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Journal

International Journal of Radiation Biology, 61(6)

ISSN

0955-3002

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Publication Date

1992

DOI

10.1080/09553009214551661

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INT. J. RADIAT. BIOL., 1992, VOL. 61, NO. 6, 791-796

Membrane oxidative damage induced by ionizing radiation detected by diphenylhexatriene fluorescence lifetime distributions

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(Received 14 June 1991; second revision received 13 January 1992; accepted 19 January 1992)

Abstract. The sensitivity of the fluorescence lifetime of 1,6-diphenyl-1,3,5-hexatriene (DPH) to the dielectric constant of its environment has been used to detect oxidative damge to phospholipid membranes induced by ionizing radiation. The DPH fluorescence decay in phospholipid vesicles is described well by a continuous distribution of lifetime values, reflecting the various DPH depths in the bilayer and related to the gradient of the dielectric constant. Ionizing radiation oxidizes unsaturated acyl residues of phospholipids, altering the dielectric constant across the bilayer, sharpening the distribution of DPH lifetimes and increasing the centre of the distribution. Ionizing radiation doses between 22 and 110 Gy were used, and were effective only in the presence of oxygen. A model based on the formation of packing defects in the bilayer describes the phenomenon.

1. Introduction

1,6-diphenyl-1,3,5-hexatriene (DPH) is a popular fluorescent membrane probe, widely used to evaluate membrane phase state (Parasassi *et al.* 1984), microheterogeneity (Fiorini *et al.* 1987, Parasassi *et al.* 1987) and order (Van der Meer 1988). The polarization and the average lifetime values show an abrupt change from the gel to the liquid-crystalline phase of the membrane bilayer (Parasassi *et al.* 1984). In membranes of unknown composition these values can indicate the average phase state or the order of the system. The fluorescence decay of DPH in membranes is described well by a continuous distribution of lifetime values (Fiorini *et al.* 1987, Parasassi *et al.* 1987). Since DPH can be located at various depths along the acyl chain residues (Lentz 1989), the distribution of its lifetime values has been interpreted as representing the sensitivity of the probe to the heterogeneity of the physical properties along the bilayer normal. The basis of this sensitivity is the dependence of the lifetime value on the polarity of solvents (Parasassi *et al.* 1991a). A gradient of polarity has been demonstrated to exist along the bilayer normal (Griffith *et al.* 1974), due to a gradient of water concentration. Discontinuities in the cooperative van der Waals interactions between the acyl residues of phospholipids locally disorder the membrane, favouring the penetration of water moleclues. The width of DPH lifetime distribution has been used to evaluate the heterogeneity of a membrane system (Parasassi *et al.* 1987, Fiorini *et al.* 1988).

Ionizing radiation induces oxidative damage to unsaturated phospholipid acyl residues, leading to the formation of hydroperoxides and acyl-chain crosslinkages (Konings 1985). The production of these modified residues in membranes can alter molecular interactions and water penetration, inducing variations in the bilayer polarity.

A previous study on irradiated erythrocyte ghosts (Parasassi *et al.* 1991b) showed a surprising sensitivity of DPH decay parameters to membrane oxidative damage. The lowest radiation dose which produced observable variations in DPH decay parameters was 0·5 Gy, well within the dose range used for other biological end-point such as mutation, transformation and cell survival. Previous spectroscopic studies on radiation-induced membrane damage using different techniques reported that the lowest dose to detect damage was more than two orders of magnitude greater (Bonincontro *et al.* 1987, Cantafora *et al.* 1987). DPH steady-state polarization and dynamic anisotropy are also sensitive to oxidative damage in membranes, but they require much higher ionizing radiation doses (Joshi *et al.* 1982).

With the aim of understanding the origin of the variation of DPH decay parameters after oxidative damage to membranes, we irradiated unsaturated phospholipid vesicles with various degrees and positions of unsaturation in the acyl residues. The results have been compared with those obtained in the

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absence of oxygen and with those obtained using saturated phospholipid vesicles.

2. Materials and methods

2.1. *Sample preparation*

Dipalmitoyl- (DPPC), dipetroselinoyl- (PEPC), dioleoyl- (DOPC) and dilinoleoyl-phosphatidylcholine (DLOPC) were from Avanti Polar Lipids Inc. (Pelham, AL). 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes Inc. (Eugene, OR). Multilamellar phospholipid vesicles were prepared by evaporating a chloroform stock solution under nitrogen, resuspending the dried samples in phosphate buffered saline (PBS), warming above the transition temperature and vortexing.

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. For experiments performed in the absence of oxygen, 3 ml of the vesicles were placed in a glass vial capped with a silicone rubber gasket, through which input and output needles were inserted. The samples were degassed with a mixture of 95% nitrogen and 5% CO_2 for about 20 min and the vials were sealed and irradiated immediately. Vesicle preparation, deoxygenation and irradiation were performed at 0°C in the dark or under red light.

After irradiation, the samples were transferred to vials where an aliquot of a stock solution of DPH in chloroform had been dried, incubated overnight at 0–4°C and then measured. The final concentrations of phospholipids and DPH were 0·3 mM and 0·5 μM, respectively.

2.2. *Fluorescence measurements and analysis*

Lifetime measurements were performed using a GREG 200 phase fluorometer (ISS Inc., Champaign, IL) equipped with a xenon arc lamp. Excitation was at 357 nm with a 16 nm bandwidth. Emission was observed through a 420 nm cut-off filter (Andover Co., New Hampshire). The background fluorescence of unlabelled vesicles was less than 0·1% of the total fluorescence. A solution of 2,2′-*p*-phenylene-*bis*(5-phenyl)oxazole (POPOP) in ethanol was used as the reference (lifetime = 1·35 ns). Phase and modulation data were collected for 9 to 11 modulation frequencies, in the range from 2 to 90 MHz. In all experiments the sample cell holder was kept at 20°C by a water-circulating bath. Data were analysed using the Globals Unlimited software from the Laboratory for Fluorescence Dynamics (University of Illinois at Urbana-Champaign) (Beechem and Gratton 1988). The goodness of fit was evaluated by the value of the reduced chi square. Chi square minimization was performed using a Simplex algorithm (Alcala *et al.* 1987). The fitting function was the sum of two continuously distributed Lorentzian components. The two distributions were characterized by three parameters: the centre, the full width at half-maximum (referred as width in the followings) and the fractional intensity. Some experiments were performed using a K2 fluorometer (ISS Inc.) with the same experimental set-up. The error in the width and centre determinations was estimated by the standard deviation of several measurements repeated at the same dose, resulting in 0·2 ns and 0·05 ns for the width and centre, respectively.

3. Results

The DPH lifetime distributions in phospholipid multilamellar vesicles can be described well by two components, each distributed following a Lorentzian function. At 20°C the main component has a fractional intensity of about 95%, a centre of about 8 ns and a width of about 2 ns. The remaining 5% originates from a short-lived component, with centre and width of about 0·1 ns. The reported centre and width values show variations depending on the phospholipid composition of the vesicles, as shown in Figures 1 and 2. Generally, the centre value of the distribution of the main component of DPH decay decreases to shorter lifetimes as the unsaturation of the acyl residues increases. The width is greater in vesicles composed of phospholipids with unsaturated acyl residues; this can be explained by an increase in the heterogeneity of the polarity along the bilayer normal, due to packing defects introduced by the *cis* double bond. The DPH lifetime is also inversely related to the polarity of the environment, and this property can explain the behaviour of the centre values reported above.

Multilamellar vesicles composed of phospholipids with different degrees of unsaturation in their acyl residues were irradiated in the dose-range from 22 to 110 Gy. No variation of the DPH lifetime distribution was observed in irradiated DPPC samples (Figures 1 and 2). Figure 3 shows the DPH lifetime distributions in DOPC-irradiated vesicles. With doses greater than 22 Gy the distribution width of the main component of the decay decreased linearly

with the logarithm of the dose (Figure 1) while the centre of the distribution increased linearly with the dose (Figure 2). A small increase in the fractional intensity and in the centre value of the short-lived component of DPH decay was observed as a function of radiation dose, as shown in Figure 3. The increase of intensity and centre value of this short-lived component as a function of radiation dose is even more evident in experiments performed using DLOPC vesicles (Figure 4). When the irradiation was performed under anoxic conditions neither variations in the distribution width nor in the centre value were observed (data not shown).

Figure 1 shows that the width of the main component decreases as a function of the logarithm of the radiation dose with a different slope in vesicles of different composition. For DOPC, the decrease in the width of the DPH lifetime distribution is faster than for PEPC vesicles. The greatest effect on narrowing of the width after irradiation with 22 Gy was obtained using DLOPC vesicles.

The centre value of the main component of DPH decay varied as a function of radiation dose (Figure 2). The centre values in the absence of irradiation are different, depending on the composition of the vesicles. Their variation following irradiation treatment showed a dependence on vesicle composition in terms of unsaturation of acyl residues. Again, for DOPC the slope is higher than for PEPC vesicles.

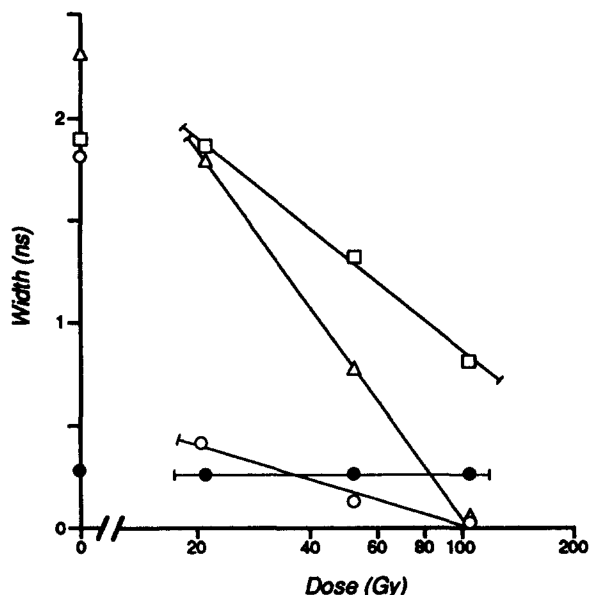

Figure 1. Width variation of the continuous distribution of the main lifetime component of DPH decay as a function of radiation dose in vesicles composed of DPPC (●), PEPC (□), DOPC (△) and DLOPC (○).

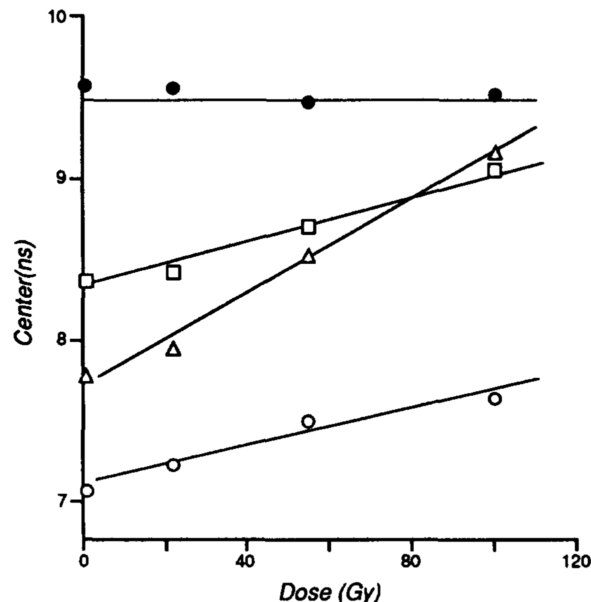

Figure 2. Centre variation of the distribution of the main component of DPH decay as a function of radiation dose in vesicles composed of DPPC (●), PEPC (□), DOPC (△) and DLOPC (○).

4. Discussion

The variation of DPH decay parameters observed in irradiated phospholipid multilamellar vesicles is caused by oxidative damage occurring in unsaturated acyl residues. DPH decay is not affected by irradiation when the vesicles are composed of saturated phospholipid, or when the unsaturated phospholipid samples are deoxygenated.

According to Konings (1985), oxidation of unsaturated lipids leads to chain reactions, locally amplifying the initial damage, resulting in the formation of hydroperoxides and acyl-chain crosslinkages. These new residues induce a disordering of the upper region of the bilayer, close to the aqueous phase, by decreasing the cooperativity of van der Waals interactions between adjacent acyl chains. Water penetration and concentration at the bilayer surface will then increase, and will quench the fluorescence of DPH molecules closer to the surface of the bilayer. The residual fluorescence will then originate from DPH molecules located in the inner hydrophobic region of the bilayer, unaffected by oxidation, then with lower water concentration. The resulting width value of the DPH lifetime distribution is lower, due to the relative homogeneity of the remaining fluorescence from this hydrophobic environment (Griffith *et al.* 1974), and the centre value increases due to the low polarity.

Considering each double bond as a target for

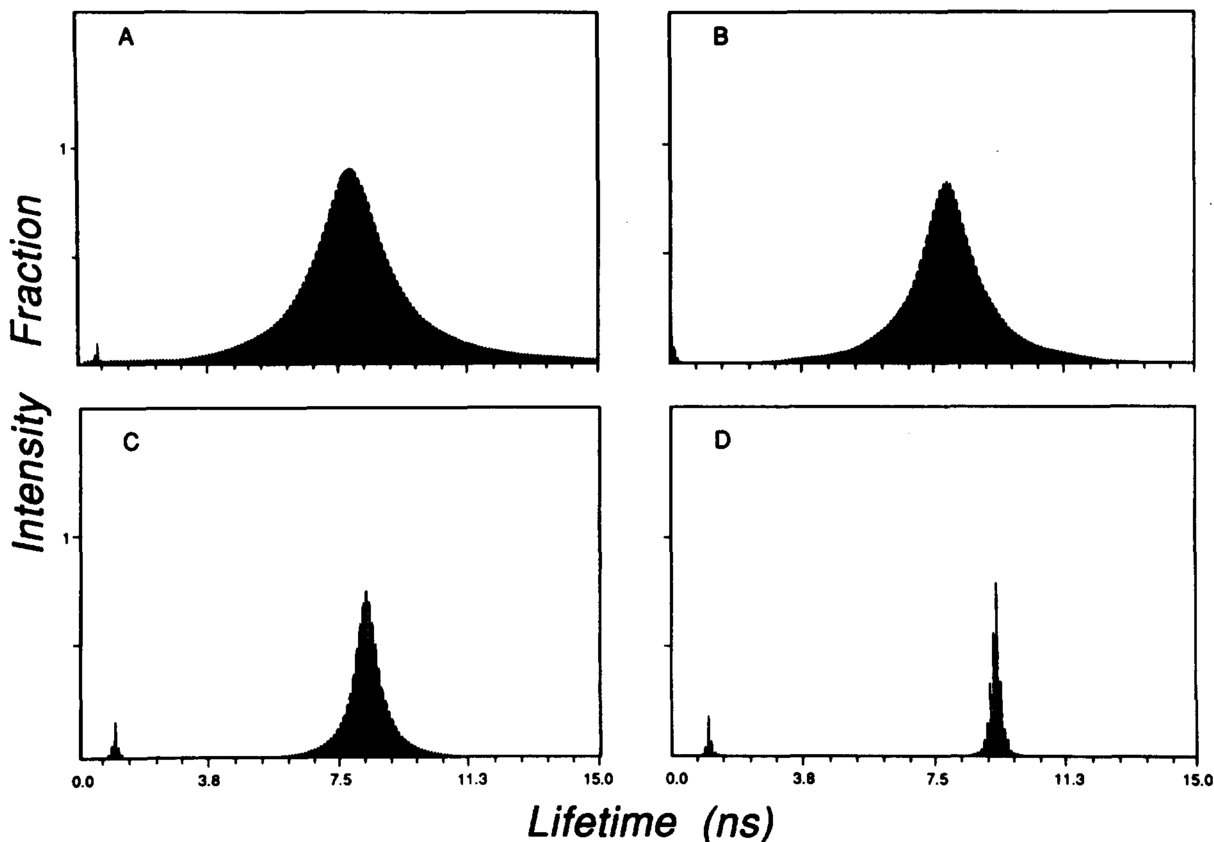

Figure 3. DPH lifetime distributions in DOPC vesicles unirradiated (A) and irradiated with doses of 22 Gy (B), 55 Gy (C) and 110 Gy (D).

ionizing radiation, and assuming the width of the DPH lifetime distribution to be related to the number of undamaged targets, then the measured width value, W_D after a dose D irradiation, will be:

$$W_D = W_0 - k \log D \quad (1)$$

This experimentally based equation (1) describes our results in the dose range used, and W_0 represents the width value at the beginning of its linear decrease. In this simple model the width decrease is a linear function of the logarithm of the dose, and the slope of the plot depends on k (a function of the position and the number of unsaturations in the acyl residues). The closer the unsaturated bonds of the acyl residues are to the bilayer aqueous surface, the lower is the value of k. Similarly, increasing the number of unsaturated bonds along the acyl residue increases the k value. Our results show that using PEPC (C18:1, one unsaturated bond at carbon 6), the slope of the decrease in width is smaller than that obtained with DOPC (C18:1, one unsaturated bond at carbon 9). Using DLOPC vesicles (C18:2, two unsaturated bonds at carbons 9 and 12), a more dramatic effect is obtained after a low dose ($\leq$22 Gy) the width of the distribution decreasing from 1·8 to 0·4 ns. With higher doses the width continues to decrease, to a minimum value, different from zero. Moreover, a radiation dose of 22 Gy affects the decrease in width differently, depending on the position of the double bonds in the acyl residues. The closer the unsaturated bond is to the aqueous surface (PEPC), the higher is the minimum dose required to observe a given decrease in width. Conversely, low doses more readily affect the system when the unsaturated bonds lie deep in the bilayer (DOPC), and even more so when such unsaturation is multiple (DLOPC).

In accord with our previous results (Parasassi *et al.* 1991a), our model is based on the assumption that the polarity gradient along the bilayer is flat in the inner hydrophobic region (Griffith *et al.* 1974). The closer the double-bond position is to the surface, the greater will be the polarity gradient that will be experienced by the fluorescent probe after a dose D of radiation. The peculiar sensitivity of DLOPC vesicles to irradiation can be explained by the number and the position of unsaturations along the acyl residues. The two unsaturated bonds are very close to the centre of the bilayer. When these bonds are damaged by radiation the remaining portion of

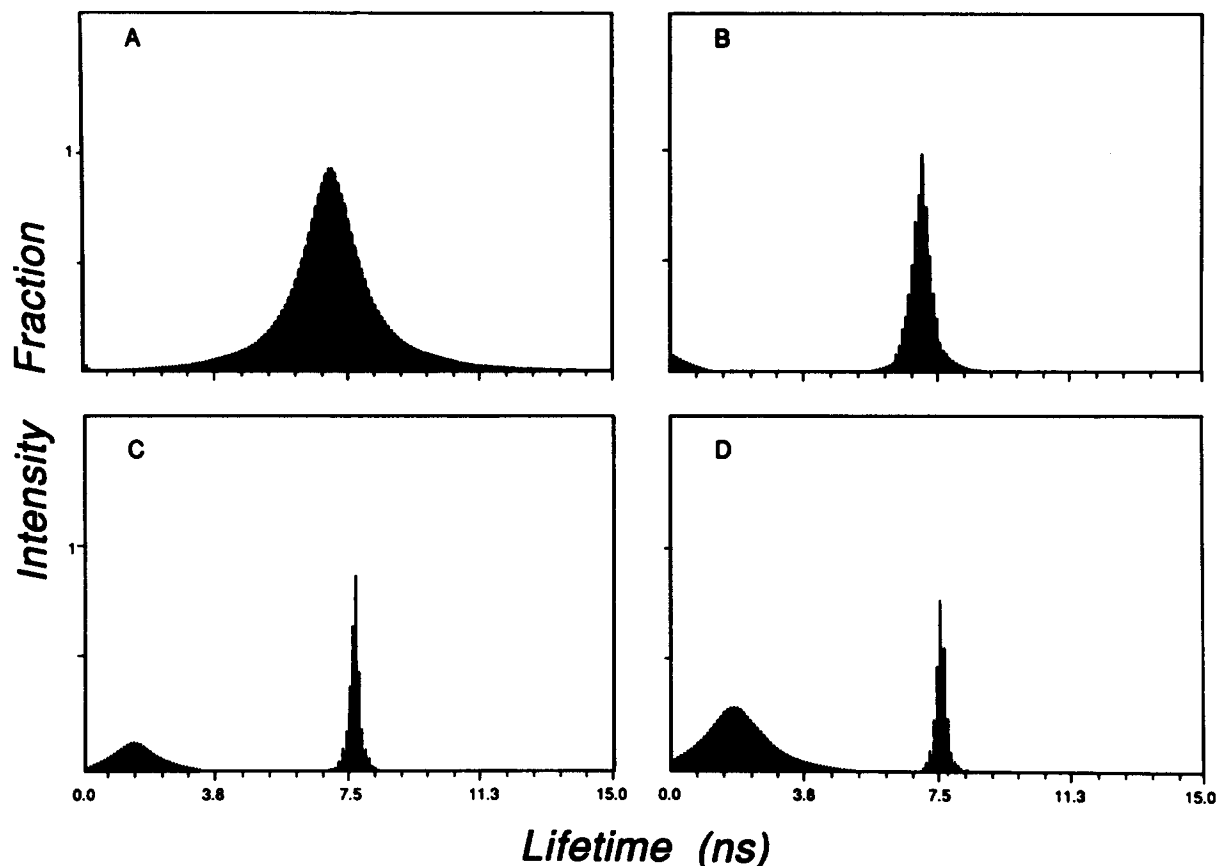

Figure 4. DPH lifetime distributions in DLOPC vesicles unirradiated (A) and irradiated with doses of 22 Gy (B), 55 Gy (C) and 110 Gy (D).

the bilayer available for fluorescent DPH molecules is quite small and homogeneous.

On this model the total fluorescence intensity will decrease with increasing radiation dose. Since each sample was prepared separately, and the fluorescent probe was added after irradiation, very precise measurements of the variation in intensity due to the various radiation doses were not possible. Nevertheless, in DOPC samples we observed a decrease in fluorescence intensity of about 50% after irradiation with 22 Gy, and of about 75% after irradiation with 110 Gy.

The short-lived component of DPH decay has been attributed to the photophysical properties of DPH (Parasassi *et al.* 1991a). Although its fractional intensity is quite small, it has been previously observed to increase with illumination in the presence of oxygen (Parasassi *et al.* 1984). Thus the presence of this second short-lived component is due both to peculiar DPH photophysical properties and to photo-oxidation processes favoured by the presence of unsaturated phospholipids. In previous work on erythrocyte ghosts (Parasassi *et al.* 1991b), the fractional intensity associated with this component, together with the width and centre values of its lifetime distribution, has been shown to increase after irradiation with doses greater than 110 Gy—doses larger than those used in this work. In the present experiments an appreciable increase in the fractional intensity and lifetime distribution width of this second, short component is clearly observable in the highly unsaturated DLOPC vesicles after irradiation, in accordance with previous observations.

The results presented can also explain the origin of the decrease in width of the DPH lifetime distribution observed in erythrocyte membranes after treatment with ionizing radiation (Parasassi *et al.* 1991b). In the present work, model systems were studied, and because we were not concerned with the limiting sensitivity of the method, the radiation doses used were at least 20-fold greater than the minimum dose at which width variations were observed on natural membranes. On the basis of the results presented we can anticipate that, in natural membranes, the minimum dose required to observe width variations, and the amount of those variations, will depend on the complexity of composition of the

membrane, especially of the various possible phospholipid acyl residues. The presence of proteins and cholesterol may also be expected to influence the bilayer packing and the water penetration, with consequences on the production of oxidative damage to unsaturated acyl residue via the penetration of damaging radicals produced in the aqueous phase, and on modifications of the width of the DPH lifetime distributions.

Acknowledgements

We thank Prof. Filippo Conti for the use of the K2 fluorometer. This work was supported by CNR (T.P., G.R.), ISS (O.S.) and NIH RR03155 (E.G.).

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