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# Evaluation of Antioxidant Activity: II. Application of a Heme-catalyzed System

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## ABSTRACT

A study was made of the inhibitory effect of the antioxidants propyl gallate, *tert*-butylated hydroxyanisol, α-tocopherol and ethoxyquin on the hemoglobin-catalyzed oxygenation of linoleic acid. The concentration of unchanged fatty acid after varying incubation periods and at varying concentrations of antioxidant was measured by gas chromatography. The effect of the antioxidants is compared with results obtained previously from the lipoxygenase-catalyzed oxidation of a linoleic acid emulsion. It is concluded that all 4 antioxidants are good inhibitors of fatty acid oxidation catalyzed by hemoglobin.

## INTRODUCTION

In a previous communication (1), we described a gas chromatographic method for evaluating the effect of antioxidants. The inhibitory effect of *tert*-butylated hydroxyanisol (BHA) and ethoxyquin (EMQ) on a lipoxygenase-catalyzed oxidation of linoleic acid was determined. In the first paper of this series (2), the system was modified and linolenic acid was applied as substrate. Linoleic acid appeared to be the more suitable substrate and propyl gallate (PG) a much more efficient antioxidant than BHA, EMQ and α-tocopherol (TO) when tested in the lipoxygenase-catalyzed system (1-3).

Lipoxygenase is a biological catalyst present mainly in plant tissues. It was therefore decided to extend measurements on antioxidative effect by applying a catalyst of animal origin and the results are described in this paper. Acceleration of the oxidation of animal fat depends on the presence of hemoglobin, myoglobin and their derivatives, which are distributed throughout the animal organism.

Tappel (4,5) has reviewed the catalytic activity of hematin compounds in biological systems. He and his coworkers studied the reaction rate and the mechanism of the hematin-catalyzed lipid oxidation (6,7) and the decomposition of lipid hydroperoxides (8,9).

More recently, Gardner (10) reviewed the decomposition of lipid hydroperoxides and the influence of heme compounds on the degradation process. Several workers have shown great interest in the reaction mechanism of the breakdown reaction, as well as the identification of the secondary products (11-17). It has been concluded that heme and metal ion catalysis plays an important role in the initiation and propagation steps of the reaction.

A variety of methods has been devised for testing the activity of food antioxidants on the heme-catalyzed oxidation of unsaturated lipids (6,18-20). Hamilton and Tappel (19) oxidized a methyl linoleate emulsion with hemoglobin and measured the oxygen consumption by a rapid polarographic method. This procedure was modified by Cort (20), who determined the activity of several food antioxidants by measuring oxygen removal with an oxygen analyzer. Tappel (4) has proposed a reaction mechanism for the antioxidant effect on the heme-catalyzed oxidation.

The measurement of oxygen uptake in a dynamic system has, however, proven difficult to standardize and reproduce (21). We therefore carried out a study on the effects of BHA, PG, TO and EMQ on the hemoglobin-catalyzed peroxidation of linoleic acid using the method developed in our laboratory (1).

## EXPERIMENTAL PROCEDURES

Instrumental techniques have already been described (1).

### Reagents

Hemoglobin from beef blood (type I) and DL-α-tocopherol (Vitamin E) were obtained from Sigma Chemical Co. (St. Louis, MO). The other reagents were as described previously (1,2).

### Analytical Procedure

Hemoglobin stock solutions (5 mg/ml, 3 mg/ml and 0.5 mg/ml) were prepared in ice-cold 0.2 M phosphate buffer (pH = 7.0), stored at 5 C and kept in an ice-water bath while in use. About 0.2 ml was added to the reaction mixture. The amount of hemoglobin was calculated from constant weight ratio of substrate and catalyst. Stock solutions of fatty acid, antioxidants and internal standard

were prepared as previously described (1). The catalyzed reaction, analysis of the reaction mixture and processing of the data were also performed as previously described (1). Antioxidant concentrations of 8% and 0.8% w/w of fatty acid were tested. The addition of catalyst to the reaction mixture was also varied, 16.7% ($4.9 \times 10^{-6}$ M), 10% ($2.9 \times 10^{-6}$ M) and 1.7% ($4.9 \times 10^{-7}$ M) w/w of linoleic acid.

## RESULTS

The percentage of unchanged fatty acid in the hemoglobin-oxidized system after various incubation periods and at various concentrations of the antioxidants and the catalyst was calculated from the gas chromatograms. The data are presented in Tables I and II. The calculated standard deviation of the modified method was $s_d = \pm 0.88\%$, as compared to $s_d = \pm 0.86\%$ for the lipoxygenase-catalyzed oxidation of linoleic acid.

The rate of oxidation of fatty acid catalyzed by heme compounds is greatly influenced by the concentration ratio of hemoprotein-to-fatty acid (22-26). Our results show that 10% hemoglobin concentration oxidized ca. 39% of the fatty acid when the mixture was incubated for 15 min, whereas a reduction of the quantity of catalyst to 1.7% resulted in ca. 78% unchanged linoleic acid. An increase in the hemoglobin concentration to 16.7% caused the inhibition of oxidation of fatty acid to a degree corresponding to the effect of the antioxidants.

Within the limits of experimental error, inhibition of the catalyzed oxidation of linoleic acid by the 4 antioxidants at the 2 concentration levels appeared to be about the same.

Alteration in the concentration ratio of hemoglobin and fatty acid did not influence the effect of the antioxidants. No further inhibition of the reaction catalyzed by 16.7% hemoglobin was obtained in the presence of antioxidant. A selection of the experimental data at varying concentrations of catalyst is included in Table II.

A Student's t-test (27) at 99% confidence level showed significant differences between the results obtained with and without antioxidants when 10% and 1.7% hemoglobin were added. No significant differences were obtained between corresponding results at 16.7% hemoglobin addition.

In order to facilitate the comparison of antioxidant activity we have previously defined a protective index (PI) as the time required to oxidize 30% of the fatty acid in the antioxidant system divided by the time required to oxidize 30% of the fatty acid in the system without antioxidant.

The oxidation in the antioxidant system was extended to 90 min to obtain 30% oxidation of linoleic acid when catalyzed by 10% hemoglobin. The data in Table III show that PI for both concentrations of TO are slightly lower than for the other antioxidants. The induction time in the accelerated antioxidant system is ca. 3-4 min.

## DISCUSSION

The antioxidants PG, BHA, TO and EMQ have more pronounced inhibitory effects on the heme-catalyzed oxidation of unsaturated fatty acids than on the lipoxygenase-catalyzed oxidation. This can be reasonably explained by the different reaction mechanisms of the 2 catalysts. Heme compounds catalyze both oxidation to lipid hydroperoxides and further decomposition to secondary products such as volatiles, polymers and oxygenated

TABLE I

Relative Quantities of Unchanged Linoleic Acid at Varying Incubation Times and Concentrations of Antioxidant (Hemoglobin Concentration 10%)[a]

| Incubation time (min) | Without antioxidant | BHA | | EMQ | | PG | | TO | |
|---|---|---|---|---|---|---|---|---|---|
| | | 8% (0.93 M) | 0.8% (0.09 M) | 8% (0.77 M) | 0.8% (0.08 M) | 8% (0.78 M) | 0.8% (0.08 M) | 8% (0.39 M) | 0.8% (0.04 M) |
| 1 | 94.7 | 99.0 | 98.7 | 99.1 | 99.3 | 99.4 | 99.8 | 99.4 | 99.0 |
| 3 | 86.1 | 98.3 | 97.9 | 98.7 | 97.9 | 99.2 | 97.6 | 98.4 | 98.2 |
| 5 | 78.6 | 95.5 | 95.3 | 97.2 | 96.4 | 98.0 | 97.6 | 97.6 | 97.4 |
| 7 | 74.4 | 95.3 | 95.0 | 96.3 | 96.5 | 95.7 | 95.6 | 96.3 | 94.2 |
| 10 | 70.5 | 94.4 | 94.8 | 95.1 | 96.2 | 94.3 | 94.4 | 95.6 | 94.5 |
| 12 | 66.3 | 93.4 | 92.8 | 93.5 | 94.1 | 93.8 | 93.0 | 92.8 | 92.6 |
| 15 | 61.4 | 91.0 | 89.9 | 92.4 | 92.3 | 91.9 | 91.5 | 89.8 | 86.7 |

[a]Concentrations are expressed as w/w of initial amount of linoleic acid.

[b]Antioxidants are expressed as: BHA = butylated hydroxyanisol; EMQ = ethoxyquin; PG = propyl gallate; TO = α-tocopherol.

TABLE II

Relative Quantities of Unchanged Linoleic Acid at Varying Incubation Times and Concentrations of Antioxidant and Hemoglobin[a]

| Incubation time (min) | Hemoglobin 16.7% | | | Hemoglobin 1.7% | | | | |
|---|---|---|---|---|---|---|---|---|
| | Without antioxidant | BHA | PG | Without antioxidant | PG | | α-TO | |
| | | 8% | 8% | | 8% | 0.8% | 8% | 0.8% |
| 1 | 99.5 | 99.8 | 99.5 | 96.9 | 99.9 | 100.1 | 98.8 | 99.7 |
| 3 | 99.1 | 99.0 | 99.9 | 92.7 | 99.7 | 99.2 | 99.3 | 98.7 |
| 5 | 97.7 | 98.3 | 97.5 | 89.0 | 97.9 | 97.9 | 98.1 | 97.3 |
| 7 | 95.8 | 95.9 | 97.5 | 86.4 | 97.8 | 97.6 | 98.5 | 96.7 |
| 10 | 95.4 | 94.8 | 95.3 | 82.8 | 96.6 | 96.0 | 96.4 | 95.6 |
| 12 | 92.9 | 93.9 | 93.5 | 80.9 | 92.6 | 92.9 | 94.7 | 93.6 |
| 15 | 91.1 | 92.0 | 92.1 | 78.2 | 92.2 | 92.9 | 92.4 | 92.1 |

[a]See footnotes in Table I.

TABLE III

The Effect of the Concentration of the Antioxidant on the Protective Indices (PI) in a System Catalyzed by 10% Hemoglobin[a]

| Antioxidant | Concentration of antioxidant | |
|---|---|---|
| | 8% | 0.8% |
| PG | 7.8 | 6.7 |
| BHA | 7.9 | 7.5 |
| EMQ | 7.9 | 7.0 |
| TO | 5.1 | 5.1 |

[a]See footnotes in Table I.

compounds.

Various reaction mechanisms have been proposed for the heme-catalyzed peroxidation of unsaturated fatty acids. Several authors (4,12,14,25) have suggested that in the initial step, a reactive complex is formed between the acid and the hemoprotein which activates the fatty acid for rapid oxidation. No valence change of the protein iron in the oxidation reaction was considered to have taken place although Haurowitz et al. (12) suggested that the heme catalysts act like lipoxygenase (28). An alternative reaction mechanism has been proposed by O'Brien (16), in which the protein metal catalyzes peroxidation by activation of oxygen or by forming a complex with oxygen.

The fact that the phenolic antioxidants (BHA, PG, TO) and EMQ readily inhibit the heme-catalyzed peroxidation of unsaturated fatty acids supports the view that free radicals are involved. We therefore suggest the following mechanism for the different activities of the antioxidants on the heme-catalyzed oxidation as compared to the lipoxygenase-catalyzed reaction.

In the lipoxygenase-catalyzed oxidation, the fatty acid radicals form complexes with the enzyme to combine stereospecifically with oxygen. The complex-bound radicals are less available for attack by antioxidant. In contrast to lipoxygenase, the heme proteins are unspecific in the fatty acid oxidation. Thus, it is reasonable to assume that the heme catalyst will not protect the fatty acid radicals in the same way as lipoxygenase, and that the fatty acid/catalyst complex will dissociate as soon as the radicals are formed. The free radicals will be attacked by the antioxidant and further oxidation will be inhibited.

The slight oxidation of linoleic acid in the presence of both antioxidant and inhibitory concentration of hemoglobin may have resulted from other autoxidative reactions in the accelerated system. This level must therefore be the basis for comparison with hemoglobin-catalyzed reactions inhibited by antioxidants.

Our results show that the efficiency of the antioxidants in the heme-catalyzed reaction is independant of the concentration of the antioxidant. Quite low concentrations of the antioxidants inhibit oxidation, a situation which contrasts with that in the enzyme-catalyzed reaction (1-3). The antioxidant effect is almost identical for structurally different antioxidants, such as BHA, PG, TO and EMQ.

The effect of antioxidants on heme-catalyzed oxidations as measured in our system appears to differ from previous findings (18-20). Both Hamilton et al. (19) and Cort (20) measured the efficiency of the antioxidants on the basis of oxygen uptake in a dynamic lipid emulsion system. Such a system is difficult to reproduce and their results are therefore not directly comparable with ours. In our system, we measured the ability of the antioxidants to inhibit the formation of fatty acid radicals either in the initial step or the propagation step. The hydroperoxy radicals that are formed in the propagation steps may, in the presence of antioxidants, be inhibited from reacting further with fatty acid molecules.

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