Studies of Differently Induced Peroxidation Phenomena in Lecithins

Adriana Memoli,*,† Luisa G. Palermiti,† Valter Travagli,‡ and Franco Alhaique†

Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Facoltà di Farmacia, Università di Roma (La Sapienza), P.le A. Moro 5, 00185 Rome, Italy, and Dipartimento Farmaco Chimico Tecnologico, Università di Siena, Siena, Italy

Egg and soya phospholipids, in the form of liposomes, were analyzed to evaluate lipid peroxidation by means of UV and fluorescence measurements. Oxidation processes were induced by different methods: sonication, UV radiation, and incubation with ferrous ions/ascorbic acid. Aging effect and pH influence were also investigated. The obtained results showed that tested vegetable phospholipids, because of their higher content in polyunsaturated fatty acids, were in all cases much more rapidly oxidized with respect to the animal ones; furthermore, an inverse relationship between the starting hydroperoxide level and the oxidation rate of phospholipids was observed. In the case of soya lecithin a sharp increase in the peroxidation rate was appreciated in slightly alkaline environmental conditions. No effects on peroxidation were ever observed when UV radiations with $\lambda = 254$ nm were used.

Keywords: *Lipid peroxidation; egg lecithins; soya lecithins*

INTRODUCTION

In recent years considerable attention has been devoted to the chemical stability of dietary lipids with particular emphasis on the need of a continuous control over manufacturing, handling, and storing conditions in order to limit oxidation phenomena. Oxidation in fact can induce changes in the taste and/or in the odor of lipids and can affect membrane functions (Halliwell and Gutteridge, 1985; Wolff et al., 1986); furthermore, atherogenic, cytotoxic, and carcinogenic effects have been related to the presence of oxidized lipids in food (Addis and Warner, 1991).

Most studies in this field are focused on the oxidative degradation, and in particular peroxidation, that takes place in edible oils (Nourooz-Zadeh et al., 1995) and in polyunsaturated fatty acids (PUFA) of lecithins (Lang and Vigo-Pelfrey, 1993). PUFA peroxidation, whose mechanism was deeply investigated (Yamamoto et al., 1984), is a mixed chemical process, involving the formation and the propagation of lipid radicals with a final rearrangement of the original double bonds.

On the basis of preliminary studies (Memoli et al., 1993), the aim of this work is to compare the peroxidation rates of pure and commercial grade (much less expensive) L-α-phosphatidylcholine of animal (egg yolk) and vegetable (soybean) origin. Owing to the insolubility of lecithins in water, a useful tool for the study of PUFA moiety peroxidation in these substances is the preparation of homogeneous dispersions of aggregated structures such as liposomes in the form of multilamellar vesicles (MLV) or small unilamellar vesicles (SUV). This type of approach acquires a particular interest if we consider the ever increasing use of liposomes in the field of the agrofood industry (Arnaud, 1995; Quinn and Perret, 1996).

Experiments on SUV and MLV preparations were carried out in different environmental conditions, and the effect of the presence of tocopherol on the actual stability of the tested phospholipids was also investigated.

UV absorbance and fluorescence measurements were used for the quantitative evaluation of lipid peroxidation.

MATERIALS AND METHODS

Materials. A 90% amount of pure enriched egg phosphatidylcholine (Phospholipon 90 Egg, P90 egg; Nattermann Phospholipids GmbH) and 90% pure enriched soya phosphatidylcholine (Phospholipon 90, P90 soya; Nattermann) were the generous gift of M.I.R.E.D. (Milan, Italy); 99% pure L-α-phosphatidylcholine from egg yolk (EPC) and 99% pure L-α-phosphatidylcholine from soybean (SPC) (type V-E, 100 mg/mL chloroform:methanol (9:1) solution and type III-S, 100 mg/mL chloroform solution, respectively) were purchased from Sigma Chemical Co. Further information about the composition of the phospholipids used for the present investigation is given in Table 1.

KCl (10^{-1} M) or pH 7.4 HEPES buffer solution (10^{-3} M), obtained with freshly distilled and deaerated water, were used.

Cholesterol, *d,l*-α-tocopherol, ascorbic acid, $FeSO_4 \cdot 7H_2O$, glycine, absolute EtOH, KCl, and all the other products used for the present investigation were of analytical grade.

Apparatus. UV measurements were carried out by means of a Perkin-Elmer Lambda 3A spectrophotometer equipped with 10 mm quartz cells.

Fluorescence measurements were obtained by means of a Perkin-Elmer LS5 spectrofluorometer.

Shaking for MLV preparation was carried out with a vortex (VELP scientifica, ZX type).

Sonication was performed with a Soniprep 150 apparatus (MSE, Crowley) equipped with an exponential microprobe, operating at 23 kHz and an amplitude of 6 μm.

UV irradiation was performed by means of APQ 40 and APB 40 annular photoreactors (Applied Photophysics Ltd.) equipped with mercury lamps (low-pressure lamp, 16 W emitting at $\lambda = 254$ nm, or medium-pressure lamp, 125 W, emitting at $\lambda = 365$ nm).

Phospholipid Dispersion. The techniques used to obtain a homogeneous dispersion of the phospholipids led to multilamellar and unilamellar vesicles. Furthermore, since sonication carried out in the presence of air causes faster peroxidation of the phospholipids, this technique was chosen both

* Author to whom correspondence should be addressed.

† Università di Roma.

‡ Università di Siena.

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Table 1. Fatty Acids (% w/w) in the Tested Phospholipids

fatty acid (C atoms:double bonds)	EPC and P90 egg[a]	SPC and P90 soya[b]
14:0	0.3	0.1
16:0	28−38	10−15
16:1	1.1	0.2
18:0	9−18	1−5
18:1	25−37	6−13
18:2	5−18	61−71
18:3	0.5	4−8
20:4	1−7	

[a] Maximum percentage (w/w) of the other main components of P90 egg: lysophosphatidylcholine, 4%; sphingomyelin, 4%; phosphatidylethanolamine, 1%; water, 1%; tochopherol, 0.2% [b] Maximum percentage (w/w) of the other main components of P90 soya: lysophosphatidylcholine, 6%; water, 1.5%; ethanol, 0.5%; tocopherol, 0.2%.

for lecithin dispersion (in the form of SUV) and as an oxidation-inducing method.

(A) Multilamellar Vesicles. A 80 or 45 mg sample of the phospholipid (according to the specific measurement carried out on the preparation as indicated in each case) was dissolved in 2.5 mL of $CHCl_3$. The solvent was vacuum evaporated to form a thin film inside the vessel, and the residue, after dispersion in 5 mL of the HEPES buffer (pH = 7.4) or of the KCl aqueous solution (pH = 6.0), was shaken with the vortex for 30 min at room temperature. The final phospholipid concentrations were 16 and 9 mg/mL, respectively. As indicated below, for some experiments the preparations needed a further dilution with the dispersing solution.

(B) Small Unilamellar Vesicles. MLV (16 mg/mL of phospholipid) were sonicated for different intervals of time; the temperature was maintained at 15−20 °C by means of a water bath.

Evaluation of Lipid Peroxidation. Lipid peroxidation was determined by means of two different techniques based on the determination of the "oxidation index" (Klein, 1970) and on the formation of fluorescent products during the induced peroxidation reactions (Ohyashiki et al., 1991; Shimasaki, 1994).

The "oxidation index" was expressed by the ratio between the absorbance maximum near 233 nm (slight differences of wavelength maxima have been detected with the various tested products), directly related to peroxidation, and the phospholipid absorbance at 210 nm. For this purpose 100 μL samples (16 mg/mL in 0.1 M KCl) were taken at fixed time intervals during sonication from the SUV dispersion and appropriately diluted with absolute EtOH for UV absorbance determinations. The oxidation index was also determined during UV irradiation of the phospholipids in the form of a thin film and as MLV dispersions.

The formation of fluorescent products during lipid peroxidation was obtained by incubation of a diluted dispersion of MLV (phospholipid concentration, 3 mg/mL) in a solution containing ascorbic acid (100 μM), $FeSO_4$ (10 μM), and glycine (100 mM). The mixture was kept at 37 °C, under constant stirring. At fixed time intervals aliquots of the incubated samples, diluted 1:10 with absolute EtOH or HEPES, were analyzed with the spectrofluorometer. Excitation and emission wavelengths were set at 360 and 437 nm, respectively.

Dissolution in EtOH allowed avoidance of the use of the cutoff filter (Ohyashiki et al., 1991) that would have been necessary to prevent the error due to the light scattering of the sample in the aqueous solvent. Appropriate measurements were previously carried out in order to verify the consistency of the results obtained from the measurements in the water dispersion and in EtOH.

UV Irradiation. Irradiation was performed on samples both in aqueous dispersion and in a dried thin film state.

In the first case, the UV absorbance of appropriately diluted (phospholipid concentration, 0.5 mg/mL) MLV aliquots was investigated after exposing the samples, contained in quartz vessels, to UVA (λ = 365 nm) or UVB (λ = 254 nm) by means of a continuous magnetic stirring system.

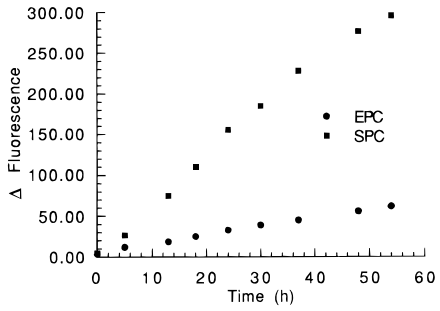

Figure 1. Fluorescence development in time in EPC and SPC liposomes incubated with ascorbic acid/Fe^{2+}/glycine. Δ fluorescence is the difference between the fluorescence intensities determined with and without glycine (λ_{ex} = 360 nm; λ_{em} = 437 nm).

Lipid films were obtained by spreading a $CHCl_3$ solution of phospholipid on a fixed surface area of identical glass slides and gently evaporating the solvent under a nitrogen atmosphere. The final $CHCl_3$ residue was then removed by vacuum evaporation. Lipid films (0.30 ± 0.03 mm) were then directly dissolved, at different times, in absolute ethanol and appropriately diluted for UV analysis.

All of the results here reported represent the mean values obtained from at least four separate experiments that always presented good reproducibility (CV ≤ 5%).

RESULTS AND DISCUSSION

Fluorescence Measurements. Lipid peroxidation was induced by means of $FeSO_4$ and ascorbic acid (Ohyashiki et al., 1991; Shimasaki, 1994). Figure 1 shows the results obtained with MLV prepared from pure soybean or egg phospholipids in the HEPES solution. As can be observed, the vegetable phospholipids were much more rapidly oxidized with respect to the animal ones; this result is to be related to the different percentage of PUFA in the tested phospholipids, in particular those sited at the sn-2 position (i.e., oleic acid between 25 and 37% in egg yolk PC and between 6 and 13% in soya PC; linoleic acid between 5 and 18% in egg PC and between 61 and 71% in soya (Läutenschlager et al., 1988; Pikul and Kummerow, 1991).

When the commercial products (P90 egg and P90 soya), which contained 0.2% of *d,l*-α-tocopherol as antioxidant, were tested in the experimental conditions reported above, comparable results allowed confirmation of the different behavior according to the origin of the phospholipid.

Since the peroxidation rate of egg phosphatidylcholine was always negligible in the tested experimental conditions, the effect of *d,l*-α-tocopherol on the chemical stability of phosphatidylcholine was studied only with the soya products. Results, reported in Figure 2, indicate that as the tocopherol concentration increased, a corresponding decrease of the oxidation rate was obtained. It is interesting to point out that no difference was ever observed when *d,l*-α-tocopherol was already present during the liposome preparation or when it was added to the preformed liposomes which were kept overnight under a nitrogen atmosphere.

Oxidation Index Measurements. The variation, as a function of time, of the oxidation indexes obtained during sonication of aqueous dispersions of the vegetable phospholipid (16 mg/mL) was studied. Sonication was carried out in the presence of air in order to induce more rapid peroxidation processes. In these experi-

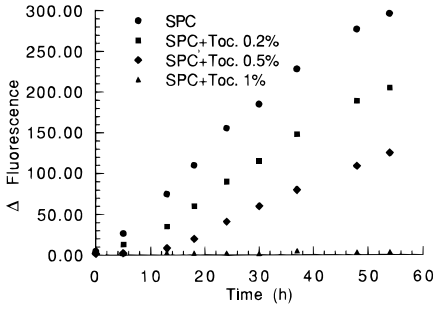

Figure 2. Effect of increasing *d*,*l*-α-tocopherol concentration (Toc. % (w/w)) on the fluorescence development of SPC liposomes incubated with ascorbic acid/Fe^{2+}/glycine. Δ fluorescence is the difference between the fluorescence intensities determined with and without glycine ($\lambda_{ex} = 360$ nm; $\lambda_{em} = 437$ nm). No differences in oxidation were observed for Toc. concentrations of 1% or above (e.g., 2 and 3%).

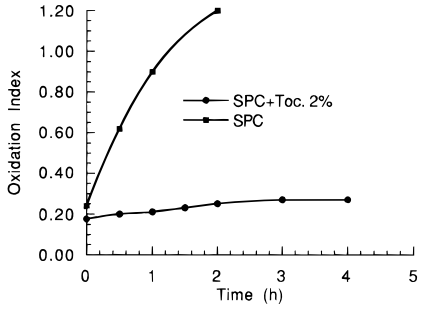

Figure 3. Oxidation index determined during sonication of SPC, alone or in the presence of *d*,*l*-α-tocopherol (2% (w/w)).

ments both pure and 2% *d*,*l*-α-tocopherol-added soya phosphatidylcholine (SPC) were used. The plots reported in Figure 3 show how, as expected, the oxidation rate was once more remarkably reduced by the presence of the antioxidant. Furthermore, the results obtained indicate that small amounts of oxidized PUFA moyeties (oxidation index ≅ 0.2) were already present since the very beginning of the experiment, i.e., as the sealed container was opened.

Also in these experimental conditions the oxidation index of liposomes obtained from egg phospholipids was negligible with respect to that determined with soya phospholipids.

Aging Effect. For this purpose several soya PC samples, kept in the original sealed container at 0 ± 2 °C for long periods of time, were used and tested for the oxidation index against the corresponding fresh product (less than 2 months from the preparation as it was possible to assume from the batch number of the products). As in the previous experiments, the phospholipid aqueous dispersions (0.1 M KCl, 16 mg/mL) were sonicated and the oxidation index was determined at different time intervals. Results reported in Figure 4 (which refers to P90 soya products) indicate that although the product was stored in optimized conditions (i.e., dry and sealed at a maximum temperature of 4 °C, as indicated by the manufacturer), as the containers were opened after 1 and after 2 years, a remarkable increase of the initial oxidation index was detected with respect to the more recently-obtained product. Furthermore, quite unexpectedly but in accordance with the recent observations of Bose and Chatterjee (1994) on lipid peroxidation following UV exposure, the obtained results showed an inverse relationship between the initial level of hydroperoxides and the oxidation rate during sonication. This last effect can be related to the

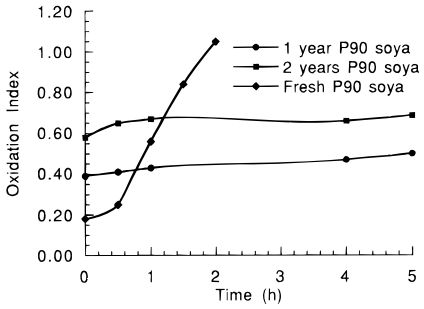

Figure 4. Oxidation index determined during sonication of differently-aged P90 soya samples.

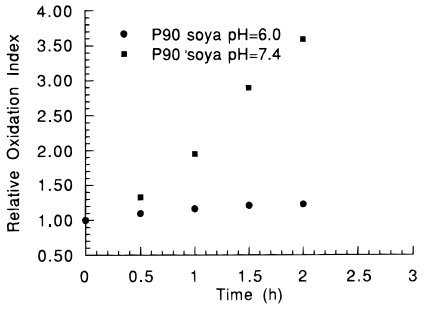

Figure 5. Relative oxidation index of P90 soya during sonication at different pH conditions. Results are expressed as the ratio between the values of oxidation index obtained at different times and those of the references determined just before sonication.

higher concentration, in the older samples, of free radicals that increase the probability of a termination step of the overall oxidation reaction. The same results were obtained when pure soya phospholipid was tested; consequently, it can be assumed that the impurities present in P90 did not affect these oxidation processes.

pH Effect. Hydrogen ion concentration has been reported to be capable of affecting biological free radical reactions (Ohyashiki et al., 1993; Fernandes et al., 1995); thus sonication experiments were repeated at different environmental pH conditions (i.e., pH 6.0 KCl, according to Klein (1970), and pH 7.4 HEPES). Figure 5 shows the increase of the oxidation index, during sonication of the SUV obtained from P90 soya in the two solutions. As can be observed, a remarkably different oxidation rate was obtained: in the alkaline medium a sharp increase of the peroxidation rate occurred, while in the acidic pH conditions the oxidation became almost negligible.

No significant difference in the oxidation rate was detected when the egg phospholipid was used, probably because of the slower oxidation rate of this last product.

UV Irradiation Effects. Lecithins, commonly used alone or in food preparations, can be occasionally exposed to sunlight (e.g., during storage, transport, shelf life, etc.); at the same time the influence of UV radiation on radical reactions, and consequently on the oxidation of these substances, is well-known; thus, we studied the possibility of inducing peroxidation processes by phospholipid irradiation at 254 nm (UVB) or at 365 nm (UVA), both in the solid state and in an aqueous dispersion.

Figure 6 shows the effects of UVA radiation on the oxidation of P90 soya and P90 egg in the form a thin film (solid state). Both phospholipids show a remarkable UV-induced peroxidation rate, and once more the vegetable phosphatidylcoline was oxidized faster and in a higher degree than the animal one.

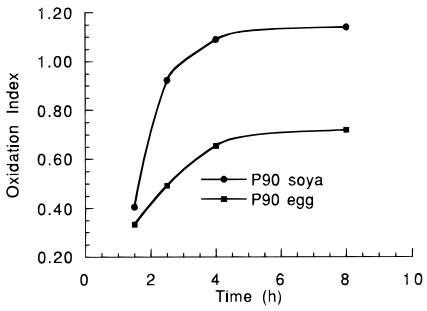

Figure 6. Effect of UVA radiations on the oxidation of P90 soya and P90 egg in the form of a thin film.

When aqueous dispersions of phospholipids (in the form of MLV) were exposed to UVA radiations, the rate of the process (followed by means of the oxidation index) almost doubled with respect to that observed with the thin film, and again a difference between egg and soya phospholipid was found. The different peroxidation rate between the solid state and the vesicular preparation can be related to the larger surface area of the irradiated samples and to the presence of oxygen also in the MLV dispersion (Barclay, 1993).

No effects were observed in the UVB-irradiated samples. Dried films and aqueous dispersions were exposed to UVB for periods of time ranging from 0.5 to 24 h.

All data reported emphasize the importance of optimizing handling, storage, and environmental pH conditions, in order to avoid or, at least, minimize the peroxidation phenomena of lecithins.

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