Antioxidative Activity of New *N*-Oxides of Tertiary Amines: Membrane Model and Chromogen Studies

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Potential antioxidative activities of three series of newly synthesized *N*-oxides were studied. Individual components in each of the series differed in the lipophilicities and number of free radical scavenging groups. Various methods were used to determine their antioxidative efficiencies: Prevention of erythrocyte membrane lipid oxidation induced by UV irradiation and chromogen experiments in which antioxidative efficiencies of compounds were compared to that of the standard antioxidant Trolox (a water-soluble vitamin E analogue). Additionally, some hemolytic (pig erythrocytes) and differential scanning calorimetry (DSC) measurements were performed to determine a mechanism of the interaction between membranes and *N*-oxides.

It was found that *N*-oxides, especially those of long alkyl chains (> $C_{12}H_{25}$), readily interacted with both, erythrocyte and liposomal membranes. No marked differences were found in their protection of erythrocytes against oxidation. In most cases inhibition of oxidation changed between 15% and 25%. Still, it was far better than in chromogen experiments where suppression of free radicals reached 20% in the best case. It may be concluded that antioxidative capabilities of *N*-oxides are moderate.

Studies on the interaction mechanism showed that incorporation of particular compounds into model membranes varied. Hemolysing activities of compounds increased with the elongation of the alkyl chain but differed for corresponding compounds of particular series indicating that lipophilicity of compounds is not the only factor determing their interaction with erythrocyte membranes.

DSC experiments showed that *N*-oxides, upon incorporation into 1,2-dipalmitoyl-3-sn-phosphatidylcholine liposomes, shifted the subtransition (Tp) and the main transition (Tm). The shifts observed depended on the alkyl chain length. The effects differed for each series. It seems that in the case of long alkyl chain compounds the domain formation may take place. Generally, the decrease of Tm was greatest for the same compounds that exhibited the best hemolytic efficacy. The same conclusion concerns the decrease of cooperativity of the main transition and the observed changes suggest an increase in membrane fluidity. Both, erythrocyte and DSC experiments seem to indicate that compounds of particular series incorporate in a somewhat different way into membranes.

Key words: *N*-Oxides, Antioxidative Activity

Introduction

Antioxidant activity which enables compounds to protect biological objects against harmful effects of excessive oxidation, leading to severe diseases (Braughler and Hall, 1989; Piotrowski *et al.*, 1990; Adams and Odunze, 1991; Gey, 1993; Smith *et al.*, 1994), is a very important parameter characterising various plant materials (Arnao, 2000). Toxic effects also concern lipids of a cell membrane (Pryor, 1976). Antioxidative properties, beside natural plant extracts, exhibit some synthesized compounds. This is the reason why these new compounds are checked for their antioxidative efficiency and some are synthesized specifically for that reason. In this work we studied a group of *N*-oxides of tertiary amines (NTA) that were intended to be used as antioxidants.

It is evident that to protect cell membrane lipids against free radical damage compounds must be incorporated into membranes. Hence, our prelimi-

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nary studies concerned the interaction between a group of newly synthesized NTA and model and biological membranes (liposomes and erythrocytes). To evaluate the strength of this interaction the hemolytic efficacy of particular compounds and their potency to influence DPPC (1,2-dipalmitoyl-3-sn-phosphatidylcholine) phase transitions in multilayer liposomes were studied. Both types of measurements were proved to give some insight into the interaction mechanism in several cases (O'Leary *et al.*, 1986; Cevc and Marsh, 1987; Kleszczyńska *et al.*, 2000, 2002, 2003; Kleszczyńska and Sarapuk, 2001; Heimburg, 2000; Hendrich *et al.*, 2002), and especially to give information about a mode of incorporation into the lipid phase of model membranes used.

The red cell ghosts were used in measurements of antioxidative efficiency of NTA and the results obtained were then compared to the chromogen experiments where the same compounds reduced the radical $ABTS^{+\cdot}$ [ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid] to a nonreactive form. In turn, the chromogen experiments were compared to $ABTS^{+\cdot}$ reduction by Trolox, an analogue of a vitamin E known for its antioxidant activity (Niki *et al.*, 1986).

Materials and Methods

N-Oxides of tertiary amines

General structures of compounds studied are shown in Fig. 1. They were synthesized in the Institute of Organic Chemistry, Biochemistry and Biotechnology, University of Technology, Wrocław, Poland. Synthesis procedures of series I and II were described in patent announcements (Piasecki and Karczewski, 2001a, 2001b; Piasecki *et al.*, 2003a, 2003b) and synthesis of series III was described by Nikawitz (1951) and Miller (1967).

Hemolytic studies

The experiments were conducted on fresh, heparinized pig blood. For washing the erythrocytes and in the experiments performed, an isotonic phosphate solution of pH 7.4 (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.80 mM $Na_2HPO_4 \cdot 2H_2O$, 1.80 mM $NaH_2PO_4 \cdot H_2O$) was used. Upon removal from the plasma, the erythrocytes were washed four times in a phosphate buffer and then incubated in the same solution but containing proper amounts of the compounds studied. The modification was conducted at 37 °C for 0.5 h, each sample containing 10 ml of erythrocyte suspension of 2% hematocrit, stirred continuously. After modification 1 ml samples were taken, centrifuged and the supernatant assayed for hemoglobin content using a spectrophotometer (Spekol 11, Carl Zeiss, Jena) at 540 nm wavelength. The hemoglobin concentration in the supernatant, expressed as a percentage of hemoglobin concentration in the supernatant of totally hemolyzed cells, was assumed as the measure of the extent of hemolysis.

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 at a protein concentration of ca. 1 mg/ml. Two kinds of suspensions were prepared. One contained erythrocyte ghosts only, and the 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). 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. Supernatant absorption was determined spectrophotometrically at 532 nm (Spekol 11, Carl Zeiss) – increased absorption indicated increased lipid peroxidation. The experimental procedure was described earlier (Kleszczyńska *et al.*, 2002).

Chromogen experiments

The standard TEAC (Trolox Equivalent Antioxidant Capacity) assay has been used. This assay assesses the total radical scavenging capacity of compound to scavenge the bluegreen chromogen radical $ABTS^{+\cdot}$ in 20 min followed by a transition of chromogen to a colorless form monitored spectrophotometrically at 414 nm. The absorbance decrease is proportional to the antioxidant property. The detailed procedure was described earlier (Van der Berg *et al.*, 1999; Re *et al.*, 1999; Arnao, 2000). The results obtained were compared to the same effect caused by the standard antioxidant Trolox. The TEAC equivalent of the compound was calculated as the concentration (mM) of Trolox showing an antioxidant capacity equivalent to 1 mM of the tested compound. The results obtained in these experiments are a direct indication of compound's antioxidative activity.

Differential scanning calorimetry (DSC) studies

DPPC was purchased from Sigma Aldrich. Samples for DSC were performed on multilamellar vesicles (MLVs). DPPC dissolved in chloroform was evaporated. Traces of chloroform were removed with a stream of dry nitrogen under vacuum for 2 h. The lipid film formed was dispersed by adding a solution of compounds I, II or III in water of appropriate concentration, and agitating the flask on a vortex mixer to give a milky suspension of liposomes. The mixture was heated at about 60 °C for 15 min and finally cooled down to room temperature. The final lipid concentration was 25 mg/ml. The lipid suspension was loaded into the sample cell of a DSC microcalorimeter (Mettler Toledo Thermal Analysis System D.S.C. 821^e). Employed scan rates were 2 °C/min, while time of incubation performed at 4 °C was 48 h.

Results and Discussion

The measure of the intensity of the interaction of *N*-oxides (NTA) with pig erythrocytes (RBC) was their hemolytic efficacy. The values of concentrations of NTA causing 50% hemolysis (C_{50}) of RBC as well as other experiments performed are collected in Table I. The results obtained indicate that *N*-oxides of series I and III (Fig. 1) readily interacted with erythrocyte membranes and caused hemolysis when used at micromolar concentrations. This conclusion concerns compounds whose alkyl chains were long enough (> $C_{12}H_{25}$). Hemolytic efficiency of series II was significantly weaker even for long alkyl chain compounds. This indicates that there are other factors, beside lipophilicity, that influence NTA interaction with the RBC membrane. Since corresponding compounds of series I and II vary by addition of a methylene

Table I. The values of concentration of *N*-oxides causing 50% hemolysis of erythrocytes (C_{50}), inhibiting oxidation of erythrocytes by 50% (IC_{50}), the percentage of inhibition after 120 min (%) caused by compounds used in 10^{-4} M concentration, the percentage of suppression of chromogen and molar equivalent of Trolox inducing the same effect (1 M of compound is equivalent to *x* mM of Trolox). Standard deviations for C_{50} and IC_{50} did not exceed 8%.

Compound	Hemolysis C_{50} [mM]	Oxidation inhibition IC_{50} [mM]	(%)	Suppresion of ABTS (%)	*x* mM of Trolox ± SE
IA	> 10	0.20	33.0	18.3	16.4 ± 0.8
IB	> 10	0.65	27.5	2.5	3.64 ± 0.26
IC	0.95	0.60	27.0	4.1	5.25 ± 0.36
ID	0.04	0.10	40.0	20.4	19.8 ± 1.0
IE	0.02	0.75	25.0	13.7	14.0 ± 0.67
IF	0.01	1.50	12.0	4.5	4.10 ± 0.29
IIA	8.0	0.90	20.0	4.5	5.93 ± 0.40
IIB	2.0	0.35	26.0	9.2	9.72 ± 0.60
IIC	3.0	0.75	23.0	5.5	6.41 ± 0.42
IID	1.0	1.50	12.0	6.7	7.48 ± 0.48
IIE	0.70	0.80	23.5	6.0	6.82 ± 0.44
IIIA	0.40	0.85	23.5	4.4	5.35 ± 0.36
IIIB	0.70	1.00	15.0	17.1	16.6 ± 1.0

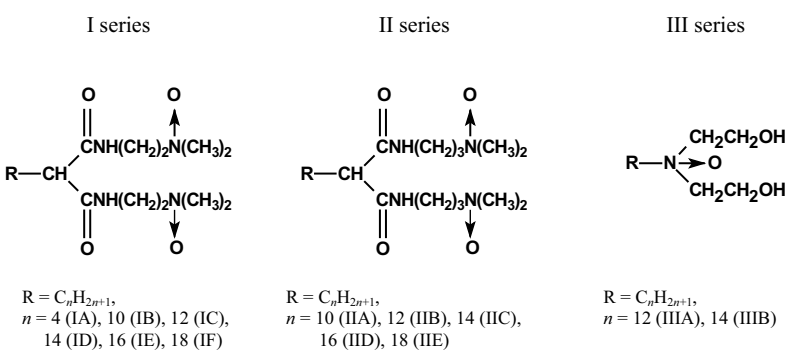

Fig. 1. General structures of *N*-oxides.

group between carboxyl and *N*-oxide groups (see Fig. 1) this methylene group must be responsible for weakened interaction with the RBC membrane and decrease in the hemolytic ability of series II in comparison with series I. The presence of a slightly polar hydroxyl group at the tertiary amine does not seem to weaken interaction of compounds III with RBC, especially of compound IIIB ($C_{14}H_{29}$-alkyl chain). However, it must be underlined that any increase in lipophilicity determines the activity of the compound inside each series. Its importance in the interaction of NTA with membrane lipids was confirmed by DSC measurements (Fig. 2). Both subtransition (Tp) and main transition (Tm) shifting in 1,2-dipalmitoyl-3-sn-phosphatidylcholine liposomes depended on the alkyl chain length of the incorporated compound, thus pointing to a disordering of the lipid bilayer. The sensitivity of transition temperatures on the presence of various compounds penetrating the liposome bilayer was shown earlier (O'Leary *et al.*, 1986; Hendrich *et al.*, 2001, 2002). Generally, the decrease of Tm was greatest for the same compounds that exhibited the best hemolytic efficacy. The same conclusion concerns the decrease of cooperativity of the main transition, and the observed changes suggest an increase in membrane fluidity. Splitting of the main transition peak suggests that there is a possible domain formation in the case of longer alkyl chain compounds. The splitting was observed for compounds of all series. They also liquidated the pretransition peak that may also be connected with changes in the hydration of polar heads of lipid bilayers induced by incorporated compounds (Heimburg, 2000) or their interaction with water molecules in the hydration layer (Koynova *et al.*, 1997). All these results taken together point at fluidization of lipid bilayers on incorporation of *N*-oxides.

The studies on antioxidative activity of *N*-oxides showed that inside each series, as in the hemolytic and phase transition experiments, there was a depen-

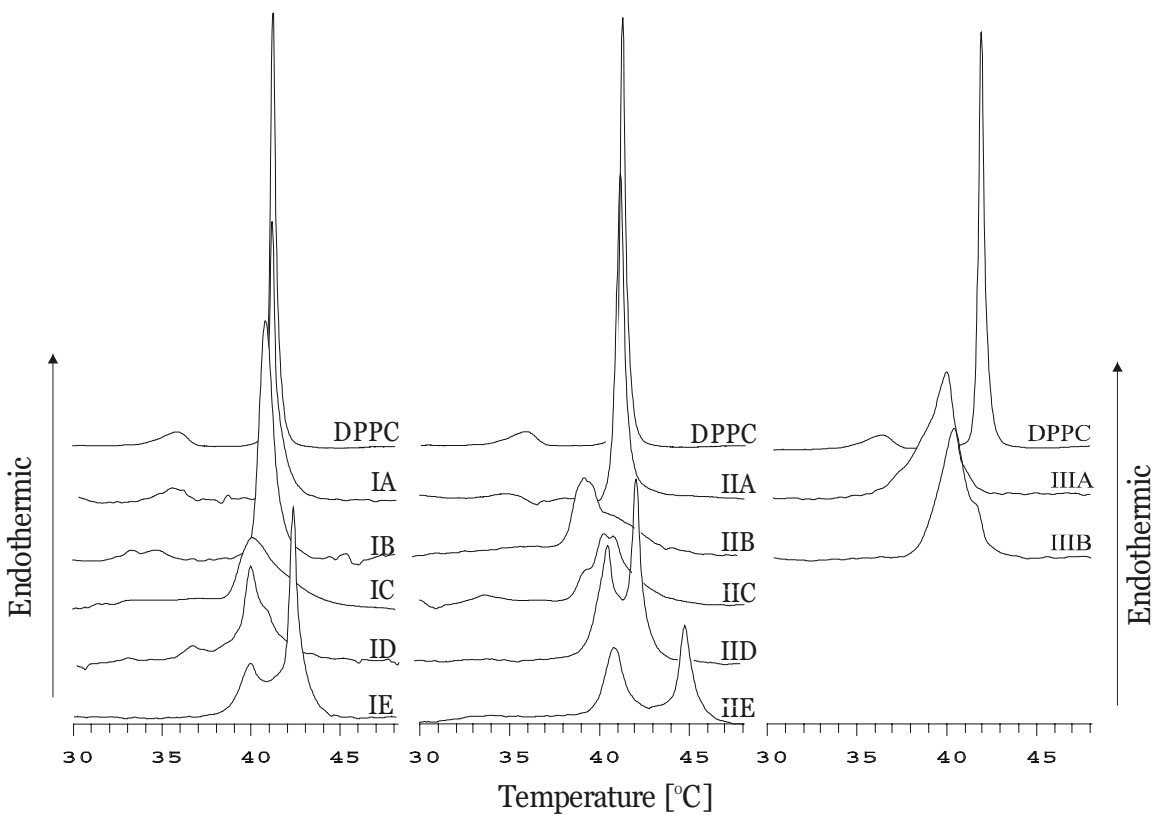

Fig. 2. Phase transitions in DPPC liposomes induced by *N*-oxides.

dency on the lipophilicity of compounds. However, no such significant differences were found between activities of particular series. The weakest antioxidative activity of compounds III, in comparison with corresponding compounds of series I and II (see Table I) – in other words they have the same alkyl chain – is probably the result of the presence of the polar hydroxyl group that may influence molecule incorporation and the fact that these compounds have only one scavenger group while all other compounds have two *N*-oxide groups each (see Fig. 1).

The antioxidative activity of NTA was also verified in chromogen experiments where suppression of the bluegreen chromogen radical $ABTS^{+\cdot}$ by *N*-oxides was studied. The results obtained showed that it was following qualitatively the results of antioxidative activity found in RBC experiments. However, the antioxidative efficiency of compounds determined in these experiments was weaker.

The general conclusion evolving from these presented studies is that some synthesized *N*-oxides of tertiary amines may be used as antioxidants in biological systems.

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