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Antioxidant activity of albumin-bound bilirubin

(reactive oxygen species/plasma antioxidants/biliverdin/evolution)

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ABSTRACT **Bilirubin, when bound to human albumin and at concentrations present in normal human plasma, protects albumin-bound linoleic acid from peroxyl radical-induced oxidation *in vitro*. Initially, albumin-bound bilirubin (Alb-BR) is oxidized at the same rate as peroxyl radicals are formed and biliverdin is produced stoichiometrically as the oxidation product. On an equimolar basis, Alb-BR successfully competes with uric acid for peroxyl radicals but is less efficient in scavenging these radicals than vitamin C. These results show that 1 mol of Alb-BR can scavenge 2 mol of peroxyl radicals and that small amounts of plasma bilirubin are sufficient to prevent oxidation of albumin-bound fatty acids as well as of the protein itself. The data indicate a role for Alb-BR as a physiological antioxidant in plasma and the extravascular space.**

In mammals, the conversion of heme to bilirubin involves the combined action of heme oxygenase (decyclizing) [heme, hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3] and biliverdin reductase [bilirubin:NAD(P)$^+$ oxidoreductase, EC 1.3.1.24] (1). Because of its intramolecular hydrogen bonding, the bilirubin produced is sparingly soluble in water at physiological pH and ionic strength (2, 3) and is tightly bound to albumin in order to be transported within the blood circulation (3). Under physiological conditions, plasma bilirubin concentrations in humans range from $\approx$5 to 17 μM (4), practically all of which is unconjugated pigment bound to albumin (2, 3, 5). Plasma concentrations >300 μM are associated with the risk of development of neurologic dysfunctions (6) as a result of the preferential deposition of bilirubin in brain and its enhanced toxic effects on cellular functions in this tissue (7). The precise mechanism of cellular bilirubin toxicity is still uncertain but may include selective interference with energy metabolism, protein synthesis, and carbohydrate metabolism within the target cell (7). Available evidence suggests that the formation of bilirubin from biliverdin was introduced in mammals during evolution (8). The purpose of biliverdin reduction in mammals has been obscure since no physiological function(s) have been attributed to the potentially toxic bilirubin that derives from its nontoxic metabolic precursor biliverdin in an energetically expensive reaction.

Recently, we have proposed that one beneficial role of bilirubin may be to act as a physiological antioxidant since, under low oxygen concentrations (2%) and when incorporated into liposomes, it scavenges peroxyl radicals as efficiently as α-tocopherol (9), which is regarded as the best antioxidant of lipid peroxidation. Although free bilirubin has been shown to interact with purified plasma membranes and microsomes (10), a possible physiological function of bilirubin as a membrane-bound chain-breaking antioxidant has to be questioned because of its toxic properties mentioned above. However, binding of bilirubin to albumin not only sequesters the molecule into a nontoxic form (3, 6, 10, 11) but also distributes the pigment throughout the entire circulation and extravascular space (3, 12). Therefore, we tested the antioxidant properties of albumin-bound bilirubin (Alb-BR) toward peroxyl radicals, generated chemically by the thermal decomposition of the water-soluble azo compound 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) (13, 14).

MATERIALS AND METHODS

Preparation of Alb-BR. Recrystallized (15) bilirubin (Sigma) was dissolved in 0.05 M NaOH immediately before it was added to a phosphate-buffered solution of essentially fatty acid-free human albumin (Sigma). For the control sample (no bilirubin) the appropriate amount of NaOH was added. Purified (16) linoleic acid (Sigma) was added to the albumin solution as an aqueous dispersion and stirred at 4°C until the solution was clear. Cold AAPH (Polyscience, Warrington, PA) was then added and the reaction was initiated by placing the reaction tube in a waterbath equilibrated at 37°C. Final concentrations were as follows: albumin, 500 μM; linoleic acid, 2 mM; AAPH, 50 mM in 50 mM phosphate buffer/0.154 M NaCl, pH 7.0. Bilirubin concentrations were as indicated in the figure legends.

Quantitation of Linoleic Acid Hydroperoxide (18:2-OOH). Aliquots were removed and the fatty acids were extracted from the albumin by the addition of 1 vol of reaction mixture to 10 vol of cold chloroform. The two phases were separated by a 2-min centrifugation at 11,000 $\times$ g, and a known amount of the organic phase was removed, dried under a stream of nitrogen, and resuspended in methanol. Drying of 18:2-OOH in the presence of bilirubin did not result in any significant loss of the hydroperoxide. Quantitation for 18:2-OOH was done at 234 nm by HPLC on an analytical LC-NH_2 column (Supelco, Bellefonte, PA) with methanol and 40 mM NaH_2PO_4 (9:1, vol/vol) (1 ml/min) as the mobile phase (16). Standards of 18:2-OOH were prepared as described (16).

Quantitation of Bilirubin and Its Oxidation Product. Aliquots were removed and the bile pigments were extracted by addition of 1 vol of reaction mixture to 4 vol of cold methanol. The protein was pelleted, the supernatant was removed, and an aliquot was analyzed and quantitated for bilirubin and its oxidation product by HPLC at 460 nm and 650 nm, respectively. Using an analytical C_{18} column (Supelco) with 0.1 M di-*n*-octylamine acetate in methanol and H_2O (96:4, vol/vol) (1 ml/min) as the mobile phase (17), the retention times for biliverdin and bilirubin were 5.4 and 12.5 min, respectively. Biliverdin IX dihydrochloride (Porphyrin Products, Logan, UT) was used as a standard without further purification, and the area of the main peak was used for quantitation.

Characterization of the AAPH-Induced Oxidation Product of Alb-BR. A solution containing 500 μM human albumin, 2

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; Alb-BR, albumin-bound bilirubin; Alb-BV, albumin-bound biliverdin; 18:2-OOH, linoleic acid hydroperoxide.

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mM linoleic acid, and 250 μM bilirubin in phosphate-buffered saline (pH 7.0) was thermostated at 37°C and the oxidation of bilirubin was initiated by the addition of 50 mM AAPH. The extent of formation of the oxidation product was followed spectroscopically as an increase in absorbance at 650 nm. After reaching maximal absorbance, the reaction was stopped by the addition of 4 vol of cold methanol. The albumin was pelleted and the supernatant containing the oxidation product was injected on a semipreparative C_{18} column (Supelco) using 0.1 M di-*n*-octylamine acetate in methanol and H_2O (97:3, vol/vol) (2 ml/min) as eluant and monitoring at 650 nm. Eluting fractions corresponding to the single peak derived from the AAPH-induced oxidation of Alb-BR and the major peak of injected biliverdin standard were collected. After recording a spectrum of these fractions the solvents were dried under reduced pressure and the remainder was resuspended in methanol and H_2O (1:1, vol/vol) before being applied to a Sep-Pak C_{18} cartridge (Waters Associates), equilibrated previously with the same solvent system. Most of the contaminating di-*n*-octylamine was washed off the column with 50% methanol, while the oxidation product and the biliverdin standard remained bound to the column as a clearly visible green band. The bile pigments were eluted with 100% methanol, and the molecular weight of the dried compounds was determined by positive fast atom bombardment mass spectrometry using nitrobenzyl alcohol as the liquid matrix.

AAPH-Induced Oxidation of Bilirubin in Human Plasma. Heparinized blood obtained from healthy male donors was centrifuged initially at 1500 × *g* for 10 min and the supernatant was recentrifuged at 11,000 × *g* for 5 min to remove contaminating erythrocytes and platelets; 900 μl of the resultant plasma was incubated at 37°C for 5 min before oxidation was initiated by the addition of 100 μl of 500 mM AAPH. At various time points, aliquots of the reaction mixture were removed and analyzed for bilirubin and biliverdin as described above. Plasma ascorbate levels were determined as described (16).

RESULTS

The thermal decomposition of AAPH under air produces peroxyl radicals at a constant rate (13, 14) and these radicals oxidize linoleic acid quantitatively at the initial stage to give 18:2-OOH (18). The extent of oxidation may be followed simply by measuring the formation of 18:2-OOH. Any compound possessing peroxyl radical scavenging activity will decrease the rate of formation of 18:2-OOH. The effect of bilirubin at concentrations normally found in human plasma and when bound to human albumin on the peroxyl radical-induced oxidation of albumin-bound linoleic acid in homogeneous solution and under air is shown in Fig. 1. In the absence of the bile pigment, the accumulation of 18:2-OOH proceeded without delay and at a constant rate. Bilirubin at 20 μM inhibited the formation of 18:2-OOH initially by >80% (Fig. 1*A*) and the extent of this inhibition was dependent on the initial concentration of bilirubin present (Fig. 1*D*). Within minutes of initiation of the AAPH-induced oxidation of Alb-BR the color of the reaction mixtures changed from yellow to green, as indicated by the decrease in absorbance at 460 nm and the increase in absorbance at 380 and 650 nm (Fig. 1*B*). Maximal absorbances at 380 and 650 nm are spectral features typical of biliverdin. HPLC analysis revealed that, in the presence of AAPH, the original 20 μM bilirubin disappeared at an initial rate of 1.6 μM/min and was oxidized completely within the first 25 min (Fig. 1*C*). In the

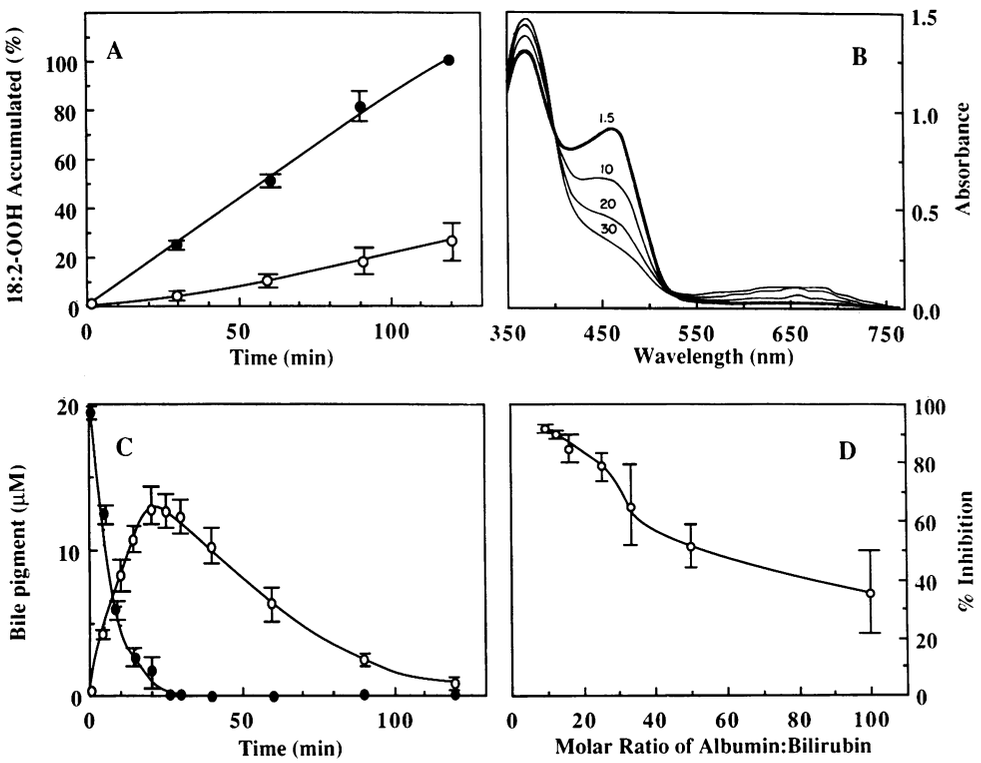

FIG. 1. Effect of physiological amounts of Alb-BR on the AAPH-induced oxidation of albumin-bound linoleic acid. (*A*) Time-dependent oxidation of linoleic acid in the absence (●) and presence (○) of 20 μM bilirubin. The total amounts of 18:2-OOH formed during the 2-hr incubation period (i.e., 100%) varied between 101 and 235 μM. (*B*) Time-dependent spectral changes associated with the AAPH-induced oxidation of 20 μM Alb-BR in *A*. The numbers indicate the time in minutes after addition of AAPH. (*C*) Time-dependent oxidation of bilirubin (●) in *A* with concomitant formation of biliverdin (○). (*D*) Bilirubin concentration-dependent inhibition of AAPH-induced oxidation of albumin-bound linoleic acid in the presence of 500 μM albumin, the concentration present in human blood. Samples were incubated for 30 min and the extent of oxidation of linoleic acid expressed as percentage of that obtained in the absence of bilirubin. Data in *A*, *C*, and *D* represent means ± SD of three or four separate experiments, while data in *B* are representative of a typical result obtained in three separate experiments.

absence of AAPH, the concentration of Alb-BR did not change significantly during incubation at 37°C for up to 4 hr (data not shown). The initial rate of AAPH-induced oxidation of bilirubin did not change significantly whether the albumin contained added fatty acids or not (data not shown). Concomitant with the oxidation of Alb-BR a single product was formed and reached maximal concentration at a time coinciding with that of complete disappearance of bilirubin (Fig. 1*C*). This oxidation product, which was identified as biliverdin (see below), accumulated to maximally 13.5 μM and was oxidized subsequently at a rate of 0.2 μM/min.

HPLC analysis showed that the oxidation product of Alb-BR eluted at 9.4 min from a semipreparative C_{18} column (Fig. 2*A*), while, under identical conditions, biliverdin standard eluted at 9.2 min (Fig. 2*B*). This difference in the relative retention times appeared to be due to the presence of water in the aqueous methanol extract of the Alb-BR-derived sample but not in the biliverdin standard (which was dissolved in the mobile phase) since co-injection of the two

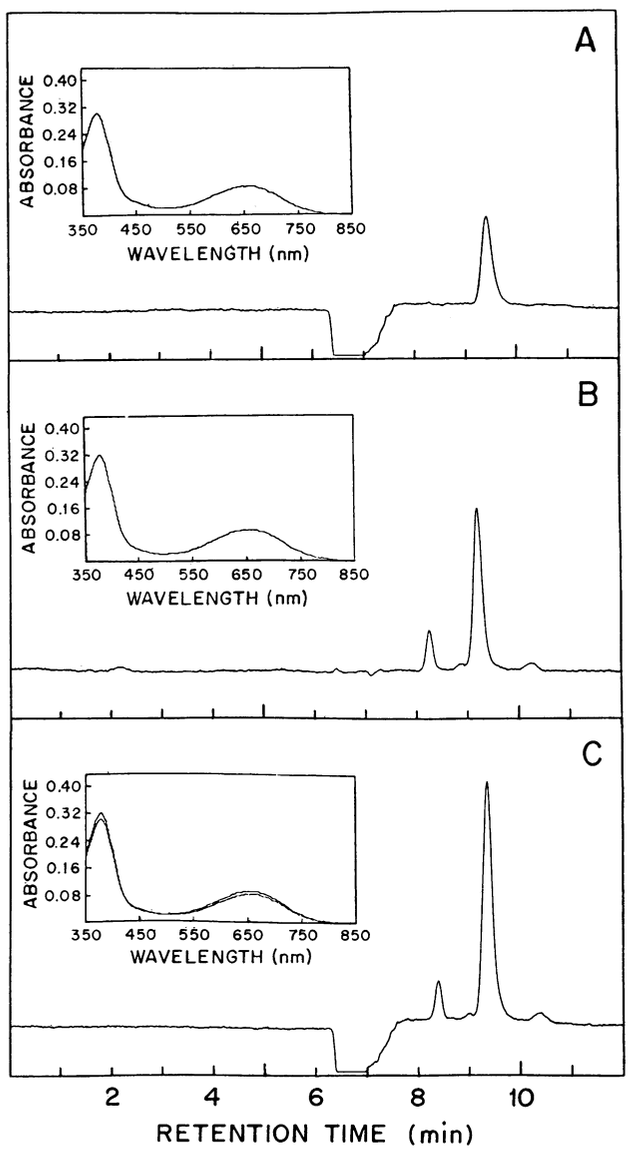

FIG. 2. HPLC characterization of the AAPH-induced product of Alb-BR (see *Materials and Methods*). (*A*) Aqueous methanol extract containing the reaction product derived from AAPH-induced oxidation of Alb-BR. (*B*) Biliverdin standard (50 nmol). (*C*) Sample *A* mixed with 50 nmol of biliverdin standard. (*Insets*) Visible spectra of the eluted fractions containing the oxidation product and/or biliverdin standard.

samples resulted in only one major peak eluting at 9.4 min (Fig. 2*C*). The identity of the compounds responsible for the major peaks in Fig. 2 *A* and *B* was also indicated by their identical visible absorption spectra (Fig. 2 *Insets*). Furthermore, positive fast atom bombardment mass spectrometry of the isolated and purified oxidation product of Alb-BR revealed a MH^+ peak of M_r 583 with >90% relative intensity, identical to that of a biliverdin standard treated the same way.

To assess the biological importance of Alb-BR as a plasma antioxidant, we examined its peroxyl radical trapping activity in the presence of physiological amounts of known plasma antioxidants by measuring the rate of bilirubin oxidation as the decrease in absorbance at 460 nm. In the presence of 50 μM ascorbate, the initial AAPH-induced oxidation of Alb-BR was inhibited almost completely, producing a clear induction period after which the rate of bilirubin oxidation was nearly identical to that observed in the absence of any additional antioxidant (Table 1). In contrast to ascorbate, the presence of 300 μM uric acid did not result in an induction period and bilirubin oxidation proceeded smoothly and at a constant rate. From the initial rates of bilirubin oxidation and the initial concentrations of the antioxidants present, it was calculated that peroxyl radicals reacted with Alb-BR at a rate 12.5 times slower and 3.1 times faster than with ascorbate and uric acid, respectively.

The physiological relevance of the above-mentioned chemical studies was examined by the addition of 50 mM AAPH to fresh human plasma containing 40–55 μM endogenous ascorbate. Oxidation of plasma bilirubin was preceded by an induction period of ≈30 min, after which disappearance of bilirubin progressed (Fig. 3). The initial rate of bilirubin oxidation seemed to be directly proportional to the original concentration of the bile pigment present in plasma, since 80 and 47 pmol of bilirubin were oxidized per min when the original plasma concentrations were 12.5 and 6.4 μM, respectively. Biliverdin was formed and accumulated concomitant with bilirubin oxidation.

DISCUSSION

Under physiological conditions, two molecules of bilirubin dianion can bind to one molecule of albumin with binding constants of $5.9 \times 10^7\ M^{-1}$ and $4.4 \times 10^6\ M^{-1}$ for the primary and secondary binding site, respectively (3). Long-chain fatty acids are also important ligands of albumin, and in the plasma of newborns they are present in amounts ranging from 0.5 to 2.0 mol per mol of albumin. Since the primary binding site affinity for bilirubin to human defatted albumin is not changed by cobinding of up to four molecules of fatty acids

Table 1. Initial rates of AAPH-induced oxidation of Alb-BR at 37°C in the presence and absence of physiological amounts of known plasma antioxidants

Alb-BR	Alb-BR + ascorbate*	Alb-BR + urate†
858 ± 50	28 ± 5‡	178 ± 8

Experimental conditions were as follows: albumin (500 μM), bilirubin (20 μM), AAPH (50 mM) in 50 mM phosphate buffer/0.154 M NaCl, pH 7.0. Initial rates are expressed as pmol of bilirubin oxidized per min using an extinction coefficient of 40.4 mM at 460 nm. Rates are relative and are not corrected for contributions at 460 nm due to product. The results represent the mean ± SD of three independent experiments.

*The initial ascorbate concentration was 50 μM.

†The initial urate concentration was 300 μM.

‡The rate given is the initial rate of bilirubin oxidation. This rate was maintained during an "induction period" (defined as the time between the initiation of bilirubin oxidation and a sharp change in the rate) of 23.9 ± 0.8 min. After the induction period, the rate of bilirubin oxidation was 955 ± 50 pmol/min.

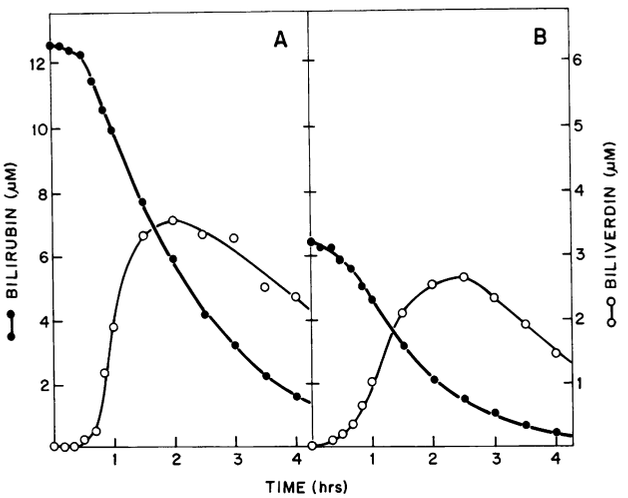

FIG. 3. AAPH-induced oxidation of endogenous bilirubin (●) in fresh human plasma with concomitant formation of biliverdin (○). Representative of typical results obtained with plasmas containing different initial concentrations of bilirubin.

(3) and bilirubin dianion binds to the primary binding site on albumin when the pigment is added in a sodium hydroxide solution to the albumin (3), the experimental model system used in this study is representative of the *in vivo* form of bilirubin bound to the primary binding site on human albumin.

The results presented clearly show that Alb-BR at concentrations found in plasma of healthy adults is a very efficient peroxyl radical scavenger and protects fatty acids transported on albumin from oxidation by these radicals. The rate constant of the reaction between Alb-BR and the AAPH-derived alkylperoxyl radical can be estimated indirectly from the rate of AAPH-induced oxidation of Alb-BR in the presence and absence of ascorbate (Table 1) and the known rate of reaction between ascorbate and alkylperoxyl radical [i.e., 2.2×10^6 $M^{-1}\cdot sec^{-1}$, (19)]. The obtained rate of 1.7×10^5 $M^{-1}\cdot sec^{-1}$ is >30 times higher than that calculated for the reaction of free bilirubin with peroxyl radicals generated from the lipid-soluble analog of AAPH, 2,2′-azobis-(2,4-dimethylvaleronitrile), in chloroform [i.e., 5×10^3 $M^{-1}\cdot sec^{-1}$ (9)]. 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 dipyrroles of the bilirubin molecule in an out-of-plane position (3). This model is supported by the fact that Alb-BR but not free bilirubin gives rise to a bisignate circular dichroism spectrum (20). Such asymmetric positioning of the bilirubin molecule on albumin is expected to expose the reactive hydrogen atom at C-10 for initial hydrogen abstraction by peroxyl radicals while protecting the two dipyrroles moieties from oxidation. This is consistent with the observations that during the AAPH-induced initial oxidation of Alb-BR biliverdin is formed stoichiometrically as the oxidation product (Fig. 1*C*), while oxidation of unbound bilirubin in chloroform by 2,2′-azobis(2,4-dimethylvaleronitrile) results in the formation of at least five polar reaction products without significant amounts of biliverdin being formed (R.S. and B.N.A., unpublished data). Therefore, binding to albumin seems to confer both increased specificity and reactivity of bilirubin toward peroxyl radicals. The results further indicate that each molecule of Alb-BR can donate two hydrogens to scavenge two molecules of peroxyl radicals, giving rise to albumin-bound biliverdin (Alb-BV) as the reaction product (Reaction **1**). Biliverdin has been shown to bind to the primary bilirubin binding site on human albumin (21)

$$\text{Alb-BR} + 2\text{ROO}\cdot \rightarrow \text{Alb-BV} + 2\text{ROOH} \qquad [1]$$

A comparison between the rate of oxidation of Alb-BR (Fig. 1*C*) and the calculated rate of radical production[¶] indicates that, initially, all radicals formed are scavenged by bilirubin, thereby completely protecting albumin-bound fatty acids and, most likely, the protein itself from oxidation. The latter notion is supported indirectly by the finding that photooxidation of Alb-BR resulted in substantial oxidation of bilirubin, whereas no oxidation of the protein was observed as judged by amino acid analysis (23).

It is apparent from our present results that Alb-BR reacts several times faster with peroxyl radicals than Alb-BV (Fig. 1*C*). This finding is in contrast to the results obtained with the unbound pigments where biliverdin is a much better peroxyl radical trap than bilirubin (9) but may represent a significant selective pressure favoring the conversion of biliverdin to bilirubin during the evolution of heme metabolism in mammals.

Since 40% of the human albumin occurs within the blood circulation (24), it was of interest to compare the antioxidant activity of Alb-BR with that of the known water-soluble plasma antioxidants ascorbate and urate. The results presented clearly show that Alb-BR is able to compete successfully for peroxyl radicals with urate but not with ascorbate. The addition of ascorbate to the Alb-BR system resulted in a clear induction period in the oxidation of bilirubin (Table 1), indicating that as long as the vitamin is present it reacts preferentially with the peroxyl radicals. This was confirmed by the induction periods observed after the addition of AAPH to freshly isolated human plasma samples (Fig. 3). The induction periods observed in the plasma samples were slightly longer than what would have been expected from their endogenous contents of ascorbate, indicating that, besides ascorbate, there may be a small amount of an additional antioxidant present that reacts with peroxyl radicals faster than Alb-BR does. Biliverdin was produced as a reaction product of bilirubin oxidation in plasma, although to a lesser extent than what would have been expected from a stoichiometric interconversion of the two pigments. The reason(s) for this observation are not clear at present. Exogenous biliverdin is stable in human plasma *in vitro* for at least 4 hr (R.S. and B.N.A., unpublished data), indicating that there is no biliverdin-reducing activity present in plasma. Independent of the amounts produced, Alb-BV formed by peroxyl radicals *in vivo* is unlikely to accumulate in the blood due to its rapid clearance from the circulation and its subsequent reduction to bilirubin by the liver.

The relative importance of Alb-BR as a plasma antioxidant is expected to increase as its concentration increases or under conditions where plasma ascorbate is low. It is interesting to note that the activity of heme oxygenase, which is the rate-limiting enzyme in bilirubin formation, has been shown to be increased in animals deprived of ascorbate (25). In addition, heme oxygenase is induced by a number of conditions known to exert an oxidative stress, including exposure of rats to certain metal ions (26), sulfhydryl reactive compounds (27, 28), and endotoxin (29), as well as depriving mice of selenium (30). Cigarette smoking, known to be associated with enhanced production of oxygen and carbon-centered radicals (31), significantly lowers plasma levels of bilirubin in humans (32). It would be of interest to see if increased bilirubin and other antioxidants could mitigate some of the toxic effects of cigarette smoking. Further evidence for an *in*

[¶]The obtained rate of 3.0×10^{-6} mol peroxyl radicals formed per min was calculated using $R_i(aq) = 1.0 \times 10^{-6}$ [AAPH] as described (22) for an aqueous solution of albumin.

vivo function of Alb-BR as a natural antioxidant comes from a report demonstrating that in patients with acute viral hepatitis the levels of serum bilirubin were correlated positively with the total activity of serum antioxidants and negatively with diene conjugates, an index of lipid peroxidation, present in blood (33).

The antioxidant activity of Alb-BR is not expected to be limited to the blood stream, since 60% of human albumin is located in the extravascular space (24) and the extrahepatic extravascular pool of bilirubin is correlated roughly with the extrahepatic albumin (12). Albumin has been reported to leave the blood stream and to appear in inflammatory exudate (34), thereby transporting bound substances, such as bilirubin, across the vascular wall into these sites of increased production of oxygen radicals by phagocytic cells (35). A further plausible hypothesis is that bilirubin bound to proteins other than albumin may also possess antioxidant activity. Bilirubin binds to glutathione transferase and the Z-protein in the liver and the intestinal mucosa (36) and may thus contribute to the cytosolic antioxidant activities of the cells in these tissues.

We have shown previously that unbound bilirubin, in solution or when incorporated into liposomes, efficiently scavenges peroxyl radicals (9) and have now extended this property to the albumin-bound form of bilirubin. Preliminary studies with conjugated bilirubin, the form of the pigment present in large quantities in bile and intestine, have shown its reactivity toward peroxyl radicals (R.S. and B.N.A., unpublished data). We believe that these findings are strong support for the view that bilirubin serves a beneficial role as an endogenous antioxidant.

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1. Schmid, R. & McDonagh, A. F. (1975) *Ann. N.Y. Acad. Sci.* **244,** 533–552.
2. McDonagh, A. F. (1979) in *The Porphyrins*, ed. Dolphin, D. (Academic, New York), Vol. 6, pp. 293–491.
3. Brodersen, R. (1979) *CRC Crit. Rev. Clin. Lab. Invest.* **11,** 305–399.
4. Bloomer, J. R., Berk, P. D., Howe, R. B. & Berlin, N. I. (1971) *J. Am. Med. Assoc.* **218,** 216–220.
5. Lamola, A. A., Eisinger, J., Blumberg, W. E., Patel, S. C. & Flores, J. (1979) *Anal. Biochem.* **100,** 25–42.
6. Meuwissen, J. A. T. P. & Heirwegh, K. P. M. (1982) in *Bilirubin*, eds. Heirwegh, K. P. M. & Brown, S. B. (CRC, Boca Raton, FL), Vol. 2, pp. 39–83.
7. Schenker, S., Hoyumpa, A. M. & McCandless, D. W. (1986) in *Bile Pigments and Jaundice*, ed. Ostrow, J. D. (Marcel Dekker, New York), pp. 395–419.
8. Colleran, E. & O'Carra, P. (1977) in *Chemistry and Physiology of Bile Pigments*, eds. Berk, P. D. & Berlin, N. I. (GPO, Washington, DC), pp. 69–80.
9. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N. & Ames, B. N. (1987) *Science* **235,** 1043–1046.
10. Meuwissen, J. A. T. P. & Heirwegh, K. P. M. (1978) in *Transport by Proteins*, eds. Blauer, G. & Sund, H. (de Gruyter, Berlin), pp. 387–401.
11. Odell, G. B. (1973) *Ann. N.Y. Acad. Sci.* **226,** 225–237.
12. Brodersen, R. (1972) *Scand. J. Clin. Lab. Invest.* **30,** 95–106.
13. Yamamoto, Y., Haga, S., Niki, E. & Kamiya, Y. (1984) *Bull. Chem. Soc. Jpn.* **57,** 1260–1264.
14. Barclay, L. R. C., Locke, S. J., MacNeil, J. M., VanKessel, J., Burton, G. W. & Ingold, K. U. (1984) *J. Am. Chem. Soc.* **106,** 2479–2481.
15. McDonagh, A. F. & Assisi, F. (1972) *Biochem. J.* **129,** 797–800.
16. Yamamoto, Y., Brodsky, M. H., Baker, J. C. & Ames, B. N. (1987) *Anal. Biochem.* **160,** 7–13.
17. McDonagh, A. F., Palma, L. A., Trull, F. R. & Lightner, D. A. (1982) *J. Am. Chem. Soc.* **104,** 6865–6876.
18. Yamamoto, Y., Niki, E. & Kamiya, Y. (1982) *Bull. Chem. Soc. Jpn.* **55,** 1548–1550.
19. Packer, J. E., Willson, R. L., Bahnemann, D. & Asmus, K. D. (1980) *J. Chem. Soc. Perkin Trans.* **2,** 296–299.
20. Blauer, G. & King, T. E. (1970) *J. Biol. Chem.* **245,** 372–381.
21. Ahlfors, C. E. (1981) *Anal. Biochem.* **110,** 295–307.
22. Niki, E., Saito, M., Yoshikawa, Y., Yamamoto, Y. & Kamiya, Y. (1986) *Bull. Chem. Soc. Jpn.* **59,** 471–477.
23. Pedersen, A. O., Schønheyder, F. & Brodersen, R. (1977) *Eur. J. Biochem.* **72,** 213–221.
24. Rothschild, M. A., Bauman, A., Yalow, R. S. & Berson, S. A. (1955) *J. Clin. Invest.* **34,** 1354–1358.
25. Walsch, S. & Degkwitz, E. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361,** 1243–1249.
26. Maines, M. D. & Kappas, A. (1977) *Science* **198,** 1215–1221.
27. Maines, M. D. & Kappas, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1875–1878.
28. Kikuchi, G. & Yoshida, T. (1983) *Mol. Cell. Biochem.* **53/54,** 163–183.
29. Gemsa, D., Woo, C. H., Fudenberg, H. H. & Schmid, R. (1974) *J. Clin. Invest.* **53,** 647–651.
30. Reiter, R. & Wendel, A. (1983) *Biochem. Pharmacol.* **32,** 3063–3067.
31. Church, D. F. & Pryor, W. A. (1985) *Environ. Health Perspect.* **64,** 111–126.
32. Chan-Yeung, M., Ferreira, P., Frohlich, J., Schulzer, M. & Tan, F. (1981) *Am. Soc. Clin. Pathol.* **75,** 320–326.
33. Blyuger, A. F., Dudnik, L. B., Maiore, A. Ya. & Mieze, I. É. (1985) *Byull. Éksp. Biol. Med.* **99,** 166–168.
34. Tögl-Leimüller, A., Egger, G. & Porta, S. (1986) *Exp. Pathol.* **30,** 91–96.
35. Fantone, J. C. & Ward, P. A. (1982) *Am. J. Pathol.* **107,** 397–418.
36. Levi, A. J., Gatzmaitan, Z. & Arias, I. M. (1969) *J. Clin. Invest.* **48,** 2156–2167.