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Free Radical Formation and Erythrocyte Membrane Alterations during MetHb Formation Induced by the BHA Metabolite, *tert*-Butylhydroquinone

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Erythrocyte membranes are altered as a consequence of oxidative stress following the incubation of intact erythrocytes with one of the major metabolites of the antioxidant butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (*t*BHQ). A rather persistent semiquinone radical was observed by electron spin resonance (ESR) spectroscopy when *t*BHQ was incubated with either homogeneous oxyhemoglobin solutions or suspensions of intact erythrocytes. Erythrocyte ghosts prepared from fresh control erythrocytes and ghosts from erythrocytes preincubated with BHA and its metabolite, *t*BHQ, were subjected to polyacrylamide gel electrophoresis (SDS-PAGE). Only minor changes of the electrophoresis pattern relative to the control was observed in the BHA incubations whereas *t*BHQ significantly increased the amount of high molecular weight degradation products of erythrocyte membrane constituents. These changes were only observed when incubations were performed in the presence of oxygen. In control experiments where heme oxygen was replaced by carbon monoxide, no membrane degradation products appeared. These observations can be interpreted in terms of metabolic activation of the antioxidant BHA via *t*BHQ to the *tert*-butylsemiquinone free radical and finally to the corresponding quinone, thereby leading to harmful effects on erythrocyte membrane structures. Moreover, deleterious effects on other biological membranes are also likely to occur.

Keywords: ESR, SDS-PAGE, erythrocyte membrane alteration, BHA metabolite, *tert*-butylhydroquinone, antioxidants, food additives

Abbreviations: BG I, minor bovine glycoprotein; BG II, major bovine glycoprotein; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DEAE, diethylaminoethyl-; DTPA, diethyltriaminepentaacetic acid; DTT, dithiothreitol; ESR, electron spin resonance; GSH, glutathione (reduced form); HbO_2, oxyhemoglobin; HMWC, high molecular weight compounds; MetHb, methemoglobin; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; *t*BHQ, *tert*-butylhydroquinone

INTRODUCTION

Phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or .the hydroquinone *tert*-butylhydroquinone (*t*BHQ) are frequently used as food preservatives, especially in fat- and oil-containing foods.[1] *t*BHQ has also been reported to be one of the major metabolites of BHA *in vivo*[2–4] and in

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experiments with rat liver microsomes *in vitro*.[5] It was found that the addition of only 0.02% *t*BHQ to oxygen exposed fish oil, which is easily oxidized due to its high content of polyunsaturated fatty acids, prevented oxidative alterations for more than 72 h whereas peroxide levels increased 12-fold within 48 h in the absence of antioxidants.[6] *t*BHQ has also been shown to inhibit superoxide-dependent chemiluminescence, to prevent lipid peroxidation, and to have antitumor effects.[7] On the other hand, there are also reports on toxic side effects of *t*BHQ.[8] More recently, Okubo *et al.*[9] reported on the formation of oxygen radicals leading to DNA cleavage, and Zhang *et al.*[10] questioned the application of *t*BHQ as an antioxidant due to possible carcinogenic effects. Especially in two-stage carcinogenesis model experiments together with $NaNO_2$, *t*BHQ showed a strong enhancing effect on the thickness of the forestomach mucosa of rats.[11] An inhibitory effect of *t*BHQ on the mitochondrial electron transport chain before cytochrome b has also been suggested.[12]

We have previously reported on the formation of reactive intermediates such as free radicals detectable by ESR[13–18] or compound I ferryl heme species observed by low-level chemiluminescence[19–21] which are formed in the reaction of hydroxylamines[15–17] or phenolic compounds[13,14] with oxyhemoglobin or oxymyoglobin.[18] While xenobiotics have direct access to the heme center in homogeneous solution, the reactivity of erythrocyte-bound hemoglobin does also depend on the ability of the respective xenobiotic to permeate the erythrocyte membrane. In addition to our earlier findings on the formation of MetHb and the observation of toxic reaction intermediates such as free radicals or ferryl hemoglobin, reducing xenobiotics such as BHA or *t*BHQ, might also alter the erythrocyte membrane, eventually leading to partial or complete hemolysis and the formation of Heinz bodies.[22]

We have previously shown that lipophilic compounds such as BHA or BHT have a severe impact on the membrane characteristics of both, heart mitochondria[23] and bovine erythrocytes.[24] In addition, ESR spin-labeling investigations by Butterfield *et al.*[25–29] have shown that different xenobiotic-sensitive sites of the erythrocyte membrane exist, the lipid bilayer,[28] the polar headgroups,[29] SH-groups on the spectrin web,[26] and different sugar moieties[27] located on the outer side of the membrane.

The aim of the present study was to investigate if, and to what extent the reactive metabolites recently detected in homogeneous oxyhemoglobin solution [13–17,19,20] as well as with intact erythrocytes[24] can alter the pattern of erythrocyte membrane constituents, which are detectable by means of polyacrylamide gel electrophoresis in the absence and presence of sodium dodecylsulfate (SDS-PAGE).

MATERIALS AND METHODS

Chemicals

BHA (2- and 3-*tert*-butyl-4-hydroxyanisole) and *tert*-butylhydroquinone (*t*BHQ) were purchased from Aldrich (Steinheim, Germany) and were recrystallized from hexane before use. Ammonium persulfate, bromophenol blue sodium salt, dithiothreitol, SDS and tris-(hydroxymethyl)-aminomethane were from Merck (Darmstadt, Germany), Sephadex G 25, LMW and HMW-SDS electrophoresis calibration kit were from Pharmacia (Uppsala, Sweden), glycerol, N,N′-methylene-bis-acrylamide, and N,N,N′, N′-tetramethyl-ethylenediamine were from Sigma (Deisenhofen, Germany), Celite and $DEAE_{25}$-cellulose were from Serva (Heidelberg, Germany).

Isolation of Erythrocytes

Bovine erythrocytes were prepared in a modified procedure described by Eyer *et al.*[30] for human hemoglobin. After removal of plasma and leucocytes by centrifugation for 10 min at $2200\times g$ the cells were washed five times by addition of twice

the amount of phosphate-buffered saline (PBS: 140 mM NaCl, 10 mM sodium/potassium phosphate, pH 7.4) and subsequent centrifugation (10 min 2200× *g*). The hemoglobin content was determined as follows: 100 µL of homogeneous cell suspension was hemolyzed in an adequate amount of hypotonic buffer (5 mM sodium/potassium phosphate, pH 7.4) and the absorbance was measured at 540 nm according to Grisk.[31] The erythrocytes were stored at 4°C and used within three days.

Preparation of Erythrocyte Ghosts

After washing the erythrocytes with PBS buffer in order to remove excess xenobiotic, erythrocyte ghosts were prepared by hemolysis of packed erythrocytes in a 5–10-fold excess of phosphate buffer (5 mM, pH 7.4). The resulting hemolysate was centrifuged for 10 min at 11000× *g*, the supernatant was subsequently removed by careful aspiration. This procedure was repeated 3–4 times, until the supernatant appeared colorless. After each centrifugation, the reddish button on the bottom of the membrane fraction was carefully removed. Membrane protein content was determined according to Lowry *et al.*[32]

Isolation of Hemoglobin

For the isolation of hemoglobin,[30] the erythrocytes were hemolyzed in phosphate buffer (5 mM, pH 7.4) and 10 g of Celite was added to 250 mL of the hemolysate. The mixture was stirred for 20 min and then centrifuged for 30 min at 15,000× *g*. Purified hemoglobin was prepared by repeated treatment of the crude hemolysate with $DEAE_{52}$-cellulose for 30 min followed by centrifugation at 15,000× *g*. The fractions were tested for catalase[33] and SOD activity:[34] only those with a catalase activity $k < 1$ and no detectable SOD activity were pooled. The pooled fractions were dialyzed several times against 1.5 mM phosphate buffer, pH 7.4 until no traces of glutathione were detectable in the dialysis buffer. Oxyhemoglobin was determined at its absorption maximum at 540 nm as described above.

ESR Experiments

All ESR experiments were carried out in a Bruker ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a rectangular TM_{110} microwave cavity and a Bruker data system ESP 1600.

Incubation of Erythrocytes and Erythrocyte Ghosts with BHA or *t*BHQ

Erythrocytes were incubated in PBS pH 7.4 and erythrocyte ghosts were incubated in phosphate buffer (5 mM, pH 7.4) alone (control) or in the presence of the respective xenobiotic: BHA (1 mM, 4 h at 25°C), or *t*BHQ (0–2 mM, 4 h at 25°C). After the incubation, excess xenobiotic was removed by centrifugation. From intact erythrocytes, membrane ghosts were prepared according to the procedure described above.

SDS-PAGE

Electrophoresis was performed with a Biorad Protean II slab cell apparatus. A neutral discontinuous buffer system according to Laemmli[35] was used on 200 × 200 × 0.75 mm polyacrylamide slab gels with a 5–20% gradient and containing 0.1% SDS. Samples were diluted 1 : 2 with either non-reducing or reducing sample buffer (containing 200 mM DTT). 20–30 µl per track were applied to the gels with a Hamilton syringe after equilibration for about 15 min (or heating to 60°C for 10 min). Stacking usually lasted 45 min at 13 mA, while the run was 4–5 h at 20 mA, leading to a 15 cm migration of the buffer front on the analyzing gel. The slab gels were stained overnight with Coomassie brilliant blue in a 40% methanol/10% acetic acid solution. After destaining densitometric measurements were performed at 590 nm on a Camag TLC Scanner with transient light option.

RESULTS

ESR Experiments

When erythrocytes (4.2 mM HbO_2) were incubated in the presence of *t*BHQ (8.3 mM), an 8-line ESR spectrum appeared within a few minutes (Figure 1A), whose coupling constants ($a_H = 2.90$, 2.15, and 1.65 G, PBS, pH 7.4) were consistent with those reported for the 2-*tert*-butyl-*p*-benzosemiquinone free radical ($a_H = 3.05$, 2.15, and 1.70 G; alkaline water solution).[36] Within about 1 h a broad, unresolved secondary species appeared which was superimposed to the original semiquinone radical (Figure 1B). The 2-*tert*-butyl-*p*-benzosemiquinone radical was formed immediately when *t*BHQ was mixed with a homogeneous oxyhemoglobin solution at pH 7.4 (Figure 2A), after prolonged incubation the above-mentioned secondary radical appeared gradually (Figure 2B). In the absence of oxyhemoglobin no ESR signal was detectable during the first 10 min (Figure 2C) and even after prolonged incubation only a very weak signal of the 2-*tert*-butyl-*p*-benzosemiquinone radical appeared which was due to autoxidation of *t*BHQ in the presence of oxygen (not shown), the secondary product was, however, not formed.

Pretreatment of oxyhemoglobin (6 mM) with the thiol-blocking agent N-ethylmaleimide (NEM) enhanced the intensity of the 2-*tert*-butyl-*p*-benzosemiquinone signal about 2-fold and prevented the formation of the secondary species (experiment not shown). This indicates that in the absence of NEM part of the 2-*tert*-butyl-*p*-benzosemiquinone radicals react with an easily accessible SH-group of the globin moiety, possibly at position β-93,[37] thereby giving rise to the more stable secondary radical species. The ESR spectrum of this immobilized secondary radical could

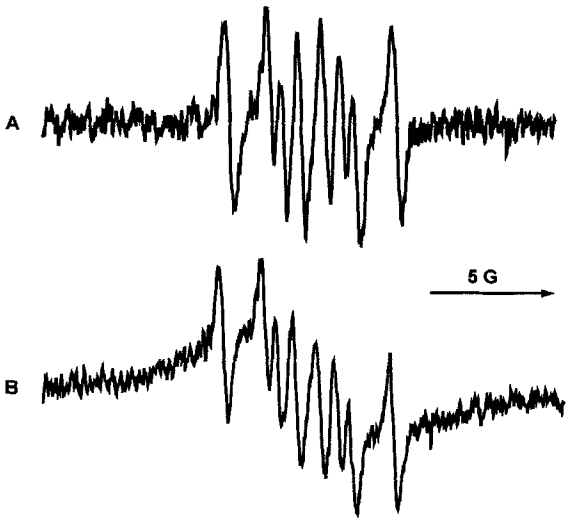

FIGURE 1 *Formation of the 2-tert-butyl-p-benzosemiquinone free radical in whole erythrocytes.* A: Erythrocytes (4.2 mM heme content, suspended in isotonic PBS buffer, pH 7.4), and *t*BHQ (8.3 mM) were incubated and measured in a quartz flat cell. ESR settings: sweep width, 20 G; modulation amplitude, 0.4 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 1×10^5; scan rate, 14.8 G/min. B: Same incubation as in A but recorded after 60 min at room temperature (same ESR settings as in Figure 1A).

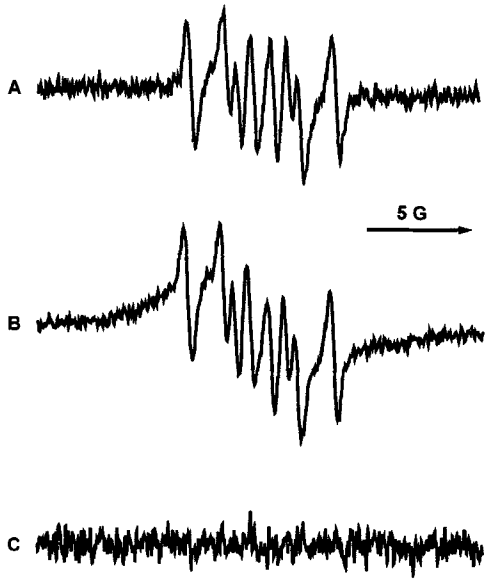

FIGURE 2 *Formation of the 2-tert-butyl-p-benzosemiquinone free radical in homogeneous oxyhemoglobin solution.* A: Oxyhemoglobin (2.5 mM heme content, dissolved in isotonic PBS buffer, pH 7.4, containing 1 mM DTPA), and *t*BHQ (8.3 mM) were incubated and measured in a quartz flat cell. ESR settings: sweep width, 20 G; modulation amplitude, 0.25 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 1×10^5; scan rate, 14.8 G/min. B: Same incubation as in A but recorded after 5 h at room temperature (same ESR settings as in Figure 1A). C: Incubation of *t*BHQ (8.3 mM) in the absence of oxyhemoglobin under otherwise identical conditions.

be obtained either by subtraction of the pure semiquinone radical spectrum (recorded in the presence of NEM) from the superimposed spectrum obtained without the addition of NEM (Figure 3A) or after rapid Sephadex G-25 column chromatography of the mixture (Figure 3B), where the immobilized secondary species coeluted with the hemoglobin fraction. Its elution profile (ESR intensity of the fractions) correlated with the respective hemoglobin fractions (measured spectrophotometrically as the MetHb–CN complex, data not shown). These data suggest that the secondary species is firmly attached to the hemoglobin molecule via a reactive SH-group of the globin moiety, possibly at position β-93.[37]

In order to get additional information about the binding site of the radical, an attempt was made to overcome the immobilization of the radical by means of partial degradation of the globin protein by the enzymes proteinase K or pronase. The best result is shown in Figure 4A. Unfortunately it was not possible to obtain the well resolved ESR spectrum of a fully mobilized radical species which could be expected after complete digestive degradation. A possible explanation for this is the considerable stability decrease of the resulting product as the digestive degradation removes the protective protein environment from the radical site and exposes it to redox partners in the solution, a second aspect to

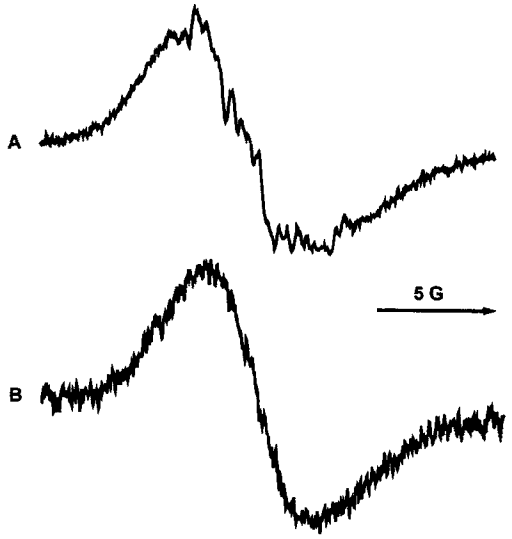

FIGURE 3 *ESR spectrum of the immobilized hemoglobin adduct.* A: The ESR spectrum of the immobilized species was obtained by computational subtraction: The ESR spectrum of an incubation mixture containing *t*BHQ (8.3 mM) and oxyhemoglobin (4.9 mM) pretreated with the thiol-blocking agent NEM (20 mM) was subtracted from the corresponding ESR-spectrum obtained from a control incubation (8.3 mM *t*BHQ, 4.9 mM oxyhemoglobin, no NEM pretreatment). ESR settings: sweep width, 20 G; modulation amplitude, 0.25 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 1×10^5; scan rate, 14.8 G/min. B: Spectrum obtained by short column separation of the incubation mixture from Figure 2B on a Sephadex G-25 column. ESR settings: sweep width, 20 G; modulation amplitude, 0.25 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 1×10^5; scan rate, 14.8 G/min, 2 scans accumulated.

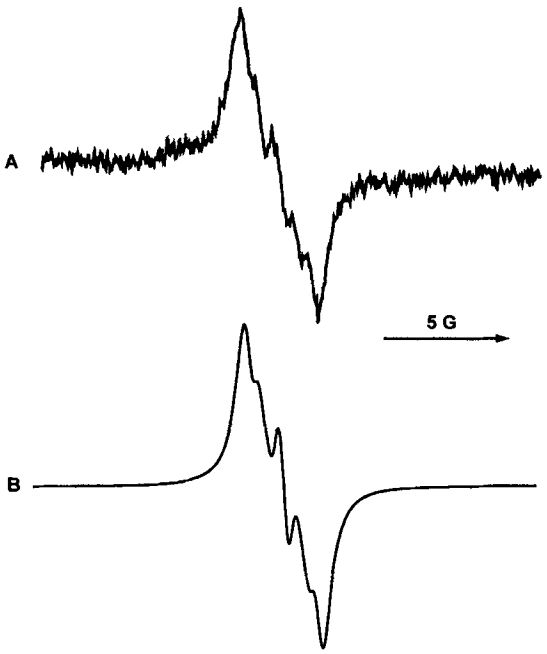

FIGURE 4 *ESR spectrum obtained after treatment of the immobilized species with proteinase K.* A: 1 ml of the incubation mixture of Figure 3B was incubated with 1.2 mg of proteinase K for 30 min. The ESR spectrum was recorded using the following settings: sweep width, 20 G ; modulation amplitude, 0.35 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 1×10^5; scan rate, 14.8 G/min, 10 scans accumulated. B: Computer simulation of the ESR spectrum shown in Figure 4A. The best fit was obtained using the following parameters: $a_H = 1.38$, 0.77, and 0.46 G, linewidth 0.58 G, 100% Lorentzian line shape.

be considered is the contribution of unresolved hyperfine splittings of the *tert*-butyl group protons which are expected to be in the range between 0.06 and 0.10 G.

An ESR simulation attempt was made to determine the spectral parameters of the partially mobilized degradation product (see Figure 4B). The best fit between experimental and simulated spectra was obtained with the following parameters: ($a_H = 1.38$, 0.77, and 0.46 G, linewidth 0.58 G, 100% Lorentzian line shape).

These values correspond with data expected for 2-alkylmercapto-5-alkyl-*p*-benzosemiquinone radicals. The coupling constants of the 2-(isopropylmercapto)-5-methyl-*p*-benzosemiquinone radical have previously been reported in the literature[36] ($a_H = 1.0$ and 1.6 G (protons of the aromatic ring), $a_H = 0.6$ G (CH group), $a_{H(3)} = 2.6$ G). An assignment of the aromatic ring protons has not been made, but considering experimental data for other sulfur-substituted *p*-benzosemiquinones such as 2-benzylmercapto-*p*-benzosemiquinone or 2,5-bis-(isopropylmercapto)-*p*-benzosemiquinone[36] we suggest the tentative assignment of the coupling constants shown in Figure 5.

SDS-PAGE Experiments

In Figure 6, the typical pattern of erythrocyte membrane proteins after SDS-PAGE can be seen in the control experiments (0 mM *t*BHQ). No changes relative to the controls were obtained upon incubation with purified (*t*BHQ-free) BHA (experiment not shown). When ghosts were prepared from erythrocytes, which had been aerobically incubated for 4 h at 25°C with *t*BHQ (left side: 0, 0.5, 1, and 2 mM) and subjected to SDS-PAGE under reducing conditions (200 mM DTT), the amount of high molecular weight compounds (HMWC) formed (above the spectrin bands at 250 kDa) increases in direct relation to the *t*BHQ concentration used. At the same time the intensities of the other bands decrease indicating a degradation process under the influence of *t*BHQ leading to polymerized products. Only minor changes of the SDS-PAGE pattern were observed when the experiments were performed under anaerobic conditions (*t*BHQ incubations under nitrogen with CO-pretreated erythrocytes, see Figure 6, right side: 0, 0.5, 1, and 2 mM) whereas incubation with 2-*tert*-butyl-*p*-benzoquinone (experiment not shown) resulted in membrane alterations comparable to those obtained with aerobic incubation in the presence of *t*BHQ, thus indicating that oxygen-activation of *t*BHQ is a

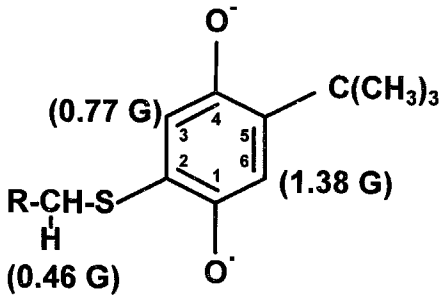

FIGURE 5 *The suggested structure of the proteinase K degraded secondary species and a tentative assignment of the computed coupling constants.*

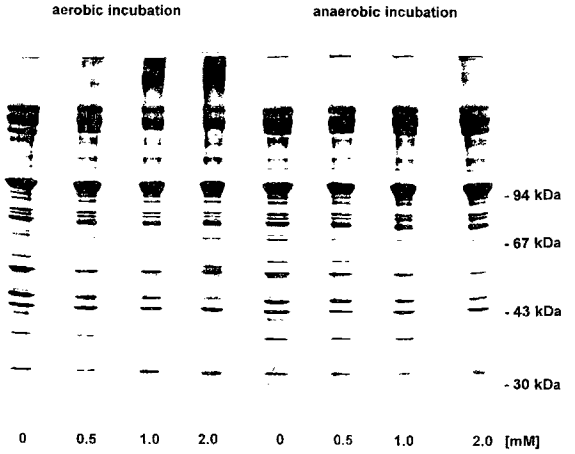

FIGURE 6 *SDS-PAGE of erythrocyte membrane proteins after treatment with tBHQ under aerobic and anaerobic conditions.* Erythrocytes were incubated in PBS buffer pH 7.4 aerobically (left side) or anaerobically (under N_2/CO atmosphere, right side) with *t*BHQ (0, 0.5, 1, or 2 mM) for 4 h at room temperature. After washing and centrifugation, membrane ghosts were prepared and subjected to SDS-PAGE as described in the Methods section.

prerequisite for membrane alterations to occur. Quantitation of these changes was done by computer integration of the individual lanes using a CAMAG scanner in the absorbing mode at 590 nm (Coomassie blue). A typical integration profile with the integration limits indicated can be seen in Figure 7A. In Figure 7B the relative changes of the band intensities (means of 5 independent experiments) are depicted including the high molecular weight compounds. The intensities are expressed as % of the total lane integral. As can be seen, the amount of HMWC increases from 3.5% (control) to 28.2% after incubation with 2 mM *t*BHQ. At the same time the relative amount of bands 1 and 2 decreases from 29.0% to 18.7%, the area around band 4 from 8.7% to 5.7% and the band marked "8" (the minor bovine glycoprotein (BG I) around 35 kDa, see Figures 6 and 7A) disappears completely. On the other hand, the band 3 intensity is not decreased, its relative increase from about 13.9% to 19.3% most probably results from an absolute decrease of the other proteins to degradation products which do not contribute to the integrated intensity, i.e. to degradation products which do not show up within the range of the SDS-PAGE experiment (20–500 kDa).

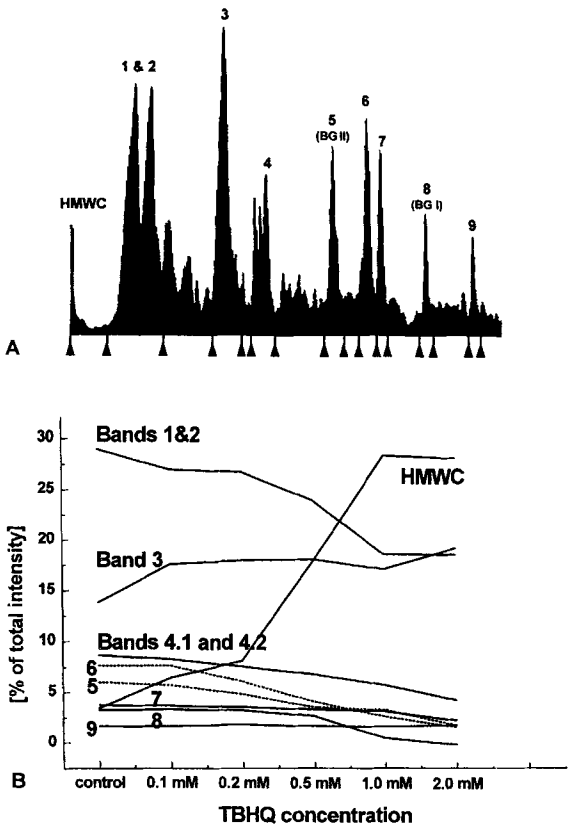

FIGURE 7 *Relative changes of the erythrocyte membrane protein contents after treatment with tBHQ* (0–2 mM). A: Typical distribution of membrane protein intensities after integration of the Coomassie Blue-stained SDS-PAGE lanes (control experiment). Integration limits are indicated by arrows. B: Relative changes (% of total integral) of protein contents: HMWC: high molecular weight compounds (aggregated proteins above 250 kDa); 1 and 2: sum of bands 1 and 2; 3: area around band 3; 4: area around bands 4.1 and 4.2; additional membrane proteins; 5 (major bovine glycoprotein: BG II), 6, 7, 8 (minor bovine glycoprotein: BG I), and 9.

DISCUSSION

Tert-Butylhydroquinone is oxidized to its semiquinone free radical in the presence of oxyhemoglobin (Equation (1)):

$$3\mathrm{Ar{-}OH} + \mathrm{HbO_2} \rightarrow 3\mathrm{Ar{-}O^{\bullet}} + \mathrm{MetHb} + \mathrm{H_2O} + \mathrm{OH^-}$$

Part of the semiquinone radicals react with a reactive thiol group located on the globin moiety, thereby forming a paramagnetic adduct, most probably in the position 5 of the aromatic ring, as was deduced from the coupling constants found after partial degradation of the globin adduct by proteinase K (see Figure 8).

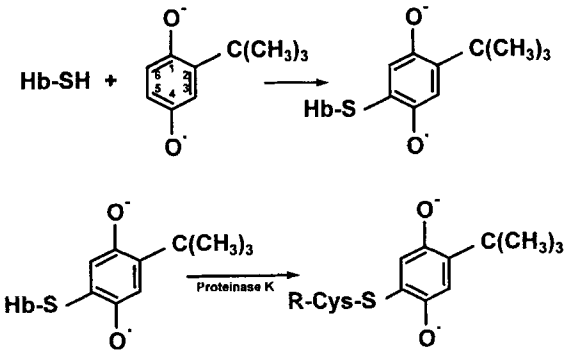

FIGURE 8 *Reaction sequence of the formation of the secondary product and protein degradation by proteinase K.*

In addition, erythrocyte membrane constituents are also attacked by *t*BHQ or its semiquinone, *t*BSQ$^{\bullet}$, especially spectrin (bands 1 and 2) and band 4, thereby forming HMWC above 250 kDa. This shows that not only the formation of S-S-bridges is involved in polymerization and adduct formation reactions since SDS-PAGE experiments were performed under reducing conditions, i.e. in the presence of dithiothreitol (DTT), which reduces S-S-bridges to the SH form. It is possible that the reaction of *t*BSQ$^{\bullet}$ radicals and/or the respective quinone with reactive SH groups of the erythrocyte membrane is responsible for the observed formation of HMW membrane degradation products via multiple SH-adduct formation but the involvement of other reactive groups cannot be excluded.

It can be assumed that these reactions do also take place *in vivo* and therefore cannot be completely prevented by the presence of thiol-containing reductants such as GSH.

Although the toxicity of *t*BHQ has been evaluated by van Esch[8] in order to establish a safe concentration range as a food antioxidant, Okubo *et al.*[9] presented *in vitro* evidence on DNA single and double strand breaks induced by *t*BHQ detectable with concentrations as low as 1 μM. These observations are especially important in view of the fact that *t*BHQ has been found to be one of the major *in vivo* metabolites of BHA in dogs, rats, and man.[2–4] The pathway seems to be oxidative demethylation of BHA which has recently been demonstrated *in vitro* with rat liver microsomes.[5]

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