

# Antioxidative Activity of Some Quaternary Ammonium Salts Incorporated into Erythrocyte Membranes

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The antioxidative activity of two series of amphiphilic compounds from a group of quaternary ammonium salts has been investigated. They were so-called bifunctional surfactants synthesized to be used as common pesticides or as antioxidants. The latter application was to be ensured by providing the compounds studied with an antioxidant group. Studies on antioxidative possibilities of those compounds were performed on pig erythrocytes. Due to their hydrophobic parts, they anchor in the erythrocyte membrane and influence the degree of lipid oxidation in the erythrocyte membrane subjected to UV radiation. It was found that compounds of both series decreased the oxidation of the membrane lipids. The inhibition of this oxidation increased with the length of their hydrophobic chains up to fourteen carbon atoms. The compounds of the longest hydrophobic chains showed a somewhat weaker antioxidative activity. Of the two series studied compounds were more effective having bromide ions as counterions. The corresponding compounds of a second series (chlorides) protected erythrocyte significantly weaker against oxidation. The effect of the compounds on fluidity of the erythrocyte membrane has been studied in order to explain the oxidation results. Change in fluidity of the erythrocyte ghost membranes was found also dependent on length of the hydrophobic part of the compounds and was more pronounced in the case of bromide surfactants.

The final conclusion is that the compounds studied can be successfully used as antioxidant agents of good efficacy.

## Introduction

It has long been known that peroxidation of the lipid phase of biological membranes may lead to serious damage of the membranes leading in the extreme to cell death or serious disturbance in its metabolic processes (Bartosz, 1995; Halliwell and Gutteridge, 1989). This is the reason for continuous search for more effective antioxidants that could be incorporated into the cell membrane to protect it against oxidation and, at the same time, not impairing its biological functions. Investigations published in recent years on this topic are concerned both with natural and synthetic antioxidants (Ariga and Hamano, 1990; Kilinc and Rouhani, 1992; Rios *et al.*, 1992; Chen *et al.*, 1996; Rasetti *et al.*, 1996/97; Vaya *et al.*, 1997; Karen *et al.*, 1997).

The aim of the present investigations was to determine the antioxidant activity of two series of new compounds from a group of quaternary ammonium salts with a hindered phenol substituent as an antioxidant group function. Both series belong to the so-called bifunctional surfactants, which at higher concentrations can be used as common pesticides (Witek *et al.*, 1997), and differ in the halide counterion. Both series are derivatives of dihydrocinnamic acid. One series consisted of chlorides (DEE-n) while the other one of bromides (DEA-n). The hemolysis of pig erythrocytes (RBC) and the degree of the lipid oxidation in RBC membranes subjected to UV irradiation were studied in the presence of both series. Also, the measurements of fluidity changes of erythrocyte ghosts were performed at sublytic concentrations of the compounds studied. Erythrocytes were

chosen as a model to study the antioxidative properties of the bifunctional surfactants because the latter were shown to incorporate into the lipid phase of erythrocyte membranes (Kleszczyńska *et al.*, 1998; 1999; Kleszczyńska and Sarapuk, 1998). It is generally assumed that hemolytic effects, especially caused by amphiphilic compounds, take place at the lipid phase of RBCs.

The results obtained allowed to evaluate the potential antioxidant activity of the compounds studied and may be helpful to synthesize new, more potent antioxidants.

## Materials and Methods

### Reagents

The bifunctional quaternary ammonium salts with antioxidant function are presented in Fig. 1. They were synthesized in our laboratory. The structure and purity of the compounds were confirmed by  $^1\text{H}$ -NMR spectra (Bruker Avance DRX300 instrument, in deuteriochloroform, TMS as internal standard). The spectral data of the DEA-10 and DEE-12 compounds are given as examples:

DEA-10: N-(3,5-di-*tert*-butyl-4-hydroxy)phenylethylcarboethoxy-N-decyloxymethyl-N,N-diethyl-

ammonium bromide.  $^1\text{H}$  NMR spectrum: (300 MHz,  $\text{CDCl}_3$ ,  $\delta$  ppm); 0.881 (3H, t,  $\text{CH}_3$ -alkyl); 1.239 (16H, s,  $(\text{CH}_2)_8$ ); 1.427 (24H, s, *t*-Bu i  $(\text{CH}_3)_2$ ); 2.660–2.687 (2H, m,  $-\text{CH}_2\text{COO}$ ); 2.842–2.869 (2H, m,  $-\text{CH}_2\text{N}$ ); 2.840–2.868 (4H, m,  $\text{N}(\text{CH}_2)_2$ ); 3.141 (2H, m,  $\text{NCH}_2$ -alkyl); 3.383–3.428 (2H, m, Ar- $\text{CH}_2$ ); 4.608 (2H, t,  $\text{COOCH}_2$ ); 5.098 (1H, s, OH); 6.978 (2H, s, Ar).

DEE-12: N-(3,5-di-*tert*-butyl-4-hydroxy)phenylethylcarboethoxy-N-dodecyloxymethyl-N,N-diethylammonium chloride.  $^1\text{H}$  NMR spectrum: (300 MHz,  $\text{CDCl}_3$ ,  $\delta$  ppm); 0.881 (3H, t,  $\text{CH}_3$ -alkyl); 1.239 (20H, s,  $(\text{CH}_2)_{10}$ ); 1.427 (24H, s, *t*-Bu i  $(\text{CH}_3)_2$ ); 1.555–1.578 (2H, m, N- $\text{CH}_2$ -alkyl); 2.634–2.685 (2H, m,  $\text{CH}_2\text{COO}$ ); 2.840–2.891 (2H, m,  $-\text{CH}_2\text{N}$ ); 3.195–3.152 (4H, m,  $\text{N}(\text{CH}_2)_2$ ); 3.250–3.267 (2H, m, Ar- $\text{CH}_2$ ); 3.498–3.542 (2H, m,  $-\text{OCH}_2$ -alkyl); 4.601 (2H, t,  $\text{COOCH}_2$ ); 5.067 (1H, s, OH); 6.977 (2H, s, Ar).

The fluorescent probe TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate)] was from Molecular Probes Inc. (Eugene, Oregon, USA). TBA – thiobarbituric acid was obtained from Chemical Company (St. Louis, Missouri, USA) and TCA – trichloroacetic acid was obtained from Fluka Chemie AG (Buchs, Switzerland).

### Hemolytic studies

Fresh heparinized pig blood was used in the hemolytic experiments. Erythrocytes (RBC) were washed four times in phosphate buffer of pH 7.4 and incubated in it, after adding chosen concentrations of DEA-n and DEE-n compounds (0.5, 1.0, 1.5, 2.0 and 2.5 mM), at 37 °C for 4 h. The hematocrit was 2%. Percent of hemolysis was measured of 1 ml samples taken after 0.5, 1, 1.5, 2, 3 and 4 h of incubation. They were centrifuged and the hemoglobin content in the supernatant was measured with a Specol 11 spectrophotometer (Carl Zeiss, Jena, Germany) at 540 nm. Kinetic curves of hemolysis (not included into this paper) were then used for calculation of the concentration of compound inducing 50% hemolysis ( $C_{50}$ ).

### Oxidation studies

Erythrocyte membranes were prepared, according to Dodge *et al.* (1963) from fresh heparinized pig blood. Erythrocytes were suspended in a phos-

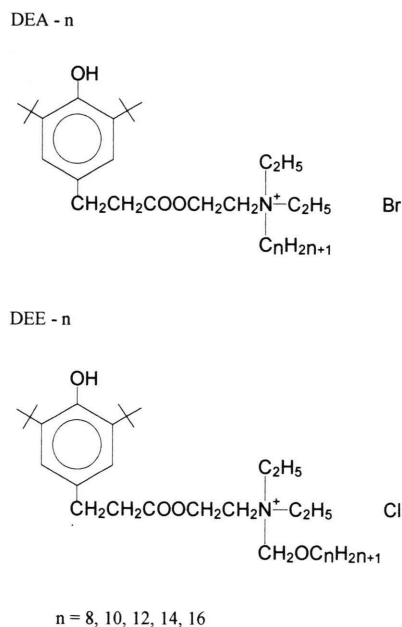


Fig. 1. Structure of the compounds studied.

phate solution of pH 7.4. The bifunctional surfactants were added to the suspension containing erythrocytes and their protective antioxidative effect compared with a control solution containing no compound. The lipid peroxidation in the erythrocyte membrane was induced by UV radiation (bactericidal lamp, intensity 3.5 mW/cm<sup>2</sup>). The measure of the antioxidant efficacy of the compounds studied was their concentration inhibiting by 50% lipid oxidation (IC<sub>50</sub>) relative to the control sample oxidation. The degree of lipid peroxidation was determined by measurement of malonic dialdehyde concentration released in the samples, using its colour reaction with thiobarbituric acid (Bartosz, 1995; Stock and Dormandy, 1971). In order to do it, 1 ml samples were taken and 1 ml of trichloroacetic acid (TCA; 15% TCA in 0.25 M HCl) and 1 ml of thiobarbituric acid (TBA; 0.37% TBA in 0.25 M HCl) were added. The samples taken were stoppered with a ball and heated at 100 °C for 15 min, and then cooled fast and centrifuged, the absorption of supernatant was measured at 535 nm.

#### Fluorimetric studies

Fluorimetric studies were performed on erythrocyte ghosts. Erythrocyte membranes were prepared according to Dodge *et al.* (1963) method from fresh heparinized pig blood. Erythrocyte membranes subjected to the action of the antioxidants studied or erythrocyte ghosts oxidized in the absence or presence of antioxidant compounds were labelled with the fluorescent probe TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate)] at 1 μM concentration. The concentration of the compounds of series DEA-n was 6 μM and that of series DEE-n was 10 μM, respectively. These concentrations correspond to those found in the oxidation studies for compounds that were the most potent antioxidant inside each series. Protein concentration in the samples was ca. 100 μg/ml. The measurements were performed with an SFM 25 spectrofluorimeter (KONTRON). On the basis of fluorescence intensity measurements the anisotropy coefficient was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz 1988):

$$A = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + 2GI_{\perp})$$

where  $I_{\parallel}$  – intensity of fluorescence emitted parallel to the polarization plane of the exciting light,  $I_{\perp}$  – intensity of fluorescence emitted perpendicular to the polarization plane,  $G$  – diffraction constant, dependent on wavelength.

#### Results and Discussion

The results of the experiments performed are presented in Figs. 2 and 3, showing the values of IC<sub>50</sub> and anisotropy coefficient ( $A$ ) found for DEA-n and DEE-n compounds, respectively. The results of hemolytic experiments are collected in Table I. Note that concentrations of the compounds causing 50% hemolysis ( $C_{50}$ ) are at least two orders of magnitude higher than those at which fluidity experiments were done or concentrations inhibiting by 50% the membrane lipid oxidation (IC<sub>50</sub>) calculated relative to non-protected control samples. Practically, this means that the compounds concentration used in the latter experiments were far from concentrations at which bifunctional surfactants would be applied as pesticides or far from those destroying membranes and/or impairing their function. It also means that the compounds studied can be safely used in micromolar concentrations as antioxidants without a danger to the erythrocyte membrane.

It can be seen in Fig. 2 that the antioxidant efficacy of both series depended on the length of the hydrophobic parts of the compounds and that there are efficacy maxima for DEA-14 and DEE-14 compounds. The poor protective antioxidant potency of the longest compounds of both series in comparison with fourteen carbon atoms ones reminds of the loss of bactericidal activity found for several homologous series of pesticides (Devinsky *et al.*, 1990; Uhrikova *et al.*, 1993; Balgavy and Devinsky, 1994). A possible explanation may

Table I. Concentrations that cause 50% hemolysis of the erythrocytes ( $C_{50}$ ). Numbers mean carbon atoms in the hydrocarbon chain.

| Compounds | $C_{50}$ | Compounds | $C_{50}$ |
|-----------|----------|-----------|----------|
| DEA-8     | 1.25     | DEE-8     | 1.40     |
| DEA-10    | 0.80     | DEE-10    | 1.05     |
| DEA-12    | 0.65     | DEE-12    | 0.95     |
| DEA-14    | 0.40     | DEE-14    | 0.60     |
| DEA-16    | 0.35     | DEE-16    | 0.45     |

Standard deviation was  $\pm 0.05$ .

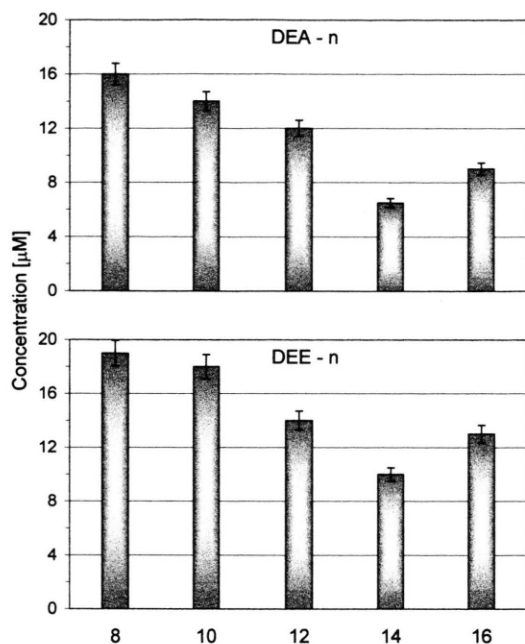


Fig. 2. Bar diagram of compounds' concentrations that cause 50% inhibition of oxidation of erythrocyte membrane lipids ( $IC_{50}$ ). DEA- N-(3,5-di-*tert*-butyl-4-hydroxy) phenylethylcarboethoxy-N-alkyloxymethyl-N,N-diethylammonium bromide. DEE- N-(3,5-di-*tert*-butyl-4-hydroxy)phenylethylcarboethoxy-N-dodecyloxymethyl-N,N-diethylammonium chloride.

be the appearance of an interdigitated structure where a compound, also an amphiphilic one, incorporated into one monolayer of the lipid bilayer can extend its hydrocarbon chain into the opposite monolayer thus stabilizing the bilayer. In order to explain such possibility, fluidity experiments were performed. Fluidity of erythrocyte ghosts, measured by change of the anisotropy coefficient ( $A$ ), was found to depend on the length of the hydrophobic part of the compounds (Fig. 3). However, contrary to the oxidation studies, no maxima of the fluidity change were found. The greatest fluidity changes found for the DEA-16 and DEE-16 imply that those compounds, like all others, did not stabilize the lipid bilayer. This problem needs further investigation. Perhaps the concentrations used ( $6 \mu M$  and  $10 \mu M$  for the DEA-n and DEE-n series, respectively) were too low. They were chosen on the basis of the oxidation studies with DEA-14 and DEE-14 causing 50% inhibition of the lipid oxidation.

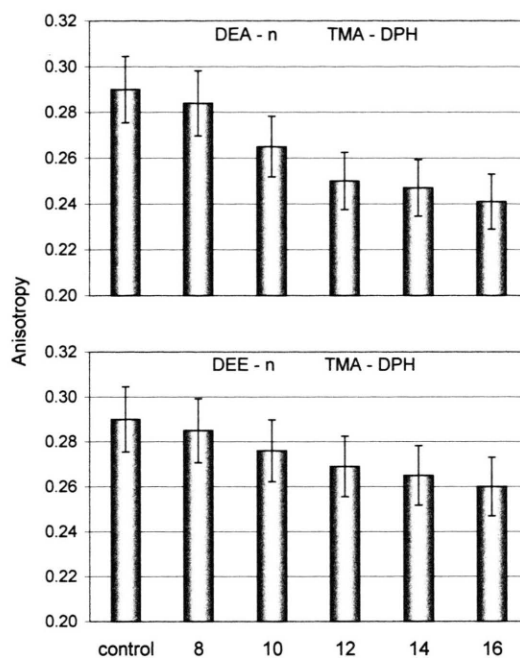


Fig. 3. Bar diagram of the anisotropy coefficients in the presence of DEA-n and DEE-n at  $6 \mu M$  and  $10 \mu M$ , respectively. Fluorescent probes used was TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate)].

The antioxidant activity depends mainly on number and localization of its hindered hydroxyl groups (Chen *et al.*, 1996). The investigations presented here have shown that hydrophobicity has a strong influence on antioxidant properties because, among others, it decides on the depth of incorporation of the compound into the lipid bilayer. The result of different embedding of a bifunctional surfactant is a different localization of its antioxidant fragment. It seems that the closer the antioxidant function is localized at the bilayer surface the more pronounced is the antioxidative efficiency of the surfactant.

Corroborating previous results, bromide surfactants protect more effectively erythrocyte membranes against peroxidation than the chloride ones. Apparently, such difference between the bromide and chloride anions is due to different possibilities to modify the electrostatic potential at the lipid bilayer surface (Kleszczynska and Sarapuk, 1998; Sarapuk *et al.*, 1998).

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