can be
854 transported from blood to the intestinal lumen directly through the bowel wall (382, 406). Higher
855 amounts of UCB are found in the feces of Gunn rats and Crigler-Najjar patients than in control
856 subjects (382). Such transmural transfer is likely to be the result of equilibration of UB in blood with
857 bilirubin in the intestinal lumen. Indeed, treatment of Gunn rats with Orlistat® has been shown to
858 reduce TSB levels, probably due to capture of bilirubin by intestinal fats following transmucosal
859 clearance of UB (268, 410). Transmucosal clearance of bilirubin is also supported by the observation
860 that oral calcium phosphate reduces TSB levels both in Gunn rats and in Crigler-Najjar patients (662,
861 663).

862 B. Role/Function of Bilirubin in Intestines

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

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

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

888 *1. Breast milk jaundice*

889 Bilirubin diglucuronide is not reabsorbed in significant quantities from the intestines, while the
890 monoglucuronide may be absorbed, albeit in rats only one fifth is absorbed and re-excreted (407,
891 408, 561). However, UCB may be reabsorbed and may contribute to hyperbilirubinemia in neonates
892 (122, 407, 408).

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

897 days of the study (543). Thus, reabsorption of bilirubin from the intestine may be an important
898 contributor to physiologic jaundice in the neonate, as previously also suggested by others (122).
899 Factor(s) in breast milk may contribute to or enhance intestinal reabsorption of bilirubin (18).
900 This phenomenon is often referred to as 'breast milk jaundice', and must be distinguished from
901 'breastfeeding jaundice', in which insufficient breast milk causes delays in the transit of intestinal
902 contents (228). Thus, breastfeeding is likely to be the most important contributor to the increased
903 incidence of significant NNJ, increased postnatal weight loss, and decreased urine and stool outputs
904 observed in infants who are exclusively breastfed compared with those receiving partial or complete
905 formula nutrition (142).
906 The mechanism(s) underlying breast milk jaundice remain under investigation, with several
907 proposed candidate explanations. While NNJ in the majority of infants is a transient phenomenon in
908 which TSB values peak at around 2–4 days of life (441), in about 1/5 to 1/3 of exclusively breastfed
909 infants, unconjugated hyperbilirubinemia, manifesting as visually apparent jaundice [typically
910 requiring TSB > 85 µmol/L (5 mg/dL) for the eye to perceive], may persist for more than 4 weeks (141,
911 440, 645) and in some for up to 3 months (228).
912 Several studies have shown that breast milk in itself may contribute to the 'breast milk
913 jaundice' phenomenon (246, 248, 249). In healthy, term, vaginally-delivered infants randomized to
914 receive either breast milk or one of two formulas (a casein hydrolysate product and a standard whey-
915 predominant formula) ad libitum, the jaundice index (measured by TcB) was lowest in the casein
916 hydrolysate group and highest in the breast milk group (249). In 4 groups of breastfed infants, the
917 control group received breast milk only, while the study groups, in addition to breast milk, received
918 six 5-mL aliquots daily of either L-aspartic acid, enzymatically hydrolyzed casein, or whey/casein
919 protein (ratio 60/40) (248) (248). All intervention groups had significantly lower TcB levels than the
920 control group (75.8%, 69.6%, and 69.2%, respectively, of the control mean [146 µmol/L (8.5 mg/dL)])
921 at peak bilirubin on day 4. L-aspartic acid and hydrolyzed casein were chosen for the intervention
922 because of their known ability to inhibit β-glucuronidase, with L-aspartic acid being the principal β-

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

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

949 A genetic contribution to breast milk jaundice has also been suggested (10, 11). Thus, the
950 allele frequency of the missense mutation G-to A at nucleotide 211 in the UGT1A1 gene, causing an
951 amino acid change of glycine to arginine at codon 71 (Gly71Arg) (which manifests clinically as Gilbert
952 syndrome in older children and adults), was significantly higher in Japanese newborns with
953 hyperbilirubinemia than in healthy controls (10). Neonates with this mutation had a gene dose-
954 dependent increase of TSB levels on days 2–4, the time around which NNJ typically peaks, and the
955 frequency of the same mutation was significantly higher in neonates who needed phototherapy than
956 in infants who did not need treatment (11). This was confirmed in 170 Japanese infants with breast
957 milk jaundice in whom more than half were homozygous for the Gly71Arg mutation (UGT1A1*6
958 allele) (452). In these infants, TSB levels were significantly higher than in infants with other
959 genotypes. However, breast milk jaundice was observed in almost 50% of infants who did not have
960 the UGT1A1*6 allele, and must be due to other causes (573). In 240 term breastfed Chinese infants
961 followed prospectively to investigate potential risk factors for significant hyperbilirubinemia, only
962 predischarge TSB levels on the third day and the variant UGT1A1 gene at nucleotide 211 (Gly71Arg)
963 were predictors of significant hyperbilirubinemia (140). In addition, in the same ethnic group male
964 breastfed infants with the Gly71Arg variant had a higher risk than females for prolonged
965 hyperbilirubinemia (141). Ethnic variations in gene allele frequency likely explain some of the variable
966 results from different groups. Thus, a Gilbert syndrome genotype (TA7/7), which did not involve the
967 Gly71Arg mutation seen in Japanese, Chinese and Korean infants, appeared to be related to
968 prolonged unconjugated hyperbilirubinemia in European breastfed term neonates (485).
969 In an early study of breast milk jaundice, pregnane-3 (α) 20 (β)-diol was identified in the
970 breast milk of 7 infants with prolonged jaundice and shown to competitively inhibit UGT1A1 activity
971 in vitro (42). However, later studies had not been able to confirm these findings (496, 549). This issue
972 was recently revisited with newer genetic techniques by analyzing the inhibitory effect of
973 pregnanediol on the transcriptional and enzyme activities of UGT1A1 (525). In the presence of 100-
974 μM pregnanediol, bilirubin glucuronidation by G71R-UGT1A1 was reduced to 51% of wild-type levels.

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

978 *2. Effects of perturbed intestinal transit*

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

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

990 In transit through the intestines, bilirubin may be metabolized through processes that may be related
991 both to intestinal contents and to bowel tissue per se. In an early analysis of bilirubin and its
992 conjugates in feces from newborn infants during the first 1–2 weeks of life, the bilirubin found was
993 mostly unconjugated and concentrations were higher than in blood plasma (122). It was speculated
994 that the low degree of bilirubin conjugation present after intestinal passage was due to the activity of
995 β-glucuronidase, which was quantitated and studied, concluding that the enzyme likely came from
996 the bowel itself and not from microbes (122). Epithelial cells in the villi of the jejunum have UGT
997 activity, which show a progressive increase in concentration from the crypt to the villar tip (147). The
998 contribution of bilirubin glucuronidation in the proximal small intestine to overall intestinal bilirubin
999 metabolism in normal physiology remains unknown, but 2 animal studies suggest that this function
1000 may be relevant (473, 637). When Gunn rats, who are congenitally deficient in UGT activity, were

1001 transplanted orthotopically with small bowel from normal Wistar rats, both TSB and UCB levels
1002 dropped rapidly and in a sustained fashion (473). The reduction occurred more slowly and was not
1003 well sustained in animals who received heterotopic transplants. Gunn-to-Gunn transplants did not
1004 evince reductions in bilirubin levels. These findings have been confirmed by others (637). Apparently,
1005 the transplanted bowel contains UGT activity and enhances the metabolism and clearance of
1006 bilirubin.

1007 Evidence supporting a role for intestinal UGT1A1 in bilirubin metabolism was strengthened in
1008 a study of UGT1A1 expression in humanized Ugt1$^{-/-}$ mice, which included the human UGT1 locus and
1009 encoded all 9-UGT1A genes (223). During the first 2 weeks of life TSB levels in the pups increased in
1010 both *Tg(UGT1A1*1)Ugt1*$^{-/-}$ and *Tg(UGT1A1*28)Ugt1*$^{-/-}$ mice and in some pups exceeded 255 µmol/L
1011 (15.0 mg/dL). However, during the 3rd week of life, TSB levels fell quickly to adult levels (223). This
1012 rapid reduction in TSB levels did not reflect increased hepatic UGT1A1 activity, but UGT1A1 gene
1013 expression and protein in the small intestine increased significantly during this period, concordant
1014 with changes in TSB. Thus, at least in humanized UGT1A1 mice, glucuronidation of bilirubin by
1015 UGT1A1 in the intestine may play a role in bilirubin clearance in the neonatal period (223). Further
1016 studies using the same genetic model showed that while formula feeding will induce UGT1A1 activity
1017 and reduce hyperbilirubinemia, breast milk will inhibit UGT1A1 and augment jaundice (222).
1018 Furthermore, deletion of the intestinal nuclear receptor co-repressor 1 (NCoR1) was recently shown
1019 to almost abolish hyperbilirubinemia in newborn hUGT1 mice, and control of NCoR1 function/de-
1020 repression was linked to the function of the inhibitor of NF-κB kinase subunit β (IKKβ) (144). NCoR1
1021 played a significant role in repressing intestinal developmental maturation. It is intriguing that the
1022 function and regulation of NCoR1 appears dependent on phosphorylation events (347, 490). Bilirubin
1023 has previously been shown to inhibit phosphorylation of several proteins/peptides (292, 296, 297,
1024 305) although its interaction with NCoR1 appears not to have been studied specifically. Thus, several
1025 processes in bowel homeostasis appear to be linked, contributing to control of the developmental
1026 repression of UGT1A1 and to the modulation of hyperbilirubinemia (144).

1027 Intestinal bilirubin metabolism is also influenced by the gut microbiota. However, as the gut
1028 of the newborn infant is sterile, the role of microbes in bilirubin metabolism is likely to be very
1029 limited in the first few days of life. In apparent confirmation of this, urobilinoids were found in the
1030 stools of 57% of newborn human infants on the 5th day of life, but not earlier (670). At this early time
1031 point urobilinoid production was low and unlikely to contribute much to bilirubin removal. UCB in
1032 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
1033 decreased 15-fold as the colonic microbiota was established and urobilinoid production approached
1034 adult levels (670). Thus, the newborn intestinal microbiota favors deconjugation of UCB over
1035 urobilinoid production, leading to increased enterohepatic circulation of UCB. Microbiologic analyses
1036 of the neonatal stools were done in some of the cases, and the activity of isolated bacteria as far as
1037 ability to metabolize bilirubin was studied in vitro. Two strains of *Clostridium perfringens* and *difficile*
1038 were both able to reduce bilirubin to urobilinoids (670), apparently different from the two strains of
1039 microbes previously shown to convert bilirubin to urobilinoids – *C. ramosum* (477) and *Bacteroides*
1040 *fragilis* (201).

1041 Gunn rats received either clindamycin/neomycin or co-trimoxazole orally for 4 days, resulting
1042 in the disappearance of fecal urobilinoids and a significant increase in TSB levels in the
1043 clindamycin/neomycin-treated rats, while co-trimoxazole had no effect on either level (673). When
1044 the intestines were re-colonized with *C. perfringens*, which has been shown to reduce bilirubin,
1045 urobilinoids reappeared in the feces and TSB decreased significantly, although less impressively than
1046 the increase which had followed sterilization of the gut. In comparison, re-colonization with *C.*
1047 *pasteurianum*, which does not reduce bilirubin, had negligible effects (673). Thus, metabolism of
1048 bilirubin by the intestinal microbiota significantly influences overall bilirubin metabolism.

1049 A strain of *C. perfringens* from neonatal stools was incubated under anaerobic conditions
1050 with both native and synthetic bile pigments, which were then separated by thin layer
1051 chromatography and analyzed by spectrophotometry, spectrofluorometry, and mass spectrometry
1052 (671). Several bilirubin reduction products were found, representing different urobilinogen species,

1053 but the reduction process apparently does not proceed to stercobilinogen. Bilirubin diglucuronide
1054 was not reduced to urobilinoid conjugates, suggesting that hydrolysis of glucuronides must take
1055 place before reduction of the double bond (671). For this reason, UCB may be reduced more rapidly.

1056 The above data suggest that during the first few days of life bilirubin is metabolized in the
1057 intestines primarily through deconjugation by enterocytes, thereby contributing to NNJ (212, 670).
1058 Further breakdown to colorless urobilinoids depends on the establishment of the intestinal microbial
1059 flora (671), and thus will come later (212, 646). These latter processes may take place mainly in the
1060 distal ileum and colon (646). Infants who are formula-fed appear to excrete urobilinoids earlier than
1061 infants who are breastfed, and it has been speculated that that this may be due to the effects of
1062 formula on the establishment of intestinal flora (730).

1063 **E. Fecal Excretion**

1064 The content of bilirubin in meconium represents about 5 to 10 times the daily production (249). Of
1065 this, approximately 50% is unconjugated and may be reabsorbed. In the meconium that is passed
1066 first, bilirubin-IXβ is the predominant bile pigment. It decreases during the first weeks of life, though
1067 more slowly in preterm infants. However, along with zinc coproporphyrin, it can be considered a
1068 biochemical marker of meconium (49, 50).

1069 Delayed meconium passage has been thought to cause NNJ, but studies involving facilitation
1070 of meconium passage have yielded conflicting results. Infants who received rectal stimulation due to
1071 rectal temperature measurement passed yellow stools significantly earlier than those assigned to
1072 axillary temperature control, and at about 3d of age TSB levels in the rectally-stimulated group were
1073 on average 17 µmol/L (1.0 mg/dL) lower than in controls ($p = 0.042$) (159). However, in another study
1074 TSB levels during the first 72 hrs of life in infants randomized to receive a glycerin suppository every 4
1075 hrs were no different from those in untreated controls, although meconium was evacuated earlier in
1076 the treatment group (698). Others have found similar results (51, 143).

1077 Manna is a plant extract with purgative effects that was used to treat NNJ in the 18th and 19th
1078 centuries in Europe (307), and it is still being used for this indication in Iran and South East Asian

1079 countries (205). A recent study compared infants on phototherapy with two other study groups
1080 (n=30 per group) as far as reduction of TSB levels and length of hospital stay – one intervention group
1081 received drops of manna as a laxative and the other group received glycerin suppositories in addition
1082 to phototherapy (205). TSB levels at 12, 24, and 48 hrs were significantly lower in both intervention
1083 groups than in controls, and length of hospital stay was shorter. However, a recent review of
1084 randomized controlled trials concluded that there was no evidence of effects of rectal purging on
1085 NNJ (621). Thus, the current evidence that suppositories, enemas, laxatives, or rectal stimulation are
1086 effective in decreasing TSB levels is equivocal. Therefore, until larger and appropriately designed and
1087 powered studies become available, routine clinical use cannot be considered evidence based.

1088 Loose stools during phototherapy for NNJ were described in several early studies (126, 348,
1089 427, 428). Several groups have tried to elucidate the mechanism(s) behind this phenomenon. Thus, a
1090 carmine test was performed on 3 groups of newborn infants: *1)* healthy full-term newborns; *2)*
1091 jaundiced full-term newborns who had received phototherapy for 24 hrs at the time of the test; and
1092 *3)* non-jaundiced full-term newborns who also received phototherapy with carmine testing after 24
1093 hrs (577). Significantly shortened intestinal transit time (from about 14 to 7 hrs) was observed *only* in
1094 the newborns that were jaundiced *and* were receiving phototherapy. The authors speculated that
1095 the photoisomers of bilirubin might be responsible for the diarrhea, and that reduced intestinal
1096 transit time might even be beneficial in reducing the enterohepatic circulation of bilirubin (577).
1097 Intestinal lactase activity in duodenal biopsies obtained with a hydraulic capsule was compared in 6
1098 jaundiced newborn infants with diarrhea on phototherapy and 8 healthy controls (52). The infants
1099 were also subjected to lactose tolerance tests. Both results from the lactose tolerance tests and the
1100 biopsies were judged to confirm a diagnosis of lactase deficiency. A normal biopsy from a 2-year-old
1101 girl was exposed to bilirubin in vitro, and no lactase activity was found. Stools normalized after
1102 introduction of a lactose-free breast milk substitute, but recurred when breast milk was re-instituted
1103 in infants who were still on phototherapy (52). However, other groups have not been able to confirm
1104 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

1131 century, cases were reported of infants who survived extreme jaundice, and who later exhibited
1132 neurologic sequelae with choreoathetosis (262). Subsequent case reports and case series contributed
1133 to the understanding that the clinical manifestations of KSD evolved over time (131). Hypertonicity,
1134 absent Moro reflex, opisthotonos, high-pitched cry, and poor feeding dominated the clinical picture
1135 during the first 2 to 3 months. Decreased muscle tone, hyperreflexia, and persistence of immature
1136 postural patterns was accompanied by significantly delayed motor development, which then became
1137 increasingly manifest during the first 2 years of life. Athetosis could be variably present, ranging from
1138 hardly noticeable to completely disabling, and with age of presentation ranging from the end of the
1139 second year of life until 8-9 years of age. Hearing loss of varying degree was noted in the majority. A
1140 fairly obligatory paresis of upward gaze appeared to distinguish KSD from other types of cerebral
1141 palsy. Few patients had developmental delay and/or intellectual deficits. KSD is described in similar
1142 terms even in the most recent discussions of this condition (678).

1143 The chronic sequelae of bilirubin brain toxicity may be multifaceted, and involve both the
1144 clinical findings, changes that occur over time, and severity (603). The recent proposal to use the
1145 overarching term KSD reflects the idea that the effects of bilirubin on the brain can take different
1146 forms as far as both the type and severity of the insult (397). While the term ABE describes
1147 neurological symptoms which are caused by ongoing bilirubin exposure, in the case of KSD bilirubin
1148 exposure had happened in the past, and it is the long-term sequelae of that time-limited exposure
1149 which causes the signs and symptoms currently observed in the patient (397). In addition to KSD,
1150 modifier terms for severity can be used to describe the condition as mild, moderate, and severe.
1151 Further, subtype modifiers such as auditory, motor, and classical can be used to describe auditory-
1152 predominant, motor-predominant, and combined auditory and motor sequelae. Finally, the term
1153 *'subtle kernicterus'* can be applied to infants with neurodevelopmental disabilities that, having
1154 excluded other diagnoses, may perhaps be understood on the background of a history of extreme
1155 NNJ and/or ABE (397).

1156 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 µmol/L (20 mg/dL) (597). In a cohort study 128 term Finnish children who had experienced NNJ with TSB >340 µmol/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

1183 NNJ subjects who had neurobehavioral issues, had significantly lower results on all cognitive function
1184 tests, and continued to have problems in adulthood, with lower academic achievement and lower
1185 ability to complete secondary and tertiary education (319). Persisting cognitive challenges affected
1186 both reading, writing, and mathematics. A registry-based follow-up study of 733826 Danish children
1187 showed that those with a recorded diagnostic code of NNJ (4.87% of the total cohort) had a 56%–88%
1188 increased risk of developing a psychological development disorder (434). The risk for autism
1189 spectrum disorders was significantly increased only for boys, and was present only for infants born in
1190 winter months, a finding the authors speculate could be due to more prolonged exposure to NNJ
1191 because of shorter daylight hours. However, prospective data from a 1-year study of phototherapy in
1192 Norwegian NICUs did not show evidence for increased need for phototherapy in the darkest months
1193 of the year (Mreihil K, personal communication). In contrast to these findings, in another Danish
1194 study, in which 463 army conscripts with a recorded diagnosis of NNJ were compared to 12,718 non-
1195 jaundiced conscripts, no association was found between level of hyperbilirubinemia and cognitive
1196 scores (190). These apparently contradictory findings suggest that studies based on registries may
1197 vulnerable to precision in diagnostic coding. Thus, while in the latter study the population with a
1198 diagnostic code for NNJ constituted 3.51% of the total, in the former study 4.87% had been so coded.
1199 Some studies suggest that even moderate degrees of NNJ may have long-term effects on
1200 behavior and neurodevelopmental outcomes (28, 429, 430, 604, 619). However, both patient
1201 selection and sensitivity *versus* specificity of the methods used for assessment must be carefully
1202 considered (429). The concept of minor neurological dysfunction (MND) has evolved, and it is
1203 possible that those with complex MND are of special interest as regards sequelae of moderate
1204 neonatal hyperbilirubinemia (266, 430). Thus, more studies will be necessary to delineate the details.
1205 Present-day bilirubin researchers seem to agree that bilirubin in brain cells causes a primary
1206 toxic event, it is not just a dye or marker. In clinical experience tolerance for bilirubin brain toxicity
1207 varies between infants (20, 187, 276, 431, 443, 476). Some term infants may tolerate extremely high
1208 TSB levels [> 500 to 600 µmol/L (>29.2 to 35.1 mg/dL)] without suffering any long-term toxicity, while

1209 others develop KSD with TSB just slightly above 350 µmol/L (20.5 mg/dL). In preterm infants, this has
1210 happened at even lower TSB levels (310, 322, 484, 693). While in some cases increased vulnerability
1211 may be due to illness or immaturity, variable vulnerability is also seen in infants judged to be healthy
1212 and mature. More research is needed to elucidate the mechanisms behind such differences, which
1213 could be due to altered or disrupted bilirubin metabolism in the brain or to bilirubin passage through
1214 the BBB, e.g. through both genetic, intrinsic, and extrinsic modulation of membrane transporters.

1215 Biological risk factors for ABE and KSD involve both those that impact production,
1216 transport/binding, and/or excretion of bilirubin, those that involve transfer across the BBB as well as
1217 BBB permeability, and probably also characteristics/vulnerability of brain cells as well as bilirubin
1218 processing by those cells. Further details of ABE and KSD biology and risk will be discussed below. On
1219 a larger scale, the risk of an infant with severe hyperbilirubinemia going on to develop ABE and KSD
1220 depends on the availability of healthcare services, delays in seeking care, and structural as well as
1221 organizational roadblocks within health care systems (15, 179, 521, 555, 656). A further discussion of
1222 these challenges is beyond the scope of this paper.

1223 Below we will discuss the merits versus weaknesses of theories regarding the '*basic*
1224 *mechanism(s) of bilirubin neurotoxicity*', as well as research on bilirubin effects in, and interactions
1225 with, the brain. These studies have used a wide range of methods, from 'pure' in vitro, to cell
1226 cultures, ex vivo organotypic cultures, and animal models. This topic has recently been extensively
1227 reviewed, and will only be briefly outlined here (91, 460). Studies in cell cultures first tended to use
1228 immortalized cell lines, such as e.g. mouse or human neuroblastoma cells, human astrocytoma cells,
1229 and differentiated human NT2-N neurons (35, 151, 273-275, 344, 504, 505, 546, 570). Later, cell
1230 cultures obtained by primary culture techniques have increasingly been used, including both neurons
1231 and several types of glia (107). These cells have yielded many interesting data on differential
1232 sensitivity to bilirubin toxicity, as well as insight into possible pathways and mechanisms. Common
1233 challenges to all in vitro studies is bilirubin solubility and stability in solution, leading to discussions
1234 about 'physiologically relevant' bilirubin concentrations for such studies (464, 523). This has been

1235 particularly challenging when studying the toxicity of bilirubin photoisomers (304). Also, cultures
1236 involving a single cell type will not be able to study the interplay between e.g. neurons and glia which
1237 appears to modulate bilirubin cell toxicity (107, 202, 203).

1238 Organotypic cultures consist of slices of brain regions of interest, which for a time retain the
1239 complexity of the brain tissue of origin. Bilirubin toxicity has primarily been studied in hippocampal
1240 slices, both as far as synaptic function and plasticity as well as the interplay between glia and neurons
1241 (139, 164, 297, 613, 687). Different experimental setups and conditions have led to apparently
1242 contradictory results, potentially opening door to new discoveries through refinement of the
1243 paradigms (164, 166).

1244 The classical in vivo model of kernicterus is the Gunn rat, a spontaneous mutant discovered
1245 in the 1930s (259). These rats have a complete deficiency of UGT1A1, leading to hyperbilirubinemia
1246 which peaks around 2 weeks of age, and with brain damage in the form of cerebellar hypoplasia (91,
1247 460). Deaths from kernicterus and TSB levels corresponded reasonably well (351). Later on,
1248 crossbreeding to other strains resulted in two strains (ACI/N-j and RHA/N-j) with very different
1249 mortality rates, which thus far is unexplained (303). The Gunn rat has been extensively used as a
1250 model of bilirubin neurotoxicity, e.g. by ABRs which have yielded important information on the
1251 importance of UB (22, 24, 26, 27, 31, 260, 294, 390, 603, 714).

1252 New methods for genome manipulation have been used to create constitutive and
1253 conditional knock-out, knock-in, and transgenic strains. These models have been reviewed quite
1254 recently (91). In brief, it has been possible to model the human Crigler-Najjar syndrome, and to study
1255 interactions between UGT1A and the pregnane X and constitutive androstane receptors by mating
1256 with null strains of the latter (see Bortolussi and Muro 2019 for a review). UGT1A null mouse
1257 mutants exhibit more severe neurological damage than Gunn rats and eventually die, though the
1258 reasons for the observed differences in mortality between strains is as yet unexplained, as remains
1259 true of the Gunn ACI/N-j and RHA/N-j mutants mentioned above. Thus, further studies of these
1260 models appear to be of great interest.

1261 **B. The Role of the BBB**

1262 *1. Permeability and its modulation*

1263 For bilirubin brain toxicity to occur, bilirubin must gain entry into brain. Bilirubin in the blood vs the
1264 brain is in an unstable equilibrium, influenced by several factors (**FIGURE 4**). Chemically speaking
1265 bilirubin is amphipathic, but it behaves in many respects as lipophilic, binding to and crossing
1266 phospholipid membranes (120, 293, 331). Although this characteristic is thought to enable UB to
1267 cross the intact BBB and gain access to the brain (281), it does so to a lesser extent than one would
1268 expect of a typical lipophilic molecule (332). Thus, the blood-brain gradient is high – in study animals
1269 with an intact BBB the brain bilirubin concentration is only 1% to 2% of serum concentrations (283).
1270 The characteristics of molecules that can cross the BBB in significant amounts include: *1)* molecular
1271 weight <400 Daltons; *2)* lipid solubility; and *3)* not being a substrate for an efflux transporter (534).
1272 Bilirubin does not really satisfy any of these criteria: its molecular weight is 585, lipid solubility varies
1273 with the isomeric form, and it is a substrate for phosphoglycoprotein (P-gp) (690). This may explain
1274 why bilirubin entry into brain is limited.

1275 Albumin-bound bilirubin probably does not cross an intact BBB (118, 300) (see Section IV).
1276 Nanomolar concentrations of UB are always present in plasma during significant neonatal
1277 ~~hyperbilirubinemia~~. The "free bilirubin theory" posits that UB is the moiety which enters the brain
1278 and causes injury (702). The first clinical observations that supported this theory came from the use
1279 of drugs that turned out to compete with bilirubin for its albumin binding, thus increasing the UB
1280 concentration (37). Subsequently, many studies have provided both clinical and experimental
1281 evidence for how UB impacts the risk for kernicterus/KSD (7-9, 31, 137, 514, 515, 518, 733).
1282 According to the laws of equilibrium, UB in high serum concentrations will force more bilirubin into
1283 tissues, such as the brain (**FIGURE 4**). Increased concentrations of UB occur in the presence of altered
1284 albumin binding characteristics or exo-/endogenous binding competitors and increase bilirubin entry
1285 into brain (80, 100, 118, 135, 707). Many drugs act as binding competitors with bilirubin relative to

1286 serum albumin (37, 455). Increased entry of bilirubin into brain following administration of bilirubin-
1287 displacing substances has also been documented in several animal studies (118, 281, 283, 293, 702).
1288 The BBB in newborn animals appears to behave differently relative to bilirubin passage than
1289 it does in more mature subjects (399, 400, 409). It is possible that expression of P-gp, which appears
1290 to evolve with increasing maturity, may contribute to the apparent increase of bilirubin passage
1291 through the BBB in immature subjects (649). Albumin permeability may also be reduced with age,
1292 though not all published data are in agreement on this (263, 399, 519).
1293 The function of biologic membranes may be impacted by bilirubin, leading to questions of
1294 whether BBB function could also be perturbed by bilirubin (35, 160, 459). The permeability of the
1295 BBB both to a dye and to bilirubin itself was increased by pre-exposure to bilirubin (256, 568). Glial
1296 cells, particularly astrocytes, play a fundamental role in the maintenance of the BBB (111). Therefore,
1297 toxicity of bilirubin to glial cells could also contribute to effects on BBB function (33, 635). UCB
1298 disturbs homeostasis in endothelial cells by inducing increased eNOS expression, which is followed by
1299 accumulation of nitrites, suggesting nitrosative stress (531). In the same experiments, brief exposure
1300 of brain endothelial cells to UCB first inhibited the release of IL-6, IL-8, and vascular endothelial
1301 growth factor (VEGF), but this was later followed by an increased release, first of IL-6, then of IL-8
1302 and later on of VEGF. Thus, UCB may cause injury to endothelial cells, affecting important mediators
1303 that may increase inflammation in the brain and perhaps affect BBB integrity (111). The interaction
1304 between glial cells and bilirubin is discussed further below.
1305 Several conditions that occur quite frequently in seriously ill newborn infant may affect BBB
1306 permeability (**FIGURE 4**). The hypercarbia that accompanies respiratory failure, the hyperosmolality
1307 seen in severe hyperglycemia, IV hyperalimentation, or renal failure, and the damage caused by
1308 asphyxia have all been shown to increase bilirubin entry into brain (129, 130, 281, 283, 300, 330, 704).
1309 This seems clearly relevant for management of jaundice in NICU infants. Hypercarbia increases brain
1310 blood flow, and most of the bilirubin enters brain as UB; while in hyperosmolality albumin also enters

1311 the brain in significant amounts along with bilirubin (130, 300). Furthermore, in hypercarbia, the
1312 acute entry of bilirubin into brain is increased compared to control conditions (129, 283).
1313 In vitro studies appear to show that albumin in equimolar concentrations blocks the toxic
1314 effects of bilirubin (97, 102, 160, 414). When the BBB is opened, bilirubin neurotoxicity increases
1315 (301, 330, 704). In the presence of an open BBB, both albumin-bound and UB enter the brain, but in
1316 the immature subject entry of albumin-bound bilirubin may occur even with an intact BBB (520).
1317 Total brain bilirubin content appears to be the best predictor of toxicity, while increased albumin
1318 binding of bilirubin may be protective (704).

1319 *2. Transport mechanisms*

1320 *a. The role of 'flippases'*

1321 P-gp is an ATP-binding membrane transporter. Expression of such transporters has been observed
1322 both in normal and in diseased tissues and limit the entry of xenobiotics into cells (238). In a
1323 preliminary report UCB was reported to be a weak substrate for multi-drug resistance protein (MDR1)
1324 (245). Bilirubin has many properties in common with P-gp substrates, including hydrophobicity when
1325 unconjugated as well as certain structural elements that facilitate interaction with P-gp (596, 691).
1326 These elements involve electron donor groups with a spatial separation of about 2.5±0.3 Å (691). In
1327 vitro verapamil, a P-gp inhibitor, inhibited [^{3}H]bilirubin transport through human Caco-2 monolayer
1328 cells, providing further support that bilirubin is a substrate for P-gp (433).
1329 However, the interaction between bilirubin and P-gp may have more than one perspective – as
1330 bilirubin may inhibit P-gp function (345, 392, 690). Therefore, it was hypothesized that changes in P-
1331 gp expression/function could modulate bilirubin entry into brain. To test this hypothesis the entry of
1332 bilirubin into brain was first compared between P-gp knockout and wild-type mice, showing that
1333 brain bilirubin content was almost two-fold higher in knock-out mice (690). Then, drugs known to
1334 inhibit P-gp function were shown to significantly increase bilirubin entry into rat brain (219, 220, 277).
1335 Studies in postmortem brain tissue sections from human infants born at 22 to 42 weeks' GA showed
1336 P-gp to be expressed in a regionally and cell specific pattern which was also dependent on

1337 maturation, findings clearly relevant to the putative role of P-gp in modulating bilirubin uptake into
1338 and/or extrusion from brain (167).

1339 *b. Other BBB molecules with relevance for brain bilirubin uptake and excretion*

1340 MRP1 (encoded by the ABCC1 gene) may also limit bilirubin access to cells (559). Cytotoxicity and
1341 intracellular accumulation of [^{3}H]bilirubin were higher in fibroblasts from MRP1 knockout mice than
1342 in than in cells from wild-type controls (132). Bilirubin appeared to upregulate MRP1 in cultured
1343 astrocytes, thus reducing sensitivity to bilirubin toxicity (237). Given the important role of astrocytes
1344 in BBB function and the apparent ability of bilirubin exposure to increase both BBB and blood-
1345 cerebrospinal fluid (CSF) barrier permeability to bilirubin itself, the interplay between bilirubin,
1346 barrier elements, and transport proteins appears complex and clearly in need of further study (111,
1347 568).

1348 **C. Brain Blood Flow**

1349 Hypercarbia leads to increased brain blood flow, which in experimental animals is followed by
1350 increased bilirubin entry into brain (129, 283, 300). There will always be a significant blood-to-brain
1351 bilirubin concentration gradient, thus when brain blood flow increases, each circulating bilirubin
1352 molecule will pass the BBB more often, consequently the opportunities to equilibrate with bilirubin in
1353 the brain will increase.

1354 **D. Excretion**

1355 *1. The CSF "sink"*

1356 Yellow coloring of the CSF was noticed early on during autopsies of neonates with kernicterus (587,
1357 588). When CSF bilirubin was analyzed in infants with 'physiologic' NNJ as well as due to
1358 isoimmunization, CSF bilirubin was predominantly unconjugated and bilirubin values were mostly
1359 1%–3% of TSB, corresponding remarkably well to brain tissue data from later animal studies (281,
1360 283, 623). A subsequent study showed that CSF bilirubin correlated with total protein levels in CSF.
1361 The relationship between CSF bilirubin concentrations and TSB was not linear, suggesting that there
1362 are individual variations in BBB permeability during the first days of life (624).

1363 A scatterplot of the relationship between TSB and CSF bilirubin in 100 newborn infants, of
1364 whom 34 were normal term infants, 49 were normal preterm, and 17 had erythroblastosis due to Rh-
1365 or AB0-incompatibility, showed a correlation coefficient of 0.58 (501). While CSF bilirubin in the
1366 preterm infants was on average 4.4% of the TSB, with wide range of variation (from 1.7%–15.6%), in
1367 term infants CSF bilirubin was 3.0% of the TSB (ranging from 0.95%–11.9%). In older jaundiced
1368 control subjects, CSF bilirubin was 0.65% of TSB values. The higher CSF bilirubin:TSB ratio in the
1369 premature infants is likely to reflect increased permeability of the BBB for bilirubin, as also shown in
1370 animal studies (399, 400, 409).

1371 Rabbit choroid plexus in vitro accumulates ^{3}H-bilirubin, suggesting that the choroid plexus
1372 may be involved in transporting bilirubin from the CSF to blood (340). An active mechanism for
1373 bilirubin transport out of the CSF also seemed compatible with studies of 45 newborn infants (475).
1374 CSF bilirubin correlated linearly with UB concentration in serum, but CSF bilirubin values were
1375 approximately 150 times greater than the concentrations of UB in serum, indicating a pronounced
1376 CSF-to-serum concentration gradient (474).

1377 P-gp/ABCB1 and MRP1/ABCC are both expressed in the choroid plexus epithelium in the
1378 developing human CNS (167). It was suggested that the complementary patterns of P-gp/ABCB1 and
1379 BCRP/ABCG2 at the BBB with MRP1/ABCC1 at the blood-CSF barrier may limit CNS uptake and
1380 retention of drugs and toxins in neonates (167). However, P-gp/ABCB1 seems to be localized to the
1381 apical membrane of the choroid plexus and from that position might be expected to mediate
1382 transport of substrates from blood into the CSF. The implication of such P-gp/ABCB1 expression for
1383 bilirubin brain toxicity appears not to have been studied (405, 538).

1384 Organic anion transporters, such as MRPs, have also been hypothesized to limit bilirubin
1385 entry into the CSF (523). In choroid plexus epithelial cells from Gunn rat pups exposed to bilirubin in
1386 vitro, MRP1 protein was down-regulated, a phenomenon also observed in choroid plexa isolated
1387 from homozygous (*jj*) Gunn rats when compared to their non-jaundiced (*Jj*) littermates (230). The
1388 authors speculated that down-regulation of Mrp1 protein at the blood-CSF barrier, which probably

1389 results from a direct effect of bilirubin on epithelial cells, can perturb the MRP1-mediated
1390 neuroprotective functions of the blood-CSF barrier and possibly accentuate bilirubin neurotoxicity
1391 (230).

1392 *2. The BBB*

1393 Bilirubin is not static once it has entered the brain. The half-life has been estimated with different
1394 techniques yielding different results. This first study performed with unilateral hyperosmolar opening
1395 of the BBB induced by arabinose infusion into one carotid artery yielded a half-life of bilirubin in
1396 brain of 1.7 hrs and in blood 1.6 hrs (409). This technique opens the BBB only ipsilaterally and was
1397 thought to be reversible within 1 hr (409). However, later studies reduced the estimate of the time to
1398 reversibility of permeability changes to 10 min (550). Serum osmolality during or after arabinose
1399 infusion was not reported. Later work estimated a much lower bilirubin half-life in brain - 16–18 min
1400 during baseline conditions and approximately 38 min during general hyperosmolality induced by urea
1401 injection (283, 293). Serum osmolality was increased from the normal 290 to 395 mosm/L, which was
1402 sustained until sacrifice (283). As both UB and albumin-bound bilirubin enter the brain when the BBB
1403 is opened, with rapid closure the much larger bilirubin-albumin complex will probably be 'trapped'
1404 on the brain side of the BBB, while clearance of UB will be more rapid. This may explain the
1405 differences between the half-life data from earlier vs later studies (283, 293, 409). Time-limited
1406 unilateral opening of the BBB provides important experimental data, but sustained global opening is
1407 more likely to be representative of conditions in vivo. However, in both models, bilirubin retention in
1408 the brain is clearly more prolonged than during control conditions. It is not clear whether prolonged
1409 exposure increases toxicity, but limited experimental and clinical data do suggest that, in addition to
1410 the level of TSB, the duration of hyperbilirubinemia may impact the risk for neurologic sequelae (301,
1411 578). In hypercarbia both acute brain entry of bilirubin and clearance are rapid (129, 283).
1412 Hypothetically, the more rapid clearance might be explained by increased opportunities for
1413 equilibration across the BBB pursuant to increased brain blood flow.

1414 **E. Bilirubin Metabolism in the Brain**

1415 Experimental studies showed that clearance of bilirubin from the brain could be more rapid than
1416 from blood, suggesting that additional mechanisms might contribute to bilirubin clearance from the
1417 brain (289, 293, 409). An enzyme on the inner mitochondrial membrane of brain cells as well as in
1418 other tissues capable of oxidizing bilirubin had been described (121) and verified by others (17, 280,
1419 287, 288, 290, 295, 302, 303). The activity has enzyme characteristics, such as pH and temperature
1420 maxima for activity, and denaturability (288). Cytochrome P-450 2A5 may contribute to hepatic
1421 oxidation of bilirubin, but it is not known whether brain and hepatic metabolism are the same (2).
1422 The activity of the brain enzyme is lower in the immature organism and in neurons *versus* glia (290),
1423 observations that seem compatible with the clinical impression that infants are more vulnerable to
1424 bilirubin toxicity than older individuals, and the same applies to neurons *versus* glia. This enzyme
1425 possesses some of the characteristics of the cytochrome oxidases, but has not with certainty been
1426 identified as such (284, 308).

1427 Oxidation of bilirubin will reduce the concentration of toxic bilirubin, but it is not known with
1428 certainty whether the oxidation products are more or less toxic than bilirubin itself. Genetic
1429 variability exists between different strains of Gunn rats, however the Gunn rat strain with higher
1430 specific bilirubin-oxidizing capacity was also the strain that exhibited significantly higher early
1431 spontaneous mortality (302). However, there could be reasons for these differences in mortality
1432 rates that have no connection with bilirubin.

1433 Molecules with structures similar to bilirubin oxidation products are found in the CSF of
1434 patients with cerebral vasospasm following subarachnoid hemorrhage, and bilirubin oxidation
1435 products have similar effects on blood vessels both in vitro and in vivo (154). In an in vitro model of
1436 subarachnoid hemorrhage the production of bilirubin oxidation products was significantly enhanced
1437 when cytochrome oxidase was stimulated, but was attenuated by cyanide (425). In support of
1438 previous data from others the authors suggested that mitochondrial cytochrome oxidase could be a
1439 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

1466 mitochondria from both glia and neurons. However, in one study they compared the mixed
1467 mitochondrial membranes with membranes from a pure neuronal source (synaptosomes) and found
1468 that the bilirubin oxidation rate per milligram protein was significantly lower using the pure neuronal
1469 membranes, leading the authors to speculate that this might explain the greater vulnerability of
1470 neurons compared with glial cells to bilirubin toxicity (290). Another clue that different enzymes may
1471 be involved is that while one group needed NADPH to start their bilirubin oxidation reactions (226),
1472 the other group showed that in their assay, neither NAD, NADP, NADH, NADPH, GSH, nor GSSH had
1473 any effect on oxidation velocity (291). The reaction was cytochrome c dependent, and it could also
1474 be inhibited by clotrimazole and ketoconazole, both known to inhibit the cytochrome P450 oxidase
1475 group of enzymes (291). While data from the pure glial source also pointed to the cytochrome P450
1476 oxidases, specifically Cyps 1A1 and 1A2 (226, 232), known inhibitors of these enzymes (omeprazole
1477 and fluvoxamine) were unable to inhibit the activity measured in mixed brain mitochondrial
1478 membranes (308). Recently CYP1A2 mRNA levels were found to increase with maturation in rat brain
1479 and liver microsomes, but microsomal fractions did not affect bilirubin or its metabolites, suggesting
1480 that physiologically this CYP may not have a role in bilirubin oxidation (411). Also, recent attempts to
1481 purify the mixed mitochondrial enzyme activity by salt fractionation showed peak activity in fractions
1482 around 205 mM NaCl (308). Parsing of proteomics data from these fractions yielded several
1483 candidate proteins in the cytochrome oxidase group. However, testing with antibodies currently
1484 available against these enzymes has as yet not resulted in a firm conclusion (308).

1485 We do not as yet know what, if anything, metabolism of bilirubin in brain might mean for
1486 clinical practice, thus more studies are needed. It should be noted, however, that the term *'bilirubin*
1487 *oxidase'* does not identify this activity (288). Commercial *'bilirubin oxidase'* behaves differently from
1488 the mitochondrial enzyme discussed above (17, 121, 284, 287, 288, 291, 302, 303). There appears to
1489 be no evidence that *'bilirubin oxidase'* catalyzes bilirubin oxidation in brain (291).

1490 *F. Regional and subcellular localization*

1491 The question of how bilirubin localizes to the basal ganglia is likely to be important for a full

1492 understanding of bilirubin brain toxicity, as the clinical syndrome of KSD in survivors of ABE in the
1493 newborn period involves movement disorders associated with basal ganglia dysfunction (262, 397).
1494 Extraction of bilirubin from the brains of four infants who died with severe jaundice found
1495 concentrations of about 35 nmol/g in the basal ganglia and 8 nmol/g in the rest of the brains. Much
1496 higher bilirubin concentrations were thought to be present in the most intensely stained regions of
1497 the nuclei, but could not be quantitated (153).

1498 Many have tried to recreate this staining pattern in animal models, but success has been
1499 limited. Though regional differences in brain bilirubin concentration were found in piglets following a
1500 [^{3}H]bilirubin infusion during conditions of hypercarbia or hyperosmolality (129, 130), such differences
1501 could have been due both to variations in bilirubin entry into or disappearance from the brain, or to
1502 redistribution, or to binding to tissue or cell elements. Significant differences were found in brain
1503 regional bilirubin concentrations in 15–19-day-old Gunn rat pups pretreated with sulfadimethoxine
1504 (133). The concentration of bilirubin in the cerebellum was 32 nmol/L; while the brainstem and
1505 cortex contained 18 and 8 nmol/L respectively. But while higher brain bilirubin concentrations were
1506 found in male pups, suggesting that sex could influence brain bilirubin uptake or clearance, the
1507 regional differences did not correspond to a typical kernicteric staining pattern, nor is it clear how
1508 they arose. Studies in Sprague-Dawley rats who received infusions of bilirubin-containing solutions
1509 stabilized with albumin and accompanied by manipulations of bilirubin binding, BBB opening, and
1510 brain blood flow, have not succeeded in mimicking the basal ganglia accumulation (281, 283, 293,
1511 300). Furthermore, no inter-regional differences between bilirubin entry and clearance from brain
1512 were shown. Regional differences in brain bilirubin metabolism did not explain the kernicteric
1513 staining pattern (288).

1514 In order for the classical theory of bilirubin inhibition of mitochondrial respiration to be
1515 supported, bilirubin concentrations in the mitochondria during relevant levels of hyperbilirubinemia
1516 must be high enough to cause toxicity (196). Only one study appears to have addressed this question.
1517 [^{3}H]bilirubin was given as an IV bolus to rats and the brains of animals sacrificed after 10 and 30 min

1518 were processed by subcellular fractionation (280). Absolute values for bilirubin could not be
1519 calculated, but bilirubin content related to protein concentration was much lower in mitochondria
1520 than in the cytoplasmic and membrane fractions.

1521 **G. Mechanism(s) of Bilirubin Neurotoxicity**

1522 *1. Transient versus permanent effects*

1523 A discussion of the mechanisms of bilirubin-induced brain injury must include events associated with
1524 cell death. However, bilirubin also appears to have transient effects on the brain. Jaundiced neonates
1525 may be lethargic/drowsy, have reduced muscle tone, and difficulties with feeding (20, 198, 678).
1526 Auditory brainstem response (ABR) studies, both in humans and in animal models, have yielded more
1527 objective evidence of transiently altered neuronal function, although some have also found more
1528 permanent changes (22, 24, 243, 260, 294, 390, 603, 714). Both acute and chronic auditory
1529 neuropathy has been shown to be associated with levels of UB, but not TSB (22, 24, 26, 27, 31). An
1530 increased incidence of apneas in jaundiced premature infants compared to less jaundiced controls
1531 gradually disappears from the second week of life, and that is also associated with UB levels as well
1532 as appearing to follow changes in the ABR (24, 30). Such changes in neuronal function and behavior
1533 likely reflect bilirubin effects on the brain, and although these apparently transitory effects of
1534 bilirubin on the brain may be referred to as early phase ABE, clinicians probably would not use the
1535 term kernicterus/KSD about such reversible toxicity, nor is there a diagnostic code for this condition
1536 in the diagnostic coding systems (20, 62, 656, 715).

1537 The signs of early phase ABE versus KSD could represent extreme ends of a continuum of
1538 toxicity. But one must also consider the possibility that separate and distinct mechanisms are
1539 involved in cell death versus milder, and perhaps transitory, perturbation of neuronal signaling (285).
1540 The term '*subtle kernicterus spectrum disorder'* was part of the recent proposal for a revision of
1541 terminology and could possibly be applicable to the behavioral and neurodevelopmental effects
1542 mentioned above (397). Herein, we discuss bilirubin effects on brain in a wide sense, and also include
1543 processes that may not lead to cell death and lasting damage.

1544 *2. Inhibition of cell respiration*

1545 The first in vitro studies of bilirubin toxicity showed that bilirubin inhibited respiration in a rat brain
1546 homogenate (171). In rat liver mitochondria bilirubin [300 µmol/L (17.5 mg/dL)] partially inhibited
1547 respiration and almost completely inhibited phosphorylation (196). Uncoupling of oxidative
1548 phosphorylation, causing energy failure, was for many years the main theory on the 'basic
1549 mechanism' of bilirubin toxicity (171, 196, 460, 523). Evidence for bilirubin toxicity to mitochondria
1550 has been found in several in vitro studies. Bilirubin perturbed the mitochondrial membrane leading
1551 to increased permeability, decreased potential, release of cytochrome c, and triggering of apoptosis
1552 (563-566). Mitochondrial toxicity was also suggested from in vivo observations. Ultrastructural
1553 changes were found in the brain mitochondria of Gunn rats (346, 590, 591). Significantly lowered
1554 phosphocreatine and ATP were found in the brains of rats with hyperbilirubinemia induced by IV
1555 infusion; although opening of the BBB with hyperosmolality was required to elicit such changes (705).
1556 Magnetic resonance spectroscopy in five infants with severe NNJ showed that one of the infants had
1557 an abnormally high lactate:N-acetyl aspartate ratio (252). Only this infant had abnormalities in the
1558 basal ganglia on MRI and, along with one other infant, at follow-up was neurologically abnormal. The
1559 increased ratio of lactate:N-acetyl aspartate could have been due to changes in mitochondrial
1560 function. A cartoon which shows some of the different effects and interactions of bilirubin with cells
1561 is shown in **Figure 5**.

1562 However, other data are not compatible with mitochondria as the principal targets of in vivo
1563 bilirubin toxicity. Electron microscopy of Gunn rat brains appeared to show that changes in the
1564 mitochondria was most likely caused by prior changes in the cytoplasm, however this interpretation
1565 has been critiqued (460, 590). In cultured L-929 cells bilirubin effects, when compared to compounds
1566 known to uncouple oxidative phosphorylation and inhibit reduced NAD, were more likely on the cell
1567 membrane than on the respiratory chain (160). Newborn pigs with brain bilirubin levels comparable
1568 to those found in infants with kernicterus did not show changes in cerebral oxygen, glucose, and
1569 lactate metabolism, as would have been expected if mitochondrial function were disturbed (96).

1570 Bilirubin perturbed neurotransmitter metabolism in permeabilized synaptosomes in vitro with high
1571 exogenous ATP present, thus loss of endogenous ATP is unlikely to have caused the observed effects
1572 (295). Of six infants with extreme jaundice, of whom four were neurologically abnormal at 1 year of
1573 age, none had demonstrated high brain lactate levels during the period of hyperbilirubinemia (511).
1574 Indeed, the reported findings seemed more likely to be due to changes in the sensitivity of the N-
1575 methyl-D-aspartate (NMDA) receptor.

1576 When comparing neurons and astrocytes from young versus old rats, the immature cells
1577 were most vulnerable to bilirubin toxicity, albeit the mitochondria from the young animals were
1578 more resistant to toxicity than those from the older rats (567). Thus, mitochondrial injury may be
1579 neither the only nor the primary mechanism for bilirubin neurotoxicity. Also, although the
1580 experimental data is limited, it has not been shown that sufficient quantities of bilirubin get to
1581 mitochondria in vivo to disturb their function (280). However, more bilirubin was found in the
1582 mitochondria after hyperosmolar opening of the BBB, which may be noteworthy in light of the lower
1583 phosphocreatine and ATP found in the brains of rats with infusion-induced hyperbilirubinemia
1584 following hyperosmolar opening of the BBB, but not when the BBB was intact (705). However,
1585 bilirubin likely has more direct access to mitochondria in in vitro cell cultures than in the intact brain
1586 in vivo. Thus, whether in vitro observations are necessarily relevant to the quest for the mechanisms
1587 of bilirubin toxicity in the living organism is uncertain and must await further study.

1588 *3. Membrane effects*

1589 Evidence from several sources suggests that bilirubin interacts with biological membranes (35, 160,
1590 459). In rats who were given [^{3}H]bilirubin IV, the bilirubin concentration (relative to protein) was
1591 higher in membranes than in other subcellular fractions (280). Autoradiography of brain slices
1592 exposed to [^{3}H]bilirubin in vitro showed that bilirubin bound most strongly to neurons, suggesting a
1593 stronger affinity for neuronal cell membranes (165).

1594 Bilirubin interaction with membrane polar lipids may play a role in the mechanism of toxicity
1595 (119). In cell cultures containing bilirubin cytosolic enzymes leaked into the medium, suggesting

1596 increased membrane permeability (160). Scanning electron microscopy of erythrocytes from
1597 jaundiced neonates showed crenation of the surface, suggesting that bilirubin interacted with the
1598 outer half of the erythrocyte plasma membrane bilayer complex (373). The membrane changes were
1599 reversed by phototherapy, an interesting observation in light of hypotheses that bilirubin
1600 photoisomers may be less able to cross the BBB and perhaps also are less toxic (304). Crenation of
1601 the RBC outer surface has been shown in the presence of bilirubin concentrations ranging from $1x10^{7}$
1602 to $1x10^{-5}$ mol/L (108). Bilirubin-induced depression of the synaptosome membrane potential was
1603 thought to involve changes in ion permeability (195). However, in synaptosomes permeabilized with
1604 streptolysin O (which excludes plasma membrane polarity), bilirubin inhibited Ca^{2+}-dependent
1605 neurotransmitter exocytosis and disrupted norepinephrine storage in vesicles at higher bilirubin
1606 concentrations (295).

1607 Bilirubin apparently has high affinity for cell membrane phospholipids and may form
1608 complexes with these (116, 117, 403). Partitioning into membranes was increased when these
1609 contained proteins, but specific binding sites were not involved (403). Bilirubin acid may precipitate
1610 when acidosis is present, leading to irreversible aggregation (120, 701). Bilirubin aggregation and
1611 precipitation is a theory that still has support (523). However, binding of the monovalent anion of
1612 bilirubin acid to membranes may be reversible (703). The finding that bilirubin binding to liposomes
1613 and RBCs is reversible is in line with this speculation, as are clinical and experimental observations
1614 showing that the milder signs of bilirubin influence on the brain may be reversed (456, 508, 701, 703).
1615 However, both the model and the experimental conditions seem to influence the reversibility of
1616 membrane changes. For example, washing of erythrocytes with albumin did not completely reverse
1617 bilirubin-associated membrane toxicity (112).

1618 Bilirubin may also interact with membrane-localized enzymes, pumps, or transporters, and it
1619 is possible that some of the observed effects of bilirubin on cell membranes could involve such
1620 elements. For example, bilirubin changed the temperature dependence of Na^{+}-K^{+}-ATPase to lower
1621 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 Na^+-K^+-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. Mg^{++}-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

1648 transverse hippocampal slices and Müller cells acutely in vitro by cell clamp technique (687).
1649 Therefore, although some of the published data seem to support a role for NMDA-mediated
1650 excitotoxicity in ABE, apparent contradictions will need to be reconciled (**Figure 5**).

1651 Using voltage-clamp recordings from bushy cells in the ventral cochlear nucleus from rat
1652 pups, it was shown that acute administration of bilirubin increased voltage-gated calcium channel
1653 currents mediated by high voltage-activated P/Q-type calcium currents (413). This appeared to occur
1654 via Ca^{2+} and calmodulin dependent mechanisms, causing excessive Ca^{2+} within the neurons. There
1655 are more P/Q-subtype calcium channels in neonatal neurons than at more mature stages, thus
1656 subtype-specific increase of P/Q-type Ca2+ currents may be involved the vulnerability of neurons to
1657 bilirubin toxicity in auditory as well as other brain regions (413). A role for Ca^{2+} channels in bilirubin
1658 neurotoxicity was also supported by studies of recombinant $Ca_v2.3 + \beta_3$ channel complexes and ex
1659 vivo electroretinograms from wildtype and $Ca_v2.3$-deficient mice (12). Thus, 10 µM UB produced
1660 changes in the voltage-dependence of activation and prepulse inactivation. Also, exposure of mouse
1661 retina to UCB suppressed responses of the inner retina from wildtype compared to $Ca_v2.3$-deficient
1662 mice, and recovery after washout was more complete, and occurred more rapidly in retinae that did
1663 not have $Ca_v2.3$ channels (12).

1664 *4. Neurotransmitter metabolism*

1665 In studies of brain specimens from human kernicterus cases, both of ABE and in cases with chronic
1666 sequelae, changes have been found in the expressions of neurotransmitters and neuropeptides as
1667 well as calcium-binding proteins, suggesting that bilirubin neurotoxicity may impact these important
1668 molecules and processes (264). Thus, in cases who had died with ABE during the newborn period, the
1669 expression of tyrosine hydroxylase was reduced both in the putamen and in the globus pallidus, and
1670 in the latter nucleus also in cases of chronic post-kernicteric bilirubin encephalopathy. The expression
1671 of methionine–enkephalin was also reduced in the external segment of the globus pallidus, both in
1672 cases of acute and chronic post-kernicteric BE. Finally, immunoreactivity for substance P was

1673 significantly reduced in both internal and external segments in cases of chronic post-kernicteric BE,
1674 but only mildly affected in cases who had died in the newborn period (264).

1675 In the experimental setting bilirubin reversibly inhibited both evoked potentials and synaptic
1676 activation in rat transverse hippocampal slices in vitro, which suggests an effect of bilirubin on
1677 neurotransmitter metabolism (297). Although this study may be critiqued because of the very high
1678 bilirubin concentrations used to elicit the reported changes, others have also found changes in
1679 stimulus-response in in vitro hippocampal slices, specifically in the Schaffer collateral CA1 synapses,
1680 and at bilirubin concentrations as low as 10 µmol/L (139). Phosphorylation of synapsin I is an
1681 important step in synaptic neurotransmitter release. Bilirubin inhibits synapsin I phosphorylation,
1682 which also points to an effect of bilirubin on neurotransmitter cycling (292).

1683 Reduced neurotransmitter uptake into synaptic vesicles may be due to an inhibitory
1684 interaction between bilirubin and transport proteins in vesicle membranes, causing decreased
1685 synaptic function in the jaundiced brain (574). But uptake of dopamine and glutamate were equally
1686 inhibited, which is interesting in light of the assumption that these are driven by different
1687 mechanisms (proton gradient and membrane potential, respectively). This suggests that bilirubin
1688 inhibition of neurotransmitter uptake may be mediated by an effect on the transmembrane domains
1689 of the transporter proteins, possibly at the protein/lipid interphase (574).

1690 It was suggested that perturbations of membrane function by bilirubin trigger a cascade that
1691 leads to excitotoxicity and energy failure in mitochondria (688). However, this hypothesis may be
1692 contradicted by the observation that bilirubin inhibited both exocytotic release and synaptic vesicular
1693 storage of brain catecholamines in permeabilized synaptosomes (295). Given the characteristics of
1694 the model, these effects were clearly dependent neither on endogenous ATP nor on the membrane
1695 potential (295). Thus, bilirubin may have (at least) two distinct effects on transmitter release in
1696 presynaptic catecholaminergic terminals: *i)* by decreasing the efficiency of Ca^{2+}-dependent secretion;
1697 and *ii)* at higher bilirubin concentrations, disrupting vesicular norepinephrine storage. In intact
1698 neurons these effects would decrease the evoked release of the neurotransmitter. Furthermore, in in

1699 vitro studies with purified kinases and peptides, in the absence of membranes and in the presence of
1700 excess ATP, widespread inhibitory effects of bilirubin on peptide-kinase interactions were still
1701 present (288).

1702 Inhibitory effects of bilirubin were detectable in synaptosomes, both as far as the uptake of
1703 tyrosine (dopamine precursor), and formation of dopamine (34). Bilirubin was also shown to inhibit
1704 the direct uptake of dopamine into synaptosomes, but not release (513). Endogenous acetylcholine
1705 release was inhibited and the synaptosomal membrane was depolarized. However, inhibition of
1706 dopamine release by bilirubin was observed by other investigators using a different stimulus for
1707 release (136).

1708 Bilirubin inhibited uptake of glutamate in rat cortical astrocytes in vitro (610). Release of
1709 glutamate and cell death followed bilirubin exposure of astrocytes, microglia, as well as neurons in
1710 culture, and immature cells were more vulnerable to loss of glutamate (202, 210, 244). L-carnitine
1711 protects neurons in culture from glutamate toxicity, and its presence significantly reduced bilirubin
1712 toxicity in cultured cerebellar granule cells, lending further credence to the putative role of
1713 glutamate and excitotoxicity in bilirubin cell toxicity (639).

1714 *5. Enzyme induction*

1715 As already discussed, bilirubin appears to inhibit a wide range of enzyme activities. However,
1716 bilirubin may apparently also induce some enzymes Thus, in oligodendrocytes from newborn rats
1717 bilirubin induced NOS mRNA expression, leading to increased nitrite production (236). This was
1718 accompanied by apoptosis of the oligodendroglia, which was dependent on bilirubin concentration
1719 and time of exposure. Exposure of rat cerebellar granule neurons in vitro to bilirubin led to activation
1720 of p38 MAP kinase, followed by cell death (419). Pretreatment of the cells with a p38 MAP kinase
1721 inhibitor (SB 203580) significantly reduced bilirubin neurotoxicity. In contrast to the widespread
1722 inhibitory effects of bilirubin on protein phosphorylation, p38 MAP kinase phosphorylation was
1723 upregulated by bilirubin exposure, and seemed to be the trigger for bilirubin-induced neuronal death
1724 (419).

1725 *6. Apoptosis and necrosis*

1726 Neuronal loss is a key event in kernicterus and in the brain lesions of homozygous Gunn rats, in
1727 whom cerebellar Purkinje cells appear to be particularly vulnerable (155, 169, 272, 460, 532, 656,
1728 678). Bilirubin caused apoptosis in rat cerebellar granule cells (417). Cell death could be blocked by
1729 inhibiting the synthesis of RNA and proteins, suggesting that de novo synthesis of RNA and protein
1730 may be necessary to initiate cell death. The sequence of events involved in cell death was examined
1731 in developing rat brain astrocytes and neurons (565, 566). Signs of impaired mitochondrial
1732 metabolism and membrane perturbations in the form of altered lipid polarity and fluidity, protein
1733 order, and redox status were observed before apoptosis became apparent. The effect on cell
1734 membranes, evinced by increased lipid polarity, was noted almost immediately. Neurons were
1735 around 30% more vulnerable than astrocytes (565). UDCA, a mitochondrial-membrane stabilizing
1736 agent, and cyclosporine A, an inhibitor of the permeability transition, prevented apoptosis which
1737 appeared to be triggered by mitochondrial depolarization and Bax translocation (563, 566).
1738 Cytochrome c was released from the intermembrane space of the mitochondria. This suggested that
1739 bilirubin interacted directly with mitochondria by influencing membrane lipid and protein properties,
1740 redox status, and cytochrome c content, and that induction of apoptosis by bilirubin might be
1741 mediated, at least in part, by physical changes in the mitochondrial membrane (563, 566).

1742 The above studies were carried out in cells of animal origin. However, NT2-N neurons (a
1743 human neuron-like cell line) have also been used to assess the ability of bilirubin to cause apoptosis
1744 and/or necrosis and showed that induction of apoptosis, as opposed to necrosis, may depend on
1745 bilirubin concentration. Thus, high bilirubin concentrations (100 µmol/L) induced early necrosis while
1746 low-to-moderate concentrations (0.66–25 µmol/L) predominantly induced delayed apoptosis (273).
1747 Using a specific caspase-3 inhibitor (zDEVD.FMK), a general caspase inhibitor (zVAD.FMK), and an
1748 NMDA receptor antagonist (MK-801) bilirubin-induced cell death was shown to involve both NMDA
1749 receptor-mediated and caspase-mediated pathways (274). Synergistic protection was seen following
1750 concurrent inhibition of both pathways. While caspase inhibition did not positively impact cell

1751 survival after short-term bilirubin exposure in these cells, the number of undamaged nuclei was
1752 significantly increased by NMDA blockade without effects on 3-(4,5-dimethylthiazol-2-yl)-2,5-
1753 diphenyltetrazolium bromide (MTT) reduction, another measure of cell viability (275). A possible role
1754 for NMDA receptors has also been confirmed in rat transverse hippocampal slices in vitro (139).

1755 *7. Cell metabolism*

1756 Bilirubin inhibits protein synthesis; however, one recent study did not confirm this finding (35, 250,
1757 261, 274). Inhibitory effects of bilirubin on carbohydrate metabolism were shown in older studies,
1758 but these findings do not appear to have been replicated (372, 542, 607). Bilirubin was shown to
1759 inhibit DNA synthesis, while strand breakage in DNA was increased when bilirubin exposure was
1760 combined with phototherapy (35, 571, 572, 586, 643). In a mouse model of NNJ ($UGT1^{-/-}$) DNA
1761 damage in the cerebellum was shown to occur in vivo (553). In vitro, SH-SY5Y cells which are derived
1762 from human neuroblastoma and often used to model neuronal function in vitro, evinced DNA
1763 damage when exposed to 140nM UB, as determined by Western blot and immunofluorescence
1764 analyses. If these cells were concomitantly exposed to N-acetyl-cysteine, a scavenger of free radicals,
1765 DNA damage was prevented. This was seen to support the concept that DNA damage was caused by
1766 bilirubin-induced oxidative stress. Exposure of the cells to bilirubin also activated the main DNA
1767 repair pathways through homologous recombination and non-homologous end joining (553).

1768 Exposing SH-SY5Y cells to 140 nM UB increased intracellular oxygen-free radical levels and
1769 accumulation of Nrf2 protein, which regulates the expression of antioxidant proteins, in the nucleus
1770 (546). This resulted in increased expression of multiple antioxidant response gene mRNAs. Thus, in
1771 these cells the response against bilirubin-mediated oxidative stress involves activation of antioxidant
1772 defenses, partly through the Nrf2 pathway.

1773 Exposure of PC12 cells (from rat pheochromocytoma) and rat cerebellar granule cells to
1774 bilirubin (0.5–10 μM) significantly decreased nerve growth factor and brain-derived neurotropic
1775 factor signaling to Akt and extracellular signal-regulated kinases, showing that bilirubin can interfere

1776 with important pro-survival signaling pathways (449). This involved reduced phosphorylation, and
1777 thus reduced activation, of important downstream effectors, which could be partially reversed by a
1778 phosphatase inhibitor. The possible role of inhibition of peptide/protein phosphorylation as a basic
1779 mechanism of bilirubin toxicity is discussed further in Section G.13 – 'A common mechanism?'.

1780 *8. Infection and immunology*

1781 Infection/sepsis has long been considered a risk factor for ABE/KSD, but published support was
1782 limited and equivocal (378, 539, 652). However, newer data provide stronger support for an
1783 association between sepsis and kernicterus (225, 380, 511, 699). Among 100 Pakistani infants with
1784 ABE, 52 had been diagnosed with (unspecified) sepsis (380). Among 288 Taiwanese infants with TSB >
1785 342 µmol/L (20 mg/dL) 15 developed ABE and/or KSD. The OR for adverse outcomes in infants with
1786 sepsis was 161.7 (95% CI: 11.7–2242.8), a higher OR than for any other potentially contributing factor
1787 (699). Among 249 newborn infants with TSB ≥ 427 µmol/L (25 mg/dL) admitted to Cairo University
1788 Hospital with ABE on admission (n=44) and/or neurological evidence of KSD at the time of death or
1789 discharge (n = 35), rigorously defined sepsis greatly increased the risk for bilirubin neurotoxicity (OR =
1790 20.6) although the highest risk for ABE/KSD was found among infants with Rh-incompatibility (OR =
1791 48.6) (225). Similarly, in a cohort of 21 infants with ABE from Nigeria, 15 had septicemia (512).

1792 Infected infants tend to develop higher TSB levels, which may contribute to their increased
1793 risk of ABE/KSD (181, 527, 528). In endotoxemic rats, both total and UB were elevated, leading to
1794 increased net accumulation of bilirubin in brain (298). Infected human infants had lower total
1795 albumin concentrations and also a lower reserve albumin binding capacity for bilirubin as estimated
1796 by the MADDS method (192). It is a reasonable assumption that these infants also had higher UB
1797 concentrations.

1798 Inflammatory cytokines may increase BBB permeability, which might then facilitate bilirubin
1799 passage into the brain (499). Changes in BBB permeability may be secondary to disruption of the
1800 barrier, involving modifications of tight junctions, endothelial damage, degradation of the glycocalyx,
1801 breakdown of the glia limitans, and changes in the astrocytes (665). However, changes in barrier

1802 permeability may also be non-disruptive and involve membrane transporters, cytokines,
1803 prostaglandins, and cellular transmigration (665). The complexity of this system, and the possibility
1804 for both unintended as well as perhaps planned perturbation of such processes, may be illustrated by
1805 the finding that acetaminophen, a drug commonly used for pain relief in sick neonates, can cause
1806 upregulation of P-gp protein expression through the constitutive androstane receptor pathway (616).
1807 While the interactions of some of these processes/mechanisms with bilirubin have been discussed in
1808 this review and also reviewed by others, other areas have apparently not been studied with a specific
1809 focus on the BBB (231).

1810 Endotoxin and TNF-α increased the cytotoxicity of bilirubin in mouse fibroblasts in vitro (504).
1811 When astrocytes were exposed to bilirubin in conditions that induced < 10% cell death, the release of
1812 TNF-α and IL-1β was significantly increased (210). Young astrocytes in culture were more vulnerable
1813 to bilirubin-induced cell death than older cells and also showed greater inflammatory response (202).
1814 As in the fibroblast model (504), endotoxin increased bilirubin cytotoxicity in astrocytes (202). When
1815 exposed to bilirubin in vitro, microglia were activated and released high levels of TNF-α, IL-1β, and IL-
1816 6, suggesting that bilirubin-induced cytokine production may increase neurotoxicity (244).

1817 The response of the developing cerebellum to bilirubin toxicity was studied in $UGT^{1-/-}$ mouse
1818 pups (675, 676). Notable findings were early activation of oxidative stress, endoplasmic reticulum (ER)
1819 stress, and inflammatory markers. TNFα and NFKβ were important mediators of and inflammatory
1820 reaction, which led to apoptosis and eventually opening of the autophagy pathway. During this
1821 process M1 type microglia were activated (676). Using the same model these processes were later
1822 shown to be amenable to modification by minocycline treatment (675). Reduction of
1823 neurodegeneration, neuroinflammation, and apoptosis of cerebellar neurons translated into a dose-
1824 dependent reduction of lethality. Further, decreased M1 microglia activation was accompanied by a
1825 reduction in oxidative and ER stress markers in these cells. These data support the concepts that
1826 neurodegeneration and neuro-inflammation are important elements in bilirubin-induced neonatal
1827 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-κB and TNFα 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-κB (209). Therefore, NF-κB 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-κB 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 PGE_2 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

1854 further. Also, endotoxemia or sepsis did not affect bilirubin metabolism in rat brain, though whether
1855 this is relevant for the putative connection between septicemia and ABE/KSD is as yet unknown (17).
1856 It is also possible that innate immunity signaling may ameliorate bilirubin neurotoxicity. Very
1857 high TSB levels in *UGT1A1*28* mice caused systemic oxidative stress, as shown by a decreased ratio
1858 of glutathione/glutathione disulfide, and by activation of the NADPH oxidase complex and brain
1859 antioxidant response genes in the brain (731). Very high TSB levels led to inflammation in neurons,
1860 shown by activation of microglia and astrocytes. Apparently, the toll-like receptor 2 signaling
1861 pathway was key to the regulation of gliosis, pro-inflammatory mediators, and oxidative stress, and
1862 served as a protective mechanism in the presence of severe hyperbilirubinemia (731). This was
1863 shown by the significantly higher mortality rates in jaundiced *hUGT1A1*28/Tlr22/2* mice pups, who
1864 failed to activate glial cells, pro-inflammatory cytokines, and stress response genes (731).
1865 As a further example of the yin-and-yang of bilirubin biology, there is also evidence that
1866 bilirubin may have anti-inflammatory activities in brain tissue. Thus, in rodent experimental
1867 autoimmune encephalomyelitis bilirubin, injected intraperitoneally to produce TSB levels of ~60
1868 µmol/L 30 min after injection, delayed the onset and alleviated the severity of the chronic form of
1869 the disease (423). Conversely, depleting endogenous bilirubin by treatment with zinc protoporphyrin
1870 exacerbated the disease. The authors suggest that the effect of bilirubin cannot have been due solely
1871 to its antioxidant effect as α-tocopherol, a compound with antioxidant effects similar to bilirubin,
1872 was much less effective than bilirubin in changing the course of the disease. They also investigated
1873 the effect of bilirubin (20 and 150 µM) on the proliferative responses of naive SJL/J-mouse $CD4^+$ T
1874 cells and protein lipid peptide (PLP)-specific $CD4^+$ T cells following stimulation with Con A, anti-CD3
1875 mAb with or without anti-CD28 mAb, or PLP in vitro. At these concentrations, bilirubin inhibited CD4
1876 T cell reactivity across a range of actions which included inhibition of costimulatory activities,
1877 suppression of immune transcription factor activation, and down-regulation of inducible MHC class II
1878 expression. The authors suggested that bilirubin actions were direct, and not through induction of
1879 immune deviation or regulatory T cells (423).

1880 Thus, the evidence points to a role for immunology, inflammation, and/or infection in the
1881 pathophysiology of ABE/KSD, as well as to a possible modulatory effect of bilirubin on immune
1882 mechanisms. However, a number of questions still remain to be addressed and explored. Our
1883 discussion herein concerning the role of inflammation/infection in the genesis of bilirubin
1884 neurotoxicity is by necessity limited. For a more complete discussion of these aspects, the reader is
1885 referred to an extensive review by Brites (107), in whose laboratory much of the important work
1886 regarding these questions has been carried out.

1887 *9. Differential sensitivity*

1888 With very few exceptions, extreme unconjugated hyperbilirubinemia occurs in newborn infants.
1889 Therefore, descriptions of ABE and KSD are focused on this age group, giving the impression that
1890 bilirubin neurotoxicity is primarily related to increased vulnerability in the immature brain. Within
1891 this age group, however, some infants can suffer neurologic sequelae at moderately elevated TSB
1892 levels, while others seem to tolerate extreme hyperbilirubinemia without brain damage.
1893 Furthermore, preferential accumulation of bilirubin in e.g. basal ganglia suggests greater sensitivity in
1894 some brain regions and cell populations than in others.

1895 However, brain damage due to bilirubin can also occur in more mature individuals, as shown
1896 by a few patients with Crigler-Najjar syndrome who escaped neurological sequelae for many years
1897 due to meticulous follow-up and continued phototherapy, only to suffer neurological damage during
1898 a medical or surgical emergency which caused significant increase of TSB (393, 653, 664, 678).
1899 Apparently, in these individuals, cerebellar sequelae were predominant, which is more unusual when
1900 ABE occurs in the newborn period.

1901 In vitro, most cells appear vulnerable to bilirubin toxicity, but some more so than others. The
1902 age of the cells may modify their vulnerability, and different cellular processes vary in sensitivity.
1903 Glial cells appear to be more resistant to lethal bilirubin toxicity than neurons. For example, bilirubin
1904 toxicity was seen in mouse neuroblastoma cells in vitro, but not in rat astrocytoma cells (507). When
1905 these neuroblastoma cells had differentiated under exposure to PGE_1 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 PGE_1 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 [^{3}H]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 µM for 24h. Auditory nerve fibers and vestibular nerve endings were most sensitive, evincing toxicity at bilirubin concentrations of 10-50 µM. 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 µM 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 glycoursodeoxycholic 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-µM
2028 bilirubin in the presence of 100-µM HSA, either alone or in combination with 100 µM N(G)-nitro-L-
2029 arginine methyl ester (NAME), an inhibitor of NOS, or with 50-µM 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

2036 [anti-inflammatory], $MgCl_2$ [glutamate channel blocker], curcumin [antioxidant], or a cocktail of these
2037 three drugs (164). Minocycline was used as a control considered at present to be the 'gold standard'
2038 for protection against bilirubin neurotoxicity. Single drug treatment (indomethacin, curcumin, or
2039 $MgCl_2$) improved cell viability in all regions studied, while the three-drug cocktail almost completely
2040 prevented toxicity in the most affected area (hippocampus). These findings seem to indirectly
2041 support the roles both of inflammation, glutamate toxicity, and oxidant injury as mechanisms of
2042 bilirubin neurotoxicity, but also point the way towards the possibility of intervening with drug
2043 treatments in infants with signs of ABE. In support of this, anti-inflammatory compounds which
2044 interfere with NF-κB and TNFα signaling were recently shown in a new, acute mouse model of ABE,
2045 to protect the auditory system against bilirubin toxicity (585). Thus, this avenue is in need of further
2046 exploration.

2047 Hypothermia is neuroprotective following asphyxia, both in animals and in humans (258). The
2048 same was found for meningitis and traumatic brain injury (180, 661). Based on the effects of
2049 therapeutic hypothermia on neuronal metabolism and on the progression of events in brain injury a
2050 hypothesis of protection in bilirubin neurotoxicity may be postulated. Newborn pigs were divided
2051 into a hypothermic (34.0°–35.0°C) and a normothermic (38.0°–39.0°C) group, and hyperbilirubinemia
2052 was induced by infusion of bilirubin over a 4-hr period, followed by a displacer to increase bilirubin
2053 entry into brain. Decreased cerebral cortical cell membrane Na^+-K^+-ATPase activity and increased
2054 lipid peroxidation products evinced bilirubin neurotoxicity in the normothermic group, while such
2055 changes were significantly attenuated in the hypothermia group, which also showed less evidence of
2056 reduction in brain ATP. The changes in phosphocreatine and blood and brain lactate levels seen in
2057 the control group were also less pronounced with hypothermia (536). Recently, moderate
2058 hypothermia was also shown to protect against bilirubin-induced cell death in mouse neurons in vitro
2059 (391).

2060 Thus, therapeutic hypothermia appears promising as a strategy to ward off bilirubin
2061 neurotoxicity, but no clinical studies have been published, and no ongoing clinical trials are recorded

2062 in the registries we have accessed (Clinicaltrials.gov, Australian New Zealand Clinical Trials Registry,
2063 EU Clinical Trials Register, ISRCTN registry, Health Canada's Clinical Trials Database, ICTRP Search
2064 Portal). Also, no case reports could be found in PubMed or Medline. This suggests that recruitment
2065 or selection of suitable patients, either for a trial or for compassionate use, is challenging. Indeed, in
2066 the animal study discussed above, hypothermia was induced concurrently with bilirubin infusion, a
2067 scenario not applicable to clinical practice. Also, given the difficulty of selecting an entry point for
2068 testing this potential therapy and the rarity of ABE in most settings, extensive scientific scrutiny and
2069 discussion will be necessary before proceeding. Then, a setting in a low- or middle-income country,
2070 where ABE unfortunately continues to be reported with some regularity, may be most feasible.

2071 The predominant bilirubin isomer in humans is bilirubin-IXα (*Z,Z*), found either in the charged
2072 dianion form, or as bilirubin acid. While the dianion is to some extent water soluble at neutral pH due
2073 to its eight hydrophilic groups, the acid form is nearly insoluble because of intramolecular hydrogen
2074 bonds (117, 218). Photoisomerization of bilirubin yields more polar forms of the molecule (442).
2075 While the lipophilic bilirubin-IXα(*Z,Z*) isomer is associated with toxicity, water-soluble isomers have
2076 been hypothesized to be nontoxic (442, 609, 643). Photoisomers have in some cell culture studies
2077 nevertheless increased bilirubin toxicity (150, 569, 572, 608). However, it is uncertain whether in
2078 vitro cell models are appropriate for testing a hypothesis relating to phototherapy and bilirubin
2079 neurotoxicity. Before embarking on further in vitro studies of bilirubin photoisomer toxicity it is
2080 necessary to ascertain whether photoisomers actually cross the BBB and gain access to brain cells.
2081 While a hypothesis that these polar isomers do *not* cross the BBB seems supported by our knowledge
2082 of the physicochemical characteristics of these molecules, no in vivo studies to support these
2083 speculations are on record (304, 466). Clinical evidence suggests that aggressive phototherapy, in
2084 some cases used in combination with other therapeutic tools such as exchange transfusion and IV
2085 immune globulin (IVIG), can reverse acute intermediate-to-advanced BE, but it is not clear whether
2086 decreased BBB penetration of bilirubin photoisomers plays any role in these fortuitous outcomes
2087 (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, AB0
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 AB0 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

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

12. Bilirubin binding

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

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

13. A common mechanism?

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

2140 produce brain bilirubin levels ranging from 14–51 nmol/g tissue (488). As noted above, these brain
2141 bilirubin levels are comparable to those observed both in experimental animals as well as human
2142 infants with kernicterus. Bilirubin inhibition could be partly reversed by aminophylline, suggesting
2143 that bilirubin effects on brain involve both synaptic transmission and nuclear activation through
2144 histone phosphorylation. Bilirubin also inhibited phosphorylation of endogenous proteins in
2145 fibroblasts (32).

2146 In vitro, bilirubin inhibited phosphorylation of synapsin I, a protein that is preferentially
2147 localized to presynaptic vesicles (292). Phosphorylation of synapsin I promotes neurotransmitter
2148 release at the synaptic cleft, while dephosphorylated synapsin I inhibits such release. Bilirubin
2149 inhibited synaptic activation in transverse rat hippocampal slices, which might be due to a lower
2150 degree of synapsin I phosphorylation (297). Drowsiness in jaundiced infants, along with reduced
2151 brain stem auditory evoked responses, could also be due to reduced synapsin I phosphorylation (605).
2152 Many other protein-kinase interactions are also inhibited by bilirubin (296). Thus, a speculation that
2153 inhibition of protein-peptide phosphorylation might contribute to the effects of bilirubin in biological
2154 systems does not seem far-fetched. Hypothetically, lysine binding could be involved in these
2155 reactions.

2156 As discussed elsewhere in this review, P-gp is a membrane pump that may limit the
2157 intracellular and intracerebral accumulation of bilirubin and is regulated through phosphorylation
2158 (103, 277, 326, 327, 345, 401, 690, 735). Both protein kinases A and C are implicated in P-gp
2159 phosphorylation, and the interaction between these kinases and other peptide-protein substrates is
2160 inhibited by bilirubin (296). Conceivably, inhibition of P-gp phosphorylation may have a role in
2161 bilirubin toxicity. For example, pre-exposure to bilirubin has been shown to increase the permeability
2162 of the BBB for bilirubin itself (568). Reduced P-gp activity has already been shown to increase
2163 bilirubin entry into brain (277, 690). Although this has not been studied specifically for the bilirubin P-
2164 gp interaction, another BBB transport protein, breast cancer resistance protein (BCRP/ABCG2), is co-
2165 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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2193 **References**

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3955

3956 **FIGURE LEGENDS**

3957 FIGURE 1. Bilirubin metabolism in the body. ALB, albumin; B, bilirubin; BCRP, breast cancer resistance
3958 protein (also referred to as ATP-binding cassette super-family G member 2 [ABCG2]); BDG,
3959 bilirubin diglucuronide; BG, bilirubin glucuronides; BMG, bilirubin monoglucuronide; BSEP,
3960 bile salt export pump (also referred to as ATP-binding cassette, sub-family B member 11
3961 [ABCB11] or sister of P-glycoprotein [sP-gp]); BT, proposed bilirubin transporter; BVR,
3962 biliverdin reductase; COHb, carboxyhemoglobin; ER, endoplasmic reticulum; GST,
3963 glutathione-S-transferase; Hb, hemoglobin; HO, heme oxygenase; NADP, nicotinamide
3964 adenine dinucleotide phosphate; MATE1, multidrug and toxin extrusion 1; MRP2 and 3,
3965 multidrug resistance–associated proteins 2 and 3 [also referred to as 'ATP binding cassette
3966 subfamily C members 2 and 3' (ABCC2, ABCC3)]; OATP1B1/B3, organic-anion-transporting
3967 proteins 1B1 and 1B3; O_2, oxygen; UB, unbound bilirubin; UCB, unconjugated bilirubin; UCB-
3968 A, albumin-bound bilirubin; UGT1A1, bilirubin-UDP-glucuronosyltransferase.

3969 FIGURE 2. Bilirubin structure. **A.** Planar structure of bilirubin as first presented by Fischer and
3970 Plieninger (redrawn from reference 216); **B.** Bis-lactam structure of bilirubin according to
3971 Bonnett, Davies, and Hursthouse (redrawn from reference 87); **C.** Bilirubin ridge-tile
3972 conformation (courtesy of Professor Antony F. McDonagh, PhD [deceased]); **D.** Ridge-tile
3973 structure of bilirubin (from reference 415, with permission).

3974 FIGURE 3. Bilirubin diastereomers. Linear drawings of the four diastereomers of bilirubin (from
3975 reference 415, with permission).

3976 FIGURE 4. Bilirubin-brain interaction. A, albumin; ABR, auditory brainstem response; ADHD, attention
3977 deficit hyperactivity disorder; ASD, autism spectrum disorder; B, bilirubin; B-A, albumin-
3978 bound bilirubin; BCRP, breast cancer resistance protein (also referred to as ATP-binding
3979 cassette super-family G member 2 [ABCG2]); BV, blood vessel; CSF, cerebral spinal fluid;
3980 CPEC, choroid plexus epithelial cells; MRP1, multidrug resistance–associated protein 1 [also
3981 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-1β, Interleukin 1 beta; NADPH,
3985 dihydronicotinamide-adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-light-
3986 chain-enhancer of activated B cells; NMDA, N-methyl-D-aspartate receptor; TNFα, 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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3997
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Figure 1. Bilirubin metabolism in the body.

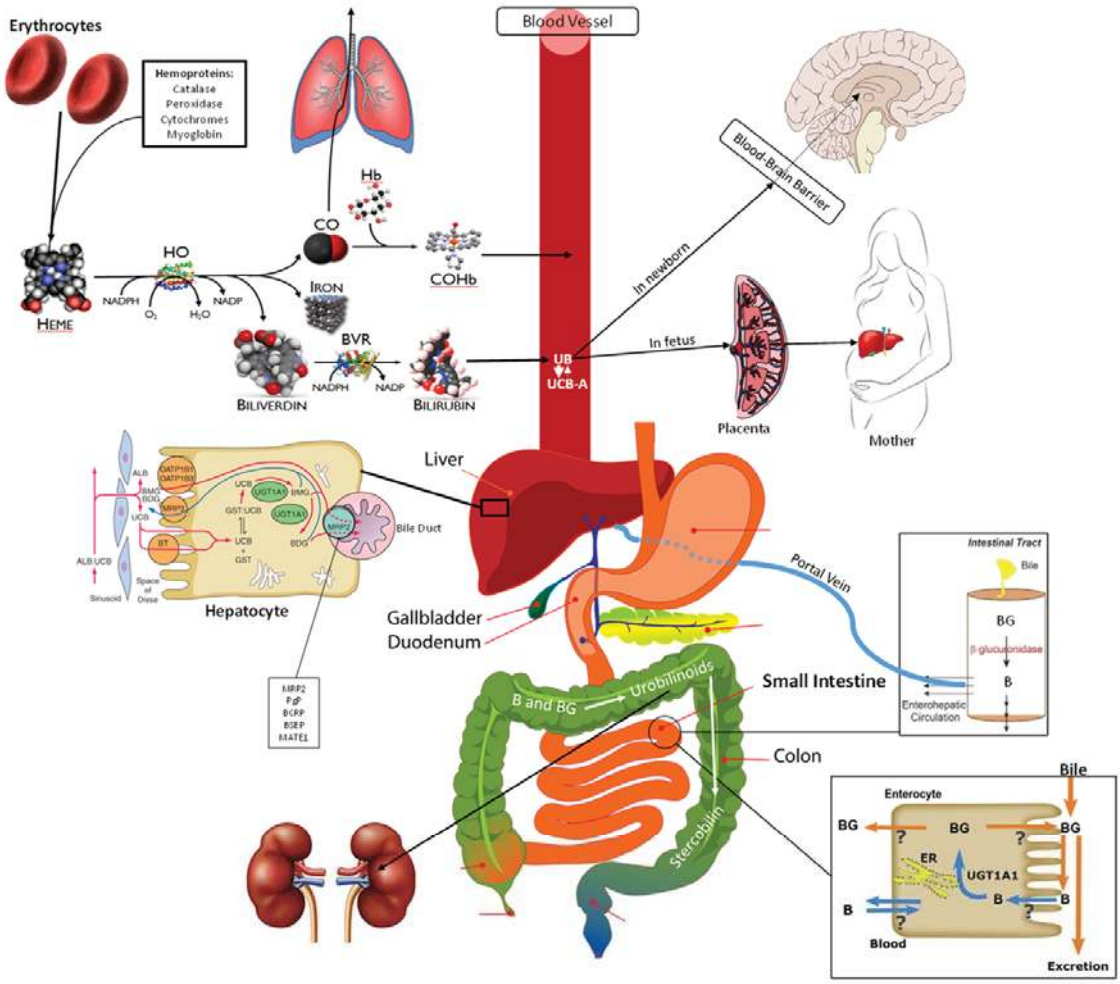
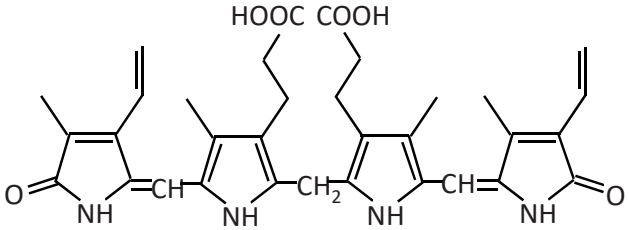


Figure 2. Bilirubin structure

A



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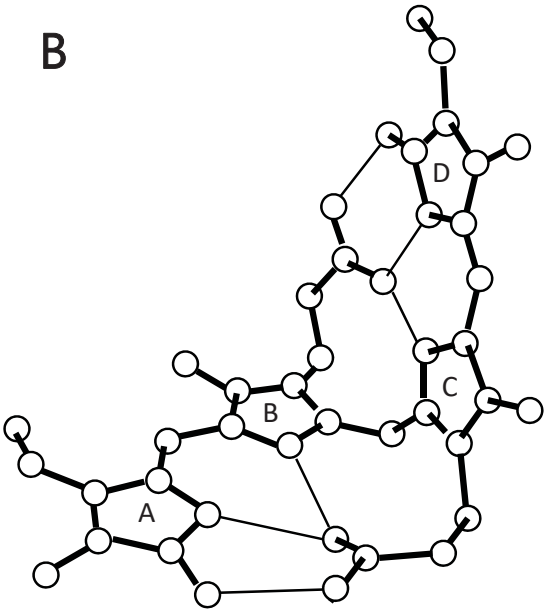

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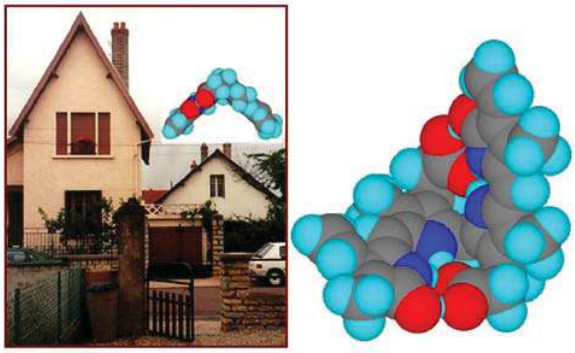

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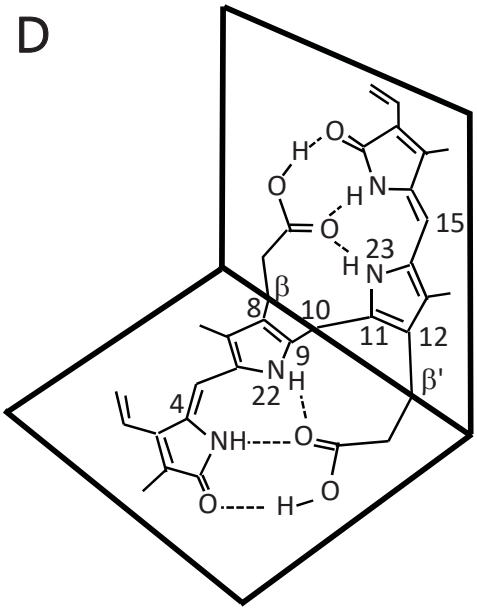
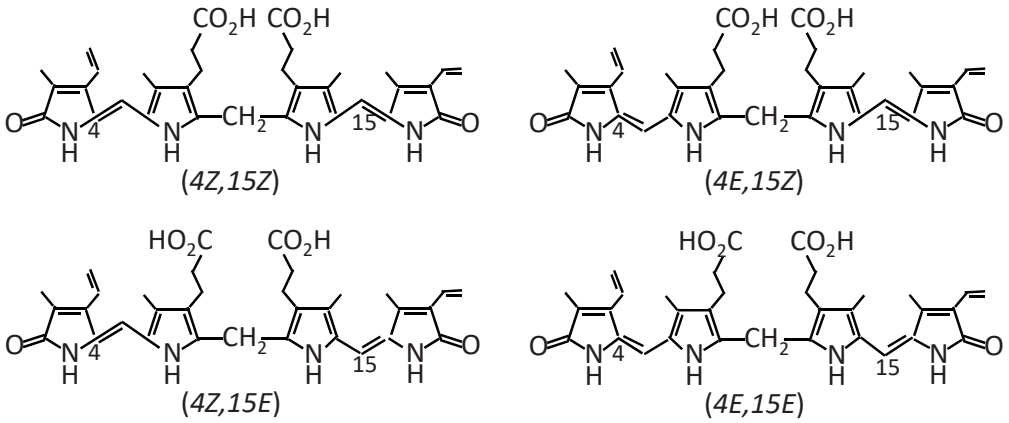


A. Planar structure of bilirubin as first presented by Fischer and Plieninger (redrawn from reference 216); **B.** Bis-lactam structure of bilirubin according to Bonnett, Davies, and Hursthouse (redrawn from reference 87); **C.** Bilirubin ridge-tile conformation (courtesy of Professor Antony F. McDonagh, 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).

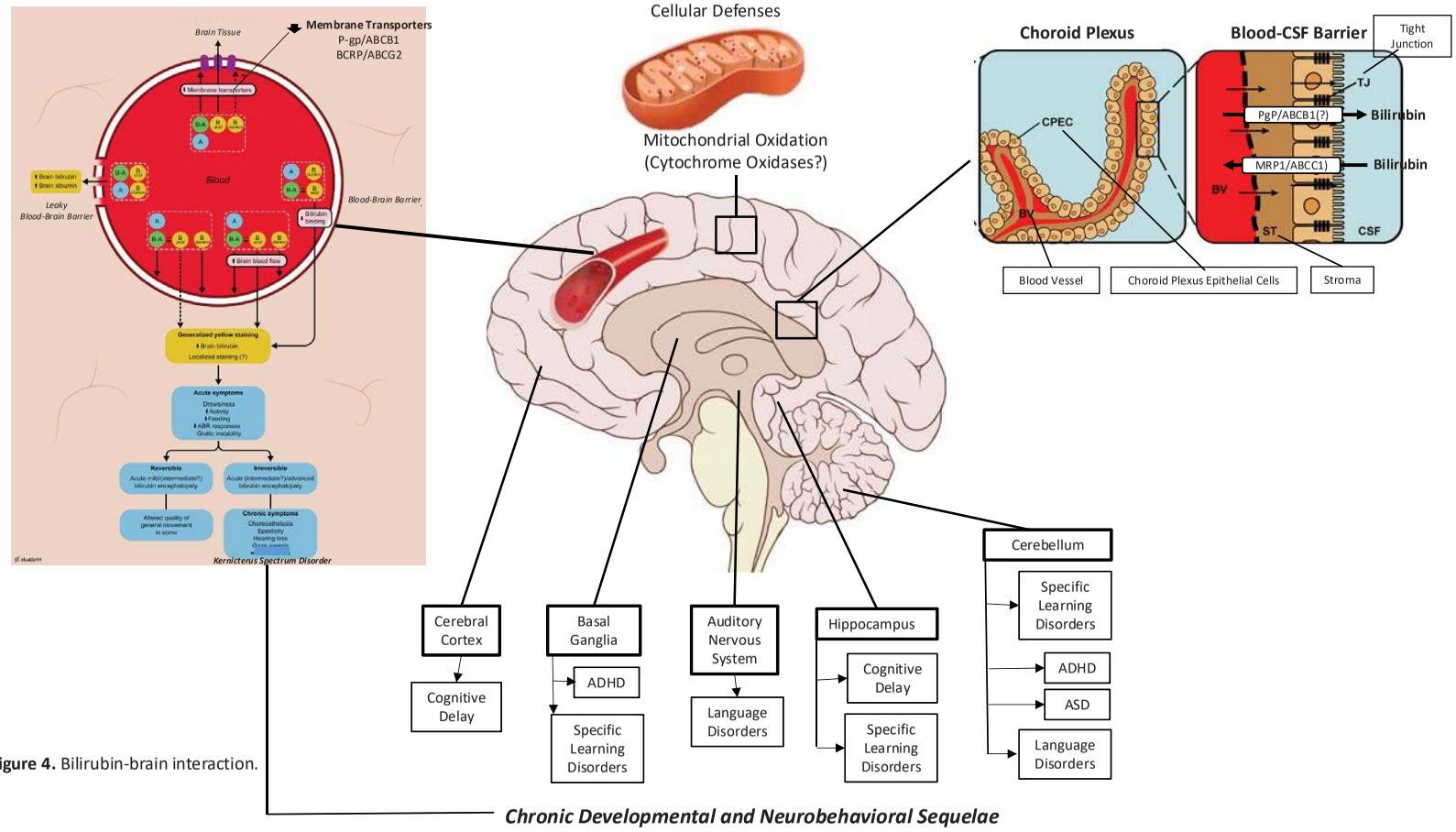
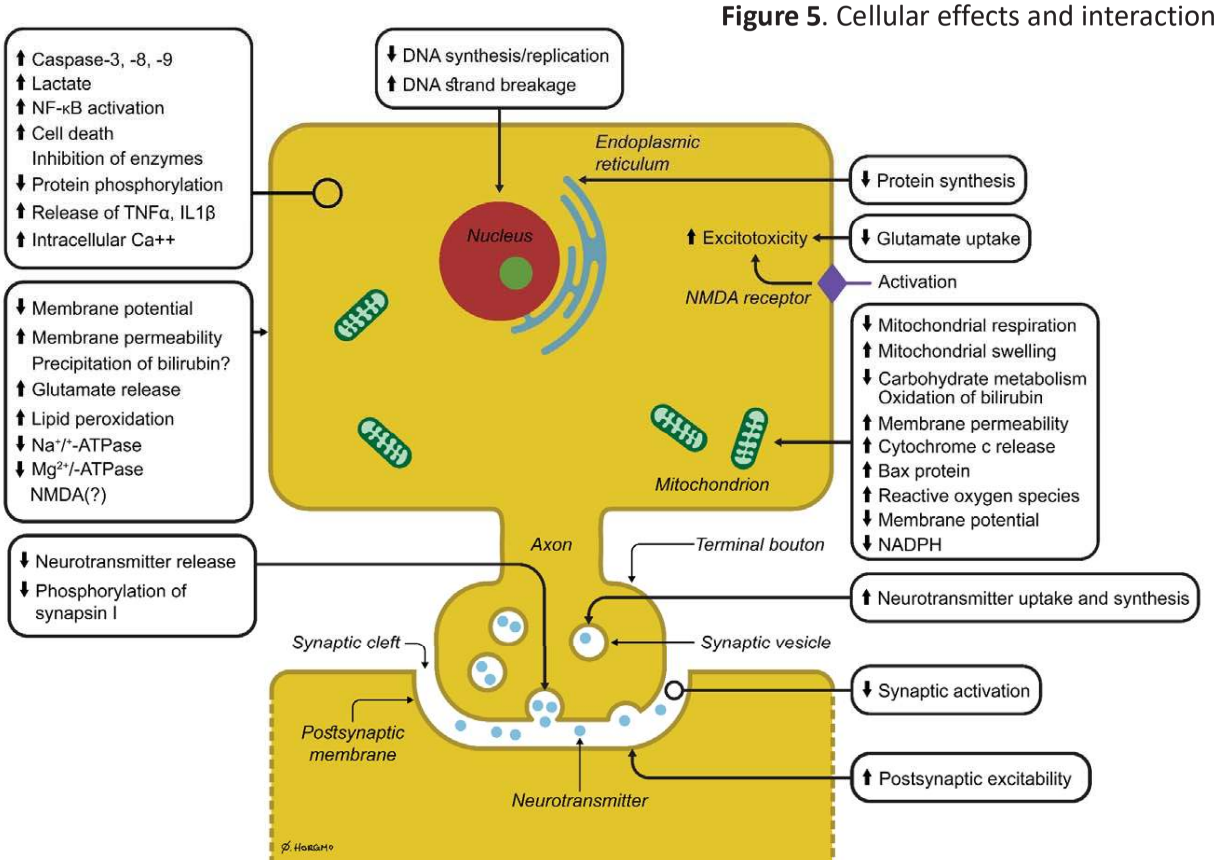
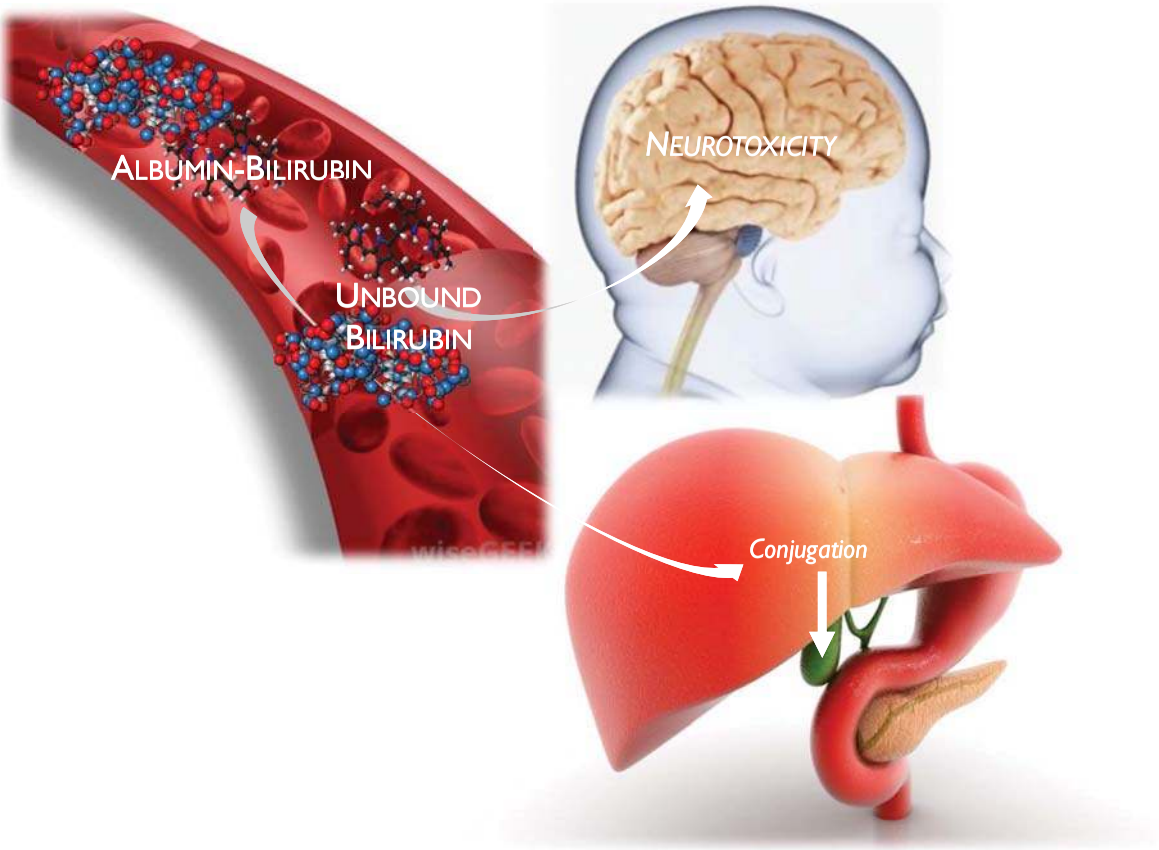


Figure 4. Bilirubin-brain interaction.

Figure 5. Cellular effects and interactions of bilirubin.




Albumin-Bilirubin
Unbound Bilirubin
Neurotoxicity
Conjugation