Free Rad. Res. Comms., Vol. 6, No. 4, pp. 243-250
Reprints available directly from the publisher
Photocopying permitted by license only

BUFFERS CAN MODULATE THE EFFECT OF SONICATION ON EGG LECITHIN LIPOSOMES

DIANA FIORENTINI, LAURA LANDI, VANNI BARZANTI and LUCIANA CABRINI

Dipartimento di Biochimica, Universita' di Bologna, Via Irnerio 48, 40126 Bologna, Italy

(Received 6 September 1988; in revised form 16 November 1988)

When model membranes are prepared by ultrasonic irradiation of polyunsaturated phospholipids, radical production can induce a partial degradation of the polyunsaturated fatty acyl chains and the formation of lipid hydroperoxides. A suitable buffer employed during liposome preparation, like Hepes or Tris, is able to exhibit a protective effect against lipid peroxidation. Hepes has been found to be the most effective: a 10 mM concentration provides a 70% protection after 30 min sonication. Tris, in the same conditions, exhibits a 50% protection. These findings may be explained on the basis of the rate constants of these organic buffers with hydroxyl radicals.

KEY WORDS: Buffers, liposomes, lipoperoxidation, free radicals.

ABBREVIATIONS: PC, egg lecithin.

INTRODUCTION

A number of review articles have described a) the general properties of liposomes and their use as model membranes[1,2] b) the reconstitution of membrane proteins into vesicles[3,4] c) the use of liposomes as carriers of drugs and macromolecules *in vivo* and their interaction with eucaryotic cells *in vitro*[5,6] d) the use of liposomes as model system for the study of lipid peroxidation.[7]

Generally the method of choice for the preparation of liposomes is ultrasonic irradiation with a probe or in a bath. On the other hand ultrasonic waves are known to initiate redox reactions in aqueous solutions, which are often similar to those produced by ionizing radiations.[8]

Among the reactive species formed by water sonolysis is the hydroxyl radical, a very strong oxidant and an efficient initiator of lipid peroxidation.[9] Thus, when model membranes are prepared by ultrasonic irradiation of phospholipids with polyunsaturated fatty acid residues like egg or soy-bean lecithin, radical production can induce a partial degradation of the polyunsaturated fatty acyl chains and the formation of lipid hydroperoxides. The peroxidation products increase the degree of disorder in the bilayer, and affect permeability,[10] fluidity[11] and stability behaviour[12] of the vesicles. These changes may affect the lipid-protein interaction when reconstituted liposomes are studied, or alter the transport characteristics of drug-carrier liposomes, or be of importance in the storage of vesicles.

Peroxidation may be completely prevented by adding a natural antioxidant like α-tocopherol or a synthetic one, like butylated hydroxytoluene, during the prepara-

Address for correspondence: Dr. Diana Fiorentini, Dipartimento di Biochimica, Universita' di Bologna, Via Irnerio, 48, 40126 Bologna, Italy.

tion of liposomes.[12] Sometimes the presence of an antioxidant may interfere with the experimental system under investigation. Therefore we propose another possibility: the careful choice of the buffer.

Since their introduction by Good *et al.*,[13] a range of organic buffers have been employed because of their efficient buffering capacity near physiological pH. Recently Hicks and Gebicki[14] reported that Hepes, Tricine and Tris are efficient scavengers of $^{\cdot}OH$ radicals and they suggested that probably other organic buffers exhibit this activity.

In this work we investigate the effect of sonolysis on egg lecithin dispersed in various buffers and show that the use of an appropriate buffer can considerably lower the extent of peroxidation.

MATERIALS AND METHODS

Egg lecithin (PC) was obtained from Lipid Products (Redhill, U.K.) and used without further purification. 4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid (Hepes) was supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.), Tris(hydroxy-methyl)-amino methane and phosphate were from Merck (Darmstadt, West Germany). Stock solutions of PC in chloroform stored at $-20°C$ were dried under nitrogen before use. The dried mixture was dispersed with a vortex mixer in 8 ml of one of the following: a) double distilled water b) 10 or 50 mM Hepes buffer, pH 7.2 c) 10 mM Tris/HCl buffer, pH 7.4 d) 10 mM K-phosphate buffer, pH 7.2. The final concentration of PC was usually 3.2 mg/ml. In other experiments PC concentration ranged from 0.5 to 8 mg/ml. The suspensions were then sonicated for different time spans up to 30 min with continuous flushing N_2, using a Labsonic 2000 (B. Braun Melsungen AG, West Germany) with a titanium probe, 127 mm length, 9.5 mm diameter, at a power flow of 20 $W \cdot cm^{-2}$, as measured by a calorimetric method. In a typical run, 8 ml of aqueous phospholipid dispersion were pipetted in a Pyrex tube (volume 40 ml). The irradiation vessel, thermostated at 0–4°C, was fitted at a constant position and the probe tip was held at a constant position in the dispersion. Sonication was carried out intermittently for 30 s, following by a 30 s resting period. The sonicated samples were centrifuged to remove any probe particles. After different irradiation times the absorption spectra of conjugated dienes were recorded in the wavelength range 320–215 nm by a Perkin-Elmer 559 UV-VIS spectrophotometer. The increase of absorption at 234 nm was an indication of the appearance of conjugated dienes and the absorption at 300 nm was taken as zero. Sonicated samples were read against a blank containing the same amount of non sonicated vesicles. Deoxycholate (pH 8.29) was added to vesicles before recording, to give a final concentration of 1% (w/v).

Alternatively fatty acid composition of phospholipid vesicles before and after sonication was determined after methanolysis in acid methanol by gas-liquid chromatography.[15]

RESULTS

First the effect of sonolysis on aqueous dispersion of egg lecithin, in the absence of buffers, was tested. Figure 1 shows that, after 30 min sonication, the production of conjugated dienes increases in relation to the amount of PC used. The scatter of the

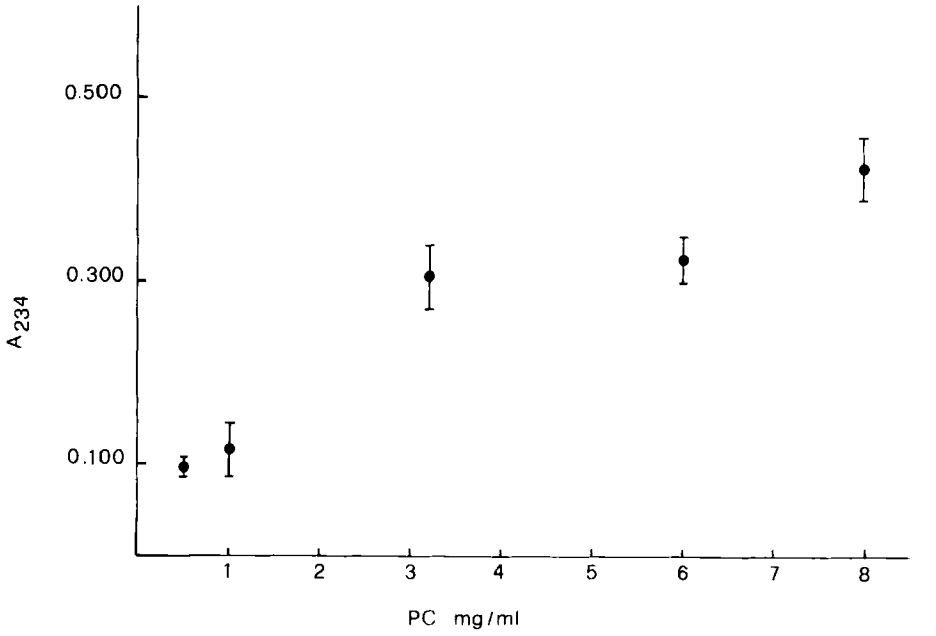

FIGURE 1 Conjugated diene production versus egg lecithin content of liposomes. The ultrasonic irradiation was performed in double distilled water for 30 min. Data are the means of three experiments with different liposome preparations.

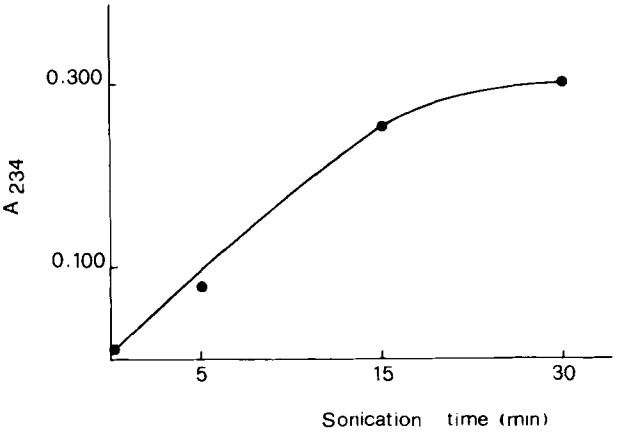

FIGURE 2 Conjugated dienes produced in liposomes as a function of sonication time. Egg lecithin content, in double distilled water, was 3.2 mg/ml. Data are the means of ten experiments with different liposome preparations.

experimental points in Figure 1 may be due to the great number of parameters involved in ultrasonic irradiation (see discussion). Among the concentrations of PC, we selected 3.2 mg/ml, extensively utilized in our previous works.[16,17]

The extent of oxidation as a function of sonication time is shown in Figure 2. It can be seen that, as liposomes are forming under constant ultrasonic intensity, the production of conjugated dienes considerably increases. In Table 1 the effect of Hepes, Tris and phosphate on the production of conjugated dienes in forming liposomes as a function of sonication time is reported. These data are compared to those in water. Hepes strongly inhibits the oxidative damage of liposomes, and its protective effect increases with increasing time of insonation. Tris is less effective than

TABLE 1
Effect of H_2O, Hepes, Tris and phosphate on the production of conjugated dienes in liposomes after different times of sonication

Sonication time (min)	A_{234}			
	H_2O	Hepes	Tris	Phosphate
0	0.002	0.001	0.002	0.004
5	0.078	0.034	0.070	0.090
15	0.256	0.080	0.154	0.205
30	0.300	0.094	0.170	0.251

Egg lecithin concentration was 3.2 mg/ml. Buffer concentration was 10 mM in all experiments. Data are the means of four experiments with different liposome preparations.

TABLE 2
Fatty acid composition of egg lecithin liposomes prepared in H_2O, Hepes and Tris before and after 30 min sonication

Fatty acid[a]	Before sonication	After sonication		
		H_2O	Hepes	Tris
C 16:0	28.709	29.970	28.846	29.576
C 16:1	2.064	2.592	2.086	2.115
C 18:0	13.550	13.559	13.773	13.641
C 18:1	28.299	27.770	28.635	29.225
C 18:2	15.580	14.730	15.456	15.405
C 20:4 (n 6)	4.393	3.519	4.270	3.954
C 22:4 (n 6)	0.287	0.190	0.272	0.236
C 22:5 (n 6)	0.884	0.600	0.893	0.713
C 22:5 (n 3)	0.240	0.090	0.243	0.120
C 22:6 (n 3)	3.458	2.570	3.260	2.873

[a]Gaschromatographic analysis was performed according to[15]. The fatty acid composition (as methylesters) are expressed as percentages of the total identified fatty acids. Buffer concentration was 10 mM. Data are the means of two analyses.

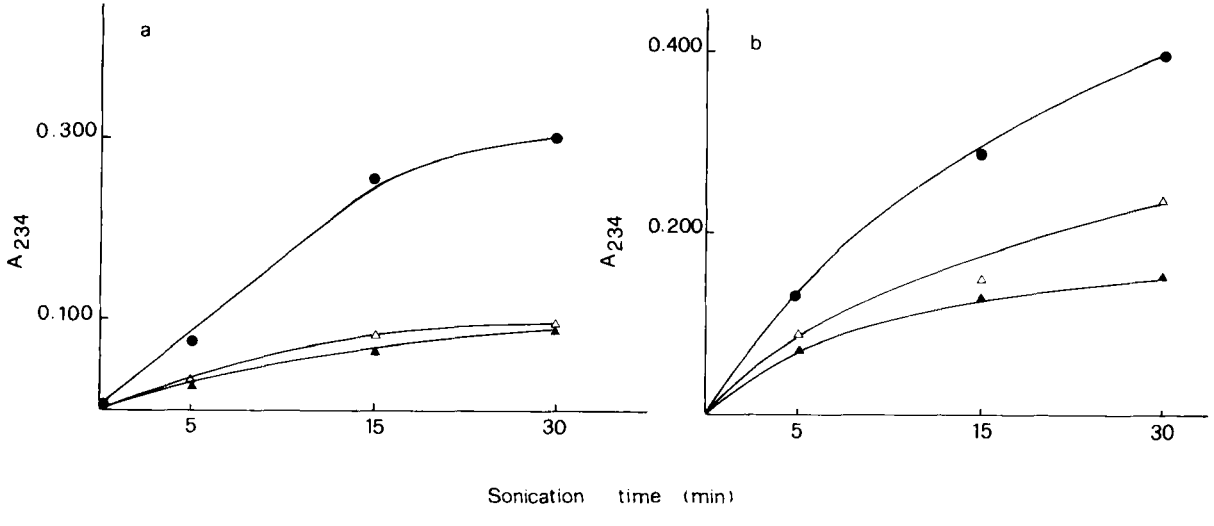

FIGURE 3 Conjugated dienes produced in liposomes versus sonication time in the presence or absence of Hepes. Data are the means of three experiments with different liposome preparations. Egg lecithin a) 3.2 mg/ml; b) 6 mg/ml. ●—●, double distilled water; △—△, Hepes 10 mM, ▲—▲, Hepes 50 mM.

Hepes in preventing the formation of conjugated dienes after 15 min insonation, whereas phosphate is quite ineffective. For this reason we tested the amount of fatty acid residues of PC vesicles before and after 30 min sonication only in H_2O, Hepes and Tris. The data reported in Table 2 confirm those shown in Table 1: the polyunsaturated fatty acid content decreases about 13% and 6% respectively in water and Tris, while it is quite unchanged when liposomes are prepared in Hepes. The effect of two different concentrations of Hepes on liposomes prepared either with 3.2 or 6 mg/ml PC, versus insonation time, is shown in Figure 3. After 30 min, both 10 and 50 mM Hepes exhibit a 70% protection against the radical attack in the presence of the lower PC concentration (Figure 3a). On the other hand when the concentration of PC is 6 mg/ml, 10 mM Hepes is still able to provide a 40% protection; however 50 mM Hepes has the same capacity to inhibit peroxidation (70%) (Figure 3b).

DISCUSSION

The effect of sonolysis on the chemical structure of egg lecithin has been previously reported by Klein[18] and Hauser.[19] Klein showed that when egg lecithin preparations in 100 mM KCl were sonicated in the presence of air, the extent of peroxidation increased with time, whereas in the presence of nitrogen it did not increase. Hauser[19] showed an appreciable chemical degradation even in the presence of N_2 and no difference between the effect of sonication on egg lecithin dispersed either in water or buffer. More recently Konings[12] presented data that demonstrated some peroxidation took place even under nitrogen gassing conditions. Experiments reported in this paper show that with continuous flushing N_2 during the preparation of vesicles in water, the polyunsaturated hydrocarbon chains may become oxidized as a function of the amount of egg lecithin present (cfr. Figure 1) as well as the sonication time (cfr. Figure 2). An explanation of the discrepancy between our findings and the previous ones may be due to the difficulty in controlling the great number of parameters during sonication. Besides the instrumental settings, the geometry of the tip of the soniprobe, volume, concentration, liquid depth and temperature were all found to influence the effect of sonication.[19] Moreover in the cited papers no accurate definition of the nature of the gas dissolved in the samples or to the use of buffers has been reported. These points are very important since the presence of oxygen induces the propagation step of peroxidation (the initiation step occurs also in its absence). A suitable buffer (Table 1 and 2), by competing with polyunsaturated fatty acid residues for $^{\cdot}OH$ radicals, can inhibit the oxidation of the residues. In fact, the data indicate that the buffer employed during the liposome preparation is essential in preventing the $^{\cdot}OH$ attack to egg lecithin. Hepes has been found to be the most effective: a 10 mM concentration provides a 70% protection after 30 min sonication, whereas Tris buffer, in the same conditions, inhibits conjugated dienes formation by 50% (cfr. Figure 3). These results may be explained on the basis of the rate constants of these organic buffers with hydroxyl radicals. In fact, as reported by Hicks and Gebicki,[14] the k values are: for Hepes $5.1 \times 10^9\,M^{-1}s^{-1}$ and for Tris $1.1 \times 10^9\,M^{-1}s^{-1}$. The value for phosphate is $< 10^7\,M^{-1}s^{-1}$.[20] Furthermore, since the buffer acts by competing with polyunsaturated fatty acid residues for $^{\cdot}OH$ radicals (k value of $^{\cdot}OH$ for linoleate is $1.1 \times 10^{10}\,M^{-1}s^{-1}$),[21] it is possible to compensate for increased amounts of unsaturated phospholipids (cf. Figure 3, a, b) by increasing Hepes concentration. The finding that both Hepes and Tris are unable to provide total protection against $^{\cdot}OH$

damages, suggests that the $^{\cdot}OH$ radicals are produced at sites not entirely accessible to the buffer molecules. In fact in the case of ultrasonic irradiation the radicals are produced in oscillating gas bubbles,[22] so that an efficient scavenger should be not very polar to enable it to enter the gas bubbles. These reasons, in addition to the lower k value, can probably explain the poor effectiveness of phosphate.

These data, taken as a whole, can provide useful suggestions to prevent oxidative degradation during the preparation of liposomes to use as membrane model systems.

Acknowledgements

This work was supported by a grant from Ministero della Pubblica Istruzione, Rome.

References

1. A.D. Bangham, M.W. Hill and N.G.A. Miller, Preparation and use of liposomes as models of biological membranes. In "Methods in Membrane Biology" vol. 1, (Korn, E.D. ed.), New York Plenum Press, 1–68, (1974).
2. D. Papahadjopoulos and N. Miller, Phospholipid model membranes. I. Structural characteristics of hydrated liquid crystals. *Biochimica et Biophysica Acta*, **135**, 624–638, (1967).
3. D. Papahadjopoulos and H.K. Kimelberg, Phospholipid vesicles (liposomes) as model for biological membranes: their properties and interaction with cholesterol and proteins. In "Progress in Surface Science" vol. 4, (Davison, S.G. ed.), Oxford Pergamon, 141–232, (1974).
4. S. Razin, Reconstitution of biological membranes. *Biochimica et Biophysica Acta*, **265**, 241–296, (1972).
5. G. Gregoriadis, The carrier potential of liposomes in biology and medicine. *N. Engl. J. Med.*, **295**, 765–770, (1976).
6. J.H. Fendler and A. Romero, Liposomes as drug carriers. *Life Science* **20**, 1109–1120, (1977).
7. S.N. Chatterjee and S. Agarwal, Liposomes as membrane model for study of lipid peroxidation. *Free Rad. Biol. & Med.*, **4**, 51–72, (1988).
8. I. El'Piner, In "Ultrasounds, Physical, Chemical and Biological Effects" Consultant Bureau, New York, (1964).
9. J.A. Raleigh and W. Kremers, Promotion of radiation peroxidation in models of lipid membranes by Coesium and Rubidium counter-ions; micellar linoleic and linolenic acids. *Int. J. Rad. Biol.*, **34**, 439–445, (1978).
10. T. Nakazawa and S. Nagatsuka, Radiation-induced lipid peroxidation and membrane permeability in liposomes. *Int. J. Rad. Biol.*, **38**, 537–544, (1980).
11. G.E. Dobrestov, T.A. Borschevskaya, V.A. Petrov and Y.A. Vladimirov, The increase of phospholipid bilayer rigidity after lipid peroxidation. *FEBS Letters*, **84**, 125–128, (1977).
12. A.W.T. Konings, Lipid peroxidation in liposomes. In "Liposome Technology" vol. I, (Gregoriadis, G., ed.), CRC Press Inc., Boca Raton, Florida, 139–161, (1984).
13. N.E. Good, W. Winget, W. Winter, T.N. Connolly, S. Izawa and M.M. Singh, Hydrogen ion buffers for biological research. *Biochem.*, **5**, 467–477, (1966).
14. M. Hicks and J.M. Gebicki, Rate constants for reaction of hydroxyl radicals with Tris, Tricine and Hepes buffers. *FEBS Letters*, **199**, 92–94, (1986).
15. W. Stoffel, F. Chu and E.H. Ahrens, Analysis of long-chain fatty acids by gas-liquid chromatography. *Anal. Chem.*, **31**, 307–308, (1959).
16. L. Landi, L. Cabrini, B. Tadolini, T. Fahmy and P. Pasquali, Incorporation of a lipophilic series, as coenzyme Q homologs, in phospholipid vesicles. *Appl. Biochem. Biotechnol.*, **11**, 123–132, (1985).
17. L. Cabrini, P. Pasquali, B. Tadolini, A.M. Sechi and L. Landi, Antioxidant behaviour of ubiquinone and β-carotene incorporated in model membranes. *Free Rad. Res. Commun.*, **2**, 85–92, (1986).
18. R.A. Klein, The detection of oxidation in liposome preparations. *Biochimica et Biophysica Acta*, **210**, 486–489, (1970).
19. H.O. Hauser, The effect of ultrasonic irradiation on the chemical structure of egg lecithin. *Biochem. Biophys. Res. Commun.*, **45**, 1049–1055, (1971).
20. M. Anbar and P. Neta, A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in

aqueous solution. *Int. J. Appl. Rad. Isotopes*, **18**, 493–523, (1967).
21. M.G.I. Heijmann, A.J.P. Heitzman, H. Nauta and K. Levine, A pulse radiolysis study of the reactions of HO /O^- with linoleic acid in oxygen-free aqueous solution. *Rad. Phys. Chem.*, **26**, 83–88, (1985).
22. A. Henglein and C. Kormann, Scavenging of HO radicals produced in the sonolysis of water. *Int. J. Rad. Biol.*, **48**, 251–258, (1985).

Accepted by Prof. G. Rotilio