Protective Effect of Quaternary Piperidinium Salts on Lipid Oxidation in the Erythrocyte Membrane

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A new series of amphiphilic compounds with incorporated antioxidant functional group has been investigated. Piperidinium bromides, differing in the alkyl chain length (8, 10, 12, 14 and 16 carbon atoms in the chain) were synthesised to protect biological and/or model membranes against peroxidation and following negative consequences. Their antioxidant activity was studied with erythrocytes subjected to UV radiation. The salts used inhibited lipid oxidation in the erythrocyte membrane. The degree of this inhibition depended on the alkyl chain length of the bromide used and increased with increasing alkyl chain length. A comparison of the results obtained for piperidinium bromides with those obtained for the widely used antioxidant 3,5-di-*t*-butyl-4-hydroxytoluene (BHT) revealed that only two shortest alkyl chain salts were less efficient than BHT in protecting erythrocyte membranes. A similar comparison with antioxidant efficiency of flavonoids extracted from *Rosa rugosa* showed that they protected the membranes studied more weakly than the least effective eight-carbon alkyl chain piperidinium bromide. The three compounds of longest alkyl chains were the most active antioxidants. Their activities did not differ significantly.

Introduction

Protection of biological and model membranes against peroxidation (Halliwell and Gutteridge, 1989; Bartosz, 1995; Janicka *et al.,* 1996) is the reason for the wide use of naturally occurring and synthesized antioxidants (Gabrielska *et al*., 1995a, 1995b, 1997; Chen *et al*., 1996; Karten *et al*., 1997; Rasetti *et al*., 1996/1997; Witek *et al*., 1997; Vaya *et al*., 1997; Kleszczyńska *et al*., 1998).

For protection antioxidants must be incorporated into membranes in such a way that their functional antioxidant group is localized in the polar part of the protected membrane while it is anchored by the hydrophobic part of a compound. Once incorporated such compound could effectively protect membranes and cells, against penetration of reactive forms of oxygen as shown for other antioxidants like those studied earlier (Kleszczyńska *et al*. 1998).

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Possibly more effective, a series of bifunctional surfactants belonging to quaternary ammonium salts were synthesized with a hindered phenol substituent as an antioxidative functional group. The studies were performed on erythrocyte membranes subjected to UV radiation and the antioxidant efficiency of piperidinium bromides studied compared to those exhibited by BHT and the flavonoids that naturally occur in *Rosa rugosa*.

Materials and Methods

Reagents

The structure of the piperidinium salts studied is shown in Fig. 1. They were of analytical grade, synthesized in our laboratory and are a new group of bifunctional compounds with antioxidant function. Their structure and purity were checked by ^{1}H-NMR spectra (Bruker Advance DRX$_{300}$ instrument, in deuteriochloroform, TMS as internal standard).

The flavonoid compound was prepared by ethyl acetate extraction and chloroform precipitation

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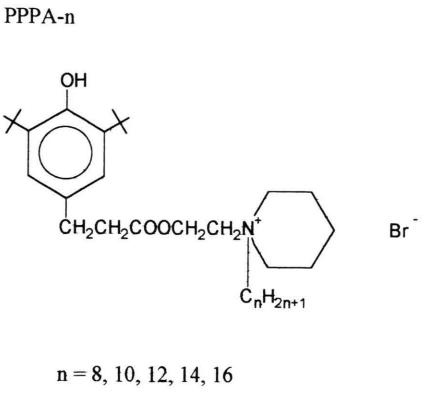

Fig. 1. Structure of the compounds studied.

from rose hip (*Rosa rugosa*) (Yoshibe *et al.*, 1992). The composition and degree of polymerization were determined by HPLC before and after thiolysis (Shibata *et al.*, 1994). The molecular weight of the flavonoid extract was determined to be 840 (Oszmiański, 1992). This value was used to specify molarity of the flavonoid extract in the erythrocyte ghosts suspension.

TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate] was from Molecular Probes Inc.(Eugene, OR).

TBA – thiobarbituric acid was obtained from Chemical Company (St. Louis, Missouri, USA)

TCA – trichloroacetic acid was obtained from Fluka Chemie AG (Buchs, Switzerland)

Oxidation studies

The erythrocyte ghosts were prepared from the washed cells according to the method of Dodge *et al.* (1963). The erythrocytes were hemolysed on ice with 14 volumes of hypotonic phosphate buffer (20 mM, pH 7.4) and centrifuged for 20 min at 4 °C at 20000×*g*. The ghosts were resuspended in ice-cold hypotonic phosphate buffer (20 mM, pH 7.4), and this process was continued until the ghosts were free of residual hemoglobin. Erythrocyte ghosts were suspended in phosphate buffer of pH 7.4 with a protein concentration of ca. 1 mg/ml. Besides the suspension containing erythrocyte ghosts only suspensions containing proper amounts of the antioxidant compounds studied were prepared and lipid peroxidation in the erythrocyte membrane was induced by UV radiation (bactericidal lamp intensity was 3.5 mW/cm^2).

The end-product of lipid peroxidation is, among others, malonic dialdehyde, and the degree of lipid peroxidation was determined on the basis of malonic dialdehyde released in the samples using its colour reaction with thiobarbituric acid (Bartosz, 1995; Stock and Dormandy, 1971).

During exposure of the ghost mixture 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 glass ball and heated at 100 °C for 15 min, then cooled fast and centrifuged, and the absorption of the supernatant was measured at 535 nm.

Fluorimetric studies

These were performed on erythrocyte ghosts. 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 at 1 μM concentration. Protein concentration in the samples was about 100 μg/ml. The fluorescence measurements were performed with SFM 25 spectrofluorimeter (KONTRON) and the polarization coefficient was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz, 1988):

$$P = \frac{I_{II} - GI_{\perp}}{I_{II} + GI_{\perp}}$$

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 of the exciting light, G – diffraction constant, dependent on wavelength.

Results

The piperidinium bromides (PPPA-*n*) studied have alkoxy substituents of different alkyl chain lengths (the number of carbon atoms in the alkyl chain was n = 8, 10, 12, 14, and 16), that differentiate their hydrophobicity, and the same polar group.

The results of studies on antioxidant activity of bifunctional piperidinium bromides are shown in Fig. 2 and contained in Table I. Part A of Fig. 2 shows the effect of one of the compounds (PPPA-

Table I. Concentrations that cause a 50% inhibition of oxidation of erythrocyte membrane lipids.

C_{50} [μM]						
PPPA-16	PPPA-14	PPPA-12	PPPA-10	PPPA-8	*Rosa rugosa*	BHT
6.6	7.2	7.6	14.0	22.0	30.0	13.0

PPPA = piperidinium bromides with alkoxy Substituents of differfant alkyl chain lengte.

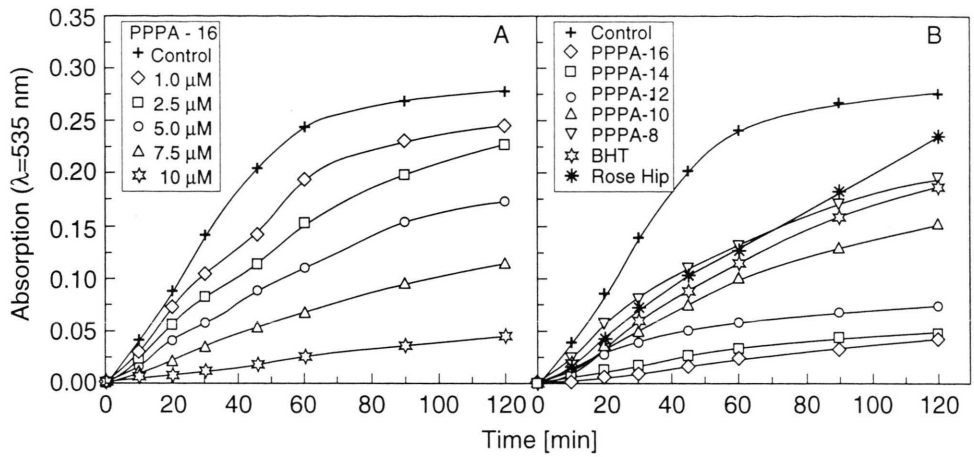

Fig. 2. Relation between absorption of light and time of lipid oxidation in the erythrocyte membrane for five concentrations of PPPA-16 (1 μM, 2.5 μM, 5 μM, 7.5 μM and 10 μM) (A). Relation between absorption of light (λ = 535 nm) and time of lipid oxidation in the erythrocyte membrane for various compounds applied at the same concentration equal to 10 μM.

16) at five different concentrations (1 μM, 2.5 μM, 5 μM, 7.5 μM and 10 μM) on lipid oxidation in the erythrocyte membrane (expressed in term of absorption) depending on UV irradiation time. As seen, the PPPA-16 salt applied at 10 μM concentration effectively protects lipids against oxidation, which is evident from the low absorption after a 120 min irradiation, compared with lipid oxidation in the control sample. Part B of Fig. 2 presents the relation between the degree of oxidation and time in the presence of all the compounds studied at the same concentration of 10 μM. Included is the well-known lipid antioxidant BHT and a flavonoid extract from *Rosa rugosa.*

Using the relations between degree of oxidation and time of irradiation, made for each of the compounds at various concentrations (1 μM – 100 μM), a relation has been obtained between percentage of oxidation inhibition and antioxidant concentration after 120 min UV irradiation. This was calculated for each compound relative to control as follows: % inhibition = $(1 - A_{535}/A_{\cdot 535}) \times 100$, where A_{535} is absorption in the control and $A_{\cdot 535}$ is absorption in samples with a compound studied after 120 min irradiation. Table I contains concentrations that cause a 50% inhibition of peroxidation of erythrocyte membrane lipids, which are regarded as a measure of their antioxidant activity. Compound PPPA-16 is the most potent antioxidant, since 50% inhibition of lipid oxidation was observed at 6.6 μM.

The antioxidant activity of the compounds follows the order:

PPPA-16 > PPPA-14 > PPPA-12 > BHT > PPPA-10 > PPPA-8 >>*Rosa rugosa*

Studies on fluidity of erythrocyte ghost membranes showed that the antioxidant efficiency sequence of PPPA-*n* compounds follows the extent of fluidity changes induced by these salts. The results of these fluorometric experiments are summarized in Tables II and III, containing the values of polarization coefficients of erythrocyte membranes as dependent on the compound concentration (Table II) and time of erythrocyte membrane oxidation at constant compound concentration (Table III).

Discussion

Our investigations have shown that the compound's hydrophobicity significantly influenced its antioxidant properties. The most hydrophobic bromide PPPA-16, of longest alkyl chain gave the best protection of erythrocyte membranes. At the concentration of 6.6 μM. it diminished the oxidation of erythrocyte lipids by 50%. The antioxidant activity of the least hydrophobic of the bromides studied, i. e., PPPA-8, with a twice shorter alkyl chain, was about three times less effective than PPPA-16 and a 22 μM concentration was required for a 50% inhibition of lipid oxidation. Antioxidant activities of other bromides studied also change with length of their alkyl chains and, as it was already mentioned.

The results obtained depended on the alkyl chain length of the compounds studied to be incorporate into the erythrocyte membranes to different depths. The longer the hydrophobic part of a compound the deeper it is embedded into the lipid bilayer due to the increase of hydrophobic interaction between hydrocarbon chains of the compound and neigbour lipid molecules. Similar behaviour of various amphiphilic compounds, forming homologous analog series which differ in the hydrophobic part length, was observed earlier (Kleszczyńska *et al.*, 1986, 1990). However, there is also a limiting factor which prevents a molecule of an amphiphile to be anchored too deep in the lipid bilayer namely, electrostatic interaction between polar parts of the incorporated molecule and surrounding lipid molecules. This factor depends, among others, on stereometry, charge and charge distribution of the hydrophilic part of compound. A localization of an amphiphilic compound in the lipid bilayer is the result of the equilibrium between these two forces and determines how far outside of the polar part of the lipid component of the erythrocyte membrane the antioxidative hydroxyl group of a compound is exposed. This in turn should reflect in different protective possibilities of particular piperidinium bromides. Additionally, the partition coefficient between membrane and aqueous environment is higher for bromides with longer alkyl chains. So, the effective concentration of such bromides is higher too which ensures a more effective protection of membrane lipids against oxidation.

Both, oxidation and fluorometric measurements seem to confirm such approach. The latter enables to study the fluidity of erythrocyte membranes, expressed by the polarization coefficient, which de-

Table II. Polarization coefficient of the erythrocyte membrane vs. concentration of the antioxidant.

Compounds	Polarization coefficient P		
	Control	Concentration [μM]	
	0	5.0	10.0
PPPA-8	0.372	0.366	0.359
PPPA-10	0.371	0.359	0.350
PPPA-12	0.373	0.366	0.348
PPPA-14	0.370	0.354	0.332
PPPA-16	0.369	0.350	0.323

(see Table I for compounds)
Values represent the mean of 3 experiments. Standard deviation was ±0.004.

Table III. Polarization coefficient of the erythrocyte membrane vs. the time of its oxidation in the presence of the antioxidant.

Compounds	Polarization coefficient P						
	Time of erythrocyte membrane oxidation [min]						
	0	10	20	30	45	60	90
Control	0.369	0.370	0.386	0.390	0.393	0.413	0.421
PPPA-8	0.373	0.375	0.387	0391	0.407	0.412	0.416
PPPA-10	0.371	0.374	0.378	0.386	0.399	0.411	0.414
PPPA-12	0.369	0.371	0.377	0.382	0.397	0.400	0.407
PPPA-14	0.370	0.373	0.379	0.383	0.399	0.401	0.404
PPPA-16	0.372	0.372	0.375	0.388	0.398	0.402	0.402

(see Table I for compounds)
Values represent the mean of 3 experiments. Standard deviation was ±0.004.

creased with elongation of alkyl chain length according to the above mentioned order.

The localization of the antioxidant fragment in the membrane depends on the depth of the incorporation of particular PPPA-*n* into membranes. Salts with longer alkyl chains incorporate into the membrane deeper than those with shorter chains, causing greater increase in the fluidity of the erythrocyte membrane. Therefore, longer alkyl chains salts protect model membranes more effectively against peroxidation. Obviously, the closer an antioxidant functional group is localized to the membrane the better it protects the membrane.

Comparison of the results for all investigated compounds indicate that PPPA-*n* salts, at least PPPA-12, PPPA-14 and PPPA-16, protect membranes more effectively than BHT or *Rosa rugosa* flavonoids.

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