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PERMEABILITY OF OXIDIZED PHOSPHATIDYLCHOLINE LIPOSOMES

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SUMMARY: Permeability of liposomes made from mixtures of unoxidized and singlet oxygen oxidized phosphatidylcholine has been related to the degree of lipid oxidation expressed as hydroperoxide moiety content in the lipids. The effect of oxidation on the liposomes permeability has been studied by fluorometry using calcein as a fluorescent probe that undergoes self quenching when highly concentrated inside liposomes. The liposomes containing 73 % and 5% of hydroperoxides retain respectively 64.5 and 96.3 % of calcein with respect to that retained by the liposomes made from unoxidized phosphatidylcholine. The fluorescence data show a linear relationship between the liposome permeability and the oxidation degree of lipids. © 1989 Academic Press, Inc.

Hydroperoxides and cyclic peroxides are known to be the primary oxidation products of unsaturated lipids. During the first stage of the oxidation process, double bond conjugation also occurs (1,2). Once formed, hydroperoxides lead to further lipid oxidation (3) and they breakdown producing many degradative products (4-6). Because of the large variety of chemical species produced by lipid oxidation, many different alterations and damages have been observed on biological and model membranes. Decrease of membrane fluidity (7,8), inactivation of membrane bound enzymes (9) and modification of ionic permeability of the phospholipid bilayer (3) are some examples of such membrane alterations. In the present study we have measured the relative permeability of phosphatidylcholine liposomes containing up to 73 mole % of hydroperoxide moieties relative to the total number of moles of liposome lipids.

MATERIALS AND METHODS :Phosphatidylcholine (from egg yolk) was obtained from Lipid Products (South Nutfield NR. Redhill, Sur-

ABBREVIATIONS. PC: phosphatidylcholine; RP-TLC: reverse phase thin layer chromatography; MLV: multilamellar vesicles; Lyso-PC: L-α-lysophosphatidylcholine.

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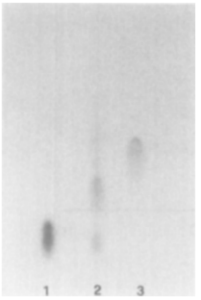
rey,England).L-α-lysophosphatidylcholine (egg yolk), ter-butyl hydroperoxide, HEPES, Florisil 100-200 mesh, Triton X-100 and calcein were obtained from Sigma. All the solvents, of the purest quality, and the RP-8 F254 HPTLC plates were obtained from Merck. 75 mg of PC were oxidized by singlet oxygen, following the procedure described elsewhere (7, 10). A modification of the oxidation method (7) was introduced using oxygen instead of air. The oxidized lipid was stored at - 80 °C. The content of hydroperoxide moieties in the oxidized PC was determined by the iodometric assay (4) using a standard curve of ter-butyl hydroperoxide and reading the absorbance values at 353 nm. Reverse phase thin layer chromatography was performed on RP-8 F254 plates using a mixture of chloroform/methanol/water (1:10:0.5, v/v/v) as developing solvent. Spots were detected by iodine vapours. UV spectra were carried out by using a Kontron Uvikon 810 spectrophotometer equipped with a Uvikon recorder 21 and a Uvikon LS printer 48. Multilamellar liposomes were made from lipid mixtures (0.5 mg of total lipids) prepared by mixing oxidized and unoxidized PC obtaining, in this way, various mole percentage of hydroperoxides referred to the total moles of lipids. The lipid mixtures were dried under a stream of nitrogen and stored at -80 °C. The liposomes were prepared from each mixture by adding 400 ul of a saturated solution of calcein containing 10mM HEPES/100 mM KCl pH 7.4 and then vortexing continuously for 10 min . The liposomes were then centrifuged (Beckman microfuge 12) for 7 min. at 10000 rpm (7000xg), then the supernatant discarded and the pellet resuspended, by 30 sec. of vortexing, in 400 ul of HEPES/KCl buffer pH 7.4. This procedure was repeated 7 times. After the last centrifugation the pellet was suspended in 3 ml of buffer and fluorescence data were collected. All fluorescence measurements were started within 90 min. of adding the calcein solution to the dried lipid mixtures. Fluorometry measurements were made at 25 °C using a Perkin-Elmer MPF-66 fluorescence spectrophotometer equipped with a P.E 7300 personal computer for data storage and elaboration. The exitation wavelength was set at 490 nm and the emission wavelength at 520 nm. The fluorescence was continuosly followed for 20 min.and then the liposomes were dissolved by adding 150 ul of a 10% Triton X-100 solution.

RESULTS AND DISCUSSION: The UV spectrum of the oxidized PC shows a strong absorption peak at 234 nm due to the conjugated double bonds (data not shown). This strong absorption reveals that lipid oxidation, under the conditions we used, was very high. This result is confirmed by the high mole percentage (73%) of hydroperoxide moieties found in the oxidized PC. This high lipid oxidation was obtained because of the use of oxygen instead of air in the reaction tube. In fact, in a previous work we obtained only 21% by mole of hydroperoxides using air as the oxygen source for PC photooxidation (7). Figure 1 shows the reverse phase thin layer chromatography of oxidized PC. From the RP-TLC pattern it appears that only a small amount of lysophosphatidylcholine was obtained in the oxidation process. The possibility that this small amount of Lyso-PC could induce a hexagonal phase on MLV, was checked by freeze fracture electron



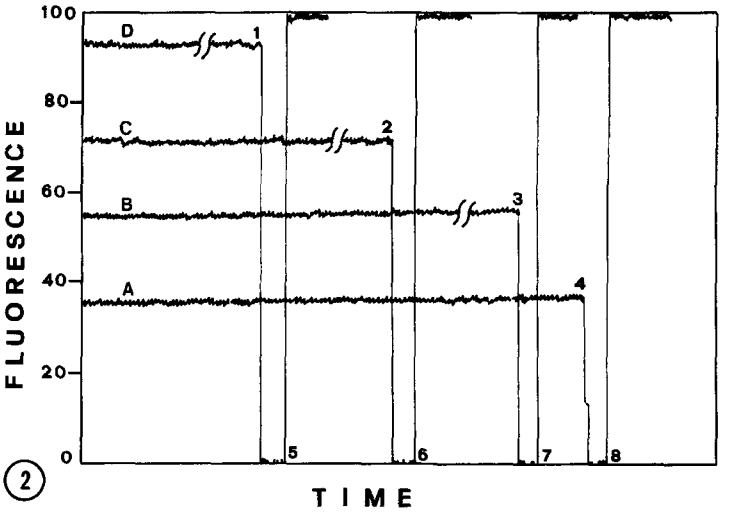


FIGURE 1: Reverse phase thin layer chromatography of: 1, unoxidized PC; 2, oxidized PC; 3, Lyso-PC.

FIGURE 2: Fluorescence of calcein trapped inside liposomes made from mixtures of PC containing : A, 0% (unoxidized PC); B, 21.24%; C, 43.04%; D, 73% of hydroperoxides. The fluorescence remained almost constant during the recording time. The initial fluorescence corresponds to that recorded at time = 0. In points 1-4 the cuvette was removed from the instrument, while in points 5-8 it was replaced inside the fluorometer after addition of Triton X-100.

microscopy which revealed only bilayer phase on the oxidized PC liposomes (data not shown) . Before preparing the samples for electron microscopy, liposomes were submitted to centrifugation at 10000 rpm for 7 min. Figure 2 shows 4 typical fluorescence experiments. For each sample, the fluorescence was normalized to a scale of 0-100 of arbitrary units using a "scale" obey program from Perkin-Elmer. The zero fluorescence value corresponds to the dark obtained when the cuvette was removed from the instrument in order to put Triton X-100 in it. The 100 fluorescence value corresponds to the maximum of fluorescence obtained after addition of Triton in the cuvette. At time equal to zero each sample showed a different initial fluorescence which is that due to the calcein still trapped inside liposomes. After normalization procedure, the initial fluorescence value of each sample was plotted against the mole % of hydroperoxides contained in the liposomes. Figure 3 shows these data from which it appears that there is a linear relationship between the initial fluorescence and the content of hydroperoxides in the liposomes. In figure 3 the graph can be seen to be divided into two parts. The lower part represents the initial fluorescence and the upper

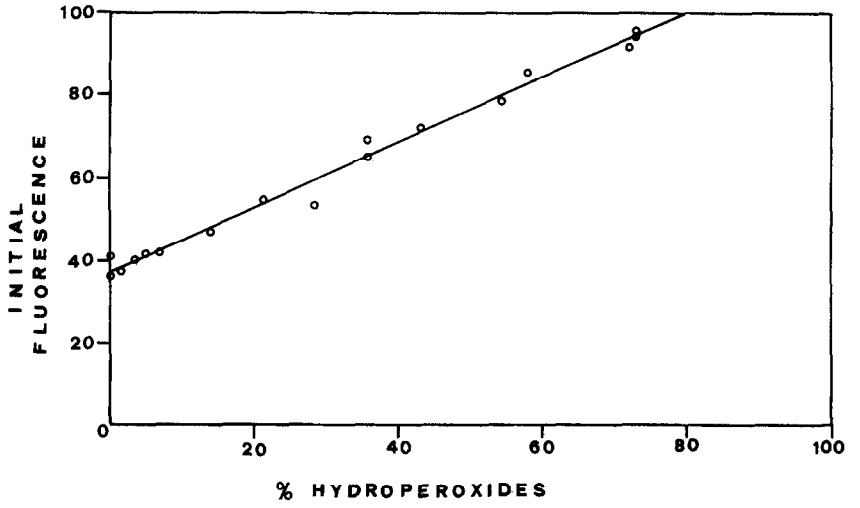


FIGURE 3: Initial fluorescence as a function of % hydroperoxides inside liposomes. The parameters of the straight line equation Y = mX + q are : m = 0.7791639; q = 37.43989. For explanation see text.

part represents the "quenched fluorescence" released from the liposomes after addition of Triton. We propose an explanation for the observed initial fluorescence. It has been shown that ufasomes (unsaturated fatty acid membranes)(11) peroxidation, induced by lipoxygenase, increases membrane permeability to water and water soluble molecules (12) and that ionic permeability of phospholipid bilayer increases after UV irradiation of the membrane (3). Our fluorescence measurements have been carried out on liposomes that already contain different amounts of oxidized lipids. Since the washing and centrifugation procedure was the same for all the samples, the different initial fluorescence found in different samples could be due to different water permeation inside liposomes, during the washings. This influx of water dilutes calcein and causes membrane swelling which can lead to membrane ruptures and calcein release. At the same time calcein could be released during centrifugation because of the gravity and mechanical stress to which the liposomes were submitted. We calculated that ,under the condition we used, the liposomes were submitted to a pressure of about 0.35-0.4 MPa. All this process should be proportional to the oxidation degree of liposomes so that, proportionally, calcein becomes more dilute and, proportionally, its self quenching is reduced. At high degree of lipid oxidation (73% hydroperoxides) the permeability of the bilayer seems to be very high because the initial fluorescence of calcein is 94% in respect to that obtained after

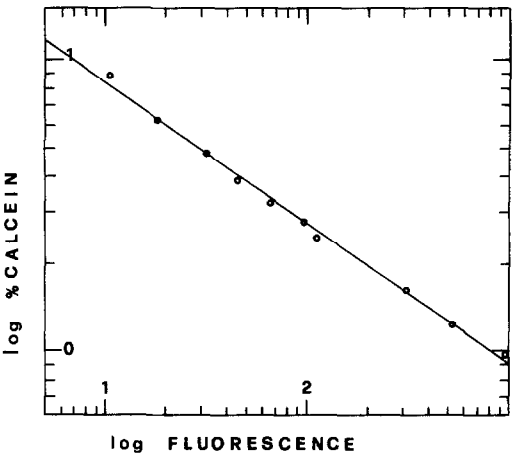


FIGURE 4: Logarithm of the fluorescence of calcein as a function of its logarithmic concentration. The parameters of the straight line equation Y = mX + q are : m = -0.4823521; q = 1.404215. For explanation see text.

addition of Triton (see figures 2,3). In order to quantify the amount of calcein trapped inside the liposomes, the initial fluorescence was related to that of a standard curve of calcein. The curve was obtained by diluting the saturated solution of calcein with buffer HEPES/KCl pH 7.4. The calcein in the diluted samples, was expressed as percentage of that present in the saturated solution to which was assigned the value of 100%. The fluorescence was measured under the same conditions used for liposomes. A linear relationship between fluorescence and % of calcein was obtained after plotting the data on double logarithmic graph paper (figure 4). From calculations, the liposomes with 73% and 5% of hydroperoxides contain respectively 64.5% and 96.3% of calcein related to that found in the unoxidized liposomes.In the latter, calcein was 4.4% related to that present in the saturated solution. In other words, during the centrifugations and washings, the saturated solution of calcein used to make liposomes, was diluted 22.8 times in the unoxidized liposomes, 23.6 times in the liposomes containing 5% of hydroperoxides and 35.3 times in liposomes at higher oxidation degree. In conclusion, from figures 2 and 3, we could measure the relative permeability of liposomes at different degree of oxidation. Our data reveal that, under the conditions we used, unoxidized PC liposomes show a high permeability to water soluble molecules due, probably, to the mechanical and gravity stress. In the liposomes containing oxidized PC, permeability increases proportionally to the oxidation degree of the lipids. At a high degree

of oxidation, (73% hydroperoxides) the liposomes still show an appreciable entrapment capacity in spite of the high content of bulky hydrophilic hydroperoxide moieties which would distort the hydrophobic membrane interior.

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REFERENCES

1. Konigs, A.W.T. (1984) in: Gregoriadis, G. (Ed.), Liposome Technology Vol.1, CRC Press Inc., Boca Raton, Florida, pp. 139-161.
2. Pryor, W.A. (1976) in: Pryor,W.A. (Ed.), Free Radicals in Biology Vol.1, Academic Press, pp. 1-49.
3. Vladimirov, Yu.A., Olenev, V.I., Suslva, T.B., and Cheremisina, Z.P. (1980) Adv. Lipid Res. 17, 173-249.
4. Buege, J.A., and Aust, S.D. Methods Enzymol., 52, 302-310.
5. Reiter, R. and Burk, R.F., (1987) Biochem. Pharmacology 36 No 6, 925-929.
6. Nair, V., Cooper, C.S., Vietti, D.E. and Turner, G.A. (1986) Lipids 21 No 1, 6-10.
7. Tanfani, F., Curatola, G. and Bertoli, E. (1989) Chem. Phys. Lipids
8. Demopoulos, H.B., Flamm, E.S., Pietronigro,D.D. and Seligman, M.L. (1980) Acta Physiol. Scand. Suppl. 492, 91-119.
9. Freeman, B.A.and Crapo, J.D. (1982) Lab. Invest. 47 No 5, 412-426.
10. Terao, J., Asano, I. and Matsushita, S. (1985) Lipids 20 No 5, 312-317.
11. Gebicki, J.M. and Hicks, M. (1976) Chem. Phys. Lipids 16, 142-160.
12. Hicks, M. and Gebicki, J.M. (1978) Biochem. Biophys. Res. Comm. (1978) 80 No 4, 704-708.