Protective Effect of Amphiphilic Ammonium Salts on the Oxidation of Lecithin Liposomes

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Z. Naturforsch. **50 c,** 840–844 (1995); received August 2/September 21, 1995

Antioxidants, Peroxidation of Phospholipid Membrane, Liposomes, Erythrocyte Membrane, Hemolytic Activity

The inhibition effect of selected amphiphilic quaternary alkyl-substituted ammonium salts on oxidation of the lecithin liposome membrane was studied, induced by ultraviolet light and Fe^{2+} ions with ascorbic acid added. The salts differed in alkyl chain length Ri having the following alkyl substitutents: R1 = C_9H_{19}; R2 = $C_{10}H_{21}$; R3 = $C_{12}H_{25}$; R4 = $CH_2OC_{14}H_{29}$; R5 = $CH_2OC_{16}H_{33}$.

It was found that all the salts used induced inhibition in oxidation of the liposome membrane, both that induced by UV light and ferrous ions. The antioxidant activity of the salts studied, dependent on the chain length and concentration, can be represented by the relation R1 < R2< R3 < R4 < R5. A similar dependence was obtained when the hemolytic activity of compounds was studied. Activity of salts increase with alkyl chain length.

Introduction

Lipid membrane oxidation is a process that in many cases results in deterioration of membrane properties (Nakazawa and Nagatsuka, 1980; Konings, 1984). Earlier studies have shown that quaternary ammonium salts bind to membrane and change the properties of the membrane (Przestalski *et al.,* 1991; Kubica and Kuczera, 1992; Gabrielska *et al.,* 1993), e.g., the transport properties of the lecithin liposome membrane (Kuczera *et al.,* 1989). Such salts, on the other hand, are to protect membranes against changes induced by the oxidation (Gabrielska *et al.,*1993). The salts studied in the present work constitute a new class of compounds that combine surface activity with antioxidant activity and can thus play a double role. The present work is concerned with the antioxidant properties of new bifunctional ammonium salts in their interaction with the lecithin liposome membrane, whose oxidation was induced by ultraviolet radiation and the Fe^{2+}- ascorbic acid complex.

The aim of the work was to determine the concentration range that insures activity of the compounds as free radical scavengers but does not destruct the liposomal membrane and to explaine the role of the alkyl chain in performing that function.

Materials and Methods

Preparation of ammonium salts

The bifunctional ammonium chlorides, of general formula shown in Fig. 1, were synthesized in the Institute of Organic and Polymer Technology, Technical University, Wrocław, Poland. The ammonium bromides containing an unbranched alkyl chain (R1 = C_9H_{19} , R2 = $C_{10}H_{21}$, R3 = $C_{12}H_{25}$) were obtained in a reaction conducted under diethyl ether using equimolar quantities of the reagents (4-hydroxy-3,5-di-*t*-butylbenzene bromide and tertiary amine dissolved in acetone). Details

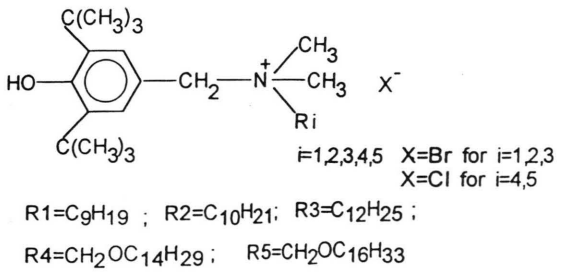

Fig. 1. Structural formulae of compounds studied.

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on the synthesis of ammonium bromides were given in application of patent (Witek *et al.,* 1994). The synthesis of ammonium chlorides, containing the alkoxymethyl chain R4 = $CH_2OC_{14}H_{29}$ and R5 = $CH_2OC_{16}H_{33}$ was performed starting with 2,6-di(*t*-butyl)-4-(N,N-dimethylaminomethyl) phenol in the reaction with R-O-CH_2Cl under ethyl diether. Details on the preparative procedure were given in application of patent (Witek *et al.,* 1994). Melting temperatures and yields of synthesis of the studied compounds having the following alkyl substituents R1, R2, R3, R4 and R5 were: 153.0–155.0°C, 119.0–120.0°C, 125.0–126.5°C, 74.0–76.0°C, 78.0–80.0°C and 67.0%, 54.0%, 86.0%, 63,5% and 68.0%, respectively. Selected ^{1}H-NMR spectra, that confirm the structure of ammonium bromide with the alkyl chain R3 = $C_{12}H_{25}$, and of ammonium bromide with the alkyl chain R4 = $CH_2OC_{14}H_{29}$ had the following signals (80 MHz, TMS, $CDCl_3$, δ ppm): 0.88 (3H, t, C**H**$_3$-alkyl); 1.25 (22H, s, (C**H**$_2$)$_{11}$); 1.44 (18H, s, *t*-Bu); 3.34 (6H, s, N(C**H**$_3$)$_2$); 4.75 (2H, s, Ar-C**H**$_2$-N); 5.53 (1H, s, **OH**); 7.31 (2H, s, C_6**H**$_2$) and 0.88 (3H, t, C**H**$_3$-alkyl); 1.26 (26H, s, (C**H**$_2$)$_{13}$; 1.45 (18H, s, *t*-Bu); 3.30 (6H, s, N(C**H**$_3$)$_2$); 4.14 (2H, s, Ar-C**H**$_2$-N); 4.94 (2H, s, N-C**H**$_2$-O); 7.33 (2H, s,C_6**H**$_2$), respectively.

Multilamellar liposome preparation and induction of the peroxidation

Chloroform solution of egg yolk lecithin, prepared in our laboratory by a method describes by Singleton (Singleton *et al.,* 1965), was evaporated under vaccum in nitrogen atmosphere. The thin film of lecithin obtained was shaken with 0.05 M Tris-HCl buffer (hydroxymethyl) aminomethane at pH 7,4 during half an hour (control liposome) and then together with the antioxidant during the next half hour. The liposome suspension contained 4.8; 3.0 and 1.5 mg phosphatidylcholine (PC) per ml in the case of the first experiment and 3.0 mg PC per ml in the remaining experiments. The concentration of the antioxidants studied changed in the range 0.005–0.2 mM in the case of R1, R2 and R3 compounds and in the range 0.005–0.05 mM for R4 and R5 compounds. Lipid peroxidation in the phospholipid liposomes was induced by: a) ultraviolet radiation – bactericidal lamp intensity 2.5 mW/cm^2; b) .0.15 mM Fe^{2+} and 1.0 mM ascorbic acid in 0.05 M Tris-HCl buffer at pH 7.4. The accumulation of phospholipid peroxidation products was estimated by the determination of 2-thiobarbituric acid (TBA)-reactive products in the incubation medium (Buege and Aust, 1978; Porębska-Budny *et al.*,1992), and expressed as the increase of absorbance at 535 nm (ΔA 535).

Hemolytic experiments

Fresh heparinized pig erythrocytes were used in experiments. The cells were washed with an isotonic phosphate solution of pH 7.4. Then the erythrocytes were modified for 0.5 h in the same solution at 37°C in the presence of various concentrations of the compounds studied and various concentrations of erythrocytes. The percent of hemolysis was measured spectrophotometricaly at 540 nm wavelength. The measure of hemolytic activity of the compound studied was the maximum concentration that do not cause the erythrocyte hemolysis. So, a compound is more active hemo-

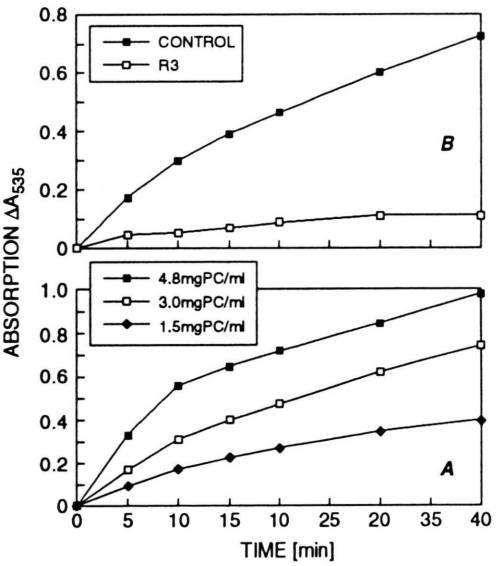

Fig. 2. A. Degree of lecithin liposome oxidation (as the increse of absorbane at 535 nm] versus time of irradiation. Egg yolk phosphatidylochline (PC) concentrations were 4.8 mg/ml, 3.0 mg/ml and 1.5 mg/ml. UV intensity 2.5 mW/cm^2.Data are the means of three experiments on different liposome preparation. B. Inhibition of oxidation of egg yolk phosphatidylocholine liposomes by antioxidant with R3 alkyl chain (R3 = $C_{12}H_{25}$) at concentration 0.2 mM. Oxidation was induced by UV radiation at intensity 2.5 mW/cm^2, concentration of egg yolk lecithin was 3.0 mg/ml. Data are the means of three experiments on different liposome preparation.

litically if its concentration is lower at which there is no hemolysis.

Results

The effect of ultraviolet-induced phospholipid peroxidation on the concentration of phosphatidylcholine is shown in Fig. 2A. This figure describes the influence of various concentrations of PC on the TBA-reactive accumulation products. Fig. 2B shows the influence of a selected antioxidant with alkyl chain R3 = $C_{12}H_{25}$ on absorbance at 535 nm, as compared with the curve without the antioxidant added. The effect of various antioxidants on peroxidation induced by UV light is shown in Fig. 3A and B. Fig. 3A demonstrates the influence of various concentrations of all studied compounds on the increase of absorbance at 535 nm, i.e. on the decrease of oxidation of the liposome membrane. Percent of inhibition of liposome peroxidation caused by the antioxidant studied versus concentration was shown in Fig.3B. On the basis of the plot in Fig. 3B, compound concentration causing 50% inhibition of lipid peroxidation were determined , and shown in Table I. The results of studies on the Fe^{2+}-ascorbic acid-induce lipid peroxidation are shown in Table II.

The results of hemolytic studies are presented in Table III. This Table contains maximum values of concentrations of the compounds studied that do not cause hemolysis of erythrocytes. They are calculated for four hematocrites of erythrocytes, i. e. 2,4,6 and 8%. The results obtained show that the hemolytic activity of the compounds studied increases with increasing alkyl chain length and is in close relationship with the antioxidant activity of the compounds.

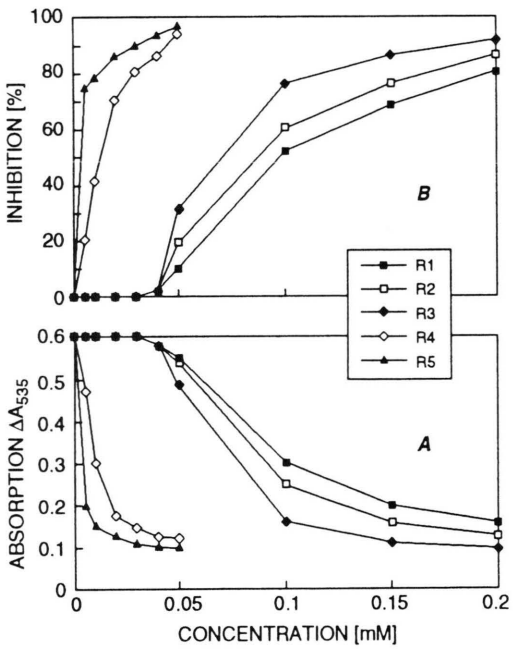

Fig. 3. A. Degree of lecithin liposome oxidation induce by UV radiation versus antioxidant concentration with different alkyl chains: R1 = C_9H_{19}; R2 = $C_{10}H_{21}$; R3 = $C_{12}H_{25}$; R4 = $CH_2OC_{14}H_{29}$ and R5 = $CH_2OC_{16}H_{33}$. Radiation intensity and concentration of egg yolk lecithin the same as in Fig. 2B. Data are the means of three experiments on different liposome preparation. B. Percent inhibition of lecithin liposome oxidation versus antioxidant concentration with different alkyl chains: R1 = C_9H_{19} ; R2 = $C_{10}H_{21}$; R3 = $C_{12}H_{25}$; R4 = $CH_2OC_{14}H_{29}$ and R5 = $CH_2OC_{16}H_{33}$. Radiation intensity and concentration of egg yolk lecithin the same as in Fig. 2.

Table I. Antioxidant (with different alkyl chain lengths Ri, where i = 1, 2, 3, 4 and 5) concentration that cause 50% inhibition of lecithin liposome oxidation. Liposomes were made in 0.05 M Tris-HCl buffer at pH 7.4 from egg yolk phosphatidylcholine at concentration 3.0 mg/ml. Lipid oxidation in liposomes was induced by UV radiation at intensity 2.5 mW/cm^2.

Antioxidant R	Antioxidant concentration that causes 50% inhibition of liposome oxidation in [μM]
$-C_9H_{19}$	96
$-C_{10}H_{21}$	86
$-C_{12}H_{25}$	70
$-CH_2OC_{14}H_{29}$	13
$-CH_2OC_{16}H_{33}$	4

Table II. Percent inhibition of lecithin liposome oxidation induced by Fe^{2+}-ascorbic acid in the presence of various antioxidants with different alkyl chain lengths Ri (where i = 1, 2, 3, 4 and 5) at different concentrations of antioxidant. Concentration of lecithin was 3.0 mg/ml. Lipid oxidation in liposomes was induced by 150 μM Fe^{2+} and 1.0 mM ascorbic acid in 0.05 M Tris-HCl buffer at pH 7.4.

Antioxidant R	% of inhibition of Fe^{2+}-ascorbic acid induced peroxidation in the presence of various antioxidant concentrations in [mM]					
	0.005	0.01	0.02	0.05	0.10	0.20
$-C_9H_{19}$	–	–	–	10	45	60
$-C_{10}H_{21}$	–	–	–	15	50	70
$-C_{12}H_{25}$	–	–	–	28	60	80
$-CH_2OC_{14}H_{29}$	7	38	49	95	95	–
$-CH_2OC_{16}H_{33}$	23	50	53	96	96	–

Table III. Maximum nonhemolytic concentrations of the compounds studied at different red blood cels hematocrite.

Antioxidant R	Concentration [mM] Hematocrit 2%	4%	6%	8%
$-C_9H_{19}$	1.100	1.400	1.8	2.000
$-C_{10}H_{21}$	1.000	1.200	1.400	1.600
$-C_{12}H_{25}$	0.140	1.600	0.180	0.200
$-CH_2OC_{14}H_{29}$	0.070	0.080	0.100	0.140
$-CH_2OC_{16}H_{33}$	0.025	0.035	0.040	0.060

Discussion

On the basis of the results shown in Fig. 2A it can be stated that the degree of lipid liposome peroxidation induced by UV light depends on phospholipid concentration . For this reason, in all subsequent investigation the lecithin concentration was assumed constant and equal to 3.0 mg/ml, as well as constant UV light intensity of 2.5 mW/cm^2. The results of subsequent experiments performed in the presence of the antioxidants indicate that the compounds cause an inhibition of the peroxidation process of the lecithin liposome membrane, and that the degree of inhibition depends on the concentration of the antioxidants used (Fig. 3A) and on the alkyl chain length in the radical Ri (Table I). Comparison of the results on inhibition of liposome peroxidation induced by UV light obtained with all the antioxidants studied allows to conclude that the degree of the inhibition increases with increasing alkyl chain length Ri. Thus, the concentration of the antioxidant used that causes 50% inhibition decreases with increasing alkyl chain length. This can be seen in Table I as well as easily follows from the results in Table II.

Analysis of the plot in Fig. 3 and data in Table I and Table II allows to conclude that the antioxidant activity (proportional to % inhibition) of the compounds with longer alkyl chains, i.e. R4 and R5, is much greater than that of compounds with shorter alkyl chains, i.e. R1, R2 and R3 . Concerning the alkyl chain length it seems correct to assume in comparative calculations (Różycka-Roszak *et.al.,* 1988), that the presence of the $-CH_2O$ group in the structure of the radicals R4 and R5 makes them longer by the equivalent length of two methylene groups. Thus the length of alkyl chains of the radicals R4 and R5 can be compared with the length of the radicals: R = $C_{16}H_{33}$ and R = $C_{18}H_{37}$, respectively. It seems likely that long alkyl chain length allows the antioxidant molecules to incorporate better (deeper) in the liposome membrane. Then the protruding hydroxyl group can be more effective as free radical scavengers and thus protect the membrane against lipid peroxidation. It must be taken in to account that antioxidant activity of the compounds possessing an alkyl chain Ri (of various length) can be the result of different values of partition coefficient of the compounds (Filipek *et al.,* 1992) which, in turn, can be connected with different molecular shapes of the compounds (Subczyński *et al.,* 1988).

The hemolytic experiments and the obtained maximum nonhemolytic concentrations of the compunds studied are the basis for determining the optimal range of concentrations where these compounds are effective antioxidants and do not destroy erythrocyte membrane and, of course, liposomal ones. Concentrations of the compounds used in the studies of inhibition of oxidation of the liposome membrane are significantly lower (for instance from 2-fold to 11-fold for 2% hematocrit) than the maximum nonhemolytic concentration. This allows to assume that they are quite safe and do not disrupt the liposome membrane.

Acknowledgements

This work was sponsored by the Polish Research Committee (KBN), grant no. 4 1746 91 01p/01.

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