Antioxidant Protection of Egg Lecithin Liposomes during Sonication

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Z. Naturforsch. **50c,** 561–564 (1995); received March 10/May 11, 1995

Lecithin, Sonication, Liposomes, Lipid Hydroperoxides, Antioxidants

When model membranes are prepared by ultrasonic treatment of polyunsaturated phospholipids, radical production can induce a partial degradation of the polyunsaturated fatty acyl chains and the formation of lipid hydroperoxides. A suitable antioxidant employed during liposome preparation is able to protect them against lipid peroxidation. This work contains the results of studies on egg lecithin liposomes with incorporated antioxidants that were supposed to play the protective role mentioned. As it has been shown the antioxidant compounds used ensured a 40–60%, i.e., satisfactory protection of liposomes after 30 min sonication. Possible practical applications are discussed.

Introduction

Since the invention of lipid model membranes (BLM and liposomes) they have been used extensively for studying various properties of biological membranes they were imitating. From several thousands of articles quite a number was devoted to the use of liposomes as model system for the study of lipid peroxidation (Konings, 1984; Chatterjee and Agarwal, 1988). However, the most common method used for the preparation of liposomes is ultrasonic treatment of a lipid dispersion in water solution with a probe or in a bath, which can lead to unwanted results as the ultrasonic waves are known to initiate redox reactions in aqueous solutions. These reactions are often similar to those produced by ionizing radiation (Jana *et al.,* 1986). One of the reactive species formed by water sonolysis is the hydroxyl radical, an efficient indicator of lipid peroxidation (O'Connel and Garner, 1983). Thus, when model membranes are prepared by ultrasonic irradiation of phospholipids with polyunsaturated fatty acid residues like egg lecithin, radical production can induce the essential stages in the oxidation including bond migration to give a conjugated diene, followed by hydroperoxide formation (Holman *et al.*, 1954). The peroxidation products affect the fluidity, permeability and stability behaviour of the vesicles (Smolen and Shohet, 1974; Dobretsov *et al.*, 1977; Konings, 1984). To prevent peroxidation and the resulting changes in lipid membrane properties one can add natural or synthetic antioxidants during the preparation stage of liposomes (Barley *et al.*, 1989; Thomas *et al.*, 1992).

In this work we have investigated the effect of sonolysis on egg lecithin vesicles with new bifunctional antioxidants incorporated in them and show that all of them can lower the extent of peroxidation. The method used for determining the extent of sample oxidation was based on measurements of absorption change due to the production of conjugated dienes (Pryor and Castle, 1984, Slater, 1984).

Materials and Methods

Egg lecithin (PC) used in the studies was prepared in our laboratory by the method described by Singleton (Singleton *et al.*, 1965). All the antioxidants studied, presented in Fig. 1, were synthesized in the Institute of Organic and Polymer Technology, Technical University, Wrocław, Poland.

Stock solutions of PC in chloroform stored at -20 °C were dried under nitrogen before use. The dried mixture was dispersed in double distilled water with the studied antioxidants added. The final concentration of PC was 4 mg per 1 ml. The concentration of antioxidants was 25 μM in one series of experiments and 25 μM, 50 μM or 100 μM in other experiments. The suspension was then sonicated for different time spans up to 30 min

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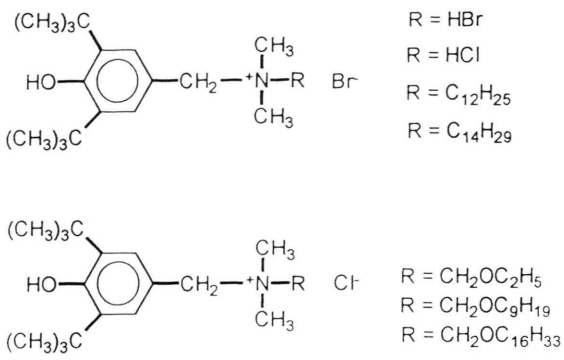

Fig. 1. Chemical formula of the compounds studied.

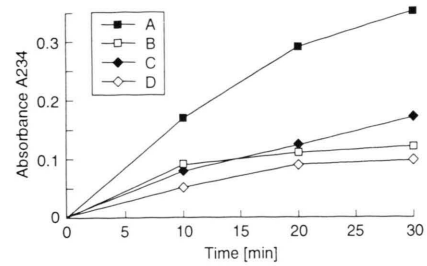

Fig. 2. Conjugated dienes produced in PC liposomes as a function of sonication time. Egg lecithin content, in double distilled water, was 4 mg/ml; A – control (no antioxidant present during the preparation of liposomes), B, C and D – BHT (butylated hydroxytoluene) and antioxidants containing -HCl and -HBr groups present during the preparation of liposomes at 10 μM, respectively.
Data are the means of three to five experiments on different liposome preparations.

with 20 kHz sonicator with a titanium probe. In a typical run, 12 ml of aqueous dispersion were pipetted in a tube (volume 30 ml). The irradiation vessel, thermostated at (0–2) °C, was fitted at constant position and the probe tip was held at constant position in the dispersion. Sonication was carried out intermittently for 30 s, followed by a 30 s resting period. The sonicated samples were centrifugated to remove any probe particles for 5 min at 690 g. After different irradiation times the absorption spectra of conjugated dienes were recorded in the 315–215 nm wavelegth range by a UV-VIS spectrophotometer (Zeiss Jena). The increase of absorption at 234 nm was an indication of the appearance of conjugated dienes, the absorption at 300 nm being taken as zero.

Planar lipid membranes (BLM) were formed from a solution of 1.5% (w/v) azolectin in n-butanol:n-decane (1:1). Antioxidants were added directly into two-compartmental chamber filled with physiological solution until their concentration reached a value which caused a breakdown of membranes in a time shorter than 5 min. Such concentrations are farther referred to as critical concentrations (CC).

Results and Discussion

The effect of sonolysis on the chemical structure of egg lecithin was previously reported by many authors (Hauser, 1971; Konings, 1984). It was shown that even nitrogen atmosphere did not prevented liposomes from some peroxidation (Konings, 1984). On the other hand, a suitable buffer employed during liposome formation is able to protect them partially against peroxidation (Fiorentini *et al.*, 1989). However, the best way to protect liposomes from peroxidation seems to be the use of antioxidants.

In this work we studied the lipid peroxidation in liposomes formed in the presence of some bifunctional antioxidants. They were synthesized as amphiphilic molecules having both a hydrophobic tail and a polar head. The hydrocarbon alkyl chain was thought to be a kind of "anchor" enabling the molecule to incorporate into the bilayer structure of liposome. An antioxidant functional group of a molecule localized in its polar head and protruding from bilayer should act as a free radical scavenger, thus limiting the possibility of initiating a chain of reactions leading to lipid peroxidation.

The effect of sonolysis on aqueous dispersion of egg lecithin (4mg/ml) in the absence of antioxidants is presented by curve A in Fig. 2. It is evident that production of conjugated dienes takes place during sonication and increases with time. By comparison, curves B, C and D present production of conjugated dienes under the same experimental conditions (constant ultrasonic intensity, sonication time etc.) but in the presence of butylated hydroxytoluene, one of very well known antioxidants and two of the compounds studied, respectively. It can be seen that all used antioxidants are significantly blocking the production of conjugated dienes. The amount of conjugated dienes decreased after 30 min of sonication to about one third of that observed for unprotected liposomes.

Table I. Effect of studied antioxidants on the production of conjugated dienes in liposomes after different time of sonication. Concentration was 50 μM. BHT – butylated hydroxytoluene used as the standard antioxidant.

Antioxidant R	A_{234} after different sonication time [min]		
	10	20	30
-HCl	0.08	0.12	0.17
-HBr	0.05	0.09	0.10
$-CH_2OC_2H_5$	0.10	0.13	0.16
$-CH_2OC_9H_{19}$	0.12	0.13	0.16
$-CH_2OC_{16}H_{33}$	0.08	0.12	0.14
$-C_{12}H_{25}$	0.09	0.12	0.13
$-C_{14}H_{29}$	0.05	0.08	0.09
BHT	0.09	0.11	0.12
Control	0.17	0.29	0.35

Similar results were obtained in the case of other studied antioxidants. They are tabularized in Table I., Table II contains the results of studies on concentration and time dependence of a chosen antioxidant and the time of its action on con-jugated dienes production. To be quite sure that the used concentrations of antioxidants do not initiate irreversible changes in liposomes (Gabrielska *et al.*, 1993) additional measurements on planar lipid membranes (BLM) were performed. Critical concentrations of the antioxidants studied were found as 4×10^{-4} M (R=HCl), 2×10^{-4} M (R=HBr), 7×10^{-3} M ($R=CH_2OC_2H_5$), $1{,}3\times10^{-4}$ M ($R=CH_2OC_{12}H_{25}$), 1.1×10^{-4} M ($R=CH_2OC_{14}H_{29}$), 5.5×10^{-5} M ($R=C_{12}H_{25}$) and 4.8×10^{-5} M ($R=C_{14}H_{29}$). Note that the highest used concentration of an antioxidant in liposome experiments was smaller than the highest one used in BLM experiments (see Table II). On the other hand, it is known from our earlier experiments (Gabrielska *et al.*, 1993) that to initiate the mentioned changes in liposomes one must use concentrations of compounds at least one order of magnitude higher than those referred to as critical ones in BLM experiments.

Table II. The concentration and time dependence of chosen antioxidant on the production of conjugated dienes in egg lecithin liposomes. BHT – butylated hydroxytoluene used as the standard antioxidant.

Antioxidant R	A_{234} after different sonication time [min] and different concentration of antioxidant [μM]			
	[min]	25μM	50μM	100μM
$-CH_2OC_{16}H_{33}$	10	0.08	0.08	0.09
	20	0.12	0.12	0.13
	30	0.13	0.14	0.14
BHT	10	0.05	0.09	0.09
	20	0.11	0.11	0.10
	30	0.12	0.13	0.13

The results obtained show that our bifunctional surfactants can be used succesfully as compounds that protect liposomes against the action of free radicals, especially during liposome formation by sonication, where those radicals appear. It must be underlined that almost all the compounds studied were found to protect liposomes better than butylated hydroxytoluene, which is well known for its antioxidative properties.

Acknowledgements

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

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