Biological Activity of New *N*-Oxides of Tertiary Amines

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Potential biological properties of newly synthesized single and double alkyl chain *N*-oxides of tertiary amines (NTA) were studied. Individual compounds in each of the series had alkyl chains of different length. Various experiments were performed to determine a mechanism of the interaction between NTA and model and biological membranes. These were measurements of hemolytic efficiencies of NTA (pig erythrocytes), their influence on the transition temperatures (DPPC liposomes), on potassium leakage from cucumber, its growth and chlorophyll content (*Cucumis sativus* cv. Krak F1), and on the resting membrane potential in alga cells (*Nitellopsis obtusa*).

Also, prevention of erythrocyte membrane lipid oxidation induced by UV irradiation was studied. Potential antioxidative properties of NTA were additionally tested in radical chromogen ($ABTS^{\bullet+}$) experiments in which antioxidative efficiencies of NTA were compared to that of the standard antioxidant Trolox.

It was found that NTA readily interacted with erythrocyte membranes. Their hemolyzing efficiency increased with the alkyl chain length. Slightly more intensive interaction was found for double alkyl chain compounds. Similar results were obtained in DSC experiments, where incorporation of NTA into liposomal membranes shifted the main transition temperatures and caused a broadening of the main transition peaks depending on the alkyl chain length. Double alkyl chain compounds were also found more efficiently influencing the growth of cucumber. Influence of NTA on the resting membrane potential of algae cells was not quite following the alkyl chain length rule found in erythrocyte and liposome experiments. Also potassium leakage and chlorophyll content determined in physiological experiments were not following the increase of lipophilicity of compounds. Most efficiently influencing those parameters were NTA having shorter alkyl chains, and efficiencies of single alkyl chain compounds were evidently stronger.

Both methods used to test the antioxidative properties of NTA showed that they depended on the alkyl chain lengths of compounds within each series, but double alkyl chain ones exhibited markedly greater efficiency.

Key words: *N*-Oxides, Membrane Activity, Antioxidative Efficiency

Introduction

Our preliminary studies concerned the influence of *N*-oxides of tertiary amines (NTA) on model and biological membranes (liposomes, erythrocytes, cucumber and algae cells). This was done by measuring the hemolytic efficacy of particular compounds and their potency in influencing the resting membrane potential of *Nitellopsis obtusa* and phase transition in multilayer liposomes formed from DPPC (1,2-dipalmitoyl-3-*sn*-phosphatidylcholine). Also, their influence on the growth of cucumber (*Cucumis sativus* cv. Krak F1), chlorophyll content and potassium leakage was measured. Such measurements were shown earlier to be useful when studying the interaction mechanism between various bioactive compounds, including antioxidants, and model and biological membranes (Kleszczyńska *et al.,* 2002, 2003, 2005; Bielecki *et al*., 2003).

Antioxidative efficiencies of *N*-oxides were determined by measuring their prevention of erythrocyte ghost membranes lipid peroxidation induced by UV irradiation. The results obtained

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were then compared to the radical chromogen experiments where the same compounds reduced the bluegreen $ABTS^{\bullet+}$ [ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid] to a colorless form. In turn, the chromogen experiments were compared to $ABTS^{\bullet+}$ reduction by Trolox, a water-soluble analogue of vitamin E known for its antioxidant activity (Niki *et al.*, 1986).

Materials and Methods

N-Oxides of tertiary amines

All *N*-oxides of tertiary amines (NTA) were synthesized in the Institute of Organic and Polymer Chemistry, University of Technology, Wrocław, Poland. Their structures are presented in Fig. 1.

Hemolytic measurements

Fresh, heparinized pig blood and 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 $NaH_2PO_4 \cdot H_2O$) were used in the experiments. Upon removal from the plasma, the erythrocytes were washed four times in a phosphate buffer and then incubated in another buffer solution containing proper amounts of the NTA at 37 °C for 0.5 h. Each sample containing 10 ml of erythrocyte suspension of 2% hematocrit was stirred continuously. After modification a number of 1 ml samples was taken, centrifuged and the supernatant assayed for hemoglobin content using a spectrophotometer (Spekol 11, Carl Zeiss, Jena) at 540 nm wavelength. Hemoglobin content in the supernatant, expressed as the percentage of hemoglobin concentration in the supernatant of totally hemolyzed cells, was assumed as the measure of the extent of hemolysis.

Oxidation studies

The red cell ghosts were used in measurements of the antioxidative efficiency of NTA. They were prepared as described by Dodge *et al.* (1963) from fresh heparinized pig blood and suspended in a phosphate solution of pH 7.4 at a protein concentration of ca. 1 mg/ml, some of them with and some without (control) addition of chosen amounts of NTA. Lipid peroxidation in the ghost erythrocyte membrane was induced by UV radiation (bactericidal lamp intensity was 3.5 mW/cm^2). The degree of lipid peroxidation was determined by measuring the concentration of malonic dialdehyde (MDA) released in the samples by using its colour reaction with thiobarbituric acid. Supernatant absorption was determined spectrophotometrically at 532 nm; increased absorption indicated increased lipid peroxidation.

Chromogen radical experiments

The standard TEAC (trolox equivalent antioxidant capacity) assay described earlier (Re *et al.*, 1999; Van den Berg *et al.,* 1999) has been used. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical ($ABTS^{\bullet+}$) in 20 min. The bluegreen $ABTS^{\bullet+}$ was produced through the reaction between 7 mM ABTS with 2.45 mM potassium persulfate in water. The concentrated $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS), pH 7.4, to a final absorbance of 0.90 at 414 nm. Stock solutions of Trolox were prepared in PBS. 20 μl of NTA solution were added to 980 μl $ABTS^{\bullet+}$ solution and the absorbance at 414 nm was measured. This was compared to a blank where 20 μl of solvent were added to 980 μl of the $ABTS^{\bullet+}$ solution of which the reduction in absorbance 20 min after addition of NTA was determined. The TEAC of the antioxidant was calculated as the concentration (mM) of Trolox showing an antioxidative potential equivalent to 1 M of the tested compound. The results obtained in these experiments are a direct indication of compound's antioxidative activity.

Differential scanning calorimetry (DSC) measurements

Multilamellar vesicles (MLVs) for DSC were prepared from DPPC purchased from Sigma Aldrich (control) and with addition of appropriate amounts of NTA dissolved in chloroform. The mixture was evaporated to form a thin film on the flask wall. Traces of chloroform were removed with a stream of dry nitrogen. Then distilled water was added and the flask was heated to 60 °C in a water bath. The lipid film was dispersed by agitating the flask on a vortex mixer to give a milky suspension of liposomes. The final lipid concentration was 25 mg/ml. This suspension was loaded into the sample cell of a DSC microcalorimeter of the Mettler Toledo Thermal Analysis System D.S.C. 821^e. Employed scan rates were 2 °C/min, and incubation performed at 4 °C lasted 3 d.

Electrophysiological experiments

Fresh internodal cells of *Nitellopsis obtusa* alga were used in electrophysiological experiments. A standard technique described earlier was applied (Trela *et al*., 2001). Single internodal cells (0.4 mm diameter, 28 mm length, mean dimensions) were incubated for 24 h in the darkness in artificial pond water (APW). The control solution of pH 7.0 contained 1 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$. The incubation solutions contained 0.05 mM of particular NTA. The membrane potential (the potential difference between the vacuole and the external medium) was measured in a routine way using a pair of Ag/AgCl microelectrodes filled with 3 M KCl; the potential microelectrode was inserted in the vacuole and the reference microelectrode was placed in the external solution. All experiments were performed in the darkness at 24 °C. The analog measurement signals were converted into digital signals using an A/D conversion card.

Physiological experiments

Cucumber (*Cucumis sativus* cv. Krak F1) was grown under constant fluorescent white light of 150 μmol m^{-2} s^{-1}. Cotyledons from 7-d-old seedlings were used for experiments. Discs of 10 mm diameter were cut avoiding the midrib. They were rinsed in double distilled water and floated 24 h under constant light on 1 mM compound solution. Potassium release was measured by an atomic absorption spectrophotometer SpectrAA-200 (Varian) with a hollow-cathode lamp (Navari-Izzo *et al.,* 1989).

Chlorophyll in cotyledon discs was extracted using 80% acetone (Lichtenthaler, 1987).

The growth tests with individual compounds were conducted in a SANYO® growth chamber with 9 h : 15 h light:dark cycle at 25 °C. Concentrations of 1 mM of each compound were used. Seeds were germinated at 25 °C for 2 d in the darkness. Fifteen uniform seedlings were transferred to Petri dishes with 2 discs of Whatman No 2 filter paper wetted with distilled water (control) or solutions of the tested compounds. The length of cucumber hypocotyls were measured after 72 h.

Results and Discussion

The results of hemolytic, oxidation and physiological studies are given in Table I. Hemolytic efficiency of *N*-oxides, being the measure of their interaction with erythrocyte membranes, increased with the length of their alkyl chains. This effect was observed for both series studied and agrees with the results of several earlier studies on the interaction of different compounds with model membranes (Kleszczyńska *et al.,* 2002, 2003, 2005). It seems that E_2-*n*(O) compounds were slightly more efficient in hemolyzing erythrocytes than DMAL-*n*(O) compounds.

The results of physiological experiments were not so clear. The longest alkyl chain compounds in both series had little influence on potassium leakage from cucumber cells and on chlorophyll content. Such results are evidence of small membrane damage caused by those compounds at the concentration used. It may also be an evidence of the cut-off phenomenon, *i. e.*, the loss of biological activity by compounds belonging to homologous series when their alkyl chain is too long (Sarapuk and Kubica, 1998). No big differences between the influence of particular compounds in series on the

Table I. Concentrations of NTA inducing 50% hemolysis of erythrocytes (C_{50}), causing 50% inhibition of peroxidation of erythrocyte membrane lipids (I_{50}), molar equivalent of Trolox inducing the same effect as NTA (1 M of NTA is equivalent to X mM of Trolox), relative chlorophyll content in cucumber cotyledon discs, potassium content in effusate, and relative hypocotyls length of cucumber. Standard deviations for C_{50} and I_{50} did not exceed 8%.

Compound	C_{50} [mM]	I_{50} [mM]	X [mM]	Chlorophyll content (% control)	Potassium content in effusate (ppm)	Hypocotyl length (% control)
DMAL-10(O)	0.60	1.20	4.14 ± 0.33	53.2	44.1 ± 2.6	88.4
DMAL-12(O)	0.40	0.70	9.18 ± 0.66	79.1	44.8 ± 2.8	79.5
DMAL-14(O)	0.35	0.50	9.83 ± 0.74	106	5.7 ± 0.46	92.3
DMAL-16(O)	0.30	0.45	8.43 ± 0.67	108	2.8 ± 0.24	94.1
E_2-11(O)	0.43	0.05	49.2 ± 2.7	56.1	28 ± 1.7	73.8
E_2-13(O)	0.25	0.07	31.3 ± 1.9	90.4	9.7 ± 0.65	76.8

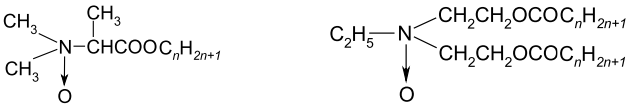

Fig. 1. General structures of *N*-oxides of tertiary amines.

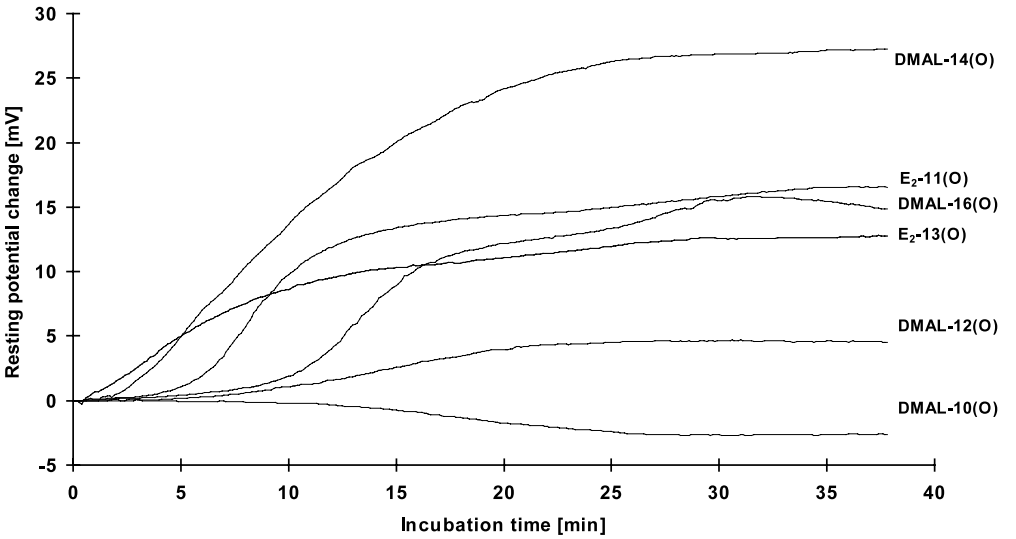

Fig. 2. Changes in the resting potential (actual potential minus the control potential) of *Nitellopsis obtusa* during incubation in the presence of NTA. Standard deviation did not exceed 10%.

growth of cucumber hypocotyles were found. However, double alkyl chain compounds were more efficiently inhibiting that growth.

The longest alkyl chain compounds in both series, DMAL-16(O) and E_2-13(O) (Fig. 1), had a weaker influence on the resting potential of alga membrane than the shorter alkyl chains DMAL-14(O) and E_2-11(O) as shown in Fig. 2. However, the shorter alkyl chain DMALs influenced the resting potential of alga membrane evidently weaker than other compounds. It can also be seen that any induced changes in the resting potential by E_2-*n*(O) compounds were greater than these induced by DMAL-*n*(O) oxides of similar *n* indices.

All these results taken together indicate that a lipophilicity of compound in its interaction with membranes is not the sole factor deciding about its capacity to change the measured parameters. Apparently, compound's greater dimensions (compare structures of NTA presented in Fig. 1) that do not facilitate its incorporation into the lipid phase of model membrane studied can be fully compensated by compound's lipophilicity as observed during the performed studies.

The dependence of membrane modifying efficiencies of NTA on the alkyl chain length was also found during DSC experiments. They showed that these efficiencies changed with compound and its concentration (Fig. 3); the measure being a shift of the main phase transition temperature (T_m). However, the changes observed were different. The short alkyl chain compounds [DMAL-10(O) and E_2-11(O)] slightly decreased or had no influence on T_m [DMAL-12(O)], while the long alkyl chain ones shifted T_m towards higher temperatures (Fig. 3A). That last effect evidences an increase of membrane rigidity. Also, all NTA caused a change of the half width of the main transition peak ($T_{1/2}$) and again, the strongest changes were observed for long alkyl chain compounds. They significantly decreased the main transition cooperativity (Fig. 3B)

The results described above and concerning the interaction of *N*-oxides with model membranes are, generally, in good agreement with those ob-

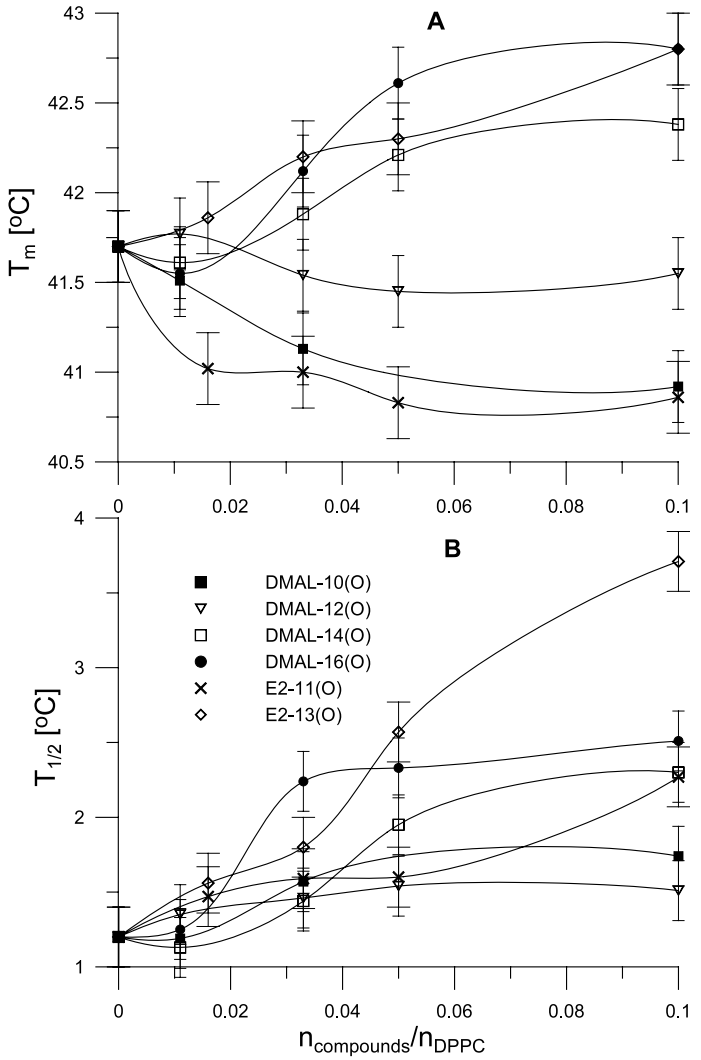

Fig. 3. The main phase transition temperatures (A) and the half width of the main temperature peaks (B) as a function of molar ratio of *N*-oxides to DPPC.

tained in tests on the antioxidative properties of NTA. This is especially well seen for the DMAL-*n*(O) series, where concentrations of compounds inhibiting membrane lipid peroxidation (I_{50}) decrease with the alkyl chain length. The effect is not so clear for the chromogen experiments but the most important is that antioxidative properties of E_2-*n*(O) compounds are significantly greater than those of the DMAL series as revealed in both types of the antioxidative experiments performed.

The results obtained indicate that potential biological possibilities of *N*-oxides studied are moderate and depend, among other things, on the combination of structural features of a compound and its lipophilicity.

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