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METHEMOGLOBIN FORMATION FROM BUTYLATED HYDROXYANISOLE AND OXYHEMOGLOBIN. COMPARISON WITH BUTYLATED HYDROXYTOLUENE AND *p*-HYDROXYANISOLE

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The widely used food additives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) react with oxyhemoglobin, thereby forming methemoglobin. The reaction rates were measured using visible spectroscopy, and second order rate constants were established for BHA and compared with *p*-hydroxyanisole. Using ESR we investigated the involvement of free radical reaction intermediates. The expected one-electron oxidation product of BHA and BHT, the phenoxyl radical, could only be detected with pure 3-*t*-butyl-4-hydroxyanisole and oxyhemoglobin. With the commercial mixture of 2- and 3-*t*-butyl-4-hydroxyanisole a very strong ESR signal of a secondary free radical species was observed, similar to the one observed earlier with *p*-hydroxyanisole and dependent on the presence of free thiol groups, so that we assumed the intermediate existence of a perferryl species, the MetHb-H_2O_2 adduct. In a second series of experiments we investigated the reactivity of this postulated intermediate with BHA and BHT, starting with a pure MetHb/H_2O_2-phenol mixture in a stopped-flow apparatus linked to the ESR spectrometer, detecting the expected phenoxyl radicals from BHA and *p*-hydroxyanisole. Due to the low solubility and decreased reactivity of BHT only traces of the phenoxyl type radical were found together with a high concentration of unreacted perferryl species. The reactivity of BHA, BHT and *p*-hydroxyanisole with free thiol groups is demonstrated by an increased reaction rate in the presence of the thiol group blocking substance NEM.

KEY WORDS: butylated hydroxyanisole, butylated hydroxytoluene, methemoglobin formation, ESR

ABBREVIATIONS: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; HbO_2, oxyhemoglobin; MetHb, methemoglobin; HbSH, reactive thiol group on the globin moiety; SOD, superoxide dismutase; NEM, N-ethylmaleimide.

INTRODUCTION

Butylated hydroxyanisole (BHA), a mixture of 2- and 3-*tert.*-butyl-4-hydroxyanisole and butylated hydroxytoluene (BHT, 2,6-di-*tert.*-butyl-4-methylphenol) are both widely used for food preservation. BHA functions as a protective agent against the toxicity of a variety of xenobiotics[1] and carcinogens.[2] On the other hand, harmful effects of BHA have been reported such as the formation of forestomach carcinoma in rats.[3] BHT may cause liver necrosis and bladder cancer[4,5] and specific binding of BHT metabolites to the SH-groups of cysteine and to microsomal protein has been reported.[6] The formation of reactive oxygen species due to metabolic activation of BHA by rat liver microsomes has recently been observed by Kahl *et al.*[7] Using a

horseradish peroxidase/H_2O_2 system, Valoti *et al.*[8] detected the formation of transient phenoxyl radicals from both BHA and BHT. Recently we investigated the reaction of the structurally related compound *p*-hydroxyanisole with oxyhemoglobin,[9] finding a concomitant formation of methemoglobin and the one-electron oxidation product of *p*-hydroxyanisole, the *p*-methoxyphenoxyl radical. This radical was shown to be scavenged by free thiol groups since thiol-blocking agents increased the steady-state concentration of the *p*-methoxyphenoxyl radical. Since both BHA and BHT are structurally related to *p*-hydroxyanisole, a similar reaction mechanism seems likely. The presence of the bulky *t*-butyl group, however, is expected to decrease the reaction rates of both the substituted phenol and the phenoxyl radical. Whether this will increase or decrease the overall reaction rate with oxyhemoglobin and free thiol groups is of toxicological interest.

MATERIALS AND METHODS

3-*tert*-butyl-4-hydroxyanisole and BHT (2,6-di-*tert*-butyl-4-methylphenol) were obtained from Fluka, BHA (2- and 3-*tert*-butyl-4-hydroxyanisole) and *p*-hydroxyanisole were from Aldrich. Bovine hemoglobin was prepared in a modified procedure described by Eyer *et al.*[10] for human hemoglobin. Bovine red cells were washed five times with twice the amount of 0.2 M phosphate buffer, pH 7.4. The cells were sonicated in distilled water 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 15000 g. Purified hemoglobin was prepared by chromatography of the hemolysate on $DEAE_{52}$-cellulose. The fractions were tested for catalase[11] and SOD activity[12] and only those with a catalase activity $k < 1$ and no detectable SOD activity were pooled. The pooled fractions were dialyzed several times against 0.2 M 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, the methemoglobin content by the absorbance increase at 540 nm after the addition of cyanide, according to Grisk.[13] The 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 TE_{102} microwave cavity. Computer simulations of ESR spectra and multi-scan experiments were carried out with the Bruker data system ESP 1600. A Hitachi model 150-20 UV-VIS spectrometer and a Shimadzu model 3000 spectrometer were used for spectrophotometry. The rate constants were calculated from initial raction rates at different concentrations of oxyhemoglobin and the phenolic compound measured at 541 nm at 25°C in 50 mM phosphate buffer containing 10% DMSO in order to increase the solubility of BHA and BHT.

RESULTS

Spectrophotometric measurements

Figure 1 shows the methemoglobin formation from oxyhemoglobin in the presence of BHA, followed by visible spectroscopy in the range between 450 nm and 650 nm. 20 repetitive scans were taken at intervals of 3 min. The initial hemoglobin concentration was 2.09 mM and the BHA concentration was 3.0 mM. Three isosbestic points can be seen, indicating that only oxyhemoglobin and methemoglobin are present in

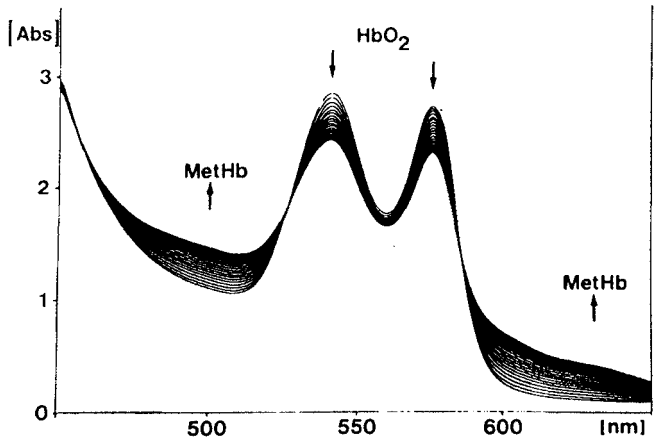

FIGURE 1 Reaction of butylated hydroxyanisole with oxyhemoglobin. 20 repetitive scans were taken at intervals of 3 min. The reaction mixture initially contained 2.09 mM oxyhemoglobin and 3.0 mM commercial BHA in 50 mM phosphate buffer, pH.7.4; 1 mm light path.

significant amounts. Other hemoglobin derivatives, such as perferryl hemoglobin mentioned below, were not detectable. Due to the high absorption of the solution a small deviation from linearity can be observed.

Figure 2 shows the dependence of the methemoglobin formation rate on the BHA concentration in the presence and in the absence of NEM. An almost linear relationship can be observed and rate constants are $k = 1.3\,M^{-1}min^{-1}$ (−NEM) and $k = 4.2\,M^{-1}min^{-1}$ (+NEM), respectively. A direct comparison of the MetHb-formation rates induced by BHA, BHT, and *p*-hydroxyanisole is shown in Table 1. In all three cases, the concentration was 80 μM, which is the maximum attainable concentration of BHT in a 10% DMSO solution. In the absence of NEM, BHA and *p*-hydroxyanisole have nearly the same reaction rates, whereas in the case of BHT no increase above the control level was observed. In the presence of NEM, however, large

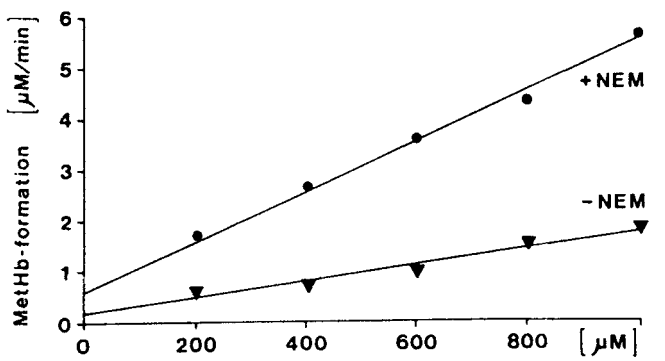

FIGURE 2 Dependence of the methemoglobin formation rate on the concentration of butylated hydroxyanisole at a final oxyhemoglobin concentration of 1.3 mM in the absence or presence of 4 mM NEM in 50 mM phosphate buffer, pH 7.4.

TABLE 1
Comparison of the reaction rates of BHT, commercial BHA and *p*-hydroxyanisole at a final concentration of 80 μM in 10% DMSO solution and an oxyhemoglobin concentration of 1·3 mM. The values represent the mean of 5 individual measurements

compound	No NEM [μM min^{-1}]	+ NEM (4 mM) [μM min^{-1}]
control	0·245	0·350
BHT	0·253	0·480
p-hydroxyanisole	0·452	0·760
BHA	0·406	1·150

differences in the reaction rates were observed. The effect of NEM was most prominent in the case of BHA, a factor of 3; with *p*-hydroxyanisole and BHT, the acceleration factor was around 2. Interestingly, even the MetHb formation of the control value was elevated by a factor of 1.4, which might be due to some structural perturbation of the globin moiety. SOD and catalase did not have any effect on the reaction rate.

ESR experiments

The expected BHA-derived phenoxyl radical CH_3O–C_6H_3 (*t*Bu)-O$^{\cdot}$ could not be detected when a flow system was used similar to our previous experiments with the unsubstituted *p*-hydroxyanisole.[9] The reason for this is twofold. First, the solubility of BHA in aqueous solution is much lower than the concentration generally used for rapid flow experiments (250 mM in the case of *p*-hydroxyanisole). Second, the formation of a secondary reaction product (see Figure 5) which gives rise to a very strong ESR signal in the same region prevents the detection of the primary species. In order to circumvent this problem in the case of BHA (a mixture of 2- and 3-*t*-butyl-4-hydroxyanisole), we used pure 3-*t*-butyl-4-hydroxyanisole, which does not form the secondary reaction product. In order to increase its concentration, we had to add 10% ethanol to the solution and to lower the phosphate buffer concentration from 50 mM down to 22.7 mM. The pH did not change significantly during the experiment. In this way, the accumulation of 120 scans in a stationary system allowed us to detect the predicted phenoxyl species (Figure 3A). The ESR spectrum is not very well resolved due to the overmodulation necessary to obtain the minimum signal-to-noise ratio. In order to increase the concentration of this species so as to allow an identification, we assumed a reaction pathway for BHA and BHT similar to the one reported for *p*-hydroxyanisole. In that case, a perferryl-type methemoglobin-hydrogen peroxide reaction intermediate was postulated which reacts rapidly with excess *p*-hydroxyanisole, thereby forming additional phenoxyl-type radicals. This reaction is much faster than the first reaction step and should therefore be detectable at lower concentrations of the hydroxyanisole compound. In Figure 3B the BHA-derived ESR spectrum can be seen, obtained in a stopped-flow experiment with methemoglobin/BHA and 10 mM H_2O_2 mixed within 5 s and recorded within the first 20 s. In order to bring adequate amounts of this compound into solution phosphate buffer concentration had to be reduced from 50 mM (which is normally used) to 22.7 mM and ethanol was added to the solution up to 10% of the total volume. Although the buffer capacity was reduced by this procedure, the pH change during the experiment was less

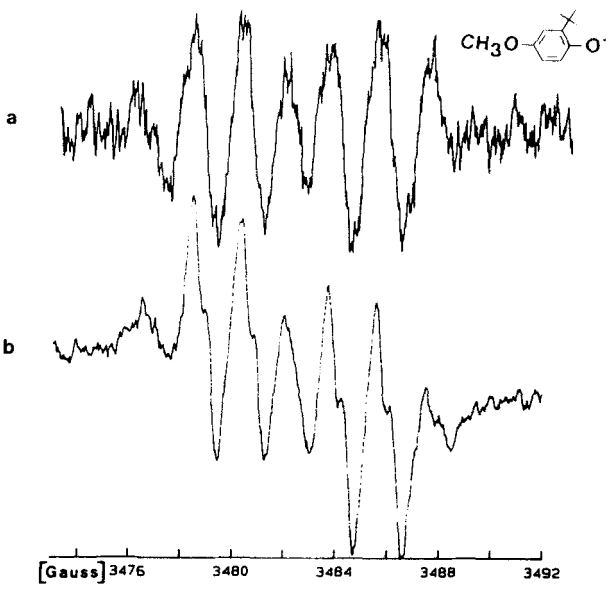

FIGURE 3 (A) ESR spectrum of the 2-*t*-butyl-4-methoxyphenoxyl radical, obtained from oxyhemoglobin (3.0 mM) and 2-*t*-butyl-4-methoxyphenol (BHA) (10 mM) in 22.7 mM phosphate buffer, pH 7.4, containing 10% ethanol. The spectrometer settings were: scan range, 20 G; modulation amplitude, 1 G; receiver gain, 2×10^6; microwave power, 40 mW; time constant, 0.33 s; scan rate, 14.3 G min^{-1}; 120 scans, baseline-corrected. (B) ESR spectrum of the 2-*t*-butyl-4-methoxyphenoxyl radical, obtained from methemoglobin (1.2 mM), 2-*t*-butyl-4-methoxyphenol (BHA) (5 mM) and H_2O_2 (5 mM) in 1.5 mM phosphate buffer, pH 6.8. The spectrometer settings were: scan range, 20 G; modulation amplitude, 0.4 G; receiver gain, 1×10^6; microwave power, 20 mW; time constant, 0.164 s; scan rate, 57.1 G min^{-1}; sum of 6 single scans.

than 0.3 pH. No interference with the perferryl species was observed. The spectrum shows the following spectral parameters, obtained by computer simulation: $a_H(1) = 5.3$ G, $a_H(3) = 1.85$ G, and $a_H(1) = 0.65$ G. These values are practically identical to the values reported by Valoti *et al.*[8] for the 2-*t*-butyl-4-methoxy-phenoxyl radical, except that the smallest proton splitting of 0.3 G was not detectable in the fast scan mode. The spectral parameters of Figure 3B including the shape of this spectrum are also identical with the ESR spectrum obtained in Figure 3A, thus confirming the existence of a phenoxyl-type radical in both cases. Similarly, an acceleration of the reaction rate of BHT oxidation can be expected when high concentrations of the postulated reaction intermediate, MetHb/H_2O_2 are used. As a consequence the steady state concentration of the phenoxyl radical intermediate should also increase. In our experiments we obtained a mixed ESR spectrum of unreacted perferryl species (MetHb/H_2O_2) superimposed to several sharp lines (not shown). In order to facilitate the interpretation of these additional lines, the well known ESR signal of the MetHb/H_2O_2 species was subtracted (Figure 4). The spectral parameters are: $a_H(3) = 11.65$ G and $a_H(2) = 1.6$ G, practically the same as reported by Valoti *et al.*[8] for the 2,6-di-*t*-butyl-4-methylphenoxyl radical.

The effect of the SH-group blocking agent NEM shown in Figure 2 suggests the participation of SH-groups in reactions with the BHA-derived phenoxyl radical mentioned above. Figure 5 shows the ESR spectra obtained in a stationary system of

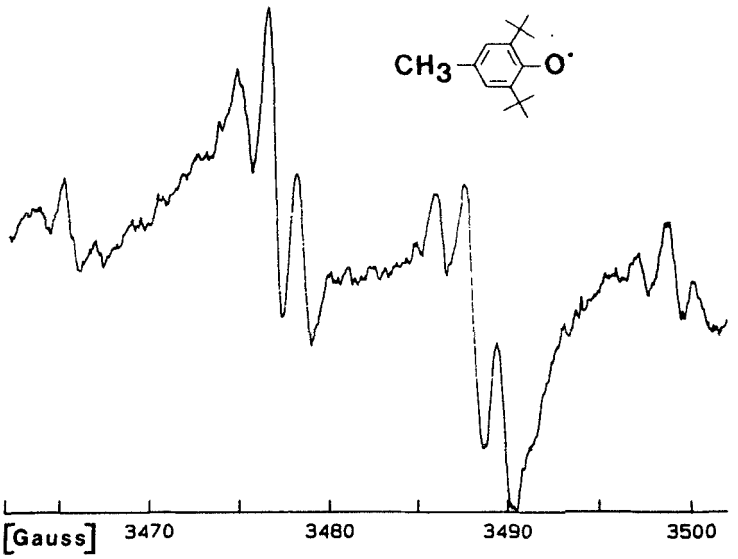

FIGURE 4 ESR spectrum of the 2,6-di-*t*-butyl-4-methylphenoxyl radical, obtained from methemoglobin (1.2 mM), 2,6-di-*t*-butyl-4-methylphenol (BHT, 0.25 mM) and H_2O_2 (5 mM) in 1.5 mM phosphate buffer, pH 6.8, containing 10% ethanol. The spectrometer settings were: scan range, 40 G; modulation amplitude, 0.8 G; receiver gain, 4×10^5; microwave power, 20 mW; time constant, 0.164 s; scan rate, 114.2 G min^{-1}; 12 scans.

oxyhemoglobin and BHA in the absence and presence of the SH-group blocking agent NEM. The intensive ESR spectrum of an immobilized species is only visible when the SH groups of the globin moiety are not blocked by NEM.

DISCUSSION

Our investigations show that both BHA and BHT are able to form methemoglobin from oxyhemoglobin, the reaction rates being similar to the one reported for *p*-hydroxyanisole.[9] In the case of BHA we observed the formation of a free radical intermediate, the 2-*t*-butyl-4-methoxyphenoxyl radical. With BHT the existence of the same type of intermediate is very likely, although it could not be proven because of the low solubility of this compound. In analogy to our results with *p*-hydroxyanisole, we assume the formation of the phenoxyl-type radical as the first reaction step:

$$[Hb^{2+}O_2] + BHA \rightarrow [MetHb^{3+}O_2^{2-}] + BHA^{\cdot} + H^+$$

As the second reaction intermediate we propose the formation of a perferryl-type species $[MetHb^{3+}\text{–}O_2^{2-}]$ where the oxygen has the oxidation state of hydrogen peroxide. In the following reaction step this species reacts with excess BHA (or BHT) thereby forming additional phenoxyl-type radicals.

$$[MetHb^{3+}O_2^{2-}] + 2BHA \rightarrow 2BHA^{\cdot} + MetHb^{3+} + 2OH^-$$

This could be proven in another set of experiments where we started with a MetHb/H_2O_2 system where both BHA and BHT immediately formed the phenoxyl radical.

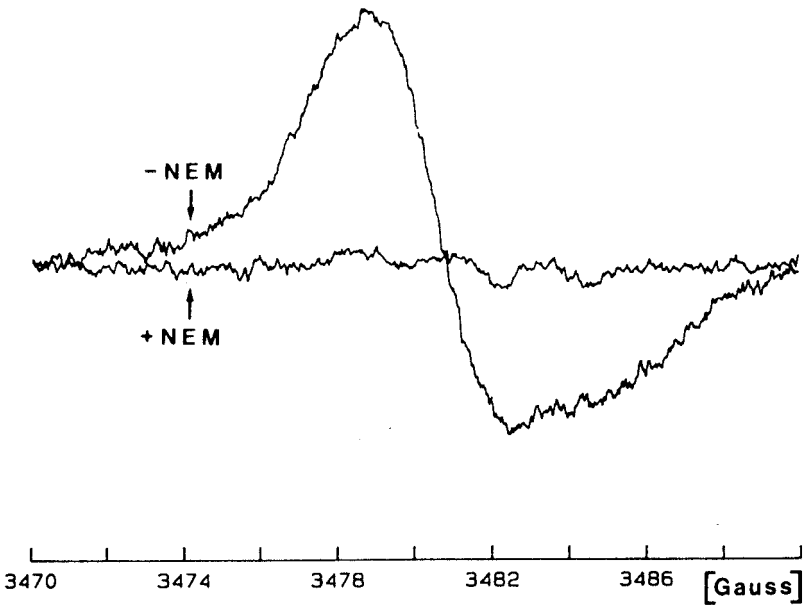

FIGURE 5 ESR spectrum of the reaction product of the 3-*t*-butyl-4-methoxyphenoxyl radical with an SH group on the globin moiety. The incubation mixture contained oxyhemoglobin (4.8 mM) and commercial BHA (1 mM) in 1.5 mM phosphate buffer, pH 6.8. The spectrum was recorded after 45 min. The spectrometer settings were: scan range, 20 G; modulation amplitude, 2 G; receiver gain, 1×10^6; microwave power, 20 mW; time constant, 0.33 s; scan rate, 14.3 G min^{-1}; 2 scans. No ESR-signal was obtained when 2 mM NEM was added to the oxyhemoglobin solution prior to the incubation.

These free radical intermediates can react in different ways. In addition to the formation of dimers as has been reported by Valoti *et al.*,[8] hydrogen abstraction from organic molecules such as biological membranes and proteins are likely. One aspect that has been investigated recently[6,14] is the reaction with reactive sulfhydryl groups on the globin moiety in the vicinity of the formation site:[14]

$$HbSH + R^{\cdot} \rightarrow HbS^{\cdot} + RH$$

In the case of BHA we have obtained direct evidence of the SH-group dependent formation of an immobilized free radical adduct. Although we were not able to clearly identify this secondary species, we observed that the formation of a SH-adduct requires a free ortho-position on the phenoxyl radical, since only phenolic compounds react with the ortho position not blocked by a *t*-butyl group (*p*-hydroxyanisole and 2-*t*-butyl-*p*-hydroxyanisole). Similar adducts have been reported by Takahashi *et al.*[15]

With BHT this type of reaction could only be deduced indirectly, since NEM had a significant influence on the reaction kinetics of methemoglobin formation (Figure 2). Although the concentrations of BHA and BHT necessary to observe the free radical species were extremely high compared to those normally used for food preservation, the additive effect of prolonged or even permanent exposure to low concentrations of reactive free radicals might cause serious damage. In addition, much higher concentrations of these compounds can be expected in biological membranes due to their lipid solubility.

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