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Evidence for an increase in water concentration in bilayers after oxidative damage of phospholipids induced by ionizing radiation

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Abstract. The two membrane fluorescent probes 2-dimethyl-amino-6-lauroyl-naphthalene (Laurdan) and 2-dimethylamino-6-propionyl-naphthalene (Prodan) have been used to study the molecular basis of the damage induced in phospholipid membranes by ionizing radiation. Laurdan and Prodan display a spectral sensitivity to the polarity of their environment, showing a red shift of both excitation and emission spectra with increase of the polarity of their environment. Owing to their chemical differences, the two probes are anchored in the membrane with different strengths. In aqueous environments Laurdan is not fluorescent while Prodan shows appreciable fluorescence. Laurdan and Prodan show an opposite response to oxidative damage produced in phospholipid bilayers by ionizing radiation. The results support the model recently developed of water penetration in the bilayer as a consequence of oxidative damage.

1. Introduction

In a recent study, oxidative damage induced on biological membranes by ionizing radiation has been detected using a dynamic fluorescence technique, sensitive enough to detect the effects of a radiation dose as low as 0·5 Gy (Parasassi et al. 1991a), well within the dose range used for cell survival studies. The molecular basis of the damage has been studied using vesicles of synthetic phospholipids containing unsaturations at different depth in the bilayer. The induced damage depends upon the position and number of double bonds in the phospholipid acyl residues (Parasassi et al. 1992a). In erythrocyte ghosts and synthetic vesicles exposed to ionizing

radiation, the fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) is modified by the penetration of few water molecules in the bilayer due to the production of hydroperoxides (Konings 1984, Wolters et al. 1987, Parasassi et al. 1991a). These studies demonstrated that membranes can be damaged by ionizing radiation in the same dose range as that used for cell lethality and DNA damage studies.

In the present work, the hypothesis that water concentration increases in the bilayer after oxidative damage induced by ionizing radiation has been further examined using two fluorescent probes with different spectral sensitivities to the polarity of their environment and a novel steady-state fluorescence technique (Parasassi et al. 1990). 2-Dimethylamino-6-lauroyl-naphthalene (Laurdan) and 2-dimethyl-amino-6-propionyl-naphthalene (Prodan) (Weber and Farris 1979, Macgregor and Weber 1981, Chong 1988) are amphiphilic molecules with a lauroyl or propionyl tail anchored into the membrane and a polar fluorescent moiety at the level of the glycerol backbone. The chemical and spectroscopic properties of the two probes have been employed to test our hypothesis that water penetrates into the membrane as a consequence of oxidative damage. The quantum yield of fluorescence of Laurdan in the membrane is quite high, while it is virtually zero in aqueous environments (Parasassi et al. 1990). In contrast, Prodan shows appreciable fluorescence in aqueous solvents (Weber and Farris 1979). Owing to the very different lengths of their acyl residues, 12 and three carbon atoms, for Laurdan and Prodan, respectively, the anchorage of the two probes in the bilayer is of very different strength.

Fluorescence excitation and emission spectra of the two probes are sensitive to the polarity of their environment (Chong 1988, Parasassi et al. 1990), due to dipolar relaxation processes (Weber and Farris 1979, Macgregor and Weber 1981). Briefly, if the molecular dynamics of the dipoles surrounding the

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fluorescent moiety of these probes is of the same order of magnitude as their fluorescence lifetimes, the solvent dipoles orientate themselves around the excited state dipole of the probes. The loss of energy required for the solvent reorientation is reflected by the red shift of the fluorescence. Laurdan and Prodan show a relevant red shift of their emission on going from the gel to the liquid–crystalline phase of phospholipids (Chong 1988, Parasassi et al. 1990), indicating that there is a remarkable difference in polarity between these two phospholipid phases. Fluorescence excitation spectra of these probes show a small blue shift but a relevant decrease of the second excitation band. This red excitation band is particularly intense in gel phase phospholipids (Parasassi et al. 1990). These spectroscopic properties of the two probes have been described using the generalized polarization (GP) (Parasassi et al. 1990), defined as $GP = (I_g - I_{lc})/(I_g + I_{lc})$, where I_g and I_{lc} are the maximum intensities observed at the wavelengths typical of the maximum emission in the gel (440 nm) and in the liquid–crystalline phase (490 nm), respectively. Since both emission and excitation spectra vary in their shape and in the wavelength of their maxima, GP can be calculated for the excitation. In this case the wavelengths for the I_g and I_{lc} intensities are chosen to achieve the best separation between the two excitation bands, 410 nm for the gel and 340 nm for the liquid–crystalline phase (Parasassi et al. 1990). Phospholipid polar-head residues have been excluded as the molecular entities responsible for the observed dipolar relaxation. The behaviour of Laurdan fluorescence is not affected by different polar residues of phospholipids in the pH range from 4·0 to 10·0 (Parasassi et al. 1991b). The different concentration and dynamics of the few water molecules present at the hydrophobic–hydrophilic interface of the bilayer are responsible for the red shift of the emission observed in the liquid–crystalline as compared with the gel phase of phospholipids (Parasassi et al. 1993a).

Modifications of water concentration in the bilayer following exposure to ionizing radiation would be reflected by the modalities of Laurdan and Prodan emission and excitation. The responses of Laurdan and Prodan are expected to be different because of their different chemical affinity with the membrane. Moreover, instead of affecting the decay of these probes, as in the case of DPH, water penetration in the membrane is expected to produce a spectral shift of Laurdan and Prodan. Using the different responses of these probes to water concentration, we have tested further our hypothesis that water penetration in the bilayer is a consequence of radiation damage.

2. Materials and methods

2.1. Sample preparation

Dioleoyl-phosphatidylcholine (DOPC) and 1-palmitoyl-2-linoleoyl-phosphatidyl-choline (PLPC) were from Avanti Polar Lipids Inc. (Pelham, AL, USA). 2-Dimethylamino-6-lauroylnaphthalene (Laurdan) and 2-dimethylamino-6-propionylnaphthalene (Prodan) were from Molecular Probes Inc. (Eugene, OR, USA). Multilamellar phospholipid vesicles were prepared by evaporating a chloroform stock solution under nitrogen flow, resuspending the dried phospholipid in phosphate-buffered saline (PBS, Sigma Chemical Co., St Louis, MO, USA), warming at 37°C and vortexing. Final phospholipid concentration was 0·3 mmol dm^{-3}. Vesicles preparation was carried out in red light. Samples (3 ml) were irradiated with a Siemens Stabilipan X-ray apparatus operating at 200 kV, 15 mA and with a 0·2 mm Cu filter, at a dose rate of 22 Gy/min. Doses > 110 Gy were never used, since we found this to be the limiting dose for the maintenance of the integrity of the bilayer structure (Parasassi et al. 1991). Irradiation was performed at 0°C. After irradiation 0·5 μl 0·25 mmol dm^{-3} solution of Laurdan or Prodan in dimethylsulfoxide (DMSO) were added to the samples under mild stirring in the dark. After 1-h incubation at room temperature, samples were transferred into a cuvette and equilibrated in the fluorometer at 20°C for about 10 min. This labelling procedure is the same as that used for the labelling of cell membranes (Parasassi et al. 1992b, 1993b) since at these DMSO concentrations the GP value was not affected. In our labelling conditions, DMSO cannot affect the oxidation induced by ionizing radiation since: (1) the final concentration in the samples is 2·3 mmol dm^{-3}, while the minimum concentration required to act as a scavenger is 0·1 mol dm^{-3} (Millar et al. 1981); and (2) DMSO is added after exposure of the samples to ionizing radiation. Moreover, the unirradiated sample is labelled by the same procedure. The actual temperature of the sample was measured using a thermocouple inserted in the cuvette.

2.2. Fluorescence measurements

Fluorescence excitation and emission spectra of Laurdan or Prodan-labelled vesicles were obtained with a GREG 200 fluorometer using a xenon arc lamp and the accompanying software (ISS Inc., Champaign, IL, USA). The fluorometer was equipped with photon counting electronics PX01

(ISS). The excitation and emission bandpass of the monochromators was 8 nm. A temperature of $20 \pm 0 \cdot 1°C$ was obtained with a water circulating bath. The spectra were only corrected for the intensity variations of the excitation source. GPs were used to evaluate the polarity of the hydrophobic–hydrophilic interface of the bilayer and its variations (Parasassi et al. 1990). Excitation GP was calculated using:

$$GP_{ex} = (I_{440} - I_{490})/(I_{440} + I_{490}), \qquad (1)$$

where I_{440} and I_{490} are the emission intensities at 440 and 490 nm, respectively, using a fixed excitation wavelength of 360 nm. These emission wavelengths were chosen on the basis of the maximum emission observed in the gel (440 nm) and in the liquid–crystalline phase (490 nm) of phospholipids, as reported previously (Parasassi et al. 1990). Excitation at 360 nm was chosen to avoid a photoselection of the probe molecules (Parasassi et al. 1990). Emission GP was calculated by:

$$GP_{em} = (I_{410} - I_{340})/(I_{410} + I_{340}), \qquad (2)$$

where I_{410} and I_{340} are the excitation intensities at 410 and 340 nm, respectively, using fixed emission at 490 nm. The excitation wavelengths were chosen to obtain the maximum photoselection of probe molecules surrounded by the gel (410 nm) and by both phospholipid phases (340 nm) (Parasassi et al. 1990). GP measurements were also performed by subtracting the excitation or emission intensities of unlabelled samples from the intensities of labelled ones. The blank subtraction affects GPs by $<3\%$ and depends on wavelength. For the calculation of GP_{ex} and GP_{em} the excitation wavelength of 360 nm and the emission wavelength of 490 nm, respectively, were chosen as the less affected by the blank subtraction.
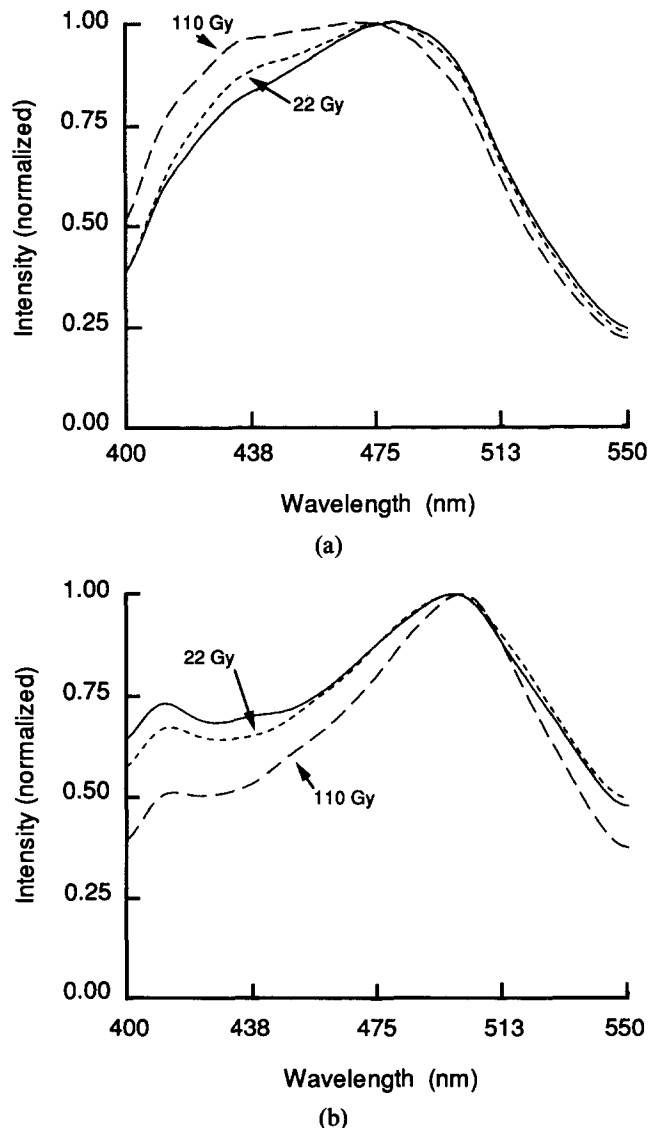

Figure 1. Fluorescence emission spectra of Laurdan (a) and Prodan (b) in DOPC multilamellar vesicles non-irradiated (continuous line) and after a dose of 22 Gy (dotted line) and 110 Gy (dashed line).

3. Results and Discussion

Laurdan and Prodan emission spectra in DOPC vesicles change after X-ray irradiation of the vesicles (Figure 1). The Laurdan emission spectrum is blue shifted in irradiated vesicles (Figure 1a), while the Prodan emission is red shifted (Figure 1b). GP_{ex}, calculated from the spectra in Figure 1 by equation (1), increase for Laurdan and decrease for Prodan as a function of radiation dose (Table 1).

In PLPC vesicles, Laurdan and Prodan fluorescence response as a function of radiation dose shows a behaviour similar to that observed in DOPC vesicles. The variation of Laurdan and Prodan GP_{ex}

as a function of the radiation dose is shown in Table 1. In Figure 2 the variation of GP_{ex} and GP_{em} of the two probes in PLPC as a function of the radiation dose is shown. GPs show opposing behaviour for the two probes and, for each probe, GP_{em} show opposing behaviour with respect to that for the GP_{ex}. Laurdan GP_{ex} increases while its GP_{em} decreases upon increasing the radiation dose. On the contrary, Prodan GP_{ex} decreases and its GP_{em} increases with increasing radiation dose. In Figure 3 the difference between the GPs measured after exposure of the samples at various radiation doses and for the unirradiated samples are shown. Laurdan GP_{ex} show larger variations than Prodan GP_{ex} (Figure 3a). Laurdan and

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Table 1. Values of Laurdan and Prodan GP_{ex} in vesicles composed of DOPC and PLPC unirradiated and after various radiation doses. Also shown is the standard deviation, calculated for four different experiments for DOPC and five experiments for PLPC, and the difference with the unirradiated samples.

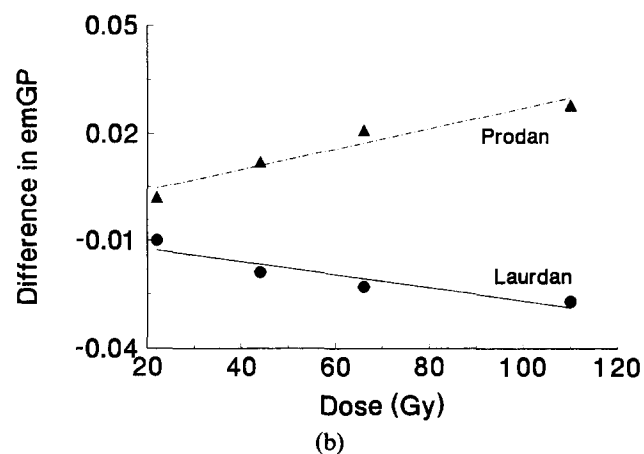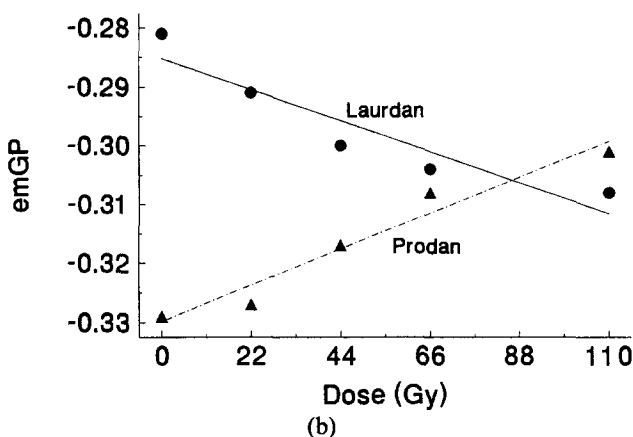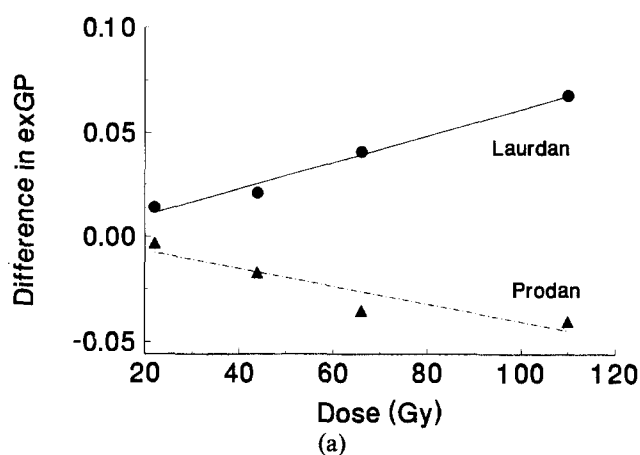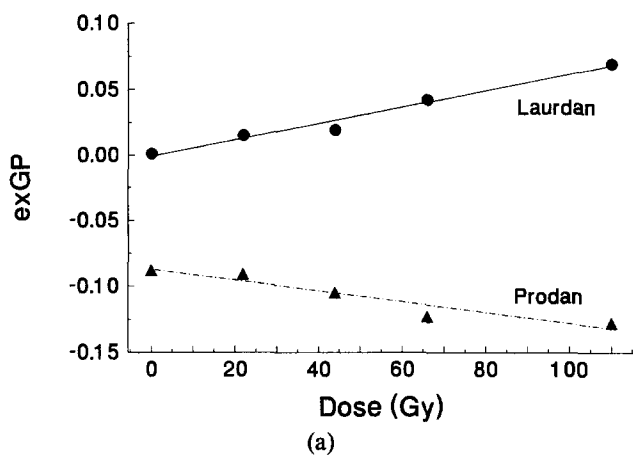| Gy	Laurdan GP_{ex}	Difference	Prodan GP_{ex}	Difference
DOPC				
0	-0.090 ± 0.006	–	-0.171 ± 0.013	–
22	-0.035 ± 0.003	0.035	-0.199 ± 0.016	-0.028
110	0.045 ± 0.002	0.135	-0.252 ± 0.012	-0.081
PLPC				
0	0.009 ± 0.002	–	-0.088 ± 0.006	–
22	0.015 ± 0.002	0.006	-0.091 ± 0.008	-0.003
44	0.019 ± 0.002	0.010	-0.105 ± 0.007	-0.017
66	0.042 ± 0.003	0.033	-0.123 ± 0.009	-0.035
110	0.069 ± 0.004	0.060	-0.128 ± 0.007	-0.040

Figure 2. Generalized polarization (GP) values of Laurdan and Prodan in PLPC vesicles as a function of the radiation dose. Excitation GP values (a) calculated following equation (1). Emission GP values (b) calculated following equation (2). Straight lines represent a linear regression fit. The data are the mean values of five separate experiments with a SD of 9%.

Figure 3. Difference between the Laurdan and Prodan GP values obtained in PLPC vesicles after various radiation doses and the GP values obtained in unirradiated samples. Difference of the GP_{ex} (a) and GP_{em} (b) values. Straight lines represent a linear regression fit.

Prodan GP_{em} show about the same variation with radiation dose (Figure 3b).

In previous studies, a model was developed to attribute a molecular origin to the modification of DPH decay observed after irradiation of erythrocyte ghosts (Parasassi *et al*. 1991a) and of phospholipid vesicles (Parasassi *et al*. 1992a). Lipid damage in erythrocyte ghosts was detected at doses as low as 0·5 Gy, well within the dose range used for cell survival studies, for the detection of DNA damage and well below the dose required to observe damage of proteins. Furthermore, damage was strictly dependent upon the presence of oxygen. To explain, at a molecular level, the origin of the observed variation of DPH decay after oxidative damage to membranes, vesicles, which were composed of phospholipids but differing in the number and position of double bonds in the acyl residues were used. The damage was found to be directly related to the number and position of unsaturated bonds and was only produced in the presence of oxygen. The prevalent final oxidation products are hydroperoxides (Konings 1984, Wolters *et al*. 1987) that disturb the hydrophobic interactions between adjacent phospholipids and favour the penetration of water molecules towards the upper region of the bilayer, i.e. from the aqueous phase to the oxidized residues. Such damage has been observed for the first time at low radiation dose (Parasassi *et al*. 1991a) due to the peculiar spectroscopic characteristics of DPH (Parasassi *et al*. 1991c). The DPH lifetime value is inversely related to the polarity of its environment. A gradient of dielectric constant exists along the normal to the surface of a phospholipid bilayer (Griffith *et al*. 1974), due to a gradient in water concentration. In the absence of oxidative damage, the relatively small DPH molecule can be distributed at various depths along the normal to the bilayer, experiencing the differences of the dielectric constant. As a consequence, its fluorescence decay was best described by a continuous distribution of lifetime values with lorentzian shape (Parasassi *et al*. 1991c). When hydroperoxides have been produced by ionizing radiation and the concentration of water in the upper region of the bilayer has increased and since DPH is no longer fluorescent in the presence of water, the residual fluorescence originates from a deeper region of the bilayer. This internal region has lower and more homogeneous values of dielectric constant (Griffith *et al*. 1974) so that DPH lifetime values are progressively higher and their lorentzian distribution is sharper with the increase of radiation dose (Parasassi *et al*. 1991a).

The model described above has been tested further using Laurdan and Prodan spectral response

to the polarity of the environment. Owing to the different length of their acyl residues, 12 and three carbon atoms, Laurdan and Prodan are located at different depths within the bilayer. Also their chemical affinity with the membrane is different, as is their fluorescence in different environments. Laurdan does not partition in aqueous environments and it does not show appreciable fluorescence in these environments, whereas it is strongly fluorescent in membranes. Prodan shows weak but appreciable fluorescence in water. Upon irradiation in the presence of oxygen, those Laurdan molecules situated in the upper region of the bilayer in the presence of relatively high concentration of water no longer fluoresce. The remaining fluorescence must then originate from Laurdan molecules driven deeper into the bilayer by their lauric acid tail. Owing to the lower polarity of this new Laurdan environment, the dipolar relaxation process is reduced, as indicated by the blue shift of the emission spectrum and by the increase of GP_{ex}. Owing to the characteristic spectroscopy of this probe (Parasassi *et al*. 1991b), a decrease in the polarity of the environment causes a blue shift of Laurdan excitation spectrum, so that its GP_{em} decreases (equation 2). Instead, Prodan is both partitioned and fluorescent in the presence of water (Chong 1988), and the increased polarity of the upper region of the bilayer increases the amount of its dipolar relaxation. This is supported by the red shift of the emission spectrum and by a corresponding decrease of GP_{ex}. The increase of Prodan GP_{em} is explained by the polarity change, as in the case of Laurdan, but with Prodan the variation of polarity causes an opposite response of GP_{em}. Prodan is still fluorescent in the presence of water. Owing to the increased average polarity of the environment, its excitation spectrum is red shifted and its GP_{em} is increased.

The variation of GP_{ex} for Laurdan and Prodan is in agreement with their different fluorescence properties and partition between the aqueous phase and the bilayer. Since Prodan is also relatively fluorescent in aqueous environment, part of its fluorescence will always arise from this aqueous environment. Laurdan is virtually non-fluorescent in water, so that following the increase of water concentration in the upper region of the bilayer, the fluorescence will arise only from the inner environment of the bilayer where the amount of dipolar relaxation is reduced. The variation of Laurdan GP_{ex} is thus larger than the variation of Prodan GP_{ex}. The variation of GP_{em} is similar for the two probes. Their excitation spectra are known to show small modifications as a function of polarity (Parasassi *et al*. 1990).

Reported results on capacitance measurements in

planar lipid membranes (Strässle *et al.* 1991) support our hypothesis of water penetration into the bilayer after oxidative damage. Irradiation was found to produce lipid peroxidation. Indeed, the increase in membrane capacitance was strongly dependent on the presence of oxygen and has been interpreted as due to an increase of the dielectric constant in the membrane interior.

When compared with the variation of DPH decay induced by oxidative damage as a function of the radiation dose in vesicles composed of the same phospholipid (Parasassi *et al.* 1992a), the variation of Laurdan GP_{ex} is of an analogous sensitivity, while the variation of Prodan GP_{ex} is of a much lower sensitivity. In DOPC vesicles, the width of DPH lifetime distribution after a dose of 110 Gy decreases by $(97 \pm 0.2)\%$ whereas in the same vesicles and after the same radiation dose Laurdan GP_{ex} increases by $(150 \pm 6)\%$, and Prodan GP_{ex} decreases by $(47 \pm 18)\%$. For DPH in erythrocyte ghosts, the lowest limiting dose to detect damage is 0·5 Gy (Parasassi *et al.* 1991a), while for Laurdan this limiting dose has not been explored. In our opinion, the present results are of relevance to and support the previously proposed model of water penetration into the bilayer after oxidative damage. Further experiments are needed to determine the limiting sensitivity of the technique for the detection of damage induced by low doses of radiation in vesicles and in natural membranes.

Acknowledgements

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