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DIFFERENTIAL EFFECTS OF ENVIRONMENTAL CHEMICALS ON LIPOSOMAL BILAYERS

FLUORESCENCE POLARIZATION AND PESTICIDE-LIPID ASSOCIATION STUDIES

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The thermal dependence of the fluorescence polarization of 1,6-diphenylhexatriene was recorded upon interaction of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and some other pesticides with dipalmitoylphosphatidylcholine liposomes. Differential effects on the gel-crystalline phase were observed. Most substances decreased probe polarization; pentachlorophenol caused an increase of this parameter. The DDT-induced change of polarization was also dependent on the vesicle concentration thus indicating the influence of light scattering. The amount of DDT and pentachlorophenol residing in the lipid bilayer was determined to confirm the localization in the membrane. Correlation with the effects on probe polarization was obtained. The difference in response of the fluorescent probe to the presence of foreign molecules in the lipid bilayer may reflect different modes of interaction.

Introduction

A number of rather stable, lipid soluble organic chemicals is widely spread in our environment. Clearance from soil and plants is a slow process. The persistence of these chemicals or their metabolites in nature inevitably renders them amenable to the uptake by animals by a variety of routes.

Interaction of these pollutants with the cell membranes of animal cells has been already the focus of investigation [1]. We set out to study the interaction of environmental chemicals with mammalian cell membranes. Our approach has been to explore the effects of pesticides on model membranes of lipid vesicles as a prerequisite to attacking the same problem with biological membranes. Shifts of thermotropic phase transitions of liposomes containing membrane-bound pesticides have been reported [2].

In the present contribution we wish to report on differential effects of DDT and pentachlorophenol on the bilayer membrane of DPPC below the phase transition temperature, i.e. in the gel-crystalline phase. An attempt has been made to refer the pesticide effects to their actual bilayer concentration. This link has turned out to be important with respect to the interpretation of the results. In addition, our work may generally be of interest with respect to the problem of molecular interaction of xenobiotics with constituents of biological membranes, the topic which has recently gained new attention [3].

Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; PCP, pentachlorophenol; DPPC, 1,2-dipalmitoylphosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; DSPC, 1,2-distearoylphosphatidylcholine.

Materials and Methods

Reagents. Phospholipids and pesticides were obtained from the sources described [2]. 1,6-Diphenylhexatriene was purchased from Serva (Heidelberg, F.R.G.). [U-^{14}C]DDT (29.7 mCi/mmol) was from Amersham Buchler (Braunschweig, F.R.G.). [U-^{14}C]Pentachlorophenol (5 mCi/mmol) was synthesized by Dr. A. Attar (Gesellschaft für Strahlen- und Umweltforschung, Institut für Ökologische Chemie, F.R.G.).

Preparation of lipid vesicles. Large multilamellar lipid vesicles called liposomes [4] were prepared as described [2]. Briefly, all compounds intended to make up the liposomes including the fluorescent probe were mixed as alcoholic solutions in the appropriate molar proportions. Probe to lipid ratio was generally 1 : 1000. The solvent was blown off under a stream of nitrogen and the resultant lipid film resuspended in buffer (phosphate buffered saline, pH 7.4) by vortex agitation at +55°C. Final lipid concentration was 1 mM in a buffer volume of 1 ml unless otherwise stated.

Measurement of fluorescence polarization. Polarization of probe fluorescence in membranes was measured in an Aminco-Bowman spectrophotofluorometer. Two commercial sheet polarizers (Melles Griot, The Netherlands) were mounted at the excitation and emission site of the cuvette. Wavelengths were set at 360 nm (excitation) and 430 nm (emission) with proper turning to the peak position in each assay. Other techniques were as described [2]. When the temperature chosen had been stabilized (after 6–8 min) the suspension was stirred with a small plastic stick to prevent sedimentation of the vesicles. The intensity of the polarized fluorescence emission was then read off a photometer. The emission polarizer was then quickly rotated by 90° and the fluorescence intensity once again determined. This procedure was sometimes repeated to check for fluctuations. The degree of polarization was calculated as $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, according to the proposed formula [5]. $I_{\parallel}$ stands for the fluorescence intensity parallel to and $I_{\perp}$ stands for the fluorescence intensity perpendicular to the plane of polarization of the excitation beam. Corrections for background intensity were not made because $I_{\parallel}$ and $I_{\perp}$ of diphenylhexatriene in buffer were 2% of $I_{\parallel}$ and 2.5% of $I_{\perp}$ of the intensity measured in liposomes. Straylight of pure vesicles was 3–5% of total fluorescence intensity. Dependence on vesicle dilution of fluorescence polarization was checked separately (Fig. 4, and Ref. 6).

Estimation of pesticide content in lipid bilayers. Liposomes were separated from unbound pesticides as follows: (1) The vesicle suspension was centrifuged at $200 \times g$ for 10 min in order to remove any unbound particulate material, and subsequently centrifuged at $20000 \times g$ for 10 min in order to sediment the lipid vesicles. (2) The vesicle suspension was chromatographed over a 2×10.5 cm column of Sephadex G-100 at room temperature. The column had been equilibrated with buffer and pre-run with a suspension of liposomes lacking the pesticide under study. Exclusion volume of Trypan blue was 13–14 ml; 3-ml fractions were collected.

Lipid content of samples was estimated through the absorption at 252 nm and quantitated by comparison with standard curves, separately established for each lipid species. Pesticides were analyzed either by counting ^{14}C-radioactivity in aliquots of labelled samples or by gas chromatography (DDT). For this, the peak fractions (cf. Fig. 3, fraction number 5) were extracted with chloroform/methanol (1 : 1, by vol.) and the organic phase collected and reduced to a small volume. Samples were injected into a Varian gas chromatograph, model 3740, with FID. Operation conditions were: 6 ft. $\times$ 2 mm i.d. glass column packed with 3% OV-101 on Chromosorb WHP 80/100 mesh. Flow rate was 30 ml of nitrogen carrier gas per min, temperature was +215°C. Retention time of DDT was 5.5 min.

Results

Fluorescence polarization of diphenylhexatriene was recorded as a function of temperature in DPPC liposomes containing various pesticides. Fig. 1 depicts the influence of DDT. The most obvious effect is the decrease of probe polarization in the temperature region below the phase transition, i.e. in the gel-crystalline phase. However, the direct interpretation in terms of the uncorrected degree of polarization is subject to restrictions (see Discussion). Further effects of DDT on DPPC

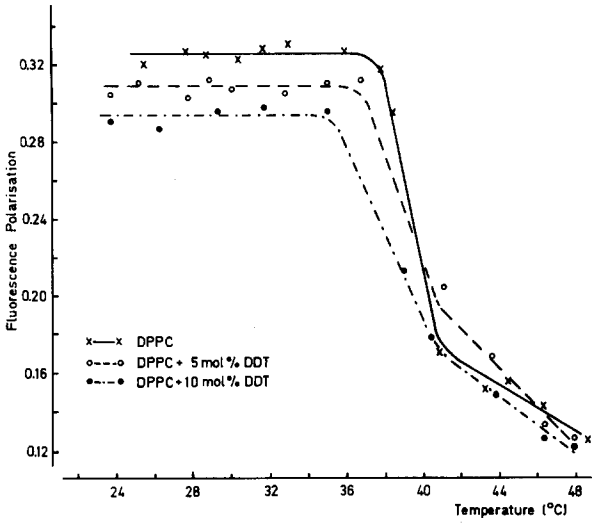

Fig. 1. The influence of DDT on the degree of polarization of diphenylhexatriene in DPPC liposomes. The intensity of the polarized fluorescence emission of diphenylhexatriene in DPPC liposomes containing DDT was measured at various temperatures. The degree of polarization was calculated [5] and plotted against temperature. Pesticide content is given as molar ratio DDT to DPPC. The T_m of DPPC was 39.7±0.3°C (n=12). The reason for this rather low value is unknown. Further experimental details are as in Materials and Methods. ×————×, DPPC; O------O, DPPC+5 mol% of DDT; ●·-·-·●, DPPC+10 mol% of DDT.

liposomes are the shift of the phase transition midpoint to lower temperatures and the broadening of the transition range, the latter being the result of the earlier onset of the transition event. Since similar results have been already obtained by recording the temperature dependence of fluorescence intensity of Parinaric acid [2], this aspect has not been studied further.

The profiles shown in Fig. 1 were also recorded with a variety of other pollutants, yet with varying intensity of the effect. Table I compiles the data of experiments at two temperatures which represent the gel-crystalline phase (+30°C) and the liquid-crystalline phase (+45°C). Most substances influenced significantly probe polarization in the gel-crystalline phase but none of them in the liquid -crystalline phase. The change of polarization is dependent on the concentration of the pesticides as exemplified in Fig. 2 with DDT and pentachlorophenol. Clear dose-related effects are apparent in the gel-crystalline phase only (Fig. 2, Panel A). The influence of light scattering of the vesicles containing DDT will be dealt with in the Discussion (cf. Fig. 4).

Some studies on the distribution of the pesticides between the lipid phase and the buffer were undertaken to evaluate the differential effects described above. Centrifugation and gel chromatography were the methods chosen to separate lipid-bound pesticides from free, unbound material. Low speed centrifugation of the original preparation cleared the vesicle preparation from any particulate material without sedimenting liposomes as judged by the 252 nm absorption. Centrifugation of the supernatant suspension at 20000×g pel-

TABLE I

THE INFLUENCE OF PESTICIDES ON THE FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN DPPC LIPOSOMES

The polarization of the fluorescence emission of the membrane probe was determined at the temperature of +30°C (representing the gel-crystalline phase of DPPC), P_{30}, and at +45°C (representing the liquid-crystalline pase), P_{45}. Experimental details are described in Materials and Methods.

Pesticide (number of determinations)		Pesticide/DPPC ratio (mol%)	P_{30}	P_{45}
None	(18)	–	0.320±0.007	0.150±0.007
Atrazine	(1)	7.5	0.325	0.147
2,4,5-Trichlorophenoxyacetic acid	(3)	10	0.315±0.002	0.147±0.002
4,4′-Dichlorobiphenyl	(3)	10	0.290±0.020	0.145±0.010
DDE	(6)	10	0.295±0.010	0.155±0.008
DDT	(10)	10	0.290±0.005	0.148±0.005
PCP	(10)	10	0.342±0.006	0.157±0.009

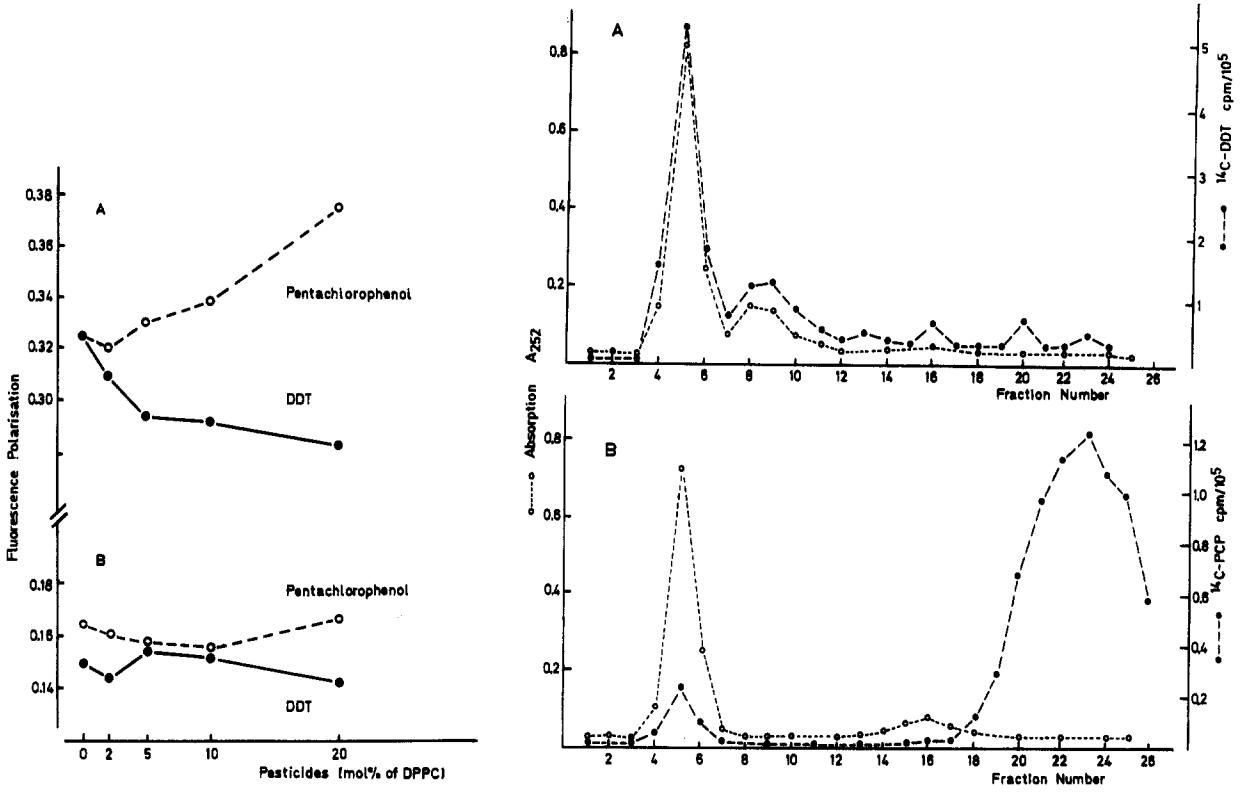

Fig. 2. Effect of DDT and pentachlorophenol on probe polarization in DPPC liposomes: dependence on the concentration of the pesticides. The preparation of liposomes and the determination of fluorescence polarization are described in the legend to Fig. 1 and in Materials and Methods. Panel A: polarization at +30°C; Panel B: polarization at +45°C.

Fig. 3. Gel chromatography profile on Sephadex G-100 of DPPC liposomes containing DDT or pentachlorophenol. Details of vesicle preparation, gel chromatography and fraction analysis are found in Materials and Methods. Pesticide concentration was 12 mol% of phospholipid. Panel A: Experiment with DPPC-DDT vesicles labelled with 2.5 μCi of [^{14}C]DDT. Lipid recovery of the peak fraction was 33%. O------O, lipid absorption at 252 nm; ●------●, radioactivity of [^{14}C]DDT. Panel B: Experiment with DPPC-pentachlorophenol vesicles labelled with 0.5 μCi of pentachloro[^{14}C]phenol. Lipid recovery of the peak fraction was 30%. O------O, lipid absorption at 252 nm; ●------●, radioactivity of pentochloro[^{14}C]phenol.

leted the lipid vesicles. The pesticide to lipid ratio of the pellets was determined and compared with the ratio of the initial preparation (Table II). Essentially, the ratio was found unchanged after centrifugation. Yet DOPC liposomes did no longer contain the full amount of DDT originally applicated. In any case, the amount of lipid-bound pesticides is well in excess of the amount that could have been bound solely due to entrapment in the water space of the vesicles (cf. Discussion, and Ref. 7). Thus, at the stage of preparation, the bulk of the pesticides was associated with bilayer lipid, presumably dissolved in the lipid phase.

Gel chromatography over Sephadex G-100 gave an indication of the tightness of lipid-pesticide interaction (Fig. 3). The large multilayered vesicles eluted in the void volume contained still and appreciable amount of DDT (Fig. 3, Panel A). On the other hand, the minor part only of pentachlorophenol of the original preparation co-chromatographed with the vesicles (Fig. 3, Panel B). The bulk of this compound was eluted from the column in subsequent low molecular weight fractions. The pesticide to lipid ratio of the peak fractions was analyzed. It dropped to 40–50% of the initial value in DPPC-DDT liposomes and to

TABLE II

INCORPORATION OF DDT AND PENTACHLOROPHENOL INTO LIPOSOMAL BILAYERS

Liposomes composed of 250 nmol of phospholipid plus the indicated amount of pesticides (labelled with 0.2 μCi of [^{14}C]DDT or 0.1 μCi of pentachloro[^{14}C]phenol) were prepared in 0.5 ml of buffer according to the standard procedure. Low speed centrifugation at 200×g removed about 8% of total DDT and about 4% of total pentachlorophenol. Lipid vesicles were sedimented at 20000×g. The pesticide to lipid ratio of the pellets is compared with the ratio of the initial preparation.

Lipid	Pesticide	Initial ratio pesticide/lipid (mol%)	Pesticide/lipid ratio of pellets (mol%)
DPPC	DDT	13	14
	PCP	9	8
DOPC	DDT	13	8
	DDT	15	10
	PCP	9	8
Egg lecithin	DDT	13	15
	PCP	9	8

5–10% in DPPC-pentachlorophenol vesicles. The release of DDT from lipid vesicles appeared to depend on both the nature of the fatty acyl chain (compare the various types of phosphatidylcholines of Table III) and the physical state of the bilayer (compare DMPC et +30°C and +5°C). The nearly total loss of pentachlorophenol beared no lipid specificity.

The polarization of diphenylhexatriene fluorescence was subsequently determined in the peak fractions which contained the lipid vesicles after gel chromatography (Table IV). The influence of pesticides was now attenuated (DDT) or eliminated (pentachlorophenol). For example, the concentration of DDT in the eluted vesicles was half the initial value. Correspondingly, the effect on probe polarization at +30°C was reduced in parallel. The miniscule concentration of pentachlorophenol in those vesicles was insufficient to cause any effect.

Discussion

In the present work, the dependence on temperature of fluorescence polarization of diphenylhexatriene has been exploited to record the influence of pesticides on the physical state of membrane lipids. The exact location of the probe in the hydrophobic interior is a matter of current discussion [8–10]. In our model system, the reliability of this fluorophore to report on the motional freedom of bilayer molecules in response to pesticide interaction has been strengthened by the broad accordance of the results obtained with diphenylhexatriene and parinaric acid [2]. The shift of the phase transition temperature and the broadening

TABLE III

GEL CHROMATOGRAPHY ON SEPHADEX G-100 OF LIPOSOMES CONTAINING DDT OR PENTACHLOROPHENOL

Liposomes were prepared with varying amounts of labelled pesticides (cf. legend to Fig. 3) and chromatographed on a column of Sephadex G-100 as described in Materials and Methods. The pesticide to lipid ratio of the peak fraction (usually fraction number 5; cf. Fig. 3) after chromatography was compared with the ratio of the initial preparation. Pesticide analysis was as described in the experimental section.

Lipid	Pesticide	Initial ratio pesticide/lipid (mol%)	Pesticide/lipid ratio of the peak fraction (mol%)	Remarks
DPPC	DDT	10	5±0.6	4 determinations
	DDT	25	10	
	PCP	12	1	2 determinations
DMPC	DDT	12	9	at +30°C
	DDT	12	2	at +5°C
DOPC	DDT	12	5	2 determinations
	DDT	25	8	
Egg lecithin	DDT	25	9	
	PCP	12	1	

TABLE IV

THE INFLUENCE OF PESTICIDES ON THE FLUORESCENCE POLARIZATION OF DIPHENYLHEXATRIENE IN DPPC LIPOSOMES AFTER GEL CHROMATOGRAPHY

DPPC liposomes (1 mM lipid in 1.5 ml of buffer) containing DDT or pentachlorophenol were prepared and chromatographed on Sephadex G-100 as detailed in Materials and Methods. Fluorescence polarization of diphenylhexatriene was determined in the peak fraction at temperatures of +30°C (P_{30}) and +45°C (P_{45}). The analysis of fractions was performed as described in the experimental section.

Pesticide	Initial ratio, pesticide/DPPC (mol%)	Pesticide/DPPC ratio of the peak fraction (mol%)	P_{30}	P_{45}
None	–	–	0.362	0.146
	–	–	0.368	0.155
DDT	12	5.3	0.335	0.149
	12	4.5	0.355	0.178
PCP	12	0.9	0.355	0.178
	12	1.0	0.373	0.191

of the transition width are likewise sensed by both probes. Superficially, it appeared that diphenylhexatriene would generally yield additional information on changes of the gel-crystalline phase by comparing directly polarization data (Fig. 1). However, this procedure seems not to hold for at least one compound, the insecticide DDT. Dilution of standard preparations of liposomal vesicles usually resulted in a higher degree of fluorescence polarization (Fig. 4). Extrapolation to zero vesicle concentration (as proposed in Ref. 6) revealed that the difference between pure liposomes and those containing DDT narrowed to a statistically insignificant amount (Fig. 4). Therefore, the observed effect of DDT on fluorescence polarization of diphenylhexatriene in DPPC liposomes at +30°C seems rather to be the result of different light scattering properties which may be based, for example, on alterations of liposomal size, shape, density or aggregation.

On the other hand, the increase of polarization upon incorporation of pentachlorophenol appears to persist throughout the dilution range examined as does the decrease of probe polarization upon interaction with 4,4'-dichlorobiphenyl (Fig. 4). Thus, the effect of DDT seems to be unique.

The relative change of probe polarization caused by pentachlorophenol and dichlorobiphenyl may be interpreted on the following assumptions. The degree of polarization of the fluorophore reflects the rate of the rotational motion of the probe ('rotational depolarization'). Probe rotation is sensitive to alterations of the structural order of surrounding phospholipid fatty acyl chains or chain segments [11,12] as well as to altered molecular

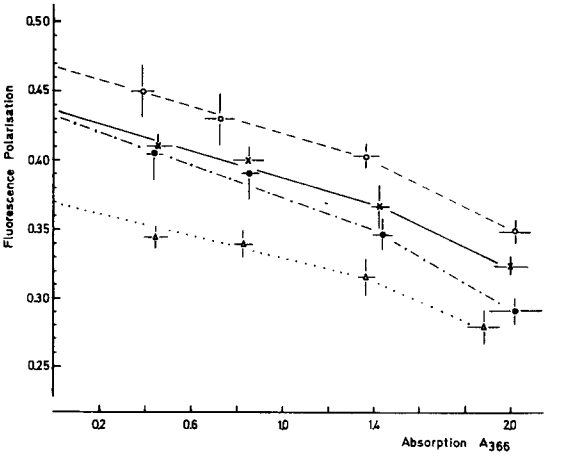

Fig. 4. Dependence on vesicle concentration of fluorescence polarization of diphenylhexatriene in DPPC liposomes. Lipid vesicles containing 10 mol% of the pesticides were prepared according to the standard procedure. Fluorescence polarization of the probe was determined at +30°C in the original suspension and in subsequent dilutions (1:1 with the same buffer). Concentration of vesicles was determined by measuring the absorbance at 366 nm [6]. ×————×, DPPC; ●-·-·-●, DPPC+DDT; O------O, DPPC+pentachlorophenol; △·····△, DPPC+4,4'-dichlorobiphenyl. The range of the S.D. is marked of both, the polarization and the absorbance.

packing of the lipids [13] influencing the motional freedom of lipid molecules in the plane of the membrane. Pentachlorophenol and dichlorobiphenyl alter these parameters in opposite directions in the gel-crystalline phase. Since diphenylhexatriene monitors an average perturbation of the bilayer, eventually composed of a weight average of all lipid domains therein, one would not expect to detect lipid phase separation in DPPC liposomes, induced for example by insertion of pesticides [5]. This question could be approached by use of other technique such as differential scanning calorimetry or NMR spectroscopy. With the method of steady-state fluorescence polarization used here, discrimination of the relative contribution of each lipid domain to the observed overall polarization is not feasible.

In order to support the conclusions derived from results of spectroscopic methods in turbid lipid suspensions we spent some effort on looking into the capacity of lipid bilayers to take up pesticides. Based on the *n*-octanol-water distribution coefficients of DDT and pentachlorophenol ($1.55 \cdot 10^6$ and $1 \cdot 10^5$, respectively [14]) more than 99% of the compounds should be dissolved in the lipid phase of the vesicles. After centrifugation, the pesticide to lipid ratio in the liposomes was not found to deviate much from the ratio of the initial preparation (Table II). The volume of the vesicles is about 1 μl [7] which corresponds to 1/500 of our sample volume. If a substantial amount of the pesticides would have been dissolved in the water phase or precipitated before uptake into the lipid bilayer this ratio would have been drastically different. Since this was not the case the results point strongly to pesticide accumulation in the lipid phase of the bilayer in line with the distribution coefficients. Further substantiation comes from the fact that the influence of the chemicals has been revealed mainly by changes of fluorescence emission of probes which are known to reside in the hydrophobic interior of bilayer membranes [5,8–10,15].

Differences of the partition coefficients take again effect during gel chromatography of the liposomes. Analysis of the eluted vesicles indicated that DDT, initially dissolved in the bilayer, is partially released from the vesicles on the gel column. The new, reduced concentration of DDT gives now rise to a diminished effect on probe polarization in parallel to the actual pesticide level in the membrane. However, sample dilution in the course of gel chromatography does also contribute to the smaller difference between pure liposomes and those containing DDT (Fig. 4). The total loss of the effect of pentachlorophenol is an unbiased, logical consequence of the vanishing concentration in the bilayer.

The distribution studies described are certainly not comprehensive. Reassessment of the in situ occurrence of DDT and pentachlorophenol in the bilayer of liposomes was necessary to support the conclusions derived from measurement of probe fluorescence polarization.

Some recent reports on the interaction of organic chemicals with phospholipid vesicles merit attention [16–19]. Reduction of fluorescence intensity of 1-anilino-8-naphthalene sulfonate by 20 mol% of chlorophenols in egg lecithin and DMPC liposomes was demonstrated to correlate with the order of toxicity [16]. A similar relation between the LD_{50} values of organic pollutants and the extent of lipid perturbation in DPPC liposomes was detected by means of differential scanning calorimetry [17]. Progressive broadening of the transition range and elimination of the pretransition were found at pesticide concentrations ranging from 5 to 20 mol% [17]. The steric structure of polychlorinated biphenyls contributed to the modification of spin-labelled DMPC liposomes pointing to an increase of membrane fluidity [18]. Antunes-Madeira and co-workers [19] inferred fluidizing effects on lipid bilayers upon interaction with 10 mol% of pesticides from recording sample turbidity. Essentially, the authors arrived at very similar conclusions as we did from our experiments. Two widely used agricultural pesticides (Parathion and Azinphos) were shown to lower the phase transition tenperature and to broaden the transition range in homogeneous and heterogeneous bilayers [19]. The appearance of a biphasic response in DMPC + DSPC bilayers upon pesticide interaction was interpreted as promotion of phase separation.

In conclusion, information on the mode of action of organic chemicals in model membranes is accumulating. The interpretation of the results relies heavily on inherent properties of the mem-

brane probes used. The validity of conclusions may differ from one compound to the other. It remains to be seen if only a similar type of interaction such as described here exists in biological membranes.

References

1 Pritchard, J.B. (1979) Fed. Proc. 38, 2220–2225
2 Buff, K. and Berndt, J. (1981) Biochim. Biophys. Acta 643, 205–212
3 Conrad, M.J. and Singer, S.J. (1981) Biochemistry 20, 808–818
4 Tyrrell, D.A., Heath, T.D., Colley, C.M. and Ryman, B.E. (1976) Biochim. Biophys. Acta 457, 259–302
5 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
6 Lentz, B.R., Moore, B.M. and Barrow, D.A. (1979) Biophys. J. 25, 489–494
7 Szoka, F., Jr. and Papahadjopoulos, D. (1980) Annu. Rev. Biophys. Bioeng. 9, 467–508
8 Petri, W.A., Jr., Pal, R., Barenholz, Y. and Wagner, R.R. (1981) Biochemistry 20, 2796–2800
9 Mely-Goubert, B. and Freedman, M.H. (1980) Biochim. Biophys. Acta 601, 315–327
10 Rockley, M.G. and Najjar, D.S. (1981) Biochim. Biophys. Acta 664, 96–100
11 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261
12 Jähnig, F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6361–6365
13 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332
14 Scheele, B. (1980) Chemosphere 9, 293–309
15 Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1977) Biochemistry 17, 819–828
16 Danner, J. and Resnick, H. (1980) Biochem. Pharmacol. 29, 2471–2474
17 Packham, E.D., Thompson, J.E., Mayfield, C.I., Inniss, W.E. and Kruuv, J. (1981) Arch. Environ. Contam. Toxicol. 10, 347–356
18 Reich, T., Depew, M.C., Marks, G.S., Singer, M.A. and Wan, J.K.S. (1981) J. Environ. Sci. Health 16, 65–71
19 Antunes-Madeira, M.C., Carvalho, A.P. and Madeira, V.M.C. (1980) Pest. Biochem. Physiol. 14, 161–169