ANTIOXIDANT ACTIVITY OF ASCORBIC ACID AGAINST PEROXIDATION OF PHOSPHATIDYLCHOLINE LIPOSOMES EXPOSED TO GAMMA RADIATION: A SYNERGISTIC INTERACTION?

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Abstract. Ionizing radiation induced damage of cellular membrane is known to alter many structural and physiological processes leading to the loss of the normal cellular function including cell death. The present study was designed to determine the changes in bilayer permeability in egg lecithin multilamellar vesicles after exposure to gamma-radiation at doses of 500 Gy and 1 kGy. Liposomal changes in permeability were monitored by measuring the leakage of pre-encapsulated 6-carboxyfluorescein (CF). The changes in permeability in the bilayer were found to be dependent on radiation dose. The presence of hydrophilic antioxidant ascorbic acid (0.1 mM) in the aqueous medium drastically increased the leakage of carboxyfluorescein from liposomes. IR and NMR studies which have been employed to reveal structural alterations in irradiated vesicles showed an increased damage upon exposure to radiation in the presence of ascorbic acid. The proton nuclear magnetic resonance (^{1}H NMR) spectrum of the irradiated lipid vesicles (in the presence of ascorbic acid) showed a preponderance of signals attributable to new compounds formed as a consequence of the degradation process. The obtained results contribute to our understanding of the mechanism of radiation oxidative damage and its modification by radical scavenging and/or organizational modulation, which emphasize the importance of structure and composition of antioxidant in developing new strategies to reduce the damaging effects of ionizing radiation.

Key words: Gamma-radiation, liposome, carboxyfluorescein, ascorbic acid, nuclear magnetic resonance, infrared spectroscopy, fluorescence, electron microscopy.

INTRODUCTION

The concept of cell membrane phospholipids has turned from their passive, structural role in the buildup of structural components [29] to the more active, functional regulation of cell functions [10, 25, 32]. They are now considered a vital element in initiating a variety of cell functions. One of the most important roles is their function as intracellular second messengers. The second messengers are essential in the control of cellular functions, like stimulation of cell proliferation

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and differentiation. There is now increasing evidence to suggest that choline phospholipids may function as second messengers and may be involved in activation of mitogenic signal transduction [1, 5, 9]. For these reasons, the experimental investigation of the radiation effects at the membrane level is of high scientific interest.

Oxidative damage of membrane is mediated by the free radicals generated in the cytosol and in the surrounding of the cell, which are believed to be involved in radical chain reactions [22]. Polyunsaturated fatty acyl chains in biological membrane are the critical targets, which display susceptibility to attack by the radiation induced free radicals. Liposomes offer a suitable model membrane system to investigate the molecular mechanism of membrane damage induced by radiation since the composition of liposomes can be easily controlled and varied [31]. Previous results have demonstrated that the presence of cholesterol in the composition of egg lecithin liposomal membrane produced a modulating effect on structural order of the membrane bilayer, which reflected in significantly protecting liposomes against radiation damage.

The antioxidant properties of ascorbic acid and of its lipophilic derivatives have been the object of several papers [6, 13]. However, there is suspicion that ascorbic acid can both act as a strong, efficient, and cheap antioxidant agent on one level, and at the same time behaves as a radical promoter on a different level, and produces dangerous species in living systems [16].

This report describes results on radiation effects on liposomes prepared from egg phosphatidylcholine (Egg Pc) and concerned the protective effect of ascorbic acid as a valuable antioxidant material. We have employed a fluorescence probe, carboxyfluorescein (CF), to detect the changes in permeability of liposomal membrane [23]. It is shown that irradiation induced membrane damage exploited by the increased release of the fluorescent material with radiation dose. Results have further shown that ascorbic acid synergizes the effect of radiation by enhancing the release of carboxyfluorescein from liposomes. Nuclear magnetic resonance and infrared study confirmed the synergistic effect of ascorbic acid in protecting the damage to liposomal membrane against gamma radiation.

MATERIALS AND METHODS

MATERIALS

Egg phosphatidylchline, carboxyfluorescin, and ascorbic acid were procured from Sigma. Sephadex G-50 coarse was a product of Pharmacia Biotech. Aqueous solutions were prepared in distilled water. Fluorescence and infrared spectroscopy (IR) measurements were made using phosphate buffer saline (PBS) filtered through 0.22 micrometer membrane filter. Organic solvents (ethanol, chloroform, ethyl acetate, diethyl ether) were obtained from Merck (Darmstadt, Germany) and used without purification.

METHODS

Preparation of multilamellar liposomes encapsulating CF

Liposomes were prepared by the film hydration method [14]. Phospholipids were dissolved in chloroform in a rounded bottom flask. The organic solvent was evaporated under vacuum leaving a uniform thin film on the inner walls of the flask. The film was hydrated with 1 ml of 10 mM PBS (pH 7.4) to give a lipid concentration of 10 μmol/ml. Multilamellar liposomes were formed by constant vortexing for 4 min in a vortex mixer and sonication in a bath sonifier for 10 min. During hydration, the temperature of egg Pc liposome suspension was maintained at 45 °C.

Ascorbic acid was dissolved in PBS buffer at a concentration of 0.1 mM and mixed with the liposome solution.

To encapsulate CF in egg Pc vesicles, the lipid film was hydrated with 100 mM CF solution prepared in PBS followed by usual procedure of sonication. The multilamellar vesicles obtained after sonication were filtered through Sephadex G-50-gel minicolumn to separate the non-encapsulated probe material [15].

Irradiation of liposomes

The multilamellar liposomes prepared from egg Pc were irradiated at room temperature by ^{137}Cs γ-rays (500 Gy, 1 kGy) at the National Institute of Standards (Egypt, Cairo). Liposome suspension was diluted to a suitable concentration with PBS buffer before irradiation. The fluorescence of CF was not changed after irradiation at the reported radiation doses.

Scanning electron microscopy

Scanning electron microscopy was performed with a JEOL Model JEM 100 S/Japan, capable of magnification up to × 200,000, a resolution power of 5 nm, and accelerating voltage from 40 to 100 kV. Vesicle size distribution was determined by measuring the diameter of fractured vesicles as previously reported [11]. The vesicle average diameter was 200 nm.

Determination of vesicle permeability

Membrane permeability in irradiated egg Pc vesicles was monitored by the leakage of CF from liposomes after exposure to a dose of 500 Gy and 1 kGy, followed by incubation at room temperature up to 3 h. Self-quenched concentration of CF (> 50 mM) in liposomes developed an intense fluorescence when it was diluted after leakage in outer aqueous environment. The fluorescence intensity of CF was measured at room temperature (λ_{ex} = 490 nm, and λ_{em} = 520 nm) by

spectrofluorimeter type Schimadzu. Permeability of liposomes was expressed in terms of relative fluorescence intensity, which was the ratio between intensity of a particular liposome sample and the total fluorescence obtained by disruption of liposomes, by Triton X-100 (0.01%) and multiplied by 100.

Infrared measurements

A film of small amount of liposome sample (~ 2 μl) was deposited between two disks of potassium bromide, avoiding the presence of air. For each sample the spectra were recorded 3 times with 16 scans from 4000 to 1000 cm^{-1} on a Jasco model v-570 infrared spectrometer.

NMR measurements

^1H NMR measurements were carried out on an Oxford XL-300 at 300 MHz. Between 16 and 128 scans were used. The ^1H chemical shifts are reported in ppm relative to tetramethylsilane (δ 0.0) as internal reference. All measurements were performed at room temperature. The solvent used was $CDCl_3$.

RESULTS

CHARACTERIZATION OF LIPOSOMES USED

Liposomes formed by egg Pc were prepared by using the hydration method yielding 200 nm liposomes. The vesicles were analyzed by electron microscopy. Figure 1 shows the size distribution analysis of the resultant liposome sample.

CF FLUORESCENCE IN IRRADIATED VESICLES

Egg phosphatidylcholine liposomes encapsulated with CF were γ-irradiated at two radiation doses (500 Gy & 1 kGy). The fluorescence intensity of liposomes measured after 3 hours of incubation at room temperature showed that the degree of CF leakage from the Pc liposomes was dependent on the applied radiation dose as is clear from Figure 2. The percent release of CF from vesicles irradiated at 500 Gy dose was 30 times higher compared to control, which was found to increase at a higher radiation dose (1 kGy). These findings are clearly illustrated in Figure 3.

Addition of ascorbic acid to the buffer medium at a concentration of 0.1 mM caused a dramatic increase in the fluorescence intensity upon gamma-irradiation. The increase in fluorescence intensity was beyond the measurable level, and therefore the precise percent of release could not be calculated.

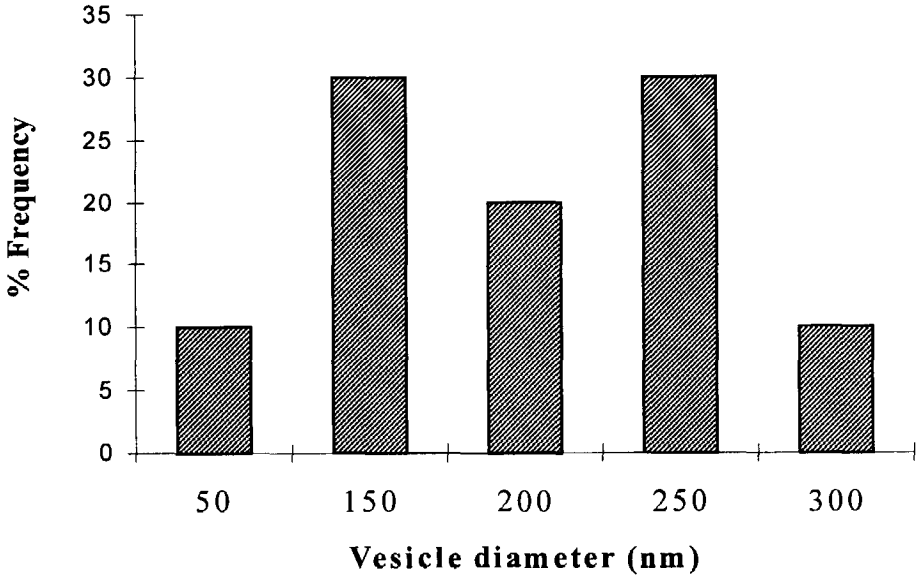


Fig. 1. – Size distribution of egg Pc vesicles obtained from electron microscopy.

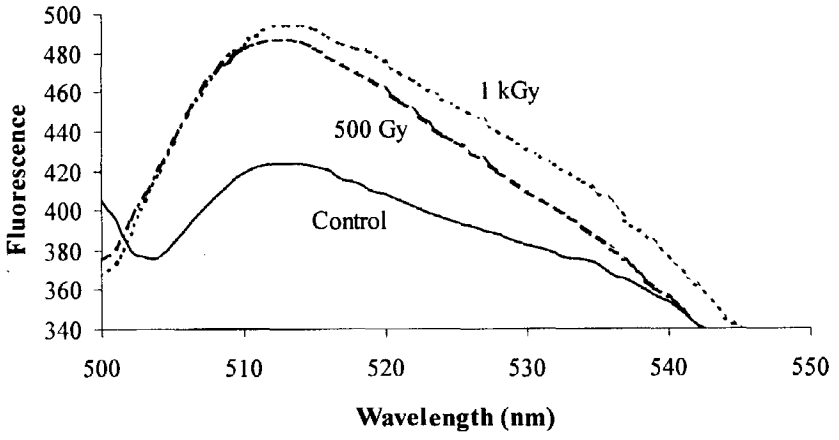


Fig. 2 – Schematic fluorescence emission spectra of 5(6)-carboxyfluorescein obtained on three differently treated liposome samples in a 0.5 cm quarts cuvette.

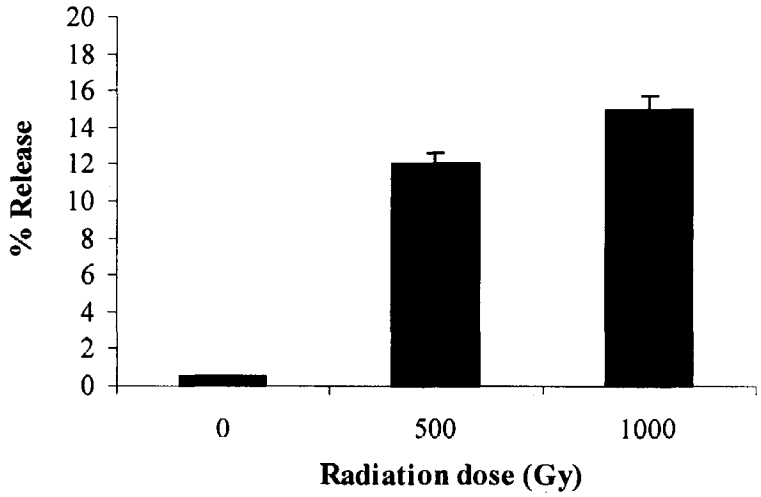


Fig. 3. – Effect of γ radiation dose on the release of 5(6)-carboxyfluorescein from liposomes composed of egg Pc.

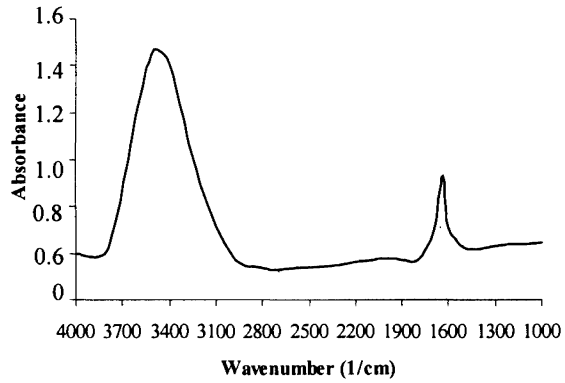
INFRARED MEASUREMENTS

The infrared spectra of membrane lipids can be separated to great advantage into spectral regions, which originate from molecular vibrations of the hydrophilic head-group and those of the hydrophobic hydrocarbon tail. Figure 4a shows the IR spectrum of control (non-irradiated) egg Pc liposome sample. The internal vibrations of the lipid acyl chains are readily assigned on the basis of the well-known dispersion curves of polymethylenes [8] and by comparison with infrared spectra of fatty acid esters and other polymethylene-chain compounds [4].

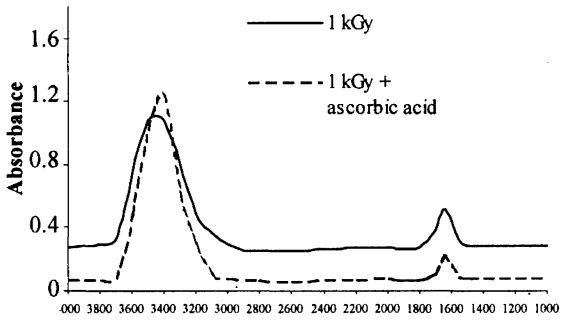
The IR spectrum in Figure 4.a shows a clear peak at 3480 cm^{-1} and another peak at 1640 cm^{-1}. The first peak is due to the carbon-hydrogen-stretching vibration. This peak is generally the strongest band in the spectra of lipids and the frequencies of these bands are conformation sensitive [3]. The second peak is characteristic for the head group modes [7] particularly the C=O stretching band.

Irradiation of the lipids at 500 Gy dose (Fig. 4b) caused a pronounced change in the frequencies corresponding to acyl chain conformation (decrease in frequency from 3480 to 3460 cm^{-1}) and in the frequencies corresponding to head group modes (increase in the frequency from 1640 to 1650 cm^{-1}).

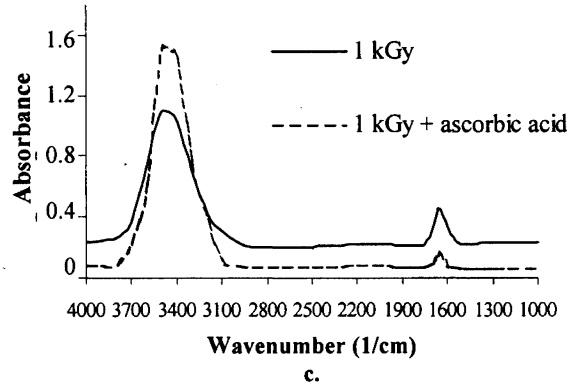
Addition of ascorbic acid to the liposome sample during γ irradiation at 500 Gy caused a dramatic decrease in the frequency of the first band to 3400 cm^{-1} and an increase in the frequency of the second band to 1660 cm^{-1}.



a.



b.



c.

Fig. 4. – Infrared spectra of aqueous dispersions of egg Pc exposed to different doses of gamma radiation. **a.** For control non-irradiated liposome sample; **b.** for samples exposed to 500 Gy in the presence and absence of 0.1 mM ascorbic acid; **c.** for samples exposed to 1 kGy in the presence and absence of 0.1 mM ascorbic acid.

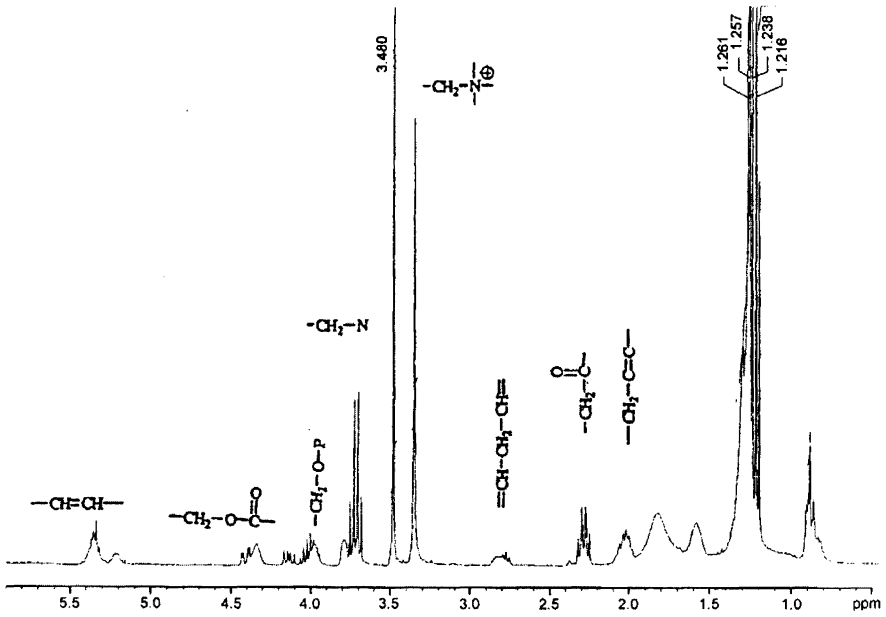


Fig. 5. – The 300 MHz ^{1}H NMR (CD Cl_3) spectrum of the non-irradiated liposome sample composed of egg Pc.

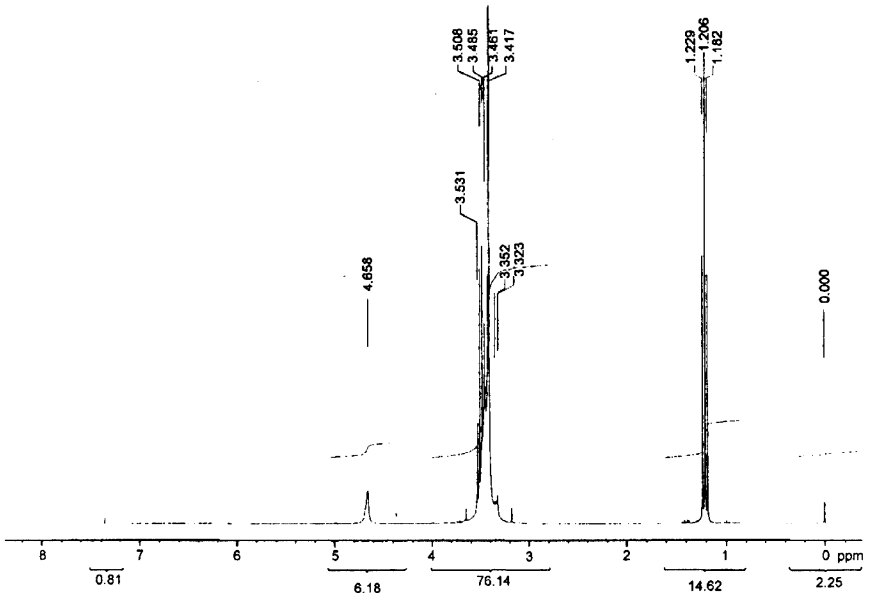
Irradiation of the lipid at 1kGy dose (Fig. 4c) did not show any change in the frequency band of the first peak. However, an increase in the second band frequency to 1650 cm^{-1} was indicated.

Addition of ascorbic acid to the liposome sample during γ-irradiation caused an increase of the frequency of the second band up to 1660 cm^{-1} without any effect on the first frequency band (3480 cm^{-1})

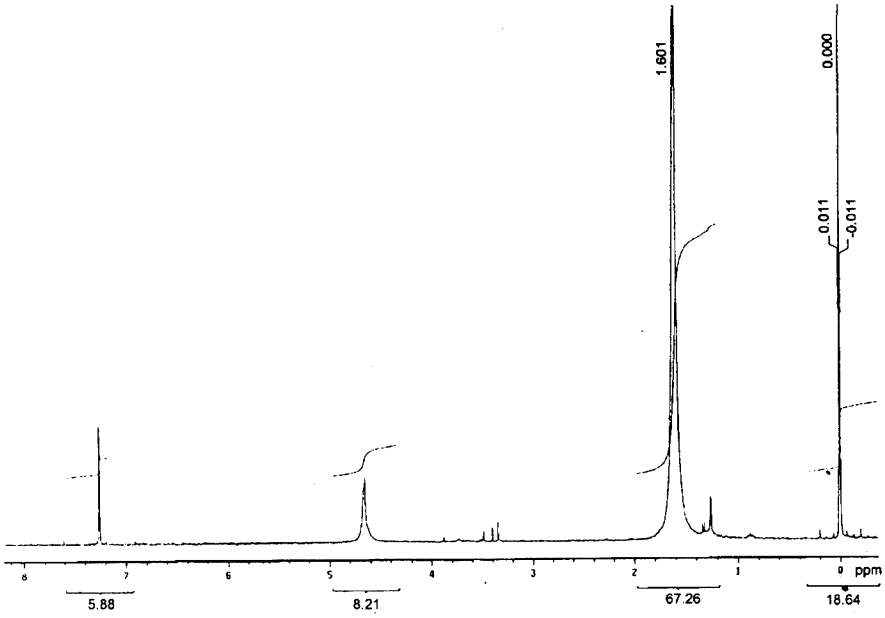
NMR MEASUREMENTS

As Pc was the major component of all the liposomes, signals from this lipid served as a reference for comparing the intact liposomes with those irradiated with gamma radiation. Figure 5 shows the ^{1}H NMR spectrum of intact Pc from a control non-irradiated sample. The singlet signal at ≈ 3.3 ppm corresponds to $(CH3)_3$–N^+, a multiplet at ≈ 3.7 ppm assigned to CH_2–N^+, and the signal at ≈ 4 ppm, attributable to the CH_2–O group. All the signals observed and indicated on the graph were assigned with the help of references and NMR-tables [17, 18].

Irradiating the lipids with gamma radiation at a dose of 500 Gy displayed the ^{1}H NMR spectrum in Figure 6a. The spectrum shows the disappearance of the peaks from 1.5 – 3 ppm and the appearance of a new singlet peak at 4.658 ppm.



a.



b.

Fig. 6. – The 300 MHz ^{1}H NMR (CD Cl_3): **a.** the spectrum of liposome sample exposed to 500 Gy γ radiation dose; **b.** the spectrum of the liposome sample exposed to the same radiation dose in the presence of 0.1 mM ascorbic acid.

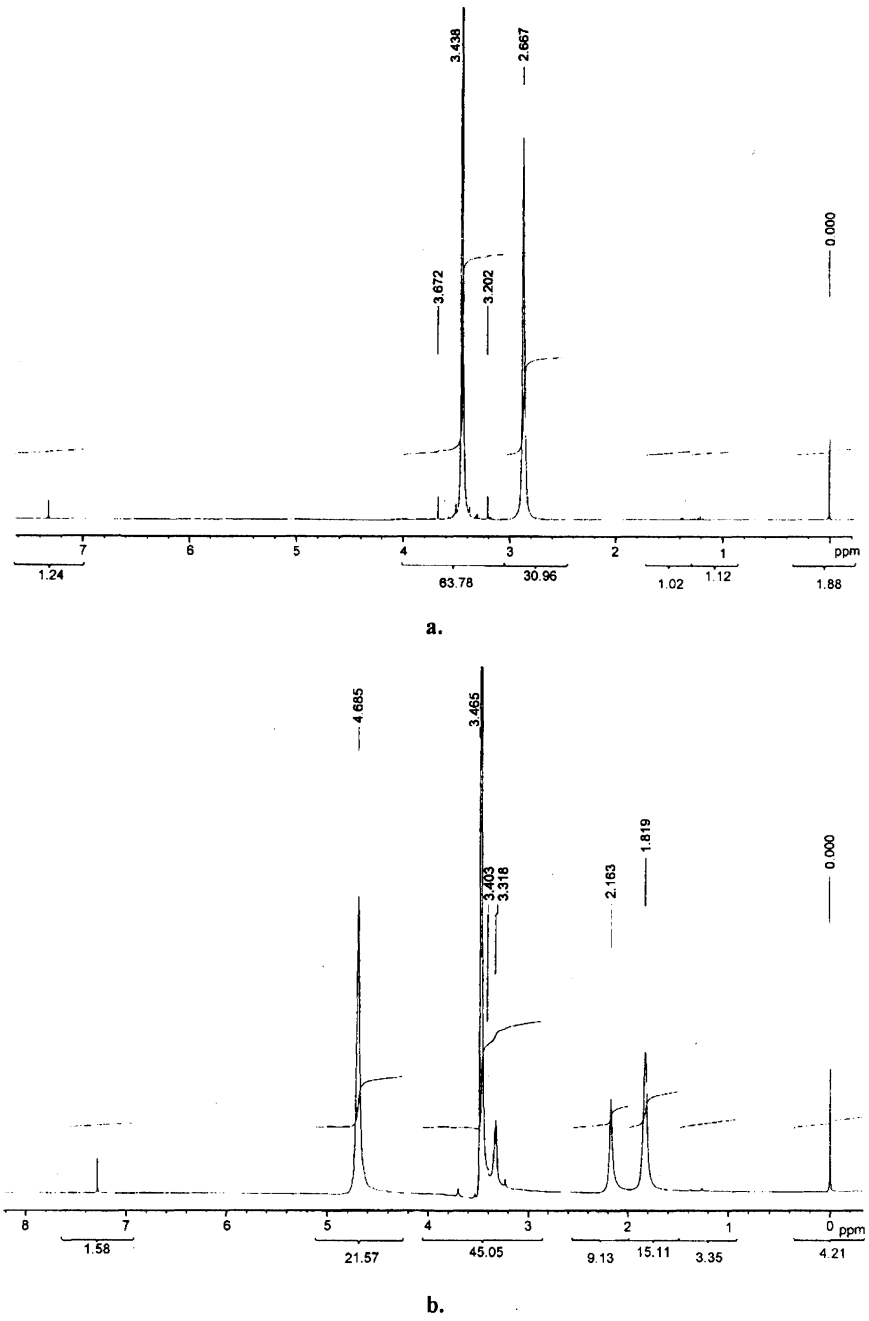


Fig. 7. – The 300 MHz ^{1}H NMR (CD Cl_3): **a**. the spectrum of liposome sample exposed to 1 kGy γ-radiation dose; **b**. the spectrum of the liposome sample exposed to the same radiation dose in the presence of 0.1 mM ascorbic acid.

Irradiating the lipids with gamma radiation at the same dose and in the presence of ascorbic acid (0.1 mM) displayed the ^{1}H NMR spectrum in Figure 6b. The spectrum shows the disappearance of the peaks in the region from 3 – 4 ppm. These are attributable to new compounds having been formed as a consequence of the degradation process originated from gamma irradiation.

More and more changes in the NMR spectrum were detected when the lipids were exposed to a radiation dose of 1 kGy, as indicated in Figure 7a. The presence of ascorbic acid (0.1 mM) during the irradiation procedure (Fig. 7b) shows a strong sharp peak at 4.685 ppm that could be attributed to the oxidation of the double bonds.

DISCUSSION

The behavior of cell growth and its transmembrane properties post-irradiation is one of the most interesting studies in biological sciences [28, 30]. However, the mechanism of radiation induced effects in biological membrane and subcellular components seem to be highly complicated.

Oxidative damage of cellular membrane by ionizing radiation has been known to cause impairment of the functional behavior of membrane and also of enzymes and receptors [22]. Modification of damage at membrane level offers an important strategy for the protection of normal cells against unavoidable therapeutic/accidental radiation exposure.

Model membranes, such as phospholipid liposome bilayers, are simple two-dimensional systems with well-characterized physical properties at their different phases [20]. Therefore, our study aimed to understand the structural behavior of phospholipid molecules presented in liposome vesicles upon exposure to gamma irradiation. The results also elucidated the protective role and antioxidant activity of ascorbic acid when exposed to ionizing gamma radiation.

Alterations in membrane fluidity in irradiated model [12, 19] as well as cellular membrane [24] have been reported employing DPH fluorescence probe. Moderate doses of gamma radiation are known to significantly increase membrane permeability of HeLa cells [31]. The increase in leakage of CF fluorescence up to a dose of 1 kGy suggests a radiation induced increase in bilayer permeability. On the other hand, addition of ascorbic acid during irradiation procedure increased the amount of CF released from liposomes. These results suggest that for a defined radiation dose, greater membrane damage occurs when using a hydrophilic antioxidant material.

The results obtained from IR and NMR demonstrated the presence of hydrolytic and oxidative processes, which was enormously enhanced in the presence of ascorbic acid.

Ascorbic acid has been shown to be rapidly destroyed by gamma radiation in dilute solutions [26]. The destruction product is called dehydroascorbic acid [21]. Both ascorbic and dehydroascorbic acids have biological activity. But these

biological activities differ [16]. Only ascorbic acid has the typical vitamin C working while the breakdown product dehydroascorbic acid is highly unstable and has no vitamin C working. If through circumstances high levels of dehydroascorbic acid are formed, then it can even damage a number of biological processes and affect health adversely [2, 27]. Our study suggests that ascorbic acid should be protected from radiation in order to perform its protective role adequately; perhaps this could be employed by encapsulating ascorbic acid in the liposome vesicles.

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