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Antioxidative Activity of Pirolidinium Salts in the Erythrocyte Membrane

H. Kleszczyńska[1], J. Sarapuk[1], M. Oswięcimska[2], S. Witek[2]

[1] Department of Physics and Biophysics, Agricultural University,
Norwida 25, 50-375 Wrocław, Poland
[2] Department of Chemistry, Technical University of Wroclaw,
Wyb. Wyspiafiskiego 27, 50-370 Wrocław, Poland

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Abstract

The antioxidant activity of two new series of pirolidinium chlorides (PAC-n) and bromides (PAB-n) was studied. The antioxidant functional group was incorporated into the polar part of the compounds. The influence of the compounds on the degree of lipid oxidation in the erythrocyte membrane subjected to UV radiation was studied. It was found that all the salts used protected erythrocyte membranes against oxidation of membrane lipids. Their antioxidant activity increased with alkyl chain length. PAB compounds were stronger oxidation inhibitors than PAC ones. Possible reasons for such behaviour are discussed, taking into account the fluidity changes in erythrocyte membrane caused by the compounds studied. In order to do this, steady-state measurements of fluorescence anisotropy were performed, enabling to calculate the anisotropy coefficient.

Keywords: lipid oxidation, erythrocyte membrane, antioxidants, oxidation inhibition, fluidity

Introduction

Peroxidation reactions are known to lead to many perturbations in living organisms. One of these is damage to the lipid component of biological membranes resulting in changes in fluidity and transport processes in membranes. Unsaturated membrane lipids are especially prone to peroxidation. Peroxidation processes should be taken into account in the designing of experiments with liposomes, whose components can undergo peroxidation. Hence, the problem of protection of both model and biological membranes is quite important and justifies research efforts to find effective compounds that could protect cells and their membranes against oxidation, mainly by blocking free radical reactions. Investigations published in recent years in this area are concerned with both natural and synthetic antioxidants [1-10].

The aim of the present studies was to determine antioxidant activity of two new series of pirolidinium salts with hindered phenol substituent as an antioxidant function. They belong to so-called bifunctional surfactants and can be used as antioxidants or as pesticides (depending on concentration). Their effect on the degree of lipid oxidation in the erythrocyte membrane (RBC) subjected to UV radiation was studied. Also, the change of fluidity of RBC membranes on incorporation of the compounds studied was determined by the steady-state fluorescence method to estimate possible membrane damage. The results obtained allow finding a correlation between the antioxidant activity of the compounds studied, their hydrophobicity and the kind of counterion as one of the series was pirolidinium chlorides (PAC-n) and the other bromides (PAB-n). The results obtained may be helpful in synthesizing new, more potent antioxidants.

Materials and Methods

Pirolidinium Compounds

The general structure of the compounds studied is presented in Fig. 1. They were synthesized in our laboratory and are of analytical grade checked by ^{1}H-NMR.

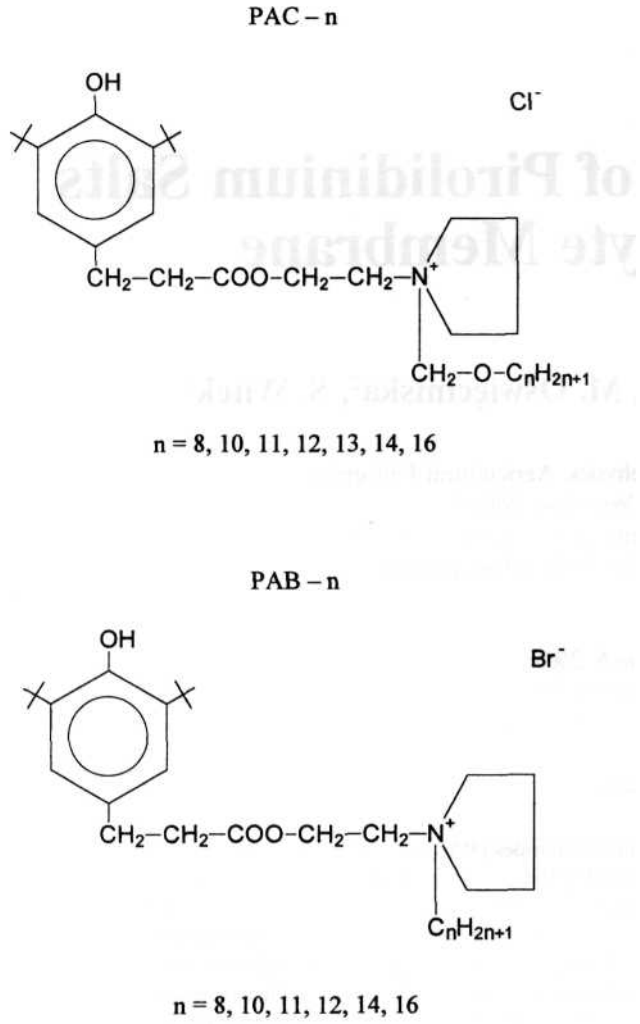

Fig. 1. Structure of the studied compounds.

Reagents and Fluorescent Probes

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

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

Oxidation Studies

Erythrocyte membranes were prepared according to the Dodge et al. method [11] from fresh heparinized pig blood. Erythrocyte ghosts were suspended in a phosphate solution of pH 7.4 at protein concentration ca. 1 mg/ml. Two kinds of suspension were prepared. One contained erythrocyte ghosts only, and other erythrocyte ghosts and chosen amounts of the antioxidants studied. Lipid peroxidation in the erythrocyte membrane was induced by UV radiation (bactericidal lamp intensity was 3.5 mW/cm^2). Concentration of malonic dialdehyde, which is one of the end-products of lipid peroxidation, was considered the measure of the lipid peroxidation process. The degree of lipid peroxidation was determined measuring the concentration of malonic dialdehyde released in the samples by using its colour reaction with thiobarbituric acid [12]. Supernatant absorption, determined spectrophotometrically at 532 nm (Spekol 11, Carl Zeiss, Jena Germany), was used as the measure of the degree of lipid peroxidatin of the erythrocyte membrane - increased absorption indicated increased lipid peroxidation. During exposure of the ghost mixture aliqouts of lml were taken, then 1 ml of trichloroacetic acid (TCA; 15% TCA in 0.25 M HC1) and 1 ml of thiobarbituric acid (TBA; 0.37% TBA in 0.25 M HC1) were added. The samples were secured with a ball and heated at 100°C for 15 min, and then quickly cooled and centrifuged for 10 min at 2500 rev/min. After centrifugation the absorption of supernatant was measured at λ = 535 nm.

Fluorescence Studies

Fluorescence measurements were performed on erythrocyte ghosts labelled with DPH and TMA-DPH using SFM spectrofluorimeter (KONTRON). The concentration of compounds in samples was 25 μM. The anisotropy (A) was calculated according to the formula [13-15]:

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

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 - is a factor used to correct for the inability of the instrument to transmit differently polarized light equally.

Results

The antioxidant activities of pirolidinium chlorides (PAC-n) and bromides (PAB-n) are presented in Fig. 2. It can be seen that chlorides efficiencies, the measure of which was compound's concentration inhibiting lipid oxidation by 50% (IC_{50}), are slightly lower than those of the corresponding, i. e. having the same carbon atom number, bromides. Both fluorescence probes used revealed that fluidity of erythrocyte ghost membranes increased with the increase of the hydrophobic part of compounds (Figs. 3 and 4). Generally, both DPH and TMA-DPH probes indicate that the fluidity of membranes is similar for compounds of the same n, but there is a tendency indicating that short-chained chlorides are more potent mem-brane-fluidizing compounds than bromides. In contrast, the long-chained bromides seem to increase fluidity of ghost membranes to a greater degree than chlorides.

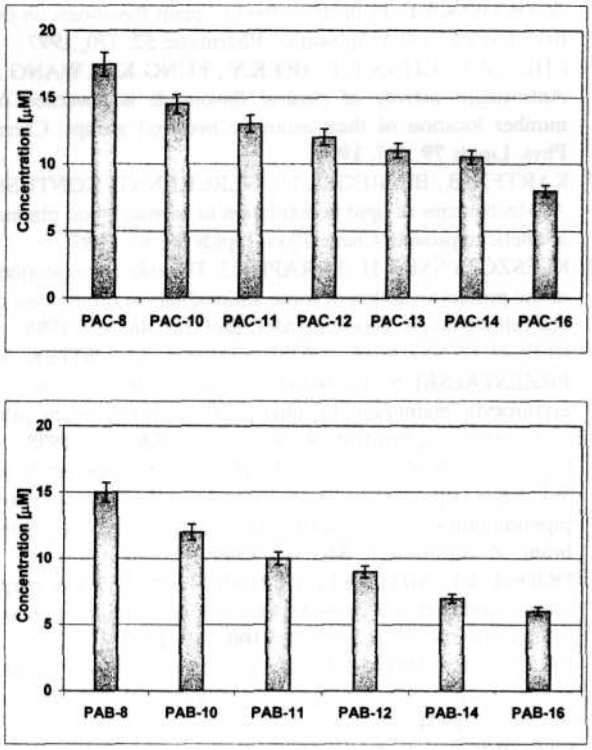

Fig. 2. The values of the concentration causing 50% inhibition of membrane lipid oxidation (C_{50}) of the compounds studied. Each experiment was repeated 3 times. Standard deviation (shown in bars) was 5%.

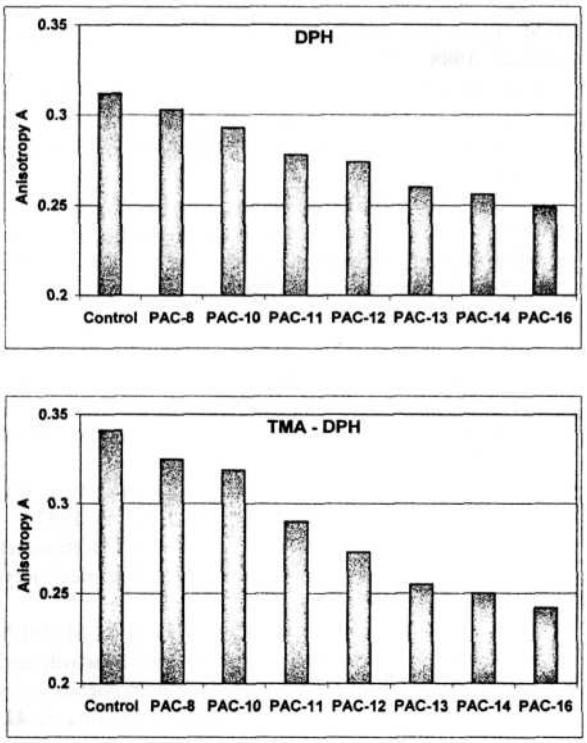

Fig. 3. The values of the anisotropy coefficient (A) for erythrocyte membranes modified by pirolidinium chlorides (PAC). Two different probes DPH (1.6-diphenyl-l,3,5-hexatriene) and TMA-DPH [l-(4-trimethylammoniumphenyl)-6-phenyl-l,3,5-hexatriene p-toluenesulfonate] were used. Standard deviation was ± 0.004.

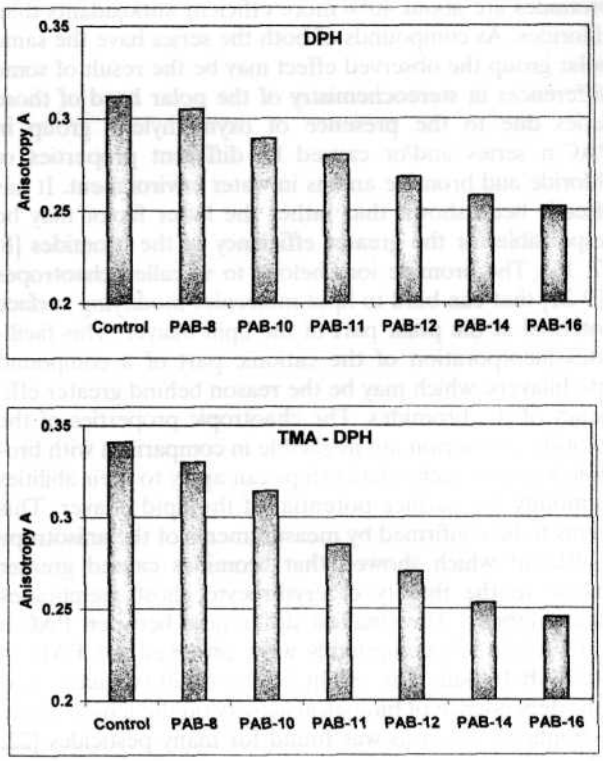

Fig. 4. The values of the anisotropy coefficient (A) for erythrocyte membranes modified by pirolidinium bromides (PAB). Two different probes DPH (1.6-diphenyl-l,3,5-hexatriene) and TMA-DPH [l-(4-trirnethylamrnoniurnphenyl)-6-phenyl-l,3,5-hexatriene p-toluenesulfonate] were used. Standard deviation was ± 0.004.

Discussion

One of the methods to protect the lipids of erythrocyte membrane against peroxidation may be incorporation of antioxidant compounds into the membrane. This is the way the compounds studied were used in the present work. Incorporation is supported by the hydrophobic chains of the compounds. However, concentrations of bifunctional surfactants cannot be too high as they could destroy the protected membranes acting as common pesticides. A different length of hydrophobic chains determines the depth of incorporation of a compound into lipid bilayer of the erythrocyte membrane. It seems that the greater hydrophobicity of a compound, the deeper it incorporates into the bilayer which, in turn, decides how far from the protected membrane the antioxidant functional of group a compound is positioned. The nearer to the lipid bilayer, the better protection (Fig. 2). A comparison of the protection efficiencies of the series studied leads to the conclusion that there must exist another factor, besides chain length, which influences those efficiencies, because compounds of PAC-n series have an alkoxy substituent of different alkyl chain length so they are more hydrophobic than the corresponding (having the same n number) compounds of the PAB-n series. Since $-CH_2O-$ group is equivalent to about two methylene groups [16], to compare antioxidant activity one should take PAC-n and PAB-(n+2) compounds. Such a comparison reveals that

bromides are about 40% more efficient antioxidants than chlorides. As compounds of both the series have the same polar group the observed effect may be the result of some differences in stereochemistry of the polar head of those series due to the presence of oxymethylene group in PAC-n series and/or caused by different properties of chloride and bromide anions in water environment. It has already been shown that rather the latter factor may be responsible for the greater efficiency of the bromides [8, 17, 18]. The bromide ions belong to so-called chaotropes [19-21] that can bind to lipid molecules modifying surface potential at the polar part of the lipid bilayer. This facilitates incorporation of the cationic part of a compound into bilayers, which may be the reason behind greater efficiency of the bromides. The chaotropic properties of the chloride counterion are negligible in comparison with bromide ions and such relationships can apply to their abilities to modify the surface potential of the lipid bilayer. This seems to be confirmed by measurements of the anisotropy coefficient which showed that bromides caused greater change to the fluidity of erythrocyte ghost membranes than chlorides. The smallest differences between PAC-n and PAB-(n+2) compounds were observed for PAC-14 and PAB-16 pair. This might be the result of quasi-parabolic dependence of biological activity on the length of the hydrophobic chain as was found for many pesticides [22, 23].

In summary, it has been found that bifunctional compounds studied display good antioxidative properties. This conclusion concerns both the series studied, but bromides were found to protect erythrocyte membranes more effectively. Comparison of the antioxidative efficiency of compounds of both the series with the efficiency of the well-known lipid antioxidant BHT (3,5-di-t-butyl-4-hydroxytoluene) [9] revealed that only a few of them (PAC-8, PAC-10 and PAB-8) were less effective in protecting erythrocytes than BHT.

Acknowledgements

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