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Forum Review

Antioxidant Activities of Bile Pigments

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ABSTRACT

Biliverdin and bilirubin are reducing species and hence potential antioxidants formed by the action of heme oxygenase and biliverdin reductase. Indeed, there is increasing evidence for the suggestion that a beneficial role of the potentially toxic bilirubin may be to act as a powerful chain-breaking antioxidant in biological systems, and that bilirubin may contribute to the cellular and tissue protection seen with increased heme oxygenase. This article reviews the *in vitro* antioxidant activities of the two bile pigments with emphasis on the different physiological forms of bilirubin and types of oxidants, and discusses these properties in light of the presence and reactivity other nonproteinaceous antioxidants. *Antioxid. Redox Signal.* 6, 841–849.

INTRODUCTION

AEROBIC EUKARYOTIC CELLS AND ORGANISMS consume most of the molecular oxygen by the mitochondrial respiratory chain via sequential reduction to water. However, during normal intermediary metabolism, both the univalent and bivalent reduction of molecular oxygen also occurs via participation of a variety of enzymes [*e.g.*, NAD(P)H oxidases, lipoxygenases, and heme proteins], different respiratory chains, and autooxidation reactions. These pathways of oxygen consumption give rise to the primary oxygen reduction products, *i.e.*, superoxide anion radical and hydrogen peroxide (12), that themselves have the potential to generate other reactive oxygen species, such as hydroxyl, alkyl, alkoxyl, and peroxyl radicals, singlet oxygen, hypochlorous acid, and lipid hydroperoxides. Reactive oxygen species may also interplay with transition metals (principally iron and copper) and nitrogen oxides (24). For example, superoxide anion and peroxyl radicals may react with nitric oxide to generate peroxynitrite and alkylperoxynitrites, respectively, that together with additional reactive nitrogen species, such as nitrogen dioxide, nitryl chloride, and nitrosothiols, may participate in different biological processes.

It is increasingly recognized that various reactive oxygen and nitrogen species (RONS) play important and useful roles in diverse biological processes, such as DNA replication, cell proliferation, antimicrobial reactions of the immune system, and cell signaling. However, RONS can also damage DNA, protein, and lipid, and may thereby contribute to the pathology of cancer, aging, tumor promotion, chronic inflammation, heart disease, and parasitic infections (24). To control the formation of RONS, as well as to repair oxidative damage to macromolecules and tissues, aerobic organisms therefore possess a complex armory of proteinaceous and nonproteinaceous antioxidants (24). The former principally include enzymes that scavenge RONS (*e.g.*, superoxide dismutase and catalase) or repair oxidized molecules (*e.g.*, phospholipase and glutathione peroxidase for phospholipid hydroperoxides), as well as proteins that sequester transition metals to prevent them from participation in inadvertent redox reactions (*e.g.*, ferritin). Nonproteinaceous antioxidants may be divided into antioxidants derived from the diet (*e.g.*, vitamins C and E) and those formed endogenously (*e.g.,* reduced glutathione, ubiquinol-10). An important aspect of antioxidants is their complex makeup and compartmentalization. For example, enzymatic antioxidants are important in cellular defense, whereas transition metal-sequestering proteins and nonproteinaceous antioxidants are comparatively more abundant in the extracellular space (Table 1).

Work from the laboratory of Bruce Ames has suggested that certain end products of oxidative intermediary metabolism (*e.g.*, uric acid and taurine) may serve as protective agents in humans. It was in this context that Alexander Glazer proposed bilirubin to also be an antioxidant of potential

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TABLE 1. ANTIOXIDANT DEFENSES

	Cellular	*Extracellular*
Enzymes	Superoxide dismutase, catalase, glutathione peroxidases/glutathione reductase, glutathione transferases, thioredoxin/thioredoxin reductase, protein disulfide isomerase, glutaredoxin/glutaredoxin reductase, heme oxygenase, phospholipase A_2, methionine sulfoxide reductase	Extracellular superoxide dismutase
Proteins	Ferritin, metallothioneins	Transferrin, albumin, haptoglobin, hemopexin, ceruloplasmin
Nonproteinaceous compounds	*Endogenous:* reduced glutathione (GSH), ubiquinol-10 *Dietary:* vitamin C (ascorbate), vitamin E (α-tocopherol)	*Endogenous:* ubiquinol-10, bilirubin, urate *Dietary:* vitamins C, E

physiological importance (59). Indeed, a growing body of evidence now supports the view that the formation of this bile pigment provides antioxidant protection in a variety of adverse conditions (43).

HEME DEGRADATION

A major metabolic pathway in mammalian systems is the degradation of protoheme derived from hemoproteins such as hemoglobin and cytochrome P450s. This pathway is initiated by heme oxygenase, which converts heme to biliverdin, carbon monoxide, and iron, and includes biliverdin reductase, which reduces biliverdin to bilirubin at the expense of NADPH (Fig. 1). As a result of this, an adult human produces ~300 mg of bilirubin per day. Because of its intramolecular hydrogen bonding, the bilirubin produced is sparingly soluble in water at physiological pH and ionic strength (36). Bilirubin is tightly bound to albumin in order to be transported within the blood circulation (8), from which it is removed mainly through uptake by hepatocytes. In the liver, bilirubin is transformed to a family of water-soluble derivatives by conjugation of one or both of its propionyl groups with glucuronic acid, glucose, or xylose (19) before its excretion into bile. Conjugated bilirubin then reaches the intestine, where it is transformed into urobilinogens and urobilins that are finally excreted into the stool. Conjugated bilirubin in the bile and intestine represents by far the biggest pool of the pigment in the body of normal humans (3). Under physiological conditions, plasma bilirubin concentrations in humans range from ~5 to 17 μ*M*, practically all of which is unconjugated pigment bound to albumin (36). Plasma concentrations of >300 μ*M* are associated with the risk of developing neurologic dysfunction due to preferential deposition of bilirubin in brain and its toxic effects on cell functions. While produced in essentially all cells, the normal range of cellular concentrations of bilirubin is unknown, although it is likely to be present at low concentrations in all cell membranes. In humans, biliverdin is normally not detectable at appreciable concentrations.

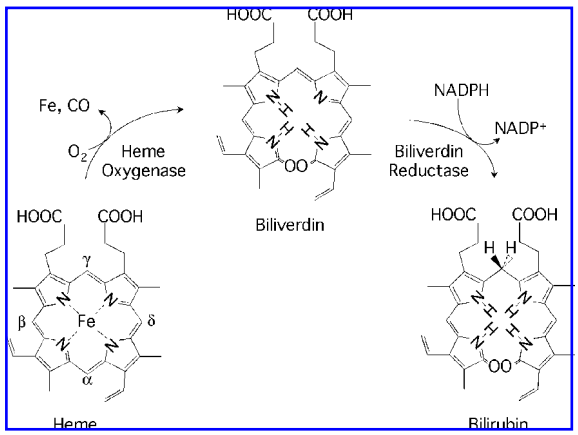

FIG. 1. Oxidative heme metabolism by heme oxygenase and biliverdin reductase giving rise to iron, carbon monoxide, biliverdin, and bilirubin.

There are three isoforms of heme oxygenase, *i.e.*, constitutive heme oxygenases-2 and -3, and inducible heme oxygenase-1 (HO-1), and it is the latter that is generally considered to represent an adaptive and protective response of cells to oxidative stress (30, 53, 64). From an antioxidant point of view, protection may result principally from the removal of reactive species or the formation of antioxidant(s). In the case of HO-1, the situation is complicated in that removal of a reactive species (*i.e.*, heme iron) not only is accompanied by the formation of antioxidant-active bile pigments, but also generates another reactive species (*i.e.*, iron). However, at least under some conditions of oxidative stress, the HO-1-dependent release of iron is linked to increased synthesis of the intracellular iron storage protein ferritin (63), so that iron-catalyzed free radical reactions would be expected to be restricted during periods of subsequent oxidative stress. Nevertheless, a fundamental yet unresolved question is whether, in conditions where induction of HO-1 provides antioxidant protection, this is achieved via elimination of heme, formation of bile pigment(s), or a decrease in the cellular pool size of redox-active iron. Similarly, another important issue that awaits clarification is whether, and if so to what extent, an increase in the antioxidant defense is responsible for the increased protection resulting from HO-1 induc-

tion. Important in this context, carbon monoxide has a number of potentially beneficial activities (43) that could contribute to, or fully explain, the protection seen with HO-1 induction, although it is presently unclear how precisely the concentrations of endogenously produced carbon monoxide relate to those required for the protective activities seen with the exogenously added gaseous molecule. Keeping these potentially important considerations in mind, let us now review the evidence in support of an antioxidant role of bile pigments, with particular emphasis on bilirubin.

ANTIOXIDANT ACTIVITIES OF BILIRUBIN

Early, indirect evidence for an antioxidant activity of bilirubin and biliverdin comes from studies showing that small quantities of the pigment stabilize vitamin A and β-carotene during intestinal uptake (4), and that animals with low plasma bilirubin show early symptoms of vitamin E deficiency (28). There have also been early reports on the reaction of bilirubin with reactive oxygen species (36, 47). Unconjugated bilirubin efficiently scavenges singlet oxygen (52) and serves as a reducing agent for certain peroxidases, including horseradish peroxidase and prostaglandin H synthase in the presence of hydrogen peroxide or organic hydroperoxides (9, 27, 48). Similarly, bilirubin glucuronides are oxidized by rat liver microsomes in the presence of lipid peroxides (15).

Bilirubin in its free, albumin-bound, and conjugated forms contains an extended system of conjugated double bonds and a pair of reactive hydrogen atoms (depicted in Fig. 1), the latter of which are most likely involved in antioxidant activity via H-donation to an incipient radical, such as lipid peroxyl radical ($LOO^{\cdot}$), to form lipid hydroperoxide (LOOH) and bilirubin radical ($Bilirubin^{\cdot}$):

$$LOO^{\cdot} + \text{Bilirubin} \rightarrow LOOH + \text{Bilirubin}^{\cdot} \quad (1)$$

By contrast, biliverdin only contains an extended system of conjugated double bonds, so that its antioxidant activity is probably due to formation of a resonance-stabilized, carbon-centered radical resulting from the addition of radicals such as $LOO^{\cdot}$ to biliverdin:

$$LOO^{\cdot} + \text{Biliverdin} \rightarrow LOO\text{–Biliverdin}^{\cdot} \quad (2)$$

Unconjugated bilirubin, conjugated bilirubin, and biliverdin

The first systematic studies on the *in vitro* antioxidant activity of bilirubin utilized chemically defined and controlled free radical chain oxidation of linoleic acid induced by thermolabile radical initiators as model systems (54, 58, 59). These experiments demonstrated unambiguously that unconjugated bilirubin, at micromolar concentrations, efficiently scavenges peroxyl radicals in homogeneous solution or multilamellar liposomes. The antioxidant activity of bilirubin in liposomes increases at physiologically relevant low oxygen tension where it can surpass that of α-tocopherol (Fig. 2), biologically the most active form of vitamin E and commonly

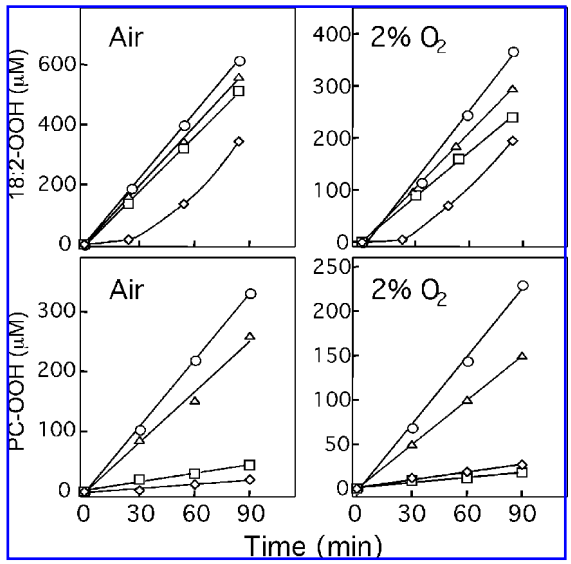

FIG. 2. Oxidation of linoleic acid (top) and soybean phosphatidylcholine (bottom) in aqueous dispersion under air (left) and 2% O_2 and 98% N_2 (right) in the absence (○) or presence of 10 μ*M trans*-β-carotene (△), bilirubin (□), or *dl*-α-tocopherol (◇) at 50°C. Progression of lipid oxidation was assessed by the accumulation of linoleic acid hydroperoxides (18:2-OOH) and phosphatidylcholine hydroperoxides (PC-OOH). Adapted from reference 59.

considered the most important lipid-soluble antioxidant in humans (10). Similarly, water-soluble, conjugated bilirubin effectively inhibits peroxyl radical-induced oxidation of lipids (54). Under these *in vitro* conditions, biliverdin scavenges peroxyl radicals more effectively than unconjugated (59) or conjugated bilirubin (54): each molecule of bilirubin and biliverdin scavenges 1.9 and 4.7 molecules of peroxyl radicals, respectively.

In addition to direct scavenging of peroxyl radicals, bilirubin and biliverdin are capable of acting in synergy with membrane-bound α-tocopherol. This can be shown with phosphatidylcholine liposomes undergoing oxidation initiated by a lipid-soluble azo-compound within the liposome membranes in the absence and presence of membrane-bound α-tocopherol and water-soluble bile pigments. In the absence of α-tocopherol, lipid peroxidation proceeds linearly and without delay, and micromolar amounts of conjugated bilirubin or biliverdin inhibit this oxidation (Fig. 3). Other water-soluble antioxidants, such as ascorbate (the antioxidant active form of vitamin C), reduced glutathione, or urate, are not protective under these conditions. The presence of α-tocopherol in liposome membranes suppresses the oxidation initially almost completely, thereby producing a clear induction period. However, in the combined presence of α-tocopherol and either of the two bile pigments, this induction period is increased substantially (Fig. 3A), and the vitamin is spared from consumption during the oxidation of phosphatidylcholine liposomes (Fig. 3B). Similar to the situation with liposome membranes and conjugated bilirubin, unconjugated bilirubin can also synergize with α-tocopherol in the

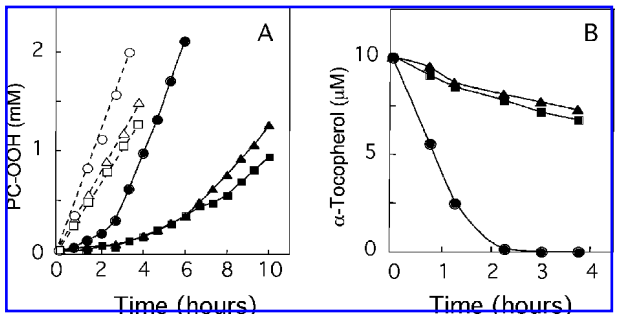

FIG. 3. Bile pigments interact in synergy with membrane-bound α-tocopherol. Oxidation of soybean phosphatidylcholine liposomes was initiated under air and at 50°C by a lipid-soluble azo-compound in the absence (open symbols) and presence of membrane-bound α-tocopherol (filled symbols), and the absence (circles) and presence of conjugated bilirubin (triangles) or biliverdin (squares). Oxidation was followed as the time-dependent (**A**) accumulation of phosphatidylcholine hydroperoxides (PC-OOH) and (**B**) consumption of α-tocopherol. All antioxidants were used at 10 μ*M* final concentrations. Adapted from reference 57.

prevention of lipoprotein lipid oxidation (41). This synergism is based on the ability of bile pigments to chemically reduce α-tocopheroxyl radical, the one-electron (1*e*)-oxidation product of α-tocopherol, in a chemically clean system, as demonstrated directly by electron paramagnetic resonance spectroscopy (68). Together, these studies demonstrate that one

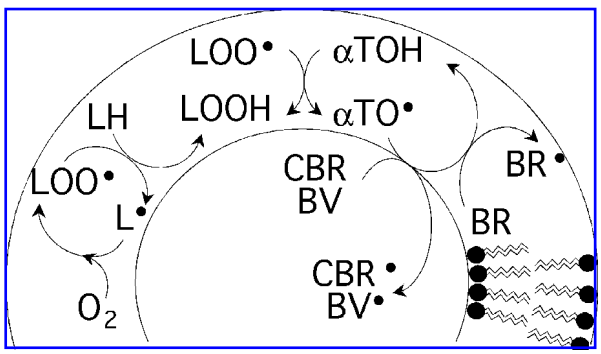

FIG. 4. Model of inhibition of membrane lipid peroxidation by bile pigments. Membrane lipid peroxidation proceeds via a chain reaction whereby the peroxidation chain-carrying lipid peroxyl radical ($LOO^{\cdot}$) abstracts a hydrogen atom from a lipid molecule, producing a molecule of lipid hydroperoxide (LOOH) and a carbon-centered lipid radical ($L^{\cdot}$). The latter adds to molecular oxygen to regenerate $LOO^{\cdot}$. The most abundant lipid-soluble antioxidant, α-tocopherol (αTO), intercepts with this chain reaction by scavenging $LOO^{\cdot}$, producing LOOH and α-tocopheroxyl radical ($\alpha TO^{\cdot}$). The latter is then reduced by water-soluble conjugated bilirubin (CBR) and biliverdin (BV), or membrane-bound unconjugated bilirubin (BR) to generate the corresponding bile pigment-derived radical, $CBR^{\cdot}$, $BV^{\cdot}$, and $BR^{\cdot}$, respectively. For simplicity, the model does not include membrane-bound ubiquinol-10 and ascorbate in the aqueous phase.

way by which bile pigments may offer cellular protection is by inhibiting membrane lipid peroxidation in synergy with vitamin E (Fig. 4).

In addition to scavenging lipid peroxyl and α-tocopheroxyl radical, bile pigments have been reported to scavenge a number of other RONS (Table 2), suggesting that bilirubin and biliverdin can scavenge both 1*e*- and 2*e*-oxidants, similar to ascorbate (11). By comparison, many other antioxidants are effective against only one class of oxidants. For example, α-tocopherol reacts rapidly with 1*e*-oxidants, but is a poor scavenger of 2*e*-oxidants (26). Examples of 2*e*-oxidants scavenged by bilirubin include singlet oxygen (52), hypochlorous acid (56), quinones (36), and peroxynitrite (29, 62).

Hypochlorous acid is a powerful and the major oxidant formed by activated neutrophils via the action of myeloperoxidase on hydrogen peroxide and chloride (25). Conjugated bilirubin and biliverdin are not able to inhibit the NADPH oxidase-dependent production of superoxide anion radical (the precursor of hydrogen peroxide), and they do not react directly with hydrogen peroxide *in vitro* or act as substrate for myeloperoxidase (56). This inability to react directly with hydrogen peroxide appears to contrast the protection against hydrogen peroxide-mediated damage in vascular endothelial cells (39), smooth muscle cells (14), and hippocampal and cortical neuronal cultures (18) seen with bilirubin added *in vitro* or derived from inducing heme oxygenase activity. However, it is important to note that hydrogen peroxide is a relatively weak oxidant and that cell cytotoxicity is likely due to more reactive oxidants derived from hydrogen peroxide that may be intercepted by bilirubin. In contrast to hydrogen peroxide, the pigments rapidly react with the powerful oxidant hypochlorous acid and can efficiently protect other biological targets of this oxidant, *e.g.*, α1-antiprotease, from oxidation (56).

Studies on the reaction of bilirubin with reactive nitrogen species are limited largely to albumin-bound bilirubin (see below). Initial studies (37, 62) showed that exposure of human plasma to reagent peroxynitrite or 3-morpholinosydnonimine, which simultaneously produces superoxide anion radical and nitric oxide, and hence peroxynitrite (32), results in the consumption of bilirubin, demonstrating that the pigment scavenges peroxynitrite or oxidants derived from it. By

TABLE 2. REACTION OF (CONJUGATED) BILIRUBIN AND BILIVERDIN WITH DIFFERENT RONS

RONS	*Scavenging*	*Reference*
Superoxide anion radical	No	56
Hydrogen peroxide, alkyl hydroperoxides	No	56
Singlet oxygen	Yes	52
Quinones	Yes	36
Peroxyl radicals	Yes	59
α-Tocopheroxyl radical	Yes	68
Mixed function oxidase/ hydroxyl radical	Yes	9, 27
Hypochlorous acid	Yes	56
Nitric oxide	Yes	29
Peroxynitrite	Yes	29, 37, 62
Nitroxyl radical	Yes	29

contrast, it is less clear whether the bile pigments react directly with nitric oxide. In support of such a notion, a study using aerobic culture medium containing 10% serum reported oxidation of bilirubin and biliverdin by the nitric oxide donor diethylamine NONOate, Angeli's salt (which decomposes to give nitroxyl), and 3-morpholinosydnonimine (29). Disappearance of the bile pigments was also seen under anaerobic conditions with bilirubin and biliverdin added to phosphate-buffered saline, pH 7.4, and using gaseous nitric oxide (29), although the overall extent of this reaction was limited, particularly in the case of bilirubin. These results were interpreted as evidence for the ability of biliverdin and, to a lesser extent, bilirubin to react directly with nitric oxide (29). Unfortunately, such interpretation is complicated by the fact that bilirubin is not soluble in aqueous solutions at physiological pH, and that phosphate buffer contains contaminating metals that could have contributed to the oxidation of bile pigments. Also, the experiments reported (29) did not include controls where bilirubin and biliverdin were incubated in the absence of gaseous nitric oxide. Therefore, additional studies are required to establish unambiguously whether the bile pigments can react directly with nitric oxide.

Albumin-bound bilirubin

As indicated earlier, most of the extracellular unconjugated bilirubin is bound to albumin. Binding of bilirubin dianion to the primary binding site on albumin is thought to involve ion pairing, hydrogen bonding, and π-interaction between amino acid side chains and the pigment, thereby fixing the two planar dipyrrole moieties of the pigment in an out-of-plane position (8). Such asymmetric positioning of the bilirubin molecule on albumin is expected to expose the reactive hydrogen atoms at C-10 for initial hydrogen abstraction by radicals (Fig. 5). Indeed, albumin-bound bilirubin at concentrations found in human plasma of healthy adults is a very efficient peroxyl radical scavenger, and it protects fatty acids transported on albumin from oxidative damage (58). In fact, the rate constant for the reaction of albumin-bound bilirubin with alkylperoxyl radicals has been estimated as ~1.7×10^5 M^{-1} s^{-1} (58), or ~30 times higher than the rate constant calculated for the reaction of peroxyl radicals with unconjugated bilirubin, *i.e.*, 5×10^3 M^{-1} s^{-1} (59). Thus, binding to albumin appears to confer increased reactivity to bilirubin. In addition, it confers increased specificity, as peroxyl radical-induced oxidation of albumin-bound bilirubin results in the stoichiometric conversion of the pigment into biliverdin (58). By comparison, little biliverdin is formed when unconjugated bilirubin is oxidized with peroxyl radicals (59).

In addition to protecting albumin-bound fatty acids from oxidation, several studies have shown that albumin-bound bilirubin also protects the protein against damage by different types of RONS. Thus, photooxidation of albumin-bound bilirubin results in the initial oxidation of bilirubin, without detectable oxidation of the protein as judged by amino acid analysis (45). Furthermore, exposure of albumin to radiolytically generated hydroxyl and superoxide anion radicals results in the loss of ~15 amino acids for each radical generated, and the presence of bilirubin decreases amino acid consumption about fourfold (42). A protective effect of albumin-bound bilirubin is also seen as inhibition of both γ-irradiation-induced cleavage of the carrier protein and formation of carbonyls and dityrosine (40). Similarly, albumin-bound bilirubin protects plasma protein amino acids from oxidation induced by peroxynitrite (37, 62). This protective action appears to be due predominantly to scavenging of secondary radicals rather than to intercepting of hydroxyl radical (42) or peroxynitrite itself (37). Finally, and similar to the situation with unconjugated and conjugated bilirubin (see above), albumin-bound bilirubin can interact with α-tocopherol by reducing α-tocopheroxyl radical (68), including that incorporated in lipoproteins (7, 41). This may be biologically important as lipoprotein-associated α-tocopheroxyl radical has prooxidant activity and can promote lipoprotein oxidation (5, 6), and lipoprotein oxidation has been linked to atherosclerosis (51).

The above studies show that binding of bilirubin to albumin confers the pigment with increased specificity and reactivity against a variety of RONS, and this can lead to increased protection of plasma proteins and lipids. This antioxidant activity of bilirubin may not be limited to plasma, as ~60% of human albumin is located in the extracellular space (50) and extrahepatic, extravascular pool of bilirubin correlates with extrahepatic albumin (8). Interestingly, albumin appears to leave the blood stream and to appear in inflammatory exudate,

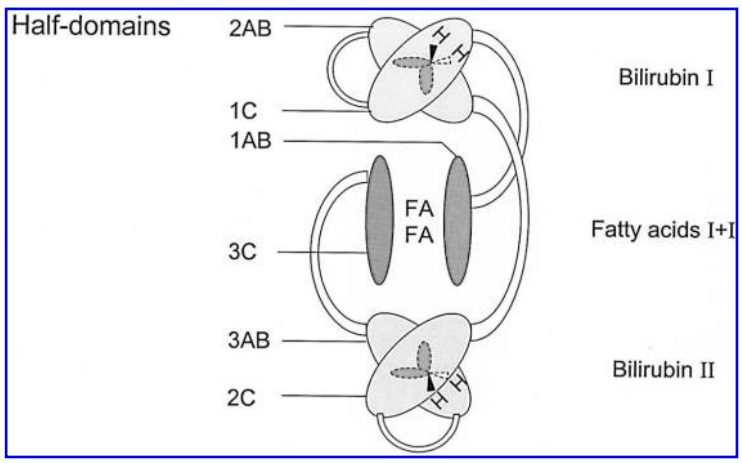

FIG. 5. Half-domain model of serum albumin with two binding sites for bilirubin and one for two molecules of fatty acid (FA). In this model, both dipyrrole moieties of bilirubin, when bound to its primary and secondary binding sites within half-domains 2AB/1C and 3AB/2C, respectively, are out-of-plane. This exposes the pair of reactive hydrogen atoms (H) at the C-10 position of bilirubin for participation in redox reactions.

raising the possibility that the pigment also provides antioxidant protection at sites of increased production of RONS by phagocytic cells. However, there are limitations to the extent of antioxidant protection by albumin-bound bilirubin. For example, in its bound form, the pigment is not able to protect albumin effectively against oxidative damage induced by hypochlorous acid (60). Albumin is thought to contribute largely to the protective effect of serum toward hypochlorous acid (66). These findings suggest that albumin's methionine, cysteine, cystine, tryptophan, and lysine residues, *i.e.*, the most reactive amino acids toward hypochlorous acid (44), outcompete albumin-bound bilirubin for this oxidant.

In vivo *antioxidant activity*

As we have seen in the preceding sections, bilirubin and biliverdin are effective reducing agents and possess antioxidant activity in a number of different *in vitro* systems and against various RONS. What is less clear is whether, and if so to what extent, this antioxidant activity extends to *in vivo* situations. At present, there is only indirect evidence in support of bilirubin as an antioxidant *in vivo*. For example, HO-1 is induced by a number of conditions known to exert an oxidative stress, such as exposure of animals to certain metal ions (33), sulfhydryl reactive agents (31, 34), and endotoxin (23). Also, heme oxygenase activity is increased in mice deprived of selenium (49), a cofactor of glutathione peroxidases, and in guinea pigs deprived of ascorbate (65). Furthermore, mice deficient in HO-1 show an increased vulnerability to endotoxin challenge, and HO-1-deficient cells have increased susceptibility to hydrogen peroxide (46). These and other findings are consistent with, although do not prove, an *in vivo* antioxidant activity of bilirubin, particularly in situations of heightened oxidative stress or when other antioxidant defenses are compromised. As we have learned, changes in heme oxygenase activity also impact on cellular heme and iron status (Fig. 1), and it is presently not possible to distinguish these changes from those in bilirubin concentration as the underlying basis for observed differences in oxidant sensitivity resulting from altered heme oxygenase activity.

To substantiate an *in vivo* antioxidant activity of bilirubin, it may be useful in the future to examine the effect of altered pigment concentrations on established *in vivo* markers of oxidative damage, such as oxidized lipids. Such approach has been used successfully to demonstrate *in vivo* antioxidant activity of other nonproteinaceous antioxidants. For example, supplementing guinea pigs with ascorbate decreases hepatic concentrations of F_2-isoprostanes (13), a nonenzymatic oxidation product of arachidonic acid commonly used as an index of *in vivo* oxidative damage (38). Similarly, increasing the concentration of ubiquinol-10 in atherosclerosis-prone, apolipoprotein E-deficient mice by dietary supplements decreases the concentration of lipid hydroperoxides in affected blood vessels (69). Unfortunately, such studies have not been carried out to date with bilirubin. However, Dennery *et al.* (17) reported plasma concentrations of thiobarbituric acid and lipid hydroperoxides to be decreased in hyperbilirubinemic Gunn rats after exposure to ≥95% oxygen for 3 days compared with nonjaundiced littermates. The Gunn rat is a mutant Wistar strain with an autosomal recessive deficiency of glucuronyl transferase, the enzyme responsible for conjugation of bilirubin, resulting in high levels of unconjugated bilirubin in homozygous animals. In this study, exposure of the animals to hyperoxia substantially increased plasma bilirubin (17), indicative of induction of heme oxygenase. Thus, in addition to increased bilirubin concentrations, altered (heme) iron metabolism may have affected the parameters used to assess oxidative damage. In addition, a potential problem is that the thiobarbituric acid assay used is an indirect measure of lipid oxidation that, when applied to plasma, bilirubin can interfere with. Similarly, lipid hydroperoxides were determined indirectly via hemoglobin-mediated metabolism coupled to the oxidation of a methylene blue derivative, the latter reaction of which could conceivably be inhibited by bilirubin. Therefore, although the studies of Dennery *et al*. (17) are generally supportive of an *in vivo* antioxidant activity of bilirubin, it would be interesting to test this more directly, *e.g.*, by using Gunn rats both without and with oxidant exposure, as well as using more direct measures of oxidative damage.

Keeping the above-mentioned limitations in mind, let us briefly consider different factors that likely determine whether bile pigments act as *in vivo* antioxidants. These factors include the local concentrations and oxidant reactivity of bile pigments relative to that of other antioxidants. Regarding availability, there is little evidence that in humans biliverdin accumulates to significant concentrations, so that its role as a physiological antioxidant is questionable. The situation may be different, however, in species such as birds, amphibians, and reptiles that do not appear to efficiently convert biliverdin to bilirubin.

As we have learned, albumin-bound bilirubin is present in the extracellular space at concentrations up to 15 μ*M*. Regarding radical scavenging in the aqueous environment of the extracellular space, the main "competitors" of albumin-bound bilirubin are ascorbate (≤50 μ*M*) and urate (≤300 μ*M*) (55). Thus, from a quantitative point of view, albumin-bound bilirubin is not a major radical scavenger in the extracellular space, as has been verified experimentally in the case of human plasma undergoing controlled lipid peroxidation initiated by aqueous peroxyl radicals (67). However, the biological relevance of such quantitative consideration is questionable as even under the most pathologic situation nonproteinaceous, low-molecular-weight antioxidants are rarely depleted. Arguably more important are qualitative considerations, such as which antioxidant is consumed first, and hence may become limiting, during conditions of increased oxidant production. To address this issue, Frei *et al.* exposed freshly obtained human plasma to a constant flux of peroxyl radicals and followed the time-dependent consumption of antioxidants in conjunction with the accumulation of lipid hydroperoxides, the latter as an index of oxidative damage (20). This and additional studies (21, 22, 41, 61) revealed that albumin-bound bilirubin represents a *second* line antioxidant defense against peroxyl radical-induced lipid peroxidation in the extracellular space; the first lines of aqueous and water-soluble antioxidant defense are provided by ascorbate and ubiquinol-10, respectively (Fig. 6). A common feature of ascorbate, ubiquinol-10, and albumin-bound bilirubin is that they all scavenge peroxyl radicals and are able to reduce α-tocopheroxyl radical. Importantly, albumin-bound bilirubin is able to inhibit lipoprotein lipid peroxidation in

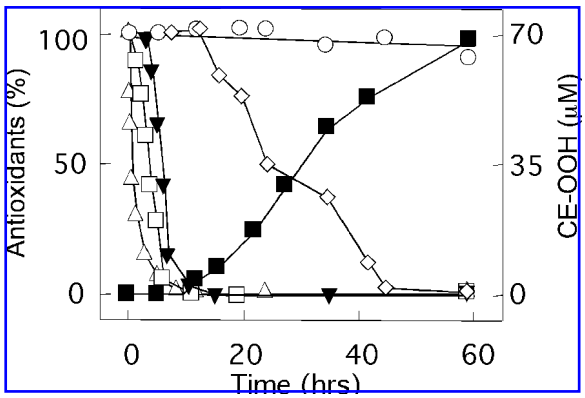

FIG. 6. Time-dependent consumption of antioxidants and accumulation of lipid hydroperoxides during oxidation of human plasma induced by lipid-soluble peroxyl radicals. Plasma was supplemented with 2 m*M* 2,2′-azobis(2,4-dimethylvaleronitrile) and incubated under air at 37°C for the indicated periods of time before the concentrations of ubiquinol-10 (△), ascorbate (□), bilirubin (▼), uric acid (◇), α-tocopherol (◯), and hydroperoxides of cholesterylesters (■) were determined as described. Adapted from reference 41.

the absence of ascorbate and ubiquinol-10 (41), so that it has the capability to act as "backup" for these antioxidants. However, ascorbate and ubiquinol-10 react faster with peroxyl and phenoxyl radicals compared with albumin-bound bilirubin, and this explains their preferred consumption under these oxidizing conditions.

Based on the above observations, it may be argued that for cell membranes, too, unconjugated bilirubin represents a "backup" line of antioxidant defense, behind that of ascorbate and ubiquinol-10. This notion is supported by the observation that, compared with albumin-bound bilirubin, the unconjugated pigment is a less efficient radical scavenger, as judged by the respective rate constants for their reaction with peroxyl radicals (see above). In biological membranes, α-tocopherol is likely far more abundant than unconjugated bilirubin, so that radical scavenging by the pigment will be limited largely to intercepting α-tocopheroxyl radical, and this will take place in competition with ascorbate and ubiquinol-10, both of which are also abundant and ubiquitous (1). The importance of bilirubin as a membrane antioxidant increases as the first line antioxidants become limited or depleted. In this context, it is perhaps important to note that cultured cells are commonly deficient in ascorbate and α-tocopherol, so that under these conditions the importance of bilirubin as an antioxidant probably increases and extends to the scavenging of primary radicals. This may contribute to the protection seen with induction of HO-1 in cultured cells exposed to oxidation stress.

It has been argued recently that bilirubin is a powerful cytoprotective antioxidant on the basis that cellular depletion of bilirubin by RNA interference with HO-1 markedly augments tissue levels of reactive oxygen species and causes apoptotic cell death, and that biliverdin reductase recycles bilirubin after its oxidation to biliverdin (2). As mentioned earlier, however, it is not possible to attribute a beneficial antioxidant activity to the bile pigment under conditions where heme oxygenase activity is interfered with. Regarding recycling of bilirubin, it is worth noting that biliverdin is not always produced upon oxidation of bilirubin. In fact, and as noted earlier, oxidative conversion of bilirubin to biliverdin is largely limited to the albumin-bound form of the pigment, and this is likely due to the specific orientation of the molecule when bound to its carrier protein (Fig. 5). In apparent contrast to the results reported by Baranano *et al.* (2), oxidation of free bilirubin by peroxyl (59) or tocopheroxyl radicals (41) yields predominantly nonspecific oxidation products. Even if cellular recycling of bilirubin from biliverdin were taking place, the pigment would still have to compete with the first line antioxidants ascorbate and ubiquinol-10, for which efficient recycling mechanisms also have been reported (16, 35). Thus, it seems important that future studies use cells with physiological concentrations of ascorbate, ubiquinol-10, and α-tocopherol to evaluate the precise role and contribution of bilirubin as a cellular antioxidant.

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ABBREVIATIONS

1*e* and 2*e*, one- and two-electron; HO-1, heme oxygenase-1; $LOO^{\cdot}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; RONS, reactive oxygen and nitrogen species.

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