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Hemolysis and Antioxidative Protection of Erythrocytes by Functionalized Quaternary Ammonium Salts

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Abstract

The results of studies on hemolytic and antioxidative activities of quaternary ammonium bromides and chlorides differing in alkyl chain length and with functionalized antioxidative group are presented. Pig erythrocytes (RBC) and their ghosts were used in experiments. The hemolytic studies permitted to determine the safe concentrations at which the compounds studied did not damage RBC membranes. RBC were then UV-irradiated and the antioxidative efficiency of the quats determined. It was found that hemolytic activities increased with lipophilicity of a compound and each bromide salt was more hemolytic than chloride ones. The antioxidant activity of the quats followed the same rule. The results obtained indicate that observed effects are the consequence of the incorporation of particular compounds to different depths into the lipid phase of the RBC membrane. The incorporation depended on lipophilicity of quats and the kind of counterions. Studies on fluidity changes induced by quats in ghost membranes confirmed the above conclusion.

Keywords: bifunctional quaternary salts, erythrocyte membrane, hemolytic activity, antioxidative activity, lipid oxidation.

Introduction

Lipid peroxidation processes in living organisms lead to many pathological events, including changes in physicochemical properties of cell membranes [1, 2]. Hence, membrane protection against peroxidation is a very important task and much time has recently been devoted to find and apply natural and synthetic compounds of antioxidative properties [3-6]. We have already presented new bifunctional quaternary ammonium salts (quats), some of which were shown to exhibit good antioxidative activities [7-11]. The compounds studied in this work belong to the same class of bifunctional quats and may be, on demand, used as pesticides or antioxidants depending on concentration. They represent two new series of ammonium bromides (DEA-n) and chlorides (PPPE-n) with an odd number of carbon atoms in the hydrocarbon chain (n = 7, 9, 11, 13 and 15) and with a phenol substituent to function as an antioxidant. Their influence on pig erythrocytes (RBC) and their ghosts (RBCG) was studied. Two kinds of experiments were performed. Hemolytic experiments permitted the determination of concentration range in which quats may act as pesticides. That concentration range was determined in antioxidation studies performed on RBCG subjected to UV irradiation. These measurements enabled us to conclude that quats may be used as effective antioxidants

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far below their hemolytic concentrations. Also, fluorescence experiments with RBCG were performed to find the correlation between structural features of the quats, that determine their interaction with RBC and RBCG membranes, and their effectiveness both as pesticides and antioxidants. Additionally, the osmotic resistance of the quats (DEA-13 and PPPE-13) was measured and compared for the same reason.

Materials and Methods

Bifunctional Quaternary Ammonium Salts (Quats)

The bifunctional antioxidants studied represent two classes of quats with a phenol substituent functioning as an antioxidant. They were synthesized in our laboratory by quaternarization of N,N-diethyl-N-ethyl esters of dihydrocinnamic acid by alkyl bromides (DEA-n) or quaternalization of piperidine ethyl esters by alkyl-chlormethylethers (PPPE-n). The structure and purity were checked by ^{1}H-NMR spectra (Bruker Avance DRX300 instrument, in deuteriochloroform, TMS as internal standard). The general structure of the salts is presented in Fig. 1.

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

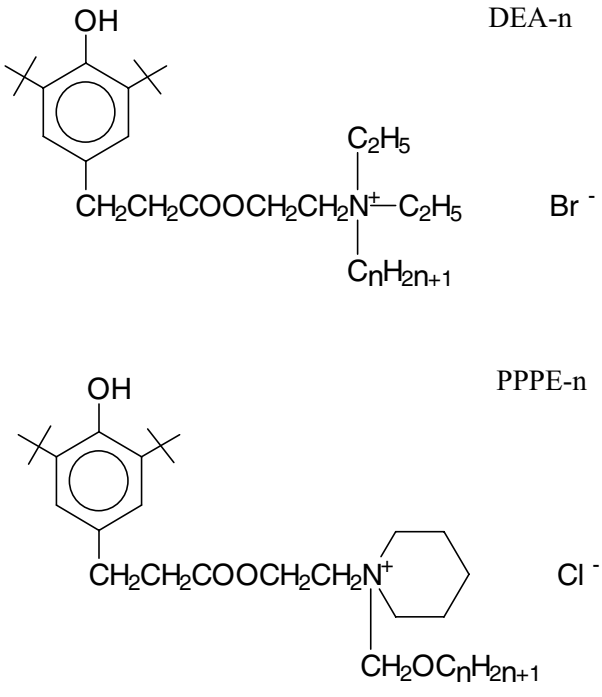

Fig. 1. Chemical structures of studied compounds, n = 7, 9, 11, 13, 15.

Oxidation Studies

Erythrocyte membranes were prepared according to Dodge *et. al*. [12] from fresh heparinized pig blood. Erythrocyte ghosts (RBCG) 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 contained RBCG only and the other contained RBCG and chosen amounts of quats. 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 a measure of the lipid peroxidation process. MDA released in the samples gives colour reaction with TBA [13]. Supernatant absorption was determined spectrophotometrically at 532 nm (Spekol 11, Carl Zeiss, Jena, Germany).

During exposure 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) was added. The samples were plugged with a glass ball and heated at 100°C for 15 min, quickly cooled and centrifuged for 10 min at 2500 rev/min. After centrifuging the absorption of supernatant was measured at 532 nm.

Fluorescence Studies

Fluorescence measurements were performed on erythrocyte ghosts labelled with TMA-DPH using a SFM spectrofluorimeter (KONTRON, Zurich, Switzerland). The concentration of compounds in the samples was 25 μM. The anisotropy was calculated according to Shinitzky and Barenholz [14]:

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

where I_{II} is intensity of fluorescence emitted parallel to the polarisation plane of the exciting light, $I_{\perp}$ is intensity of fluorescence emitted perpendicular to the polarisation 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 x *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. Hematocrit was 2%. To induce the same hemolysis (50% or 100%), different concentrations of compounds

were needed. After modification samples were taken, centrifuged and the supernatant was assayed for hemoglobin content at 540 nm. The hemoglobin concentration in the supernatant of 50% (C_{50}) or totally hemolysed erythrocyte (C_{100}) was a measure of the extent of hemolysis. Good mixing of the suspension during all stages of the procedure was insured.

Measurements of Osmotic Resistance of Erythrocytes

Osmotic resistance was measured for RBC modified for 0.5 h in solutions containing different amounts of DEA-13 and PPPE-13. The cells were then suspended in hypotonic solutions of NaCl and a percentage concentration of NaCl causing 50% hemolysis of RBC was determined.

Results and Discussion

The results obtained are summarized in Table I. Both the C_{50} and C_{100} parameters indicate that the hemolysing potency of the quats of each series increased with lipophilic chain length. Quats of longest chains (DEA-15 and PPPE-15) were over twice more effective in hemolysing RBC than those of shortest chains (DEA-7 and PPPE-7). Since compounds of PPPE-n series have additional oxymethylene spacer group between the nitrogen atom and hydrocarbon tail and piperidinium ring, it seems that these compounds should exhibit higher hemolytic activities than DEA-n compounds because of their higher lipophilicity. As it can be seen, this is not the case (see Tab. 1). Compounds of DEA-n series were found to be at least 20% stronger hemolytic agents than the corresponding PPPE-n compounds. The reason for such a difference must be the different counterions these quats have. It was already shown that bromide salts have a greater ability to destabilise biological and model membranes than chlorides, as found for other bifunctional quats [7, 15, 16]. The reason for this is the difference in the abilities of chlorides and bromides to order water molecules [17-20], which leads to different modification of the polar part region of membrane lipids and, hence, to different incorporation of these salts in the lipid bilayer. The result is the observation that DEA-n quats, being bromides, may have a more pronounced influence on RBC membranes than chloride salts. As is generally accepted, that region of the membrane is responsible for hemolysis induced by exogenous agents. The hemolysing effects were observed in a milimolar concentration range.

Measurements of the antioxidative activities of the quats showed that they followed the hemolytic sequence. This conclusion concerns a change of antioxidative potency of the compounds inside each series and comparison of potency found for the corresponding compounds of both series. Antioxidant protection of UV irradiated RBCs increased with quats lipophilicity and was about twice greater for quats of longest alkyl chain compared with the protection by those of shortest alkyl chain. The measure of the protection was the concentration of a quat that caused 50% inhibition of peroxidation after 2 h irradiadion with UV. The same effects were observed when constant (micromolar) concentration of the quats was used to inhibit oxidation (I_{20}). Such concentration of DEA-15 protected over 85% of RBC. The same protection given by PPPE-7 was only about 25% (see Tab. I). It is important to note that the concentrations of the compounds used in the oxidative studies were significantly lower than that causing

Table I. Concentration of compounds inducing 50% (C_{50}) and 100% (C_{100}) hemolysis of erythrocytes (RBC) at 2% hematocrit, and causing 50% inhibition of peroxidation of erythrocyte membrane lipids (I_{50}). I_{20} are values of percentage inhibition of peroxidation of membrane lipids subjected to 20 μM concentration of the compounds studied, and A_5 and A_{10} are values of the anisotropy coefficient measured for probe TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate] for erythrocyte ghosts treated with 5 μM and 10 μM of the compounds studied.

Parameters	Compounds										Control
	DEA					PPPE					
	7	9	11	13	15	7	9	11	13	15	
C_{50} [mM]	1.55	1.25	1.10	0.85	0.65	2.20	1.70	1.35	1.05	0.85	
C_{100} [mM]	2.20	1.85	1.65	1.30	1.05	2.85	2.30	1.90	1.65	1.25	
I_{50} [μM]	22.0	19.0	17.0	15.5	12.0	34.0	27.5	24.5	19.5	16.0	
I_{20} [%]	55	58	63	78	86	26	33	42	57	74	
A_5	0.281	0.273	0.258	0.241	0.233	0.279	0.277	0.269	0.250	0.243	0.290
A_{10}	0.272	0.258	0.247	0.235	0.224	0.280	0.271	0.257	0.244	0.238	0.290

Deviation 4%

erythrocyte hemolysis (Table 1). Thus, the compounds studied incorporated into erythrocyte membranes without evident damage, which is an essential condition for using them as an antioxidant.

In view of the results obtained it is rather obvious that the combined effect of lipophilicity of a molecule, its structural features and the kind of counterion it has decide of the depth of its incorporation in the lipid phase of a membrane, and that in turn, decide of the molecule antioxidative activity. Nevertheless, some additional experiments were needed. These were concerned with a description of the membrane state after incorporation of quats, i. e., measurements of anisotropy of RBCG membranes and the osmotic resistance of RBC. The anisotropy changes were found to follow the pattern already presented. Anisotropy decreased with lipophilicity inside each series, and DEA-n compounds induced greater order in RBCG membranes than PPPE-n compounds. Anisotropy measurements were performed for 5 M and 10 mM concentrations of quats (A_5 and A_{10}; see Tab. 1). The chosen concentrations were two to three orders of magnitude lower than those in hemolytic experiments.

The osmotic resistance measurements were performed for chosen quats of both series (DEA-13 and PPPE-13) on the assumption that they were qualitatively representative for all the rest of the quats. The osmotic resistances, expressed as a percentage concentration inducing 50% hemolysis of RBC previously modified by quats, were found to be similar and equal to 0.64% and 0.65%; control value being 0.58%. These values indicate a change in mechanical stability of erythrocyte membranes after incorporation of the quats.

In summary, it has been shown that the quats of both series studied, especially those with longer lipophilic parts, have sufficient antioxidative properties to be used as antioxidants if applied at suitable concentrations. It was also shown that the structure and lipophilicity of the compounds and their counterions were factors deciding the antioxidative property, most probably affecting the depth of incorporation of a compound in the lipid phase of membrane.

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