Antioxidative Properties of Pyrrolidinium and Piperidinium Salts

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Two series of pyrrolidinium (PYA-*n*) and piperidinium (PPPA-*n*) bromides with incorporated antioxidant function were synthesized. Both have hydrocarbon chains with odd number of the carbon atoms (*n*) ranging between 7 and 15. Pig erythrocytes (RBC) were used to study antioxidant activity of these compounds. They were incorporated into RBC membranes in sublytic (micromolar) concentrations and RBC were then subjected to UV radiation. It was found that all the salts used protected erythrocyte membranes against oxidation of membrane lipids. This protection increased with hydrocarbon chain length. Such effect may be the result of an incorporation of particular compounds to different depths into the lipid phase of RBC membrane depending on their chain length. Such possibility was checked by studies on fluidity changes induced by the compounds studied in ghost membranes by fluorimetric measurements.

The measurements showed that pyrrolidinium bromides were slightly more effective in a protection of erythrocytes than the corresponding piperidinium ones. The possible reason of such behaviour may be the difference in lipophilicity between piperidine and pyrrolidine rings.

Introduction

At the cellular level free radical mediated effects involve the lipid component of biological membranes and reflects in a change of the membrane physicochemical properties (Pryor, 1976). Also many studies have been performed using liposomes which may undergo peroxidation during appriopriate experiments. Hence, the protection of model and biological membranes is quite important and justifies research efforts to find new effective compounds that could protect cells and their membranes against oxidation by scavenging of free radicals. Intensive studies in recent years relate to both natural (Rios *et al*., 1992; Chen *et al*., 1996; Gabrielska *et al*., 1997) and synthetic bifunctional antioxidants (Kleszczyńska and Sarapuk, 1998; Kleszczyńska *et al*., 1998; 1999, 2000a; 2000b).

The objective of the present study was to determine the antioxidant activity of two new series of pyrrolidinium (PYA-*n*) and piperidinium (PPPA-*n*) bromides with odd number of carbon atoms in the hydrocarbon chain (*n* = 7, 9, 11, 13 and 15) and with a phenol substituent as an antioxidant function. They represent so-called bifunctional surfactants and can be used as antioxidants or as pesticides, depending on concentration. Their antioxidative protection of pig erythrocytes (RBC) subjected to UV irradiation against membrane lipids oxidation was studied. Also, their influence on fluidity of ghost membranes, while incorporated into these, was studied by the fluorescence method to estimate possible membrane damage. The results obtained allowed finding correlation between the antioxidative activity of the compounds and their hydrophobicity.

Materials and Methods

Pyrrolidinium and piperidinium compounds

The new antioxidants included two classes of quaternary ammonium salts with a phenol substituent functioning as an antioxidant. The salts were synthesized by quaternarization of pyrrolidine ethyl esters of dihydrocinnamic acid by *n*-alkyl bromides (PYA-*n*) or quaternarization of piperi-

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dine ethyl esters by *n*-alkyl bromides (PPPA-*n*). The general structure of salts is presented in Fig. 1. Their purity was checked by ^{1}H-NMR.

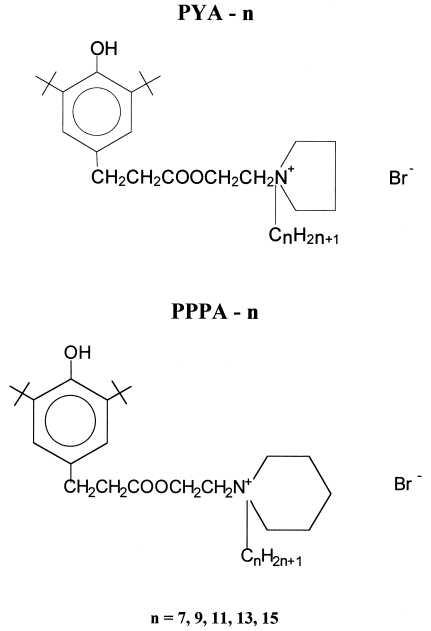

Fig. 1. The compounds studied; pyrrolidinium bromides – PYA-*n*, piperidinium bromides – PPPA-*n*.

Reagents and fluorescent probes

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

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 from Molecular Probes Inc. (Eugene, Oregon, USA).

Oxidation studies

Erythrocyte membranes were prepared according to Dodge *et. al*. (1963) from fresh heparinized pig blood. Erythrocyte ghosts were suspended in a phosphate solution of pH 7.4 with a protein concentration of ca. 1 mg/ml. Two kinds of suspensions were prepared. The control one contained erythrocyte ghosts only and the other contained erythrocyte ghosts and chosen amounts of the bromides studied. Lipid peroxidation in the erythrocyte membrane was induced by UV radiation (bactericidal lamp intensity was 3.5 mW/cm^2). Concentration of malone dialdehyde (MDA), which is one of the end products of lipid peroxidation, was considered as a measure of the lipid peroxidation process. MDA released in the samples gives colour reaction with thiobarbituric acid (Stock and Dormandy, 1971). The supernatant absorption was determined spectrophotometrically at 532 nm (Spekol 11, Carl Zeiss, Jena, Germany).

During exposure of the ghost mixture samples aliquots of 1 ml were taken, then 1 ml of trichloroacetic acid (TCA; 15% TCA in 0.25 M HCl) and 1 ml of TBA (0.37% TBA in 0.25 M HCl) were added. The samples were closed with a glass ball and heated at 100 °C for 15 min, quickly cooled and centrifuged for 10 min at 2500 rev/min. After centrifugation the absorption of supernatant was measured at 532 nm.

Fluorescence studies

Fluorescence measurements were performed on erythrocyte ghosts labelled with DPH and TMA-DPH using a SFM spectrofluorimeter (KONTRON, Zurich, Switzerland). The concentration of compounds in samples was 25 μM. The anisotropy was calculated according to Lakowicz (1983), Campbell and Dwek (1984) and Lentz (1988):

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

where I_{II} is intensity of fluorescence emitted parallel to the polarization plane of the exciting light, $I_{\perp}$ is intensity of fluorescence emitted perpendicular to the polarization plane, G is a factor used to correct for the inability of the instrument to transmit equally differently polarized light.

Hemolytic experiments

Fresh heparinized pig blood was used in hemolytic experiments. Blood was centrifuged for 3 min at 1000×*g*, the plasma removed and the cells washed twice with isotonic phosphate buffer solution (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.80 mM $Na_2HPO_4 \cdot 2H_2O$, 1.80 mM $Na_2HPO_4.H_2O$) of pH 7.4. The erythrocytes were then incubated for half an hour at 37 °C in the same solution containing different concentrations of the compounds studied. Four different hematocrits

were used (2%, 4%, 6% and 8%). To bring about the same hemolysis (100%) different concentrations of compounds were needed for different hematocrits. The linear dependence of hematocrit on the concentration enabled, by extrapolation, to calculate the unit hematocrit (1%). After the modification samples were taken, centrifuged and the supernatant was assayed for hemoglobin content at 540 nm. The hemoglobin concentration in the supernatant of totally hemolyzed erythrocytes was a measure of the extent of hemolysis. Good mixing of the suspension during all procedure stages was insured.

Results and Discussion

The results of the antioxidative studies are summarized in Table I. The antioxidative effect was measured after 2 h of irradiation of RBC with UV light and the used concentration of PYA-*n* and PPPA-*n* compounds was 10 μM. It can be seen that percentage inhibition of lipid peroxidation by such concentration of compounds (I_{10}) increased with an increase of their hydrocarbon chain length. In the best case (PYA-15) the inhibition has reached about 85% in comparison to peroxidation of unprotected (control) RBCs. The activity increase have roughly parabolic character and is more pronounced for compounds with longest chains (n = 13 and 15). Stronger antioxidative properties, for compounds with the same alkyl chains, were found for PYA series; the difference was about 10–20%. We have also determined concentrations of compounds inhibiting peroxidation by 50% (I_{50}) (Table I). It can be seen that the longest alkyl chain compounds protected RBCs about 3 (PYA-*n*) to 4 times (PPPA-*n*) better than the shortest alkyl chain ones. As already mentioned, PYA-*n* compounds exhibited better antioxidative properties than PPPA-*n* compounds. Since both series differ in the structure of the ring (piperidine or morpholine), the reason for the found difference in the antioxidative activity must be due to different lipophilicity of this part of the molecules. It was already shown that lipophilicity and steric properties of a molecule belong to factors deciding of the depth of incorporation of a compound in the lipid phase of membrane (Kleszczyńska *et al.*, 2000a; 2000c).

It is important to note that the concentrations of the compounds used in the oxidative studies were significantly lower than that causing erythrocyte hemolysis (Table I). Thus, the compounds studied incorporated into erythrocyte membranes, causing no evident damages, which is an essential condition for using them as an antioxidant. To be sure of that, hemolytic experiments were done. The results of these experiments showed that 50% hemolysis of RBCs (C_{50}) (Table I) was caused by concentrations of the compounds which were

Table I. Concentration of compounds inducing 50% hemolysis (C_{50}) of erythrocytes (RBC) for 6% hematocrit, and causing 50% inhibition of peroxidation of erythrocyte membrane lipids (I_{50}). I_{10} are values of percentage inhibition of peroxidation of membrane lipids subjected to 10 μM concentration of compounds studied, and P_T and P_D are values of the polarization coefficient measured for probes TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate] and DPH (1.6-diphenyl-1,3,5-hexatriene) for erythrocyte ghosts treated with 10 μM of compounds studied.

Parameters	Compounds										Control
	PPPA					PYA					
	7	9	11	13	15	7	9	11	13	15	
C_{50} [mM]	5.76	3.86	1.32	1.25	1.05	2-25	2.00	1.40	1.50	0.95	
I_{50} [μM]	28.0	18.0	14.2	7.5	7.0	17.6	12.2	10.0	7.0	5.5	
I_{10} [%]	28	36	42	66	72	34	44	50	71	85	
P_T	0.356	0.355	0.354	0.323	0.323	0.357	0.339	0.330	0.327	0.318	0.357
P_D	0.348	0.344	0.338	0.332	0.330	0.346	0.341	0.336	0.330	0.329	0.347

Deviation was about 8%.

about 3 to 4 orders of magnitude higher than these inhibiting peroxidation by 50% (I_{50}), depending on hematocrit and compound.

The fluorescence experiments revealed that the polarization coefficient remained essentially constant up to concentrations of bromides significantly higher than those used in the oxidation studies (20 μM). This conclusion is valid for both the used probes. The DPH probe was less sensitive to fluidity changes induced by the bifunctional compounds studied.

In summary, it has been shown that both series studied have sufficient antioxidative properties to be used as antioxidants if applied at carefully chosen concentrations. As it was mentioned above no hemolytic toxicity is expected when these concentrations do not excess micromolar values. Still, it must be underlined that no other experiments on the toxicity of compounds studied were performed.

Comparison of the antioxidative efficiency of the compounds of both the series with the efficiency of the well-known lipid antioxidant BHT (3,5-di-*tert*-butyl-4-hydroxytoluene) Kleszczyńska *et al.*, 1999) revealed that the PYA-n series, with exception of PYA-7, are better antioxidants than BHT. Also two compounds of the PPPA-*n* series (PPPA-13 and PPPA-15) exhibited better antioxidative properties in protecting erythrocytes than BHT.

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