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INHIBITION OF LIPID PEROXIDATION OF LECITHIN LIPOSOMES KEPT IN A pH-STAT SYSTEM NEAR NEUTRAL pH

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During 5 days of autoxidation of egg lecithin liposomes in nonbuffered saline pH dropped from an initial value of 7.4 to 4.5. A linear relationship between oxidation index and pH was obtained. Lipid peroxidation, monitored as conjugated diene and TBA-reactive products, was inhibited significantly by keeping the samples under pH-controlled conditions (7.4 ± 0.5), compared to controls. Obtained results indicate that the buffering capacity of Tris and Hepes buffers may play a role in their recently reported (D. Fiorentini *et al.* (1989) *Free Radical Res. Commun.*, **6**, 243) inhibitory action against lipid peroxidation of lecithin liposomes.

KEY WORDS: Lipid peroxidation, buffers, pH, liposomes.

INTRODUCTION

Chemistry of chain reactions of lipid oxidation and inhibitory action of antioxidants have been studied in great detail.[1-4] Mechanisms of antioxidant action of amino and phenolic antioxidants seems to be well understood.[3,5]

Recently, Fiorentini *et al.*[6] reported inhibitory action of Tris and Hepes buffers against ultrasonically initiated peroxidation of egg lecithin liposomes. They attributed the observed effect to the ability of these compounds to scavenge hydroxyl radicals, which has been shown earlier.[7]

The aim of the present work was to examine whether the buffering capacity contributes to the inhibitory action of the buffers.

MATERIALS AND METHODS

Chemicals

Egg yolk phosphatidylcholine (lecithin, PC) was isolated according to Singleton.[8] The antioxidant (−)-cis-2,8 dimethyl-2,3,4,4a,5,9b,-hexahydro-1H-pyrido(4,3b) indole

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dihydrochloride (stobadine)[9,10] was obtained from the Institute of Organic Chemistry and Biochemistry Prague (Czechoslovakia), the antioxidant butylated hydroxytoluene (BHT) was from Sigma (USA), 2-thiobarbituric acid (TBA) was from Fluka AG (Switzerland). All other chemicals were from commercial sources of analytical grade.

Preparation of Liposomes

10 mg of PC was hydrated with 1 ml of saline (100 mM KCl) without buffer addition. Multilamellar liposomes were prepared by vortexing the suspension for 1 min. The antioxidants stobadine and BHT were added to selected samples up to the final lipid/drug molar ratio of 316/1. In all samples pH was adjusted to 7.4 by diluted KOH solution.

Lipid Peroxidation Assay

The samples were incubated in a 50°C water bath, and the pH was measured every 24 h. Simultaneously aliquots of 1 mg of lipids were taken and lipid peroxidation was measured. After 5 days of incubation pK value was determined in the controls.

pH-Stat Lipid Peroxidation

Suspensions of liposomes, prepared as described earlier, were incubated at 50°C, mechanically stirred. Addition of diluted KOH by a computer-controlled automatic burette (pH-stat system), maintained the pH at 7.4 $\pm$ 0.5. In controls, pH was not adjusted. Each hour pH values were measured in the control and in the pH-stat-sample. Aliquots of 1 mg of lipids were taken at desired time intervals and the extent of lipid peroxidation was established.

Measurement of Lipid Peroxidation

Lipid peroxidation was measured as conjugated diene formation and expressed as oxidation index.[11] In the pH-stat experiments TBA-reactive products were also measured.[12]

RESULTS

During autoxidation of liposomal suspension the oxidation index increased (Figure 1), and simultaneously decrease of pH was observed (Figure 2). The pH in the controls dropped from an initial value of 7.4 to 4.5 over 4 days of autoxidation, and further decrease was not observed during additional incubation. Decrease of pH correlated linearly with the oxidation index (Figure 3). Phenolic antioxidant BHT and amino antioxidant stobadine inhibited both conjugated diene formation (Figure 1) and pH decrease (Figure 2). The pH-oxidation index relationship in the presence of antioxidants was again linear, and almost identical with that obtained in controls (Figure 3).

Further, an effect of pH on the rate of lipid peroxidation was studied. Autoxidation ran under pH-controlled conditions in a pH-stat system (Figure 4A). Oxidation index increase as well as TBARP accumulation were significantly inhibited in the sample kept at pH 7.4 $\pm$ 0.5, as comapred to reference control (Figures 4B and C).

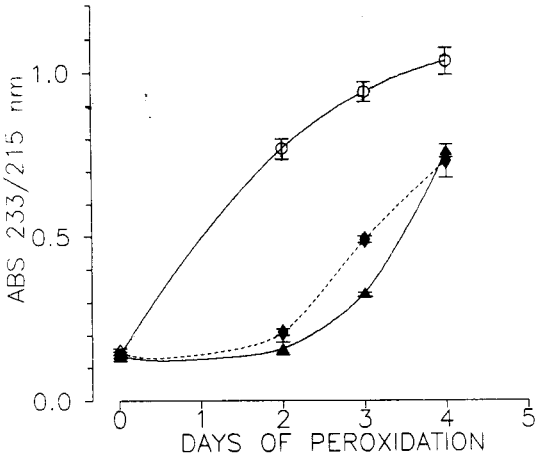

FIGURE 1 Time course of autoxidation of PC liposomes expressed as oxidation index (ABS 233/215 nm). Controls (circles), and samples in the presence of antioxidants: stobadine (diamonds), BHT (triangles).

By titration of the peroxidized control at the end of experiment, a pK value of 4.6 was obtained (not shown), a value typical for carboxyls.

DISCUSSION

The observed pH drop in the course of lipid peroxidation (Figure 2) is not unexpected, since formation of carboxylic acids in this process has been known for years.[3]

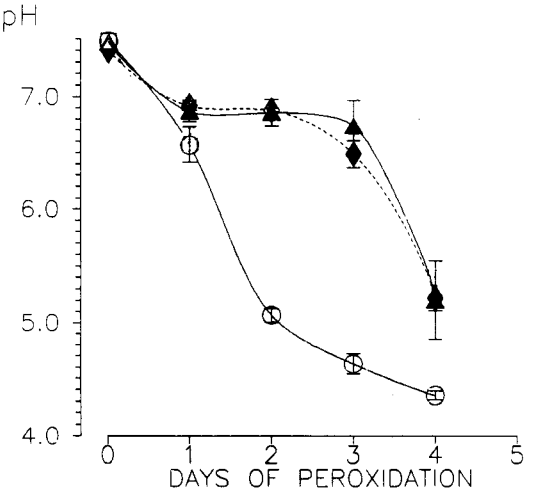

FIGURE 2 Changes in pH during autoxidation of PC liposomes. Controls (circles), and samples in the presence of antioxidants: stobadine (diamonds), BHT (triangles).

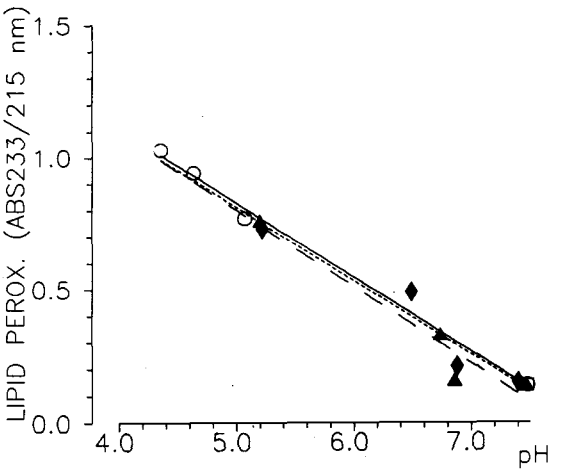

FIGURE 3 Relationship between peroxidation index and corresponding pH during autoxidation of liposomes. Controls (circles), and samples in the presence of antioxidants: stobadine (diamonds), BHT (triangles).

However the obtained linearity of the pH-oxidation index dependence is interesting (Figure 3). It makes possible in our system to determine the extent of lipid peroxidation by measurement of pH, as the same pH-oxidation index relationship was obtained when autoxidation was inhibited by antioxidants (Figure 3). The linear dependence between oxidation index and corresponding pH means that there is a nonlinear dependence between oxidation index and the concentration of free hydrogen ions. This indicates that a delay exists between the oxidation index increase (which in the early stages of peroxidation is proportional to the lipid hydroperoxide formation[14]) and the release of hydrogen ions. This time shift is probably caused by the delay between accumulation of relatively stable lipid hydroperoxides and their disproportionation to the secondary acidic compounds. Similarly, Deev *et al.*[15] reported generation of some negative charges at the surface of PC liposomes in the later phase of UV light-induced peroxidation. No negative charges were observed in the earlier stages of peroxidation. Similar to our results, titration of the charged products in their experiments revealed a pK value typical for carboxyls.

The main concern of this paper was to investigate the effect of pH on lipid peroxidation. We observed a significant inhibition of the rate of lipid peroxidation by maintaining the pH around neutral value. This result agrees with the results of Mabrouk and Dugan,[16] who reported an increase of oxidation rates of linoleic acid emulsions, with decrease of pH from 8.00 to 5.50. They attributed the observed effect to the participation of carboxyl groups in the decomposition of hydroperoxides (see [17, 18]). If true, then probably the protonated form of carboxyls is prooxidant, since linoleic acid was oxidized more rapid at acidic pH than at higher pH in their experiments. Accordingly, we may expect that carboxylic groups generated in the course of autoxidation in our system, have prooxidant effect, which should be lower at higher pH, if protonated form of carboxyls is effective.

There are several data in the literature on the effect of pH on lipid oxidation,[13,19–21] some of which are controversial. Discrepancies could be attributed to the other

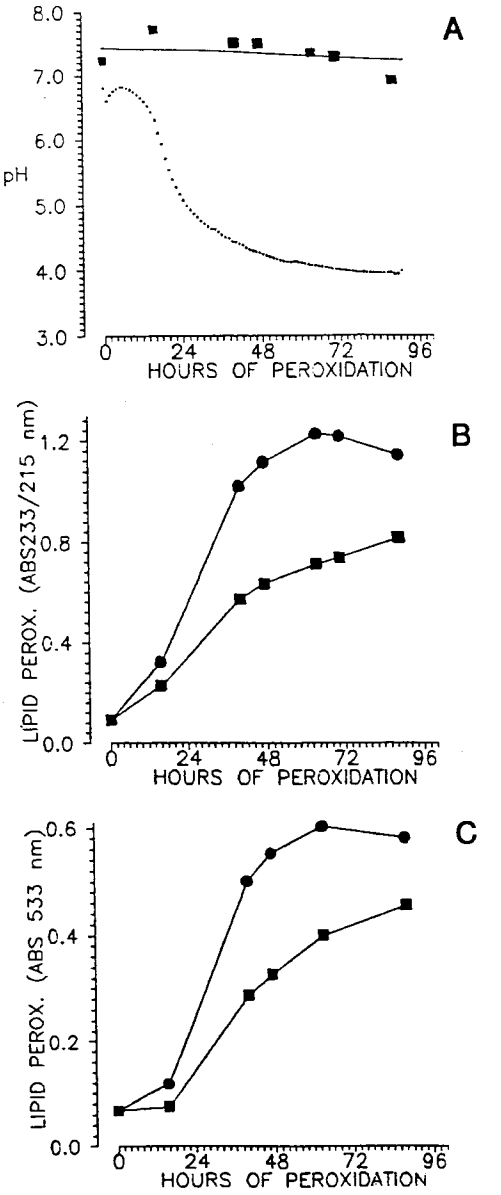

FIGURE 4 pH-changes (A), conjugated diene formation (B) and TBA reactive products formation (C) in a pH-stat-lipid peroxidation experiments. Solid lines – pH adjusted, broken lines – controls.

factors important in the oxidative reactions, which are influenced by the pH, such as oxygen solubility,[16] extent of peroxidation (prooxidant carboxyls are apparently formed in the latter stages of peroxidation[15]), and buffers used in the preparation.[13]

Based on the reported facts we suggest, that the reported inhibitory action of Hepes and Tris buffers against lipid peroxidation of lecithin liposomes,[6] may also be related to their buffering capacity. We propose that the buffering capacity of buffers, besides their known chelating and hydroxyl radical scavenging activity, may modify oxidative stability of lipids significantly.

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