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Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in bile

(xenobiotics detoxification/reactive oxygen species/biliverdin)

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ABSTRACT **Conjugated bilirubin and copper ions at their physiological concentrations in bile may play an important role in hydroperoxide and other detoxification. Conjugated bilirubin may also be an important chain-breaking antioxidant preventing lipid peroxidation. Bilirubin ditaurine (BR-DT), a water-soluble model compound of conjugated bilirubin, completely prevents the peroxyl radical-induced oxidation of phosphatidylcholine in either multilamellar liposomes or micelles. This antioxidant activity is associated with the bilirubin moiety of BR-DT, since taurine alone is inefficient in scavenging peroxyl radicals. The number of peroxyl radicals trapped per molecule of BR-DT is 1.9, compared to 4.7 trapped per molecule of biliverdin, the water-soluble physiological precursor of bilirubin. Peroxyl radical-induced oxidation of BR-DT results in a spectral shift in maximal absorbance toward shorter wavelengths; biliverdin is not formed as a major oxidation product. BR-DT, but neither taurine nor biliverdin, greatly accelerates the cupric ion-catalyzed decomposition of linoleic acid hydroperoxide. In the presence of ferric ion, BR-DT shows no lipid hydroperoxide-degrading activity. Addition of cupric ion to BR-DT results in formation of a complex with spectral features similar to that of a biliverdin–cupric ion complex, indicating that BR-DT and cupric ion undergo redox reactions.**

In adult humans, the degradation of protoheme results in the production of ≈300 mg of bilirubin per day (1). The unconjugated bilirubin produced is sparingly soluble in water at physiological pH and is tightly bound to albumin to be transported within the circulation (2), 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 (3) before its excretion into bile. Under physiological conditions, biliary bilirubin concentrations in humans range from 0.35 to 4 mM, practically all of which is present in conjugated form primarily as diglucuronide (4). Conjugated bilirubin then reaches the intestine, where it is transformed into urobilinogens and urobilins, which are finally excreted into the stool (1). Conjugated bilirubin in the bile and intestine represents by far the biggest pool of the pigment in the body of normal humans (5, 6).

We have proposed that bilirubin may function as a natural antioxidant (7, 8). Some earlier and neglected work has also suggested that bilirubin might play a role as an antioxidant (9–11). Indeed, reactions of bilirubin involving free radicals (12, 13) or toxic oxygen reduction products are well documented in the literature: unconjugated bilirubin scavenges singlet oxygen with high efficiency (14), reacts with superoxide anion (15) and peroxyl radicals (7), and serves as a reducing substrate for peroxidases in the presence of hydrogen peroxide or organic hydroperoxides (16, 17). Albumin-bound bilirubin efficiently inhibits the peroxyl radical-induced oxidation of albumin-bound fatty acids (8). At present, little is known about the potential antioxidant activity of conjugated bilirubin, although Cuypers and coworkers (18) reported a lipid peroxide-induced oxidation of bilirubin and its glucuronides by rat liver microsomes. We now show that bilirubin ditaurine (BR-DT), a commercially available model compound of conjugated bilirubin, efficiently scavenges peroxyl radicals, thereby preventing lipid peroxidation. Lipid hydroperoxides are known to be decomposed by some metal ions in a process that is accelerated by hydrogen donors such as ascorbate and cysteine (19). Since the antioxidant activity of bilirubin may be due to its hydrogen-donating activity (7, 8), and since bile is the excretory medium for >80% of the absorbed copper in humans (20), we also investigated whether conjugated bilirubin accelerates the copper-catalyzed decomposition of a lipid hydroperoxide.

MATERIALS AND METHODS

Production of Peroxyl Radicals. Peroxyl radicals were produced by the water-soluble azo compound 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH, Polyscience, Warrington, PA). AAPH decomposes thermally to yield peroxyl radicals at a known and constant rate (21, 22). R_i, the overall rate of peroxyl radical formation, is obtained by measuring the length of induction period, τ, produced by a known amount of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) via $R_i = n\,[\text{Trolox}]/\tau_{\text{Trolox}}$ (23). The stoichiometric factor n represents the number of peroxyl radicals trapped per molecule and is 2.0 for Trolox (24), a water-soluble analogue of vitamin E. The n values of some bile pigments were determined by comparing the length of induction period produced by a known amount of the compound tested with that produced by a known amount of Trolox as described (23).

AAPH-Induced Oxidation of Phosphatidylcholine. Multilamellar liposomes (7) were prepared using purified (25) soybean phosphatidylcholine (PtdCho; Sigma) at 20 mM in 50 mM phosphate buffer/0.154 M NaCl, pH 7.0. Incubations were carried out in a shaking waterbath at 37°C under air in the absence (control) or presence of various concentrations of either synthetic bilirubin ditaurine (Porphyrin Products, Logan, UT), taurine (Aldrich), biliverdin IX dihydrochloride (Porphyrin Products), Trolox (Aldrich), or xanthobilirubinic acid {1*H*-pyrrole-3-propanoic acid 5-[3-ethyl-1,5-dihydro-4-

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; BR-DT, bilirubin ditaurine; 18:2-OOH, linoleic acid hydroperoxide; PtdCho, phosphatidylcholine; PtdCho-OOH, PtdCho hydroperoxide.

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methyl-5-oxo-2*H*-pyrrol-2-ylidene)methyl]-2,4-dimethyl}. BR-DT was used as a model compound for bilirubin diglucuronide, since the latter is not readily available. Xanthobilirubinic acid, synthesized by D. A. Lightner (University of Nevada, Reno), was generously given to us by A. F. McDonagh (University of California, San Francisco). Biliverdin and xanthobilirubinic acid were dissolved in a few drops of 0.05 M NaOH before the stock solutions were adjusted to the appropriate volumes with H_2O. The quantities of NaOH used did not produce a significant change in the final pH of the reaction mixture. Oxidation of PtdCho was initiated by the addition of 10 mM AAPH. At various time points, aliquots of the reaction mixture were removed and analyzed for PtdCho hydroperoxide (PtdCho-OOH) at 234 nm by HPLC on an analytical C_{18}-DB column (Supelco, Bellefonte, PA) with methanol containing 0.02% triethylamine (Fisher Scientific, Fair Lawn, NJ) (1 ml/min) as the mobile phase (25). PtdCho micelles were prepared by the addition of 50 mM taurocholate to the reaction mixture.

Quantitation of BR-DT. Aliquots of the reaction mixture were removed and analyzed and quantitated for BR-DT by HPLC at 460 nm using an analytical C_{18} column (Supelco) with 0.1 M di-*n*-octylamine acetate in methanol and H_2O, pH 7.7 (85:15, vol/vol) (1.3 ml/min) as the eluant.

Degradation of Linoleic Acid Hydroperoxide (18:2-OOH). Purified 18:2-OOH (20 μM) (25) was added to ice-cold 50 mM phosphate buffer/0.154 M NaCl, pH 7.0, containing the additions indicated in the legends to Fig. 3 and Table 1. An aliquot was removed immediately and extracted with 2 vol of cold hexane before the reaction mixture was placed in a waterbath equilibrated at 25°C. The hexane used was prewashed with water and methanol to remove trace amounts of hydroperoxides, which can interfere with the analysis of 18:2-OOH. At various time points, aliquots were removed and also extracted with 2 vol of cold hexane. The two phases were separated by a 2-min centrifugation at 1500 × *g*, and a known amount of the organic phase was removed, dried under a stream of argon, and redissolved in methanol. Using this extraction system, the amount of 18:2-OOH extracted into the hexane phase was linear to the amount of 18:2-OOH added to the reaction mixture over a range of 0–20 μM, and the efficiency of extraction was 25% ± 5%. Quantitation of 18:2-OOH was done with an HPLC post-column chemiluminescence detection system using an analytical C_{18}-DB column and methanol containing 0.02% triethylamine as the mobile phase (25).

RESULTS

Incubation of PtdCho, dispersed as multilamellar liposomes in an aqueous solution, with the water-soluble radical initiator AAPH, resulted in the formation of PtdCho-OOH at a constant rate (Fig. 1*A*). Under oxygen concentration of air, BR-DT at micromolar concentrations initially inhibited the oxidation of PtdCho completely, thereby producing a clear induction period. The length of induction period was proportional to the initial concentration of BR-DT used. This inhibition of oxidation of PtdCho was due to the peroxyl radical trapping activity of the bilirubin portion rather than the taurine moieties of BR-DT since the presence of taurine, even at concentrations exceeding those used in Fig. 1*A*, did not result in a significant inhibition of AAPH-induced oxidation of PtdCho (Fig. 1*B*). As judged by HPLC analysis, BR-DT, when dissolved in phosphate-buffered saline and when incubated under air at 37°C, was not completely stable and disappeared at a rate of 0.03 μM/min (Fig. 1*C*). The presence of 10 mM AAPH increased the initial rate of oxidation to 0.36 μM/min. BR-DT at 20 μM initially also completely inhibited the AAPH-induced oxidation of PtdCho micelles (Fig. 1*D*), a physiological form of phospholipids

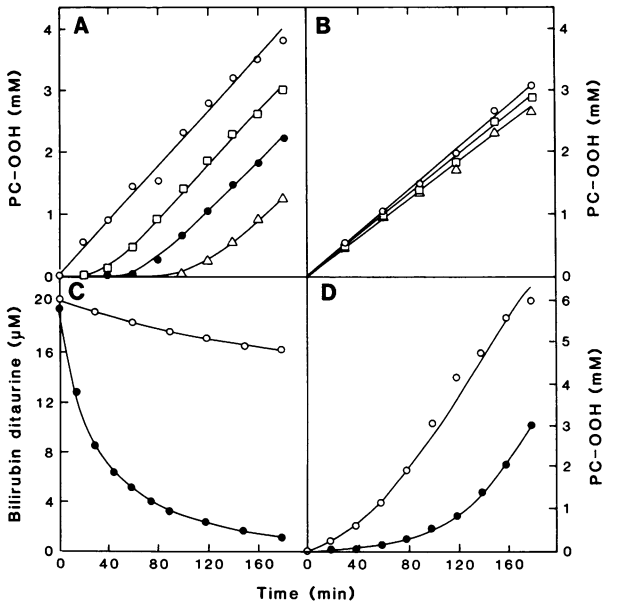

FIG. 1. Effect of micromolar concentrations of BR-DT on the AAPH-induced oxidation of purified PtdCho under air at 37°C (see *Materials and Methods*). (*A*) Time-dependent oxidation of PtdCho in an aqueous dispersion of multilamellar liposomes in the absence (○) and presence of synthetic BR-DT at 10 μM (□), 20 μM (●), and 30 μM (△). (*B*) Time-dependent oxidation of PtdCho in an aqueous dispersion of multilamellar liposomes under air in the absence (○) and presence of taurine at 50 μM (□) and 100 μM (△). (*C*) Time-dependent disappearance of 20 μM BR-DT in the absence (○) and presence (●) of 10 mM AAPH under conditions identical to those in *A*. BR-DT was quantitated by HPLC as described in *Materials and Methods*. (*D*) Time-dependent oxidation of PtdCho/taurocholate micelles in the absence (○) and presence (●) of 20 μM BR-DT. The data shown represent typical results obtained in two to five separate experiments, with a variance of <8%. PC-OOH, PtdCho-OOH.

present in bile (26). Peroxyl radical-induced oxidation of BR-DT resulted in a general bleaching of the pigment as indicated by the decrease in absorbance at 460 nm, together with a shift in the maximal absorbance toward shorter wavelengths (Fig. 2).

Under our experimental conditions the overall rate of

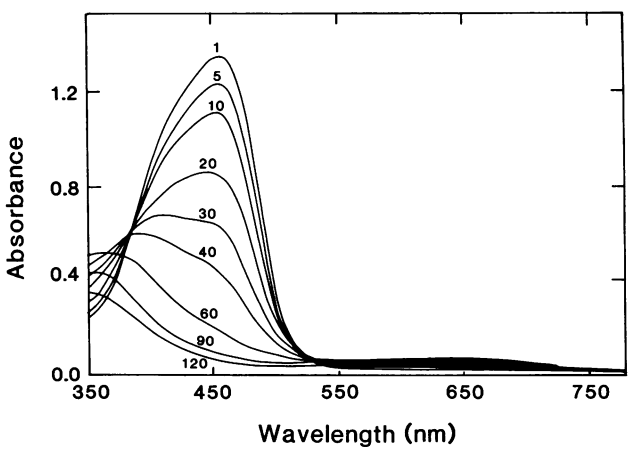

FIG. 2. Time-dependent spectral changes associated with the AAPH-induced oxidation of 20 μM BR-DT. Conditions were identical to those described for Fig. 1*A* except that no PtdCho was present. Numbers indicate the time in minutes elapsed after the addition of AAPH. Incubation of 20 μM BR-DT for 2 hr in the absence of AAPH resulted in a decrease in absorbance at 460 nm of 12% ± 3% that was not associated with any major spectral shifts (data not shown).

radical production was determined to be 0.56 μM radical per min. A comparison of the length of induction period produced by 10 μM Trolox with that produced by a known amount of BR-DT, biliverdin, or xanthobilirubinic acid yielded *n* values of 1.9, 4.7, and 0.9, respectively. An *n* value of 1.9 for BR-DT is in agreement with the initial rate of its AAPH-induced oxidation of 0.33 μM/min under conditions in which 0.56 μM radicals were formed per min.

18:2-OOH was decomposed very rapidly in the presence of both BR-DT and cupric ion (Fig. 3) at concentrations found in normal bile and duodenal juice (4, 20, 27–30). To be able to follow the degradation of 18:2-OOH quantitatively, all reactions had to be carried out at 25°C rather than 37°C. Fig. 3 shows the temporal disappearance of 18:2-OOH in the presence of 250 μM BR-DT and various concentrations of $CuCl_2$. BR-DT alone failed to decompose 18:2-OOH to any significant extent within 30 min of incubation, while BR-DT in the presence of Cu(II) degraded 18:2-OOH very rapidly within the first few minutes, after which the peroxide reached a plateau at a concentration that was inversely related to the initial amounts of Cu(II) present (Fig. 3). Decomposition kinetics similar to these were reported for the hematin-catalyzed degradation of 18:2-OOH in the presence of ascorbate (19). The presence of cupric ion alone resulted in significant hydroperoxide decomposition (Table 1). In contrast to BR-DT, taurine and biliverdin both failed to accelerate the copper-mediated degradation of 18:2-OOH. Also, no significant degradation of 18:2-OOH by BR-DT was observed when $CuCl_2$ was replaced by an equimolar amount of $FeCl_3$ (Table 1). When equimolar amounts of $CuCl_2$ were added to BR-DT in phosphate-buffered saline at pH 7.0, a general bleaching of the pigment was observed concomitant with a rapid change in the color of the reaction mixture from yellow to green (Fig. 4). The spectrum of BR-DT recorded 5 min after the addition of cupric ion was very similar to that obtained immediately after the addition of an equimolar amount of Cu(II) to biliverdin.

Table 1. Decomposition of 18:2-OOH

Addition(s)	% 18:2-OOH remaining after 10-min incubation
BR-DT	103 ± 5
$CuCl_2$	54 ± 8
Taurine + $CuCl_2$	48 ± 7
BR-DT + $CuCl_2$	1 ± 0.5
BV + $CuCl_2$	49 ± 6
BR-DT + $FeCl_3$	88 ± 12

The reaction was carried out at 25°C in 50 mM phosphate buffer/0.154 M NaCl, pH 7.0. Initial concentrations of the additives were as follows: BR-DT, 250 μM; biliverdin (BV), 250 μM; taurine, 500 μM; $CuCl_2$, 20 μM; $FeCl_3$, 20 μM; 18:2-OOH, 20 μM. If present, the metal ion was added immediately before 18:2-OOH. The amount of 18:2-OOH detected after the 10-min incubation is expressed as percentage of that detected immediately after the addition of 18:2-OOH. The results represent the mean ± SD of three or four independent experiments.

DISCUSSION

In human bile, phospholipids are transported within mixed micelles together with cholesterol and bile salts (26) or in cholesterol-containing vesicles free of bile salts (31). Bile pigments exist predominantly as self-aggregates (32) or in hybrid aggregates with bile salts (33) and do not seem to form strong associations with mixed lipid micelles. Our experimental system, consisting of BR-DT as a model compound for conjugated bilirubin in the presence of PtdCho liposomes or PtdCho/taurocholate micelles, may thus be regarded as an *in vitro* model system mimicking bile. The results clearly show that BR-DT, at concentrations well below those of conjugated bilirubin in human bile (4), efficiently protects PtdCho from peroxyl radical-induced oxidation, and that it is the bilirubin moiety and not the taurine moieties of BR-DT that possesses peroxyl radical-scavenging activity.

The mechanism by which BR-DT scavenges peroxyl radicals remains to be established. We have proposed earlier that bilirubin, when dissolved in chloroform, incorporated into liposomes, or when bound to albumin may react with the chain-carrying peroxyl radical by initial donation of a hydrogen atom attached to the C-10 of the central methene bridge of the tetrapyrrole molecule (7, 8). Such a mechanism seems unlikely for the reaction between BR-DT and peroxyl radicals, since each molecule of xanthobilirubinic acid, which can be looked at as one-half of a bilirubin molecule but which lacks the C-10 hydrogens, was able to scavenge at least half as many radicals as BR-DT. Our results are consistent with

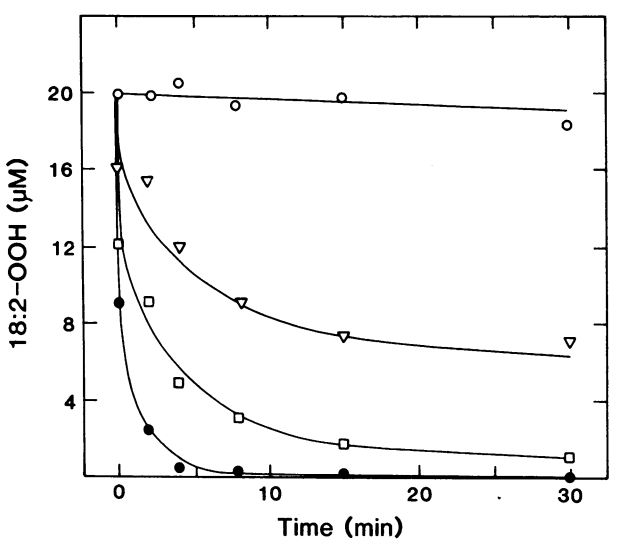

FIG. 3. Time-dependent disappearance of 18:2-OOH in the presence of 250 μM BR-DT and various concentrations of $CuCl_2$ (see *Materials and Methods*). $CuCl_2$ was used at 0 μM (○), 5 μM (▽), 10 μM (□), and 20 μM (●). Essentially identical results were obtained in two separate experiments.

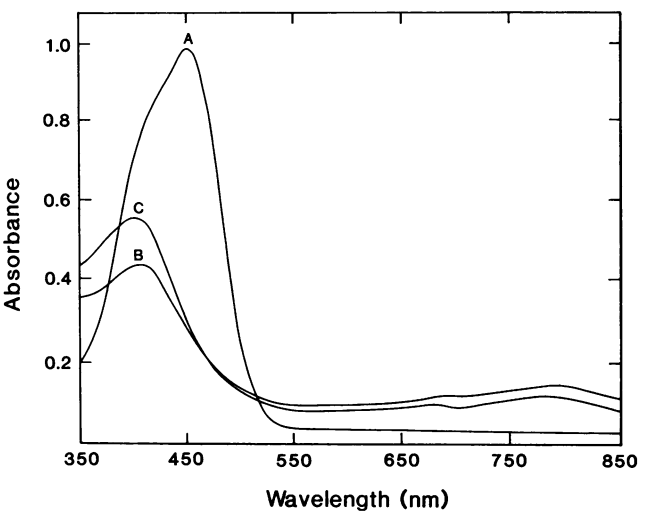

FIG. 4. Spectral changes associated with the addition of 20 μM $CuCl_2$ to an equimolar amount of bile pigment in 50 mM phosphate buffer/0.154 M NaCl, pH 7.0, at 25°C and under air. Spectrum of BR-DT recorded before (*A*) and 5 min after (*B*) the addition of Cu(II). (*C*) Spectrum recorded immediately after the addition of Cu(II) to biliverdin dihydrochloride.

an initial addition reaction of the radical to the pyrrole moiety of either xanthobilirubinic acid or BR-DT, as has been proposed as a mechanism for the free radical-mediated isomerization of unconjugated bilirubin (12, 13). Such a mechanism could explain why AAPH-induced oxidation of 20 μM BR-DT seemingly did not result in a linear and complete oxidation of the pigment within the corresponding time of induction period (Fig. 1 *A* and *C*). The HPLC conditions used for the analysis of BR-DT are based on anionic interactions between the pigment and the matrix and may therefore not distinguish readily between BR-DT and some of its addition products. Further evidence favoring an initial addition reaction comes from the observation that AAPH-induced oxidation of BR-DT did not give rise to biliverdin, in contrast to the situation with albumin-bound bilirubin (8). The finding that concomitant with the decrease in absorbance at 460 nm, maximal absorbance of the reaction mixture shifted toward shorter wavelengths may indicate that the addition product formed initially undergoes further oxidation reaction yielding di- and monopyrroles. Therefore, it seems possible that bilirubin can scavenge peroxyl radicals by either mechanism, hydrogen donation or addition reaction, depending on whether the C-10 hydrogens of the pigment are "locked," and therefore easily accessible (as is the case for albumin-bound bilirubin or for free bilirubin with intact intramolecular hydrogen bonds), or "unlocked" (as in conjugated bilirubin, where hydrogen bonds no longer exist).

It is known that hydroperoxides can be degraded by transition metals in their higher or lower oxidation state according to the following reactions (34):

$$\mathrm{ROOH + M^{+n+1} \rightarrow ROO\cdot + M^{+n} + H^{+}} \quad [1]$$

$$\mathrm{ROOH + M^{+n} \rightarrow RO\cdot + M^{+n+1} + OH^{-}} \quad [2]$$

From our *in vitro* studies with 18:2-OOH, it is apparent that BR-DT accelerates the cupric ion but not the ferric ion-catalyzed decomposition of hydroperoxides. We believe that this newly described activity of bilirubin may be of physiological importance. In humans, bile clearly represents the major physiological excretory fluid for both conjugated bilirubin (1) and copper (20), and the concentrations of copper used in our experiments are well within the range of biliary copper reported (20, 27–30). Unlike most biological tissues, in which transition metals are either part of the active site in enzymes or bound to specific transport or storage proteins, biliary copper does not seem to be protein bound (20, 27–29). Reports from several independent laboratories further indicate that in humans, conjugated bilirubin represents the ligand for at least some of the biliary copper (27–29). Furthermore, unconjugated bilirubin and bilirubin dimethyl ester are known to form complexes with a variety of metals (35, 36). In contrast to all other metals tested, complex formation of bilirubin with copper seems to be nonreversible, possibly involving a redox reaction (35, 36). Such a notion is supported indirectly by the spectral changes shown in Fig. 4. However, we do not know at present whether such a redox reaction between BR-DT and Cu(II) is necessary for the hydroperoxide-degrading activity observed. The fact that the presence of biliverdin failed to accelerate the Cu(II)-catalyzed hydroperoxide decomposition indirectly supports a model in which either cuprous ion alone (reaction **2**), or as a complex with some form of oxidized BR-DT, [Cu^{+}–BR_{ox}-DT] (reaction **3**), is the active component.

$$\mathrm{ROOH + [Cu^{+}\text{–}BR_{ox}\text{-}DT] \rightarrow RO\cdot + [Cu^{2+}\text{–}BR_{ox}\text{-}DT] + OH^{-}} \quad [3]$$

We propose that a highly active cuprous ion-containing bilirubin complex may donate electrons not only to 18:2-OOH but also to other toxic compounds derived from hepatic metabolism and known to be excreted into bile (37). Since 18:2-OOH was not completely decomposed when the initial copper concentration was smaller than that of the hydroperoxide (Fig. 3), it is unlikely that a simple and effective redox cycling of Cu(II) takes place during the reaction. A possible explanation of the results is that the Cu(II) is made unavailable by complexation with the product of the oxidation of bilirubin. Under physiological conditions, such a problem does not arise because a steady excretion of copper into bile (38) could serve as a continuous supply of copper for detoxification activities. The peroxyl or alkoxyl radicals produced in either of the reactions **1–3** would be expected to be scavenged by the large quantities of conjugated bilirubin present in bile, thereby preventing the initiation of chain reactions by these radicals.

Obviously, a necessary prerequisite for a potential physiological relevance of the hydroperoxide-degrading activity of the bilirubin–copper complex is the existence of such peroxides in bile. It was not the objective of this study to investigate this question. However, it is well established that hepatic cytochrome P-450 renders xenobiotics and a wide range of foreign substances more soluble for their excretion into bile (37). During this process, oxygen reduction products are generated that can peroxidize endogenous lipids (39). It seems possible that under normal conditions and especially under conditions of increased activity of cytochrome P-450 some of these lipid hydroperoxides escape the cellular antioxidant defense systems and are excreted into bile. Indirect evidence supporting this hypothesis comes from a study reporting increased biliary levels of malonyldialdehyde, an index of lipid peroxidation, in rats treated with ethanol and/or intron dextran (40). Excretion of lipid peroxides, toxic epoxides, carcinogens, and free radicals into bile has been hypothesized to be the cause of pancreatic disease (41).

Through bile, conjugated bilirubin and copper reach the intestine, the organ into which food and orally administered drugs pass first. Cooked food is a rich source of mutagens and carcinogens, including lipid hydroperoxides (42). In addition, free radicals are thought to be generated within the intestine as a consequence of drug metabolism (43). It seems probable that conjugated bilirubin in the intestine serves as a protective agent against these toxic compounds, in conjunction with its role as a natural antioxidant, which protects easily oxidizable substances from destruction in the intestinal tract.

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