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Bilirubin Benefits: Cellular Protection by a Biliverdin Reductase Antioxidant Cycle

Thomas W. Sedlak, MD, PhD*, and Solomon H. Snyder, MD*‡

ABBREVIATIONS. CO, carbon monoxide; BVR, biliverdin reductase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); HO1, heme oxygenase 1; HO2, heme oxygenase 2; GSH, glutathione.

Bilirubin is widely known as an end product of heme metabolism. Very high levels of serum bilirubin lead to its accumulation in the brain, causing kernicterus.[1,2] Almost all newborns display some level of jaundice, and some display high enough serum bilirubin levels that phototherapy or exchange transfusion is considered.

What most of the medical profession has not appreciated is that, from a teleologic perspective, biosynthesis of bilirubin as the key catabolite of heme does not seem to make sense. Bilirubin is a secondary degradation product of heme. Heme is best known as a constituent of hemoglobin, which is released in association with the breakdown of aging red blood cells. Heme also is contained in a wide range of enzymes whose turnover also leads to free heme release. Free heme can be toxic, so nature evolved a family of heme oxygenase enzymes to degrade heme,[3,4] and their blockade leads to greatly increased excretion of unmetabolized heme in the bile.[5] These enzymes cleave the heme ring to form biliverdin, iron, and a 1-carbon fragment as carbon monoxide (CO; Fig 1). CO is increasingly appreciated as a neurotransmitter,[6,7] and iron, itself toxic, is excreted from cells by a recently characterized pump.[8–11] Biliverdin would seem to be an appropriate end product of the pathway, being readily excreted in the bile to enter the intestine and leave the body in the feces. Indeed, in birds, reptiles, and amphibians, biliverdin is the predominant end product of heme degradation.[12] For reasons that until now have seemed obscure, in mammals, biliverdin undergoes additional metabolism, being reduced by biliverdin reductase (BVR) to bilirubin, a step that consumes the energy resource nicotinamide adenine dinucleotide phosphate (NADPH).[13] As bilirubin is more hydrophobic and insoluble than biliverdin, it is glucuronidated to facilitate excretion into the bile, costing additional cell resources.

Why have mammals evolved an energetically expensive and apparently unnecessary enzymatic step to converting the relatively innocuous biliverdin to the more toxic bilirubin? Moreover, why would nature develop a system that generates "elevated" bilirubin levels in a high proportion of all neonates? Nature may not be altogether foolhardy, as the mildly to moderately elevated levels of bilirubin in neonates are not always toxic. In 1965, in this journal, Wishingrad and associates[14,15] argued that hyperbilirubinemia of premature infants is not as deleterious as previously thought. Furthermore, some individuals with the impaired bilirubin glucuronidation system of type 2 Crigler-Najjar syndrome maintain bilirubin levels of 19 mg/dL for 50 years without detectable damage to the nervous system.[16]

Some authors have suggested that unconjugated bilirubin is physiologically useful, because it can cross the placenta, moving from the fetal to the maternal circulation easier that biliverdin.[17–19] However, isomer specificities of fetal and maternal BVR differ. The principal isomer of early fetal bilirubin is IXβ, whereas the adult forms bilirubin IXα.[20,21] Hence, adult BVR cannot have evolved to service needs of the fetus.

One possible physiologic role for bilirubin is as an antioxidant. As early as the 1950s, bilirubin was reported to protect against the oxidation of lipids such as linoleic acid and vitamin A.[22–24] In the late 1980s, Ames and colleagues[25,26] demonstrated that the antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation. Serum concentrations of bilirubin are high enough to account for a substantial portion of the total antioxidant capacity of serum.[27,28] Thus, bilirubin might alleviate oxidant stress in the blood. However, what matters most is what goes on inside cells. During the oxidant stress associated with myocardial and cerebral infarcts, infection, inflammation, and various causes of ischemia, the intracellular environment is exposed to high concentrations of reactive oxygen species. It has long been assumed that the principal cellular antioxidant is the peptide glutathione (GSH), whose tissue concentrations are millimolar, presumably sufficient to cope with most instances of oxidative stress. By contrast, levels of bilirubin in rodent tissues are only 10 to 50 nanomolar, at least 10 000 times lower than concentrations of GSH (D. Baranano and S.H. Snyder, unpublished observations).

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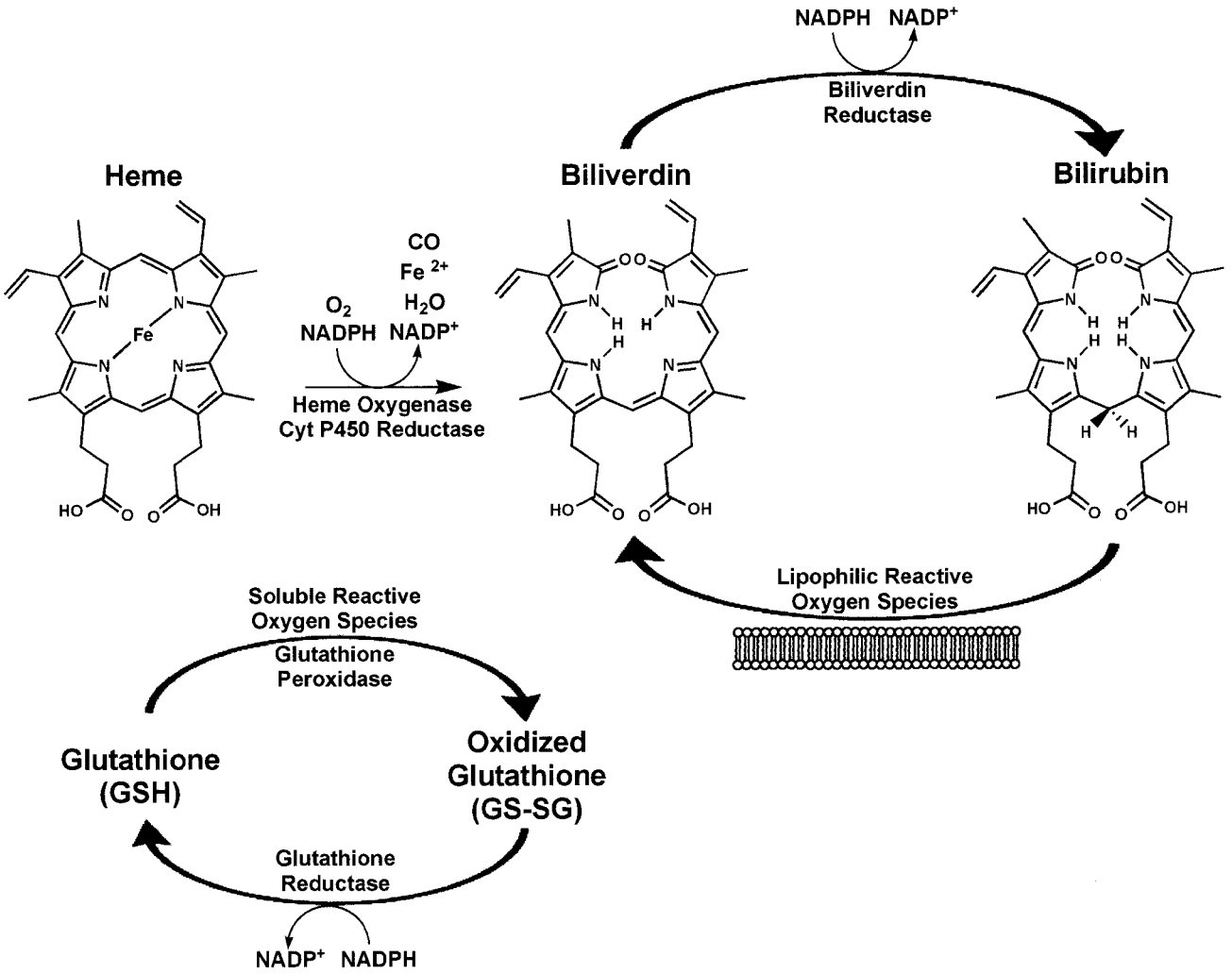

Fig 1. Oxidation-reduction cycles for bilirubin and GSH. Lipophilic reactive oxygen species act directly on bilirubin, leading to its oxidation to biliverdin. BVR catalyzes the reconversion of biliverdin to bilirubin, permitting bilirubin to detoxify a 10 000-fold excess of oxidants. Soluble oxidants are detoxified by GSH, a cycle that requires 2 enzymes, GSH peroxidase and GSH reductase.

BILIVERDIN REDUCTASE CYCLE

Insight into a mechanism whereby low nanomolar concentrations of bilirubin can protect cells came from studies of the heme oxygenase system in the brain. Heme oxygenase was first characterized by Schmid and associates in the 1960s.[29] Maines and associates[30,31] purified and cloned a family of heme oxygenase isozymes. The first known form of heme oxygenase, HO1, is highly concentrated in the spleen, the repository of aging red blood cells. HO1 is a remarkably inducible enzyme, with its synthesis rapidly and profoundly stimulated by more stimuli than almost any other known inducible enzyme.[32] Heme itself, released from degrading red blood cells, is a major stimulus to HO1, accounting for the rapid removal of free heme from the circulatory system. A second form of the enzyme, HO2, is not inducible and is highly concentrated in neurons in discrete regions of the brain, where it forms CO as a neurotransmitter.[3,33]

A role for HO2 in protecting neurons from oxidative damage comes from studies using mice with genetic deletion of HO2. Such mice are much more susceptible to stroke damage elicited by ligation of the middle cerebral artery, whereas mice with knockout of HO1, which are far more debilitated than the HO2 knockouts, do not manifest increased stroke damage.[34,35] Similarly, HO2-deficient animals demonstrate greater brain tissue loss and diminished motor function after traumatic brain injury.[36] Brain cultures of HO2 knockout mice are also exceptionally susceptible to apoptotic death elicited by oxidative stresses such as hydrogen peroxide. Remarkably, as little as 10 nanomolar bilirubin can protect cultures from the oxidant stress of 10 000 times higher concentrations of hydrogen peroxide.[37]

How might one explain this paradox? One possibility would involve cycling between biliverdin and bilirubin. According to this hypothesis, when a molecule of bilirubin acts as an antioxidant, it is itself oxidized to biliverdin. BVR is an abundant and ubiquitous enzyme with a high turnover rate. Hence, endogenous BVR should suffice to reduce newly formed biliverdin back to bilirubin. The intrinsic amplification properties of enzymes could readily augment the antioxidant effects of bilirubin 10 000-fold. Such a cycle would represent an elegant tour de force on the part of nature, making use of bilirubin's anti-

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oxidant capacity but ensuring that tissues had low endogenous levels of bilirubin, as the micromolar levels necessary for direct antioxidant actions would be toxic.

To establish the function of this cycle, we first showed that the isomer of biliverdin generated by reactive oxygen species is the IXα form, which can serve as a substrate for BVR.[38] We then directly examined whether BVR is required by cells to protect against reactive oxygen species. Using the technique of RNA interference, we depleted cells of BVR, leading to a tripling of reactive oxygen species levels and a marked augmentation of cell death. We compared the roles of cellular bilirubin and GSH, depleting the latter with buthionine sulfoximine, an inhibitor of the GSH-synthesizing enzyme Y-glutamylcysteine synthase. Lowering GSH levels 95% led to only a 50% increase in reactive oxygen species and a much more modest increase in cell death.

Might bilirubin and GSH play distinct but complementary roles in protecting cells? Being lipid soluble, bilirubin might primarily protect cells against lipid peroxidation. By contrast, the water-soluble GSH might protect soluble proteins from oxidation.

Besides bilirubin and GSH, cells seem to use several other systems to protect against oxidative stress. Examples include the enzymes superoxide dismutase and catalase, which act in concert to convert superoxide to water. Direct antioxidant actions are elicited by ascorbate and vitamin E.

CLINICAL RELEVANCE

A variety of evidence is accumulating that mild to moderately elevated serum bilirubin levels are associated with better outcome in diseases involving oxidative stress (Table 1). Neonates have long been the principal concern in studies of serum bilirubin levels. Neonatal Gunn rats, which have elevated unconjugated bilirubin as a result of autosomal recessive lack of glucuronyl transferase, are resistant to oxidative damage when reared in hyperoxia.[39] Premature neonates who are treated with supplemental oxygen often manifest retinal damage, presumably associated with oxidative stress. Heyman et al[40] and Yeo et al[41] observed a diminished incidence of retinopathy of prematurity in infants with elevated serum bilirubin, although some studies fail to detect such relationships.[42–47]

Insight into the mechanism of bilirubin protection comes in studies that have monitored the rate of rise in serum bilirubin in the first few days of life in infants with illnesses that are associated with free radical production, such as circulatory failure, neonatal asphyxia, aspiration, and sepsis.[48,49] The rate of bilirubin rise was less in patients than in a control group, suggesting that bilirubin is consumed to cope with oxidative stress. Others have found that bilirubin levels correlate with total antioxidant capacity in blood of neonates[27,28,50,51] and children with sickle cell disease,[52] although this association was not found in one study.[53]

In adults who trained extensively to participate in 50- and 80-kilometer marches, bilirubin and uric acid levels were increased as was resistance to protein oxidation.[54] Patients with longstanding amyotrophic lateral sclerosis have lower bilirubin levels than patients with more recently diagnosed disease.[55] This finding fits with abundant evidence that the pathophysiology of amyotrophic lateral sclerosis involves oxidative stressors that may consume bilirubin. Oxidative metabolites of bilirubin are increased in the urine of patients with sepsis[56] and exacerbations of atopic dermatitis.[57]

Several workers have examined a link between serum bilirubin and coronary artery disease. Two studies involving the offspring and spouses of the original Framingham cohort evaluated the relationship of bilirubin and the risk of myocardial infarction in >5000 participants.[58,59] Higher bilirubin levels, ~15.4 μM (0.9 mg/dL), were associated with lowered risk of myocardial infarction and other cardiovascular disease events compared with individuals with lower bilirubin levels of ~8.6 μM (0.5 mg/dL). In a case-control study, Hopkins et al[60] compared familial coronary artery disease patients with control subjects. The diseased individuals displayed substantially lower serum bilirubin levels than the control subjects. The protective effect of bilirubin on coronary artery disease risk in this population was comparable to that of high-density lipoprotein cholesterol. It is interesting that a history of cigarette smoking was associated with lower serum bilirubin and attenuated the protective effect of bilirubin. Another investigation evaluated the relationship of several liver enzymes and bilirubin with coronary artery disease in >800 men.[61] A 50% decrease in total bilirubin gave rise to an almost 50% augmentation in the likelihood of developing severe coronary artery disease, whereas there was no association with liver enzymes. In this study, serum bilirubin as an inverse risk factor was roughly equivalent to systolic blood pressure. Levinson[62] also observed an inverse relationship between bilirubin levels and severity of ischemic heart disease in angiographically proven cardiac disease.

A U-shaped relationship, with greater risk of ischemic heart disease at the lowest and highest bilirubin levels, was noted in an 11-year study of >7000 British men.[63] However, when individuals with evidence of liver disease were excluded from the analysis, the relationship between bilirubin levels and protection from atherosclerotic disease was linear.[64] Others have also found lower bilirubin levels associated with cigarette smoking, adiposity, and a family history of myocardial infarction.[65]

A particularly elegant approach used individuals with Gilbert's syndrome,[66] a genetic disorder of impaired bilirubin conjugation causing mild to moderate elevations of unconjugated bilirubin.[67,68] The prevalence of ischemic heart disease in a control population of middle-aged individuals was 12%, compared with 2% in those with Gilbert's syndrome. Elevated bilirubin exerted a more prominent role in protecting from ischemic heart disease then did high-density lipoprotein cholesterol levels. This work was extended to a meta-analysis of 11 studies, finding elevated bilirubin levels associated with diminished risk of atherosclerosis.[64] Genetic studies

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TABLE 1. Summary of Studies That Examined the Relationship of Bilirubin to Human Disease

Clinical Condition	Statistically Beneficial Bilirubin Effect	Study Characteristics	Reference
Antioxidant status: neonates	Yes	44 infants with free radical–producing diseases (respiratory distress, circulatory failure, sepsis, aspiration, and asphyxia) had significantly lower rises in serum bilirubin than neonates who were ill from nonoxidative disease.	48
	Yes	25 preterm infants with oxygen-radical diseases (intraventricular hemorrhage, retinopathy, bronchopulmonary dysplasia, and necrotizing enterocolitis) were found to have significantly lower levels of bilirubin than 57 control cases.	49
	Yes	Plasma bilirubin levels were closely correlated with antioxidant levels with bilirubin levels in term infants ($r^2 = 0.774$), although less so in preterm infants.	28
	Yes	In 8 newborns with hyperbilirubinemia (250–435 μM), exchange transfusion decreased both bilirubin levels and plasma antioxidant capacity.	27
	No	In 22 jaundiced preterm infants, bilirubin was not associated with plasma antioxidant status. Individuals with elevated liver function tests were not excluded.	53
	Yes	In 28 preterm infants, the antioxidants bilirubin (~71–111 μM) and uric acid were correlated with total plasma antioxidant levels.	50
	Yes	Plasma from infant blood was oxidized less than that from adult blood, correlating with their respective bilirubin levels.	51
Antioxidant status: adults	Yes	After extensive training and physical exercise, 31 male subjects demonstrated increased levels of bilirubin and uric acid and decreased measures of oxidative damage in serum.	54
	Yes	Bilirubin oxidative metabolites were significantly elevated in the urine of 19 septic patients compared with 28 control subjects.	56
Amyotrophic lateral sclerosis	Yes	Patients with longstanding disease have lower bilirubin than more recently diagnosed individuals.	55
Atopic dermatitis	Yes	13 children with exacerbations of atopic dermatitis had significantly elevated bilirubin oxidative metabolites in their urine, compared with 28 matched control subjects.	57
Cancer	Yes	In a 10-y follow-up of >10 000 Belgians, risk of death from cancer, especially nonlung cancer, decreased with increasing serum bilirubin.	75
Coronary artery disease	Yes	In 877 asymptomatic male Air Force pilots, lower bilirubin levels were correlated with greater risk and severity of coronary artery disease.	61
	Yes	A U-shaped relationship between bilirubin levels and risk of ischemic heart disease was found in 7685 British men. The greatest risk for heart disease was found with low bilirubin levels.	63
	Yes	The relationship between bilirubin level and risk of familial coronary artery disease was studied in 314 men and women. Higher bilirubin levels were associated with protection, and cigarette smoking attenuated this effect.	60
	Yes	In a 3-y genetic study of 1240 Utah adults, individuals with early-onset coronary artery disease had significantly lower bilirubin levels.	69
	Yes	An inverse relationship of bilirubin levels and risk of angiography-proven coronary artery disease was demonstrated in 254 male patients.	62
	Yes	Framingham Offspring Study: In a cohort of 5124 individuals, bilirubin levels were inversely related to risk of myocardial infarction and overall cardiovascular disease.	58,59
	Borderline	In 328 participants of the Family Heart Study, decreases in bilirubin of 1 μM were associated with increased heart disease in male participants, although the finding was not statistically significant ($P = .056$).	70
	Yes	In a 3-y study of 50 participants with hyperbilirubinemia of Gilbert's syndrome, prevalence of coronary artery disease was 2%, compared with 12.1% in a control population.	66
Peripheral vascular disease	Yes	In 31 individuals with peripheral vascular disease, bilirubin levels were significantly lower than normal population levels.	72
	Yes	In 1741 subjects who underwent screening for carotid artery disease, bilirubin levels in the highest quartile were linked to a 32% reduction in risk of plaques.	73
Retinopathy of prematurity	Yes	Lower bilirubin concentrations were associated with worsened retinopathy of prematurity in 45 infants, even when gestational age was controlled for.	40
	No	Lower bilirubin concentrations were found in more severe cases of retinopathy of prematurity, but not when gestational age was controlled.	42
	No	Bilirubin levels were not related to retinopathy of prematurity in 151 neonates.	43
	No	In 24 infants, no protective effect of bilirubin was found for retinopathy of prematurity.	44
	Yes	In 128 premature infants, lower peak bilirubin levels were associated with greater vision loss.	41
	No	No relationship was found for bilirubin levels and retinopathy of prematurity in 157 infants of gestational age 23–26 weeks.	46
	No	Bilirubin levels were unrelated to retinopathy of prematurity in 76 infants.	45
	No	Elevated bilirubin levels did not protect and possibly predisposed to retinopathy of prematurity in 240 very low birth weight infants.	47

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have identified candidate loci for the modest bilirubin elevations associated with protection from cardiac disease,[69–71] including uridine diphosphate glycosyltransferase 1, already known to harbor the mutations of Gilbert's and Crigler-Najjar syndromes.

In peripheral vascular disease, bilirubin levels are lower than in the normal population.[72] Atherosclerotic plaques of the carotid arteries predispose to stroke, and in 1741 subjects who were screened for carotid stenosis, bilirubin levels in the highest quartile were associated with a 32% reduction in risk of developing plaques.[73] An animal model of hyperbilirubinemia also is associated with protection against cerebral ischemia. Rats with a mutation in an organic anion transporter manifest hyperbilirubinemia, mimicking the human condition Dubin-Johnson syndrome.[74] Stroke damage after middle cerebral artery ligation and reperfusion is diminished in these animals. An association of serum bilirubin and cancer risk has also been noted. In a cohort of 10 000 Belgian men and women, the risk of cancer mortality declined with elevated serum bilirubin, especially for nonlung cancer.[75]

May bilirubin administration be therapeutic? Induction of HO1 by treatment with porphyrin derivatives protects against ischemic insults.[76] In perfused rat heart, as little as 100 nanomolar bilirubin reverses the effects of ischemia in cardiac function.[77] Intravenous administration of bilirubin ameliorates pulmonary fibrosis induced by bleomycin.[78] Injury to liver grafts in rats is prevented by rinsing them with bilirubin.[79] Ischemia-reperfusion injury in the rat intestine is ameliorated by intravenous infusion of bilirubin.[80,81]

CONCLUSIONS

The combined evidence from animal and human studies indicates that bilirubin is a major physiologic cytoprotectant. A protective action of modest levels of bilirubin does not alter the well-established dangers of kernicterus associated with major elevations of serum bilirubin. Also, it should be noted that many of the studies indicating beneficial effects of bilirubin involve adults, in whom bilirubin disposition may differ markedly from neonates. A substantial number of the studies, however, deal with infants.

One interesting challenge involves linking the clinical studies, which focus on serum levels of bilirubin, with intracellular mechanisms. Serum bilirubin values are 100 to 1000 times higher than intracellular values. However, ~99% of serum bilirubin is bound to plasma protein and hence unavailable for intracellular antioxidant actions. Because the clinically relevant cellular protection occurs in tissue parenchyma, it is important to understand the way in which serum and intracellular bilirubin levels interrelate. We know little about mechanisms for transporting bilirubin into and out of cells.[82,83] Bilirubin in the serum may have a direct therapeutic action in coping with oxidative stimuli within the blood stream, such as quenching oxidized low-density lipoprotein.[84]

Might there be direct therapeutic implications? In animal studies, bilirubin infusions are therapeutic. Conceivably, one could administer drugs to release bilirubin from its binding sites in serum proteins. Direct translation from animal studies may be feasible. For instance, investigations showing protective effects of bilirubin rinses on organs for tissue grafts in animals[85] may be suitable for application in human subjects. Comparing the antioxidant potential of serum from patients with Gilbert's, Dubin-Johnson, and Rotor syndromes, the last 2 of which have conjugated hyperbilirubinemia, may differentiate the protective effects of unconjugated and conjugated bilirubin. Circulating bilirubin may function most predominantly in vascular disease, whereas tissue bilirubin and BVR might be more relevant in diseases of specific organs. Uric acid was once regarded solely as a toxic metabolite responsible for gout, whereas it is now increasingly appreciated as an antioxidant.[86] Similarly, physiologic antioxidant roles for bilirubin may detoxify its traditionally nefarious reputation.

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Specter M. The extremist. *New Yorker*. April 14, 2003

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