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Perturbation of phospholipid bilayers by DDT

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The localization of the effects of DDT (5–50 mol%) addition on the acyl chain dynamics in unilamellar vesicles of two phosphatidylcholines (DPPC and egg PC) has been investigated by steady-state fluorescence polarization of a series of *n*-(9-anthroyloxy) fatty acids (*n* = 2, 6, 9, 12 and 16) whose fluorophore is located at a graded series of depths from the surface to the centre of the bilayer. The results show that DDT is a fluidizer of DPPC and egg PC bilayers. The increase in microviscosity of DPPC bilayers at 23°C begins at the centre of the bilayer (5 mol% DDT) and proceeds outward to the surface with increasing concentration of DDT (17 mol%). This pattern of effects is not evident in fluid bilayers of DPPC at 54°C or egg PC at 23°C. DDT (33 mol%) also lowers the phase transition temperature of DPPC bilayers by approximately 2 Cdeg. DDT (17 mol%) had no effect on the mean excited fluorescence life-time of 2-AP and 12-AS in DPPC, DOPC and egg PC bilayers. No quenching of 2-AP fluorescence was evident.

Introduction

Organochlorine insecticides are lipophilic compounds that generally exhibit high environmental persistence and striking biological selectivity. The basis for their toxicity is poorly understood. It is generally believed that they interfere with peripheral and central nerve conduction by alteration of the sodium and potassium conductances [1]. Recent evidence indicates that the potassium flux across non-electrogenic membranes is also affected [2,3], suggesting that DDT may be a general membrane poison.

Studies in our laboratory (W.C.) are directed at a better understanding of this pesticide-membrane interaction in order to define more fully where and how this compound affects membrane function. Fluorescent probes have provided useful information on the structure and dynamics of lipids in biological and model synthetic membranes. We have used ANS to obtain information on the state of organization in the mitochondrial membrane challenged with DDT [4]. Such membranes showed an enhanced capacity for ANS binding which was restricted only to the lipids of the membrane. Since ANS is localized mainly at the aqueous/lipid interface and hence is sensitive to changes in surface charge density and/or transmembrane electrical potential, then DDT presumably enhances ANS binding by increasing the positive

Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; ANS, 1-anilino-8-naphthalenesulphonic acid; AS, *n*-(9-anthroyloxy)stearic acid; AP, *n*-(9-anthroyloxy)palmitic acid, DOPC, dioleylphosphatidylcholine, egg PC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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surface charge density. It is therefore of interest to know whether this perturbation by DDT is confined to only the surface or extends deeper into the bilayer.

Recently a series of *n*-(9-anthroyloxy) fatty acid probes have been studied which sense the local environment at different depths in the bilayer thus providing information on the transverse fluidity gradient in the bilayer [5–7]. They have been used to assess the depth-dependent effects of anaesthetics [8] and of cholesterol [9,10] in synthetic phospholipid bilayers. Time-resolved fluorescence anisotropy measurements have shown that the fluorescent fatty acids rotate isotropically in fluid bilayers and do not show a finite anisotropy at infinite time [11,12]. They therefore reflect the kinetic component of fluidity and are not sensitive to bilayer order.

In this communication we wish to report on the effect of DDT on DPPC, egg PC and DOPC liposomes studied by single-photon counting techniques and steady-state fluorescence in conjunction with the series of *n*-(9-anthroyloxy) fatty acid probes.

Methods and Materials

DPPC, egg PC and DOPC were purchased from Sigma. The *n*-(9-anthroyloxy) fatty acids ($n = 2, 6, 9, 12, 16$) were purchased from Molecular Probes (Junction City, OR). DDT (99^+%) was purchased from Aldrich. All solvents were of the highest purity available.

Liposomes were prepared from the appropriate phospholipids by the sonication method. A chloroform solution of phospholipid with or without the indicated amount of DDT was reduced to dryness by rotary evaporation, forming a film on the inside of a round-bottom flask. The samples were stored overnight under vacuum to remove the residual solvent. The liposomes were formed by suspending the phospholipid in an appropriate quantity of 0.1 M Tris-HCl buffer (pH 7.5) and sonicating for 10 min at 55°C in the case of DPPC, or on ice under a flow of nitrogen for egg PC, using a MSE sonicator (S/W 30088) at a setting of 1.5 to 1.7 A. The suspension was incubated for a minimum of 10 min above the phase transition to ensure annealing of vesicles to prevent their aggregation [13]. The final concentration of phospholipid was 0.5 mM. The various probes were added to the liposome preparation in small volumes (10 μl) of methanolic stock solutions (1 mM) to give a final phospholipid/probe of 330:1. About 2 h were allowed for probe uptake in the dark.

Steady-state fluorescence polarization measurements were made with a single-photon counting fluorometer (Photochemistry Research Unit Department of Chemsitry, University of Western Ontario). The intensity measurements were corrected for scatter by using a blank identical to the sample but with no probe. It did contain the same quantity of methanol. The excitation wavelength was 365 nm and a Schott WG-330 filter was used in the excitation beam. A Schott WG 360 filter together with a 450 ± 40 nm interference filter (Ealing Scientific Ltd.) was placed in the path of the emitted light. The cuvette temperature was maintained by a circulating water bath. Continuous stirring of the cuvette contents was achieved using a microstirrer from Temtron Electronics (London, Canada).

The fluorescence polarization was calculated from the intensity measurements by:

$$P = \frac{I_{vv} - G(I_{vh})}{I_{vv} + G(I_{vh})}$$

where I_{vv} is the intensity measured when the polarizer and analyzer prism are in the vertical position and I_{vh} is the intensity when the analyzer prism is in the horizontal position. G is the correction factor given by the ratio of the vertical to the horizontal components when the excitation light is polarized in the horizontal direction.

Fluorescence lifetimes were measured in the Applications Laboratory of Photochemical Research Associates Inc., London, Ontario by the technique of time-correlated single-photon counting. The excitation and emission wavelengths of 363 nm and 418 nm respectively were selected by grating monochromators. The exciting light was vertically polarized and the emission polarization was set at 54.7° to the veritcal plane to eliminate the effects of polarized emission on the lifetime [14]. The results were deconvoluted as double exponentials by a nonlinear least-squares fitting decay computer program (Version 3.0). Goodness-

of-fit was assessed by the magnitude of the reduced χ^2 value and by inspection of the weighted residuals differences between the measured and calculated decay. The mean excited-state lifetime ($\langle\tau\rangle$) was calculated as $((\Sigma_i a_i\tau_i^2)/\Sigma_i a_i\tau_i)$ [15]).

Results and Discussion

The polarization of the anthroyloxy probe fluorescence in DPPC unilamellar liposomes at 25°C decreases as the fluorophore is moved further along the acyl chain (Fig. 1A), indicating greater freedom of rotation of the fluorophore because of increased fluidity of the hydrocarbon region. The steepest fluidity gradient occurs in the region of the bilayer sensed by the 6- to 12-AS probes.

The polarization of the fluorescence was decreased further when the liposomes were doped with DDT. At low concentrations of DDT (5 mol%) only the depolarization of the 16-AP and 12-AS was evident. As the concentration of DDT was increased to 9 mol% the depolarization of 9-AS was enhanced. Only at 17 mol% DDT was the depolarization of all the probes evident. Hence DDT has different effects at different depths of the bilayer, and the centre of the bilayer undergoes larger perturbations at low DDT levels than does the head-group region.

Above the transition temperature of DPPC the polarization of all the probes was much lower than when the lipid was in the crystalline state (Fig. 1B). However, the fluidity gradient was still evident and DDT caused a decrease in polarization at all levels in the membrane. The greatest change in fluidity occurred in the upper regions of the bilayer, while proportionately the effect on the hydrophobic core was less pronounce.d This differential effect of DDT on the fluidity of liposomes at 25°C and 54°C might be explained by the fact that some fluidity is essential to allow DDT to partition into the bilayer. At 25°C this requirement is met by the fluid interior, resulting in a preferential accumulation of DDT in this region of the bilayer. This in turn results in a preferential enhancement of fluidity, presumably as a result of increased disorder of the physical state in this region of the bilayer. By contrast, at 54°C the entire bilayer is sufficiently fluid to allow DDT to partition nonselectively into all depths of the bilayer. The fluidity of the interior is so large in contrast to the upper regions of the bilayer that DDT reduces the fluidity of the inter-

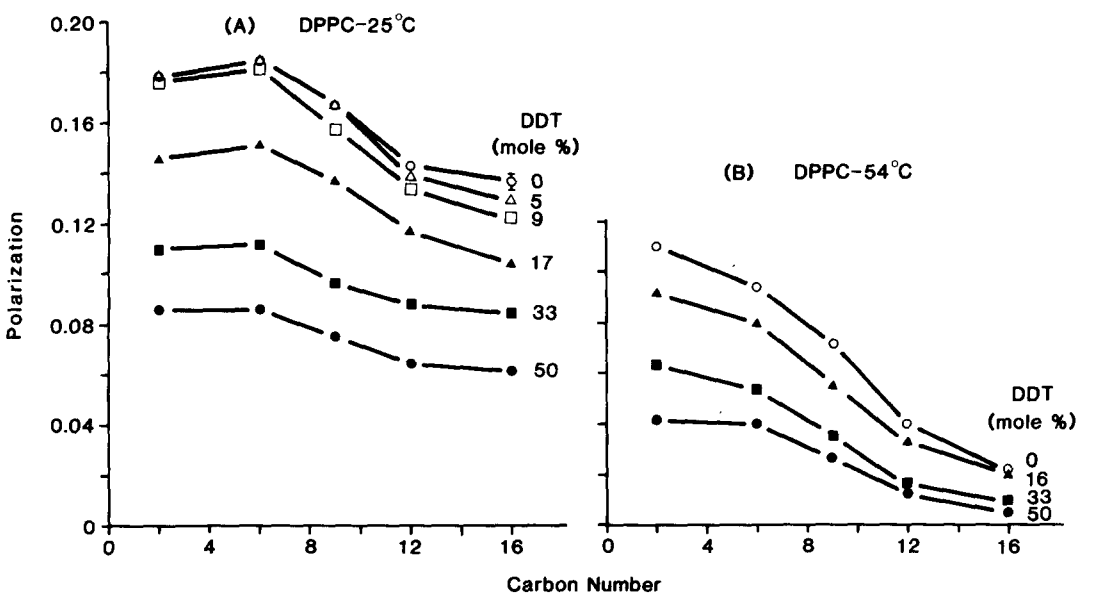

Fig. 1. Polarization gradients at two temperatures for five anthroyloxy fatty acid probes (6-, 9- and 12-AS, and 2- and 16-AP) in DPPC liposomes doped with DDT. The vertical error bar indicates error (±S.D.) of the determination of polarization.

ior proportionately less than that of the relatively less fluid upper region of the bilayer.

We also considered the possibility that the interaction of DDT with DPPC may depend on the nature of the paraffin chain of the phospholipid. Therefore, the effect of DDT on polarization of the anthroyloxy probes in liposomes of egg PC was determined. The polarization of the fluorescence of a given probe in egg PC at 25°C containing a particular DDT concentration (Fig. 2) is strikingly lower than that for the same probe in DPPC at 25°C containing the same concentration of DDT (Fig. 1A), indicating greater fluidity of the egg PC bilayer. However, a fluidity gradient is still evident. Egg PC consists of about 50% palmitic plus stearic acids and the rest of oleic acid and other polyunsaturated fatty acids [16]. The presence of DDT in egg PC reduced the fluorescence polarization of all the probes in a pattern similar to that of DPPC at 54°C. Clearly, the interaction of DDT with the bilayer does not depend on the fatty acid composition of the phospholipid as much as on the level of fluidity of the bilayer.

The anthroyloxy probes are useful in monitoring phase transitions as a function of depth. It was interesting therefore to see if DDT had any effect on the phase transition of DPPC. The data in Fig. 3 show that the polarization of 12-AS bound to unilamellar liposomes decreased sharply in the temperature range of 37.5 to 42.0°C with a

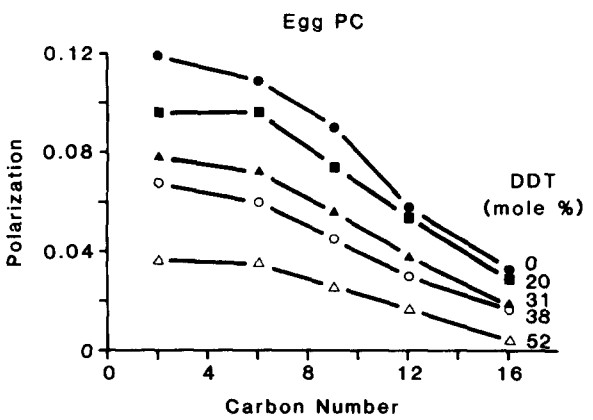

Fig. 2. Polarization gradients at 25°C for the five anthroxyloxy fatty acids in egg PC liposomes doped with various concentrations of DDT.

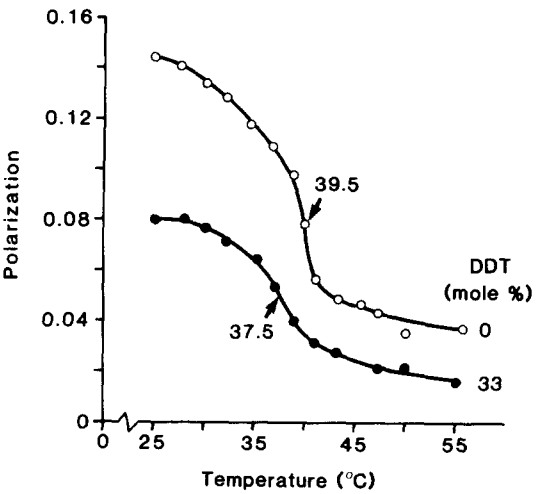

Fig. 3. Polarization as a function of temperature for 12-AS in DPPC liposomes in the presence and absence of 33 mol% DDT.

midpoint of 39.5°C. A similar temperature dependent depolarization of fluorescence of 12-AS was evident for liposomes doped with DDT except that it occurred over the temperature range of 35.5 to 39°C with a midpoint of about 37°C, resulting in a decrease in the transition temperature by about 2.5 Cdeg. A broadening of the main transition by 20 mol% DDT has also been detected by differential scanning calorimetry [17].

Table I shows the mean excited lifetimes of 2-AP and 12-AS in liposomes prepared from several phospholipids with and without 17 mol% DDT. The decay of fluorescence intensity of both probes was best fitted by a biexponential with two lifetimes, except for 2-AP in DPPC at 54°C where the decay was monoexponential. The longer lifetime component of 2-AP had about a 60% weighting in DPPC at 23°C and 87% to 90% in DOPC and egg PC. For 12-AS, the longer lifetime component had about an 85% weighting in DPPC and 91% to 92% in DOPC. The mean excited lifetime in 12-AS was greater than that of 2-AP in bilayers of saturated and unsaturated phospholipids at 23°C. However the mean excited lifetime of 2-AP but not of 12-AS was lower in DOPC than in DPPC either because of the lower viscosity of DOPC or because 2-AP is located in a more polar region of the DOPC membrane surface. The lower

TABLE I

THE EFFECT OF DDT ON THE FLUORESCENCE LIFETIMES, THE PRE-EXPONENTIALS AND MEAN EXCITED FLUORESCENCE LIFETIMES OF 2-AP AND 12-AS IN LIPOSOMES OF SEVERAL PHOSPHOLIPIDS

Determinations were made at 23°C except where indicated.

Lipid	Probe	DDT	τ_1 (a_1)	τ_2 (a_2)	$\langle\tau\rangle$
DPPC	2-AP	−	11.2 (0.22)	4.9 (0.32)	8.8
		+	10.8 (0.29)	4.3 (0.31)	8.9
	12-AS	−	13.4 (0.34)	3.7 (0.22)	12.0
		+	13.3 (0.35)	3.8 (0.21)	11.9
DPPC	2-AP	−	-	4.6 (0.67)	4.6
(54°)		+	-	4.7 (0.46)	4.7
	12-AS	−	9.9 (0.41)	2.9 (0.12)	9.3
		+	9.7 (0.39)	2.9 (0.12)	9.1
DOPC	2-AP	−	8.1 (0.36)	2.8 (0.11)	7.6
		+	7.9 (0.31)	2.8 (0.09)	7.4
	12-AS	−	12.6 (0.35)	3.4 (0.13)	11.7
		+	12.6 (0.27)	3.3 (0.10)	11.8
Egg PC	2-AP	−	7.9 (0.31)	2.4 (0.11)	7.4
		+	8.1 (0.41)	3.6 (0.14)	7.2

lifetimes of both 2-AP and 12-AS in DPPC above the phase transition than in DOPC or egg PC suggest an additional temperature effect on the polarity of the probe environment or lower viscosity of DPPC. DDT had no effect on the mean excited lifetimes nor on the relative weightings of the lifetimes of both probes, regardless of the composition and physical state of the bilayer. DDT also had no effect on fluorescence quenching of these probes in DPPC vesicles (unpublished data). Thus, the decrease in fluorescence polarization caued by DDT reflects a decrease in microviscosity of the bilayer and is not caused by an increase in fluorescence lifetime.

From these studies we conclude that DDT is a fluidizer of the lipid bilayer. The interaction of DDT with the bilayer does not seem to depend on the nature of the fatty acids of the phospholipids. It does seem to depend on the presence of fluid domains in the bilayer into which DDT may partition and which serve as centres of disorder of the more ordered region of the bilayer presumably by a weakening of the hydrophobic and electrostatic forces in the lipid bilayer.

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