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# Bilirubin as an Antioxidant: Kinetic Studies of the Reaction of Bilirubin with Peroxyl Radicals in Solution, Micelles, and Lipid Bilayers

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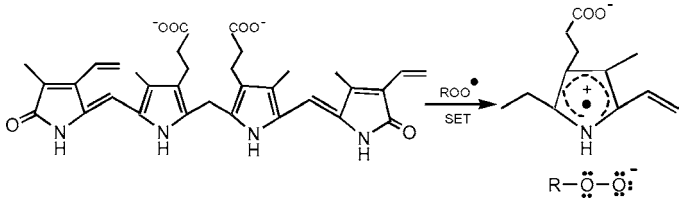
## ABSTRACT




**Bilirubin (BR) showed very weak antioxidant activity in a nonpolar medium of styrene or cumene in chlorobenzene. In contrast, BR exhibited strong antioxidant activity in polar media such as aqueous lipid bilayers or SDS micelles/methyl linoleate (pH 7.4), where the rate with peroxyl radicals, $k_{inh} = 5.0 \times 10^4$ $M^{-1}$ $s^{-1}$, was comparable to that with vitamin E analogues, Trolox, or PMHC. An electron-transfer mechanism accounts for the effect of the medium on the antioxidant properties of BR.**

Bilirubin, the common metabolite of hemoglobin, possesses both damaging and beneficial properties in biological systems. The damaging effects are evident in the appearance of jaundice, especially in premature babies when it occurs "free" in relatively high concentrations in the blood. Bilirubin is also known to sensitize photooxidation of biological molecules by singlet oxygen.[1] The beneficial effects of bilirubin are due to its effects as an antioxidant, which have been reported by various groups,[2] including a claim that it is a "major physiologic antioxidant cytoprotectant".[2a] Surprisingly, reliable quantitative kinetic data on its antioxidant activity are lacking. We now report on the rate constants and stoichiometric factor for reaction of bilirubin with peroxyl radicals, the main chain-carrying radicals in peroxidation, using quantitative methods of autoxidation, and propose a mechanism that accounts for its unusual antioxidant properties, which depend markedly on the medium used for the reaction.

The antioxidant properties of bilirubin were examined in three different systems: (1) in a homogeneous solution of chlorobenzene containing the substrates styrene or cumene; (2) in micelles of sodium dodecyl sulfate (SDS) with methyl linoleate (ML) substrate; and (3) in lipid bilayers of egg lecithin (EL) and of 1-palmitoyl-2-linoleoyl-phosphatidyl choline (PLPC). The rates of free radical initiation ($R_i$) were controlled by using azo initiators, azo-bis(isobutyronitrile) (AIBN) in solution, and either lipid-soluble azo-bis(2,4-

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dimethylvaleronitrile) (DMVN) or water-soluble azo-bis-(amidinopropane)dihydrochloride (ABAP) in aqueous systems. The inhibited oxygen uptake (IOU) method was used to determine rate constants of inhibition, $k_{inh}$[3], and the inhibition provided by bilirubin compared with known phenolic antioxidants.

Phenolic antioxidants, ArOH, inhibit peroxidation by trapping peroxyl radicals by a hydrogen atom transfer (HAT) process (reaction 1). The aryloxy radical, ArO$^\bullet$, traps a second peroxyl radical (reaction 2) so that the stoichiometric factor, $n$, for phenolic antioxidants is generally 2.

$$\mathrm{ROO}^\bullet + \mathrm{ArOH} \xrightarrow{k_{inh}} \mathrm{ROOH} + \mathrm{ArO}^\bullet \quad (1)$$

$$\mathrm{ROO}^\bullet + \mathrm{ArO}^\bullet \xrightarrow{\text{fast}} \text{non-radical products} \quad (2)$$

$$\{-\mathrm{d}[\mathrm{O_2}]/\mathrm{d}t\}_{inh} = k_p[\text{substrate}] \times R_i/nk_{inh}[\mathrm{ArOH}] \quad (3)$$

During the induction period, the reduced rate of oxygen uptake is given by eq 3, where $k_p$ is the propagation rate constant for the reaction of the substrate with peroxyl radicals and $R_i$ is the rate of free radical initiation. For quantitative studies, $R_i$ must be controlled, and this is usually done by using azo initiators that decompose at known rates to give carbon-centered radicals that react rapidly with oxygen to yield peroxyl radicals with known efficiency. The $R_i$ is measured from the length of the induction period, $\tau$, obtained by a phenolic antioxidant:

$$R_i = n[\mathrm{ArOH}]/\tau \quad (4)$$

For reliable determinations of $k_{inh}$, there must be a measurable kinetic chain length, $\nu$, during the induction period.[3] This is determined from the inhibited rate and the $R_i$ (eq 5). Values of $k_{inh}$ are calculated using the integrated form of eq 3 (eq 6).

$$\nu = \{-\mathrm{d}[\mathrm{O_2}]/\mathrm{d}t\}_{inh}/R_i \quad (5)$$

$$-\Delta[\mathrm{O_2}]_t = k_p[\text{substrate}]\ln(1 - t/\tau)/k_{inh} \quad (6)$$

A plot of $\Delta[\mathrm{O_2}]_t$ vs $\ln(1 - t/\tau)$ gives a straight line of slope $k_p[\text{substrate}]/k_{inh}$ from which $k_{inh}$ is obtained by using a substrate with a known $k_p$.

The AIBN-initiated autoxidation of styrene has advantages for quantitative studies of this kind and has been used to determine the $k_{inh}$ of a wide variety of phenolic antioxidants.[3] This method was therefore used to test the ability of bilirubin (BR) to trap peroxyl radicals in solution. Oxygen uptake traces are shown in Figure 1A for the uninhibited rate; the effects of BR and of the phenolic antioxidant 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC) are also shown during the inhibited oxidation of styrene in chlorobenzene. While PMHC gave a $k_{inh}$ of $407 \times 10^4$ $M^{-1}$ $s^{-1}$, in agreement with the literature value,[3] in contrast, BR did not give an induction period but only caused a slight reduction in oxygen uptake. In this system, BR behaved as a "retarder" rather than an active antioxidant.[4] A retarder reacts only comparatively slowly with peroxyl radicals so that chain termination also occurs by bimolecular self-reaction of peroxyl radicals. As a result, there is not a distinct induction period and the oxidation rate is slightly reduced past the time when two peroxyl radicals have been generated from the initiator for every molecule of retarder, while the retarder is only partially consumed. This was confirmed by UV−vis analysis of the reaction mixture long after the induction period was expected to terminate (eq 4), which showed that 90% of the BR was unreacted. Next we changed to cumene as the substrate. The $k_p$ for cumene is only 0.18 $M^{-1}$ $s^{-1}$ [5] compared to 41 $M^{-1}$ $s^{-1}$ for styrene[3] so that even poor antioxidants are known to give well-defined induction periods.[6] Typical results under these conditions are shown in Figure 1B. Here the relatively weak antioxidant, 3,5-di-*tert*-butyl-4-hydroxyanisole (DBHA),[3] gave a measurable induction period, whereas BR again only caused a reduction in the rate.

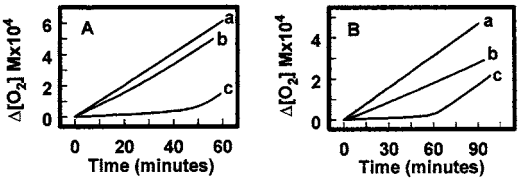


**Figure 1.** A. Oxygen uptake traces for the oxidation of styrene, 2.17 M, in chlorobenzene, initiated with azo-bis(isobutyronitrile), AIBN, 22.7 mM, at 30 °C. Curve a: uninhibited oxygen uptake. Curve b: inhibited by bilirubin (BR), 0.98 $\mu$M. Curve c: inhibited by 2,2,5,7,8-tetramethyl-6-hydroxychroman (PMHC), 3.28 $\mu$M. B. Oxygen uptake traces for the oxidation of cumene, 1.80 M, in chlorobenzene, initiated with azo-bis(isobutyronitrile), AIBN, 24.3 mM, at 30 °C. Curve a: uninhibited oxygen uptake. Curve b: inhibited by BR, 10.1 $\mu$M. Curve c: inhibited by 3,5-di-*tert*-butyl-4-hydroxyanisole (DBHA), 4.77 $\mu$M.

The antioxidant properties of BR depended markedly on the medium of the reaction. In contrast to its weak effect in a hydrocarbon−chlorobenzene solution, it displayed strong antioxidant activity in aqueous SDS micelles, phosphate buffer, pH = 7.4.[7,8] In this medium, bilirubin inhibited the oxidation of methyl linoleate initiated with ABAP using the oxygen electrode or the pressure transducer system. A typical experimental trace is shown in Figure 2 for BR along with

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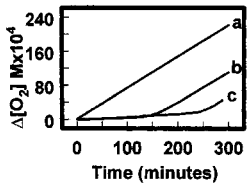


**Figure 2.** Oxygen uptake traces for the oxidation of methyl linoleate (ML) in aqueous sodium dodecyl sulfate (SDS) micelles, 0.5 M, initiated with azo-bis(amidinopropane) dihydrochloride (ABAP) at 37 °C. Curve a: uninhibited oxygen uptake with 0.437 M ML, 18.9 mM ABAP. Curve b: 0.437 M ML, 18.9 mM ABAP, inhibited by BR, 39.0 $\mu$M. Curve c: 0.495 M ML, 21.4 mM ABAP, inhibited by 2,2,5,7,8-tetramethyl-6-hydroxychroman (PMHC), 42.7 $\mu$M.

that of PMHC. Under these conditions, BR exhibited effective peroxyl trapping activity. A summary of the rate constants, $k_{inh}$, for BR, PMHC, and 2,5,7,8-tetramethyl-6-hydroxy-2-carboxychroman (Trolox) is given in Table 1. The

**Table 1.** Inhibition Rate Constants, $k_{Inh}$, and Stoichiometric Factors, $n$, of Bilirubin, BR, Trolox, and PMHC for the Inhibited Oxidation of Methyl Linoleate, ML, in 0.50 M SDS Micelles Initiated with ABAP in Phosphate Buffer, pH 7.4, at 37 °C[a]

| method[b] | inhibitor mol × $10^9$ | $k_{inh}$ $M^{-1}$ $s^{-1}$ × $10^{-4}$ [c] | $n$[d] |
|---|---|---|---|
| OE | BR[e] | | |
| | 1.85−12.0 | 5.2 | 1.5 |
| PT | BR,[f] 8.34 | 4.7 | 1.3 |
| OE | Trolox, 5.54 | 4.4 | 2 |
| PT | Trolox, 15.5 | 5.6 | 2 |
| PT | PMHC,[f] 1.94 | 4.9[g] | 2 |

[a] In all experiments, errors were less than 20%. [b] OE refers to the oxygen electrode and PT the pressure transducer. [c] Calculated for the micellar volume in 3.0 mL of 0.50 M SDS used with the OE and 2.0 mL with the PT method.[10b] [d] Stoichiometric value for phenols, Trolox, and PMHC is 2. The value for bilirubin was calculated from $n = R_i \times \tau/[BR]$, where $R_i = 2 \times [ArOH]/\tau$ and $\tau$ is the induction period of Trolox or PMHC. [e] BR was delivered in 0.05 M sodium hydroxide. [f] PMHC and BR were injected from a solution in 0.50 M SDS micelles. [g] Literature value gave $k_{inh} = 5.37 \times 10^4$ $M^{-1}$ $s^{-1}$ using linoleic acid in 0.50 M SDS.[10b] The $k_{inh}$ of PMHC was $407 \times 10^4$ $M^{-1}$ $s^{-1}$ in chlorobenzene−styrene (see text).

mean BR $k_{inh}$ , $5.0 \times 10^4$ $M^{-1}$ $s^{-1}$, is comparable with that of the active phenolic antioxidants with $n = 1.4$ somewhat lower than that of the phenols.

Lipid bilayers are frequently used as mimics of biomembranes, and BR is known to partition into bilayers,[9] so we carried out some experiments using multilamellar bilayers of egg lecithin and of PLPC in buffer, pH 7.4.[7,8] The bilayers were prepared using the coevaporation method[10a] by vortex stirring a film of the lipid containing DMVN and freeze−thaw cycles using liquid nitrogen under argon. The BR was introduced either by injecting a solution in 0.05 M sodium hydroxide during the oxidation or by coevaporating it with the lipid from chloroform. BR gave effective inhibition of lipid bilayer peroxidation, in both methods of addition, again in stark contrast to its poor activity in chlorobenzene. BR and DMVN were used in amounts designed to give long induction periods, preferably several hours, so as to minimize the error involved during temperature equilibration. Typical examples are as shown in Figure 3A with EL and Figure 3B with PLPC.

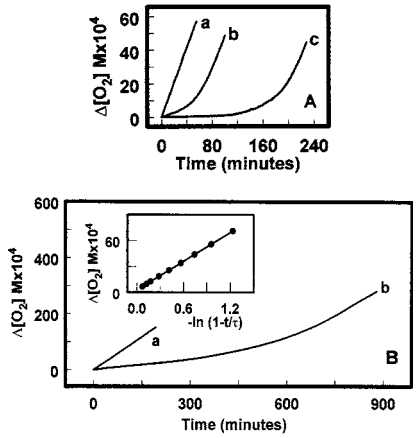


**Figure 3.** A. Oxygen uptake traces for the oxidation of egg lecithin (EL), 1.05 M, initiated with azo-bis(amidinopropane)dihydrochloride (ABAP), 0.154 M, at 37 °C. Curve a: uninhibited oxygen uptake. Curve b: inhibited by BR, 0.421 mM, injected in sodium hydroxide. Curve c: inhibited by BR, 1.01 mM, coevaporated with the EL. B. Oxygen uptake traces for the oxidation of 1-palmitoyl-2-linoleoylphosphatidyl choline (PLPC), 1.05 M, initiated with azo-bis(2,4-dimethylvaleronitrile) (DMVN), 0.225 M, at 37 °C. Curve a: uninhibited oxygen uptake. Curve b: inhibited by 0.466 mM BR, coevaporated with the PLPC. Inset: plot of data from curve b according to eq 6 (see text).

Satisfactory linear plots according to eq 6 were obtained in all cases, including those for long induction periods; for example, the inset for Figure 3B gave an $R^2 = 1.000$. Using the reported $k_p = 16.6$ $M^{-1}$ $s^{-1}$ for PLPC,[11] the $k_{inh}$ for BR was determined during inhibited oxidation initiated by DMVN and is summarized in Table 2 compared with results for Trolox. Bilirubin is about one-third as active as Trolox under these conditions from the $k_{inh}$ values but has a comparable stoichiometric factor of radical trapping. The lower $k_{inh}$ of BR in bilayers compared to Trolox or compared to the value in micelles is attributed to rate-limiting diffusion between the lipid peroxyl radicals and BR since the ionic form of BR at pH 7.4 is probably located at the membrane−water interface as suggested,[9a] while the peroxyl radicals, initiated with lipid-soluble DMVN, are formed deep in the bilayer.[12]

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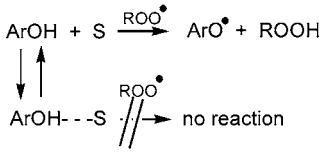
**Table 2.** Inhibition Rate Constants, $k_{inh}$, and $n$ Factors of BR and Trolox for Inhibited Oxidation of PLPC Bilayers Initiated with DMVN at 37 °C, pH 7.4[a]

| method[b] | inhibitor mol × $10^8$ | $k_{inh}$ $M^{-1}$ $s^{-1}$ × $10^{-3}$ [c] | $n$[d] |
|---|---|---|---|
| coevap | BR | | |
| | 3.02 | 3.0 | 1.9 |
| | 3.02 | 3.2 | 1.8 |
| injected in buffer | Trolox | | |
| | 1.04 | 10.4 | 2 |
| | 1.05 | 10.7 | 2 |

[a] In all experiments, variation was less than 20%. [b] Amounts used were PLPC, 6.83 × $10^{-5}$ mol; DMVN, 1.46 × $10^{-5}$ mol. [c] Calculated for the lipid volume of the bilayers.[10a] [d] Stoichiometric factors relative to $n = 2$ for Trolox; see Table 1, footnote c.

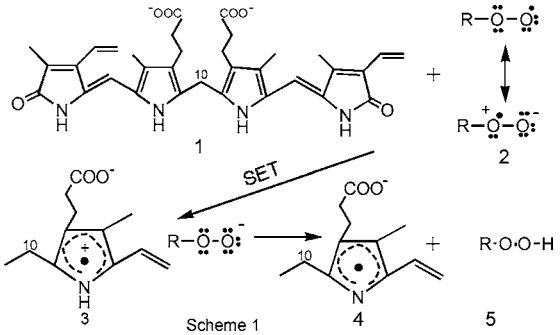
In general, phenolic antioxidants lose much of their HAT activity in polar, hydrogen-bond-accepting solvents (S) because H-bonded ArOH molecules are unreactive toward HAT.[13] Therefore, phenols have reduced HAT activities toward peroxyl radicals in aqueous lipid dispersions[10a,b] due to ArOH···S association (eq 7).




(7)

PMHC is at least 80 times more active in chlorobenzene−styrene than in aqueous SDS micelles due to this medium effect (Table 1). In contrast, the antioxidant activity of BR switched from little (or no) activity in chlorobenzene to increase markedly in aqueous SDS where it matched the rate constants, $k_{inh}$, of the vitamin E analogues, PMHC, or Trolox (Table 1). This remarkable difference for BR is attributed to the mechanism for the reaction between BR and peroxyl radicals. The hydrogen atom transfer (HAT) mechanism common for phenolic antioxidants (reaction 1) is VERY improbable for BR. For example, the proposed HAT reaction from the connecting $-C^{10}H_2-$ group[2g] is unlikely because such a process should occur whether the molecule is in the internally hydrogen bonded "ridge-tile" conformation,[14] expected in nonprotic organic solvents, or in an open conformation when the carboxyls are ionized at pH 7.4. Similarly, peroxyl radical trapping by addition to BR[2d] is an unlikely pathway because this reaction should occur just as readily in solution as in aqueous systems (e.g., in "ridge-tile" or open conformers). Further, a HAT reaction from a N−H group is improbable because this bond is orthogonal with the pyrrole $\pi$-electron system and the bond dissociation enthalpy (BDE) of this bond is several kcal higher than that of phenol.[15] We propose that BR reacts with peroxyl radicals by a single-electron transfer (SET) mechanism. A SET reaction is expected to be favored in the polar media of aqueous lipids. Known properties of pyrroles favor an SET reaction. These include the relatively low ionization potential of pyrrole compared to phenol,[16] the SET reaction observed with oxygen to form cation radicals,[17] and a transient reported for an SET reaction of dianionic bilirubin to haloperoxyl radicals in alcohols.[18] The SET reaction with ionized bilirubin, **1** , and peroxyl radicals, **2**, known to be strongly polarized,[19] is proposed for the reaction of BR with peroxyl radicals (see Scheme 1). The initial SET would form an ion

**Scheme 1**




Scheme 1

pair, **3**, and reactions of **3** such as direct proton transfer or via a separated ion pair would form a pyrrole radical, **4**, and the hydroperoxide, **5**. Since bilirubin possesses two pyrrole rings, it could deactivate two peroxyl radicals by SET giving, theoretically, a $n$ factor of 2. The factor is approximately 2 for reactions in PLPC bilayers during initiation by lipid-soluble DMVN. The $n$ factor dropped to 1.3−1.5 for reactions initiated by water-soluble ABAP (Table 1) most likely due to some direct SET oxidation[17] of BR in the aqueous phase. The proposed SET mechanism (Scheme 1) accounts for the remarkable effects of the reaction medium on the antioxidant activity of BR, and earlier reports[2] on the antioxidant properties of BR in aqueous lipid dispersions probably also involved SET reactions.

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**Supporting Information Available:** Tables S1 and S2 with additional information on the experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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