X-Ray and Fluorescence Studies on Phospholipid Bilayers. IX. Interactions with Pentachlorophenol

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Pentachlorophenol (PCP) is a widely used and highly toxic fungicide. Its toxicity is mainly expressed at the cell membrane level. It is, therefore, of interest to test its ability to alter the lipid bilayer organization. The present study was performed by X-ray diffraction techniques on dimyristoylphosphatidylethanolamine (DMPE) and dimyristoylphosphatidylcholine (DMPC) bilayers and by fluorescence on DMPC liposomes. These two phospholipids are respectively found at the inner and outer monolayers of human erythrocyte membranes. Each type of phospholipid was made to interact with different concentrations of the sodium form of PCP in absence and in presence of water. It was found that PCP significatively affected the structure of both phospholipids, being the damage much higher in DMPC bilayers.

Introduction

Pentachlorophenol (PCP) and its sodium salt are widely used fungicides, particularly for the preservation of wood. Because of their massive use, they have become dangerous environmental pollutants because of their high toxicity and resistance to degradation [1]. PCP is absorbed through the skin and the digestive and respiratory systems. Due to the extensive use it has been found in unexposed population [2]. PCP is also produced by the metabolism of the pesticides hexachlorobenzene and pentachloronitrobenzene [3], thus explaining its presence in humans from countries with little use of PCP [4].

As it is strongly lipophilic, PCP is able to partition into the lipid bilayer of cell membranes disrupting several functions [5]. It uncouples the oxidative phosphorylation [6], alters the microsomal electron transport system [7] and inhibits the amino acid transport across cell membranes [8]. In lipid bilayers, PCP induces perturbation [9] and electrical conduction [10], which is related to transmembrane proton translocation and the uncoupling effect [11]. These results indicate that PCP is able to alter the molecular organization, physical properties and functions of membranes [12], which might be the basis for its toxicity.

In the present study X-ray diffraction, steady-state depolarization and time-resolved fluorescence techniques were used to determine the perturbation induced by PCP to phospholipid bilayers. For the X-ray studies the sodium salt of PCP was made to interact in different concentrations with two types of multibilayers. One was made of dimyristoylphosphatidylethanolamine (DMPE) and the other of dimyristoylphosphatidylcholine (DMPC). They, respectively, represent the kind of phospholipids that are preferentially located in the inner and outer monolayer of human red-cell membranes [13]. Their structures, under different conditions of hydration, have been previously reported [14–15]. Chemically, their only difference is that DMPE has an $\overset{+}{N}H_3$ terminal group and DMPC an $\overset{+}{N}(CH_3)_3$. Their bilayer structures are also very similar. In fact, both have the hydrocarbon chains mostly parallel and completely extended, with the polar groups lying perpendicularly to them. However, DMPE molecules pack more tightly than those of DMPC. This effect is due to its smaller polar group and higher effective charge, resulting in a very stable structure. The addition of water, even in a large excess, does not significatively affect the structure of DMPE bilayers. However, the gradual hydration of DMPC results in water filling the highly polar spaces between the bilayers and the increasing of the interbilayer distance [16]. As a consequence, DMPC molecules become more disordered and eventually they rearrange in an hexagonal packing [17]. These bilayer

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systems had already been used in this laboratory as models for testing the structural damage produced in membranes by other compounds of biological interest [18, 19].

Fluorescence steady-state anisotropy and decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to characterize the influence of PCP in the physical properties of DMPC large unilamellar liposomes. DPH, an all-*trans* polyene with a rod-like shape, is one of the most commonly used probe for the hydrophobic regions of phospholipid bilayers. DPH fluorescence steady-state anisotropy is an useful technique to investigate dynamics of lipid bilayers as they provide a measure of the rotational diffusion of the fluorophor, restricted within a certain region such as a cone. DPH fluorescence decay was studied using multifrequency phase and modulation fluorometry. The data was analyzed using either an exponential approach and a continuous distribution analysis that took account of the bilayer microheterogeneity.

Materials and Methods

X-ray diffraction

Synthetic L-α-dimyristoylphosphatidylcholine (DMPC) from SIGMA (Lot 36F-8445, A Grade, MW 678), L-α-dimyristoylphosphatidylethanolamine (DMPE) from SIGMA (Lot 46F-8410, A Grade, MW 636) and sodium pentachlorophenol (PCP) from Merck (MW 288), a gift from Dr. Carlos Barrios, Dept. of Toxicology of the University of Concepción, were used without further purification. Powder mixtures of PCP in a 1:1 molar ratio with each phospholipid were prepared, dissolved in methanol and left to dry very slowly and carefully to facilitate their molecular interactions. The same procedure was followed with pure samples of each phospholipid and PCP. The samples thus prepared were in the form of dry and crystalline powders except for the DMPC:PCP 1:1 mixture. This had the appearance of a wet paste, even after drying it for 4 h at 85 °C over P_2O_5. All these samples were placed into low absorbing 0.7 mm diameter X-ray glass capillaries and sealed. Other specimens containing about 2 mg of each phospholipid in 1.5 mm diameter capillaries were then filled with about 100 µl of PCP aqueous solutions, sealed and left to react for about a week before being X-ray diffracted. The PCP concentration ranged from 0.01 M to 0.5 M. Similar specimens were made of DMPE, DMPC and pure water. All these samples were X-ray diffracted in flat-plate cameras provided with 0.25 mm diameter glass collimators [15] and rotating devices. The water-containing specimens were standardized by sprinkling a little calcite powder on their surfaces. Ni-filtered CuK_α from a Philips PW 1140 X-ray generator was used. The relative intensities of the reflections were measured from films in a Joyce-Loebl MK III CS microdensitometer. All experiments were carried out at room temperature and relative humidity (about 18 °C and 56% r.h.).

Fluorescence

Large DMPC unilamellar liposomes suspended in water were prepared following the vaporization method [20], to a final lipid concentration of 500 µM. DPH was incorporated by coinjection with DMPC during the liposome preparation in a probe to phospholipid ratio 1:500. Fluorescence polarization and lifetime measurements were performed with a GREG-200 steady-state and time-resolved I.S.S. spectrofluorometer interfaced to an IBM PC. Software from I.S.S. were used for data collection and analysis. All measurements were made using 1 cm path-length square quartz cuvettes at 18 °C. The temperature was controlled by an external Cole Parmer bath circulator. The actual temperature was measured in the sample cell before and after each measurement using an Omega Eng. digital thermometer. A Xenon arc-lamp was the light source; the wavelength of excitation was set at 360 nm. The emission was measured using a LL-450 Corion high pass filter, which showed negligible fluorescence. The background fluorescence, checked with blank unlabelled samples, was negligible with respect to probe fluorescence. Polarization measurements were done in the "L" configuration using Glan Thompson prism polarizers in both exciting and emitting beams. Fluorescence lifetime determination was accomplished by the multifrequency phase and modulation fluorometry. The intensity of the exciting light was modulated and the phase shift and relative demodulation of the emitted light with respect to the excitation was determined over a range of modulation frequencies. The values were analyzed assuming either a sum of exponentials or a continuous distri-

bution of lifetime components [21, 22]. For both analyses the goodness of the fit was judged by the value of the reduced chi square, defined by an equation published elsewhere [22].

Table I. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE, DMPE:PCP 1:1 and PCP obtained from dry powder samples crystallized from CH_3OH (a, b).

DMPE		DMPE:CLP 1:1		PCP	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
50.8*	1087*	50.8*	441*	–	–
–	–	32.5*	687*	–	–
25.5	21	–	–	–	–
17.0	51	16.4	30	–	–
–	–	13.7	7	–	–
12.8	35	12.4	4	–	–
–	–	–	–	11.7	16
–	–	11.0	60	–	–
10.2	7	–	–	–	–
–	–	9.89	19	–	–
9.46	11	–	–	–	–
8.74	5	8.80	4	–	–
–	–	–	–	8.01	100
7.99	18	–	–	–	–
–	–	7.89	13	–	–
–	–	–	–	7.39	9
7.32	14	7.36	11	–	–
–	–	7.08	25	–	–
6.80	6	–	–	–	–
–	–	6.53	14	–	–
6.34	5	–	–	–	–
5.95	70	5.99	20	–	–
5.71	52	5.68	15	–	–
5.47	30	5.45	17	–	–
5.20	26	–	–	5.17	9
5.09	44	5.09	32	–	–
–	–	4.91	11	–	–
4.78	133	4.77	8	–	–
4.61	77	4.64	15	–	–
–	–	4.39	64	–	–
4.23	44	4.27	46	–	–
–	–	4.19	35	–	–
4.05	544	4.06	103	4.00	18
3.80	285	3.82	116	3.82	15
–	–	3.74	51	–	–
3.64	38	3.63	33	3.62	52
3.50	11	3.53	60	–	–
–	–	3.46	18	3.46	108
3.39	29	3.33	12	3.29	42
–	–	3.26	18	3.22	21
3.18	11	3.13	19	3.11	46
–	–	3.03	11	3.03	19

a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14 cm*.
b) Several additional reflections with spacings below 3.0 Å were also observed.

Results

X-ray diffraction

The results obtained from the interactions of PCP with bilayers of DMPE and DMPC are presented in Tables I to IV and Fig. 1 to 4. All the information included in each table and figure was obtained from samples and X-ray diagrams prepared and processed under exactly the same conditions. Table I presents the interplanar spacings and the relative intensities of the reflections of dry samples of DMPE, PCP and of their 1:1 molar mixture while their diffractograms are compared in Fig. 1. From them it can be appreciated that the X-ray pattern of the mixture does not quite correspond to a superposition of those of DMPE and PCP. In fact, most of the reflections of pure DMPE and PCP are very weakened or absent. On the other hand, several new reflections that do not belong to any of these compounds were produced by their mixture. This, obviously, indicates that the phospholipid structure has been altered by PCP. However, the damage produced by PCP to dry phospholipid bilayers was much higher in the

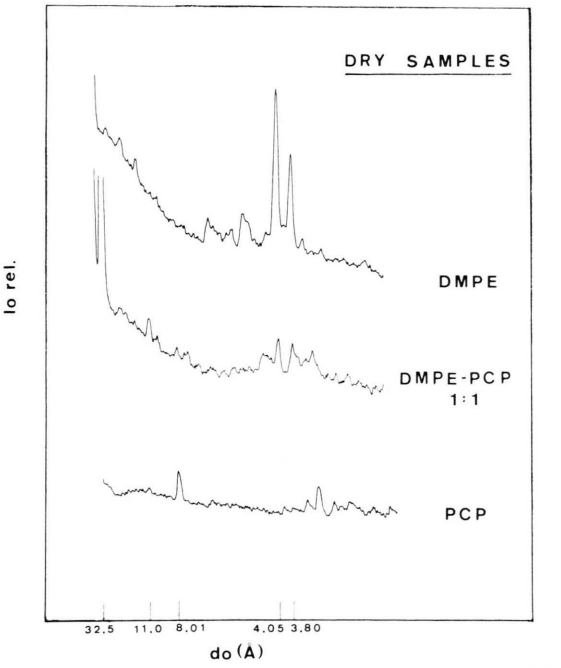

Fig. 1. Microdensitograms from X-ray diagrams of dry powder samples of DMPE, DMPE:PCP 1:1 and PCP. Flat-plate cameras (D = 8 cm).

Table II. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPC, DMPC:PCP 1:1 and PCP obtained from powder samples crystallized from CH_3OH (a, b).

DMPC		DMPC:PCP 1:1		PCP	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
54.5*	880*	–	–	–	–
–	–	41.7	1227	–	–
27.8	36	–	–	–	–
–	–	20.5	140	–	–
18.0	9	–	–	–	–
13.7	36	13.8	37	–	–
–	–	–	–	11.7	16
9.28	44	–	–	–	–
8.34	47	–	–	–	–
–	–	–	–	8.01	100
–	–	–	–	7.39	9
6.29	105	–	–	–	–
5.24	16	–	–	5.17	9
4.72	22	–	–	–	–
4.29	360	–	–	–	–
4.13	690	4.2	15	–	–
–	–	–	–	4.00	18
3.88	90	–	–	3.82	15
–	–	–	–	3.62	52
–	–	–	–	3.46	108
–	–	–	–	3.29	42
3.20	18	–	–	3.22	21
–	–	–	–	3.11	46
–	–	–	–	3.03	19

a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14 cm*.
b) Several additional reflections with spacings below 3.0 Å were also observed.

case of DMPC. As it can be seen in Table II and particularly in Fig. 2, the crystalline features of this phospholipid and PCP practically disappeared in their 1:1 molar mixture. This X-ray pattern shows only four reflections, being the first three orders of a 41.7 Å repeat, which is much shorter than the 54.5 Å bilayer width observed in dry DMPC. The fourth reflection is a 4.2 Å band, which is generally observed in lecithins with excess of water. In such a case the molecules become more disordered, being this spacing the mean separation between the hexagonally packed hydrocarbon chains [17]. This situation can be observed in the densitogram of DMPC in Fig. 4. What is surprising is the fact that this effect, which corresponds a "fluidization" of the bilayer, was observed in dry specimens below the transition temperature of DMPC.

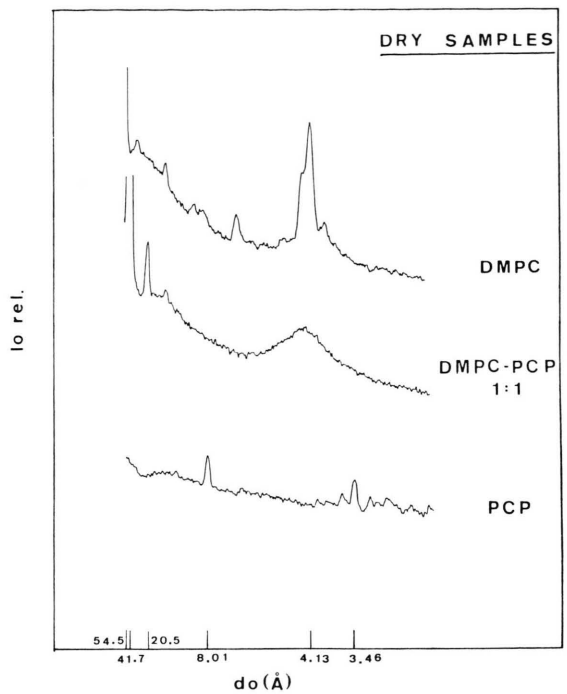

Fig. 2. Microdensitograms from X-ray diagrams of dry powder samples of DMPC, DMPC:PCP 1:1 and PCP. Flat-plate cameras (D = 8 cm).

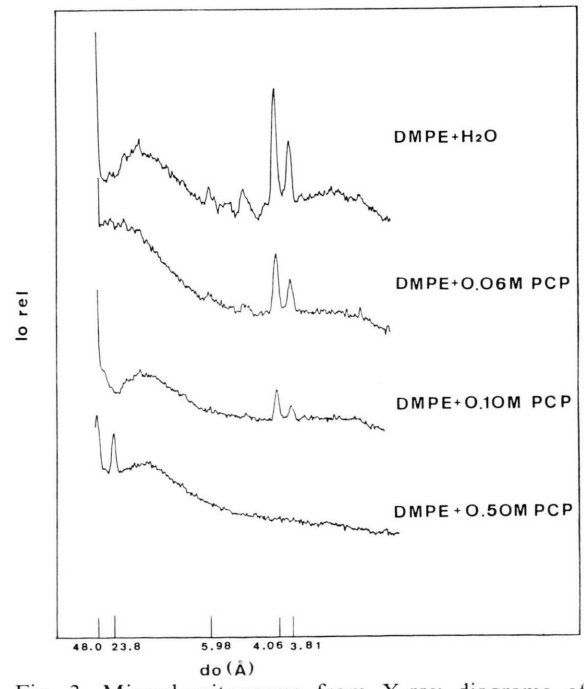

Fig. 3. Microdensitograms from X-ray diagrams of DMPE powder samples mixed with water and PCP aqueous solutions. Flat-plate cameras (D = 8 cm).

Table III. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE powder samples mixed with water and PCP aqueous solutions (a, b, c).

DMPE + H_2O in excess		DMPE + 0.06 M PCP		DMPE + 0.1 M PCP		DMPE + 0.5 M PCP	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
51.0*	1105*	50.8*	823*	51.4*	765*	48.0	230
–	–	–	–	32.5	12	32.0	11
25.7	18	25.4	26	–	–	24.0	124
17.3	33	17.2	32	17.0	9	–	–
12.7	45	12.7	20	12.7	18	–	–
7.32	21	–	–	–	–	–	–
5.98	55	5.95	24	5.97	9	–	–
5.08	34	5.10	14	5.05	6	–	–
4.78	106	4.77	24	–	–	–	–
4.64	40	4.61	20	4.63	12	–	–
4.25	44	4.25	8	4.23	3	–	–
4.06	499	4.05	240	4.05	103	–	–
3.81	266	3.81	126	3.79	54	–	–
3.63	19	3.65	6	–	–	–	–
3.39	7	–	–	–	–	–	–

a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14 cm*.
b) Several additional reflections with spacings below 3.0 Å were also observed.
c) The specimens were left for 11 days before being X-ray diffracted.

Table IV. Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPC powder samples mixed with water and PCP aqueous solutions (a, b).

DMPC + H_2O in excess		DMPC + 0.01 M PCP		DMPC + 0.02 M PCP	
do [Å]	Io rel	do [Å]	Io rel	do [Å]	Io rel
65*	733*	65*	681*	–	–
–	–	–	–	39	70
32.2	462	32.2	290	–	–
21.0	18	–	–	–	–
13.5	15	–	–	–	–
4.2	107	4.2	45	–	–

a) The interplanar spacings and intensities of the reflections were measured in X-ray diagrams obtained from flat-plate cameras. D = 8 and 14 cm*.
b) The specimens were left for 5 days before being X-ray diffracted.

Experiments were also performed in which the phospholipid bilayers, under the form of dry and insoluble powders, were made to interact with water and aqueous solutions of PCP. Table III and Fig. 3 show the results obtained when DMPE was mixed with pure water and PCP solutions of increasing concentrations. For the reasons explained in the Introduction, the bilayer structure of DMPE is not significatively affected by water. However, increasing concentrations of PCP produced a gradual diminishing in the number and intensities of the reflections. When the concentration of PCP was 0.5 M, none of the original reflections of DMPE remained. Instead, three new reflections showed up, which correspond to the second, third and fourth orders of a repeat of 96 Å. That value is about twice the bilayer width of DMPE in excess of water. Finally, Table IV and Fig. 4 present the results obtained when DMPC was mixed with pure water an aqueous solutions of PCP. Water, as already explained, produces an increase of DMPC interbilayer distance and a decrease in its molecular order. These effects are characterized here by the 65 Å bilayer width, a small number of reflec-

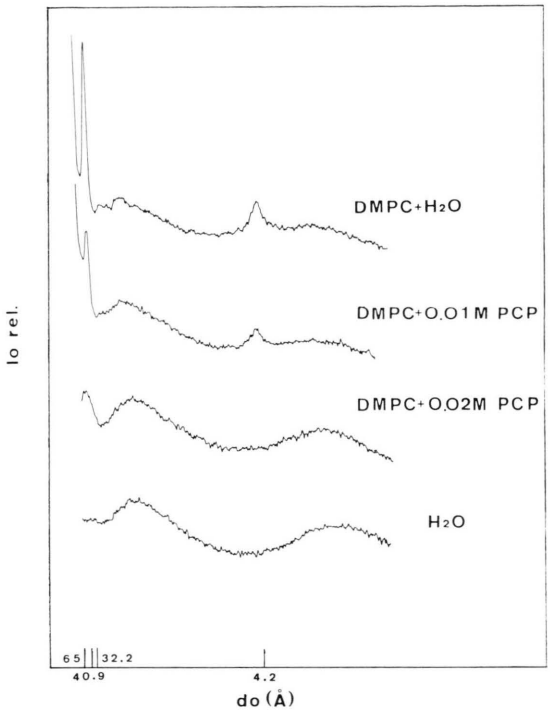

Fig. 4. Microdensitograms from X-ray diagrams of DMPC powder samples mixed with water, PCP aqueous solutions and of pure water. Flat-plate cameras (D = 8 cm).

tions and the 4.2 Å band. 0.01 M PCP produced only an attenuation of the intensities being, therefore absent the weakest reflections. However, 0.02 M PCP completely destroyed the bilayer organization of DMPC as even the 4.2 Å reflection is now absent. In fact, the resulting diffractogram is similar to that of pure water, except for the presence of a 39 Å weak band, which might be due to the diffuse scattering of a liquid-like arrangement of DMPC molecules.

Fluorescence

DPH emission steady-state anisotropy and decay was studied in DMPC large unilamellar liposomes. The anisotropy, measured in the absence and in the presence of 0.02 M PCP, was 0.33 and 0.20 respectively. This decrease indicates that the rotational mobility of the probe inside the bilayer has been enhanced by PCP. The phase and modulation were measured in the absence and in the presence of 0.02 M PCP at a number of modulation frequencies. The results are shown in Fig. 5. A solution of DMPOPOP (*p*-bis[2-(5-phenyl-3-methyloxazolyl)benzene]) in ethyl alcohol was used as the phase reference sample. Two component exponential analysis of the data yielded lifetimes of 11.5 and 4.4 ns in the absence and in the presence of 0.02 M PCP. In both cases the best fit was obtained considering a short component of 0.001 ns to take account of residual scattered light (Fig. 5).

An alternative approach to lifetime heterogeneity analysis involves the use of a distributional model [22–24]. Since DPH molecules can exist in a variety of different positions along the membrane normal, they can experience a range of environments, each of which is characterized by different lifetime values [22]. Under this approach, the same set of data was analyzed using a continuous of lifetime values characterized by a gaussian shape centered at a decay time C and having a width W. The results are shown in Fig. 6. The distribution in the absence of PCP had an average lifetime value of 11.5 ns, while in the presence of PCP it was 4.3 ns. Both average values were nearly the same of those

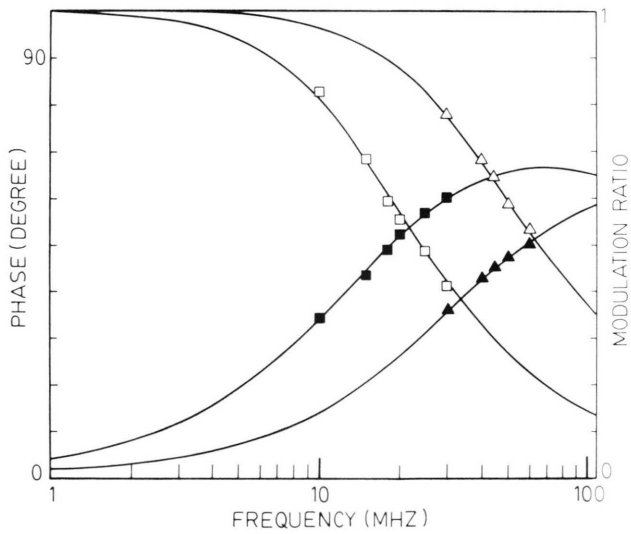

Fig. 5. Multifrequency phase (dark figures) and modulation (light figures) data for DPH fluorescence in DMPC large unilamellar liposomes at 18 °C, in the absence (squares) and presence (triangles) of 0.02 M PCP. Solid lines correspond to two component fits: for DMPC liposomes τ_1 = 11.4 ns, τ_2 = 0.001 ns and f_1 (fractional intensity) = 0.96; for DMPC liposomes + PCP, τ_1 = 4.4 ns, τ_2 = 0.001 ns and f_1 = 0.92.

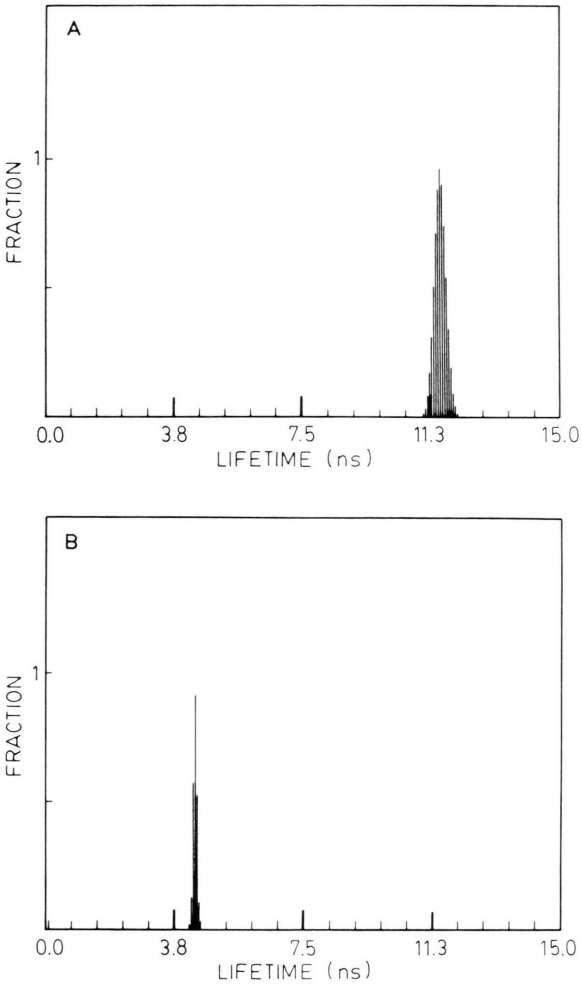

Fig. 6. Continuous distribution analysis of DPH fluorescence phase and modulation data in DMPC large unilamellar liposomes at 18 °C in the absence (A) and presence (B) of 0.02 M PCP. Center of the distribution: A = 11.5 ns; B = 4.3 ns. Full width at half maximum of distribution: A = 0.4 ns, B = 0.15 ns.

obtained in the exponential analysis. However, a difference between the widths of both distributions was observed. In the absence of PCP the full width at half maximum was 0.4 ns while upon the addition of PCP the distribution became quite narrow, with a width of 0.15 ns.

Discussion

The present study was concerned with the structural damage that PCP, in its sodium form, was able to produce to phospholipid bilayers. For this purpose, X-ray diffraction and fluorescence techniques were used. In the first, two multibilayer systems, built-up of DMPE and DMPC were made to interact with different concentrations of PCP, in the presence and absence of water. The results clearly indicate that PCP perturbed the bilayer structure of both phospholipids. This is most remarkable in the case of DMPE as this is a very stable system given its close molecular packing and high number of hydrophobic and electrostatic interactions [15]. Not surprisingly, the damage was higher in DMPC bilayers, as its molecular packing is less compact than of DMPE and, therefore, easier to penetrate. In the absence of water its 1:1 molar mixture had the consistency of a fluid paste. In fact, the X-ray pattern showed a disordered phase, with a 4.2 Å strong reflection which indicated an hexagonal molecular packing [17]. This arrangement is usually attained below the transition temperature only if water is present. On the other hand, a 0.02 M solution of PCP completely destroyed the remaining organization of DMPC bilayers.

The results of the fluorescence measurements performed on DPH large DMPC unilamellar liposomes also indicates that PCP perturbed its bilayer structure. Considering the hindered rotational model to describe the rotational behavior of DPH in bilayer systems [25], the steady-state anisotropy can be primarily related with the restriction of the rotational motion [26]. In this context, the decrease of the DPH fluorescence anisotropy upon addition of PCP would indicate a perturbation in the bilayer organization related to the packing of the hydrocarbon chains. The decrease of DPH average lifetime induced by PCP can be rationalized in terms of the sensitivity of the probe lifetime to the dielectric constant of the medium [27], enhanced by the presence of PCP ions between phospholipid polar groups and by a disruptive effect of PCP in the bilayer structure. The perturbation of the bilayer structure would also enhance the rotational and translational diffusion of the probe molecules that will rapidly average over the different environments. This assumption could support the narrowing in the lifetime distribution observed upon addition of PCP.

These results are in agreement with previous studies performed on liposomes which reported

changes in the dielectric environment of the probe following perturbation of the bilayer by PCP and other chlorinated phenols [9]. On the other hand, data obtained from natural membranes were also consistent with the assumption that PCP perturbs the lipid bilayer of cells, as they showed increases of up to 50% in fluidity [12]. From this kind of results it has been suggested that the propensity of organic pollutants to perturb the molecular organization of membranes reflect, a least in part, their toxicity [12]. In fact, it has been found that an apparent correlation exists between the extent of phospholipid perturbation and LD 50 values of the toxicants, being PCP the most toxic and most efficient bilayer "perturber" of the assayed compounds [28]. This has led the authors to suggest the possibility of formulating an "index of perturbation" which could be used to screen certain classes of organic compounds for biological toxicity on a routine basis. The present work demonstrated the feasibility of experimental techniques to measure this "perturbation index".

Acknowledgements

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