Faculty of Veterinary Medicine[1], University of Veterinary Medicine and Pharmacy, Brno, Czech Republic, and Department of Physical Chemistry of Drugs[2], Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic

Effect of cholesterol on egg yolk phosphatidylcholine peroxidation in multilamellar liposomes

J. Filípek[1], D. Uhríková[2], P. Slosarčík[2] and P. Balgavý[2]

Dedicated to Prof. Ing. P. Kovács, DrSc., Bratislava, on the occasion of his 70th birthday

Lipid peroxidation of aerated multilamellar liposomes composed of egg yolk phosphatidylcholine (EYPC) and cholesterol (CHOL) at molar ratios CHOL:EYPC = 0, 0.1, 0.2, 0.3, 0.4, 0.6 and 1.0 was studied during autooxidation and during Fe^{2+}/H_2O_2-induced peroxidation by following the formation of conjugated diene and thiobarbituric acid reactive substances. The presence of cholesterol in the fluid lipid bilayers has an inhibiting effect on the EYPC peroxidation in the propagation phase of both the autooxidation and Fe^{2+}-induced peroxidation free radical chain reactions. This inhibiting effect increases with the increase in CHOL : EYPC molar ratio. The inhibition of EYPC peroxidation by cholesterol probably originates a) from the increased lateral separation of polyunsaturated EYPC acyl chains caused by insertion of cholesterol between EYPC molecules, b) from the increased molecular packing of both the bilayer polar and hydrophobic regions due to the reduced bilayer hydration, and c) from the antioxidant properties of cholesterol.

1. Introduction

Phospholipid liposomes are widely studied because of their pharmaceutical, cosmetic and industrial applications [1–3]. Cholesterol is used in liposomal formulations to increase their stability and to reduce leakage of various solutes through the lipid bilayer in a fluid-like state [3, 4]. During liposome preparation and storage the polyunsaturated phospholipid fatty acyl chains may become peroxidized. Peroxidation of liposomes destabilizes the bilayer structure [5] and increases the rate of solute leakage [6, 7]. Cholesterol has been reported to have no effect, to increase or to decrease the rate of peroxidation depending on the liposome composition and bilayer physical properties [6–17].

Phospholipids and cholesterol are also among the main constituents of biological membranes [18]. Phospholipid bilayers in liposomes are thus useful as experimental models of lipid part of biological membranes. They are frequently used to test the antioxidant properties of various synthetic drugs [19-22], extracts [23–27] and medicines [28]. The experimental model should resemble the biological membrane as closely as possible. A convenient model is the liposome composed of egg yolk phosphatidylcholine (EYPC) and cholesterol. EYPC is a naturally occurring and readily available phospholipid with a high proportion of polyunsaturated acyl chains [17, 29, 30]. It is in the physiologically relevant fluid state with highly disordered acyl chains due to their *trans-gauche* isomerisation and laterally diffusing molecules within the bilayer over broad temperature and hydration ranges [31–35]. In the fluid bilayer, cholesterol intercalates between lipid molecules and reduces the acyl chain disorder [36–38] without influencing lipid lateral mobility [34].

In EYPC liposomes, it has been observed that cholesterol has no effect on Fe^{2+}-induced peroxidation (detected using the thiobarbituric acid reactive substances test – TBARS test) at a cholesterol : EYPC = 1 : 1 molar ratio [8], while it increases the yield of autoperoxidation products (TBARS test) at cholesterol : EYPC = 1 : 5 molar ratio during the liposome storage for up to one year [7], and decreases the amount of oxidized lipid polyunsaturated acyl chains (estimated by following changes in acyl chain composition using gas chromatography) after 3 and 6 months autoperoxidation at a cholesterol : EYPC = 1 : 10 molar ratio [17]. These inconclusive results prompted us to investigate in the present communication the autoperoxidation and Fe^{2+}-induced peroxidation of EYPC as a function of cholesterol : EYPC molar ratio up to 1 : 1 which is physiologically relevant [18].

2. Investigations, results and discussion

The UV-VIS absorption spectrum of EYPC displays a marked absorption band in the 200–220 nm region characteristic for the absorption of carbonyl groups of EYPC [39]. The intensity of the absorption in this peak does not change during EYPC autoperoxidation nor during Fe^{2+}-induced EYPC peroxidation. During both autoperoxidation and Fe^{2+}-induced peroxidation, an absorption peak appears in the EYPC spectra with a maximum around 234 nm. Its intensity increases with the time of incubation. Light absorption at this wavelength is typical of conjugated dienes [39, 40]. The structure of conjugated dienes is inherent for such products of lipoperoxidation as lipohydroperoxides. To characterize the degree of lipid oxidation, the oxidation index $\alpha = A_{233}/A_{215}$ is used where A_{233} and A_{215} are the absorbances of the EYPC solution at the wavelengths 233 nm and 215 nm, respectively. Fig.1 shows the time dependence of the oxidation index during autoperoxidation (curve A, symbols ●, t in hours) and Fe^{2+}-induced oxidation (curve B, symbols ◆, t in minutes) of EYPC liposomes. During autoperoxidation, three phases typical for chain reaction can be seen in the α vs. time curve – induction or lag phase, propagation and termination phase.

In Fe^{2+}-induced EYPC peroxidation, the oxidation index increases with no visible lag phase and the reaction proceeds at a significantly higher rate. For example, the oxidation index $\alpha = 0.6$ is reached in 60 h in the autoperoxidation reaction, but in only 60 min in the Fe^{2+}-induced reaction (Fig. 1). The amount of thiobarbituric acid reactive substances (TBARS) in the multilamellar liposomes is proportional to the absorbance A_{533} measured at 533 nm after adding 2-thiobarbituric acid, trichloroacetic acid and 2,6-di-tert-butyl-*p*-cresol (butylhydroxytoluene) to the re-

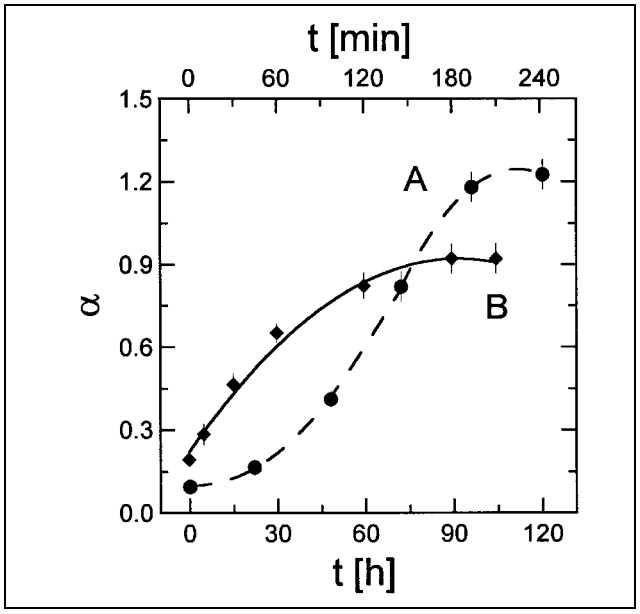

Fig. 1: Dependence of the EYPC oxidation index α on the duration t of autooxidation (curve A, symbols ●, t in hours) and Fe^{2+}-induced oxidation (curve B, symbols ◆, t in minutes). Vertical lines – error bars

action mixture. In the semi-logarithmic plot in Fig. 2, one can see a small (∼5 min) lag phase in Fe^{2+}-induced oxidation when using this parameter to characterize EYPC peroxidation. In the autoperoxidation reaction, the lag phase is clearly visible. Compared with the data in Fig. 1, it is seen that the relative experimental uncertainty of the oxidation index is significantly smaller than that of the absorbance A_{533} used in the TBARS test. Since the oxidation index and TBARS test are used to estimate different products of EYPC oxidation, it is interesting to compare them. Fig. 3 shows a linear correlation between α and A_{533} during the Fe^{2+}-induced EYPC oxidation ($r^2 = 0.982$). A linear correlation has also been observed between α and A_{533} during EYPC autooxidation (not shown).

The presence of cholesterol in bilayers of multilamellar EYPC liposomes has an inhibiting effect on both EYPC

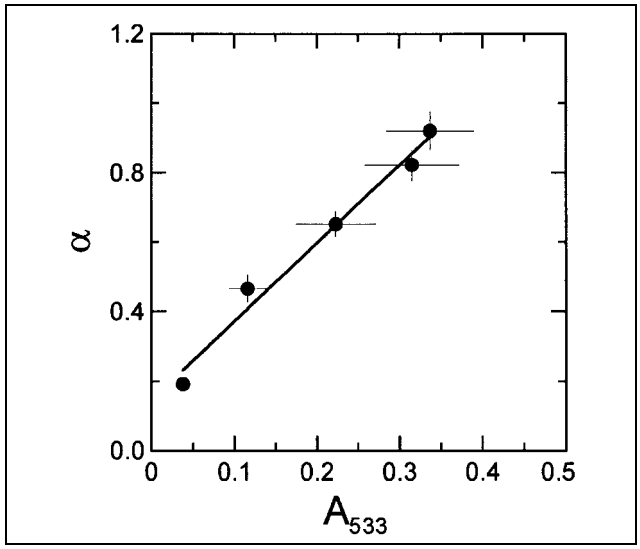

Fig. 3: Correlation between the oxidation index α and the TBARS concentration (A_{533}) during the Fe^{2+}-induced EYPC oxidation

autooxidation and Fe^{2+}-induced EYPC oxidation (Figs. 4–6). For example, an oxidation index of $\alpha = 0.47$ was observed in multilamellar EYPC liposomes in the absence of cholesterol after 48 h of autooxidation. If cholesterol was added to EYPC at a molar ratio of cholesterol : EYPC = 0.4 : 1, the oxidation index was reduced to $\alpha = 0.32$ after 48 h of autooxidation, i.e. to 68% of the control value. Similarly, the presence of cholesterol in the lipid bilayers at a molar ratio of cholesterol : EYPC = 0.4 : 1 reduced the yield of TBARS to 69% of the control value (without cholesterol) after 42 h of autooxidation. The extent and the time course of EYPC oxidation were slightly dependent on the source of the EYPC. Using another batch of EYPC, we observed $\alpha = 0.47$ and $\alpha = 0.35$ in the absence and in the presence of cholesterol (cholesterol : EYPC = 0.4 : 1 mol/mol), respectively, after 48 h of autooxidation (Fig. 6). Cholesterol also inhibits Fe^{2+}-induced EYPC oxidation. For example, at a cholesterol : EYPC = 0.4 : 1 molar ratio, the oxidation index is reduced to 85% and the TBARS concentration to 86% of the control without cholesterol after 2 h of oxida-

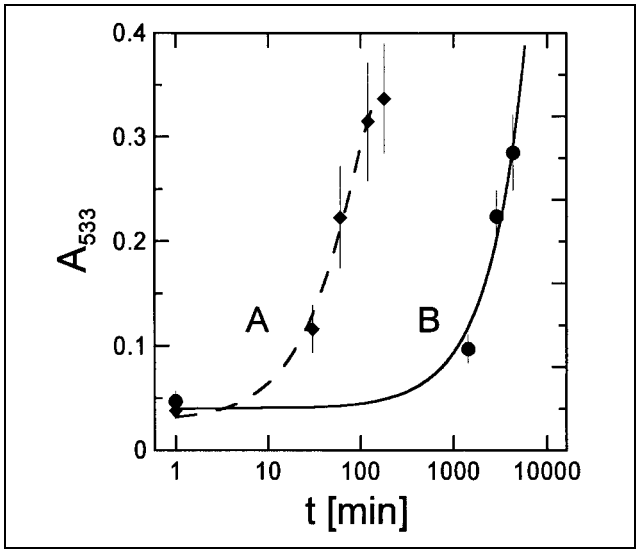

Fig. 2: Dependence of the TBARS concentration (A_{533}) on the duration t of Fe^{2+}-induced EYPC oxidation (curve A, symbols ◆) and autooxidation (curve B, symbols ●)

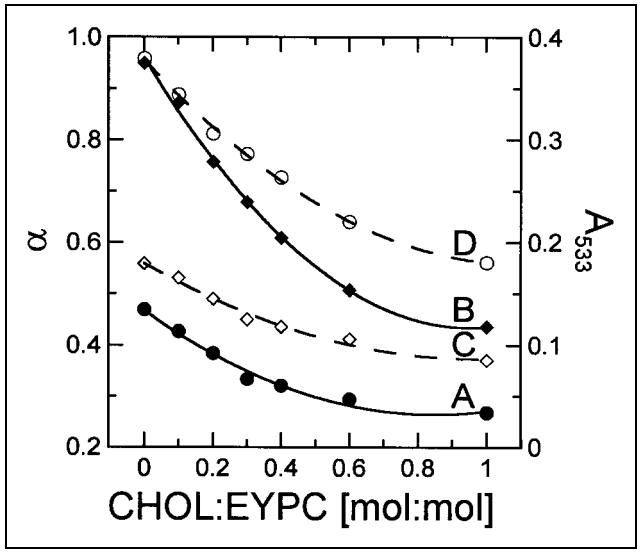

Fig. 4: Effect of liposomal composition on the oxidation index α (full curves A and B) and TBARS concentration (dashed curves C and D) after 14 (◇), 42 (○), 48 (●) and 72 (◆) hours of autooxidation

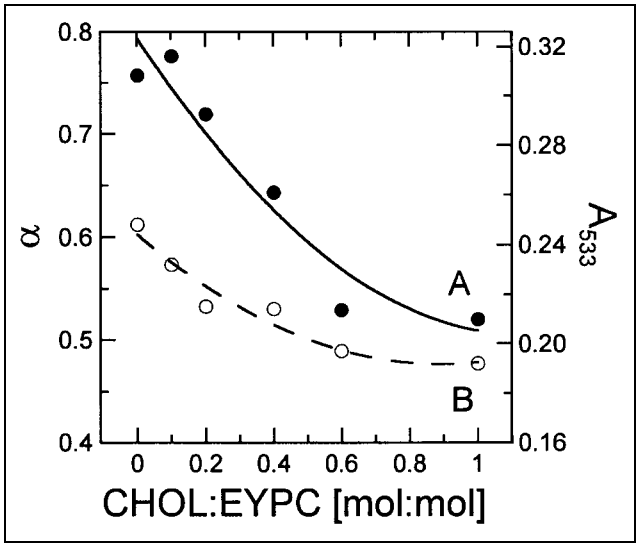

Fig. 5: Effect of liposomal composition on the oxidation index α (full curve A) and TBARS concentration (dashed curve B) after 2 hours of Fe^{2+}-induced EYPC oxidation

tion. The inhibiting effect of cholesterol on oxidation is concentration-dependent – both the oxidation index and the TBARS concentration decrease with an increase of the cholesterol : EYPC molar ratio. However, the dependence of both the oxidation index and TBARS concentration on the cholesterol : EYPC molar ratio is non-linear. The absolute values of the slopes of these dependencies are maximal at low cholesterol concentrations up to a 0.3–0.4 : 1 molar ratio and then decrease at higher concentrations (Figs. 4 and 5). In EYPC autooxidation, the duration of the reaction is a very important factor. For example, comparing the oxidation index in the control and at a cholesterol : EYPC = 0.4 : 1 molar ratio, it is seen that the oxidation index is lower in the presence of cholesterol only after ~30 h of autooxidation (Fig. 6). In Fe^{2+}-induced EYPC oxidation, the induction phase was too short to observe any reproducible effect of cholesterol on the oxidation index and TBARS concentration during its duration. Cholesterol could also have some effect on the total yield

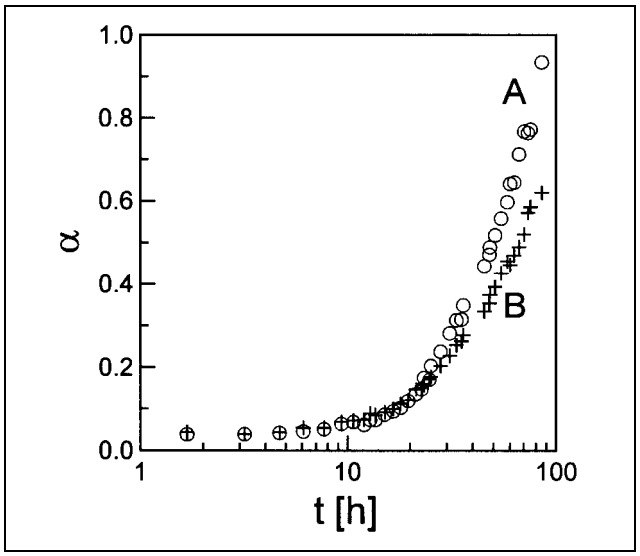

Fig. 6: Dependence of the EYPC oxidation index α on the duration t of autooxidation in the absence of cholesterol (curve A, symbols ○) and in the presence of cholesterol (curve B, symbols +) at cholesterol : EYPC = 0.4 : 1 molar ratio

of both EYPC autooxidation and Fe^{2+}-induced EYPC oxidation in the termination phase of these chain reactions. This will be a subject of our future experiments.

The inhibiting effect of cholesterol on the propagation phase of EYPC oxidation could be purely physical. In the induction phase, a hydrogen atom is abstracted from the polyunsaturated EYPC acyl chain

$$RH \rightarrow R^{\bullet} + H^{\bullet} \quad (1)$$

and the lipid free radical $R^{\bullet}$ with conjugated double bonds is formed. The lipid free radical then interacts with molecular oxygen and the reaction results in the lipoperoxyl free radical:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$

In the propagation phase, the lipoperoxyl free radical interacts with another phospholipid acyl chain RH:

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \quad (3)$$

resulting in the hydroperoxide ROOH with a conjugated diene structure and the new lipid free radical $R^{\bullet}$. The hydroperoxide decomposes forming new free radicals which can initiate subsequent chain reactions:

$$ROOH \rightarrow RO^{\bullet} + HO^{\bullet} \quad (4)$$

$$2\,ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \quad (5)$$

$$RO^{\bullet} + RH \rightarrow R^{\bullet} + ROH \quad (6)$$

$$HO^{\bullet} + RH \rightarrow R^{\bullet} + H_2O \quad (7)$$

The $R^{\bullet}$ and $ROO^{\bullet}$ free radicals then initiate new reactions 2 and 3, respectively. Since the cholesterol molecules intercalate between phospholipid molecules in the lipid bilayer and thus expand the bilayer laterally, the mean distance between phospholipid molecules is increased and the frequency of their encounters is decreased. This should decrease the rates of reactions 3, 5 and 6 above thus decreasing the overall rates of oxidation product formation in the propagation phase. The effect of cholesterol on EYPC oxidation could also be connected also with its effects on lipid bilayer hydration and increased packing in the bilayer [14, 17]. Cholesterol is largely nonpolar. According to Huang and Feigenson [41], when cholesterol molecules are intercalated into a phospholipid bilayer, phospholipid headgroups provide "cover" to shield the nonpolar part of cholesterol from exposure to water. The bilayer polar region and the space under the headgroups shared by acyl chains and cholesterols thus become tightly packed. This tight packing decreases the depth of water penetration into the bilayer [42], the hydration of the bilayer polar region [43, 44 and references therein], and the oxygen concentration in the bilayer [14]. The increased hydrophobicity of the bilayer could have an effect on reactions 5 and 7. Low concentration of oxygen could influence the yield of reaction 2 above and the tight bilayer packing could restrict the diffusion of $HO^{\bullet}$ free radical (reactions 4 and 7). The tight packing could also cause steric hindrance to all reactions involving lipid acyl chains.

However, the effect of cholesterol could be connected with its antioxidant properties [45]. It has been observed in multilamellar EYPC liposomes containing cholesterol, that Fe^{2+} and L-ascorbic acid induced loss of polyunsaturated EYPC acyl chains was accompanied by the epoxidation and hydroperoxidation of cholesterol [46]. When the same Fe^{2+} and L-ascorbic acid reaction took place in a

dispersion of dimyristoylphosphatidylcholine liposomes containing cholesterol, little oxidation was observed in the cholesterol fraction [46]. Cooxidation of polyunsaturated fatty acyl chains and cholesterol has also been observed during autooxidation in unilamellar liposomes containing bovine liver phosphatidylcholine and cholesterol [47]. These results indicate that cholesterol oxidation depends on peroxidation of polyunsaturated fatty acyl chains in liposomal phospholipids and that cholesterol can act as an antioxidant in the propagation phase.

In conclusion, we have observed that cholesterol inhibits EYPC autoxidation and Fe^{2+}-induced EYPC oxidation in multilamellar liposomes in the propagation phase of the oxidation free radical chain reaction. There are several possible mechanisms behind this effect of cholesterol. Inhibition of lipoperoxidation in liposomes by cholesterol can be used in various liposome formulations. Our results also indicate that the role of cholesterol in biological membranes is important not only in regulating of their fluidity but also in their protection against oxidative damage.

3. Experimental

3.1. Chemicals

Phosphatidylcholine from hen egg yolks (EYPC) was prepared, purified and analyzed by TLC according to Singleton et al. [48]. The Silufol chromatographic plates were from Kavalier (Sázava, Czech Republic). Cholesterol and tris(hydroxymethyl)aminomethane · HCl (TRIS · HCl) were obtained from Serva (Heidelberg, Germany), 2-thiobarbituric acid (TBA) and trichloroacetic acid (TCA) from Merck (Darmstadt, Germany) and butylhydroxytoluene (BHT) from Fluka (Buchs, Switzerland). The organic solvents and the other chemicals were purchased from Lachema (Brno, Czech Republic). For UV-VIS spectrophotometry, ethanol of spectral purity was used, the other commercial chemicals were of analytical purity. Water and the organic solvents except the ethanol of spectral purity were redistilled before use.

3.2. Multilamellar liposome preparation

EYPC and cholesterol were mixed in a chloroform-methanol (2 : 1) solution and then evaporated to dryness under a stream of pure gaseous nitrogen, followed by evacuation in a vacuum chamber using an oil vacuum pump and liquid nitrogen trap for evacuated traces of solvents. To the dry EYPC and cholesterol mixture, aqueous buffer solution (0.1 M KCl, 1 mM TRIS, pH 7.4) saturated with gaseous nitrogen was added. The glass tube with this EYPC + cholesterol dispersion was purged with pure gaseous nitrogen and sealed with Parafilm M (American National Can, Greenwich, USA). Multilamellar liposomes were prepared from this dispersion by vortexing and sonication in a Tesla UC 405 BJ-1 bath sonicator (Vráble, Slovakia) at 4 °C. The final EYPC concentration in all samples was 10 mg/ml.

3.3. Lipid peroxidation

Autoperoxidation of EYPC liposomes was conducted in glass tubes immersed in a shaking water bath thermostated at 45 °C. Immediately before immersion and heating, the tubes were opened and the liposome dispersion was aerated. At regular intervals during the reaction, the closed tubes were vortexed, opened, and aerated and small volumes were taken for analysis. Fe^{2+}-induced peroxidation of aerated EYPC liposomes in glass tubes was started by adding freshly prepared aqueous solutions of $FeSO_4$ and H_2O_2 to give a final concentration of 0.2 mM Fe^{2+} and 0.1 mM H_2O_2 in the sample. After vigorous mixing, the tubes were immersed in a shaking water bath thermostated at 20 °C. Small volumes of the final dispersion were taken for analysis at regular intervals.

The extent of EYPC peroxidation was measured by the estimation of conjugated dienes according to Klein [39] and of thiobarbituric acid reactive substances (TBARS) according to Haenen and Bast [49]. The conjugated dienes were quantified as the oxidation index $\alpha = A_{233}/A_{215}$ where A_{233} and A_{215} are the absorbances of the 0.25 g/l EYPC solution in ethanol at the wavelengths 233 nm and 215 nm, respectively. For the TBARS determination, the sample of aqueous liposome dispersion containing 1 mg of EYPC was mixed with 1.5 ml of aqueous TBA + TCA solution (4.21 g/l TBA and 168 g/l TCA) and with 150 µl of an ethanol BHT solution (1.5 g/l BHT). This mixture was heated at 80 °C for 20 min, than cooled and centrifuged (15 min at 3000 rpm). The TBARS in the supernatant were quantified by the absorbance A_{533} measured at 533 nm.

3.4. Data analysis

The experimental data were evaluated using the standard statistical methods described in [50] and the freeware PC program Nelireg [51].

Acknowledgement: We thank Prof. Ing. P. Kovács, DrSc. for his continuing interest in our work. This study was supported by the Slovak Ministry of Education grant 1/6039/99.

References

1 Lasic, D. D.: Liposomes: From Physics to Applications, Elsevier Science, Amsterdam 1993
2 Lasic, D. D.; Barenholz, Y.: Handbook of Nonmedical Applications of Liposomes, CRC Press, Boca Raton, Fl. 1996
3 Lasic, D. D.; Papahadjopoulos, D.: Medical Applications of Liposomes, Elsevier Science, Amsterdam 1998
4 Kirby, C.; Clarke, J.; Gregoriadis, G.: Biochem. J. **186**, 591 (1980)
5 Mišík, V.; Ondriaš, K.; Balgavý, P.: Gen. Physiol Biophys. **11**, 317 (1992)
6 Nakazawa, T.; Nagatsuka, S.; Yukawa, O.: Drugs Exp. Clin. Res. **12**, 831 (1986)
7 Hernandez-Caselles, T.; Villalain, J.; Gomez-Fernandez, J. C.: J. Pharm. Pharmacol. **42**, 397 (1990)
8 Deev, A. I.; Dobretsov, G. E.; Vladimirov, I.: Vopr. Med. Khim. **23**, 545 (1977)
9 Gutteridge, J. M.: Res. Commun. Chem. Pathol. Pharmacol. **22**, 563 (1978)
10 Mowri, H.; Nojima, S.; Inoue, K.: J. Biochem. (Tokyo) **95**, 551 (1984)
11 Montfoort, A.; Bezstarosti, K.; Groh, M. M.; Metsa-Ketela, T. J.: Biochem. Int. **15**, 525 (1987)
12 McLean, L. R.; Hagaman, K. A.: Free Radic. Biol. Med. **12**, 113 (1992)
13 Wiseman, H.: FEBS Lett. **326**, 285 (1993)
14 Parasassi, T.; Giusti, A. M.; Raimondi, M.; Ravagnan, G.; Sapora, O.; Gartton, E.: Free Radic. Biol. Med. **19**, 511 (1995)
15 Tirosh, O.; Kohen, R.; Katzhendler, J.; Alon, A.; Barenholz, Y.: Chem. Phys. Lipids **87**, 17 (1997)
16 Girao, H.; Mota, C.; Pereira, P.: Curr. Eye Res. **18**, 448 (1999)
17 Samuni, A. M.; Lipman, A.; Barenholz, Y.: Chem. Phys. Lipids **105**, 121 (2000)
18 Yeagle, P. L.: Biochim. Biophys. Acta **822**, 287 (1985)
19 Ondriaš, K.; Mišík, V.; Gergel', D.; Staško, A.: Biochim. Biophys. Acta **1003**, 238 (1989)
20 Mišík, V.; Švajdlenka, E.; Filípek, J.; Gergel', D.; Ondriaš, K.: Free Radical Res. Commun. **15**, 159 (1991)
21 Filípek, J.; Kovács, P.; Balgavý, P.: Biochem. Int. **26**, 79 (1992)
22 Przestalski, S.; Hladyszowski, J.; Kuczera, J.; Rozycka-Roszak, B.; Trela, Z.; Chojnacki, H.; Witek, S.; Fisicaro, E.: Biophys. J. **70**, 2203 (1996)
23 Filípek, J.: Pharmazie **47**, 717 (1992)
24 Filípek, J.: Pharmazie **47**, 393 (1992)
25 Gabrielska, J.; Oszmianski, J.; Lamer-Zarawska, E.: Pharmazie **52**, 170 (1997)
26 Gabrielska, J.; Oszmianski, J.; Zylka, R.; Komorowska, M.: Z. Naturforsch. [C.] **52**, 817 (1997)
27 Gabrielska, J.; Oszmianski, J.; Komorowska, M.; Langner, M.: Z. Naturforsch. [C.] **54**, 319 (1999)
28 Gergel', D.; Mišík, V.: Pharmazie **46**, 469 (1991)
29 Filípek, J.: PhD. Thesis, Faculty of Pharmacy, J. A. Comenius University, Bratislava 1992
30 Filípek, J.; Gelienová, K.; Kovács, P.; Balgavý, P.: Gen. Physiol. Biophys. **12**, 55 (1993)
31 Tardieu, A.; Luzzati, V.; Reman, F. C.: J. Mol. Biol. **75**, 711 (1973)
32 Galla, H.-J.; Sackmann, E.: Biochim. Biophys. Acta **339**, 103 (1974)
33 Galla, H.-J.; Hartmann, W.; Theilen, U.; Sackmann, E.: J. Membr. Biol. **48**, 215 (1979)
34 Lindblom, G.; Johansson, L. B.; Arvidson, G.: Biochemistry **20**, 2204 (1981)
35 Gallová, J.: PhD. Thesis, Faculty of Mathematics and Physics, J. A. Comenius University, Bratislava 1993
36 Hsia, J. C.; Schneider, H.; Smith, I. C.: Chem. Phys. Lipids **4**, 238 (1970)
37 Schreier-Muccillo, S.; Marsh, D.; Dugas, H.; Schneider, H.; Smith, C. P.: Chem. Phys. Lipids **10**, 11 (1973)
38 McIntosh, T. J.: Biochim. Biophys. Acta **513**, 43 (1978)
39 Klein, R. A.: Biochim. Biophys. Acta **210**, 486 (1970)
40 Rice-Evans, C. A.; Diplock, A. T.; Symon, M. R. C.; in: Burdon, R. H.; van Knippenber, P. H. (Eds.): Laboratory Techniques in Biochemistry and Molecular Biochemistry, pp. 125–128, Elsevier, Amsterdam 1991
41 Huang, J.; Feigenson, G. W.: Biophys. J. **76**, 2142 (1999)
42 Simon, S. A.; McIntosh, T. J.; Latorre, R.: Science **216**, 65 (1982)
43 Parasassi, T.; DiStefano, M.; Loiero, M.; Ravagnan, G.; Gratton, E.: Biophys. J. **66**, 763 (1994)

44 Krasnowska, E. K.; Bagatolli, L. A.; Gratton, E.; Parasassi, T.: Biochim. Biophys. Acta **1511**, 330 (2001)
45 Smith, L. L.: Free Radic. Biol. Med. **11**, 47 (1991)
46 Terao, J.; Sugino, K.; Matsushita, S.: J. Nutr. Sci. Vitaminol. (Tokyo) **31**, 499 (1985)
47 Sevanian, A.; McLeod, L. L.: Lipids **22**, 627 (1987)
48 Singleton, W. S.; Gray, M. S.; Brown, M. L.; White, J. L.: J. Amer. Oil Chem. Soc. **42**, 53 (1965)
49 Haenen, G. R.; Bast, A.: FEBS Lett. **159**, 24 (1983)
50 Kundračík, F.: Evaluation of Experimental Data, Faculty of Mathematics and Physics, J. A. Comenius University, Bratislava 1997
51 http://www.drp.fmph.uniba.sk/∼kundracik

Received June 5, 2001
Accepted July 4, 2001

Doc. Dr. P. Balgavý
Department of Physical
Chemistry of Drugs
Faculty of Pharmacy
Comenius University
Odbojárov 10
83232 Bratislava
Slovak Republik