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Interaction of 2,4-dichlorophenoxyacetic acid (2,4-D) with cell and model membranes

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Abstract

2,4-dichlorophenoxyacetic acid (2,4-D), a widely used herbicide, is a component of the 'agent orange' whose toxicity has been extensively studied without definite conclusions. In order to evaluate its perturbing effect upon cell membranes, 2,4-D was made to interact with human erythrocytes and molecular models. These studies were performed by scanning electron microscopy on red cells, fluorescence spectroscopy on dimyristoylphosphatidylcholine (DMPC) large unilamellar vesicles and X-ray diffraction on multilayers of DMPC and dimyristoylphosphatidylethanolamine (DMPE). It was observed that 2,4-D induced a pronounced shape change to the erythrocytes. This effect is explained by the herbicide interaction with the outer monolayer of the red cell membrane.

Keywords: 2,4-Dichlorophenoxyacetic acid; Agent orange; Membrane; Phospholipid bilayer

1. Introduction

The widespread use of pesticides in an attempt to increase crop production has generated a series of toxicological and environmental problems, particularly in developing countries [1]. Thus, their increased use in the last four decades has been accompanied by numerous cases of acute poisoning. The World Health Organization has stated that their toxicity is extensive and serious with as many as 500000 cases occurring annually throughout the world [2]. In general, the molecular mechanisms of pesticide action are poorly understood. However, the lipophilicity of most of them makes lipid-rich membranes a possible target of their interaction with living organisms [3–5]. In the light of extensive studies of Antunes-Madeira et al. [6], the fluidity of membranes is considered one of the most sensitive parameters to their exposure to pesticides. An unequivocal relation between changes in membrane fluidity and the pesticide toxicity has not been established so far [3]. However, this subject deserves a further study because some effects directly related to toxicity could

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; LUV, large unilamellar vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 6-dodecanoyl-2-dimethylaminonaphtalene; GP, general polarization; SEM, scanning electron microscopy.

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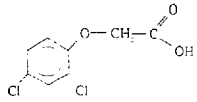

Fig. 1. Structural formula of 2,4-dichlorophenoxyacetic acid (2,4-D).

primarily be due to changes in membrane fluidity, e.g., permeability alterations for electrolytes and non-electrolytes [7], inhibition of acetylcholinesterase [8] or changes in lipid composition [9]. However, in spite of the implications that at least part of the toxic effects of pesticides could be due to the perturbation of the lipid phase of the membranes [10], very little work has been done on the possible changes in lipid fluidity due to their interactions.

2,4-dichlorophenoxyacetic acid (2,4-D), whose structural formula is shown in Fig. 1, is one of the most widely used herbicides [11]. In Vietnam it was used to defoliate jungle areas as a component of the 'agent orange' [12]. Its toxicity has been a topic of extensive research but no definite conclusions on the carcinogenicity, mutagenicity and genotoxicity have been drawn [12]. However, it is considered a moderately strong poison with a lethal dose in humans of about 28 g [13].

This paper describes the results of our studies on the interaction of 2,4-D with human erythrocyte membranes and models constituted by phospholipid multilayers and large unilamellar vesicles. These systems have previously been used in our laboratories to determine the interaction and perturbing effects on membranes by several therapeutic drugs [14–16] and the pesticides DDT [17] and pentachlorophenol [18,19]. The multilayers consisted of the phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), which represent phospholipids that are respectively located in the outer and inner monolayers of the human erythrocyte membrane [20]. Given the lipophilic nature of 2,4-D and the amphiphilic character of both phospholipids, their interactions were assayed in hydrophobic and aqueous media under a wide range of concentrations. The capacity of 2,4-D to perturb the multilayer structure of DMPC and DMPE was determined by X-ray diffraction methods.

Fluorescent steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization of 6-dodecanoyl-2-dimethylaminonaphtalene (Laurdan) were determined in DMPC large unilamellar vesicles. DPH is one of the most used probes for the hydrophobic regions of phospholipid bilayers. Its fluorescence steady-state anisotropy provides a measure of the rotational diffusion of the fluorophore restricted within a certain region due to the phospholipid acyl-chain order. On the other hand, Laurdan has a high sensitivity of excitation and emission spectra to the physical state of membranes [21]. With the fluorophore moiety located in a shallow position of the bilayer normal in the phospholipid polar head group environment, Laurdan provides information of dynamic properties in this zone of the bilayer [22,23]. Laurdan spectral shift quantification is done using the general polarization (GP) concept [24].

Finally, 2,4-D was incubated with human erythrocytes. The red cells were later observed by scanning electron microscopy to detect shape changes induced by the herbicide.

2. Materials and methods

2.1. X-ray diffraction analysis of phospholipid multilayers

Synthetic DMPC and DMPE from Sigma and 2,4-D (99%) from Aldrich were used without further purification. About 3 mg of each phospholipid were mixed with the corresponding weight of 2,4-D in order to attain DMPC:2,4-D and DMPE:2,4-D powder mixtures in the molar ratios of 10:1, 5:1, 2:1 and 1:1. Each mixture was dissolved in chloroform:methanol 3:1 v/v and left to dry. The resulting samples, under the form of crystalline powders, were introduced into special glass capillaries and diffracted in Debye-Scherrer and flat-plate cameras provided with rotating devices. The same procedure was also followed with samples of each phospholipid and 2,4-D. The aqueous specimens were prepared in glass capillaries mixing each phospholipid and 2,4-D in the same proportions as described above and then filled with about 200 μl of distilled water. These specimens were X-ray diffracted 2 and 14 days after preparation in flat-plate cameras. Specimen-to-film distances were

8 or 14 cm, standardized by sprinkling calcite powder on the capillaries surface. Ni-filtered CuKα radiation from a Phillips PW1140 X-ray generator was used. The relative reflection intensities were obtained from films by peak-integration in a Joyce-Loebl MKIIICS microdensitometer interfaced to a PC. No correction factors were applied. The experiments in water were performed at 17 ± 2°C, which is below the main transition temperatures of both DMPC and DMPE.

2.2. Fluorescence measurements on large unilamellar vesicles (LUV)

DMPC LUV suspended in water were prepared by extrusion of frozen and thawed multilamellar liposome suspension through two stacked polycarbonate filters of 400 nm pore size (Nucleopore, Corning Star Corp.) employing nitrogen pressure at 10°C over the lipid transition temperature, to a final concentration of 0.3 mM. DPH and Laurdan were incorporated into LUV by addition of small aliquots of concentrated solutions of the probe in ethanol to LUV suspension in water and gently shaken for ca. 30 min. The final probe concentration was 0.5 μM. Fluorescence spectra and anisotropy measurements were performed in a fluorolog spectrofluorometer from Spex and in a phase shift and modulation Greg-200 spectrofluorometer from ISS respectively, both interfaced to PC using ISS software. Measurements of LUV suspensions were made at 18°C employing 10 mm pathlength square quartz cuvettes. Sample temperature was controlled by an external Cole Parmer bath circulator and measured prior to and after each measurement using an Omega digital thermometer.

Table 1
Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPC, 2,4-D and of their 10:1, 5:1, 2:1 and 1:1 molar mixtures [a–c]

DMPC		DMPC:2,4-D 10:1		5:1		2:1		1:1		2,4-D	
do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel
543 *	344 *	54.7 *	83 *	54.3 *	425 *	54.7 *	221 *	55.0 *	81 *	–	–
–	–	35.5 *	2 *	35.3 *	65 *	35.9 *	68 *	35.7 *	392 *	–	–
27.2 *	6 *	–	–	–	–	–	–	–	–	–	–
18.5	2	18.6	1	18.4	7	18.7	10	18.7	5	–	–
13.7	30	13.7	5	13.7	33	13.8	28	13.8	2	–	–
9.30	4	9.33	1	9.23	7	9.31	2	–	–	–	–
8.30	2	8.25	2	8.26	5	8.30	3	–	–	–	–
–	–	–	–	–	–	–	–	–	–	7.22	7
6.29	10	6.28	8	6.25	16	6.31	7	–	–	–	–
–	–		–	–	–	–	–	–	–	5.48	5
–	–	–	–	–	–	–	–	–	–	5.23	6
4.77	1	–	–	4.78	7	4.81	2	–	–	–	–
4.66	6	4.72	7	4.61	3	4.65	2	–	–	–	–
4.32	36	4.29	28	4.26	20	4.29	5	–	–	–	–
4.13	100	4.13	80	4.12	134	4.18	61	4.2	35	–	–
–	–	–	–	–	–	–	–	–	–	3.97	6
3.88	23	3.87	14	3.86	4	–	–	–	–	3.84	5
–	–	–	–	–	–	–	–	–	–	3.57	14
–	–	–	–	–	–	–	–	–	–	3.45	6
–	–	–	–	–	–	–	–	–	–	3.36	13
–	–	–	–	–	–	–	–	–	–	3.21	10

[a] All the specimens were recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v).
[b] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flat-plate cameras with 8 and 14 * cm specimen-to-film distance.
[c] Only the main observed reflections are included.

Anisotropy measurements were done in the 'L' configuration using Glan Thompson prism polarizers in both exciting and emitting beams. The emission was measured with the aid of a WG-420 Schott high pass filter which showed negligible fluorescence. GP was evaluated by GP = (ib-ir)/(ib + ir), where ib and ir are the intensities at the blue and red edges of the emission spectrum respectively. These intensities were measured at 440 and 500 nm, corresponding to the emission maxima of Laurdan in gel and liquid-crystalline phases respectively [24]. For both probes the excitation wavelength was set at 360 nm. Blanck suspensions without probe were used to correct background light scattering. 2,4-D was incorporated by addition of small aliquots of a concentrated aqueous suspension to LUV already labeled with the corresponding fluorescent probe.

2.3. Scanning electron microscope (SEM) studies on human erythrocytes

The interaction of 2,4-D with human erythrocytes was achieved by incubating blood samples from clinically healthy male adult donors not being treated

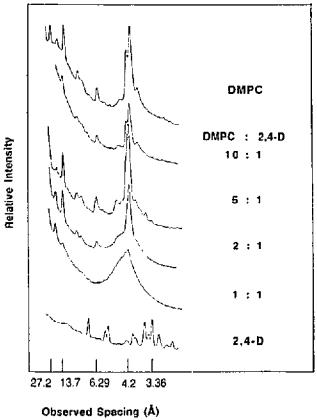

Fig. 2. Microdensitograms from X-ray diagrams of specimens recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v). Flat-plate cameras. Specimen-to-film distance 8 cm.

Table 2
Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPC, 2,4-D and of their 10:1, 5:1, 2:1 and 1:1 molar mixtures in water [a–c]

DMPC		DMPC:2,4-D 10:1		5:1		2:1		1:1		2,4-D	
do(Å)	Io rel	do(Å)	Io (rel)	do(Å)	Io rel	do(Å)	Io rel	do(Å)	Io (rel)	do(Å)	Io (rel)
63.1	205 *	63.1 *	115 *	63.1 *	86 *	63.1 *	59 *	63.1 *	46 *	–	–
31.6 *	84 *	31.3 *	55 *	31.3 *	79 *	31.6 *	51 *	31.6 *	36 *	–	–
20.9	5	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	7.22	12
–	–	–	–	–	–	–	–	–	–	5.48	14
–	–	–	–	–	–	–	–	–	–	5.23	11
4.19	100	4.20	65	4.19	66	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	3.97	15
–	–	–	–	–	–	–	–	–	–	3.84	5
–	–	–	–	–	–	–	–	–	–	3.57	21
–	–	–	–	–	–	–	–	–	–	3.45	3
–	–	–	–	–	–	–	–	–	–	3.36	21
–	–	–	–	–	–	–	–	–	–	3.21	18

[a] The samples were diffracted 2 and 14 days after preparation. No differences were observed.
[b] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from flat-plate cameras with 8 and 14 * cm specimen-to-film distance.
[c] Only the main observed reflections are included.

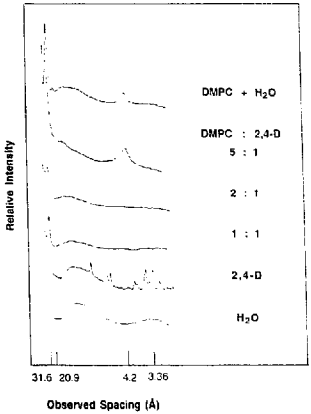

Fig. 3. Microdensitograms from X-ray diffraction diagrams of DMPC and aqueous suspensions of 2,4-D. Flat-plate cameras. Specimen-to-film distance 8 cm.

with any pharmacological agent by puncture of the ear lobule disinfected with 70% ethanol. Two drops were received in a plastic tube containing 10 ml of saline solution (0.9% NaCl) at 5°C. This blood solution was used to prepare the following samples: (a) control, by mixing 1 ml with 9 ml of saline solution, and (b) 2,4-D suspensions equivalent to 0.01 mM, 0.1 mM and 1 mM concentrations by mixing 1 ml of blood solution plus 9 ml of 2,4-D saline suspensions of adequate concentrations. These samples were incubated at 37°C for 1 h in an oven. They were then fixed with glutaraldehyde by adding one drop of each sample to a tube containing 1 ml of 2.5% glutaraldehyde in saline solution, reaching a final fixation concentration of about 2.4%. After resting overnight at 5°C, the fixed samples were placed directly on Al stubs, air-dried in an oven at 37°C for half to one hour and gold-coated for 3 min at 10-1 Torr in a S150 Edwards sputter device. The observations and photographic records were performed in an Etec Autoscan SEM.

3. Results

3.1. X-ray studies on phospholipid multilayers

The molecular interaction of 2,4-D with multilayers of the phospholipids DMPC and DMPE were studied in both a hydrophobic and an aqueous medium. Table 1 shows the interplanar spacings and relative intensities of the reflections produced by DMPC, 2,4-D and their 10:1, 5:1, 2:1 and 1:1 molar mixtures after interacting and being recrystallized from chloroform:methanol 3:1 v/v solutions. Their respective diffractograms are compared in Fig. 2. The analysis of these results indicated that the X-ray pattern of DMPC was perturbed by increasing concentrations of 2,4-D. In fact, even at such a small

Table 3
Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE, 2,4-D and of their 1:1 molar mixtures [a,b]

DMPE		DMPE:2,4-D 1:1		2,4-D	
do (Å)	Io rel	do(Å)	Io rel	dot(Å)	Io rel
51.4	331	51.4	286	–	–
25.5	3	25.4	1	–	–
17.1	5	17.1	2	–	–
12.7	5	12.8	4	–	–
–	–	7.26	8	7.22	16
5.96	14	5.92	4	–	–
5.72	5	–	–	–	–
–	–	5.48	3	5.48	11
5.22	2	5.18	6	5.23	16
5.03	3	–	–	–	–
4.78	19	4.79	8	–	–
4.66	7	–	–	–	–
–	–	–	–	4.12	3
4.04	100	4.04	66	3.97	14
3.80	52	3.81	44	3.84	11
3.64	3	–	–	–	–
–	–	3.57	9	3.57	31
–	–	–	–	3.45	12
–	–	3.36	9	3.36	29
–	–	3.21	3	3.21	22

[a] All the specimens were recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v).
[b] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from Debye-Scherrer and flat-plate cameras with 8 and 14 cm specimen-to-film distance.
[c] Only the main observed reflections are included.

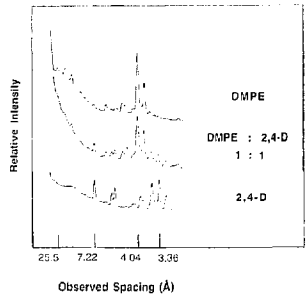

Fig. 4. Microdensitograms from X-ray diagrams of specimens recrystallized from $CHCl_3$:CH_3OH 3:1 (v/v). Flat-plate cameras. Specimen-to-film distance 8 cm.

DMPC:2,4-D molar ratio as 10:1, the reflection intensities became considerable weaker, most of them disappearing at the 1:1 ratio. On the other hand, two new reflections showed up. One, of about 35.5 Å, which might correspond to a ripple state reached by DMPC in its mixtures with 2,4-D [25]. The other, of 4.2 Å, arose from the stiff and fully extended hydrocarbon chains organized with rotational disorder in an hexagonal lattice [26]. Despite these changes, the bilayer width of DMPC remained practically constant at about 55 Å. On the other hand, in any X-ray diagram of these mixtures reflections from the herbicide were observed. These facts point to a deep penetration of 2,4-D into the hydrophobic core of DMPC.

Table 2 and Fig. 3 show the results obtained when DMPC, 2,4-D and their molar mixtures in the same ratios as above were immersed in distilled water. They were obtained 2 and 14 days after preparation without showing any significant change with time. It was observed that water produced an expansion of DMPC bilayer width from about 55 Å when dry to nearly 63 Å. The observed reflections were reduced to only the first three orders of the bilayer width and that of 4.2 Å described above. The increasing proportion of 2,4-D in the mixtures produced a diminishing

Table 4
Comparison of observed interplanar spacings (do) and relative intensities (Io rel) of DMPE, 2,4-D and of their 1:1 mixture in water [a] [c]

DMPE		DMPE:2,4-D 1:1		2,4-D	
do (Å)	Io rel	do(Å)	Io rel	do(Å)	Io rel
51.4 [b]	1051 [b]	51.4 [b]	346 [b]	–	–
25.5 [b]	2	–	–	–	–
17.0	5	–	–	–	–
12.7	10	–	–	–	–
–	–	7.20	7	7.22	19
5.93	6	5.94	3	–	–
–	–	5.43	4	5.48	21
–	–	5.24	5	5.23	18
4.78	7	–	–	–	–
4.04	100	4.04	47	3.97	23
3.79	50	3.80	31	3.84	8
–	–	3.53	30	3.57	46
–	–	3.47	2	3.45	5
–	–	3.36	25	3.36	42
	–	3.29	2	3.21	26

[a] The samples were diffracted 2 and 14 days after preparation. No differences were observed.
[b] The interplanar spacings and intensities of the reflections were measured in X-ray diagrams from flat-plate cameras with 8 and 14 cm specimen-to-film distance.
[c] Only the main observed reflections are included.

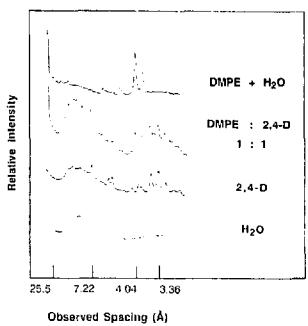

Fig. 5. Microdensitograms from X-ray diffraction diagrams of DMPE and aqueous suspensions of 2,4-D. Flat-plate cameras. Specimen-to-film distance 8 cm.

of the reflection intensities, most of them disappearing at the 2:1 ratio. Under these conditions only the 63.1 Å and 31.6 Å reflections remained. This meant a high structural perturbation of DMPC hydrophobic core. In fact, this region of its diffractograms looked very similar to that of pure water (Fig. 3).

Table 3 and Fig. 4 respectively show the interplanar spacings and X-ray patterns obtained after DMPE was made to interact with 2,4-D in the same way as described for DMPC in a hydrophobic medium. The perturbing effect of the herbicide upon the structure of DMPE multilayers was very mild. In fact, only at their 1:1 molar ratio was a change observed in the lipid pattern, which consisted of a slight diminishing of the reflection intensities. On the other hand, many 2,4-D reflections were also present in that mixture. Finally, the results of the interaction of DMPE and 2,4-D in the presence of water are presented in Table 4 and Fig. 5. As can be observed, the herbicide induced a somewhat stronger diminishing of DMPE reflection intensities than that produced in a hydrophobic medium. However, the observed effects of 2,4-D upon DMPE bilayers are much milder than those produced to DMPC.

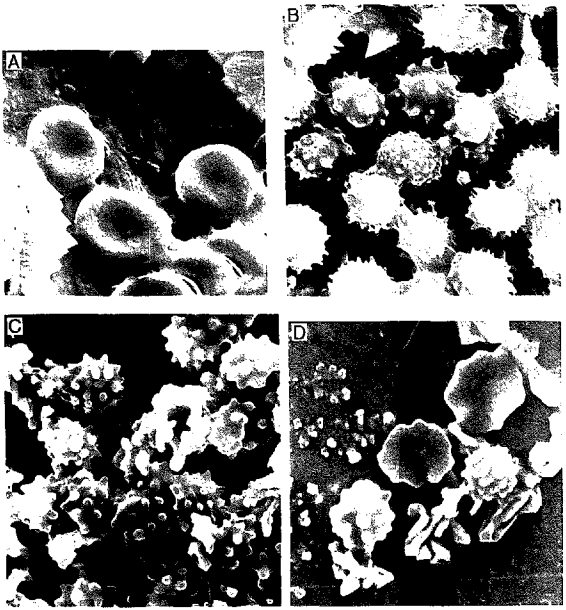

Fig. 6. Scanning electron microscope (SEM) images of human erythrocytes. ×2500. 2,4-D-free (a). Incubated with 2,4-D 1 mM (b); 0.1 mM (c) and 0.01 mM (d).

3.2. Fluorescent measurements on large unilamellar vesicles (LUV)

The effect of 2,4-D upon DMPC LUV was studied at the hydrocarbon chain and the hydrophilic/hydrophobic interface levels of the bilayer. This was achieved evaluating DPH steady-state fluorescence anisotropy (r) and Laurdan general polarization (GP). As shown in Table 5, the presence of increasing concentrations of 2,4-D produced a monotonous decrease in the fluorescence parameters of both probes with a larger effect on Laurdan GP. The DPH steady-state anisotropy is related primarily to the restriction of the rotational motion due to the hydrocarbon chain packing order. Therefore, the observed decrease in this parameter can be rationalized as a high structural disruption of the bilayer hydrophobic region produced by the incorporation of 2,4-D. On the other hand, the even larger effect of Laurdan GP indicated that the dynamics of the dipolar relaxation and/or the water penetration at the polar head group level was highly increased by the 2,4-D incorporation. There is, therefore, an agreement between these results and those obtained by X-ray diffraction on DMPC multilayers in an aqueous medium.

3.3. Scanning electron microscopy (SEM) studies on human erythrocytes

The electron microscopic examination of blood samples incubated with 2,4-D at different concentrations revealed definite red cell shape abnormalities. In contrast to normal red cells, in which the biconcave disc shape is characteristic (Fig. 6A), it was found that the erythrocytes presented a spiny configuration with numerous blebs and/or spicules in their surfaces. This shape change is known as crenation and the altered cells are referred to as echinocytes. The extent of these observed shape changes were dependent on 2,4-D concentration. Exposure of the erythrocytes to a 2,4-D suspension equivalent to a 1 mM concentration, the highest assayed, caused the most severe crenation effect. As can be observed in Fig. 6B, it resulted in a generalized shape change. The crenocytes exhibited a striking deformation of their plasma membrane consisting in numerous and prominent sharp-pointed protuberances. When 2,4-D concentration was lowered to 0.1 mM, the erythrocytes underwent similar changes. However, the shape alteration was rather minor. The cells showed a smaller number of roundish tip blebs (Fig. 6C). In the case of 0.01 mM sample incubation, it was observed that some erythrocytes did not show protuberances or spicules. However, they always displayed altered shapes consisting in polygonal or broken profiles and areas suggesting incipient bleb formation (Fig. 6D).

Table 5
Effect of 2,4-D on the anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the general polarization (GP) of Laurdan embedded in large unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (probe: lipid ratio 1:600)

2,4-D conc. (mM)	r DPH	GP Laurdan
0.00	0.274	0.513
0.01	0.261	0.464
0.10	0.127	0.119
1.00	0.092	0.021

4. Discussion

The toxicity of pesticides is a matter of great concern given their widespread use. Despite this fact, there is a generalized lack of knowledge about the molecular mechanisms that produce their toxic effects. In this paper, the toxicity of the herbicide 2,4-D towards cell membranes is described. With this aim, it was made to interact with natural and model membranes. X-ray diffraction studies were performed on multilayers of DMPC and DMPE. These are types of phospholipids that are respectively located in the outer and inner monolayers of most biological membranes. Experiments, carried out in hydrophobic and hydrophilic media, showed that 2,4-D deeply perturbed the structure of DMPC, whereas that of DMPE was much less affected.

The different type and degree of perturbation induced by 2,4-D to DMPC and DMPE can be related to their respective packing arrangements and the effects of water upon them. Chemically, they only differ in their terminal amino groups, being $+NH_3$ in DMPE and $+N(CH_3)_3$ in DMPC. Their structures are very similar in their dry crystalline phases [27]. In fact, both have the hydrocarbon chains mostly parallel and extended with the polar groups perpendicular

to them. However, DMPE molecules pack tighter than those of DMPC. This effect, due to its smaller polar group and higher effective charge, results in a very stable bilayer structure which is not significantly affected by the addition of water [27]. On the other hand, the gradual hydration of DMPC under the same conditions results in water molecules occupying the highly polar interbilayer spaces. As a consequence, there is an increase in its bilayer width from 55 Å up to about 63 Å as water fills in. This situation allowed the incorporation of 2,4-D into DMPC bilayers and its deep penetration in the hydrocarbon-chain region producing its structural disorder. These results were confirmed by fluorescence spectroscopy experiments performed on DMPC large unilamellar vesicles. In fact, it was shown that 2,4-D produced a deep structural perturbation to its hydrophobic region. Moreover, it was found that it also affected the lipid polar head arrangement.

In order to test whether 2,4-D was able to perturb the structure of cell membranes, it was incubated with human erythrocytes. The examination by scanning electron microscopy revealed that in fact the red cells changed their normal shape to a spiny configuration known as echinocyte.

Studies have shown that several compounds induce transformations from the discoid shape of erythrocytes to spiculated (echinocytes) or cupped (stomatocytes) forms [28,29]. Sheetz and Singer [30] formulated the bilayer couple hypothesis to explain these shape alterations. According to this, the shape changes induced to the erythrocytes arise from a differential expansion of the two monolayers of the membrane lipid bilayer. Thus, the echinocytes are produced by the insertion of the added compound in the outer monolayer, whereas stomatocytes are obtained by its location in the inner monolayer. The fact that 2,4-D induced the echinocytic shape in red cells is consistent with the way it interacted with phospholipid bilayers. The X-ray experiments on DMPC and DMPE clearly demonstrated that 2,4-D strongly interacted only with DMPC, which is located in the outer monolayer of the erythrocyte membrane. On the other hand, experiments performed on a neuroepithelial membrane has shown that 2,4-D interfered with Cl^- transport and total ion transport across the membrane [31]. It can, therefore, be concluded that toxic effects of 2,4-D can be related to its ability to perturb the phospholipid bilayer structure, whose integrity is essential for cell membrane functions.

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