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QUATERNARY AMMONIUM SALTS PROTECT BIOLOGICAL MEMBRANES AGAINST OXIDATION

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Antioxidative properties of a series of morpholinium quaternary ammonium salts (PPMA-n; alkyl chain carbon number n = 7, 9, 11, 13 and 15) were determined. PPMA-n compounds were incorporated into erythrocyte membranes in sublytic concentrations, erythrocytes were then UV-irradiated and efficiency of antioxidative protection of PPMA-n determined spectrophotometrically by measuring the concentration of malone dialdehyde considered as an indicator of the lipid peroxidation.

The antioxidative activity of PPMA-n was found to increase with elongation of their alkyl chain. Protection of erythrocyte membrane lipids against oxidation by PPMA-15 was found to be about four times better than that by PPMA-7.

The results obtained indicate that the compounds studied can be used at micromolar concentrations as effective antioxidants and that this antioxidative activity is directly related to compound`s lipophilicity. Since lipophilicity is, among others, a factor determining the depth of incorporation into membrane, it is rather obvious that the deeper a molecule incorporates the better its antioxidative protections.

In order to confirm this conclusion, the measurements of changes in membrane fluidity caused by incorporating PPMA-n were performed using fluorescent probes. Both the probes (TMA-DPH and DPH) showed that there is a relation between alkyl chain length and change in anisotropy.

INTRODUCTION

Peroxidation reactions are known to lead to serious consequences, one of them being damage to the lipid phase of biological membranes followed by changes in physicochemical properties of membranes, including its transport properties [Pryor, 1976; Dwight & Hendry, 1996]. Especially prone to peroxidation are all unsaturated membrane lipids. The problem of the protection of model and biological membranes is quite important and justifies research efforts to find more effective compounds that, acting as free radical scavengers, could protect cells and their membranes against oxidation. The result of the interest in the problem mentioned are intensive studies in this area concerned both with natural and synthetic antioxidants [Chen *et al*., 1996; Ferrali *et al*., 1997; Rice-Evans, 1997; Vaya *et al*., 1997; Kleszczyńska & Sarapuk, 1998; Kleszczyńska *et al*., 2000; 2002; 2003; Wang & Lin, 2000; Bonarska *et al*., 2002].

To fulfill their role antioxidants must be incorporated into membranes which may be easily done in the case of lipophilic compounds. The incorporation act usually results in changes of membrane fluidity. This may be monitored by the use of fluorescence method where the parameter observed is anisotropy or polarization coefficients of a fluorescent probe incorporated into the membrane studied. A direct indication of the peroxidation of membrane lipids is the production of malonic dialdehyde (MDA), the concentration of which is measured spectrophotometrically. We have used both techniques in the work presented, as well as measurements of hemolytic potencies of the morpholinium salts (PPMA-n) studied. Hemolytic measurements were performed to determine safe concentrations, *i.e*. such that ensure antioxidative efficiency without damaging the membranes they were expected to protect.

The aim of the presented studies was therefore to assess and to compare the antioxidative activity of a newly synthesized series of morpholinium quaternary ammonium salts with hindered phenol substituent as antioxidative agent. The salts belong to the so-called “bifunctional surfactants” and can be used as antioxidants or as pesticides dependent on demand and concentration. Their effect on lipid oxidation in the erythrocyte membrane (RBC) subjected to UV irradiation was studied. The results obtained enabled finding a correlation between the antioxidant activity of the compounds studied and their lipophilicity. They may be helpful when synthesizing new, more potent antioxidants.

MATERIALS AND METHODS

All the morpholinium quaternary ammonium salts, of general structure presented in Figure 1, were synthesized at the Institute of Organic Chemistry, Biochemistry and Biotechnology, University of Technology, Wrocław, Poland. They were purified by column chromatography and checked by ^{1}H-NMR.

Thiobarbituric acid (TBA) was obtained from the Chemical Company (St. Louis, Missouri, USA). Trichloroacetic acid (TCA) was obtained from Fluka Chemie AG (Buchs, Switzerland).

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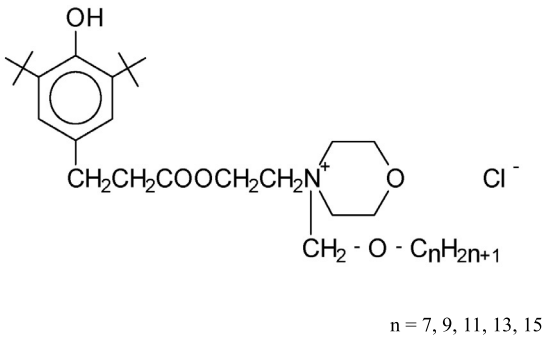
PPMA-n



n = 7, 9, 11, 13, 15

FIGURE 1. Chemical structures of compounds studied.

Fluorescent probes DPH (1.6-diphenyl-1,3,5-hexatriene) and TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate] were the products of Molecular Probes Inc. (Eugene, USA).

Erythrocyte membranes were prepared, according to the method of Dodge *et al*. [1963], from fresh heparinized pig blood. Erythrocyte ghosts were suspended in a phosphate solution of pH 7.4. Protein concentration was *ca*. 1 mg/mL. Two kinds of suspension were prepared. One contained erythrocyte ghosts only and the other contained also proper amounts of the antioxidant compounds studied. Lipid peroxidation in the erythrocyte membrane was induced by UV radiation; bactericidal lamp intensity was 3.5 mW/cm^2.

The degree of lipid peroxidation was determined by measuring the concentration of malonic dialdehyde which is, among others, the end-product of lipid peroxidation process. The concentration of malonic dialdehyde released in the samples was measured using its colour reaction with thiobarbituric acid [Stocks & Dormandy, 1971]. Supernatant absorption, determined spectrophotometrically (Spekol 11; 532 nm wavelength, Carl Zeiss Jena, Germany), was the measure of the degree of lipid peroxidation in the erythrocyte membrane, increased absorption indicating increased lipid peroxidation.

During exposure of the ghost mixture, 1 mL samples were taken and 1 mL of trichloroacetic acid (TCA; 15% TCA in 0.25 mol/L HCl) and 1 mL of thiobarbituric acid (TBA; 0.37% TBA in 0.25 mol/L HCl) were added. The samples taken were secured with a ball and heated at 100^{o}C for 15 min, and then cooled fast and centrifuged for 10 min at 2500 rev/min. After centrifugation, the absorption of the supernatant was measured at a wavelength of 535 nm.

The fluorescence measurements were performed on erythrocyte ghosts labelled with DPH and TMA-DPH using SFM spectrofluorimeter (KONTRON, Zurich, Switzerland). The anisotropy coefficient was calculated according to the following formula [Lakowicz, 1999]:

$$P = (I_{II} - GI_{\perp})/(I_{II} + 2GI_{\perp})$$

where I_{II} - 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.

Pig erythrocytes were used in the hemolytic experiments. Erythrocytes (RBC) were washed four times in phosphate buffer (pH 7.4) and incubated in the same buffer solution with the addition of chosen concentrations of PPMA-n compounds at 37°C. The samples were then centrifuged and hemoglobin content in supernatant measured with a Specol 11 spectrophotometer. The obtained hemolytic curves enabled determination of the concentrations of the compounds causing 50% (C_{50}) and 100% hemolysis (C_{100}).

RESULTS AND DISCUSSION

The results of the experiments performed are collected in Table 1 which contains the values of concentrations of particular compounds that cause 50% and 100% hemolysis (C_{50} and C_{100}), anisotropy coefficients found for both the fluorescent probes and concentrations of compounds inhibiting membrane lipid oxidation to a level of 50% (I_{50}). It is worth emphasising that the latter values are far below the hemolysing concentrations of the morpholinium salts. The hemolysis experiments showed what was expected, *i.e*. that the hemolytic potency of PPMA-n compounds ocyte membranes increased with their lipophilicity and was one-and-a half (C_{100}) to two (C_{50}) times higher for PPMA-15 compound compared to PPMA-7. An increase in hemolytic potency is connected with a stronger interaction of the compound with the lipid phase of membrane, resulting in deeper "sucking" of the compound into the membrane, as it was shown earlier for other group of quaternary ammonium salts [Kleszczyńska & Sarapuk, 1998; Kleszczyńska *et al*., 2000; 2002; 2003]. The results of hemolytic experiments indicate that the hemolytic efficiency of PPMA-n is good enough to regard them as potential pesticides.

Hemolytic efficiencies were followed by antioxidative abilities of PPMA-n compounds. As seen in Table 1, they

TABLE 1. Concentrations of compounds inducing 50% (C_{50}) and 100% (C_{100}) hemolysis of erythrocytes and causing 50% inhibition of peroxidation of erythrocyte membrane lipids (I_{50}). A_{DPH} and $A_{TMA\text{-}DPH}$ are values of the anisotropy coefficients for fluorescent probes used.

Parameters	PPMA-n					Control
	7	9	11	13	15	
C_{50} (mmol/L)	0.36	0.31	0.25	0.22	0.18	
C_{100} (mmol/L)	0.50	0.48	0.44	0.40	0.35	
I_{50} (μmol/L)	26.80	17.20	13.70	8.80	6.80	
A_{DPH}	0.279	0.270	0.263	0.255	0.243	0.288
$A_{TMA\text{-}DPH}$	0.280	0.271	0.267	0.254	0.248	0.288

Standard deviation did not exceed 5%.

were quite efficiently protected erythrocyte membranes against oxidation. That efficiency increased with compound`s lipophilicity and was about four times higher for PPMA-15 than for PPMA-7. Such results confirm the conclusion that the depth of incorporation is an important factor determining antioxidative potential of a compound.

Some additional experiments were performed to find out what was the perturbation induced by PPMA-n compound in the erythrocyte membranes. Those were measurements of membrane fluidity changes with the use of fluorescent probes. The measured anisotropy coefficients for both the probes used showed that membrane fluidity changed according to the already described way, *i.e.* increased with lipophilicity of the incorporated compound, confirming conclusions drawn from the hemolytic and oxidation experiments.

CONCLUSION

The results obtained show that morpholinium salts can be used both as membrane-active (pesticides) compounds or as antioxidants, depending on demand and concentration.

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