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The Antioxidant Role of Bile Pigments Evaluated by Chemical Tests

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Abstract—Bilirubin, biliverdin and their serum albumin complexes were tested as oxyradical scavengers (superoxide generated by the xanthine/xanthine oxidase system and peroxyl radical-trapping antioxidant ability). As superoxide scavengers the free bile pigments showed activities near to that of serum albumin, higher than the water soluble vitamin E analog Trolox and lower than ascorbic acid. The peroxyl radical-trapping antioxidant abilities of the tested bile pigments were much higher than those of the serum albumin and of the same order as their serum albumin complexes. This interaction with peroxyl radicals showed different stoichiometric factors for bilirubin (≈ 2) and biliverdin (≈ 4).

Introduction

On the basis of the general hypothesis that end products of degradation metabolic pathways may play a role as useful biological agents, bilirubin[1] (BR) has been proposed as a radical scavenger and its activity as chain-breaking antioxidant in lipid autoxidation has been shown *in vitro*.[2–4] Further, BR at biological levels has been associated with processes in which superoxide anion and oxidative stress are generated.[5–8] In this respect, BR and its biological precursor, biliverdin (BV) interact chemically with the superoxide anion.[9–12] The type of chemical interaction was recently demonstrated[11,12] to be a proton-induced superoxide dismutation,[13] where the bile pigment is N–H deprotonated. In this context, the role of the water insoluble bilirubin in lipophilic media as non-enzymatic superoxide scavenger has been suggested. The cytoprotective role of BR and BV to oxyradical damage has been shown *in vitro* through the study of the disruption of microsomal membrane fractions[14,15] and of the degree of necrosis in cell preparations.[16,17]

The understanding of the chemical processes involved in the protective effect of antioxidant scavengers to oxyradical damage is confronted by several obstacles, basically due to the different types of oxyradicals (e.g. the chemical behaviour of superoxide anion is different to that of the peroxyl radical), the different chemical environments (i.e. hydrophilic, lipophilic and lyotropic membrane structures) and the low correlation of the available chemical tests with biological models. Here we present the results of two chemical tests that evaluate the antioxidant roles of BR, BV and their complexes with bovine serum albumin (BSA) and human serum albumin (HSA)(BR-BSA, BR-HSA, BV-BSA, BV-HSA) against superoxide anion and peroxyl radicals. The reaction with superoxide (generated by the xanthine/xanthine oxidase system) was followed in competition with a reference oxidant.[18,19] The peroxyl radical-trapping antioxidant ability was measured through the consumption of dioxygen in an autoxidation test of linoleic acid. The results of both tests were compared and related to previously reported *in vitro* results.[2–8,14–17]

Experimental

Bilirubin IXα (BR), bovine serum albumin (BSA: initial fractionation by cold alcohol precipitation; fatty acid free), human serum albumin (HSA), cytochrome *c* (type III from horse heart), xanthine and xanthine oxidase were purchased from Sigma. (-)-Ascorbic acid, sodium linoleate, nitro blue tetrazolium chloride (NBT), 6-hydroxy-2,5,7,8-tetramethylchroma-2-carboxylic acid (Trolox) were supplied by Aldrich. Trimethylcetylammonium bromide (TMCA) was supplied by C. Erba and 2,2'-azo-bis(2-amidinopropane hydrochloride) (ABAP) by Polysciences Inc.

Biliverdin IXα (BV), mesobiliverdin IXα (MBV) and mesobilirubin IXα (MBR) were obtained from BR following McDonagh.[20] The bovine serum albumin (BSA) and human serum albumin (HSA) complexes[20] of BR and BV (BR-BSA, BR-HSA, BV-BSA and BV-HSA) were obtained by addition of a supersaturated aqueous solution of the bile pigment (obtained by dissolution of 1 mg of bile pigment in the minimum volume of 1 x 10^{-2} M NaOH and subsequent dilution to 2 ml with water) to an equimolecular aqueous solution (111 mg in 1 mL water) of the corresponding serum albumin, followed by equilibration in an ultrasonic bath; the pH was adjusted to 7.4 by addition of a 10 mM HCl solution when necessary, and the preparation was lyophilized.

The water insolubility of BR does not allow its direct testing, which is possible for the more soluble BV. However, BR solubilized by the cationic tensoactive TMCA could be tested for the TRAP value.

The UV/Vis spectra were recorded on a Perkin-Elmer Lambda 5 instrument.

Superoxide anion decomposition assay

The measurement was based on the competitive reaction of the superoxide anion with a reference oxidant and with the substance tested.[18,19] Cytochrome *c* is the commonly used oxidant, but it has also been described as a BR oxidant.[9,21] Previous tests showed us this interference, which also appears in the case of BV. Therefore, NBT was used instead of cytochrome *c*, as NBT does not react with the bile pigments. NBT is reduced by the superoxide anion to formazan, absorbing at 560 nm. In order to avoid the interferences due to the reaction of the bile pigments with hydrogen peroxide (generated from the superoxide dismutation) some experiments were also performed in the presence of about 1 nmol catalase: no significant difference was observed in the presence or absence of catalase. When a substance is added which competes with NBT for the superoxide anion decomposition the production of formazan is lowered. About 6 nmol xanthine oxidase was added to a spectrometric cuvette with 0.01 M phosphate buffer (pH = 7.4, 25 °C), 0.1 mM xanthine, 50 μM NBT and the substance to be tested. This addition corresponds to the zero time of the measurement and for the blank solution it leads to an absorbance increase at 560 nm of about 0.025 absorbance unit *per* minute. Results are expressed as the substance concentration which allows a 50% inhibition of the initial NBT reduction rate.

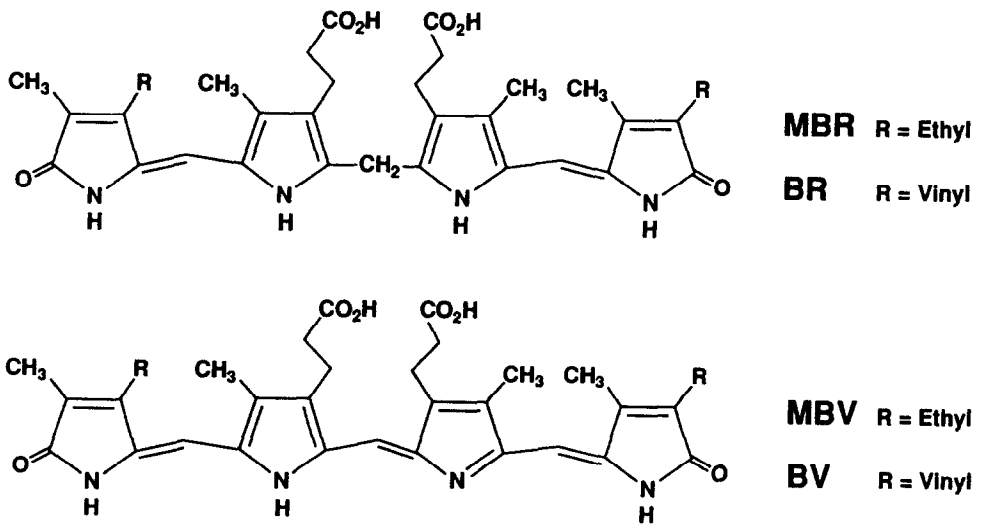
Total (peroxyl) radical-trapping antioxidant parameter (TRAP) assay

The measurement of TRAP was based on that described by Burton and Ingold.[22–25] The test is based on the decomposition to radicals at a constant rate of the water-soluble azo compound ABAP. The resulting radicals with the dioxygen present in the solution give hydroperoxyl radicals, which in turn react with an unsaturated fatty acid salt dispersed in phosphate buffer: the rate of this oxidation is monitored, using a Clark oxygen electrode, by the decrease of dioxygen in the solution (closed system). The so-called induction time, during which the fatty acid chain oxidation is broken, was measured and compared to that of the water-soluble vitamin E analog Trolox.

60 μL of a solution of 10 mg sodium linoleate in 1 mL 0.7 mM phosphate buffer pH 8.0, and 60 μL of a 0.6 M ABAP solution (phosphate buffer) were added to 3 mL of a 5 mM phosphate buffer (pH 8.0) in which a Clark oxygen electrode was dipped (Yellow Springs Scientific; biological oxygen monitor 5300, standard oxygen probe) at 37 °C: after 5 min 10 μL of a solution of the test substance (0.3–0.5 mM) was added. Just after recovery of the basal dioxygen consumption, 10 μL of 1 mM Trolox solution was introduced. The slopes at the point corresponding to the addition of inhibitor were used to calculate the rate of oxygen uptake ($-dO_2/dt$). The induction time (τ: time during which the lipid resists peroxidation in the presence of the test substance)[22,23] was determined in the usual way from the length of time between antioxidant injection and the point of the intersection of tangents to the oxidation curve corresponding to the initial inhibited and final uninhibited rates of oxidation. The TRAP values referred to Trolox, which has been extensively used as reference substance for antioxidant ability, were calculated from the ratio of the induction time of the tested substance to the corresponding molar induction time of a Trolox solution (taking into account the stoichiometric factor of 2 for Trolox)[24,25] showing a similar induction time: i.e. $TRAP_X = 2[\text{Trolox}]\,\tau_X$. Assays were performed and

$$[X]\,\tau_{\text{Trolox}}$$

evaluated for Trolox and the corresponding bile pigment in the same experiment: only relative TRAP and relative rate of oxygen consumption are given, because it was difficult in separated experiments to obtain the same basal oxygen consumption. Values of Table 1 and Figure 1 correspond at least, for each substance, to a set of three separated experiments.



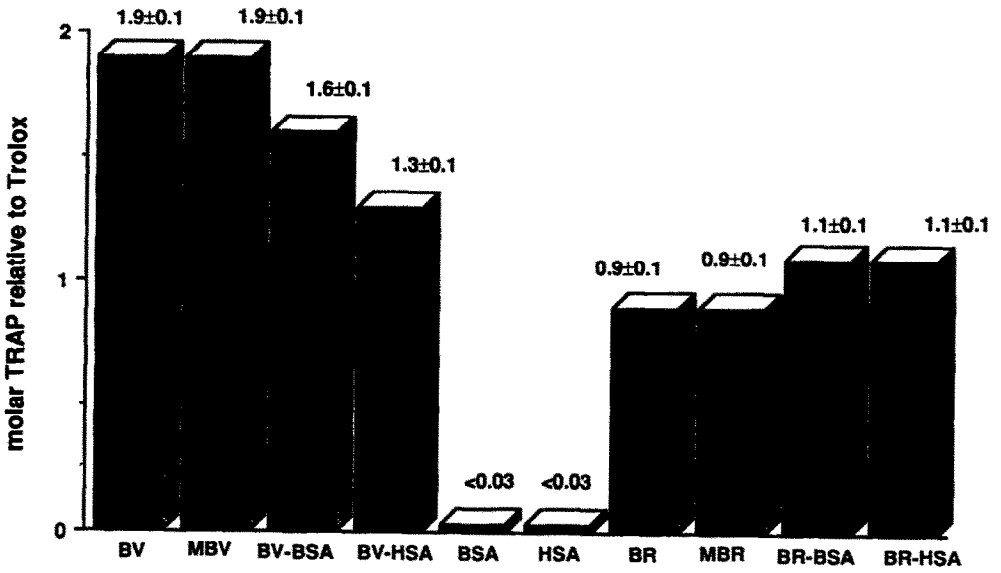


Figure 1. Oxyradical trapping antioxidant ability [expressed as TRAP parameter relative to Trolox (ref.[20–23])] of bile pigments and their albumin complexes (see Formula Scheme). TRAP values were determined from the dioxygen consumption with time in the linoleic acid (0.7 μM) chain radical peroxidation initiated by ABAP (4 mM) at pH 8.0 (Trolox typical induction time values were about 6–8 min/μM at the concentration range 1.2–1.8 μM)

Table 1. Superoxide scavenger activity in front of xanthine oxidase generated superoxide, expressed as the concentration necessary to achieve 50% inhibition (IC_{50}) in the reduction of NBT, and rate of oxygen consumption during the induction period of the TRAP assay (see Experimental)

	$O_2^{\cdot-}$ assay IC_{50} (μM) NBT oxidation [a]	Peroxyl radical-trapping assay $-dO_2/dt$ at 1.6 μM inhibitor relative to the basal oxygen consumption [b]
Bilirubin (BR)	75±6	0.10±0.02
Biliverdin (BV)	80±6	0.15±0.03
Mesobilirubin (MBR)	-----	0.13±0.03
Mesobiliverdin (MBV)	-----	0.15±0.04
BR-BSA	62±5	0.11±0.01
BV-BSA	55±5	0.12±0.02
BSA	62±5	> 0.99
Ascorbic acid	9±2	--------
Trolox	270±15	0.40±0.08

a) Commerical superoxide dismutase (SOD) in the same conditions[34] shows an $IC_{50} \approx 1 \times 10^{-3}$ μM, i.e. the IC_{50} column correspond to values between $2 \times 10^{-5} - 6 \times 10^{-4}$ SOD units/mol.
b) Typical absolute value for basal $-dO_2/dt$ was about 55 nmol-O_2 l^{-1} s^{-1}.

Results and Discussion

In the superoxide scavenger activity assay any of the tested substances do not show effect on the rate of uric acid formation (detected by the increase of absorbance at 295 nm), i.e. they do not inhibit xanthine oxidase. Further, the superoxide scavenger activities were not significantly different in the presence or absence of catalase. The bile pigments alone showed an intermediate activity between the poor value of Trolox and the high value of ascorbic acid (see Table 1). This result points to an interaction between bile pigment and superoxide anion originated by a proton induced dismutation, which has already been proposed[11,12] The inhibition activities of the bile pigment–albumin complexes do not differ significantly from the activity of the serum albumin alone, which also shows activity as superoxide scavenger, i.e. the superoxide scavenging activity could be attributed to the protein but not to the complexed bile pigment. In this case, the formation of the bile pigment–albumin complexes would result in the extinction of the proton induced dismutation capability of the bile pigment.

The bile pigments and their serum albumin complexes showed TRAP values (see Figure 1) which were of the same order of magnitude as for the vitamin E water soluble

analog Trolox. The results shown in Figure 1 are expressed relative to Trolox, $TRAP_X/TRAP_{Trolox}$, for the same concentration of product (1.6 ± 0.3 μM). The TRAP values for equimolecular solutions of BR or BV with the serum albumins did not show significant differences with the values obtained from the lyophilized preparations (see Materials and Methods), which accounts for the absence of any irreversible reaction between bile pigment and serum albumin in the experimental procedure used to obtain the serum albumin complexes. The serum albumins alone showed very low TRAP values (at the limit of detection of the method). The non vinyl-substituted bile pigments (MBR and MBV) showed the same relative TRAP values as their vinyl substituted counterparts (BR and BV).

The TRAP values (induction periods) are a measure of the stoichiometry of inhibitor radical trapping ability. Further, the rate of oxygen uptake during the induction periods can be used to compare between inhibitors the rate of trapping of the chain carrying peroxyl radicals.[27,28] The rate of oxygen uptake during the induction period is of the same order of magnitude for Trolox than for the bile pigments (see Table 1). In our experimental conditions no significant differences could be observed in the rates between bile pigments and their albumin complexes.[29] However, at the 1.5 to 3 μM concentration range Trolox showed higher oxygen uptake than the bile pigments and their albumin complexes. Further, Trolox shows a dependence of the rate of oxygen consumption on the concentration: the change of slope is higher at higher Trolox concentrations (0.08 units relative to basal oxygen consumption per μmol Trolox, in the concentration range 1 to 5 μM). For the bile pigments and their albumin complexes no dependence on the concentration could be observed, probably because in these cases the oxygen consumption is of the same order of magnitude as that originated by secondary processes, e.g. O_2 electrolysis. This result agrees with a previous one reported for BR-HSA, which showed a faster scavenging rate than the process of formation of peroxyl radicals.[4]

The differences in voltammetric oxidation potentials between bile pigments[30] (bilirubins are easier to oxidize than biliverdins) are not reflected in the superoxide scavenger and oxyradical antioxidant activity values reported here.

In contrast to the superoxide scavenger activity, the TRAP values suggest that the peroxyradical antioxidant activity of the albumin complexes would be due to the bile pigment and not to the protein part of the complex. From the binding constants of BR and BV with serum albumins[18] it can be inferred that the concentration of 'free' BR or BV in the testing conditions of the serum albumin complexes is very low, $<1 \times 10^{-8}$ M. Furthermore, the dissociation rate constants of the bile pigment serum albumin complexes[18] are not very fast ($\approx 1 \times 10^{-2}$ s). These thermodynamic and kinetic values suggest that the reaction of the albumin complexes with the generated peroxyradicals does not fit Curtin–Hammett conditions. However, it cannot be ruled out that the TRAP values measured could be at least partially due to the free bile pigment present in the solution. In this case, the bile pigment serum albumin complex would simply act as a 'reservoir' of the active bile pigment.

The high change of the rate of oxygen uptake in the TRAP test does not allow the differentiation between the vinyl and the ethyl derivatives, nor between the two different types of π system (bilirubins and biliverdins). However, BR and BV differ in their stoichiometric factors *n*. The relative TRAP values for BV and for its serum albumin complexes are higher (1.3–1.9) than those for BR and the corresponding complexes (≈1.0). Taking a stoichiometric factor of 2 for Trolox,[23–25] it results in a stoichiometric factor of 2 ± 0.2 for BR and its serum albumin complexes, as it has been already reported in a similar assay for BR-HSA.[4] However, it results in a stoichiometric factor of about 4.0 for BV, and slightly lower (near 3) for its serum albumin complexes. The $n = 2$ value for BR and its serum albumin complexes is the value commonly found for antioxidants. It comes from the fact that, globally, one radical-trapping molecule RH_2 interacts with 2 peroxyl radicals, according to the following general scheme:

$$RH_2 + LO_2^{\bullet} \rightarrow RH^{\bullet} + LO_2H$$
$$RH^{\bullet} + LO_2^{\bullet} \rightarrow LO_2H + \text{non-radical products.}$$

According to this scheme if the expected non-radical product obtained from bilirubins were biliverdins, which are also active against oxyradicals the stoichiometric factor of bilirubins should be higher than 2. The fact that a normal stoichiometric factor of 2 is observed for BR points to other oxidation pathways of bilirubins than the normal one towards biliverdins (directly towards the two ring system of propentdyopents?) as it has recently been proposed.[31] In fact, two different oxidative pathways for BR degradation have also been observed[32] in the microsomal oxidation in the presence of cytochrome P450 inducers and in Fenton chemistry conditions (H_2O_2, Fe–EDTA). A stoichiometric factor of about 4 for BV can be explained through a 2 + 2 pathway: i. e. oxidative fragmentation toward biliviolins (a three ring system) which in turn could also undergo oxidative fragmentation.[31]

In respect to the reported cytoprotective effect of bile pigments, against radicals generated by the xanthine/xanthine oxidase system, peroxyl radicals or in Fenton reaction conditions[14–17] our results point to a more important role of the serum albumin bonded bile pigments as peroxyl radical trapping antioxidants than as superoxide scavengers; e.g. against Fenton-generated radicals, serum albumin alone shows a much lower cytoprotective effect than its bile pigment complexes.[14]

Bilirubin is present at 0.26–1.4 mg% level in human non-hyperbilirubinemic plasma,[32] about 20% of bilirubin being in the form of its soluble glucoronate and the rest as serum albumin bilirubin complex, i.e. the human non-conjugated bilirubin concentration level ranges from 5 to 30 μM. The values shown in Figure 1 represent an oxyradical chain-breaking antioxidant contribution equivalent to 5–30 μM Trolox for the bilirubin concentrations in human plasma. Human plasma

shows[24,30] total antioxidant values equivalent to 300–900 μM Trolox. In conclusion, free or albumin bound bilirubin accounts for a part of serum TRAP ability values. This contribution to the TRAP value of human plasma is of the same order of magnitude as the contribution of vitamin E or ascorbate.[24]

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