ELSEVIER

Biochimica et Biophysica Acta 1190 (1994) 149–154

BBA Biochimica et Biophysica Acta

Effects of parathion on membrane organization and its implications for the mechanisms of toxicity

M.C. Antunes-Madeira, R.A. Videira, V.M.C. Madeira *

Centro de Biologia Celular, Departamento de Zoologia, 3049 Coimbra Codex, Portugal

(Received 27 July 1993)

Abstract

The effects of the organophosphorus insecticide parathion (*O,O*-diethyl *O*-(*p*-nitrophenyl)phosphorothioate) on the physical state of synthetic and native membranes was investigated by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), probing the bilayer core, and by its anionic propionic acid derivative (DPH-PA), probing the outer regions of the bilayer. Parathion disorders the gel phase of liposomes reconstituted with dimyristoylphosphatidylcholine (DMPC), broadening the transition profile and shifting the temperature midpoint of the phase transition, as detected by both probes. The insecticide strongly orders the fluid phase either in the hydrophobic core or in the outer regions of the membrane, as evaluated by DPH and DPH-PA, respectively. These ordering effects of parathion were further confirmed in fluid models of egg-yolk phosphatidylcholine. Parathion increases to some extent the ordering promoted by cholesterol in DMPC bilayers, but high cholesterol concentrations (≥ 30 mol%) prevent parathion interaction. The results in native membranes correlate reasonably with those obtained in models of synthetic lipids. Thus, parathion does not exert detectable effects in cholesterol-rich membranes, namely, erythrocytes, but moderate ordering effects of parathion are detected by both probes in brain microsomes, i.e., membranes with a lower content of cholesterol. Again, in agreement with the models of synthetic lipids, pronounced ordering effects of parathion are detected in cholesterol-poor membranes, e.g., sarcoplasmic reticulum and mitochondria.

Key words: Parathion; Cholesterol; Fluorescent probe; Membrane organization

1. Introduction

Since World War II, chemical insecticides have been widely used for reasons of health and to aid in food production [1]. Unfortunately, the benefits of use were accompanied by undesirable toxic effects on useful insects, other animals and man himself. Therefore, the exact knowledge of these insecticide effects is an imperative task. Furthermore, experimental data might help in the development of analogs with improved biological selectivity. Consequently, several attempts have been carried out to understand the physiological effects, but the precise biochemical mechanisms are not yet completely understood. Well defined biochemical actions of insecticides have been assigned only to organophosphorus and carbamate compounds and their toxic actions are commonly explained in terms of acetylcholinesterase inhibition [2]. Additionally, insecticides can also cause chronic toxicity [2] and, again, the molecular mechanisms of these effects are still not understood.

The above findings, the dynamic functions of biomembranes and the strong lipophilicity of most insecticides suggest that their effects, either acute or chronic, appear to be membrane connected. Consequently, the interaction of some popular insecticides with membrane mechanisms has been studied in our laboratory in the past few years [3–6] as an attempt to understand the molecular basis of toxicity. To further characterize the effects of insecticides on membrane mechanisms, their partitions have been determined in model and native membranes [7]. The maximal partitions of DDT, lindane, parathion and malathion in the fluid phase of

* Corresponding author. Fax: +351 39 26798.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PA, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; parathion, *O,O*-diethyl *O*-(*p*-nitrophenyl)phosphorothioate; egg-PC, egg-yolk phosphatidylcholine.

SSDI 0005-2736(93)E0334-7

egg-yolk phosphatidylcholine bilayers were 260000, 2000, 1000 and 120, respectively. However, these values were dramatically decreased when 50 mol% cholesterol was incorporated in egg-yolk phospathidylcholine bilayers, but the sequence of partition remains DDT ≫ lindane > parathion > malathion. The incorporation of these insecticides into dimyristoyl-, dipalmitoyl-and distearoylphosphatidylcholines (DMPC, DPPC and DSPC) followed also the above sequence and all the insecticides tested preferentially interacted with short-chain lipids, i.e., with DMPC. The partition studies were also extended to native membranes, namely, to mitochondria, sarcoplasmic reticulum, brain microsomes, myelin and erythrocytes, differing in intrinsic cholesterol. The partitions are higher in mitochondria and sarcoplasmic reticulum (with low cholesterol) than in brain microsomes, myelin and erythrocytes (with high cholesterol). The sequence of partition in the native membranes was identical to that found in the models: DDT ≫ lindane > parathion > malathion. DDT has the highest partition, ranging from $5 \cdot 10^5$ to $12 \cdot 10^5$, whereas malathion has the lowest, ranging from 4 to 10; lindane and parathion had intermediate partitions. These partition studies indicate that membrane fluidity *, as expressed by the fluorescence polarization of DPH probes, and insecticide structure are main parameters affecting insecticide incorporation and, potentially, insecticide toxicity. These findings and the reported gradient of order across the width of the bilayer [8] suggest different distributions of insecticides across the bilayer thickness and, consequently, different effects on membrane organization are expected. The effects of DDT, DDE and lindane on membrane organization have been extensively studied [9–14]. Following these studies, we now report the effects of the organophosphorus compound, parathion, on membrane organization.

2. Materials and methods

2.1. Materials

Cholesterol, DMPC, egg-PC and DPH were obtained from Sigma. DPH-PA was purchased from Molecular Probes. Parathion was obtained from Supelco. All these compounds were at least 99% pure.

2.2. Preparation of membranes

Synthetic membranes were prepared as described elsewhere [9]. Briefly, solutions of pure phospholipids in $CHCl_3$ were taken in round bottom flasks and the solvent was evaporated to dryness. The resulting lipid film on the wall of the round-bottom flask was hydrated with an appropriate volume of 50 mM KCl, 10 mM Tris-maleate (pH 7), and dispersed under N_2 atmosphere by hand shaking in a water bath 7–10 C° above the transition temperature of the phospholipids. Phospholipid-cholesterol bilayers were obtained by supplementing original phospholipid solutions with appropriate amounts of cholesterol. Several native membranes, namely, erythrocytes, brain microsomes, sarcoplasmic reticulum and mitochondria were prepared as described elsewhere [15]. In all cases, the final nominal concentration of membrane lipid was 345 μM (phospholipid plus cholesterol). Model and native membranes were briefly sonicated to disperse aggregates and get homogeneous suspensions with a turbidity equivalent to 0.2 A measured in a Spectronic 21 spectrophotometer at 600 nm, 1 cm light path.

2.3. Incorporation of probes and parathion into membranes

DPH in tetrahydrofuran and DPH-PA in dimethylformamide were injected (few μl) into membrane suspensions (345 μM in total lipid) to give a final lipid/diphenylhexatriene probes molar ratio of about 200. The mixture was initially vigorously vortexed for 10 s and, then, parathion was added from concentrated ethanolic solutions (50 mM). It was ascertained that added concentrations of the insecticides were within the solubility range. The mixture was incubated in the dark for 18–20 h. Control samples received equivalent volumes of tetrahydrofuran, dimethylformamide and ethanol. Added solvent volumes, always very small (few μl), had negligible effects in the measurements.

2.4. Fluorescence measurements

Fluorescence spectra were recorded in a Perkin-Elmer spectrofluorometer, model MPF-3, provided with a thermostated cell holder. The excitation was set at 336 nm and the emission at 450 nm. The excitation and emission slits were 4 and 6 nm, respectively. The temperature of the sample was checked with an accuracy of ±0.1 C°, using a thermistor thermometer. The degree of fluorescence polarization (P) was calculated according to Shinitzky and Barenholz [16] from the equation:

$$P = \frac{I_{\parallel} - I_{\perp} \cdot G}{I_{\parallel} + I_{\perp} \cdot G}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plan parallel ($\parallel$) and perpendicular ($\perp$) to that of exciting beam. A high degree of polarization represents a high structural order or low

* The term 'fluidity' is used here in an operational sense as being inversely proportional to the polarization of diphenylhexatriene probes. This fluidity is related but not identical with the physical definition of fluidity.

membrane fluidity. Although the rotational motion and structural order contribute to the steady state polarization, the latter tends to dominate. G is the correction factor for instrument polarization, given by the ratio of vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction [17].

When data are expressed in terms of the anisotropy factor, essentially the same conclusions and relationships are achieved. To be consistent with previous articles with other insecticides [9–13], polarization has been preferred in this work.

All the fluorescence measurements were corrected for the contribution of light scattering by using controls with membranes, but without added probes.

3. Results and discussion

3.1. Phospholipid bilayers

DPH and its propionic acid derivative DPH-PA have been widely used to monitor membrane organization. DPH is known to be localized within the hydrophobic core of the membrane and provides information in this region [16]. On the other hand, DPH-PA is anchored in close proximity to the bilayer surface, providing information on the bilayer organization close to the surface [18]. Thus, with these probes it is possible to compare the relative order perturbations induced by parathion in different regions across the bilayer thickness.

The effects of 50 μM parathion on the fluorescence polarization of DPH and DPH-PA embedded into bilayers of egg-yolk phosphatidylcholine, over the temperature range from 10 to 40°C are summarized in Fig. 1. Egg-yolk phosphatidylcholine bilayers remain in the fluid state over this temperature range, since the phase transition is centered at −5°C [19]. As expected, membrane fluidity increases from the outer to the central bilayer regions. Parathion exerts strong ordering effects in these fluid membranes as evaluated by DPH and DPH-PA (Fig. 1, dotted lines). These ordering effects of parathion are similar to those induced by a temperature decrease of about 18 C°, as evaluated either by DPH or by DPH-PA.

The difference of fluorescence polarization in the presence and in the absence of parathion is constant over the range of temperatures studied and is about 0.05, as detected by DPH or 0.04 as detected by DPH-PA. Thus, all the thickness of the bilayer is ordered in an identical way by parathion. However, we have previously observed [3] that insecticides, particularly parathion, increase the permeability of egg-PC membranes to non-electrolytes and to ion-ionophore complexes, without disruption of membrane structural integrity as in the case of detergents. In bilayers of mixed lipids, parathion may induce phase separations as evaluated from the thermotropic behavior of liposomes containing DMPC and DSPC, on the basis of light scattering studies [4]. Consequently, parathion apparently interacts with the lipids creating lateral heterogeneities in the plane of the membrane, thus, inducing structural defects which increase the bilayer permeability.

The effects of parathion on thermotropic phase transition of DMPC bilayers are represented in Fig. 2. The insecticide broadens the transition profile, i.e., expands the temperature range at which fluid and gel domains coexist and shifts the phase transition midpoint (T_m) to lower temperature values. Furthermore, the shift and broadening of the transition detected by both probes is concentration dependent. T_m is shifted by 0.7, 1.5 and 3 C° for parathion concentrations of 25, 50 and 100 μM, respectively, as detected by DPH.

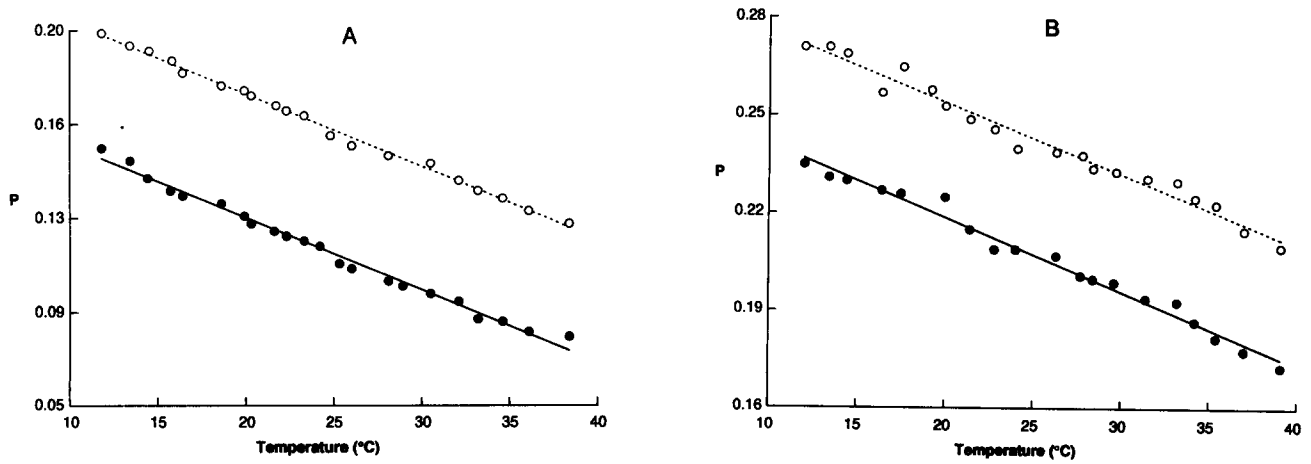

Fig. 1. Fluorescence polarization (P) of DPH (A) and DPH-PA (B) in egg-yolk phosphatidylcholine bilayers as a function of temperature, in the absence (solid symbols and lines) or in the presence (open symbols and dotted lines) of 50 μM parathion. Regression lines were calculated by means of the least-squares method. Correlation coefficients vary from −0.989 to −0.998.

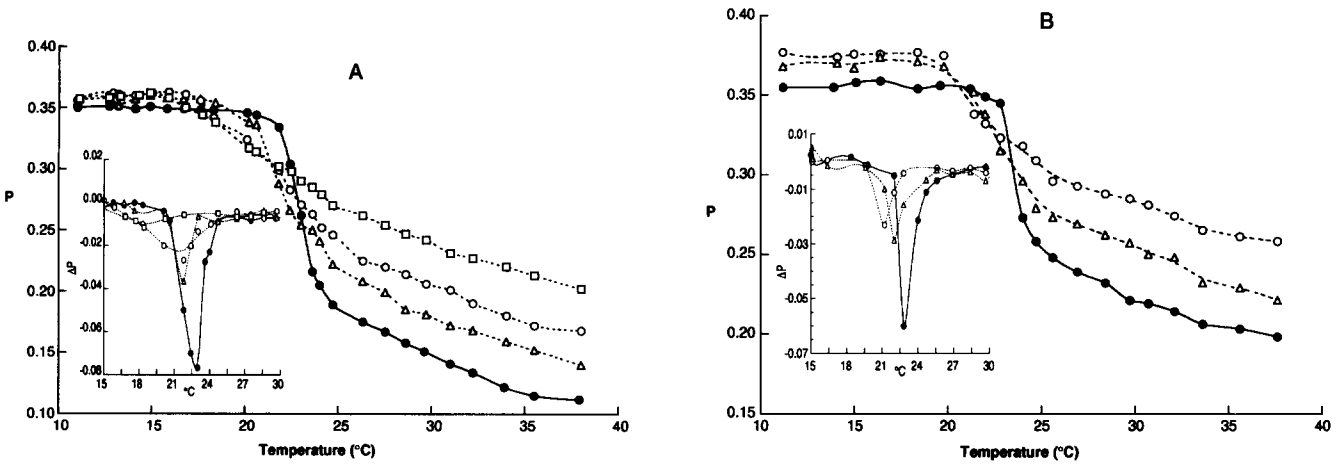

Fig. 2. Fluorescence polarization (P) of DPH (A) and DPH-PA (B) in DMPC bilayers in the absence (solid symbols and lines) or in the presence (open symbols and dotted lines) of increasing concentrations of parathion (△, 25 μM; ○, 50 μM; □, 100 μM). The insets represent differential plots of the data in the main plots.

However, disordering effects induced by parathion are only noticed at temperatures approaching that of the cooperative phase transition, which favors the incorporation of the insecticide [15]. According to Jain and Wu [20] a shifting and broadening of the transition profile suggests a localization of the foreign molecules in the vicinity of the first eight carbons of the acyl chains, i.e., in the cooperativity region. Such a localization is similar to that of the rigid rings of cholesterol, i.e., the bilayer region most perturbed by this compound [21]. Additionally, parathion is strongly displaced from the bilayers by cholesterol, being completely excluded at 50 mol% cholesterol [15]. Thus, a localization of parathion close the cooperativity region is tentatively suggested.

The main effect of parathion is, however, the ordering of the fluid phase as already detected for egg-yolk lecithin bilayers. The gel phase is also ordered to a limited extent. Furthermore, a broadening of the transition is observed accompanied with shifts in the midpoint temperature.

Data of Fig. 2 indicate that parathion interacts to a great extent with the fluid phase of DMPC (temperatures above T_m) since a significant increase in fluorescence polarization is observed. These strong ordering effects of the insecticide detected either by DPH or DPH-PA confirm and extend data, already shown in Fig. 1 for egg-PC. The overall effects of parathion (50 μM) on the thermotropic behavior of DPPC and DSPC bilayers are qualitatively similar to those described for DMPC membranes (results not shown). However, the effects of parathion are higher in DMPC as compared with DPPC and DSPC in agreement with the higher partition coefficients in DMPC bilayers [15].

Parathion–lipid interactions in the transition range may differ from those occurring in stabilized fluid and gel phases. In the gel and fluid phases of the lipid, parathion may be less buried in the hydrocarbon core of the bilayer. Therefore, in the gel and fluid phases, the geometry of the outer bilayer regions is probably more important than the hydrophobic core in deter-

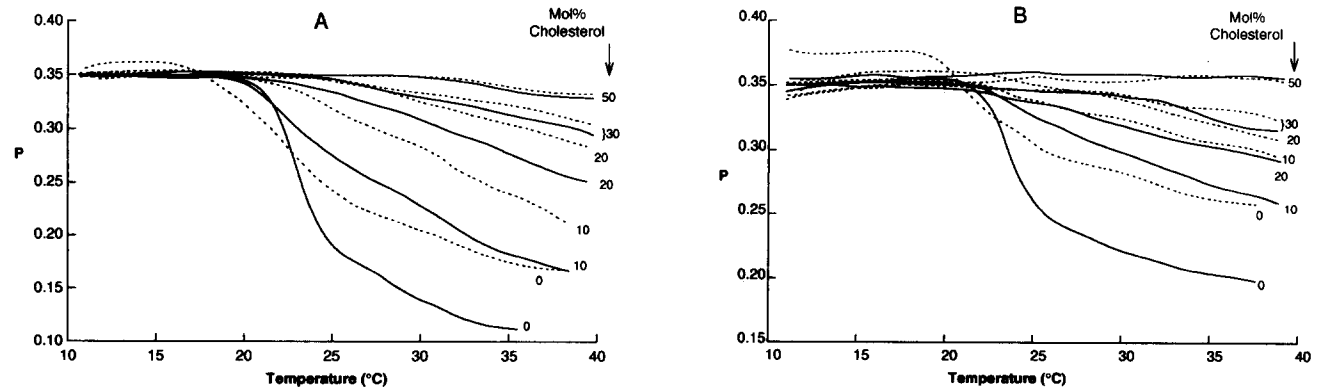

Fig. 3. Fluorescence polarization (P) of DPH (A) and DPH-PA (B) in DMPC/cholesterol bilayers, in the absence (solid lines) and presence (dotted lines) of 50 μM parathion. The numbers adjacent to the curves represent mol% of cholesterol incorporated into DMPC bilayers. Each curve was drawn across 15–17 experimental points which were removed for the sake of clarity.

mining the extent of interactions. Hydrogen bonding or dipole–dipole interactions may also take place between the nitrophenylphosphorothioate group of parathion and the headgroups of phospholipids, with a consequent decrease in the headgroup spacing. The decrease in headgroup spacing would induce a condensing effect of the bilayer. Consequently, DPH and DPH-PA detect ordering effects of parathion.

The increased polarization caused by parathion cannot be assigned to a decrease of DPH fluorescence lifetime, since the fluorescence signal of the probe DPH is not significantly affected by parathion. A signal quenching by about 10% was detected at 100 μM parathion, but 50 μM of the insecticide does not induce measurable effects. Furthermore, the increased polarization is very limited in the gel phase and almost identical for 50 or 100 μM parathion.

3.2. Phospholipid-cholesterol bilayers

As shown in Fig. 3 and according to classical observations [22], cholesterol progressively increases the molecular order in the fluid phase of DMPC bilayers. At sufficiently high concentrations (1:1, mole ratio), cholesterol completely abolishes the phase transition. Parathion induces limited effects in DMPC bilayers enriched with cholesterol, at temperatures below the phase transition of DMPC, as detected either by DPH or DPH-PA. Conversely, above the phase transition of DMPC, both probes detect significant ordering effects of parathion which gradually fade and vanish for cholesterol concentrations equal or higher to 30 mol% (Fig. 3). Previous partition data concur well with the above observations [15]. Parathion partitioning in egg-PC membranes at 24°C decreases linearly with the increase of cholesterol content, and a complete exclusion of parathion is observed for cholesterol concentrations of about 50 mol%. At these high cholesterol concentrations there are strong interactions between all cholesterol and phospholipid molecules (1:1 binding stoichiometry) allowing for maximal van der Waals contacts in the hydrocarbon region [23]. Therefore, it appears that parathion preferentially interacts with regions devoid of cholesterol since strong interactions between the sterol and the phospholipids prevent free volumes for parathion accommodation.

3.3. Native membranes

The fluorescence polarization of DPH and DPH-PA was studied in several representative native membranes (mitochondria, sarcoplasmic reticulum, brain microsomes and erythrocytes) differing in intrinsic cholesterol (Fig. 4A, B). Although in the range of the temperature studied (from 0 to 40°C) most native membranes are in the fluid state [24] the degree of fluidity depends on the temperature and on the cholesterol content (Fig. 4). Thus, membranes of mitochondria devoid of cholesterol are relatively more fluid than those of sarcoplasmic reticulum with 6 mol% cholesterol; in turn, these are significantly more fluid than those of brain microsomes and erythrocytes, where cholesterol accounts for 25 and 37 mol%, respectively. Therefore, cholesterol orders fluid membranes at physiological temperatures according to published work [25].

The effects of parathion in native membranes, probed by DPH and DPH-PA, are qualitatively similar to those described for models (Fig. 4). Thus, parathion effects are very limited in cholesterol-rich membranes,

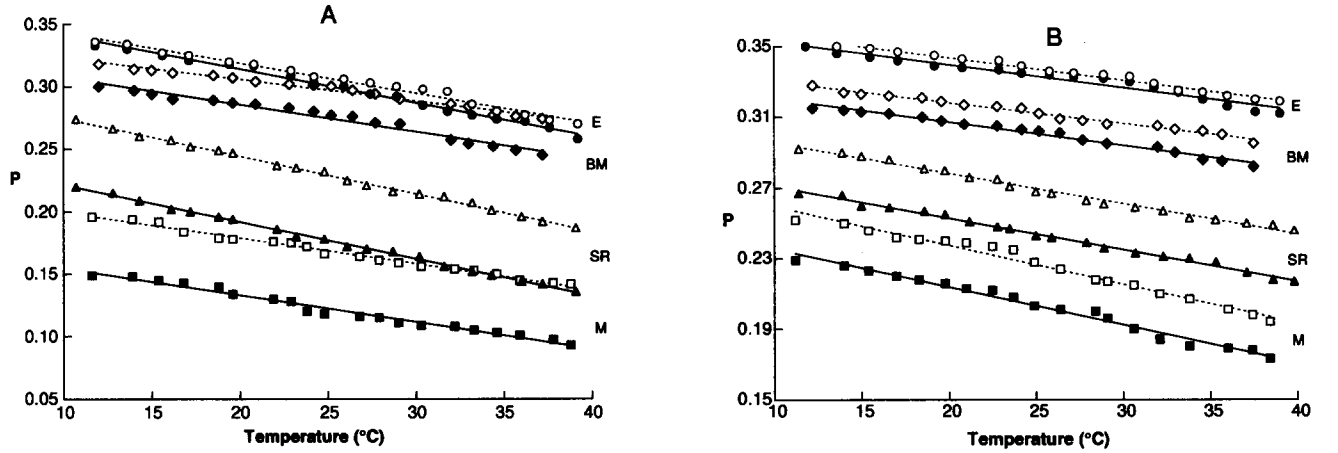

Fig. 4. Fluorescence polarization (P) of DPH (A) and DPH-PA (B) in several native membranes differing in intrinsic cholesterol, as a function of temperature, in the absence (solid symbols and lines) and in the presence (open symbols and dotted lines) of 50 μM parathion. Cholesterol/phospholipid molar ratios for mitochondria (M), sarcoplasmic reticulum (SR), brain microsomes (BM) and erythrocytes (E) are 0, 6, 25 and 37 mol%, respectively. Regression lines were calculated as indicated in Fig. 1. The correlation coefficients vary from −0.977 to −0.999. As in the previous figures, error bars are not represented, since for most experimental points they are encompassed by the size of the symbols.

namely, erythrocytes, but moderate ordering effects of parathion are already detected in brain microsomes. Again, as in the models of synthetic lipids, strong ordering effects of parathion occur in cholesterol-poor membranes, namely, sarcoplasmic reticulum and mitochondria. Also, as observed for models of synthetic lipids, cholesterol content is the main parameter controlling partition of parathion in native membranes and respective lipid dispersions [15]. Additionally, partitioning of parathion decreases linearly with the increase of intrinsic cholesterol. However, higher values of partition were detected in native membranes as compared with their respective lipid suspensions, suggesting that extra free volume is available in native membranes for insecticide incorporation. This free volume, presumably provided by the lipid-protein interfaces, is accessible to insecticides but not to cholesterol, which appears to be excluded from direct contact with integral membrane proteins [26]. Since the activities of integral proteins depend on the physico-chemical characteristics of boundary domains [27], the incorporation of parathion into these regions may affect protein structure and function. Actually, it has been previously shown that parathion perturbs the function of the Ca^{2+}-pump of sarcoplasmic reticulum by stimulating the pump turnover in terms of Ca^{2+} uptake and ATP hydrolysis [6]. Likewise, similar effects are expected for other intrinsic membrane enzymes. According to the above studies, parathion would preferentially perturb membranes or regions of the membranes where cholesterol is scarce.

In summary, it can be concluded that the fluidity changes of lipid bilayers may partially explain the alterations of membrane mechanisms induced by parathion contributing for the toxicity of the insecticide.

4. Acknowledgments

This work was supported by JNICT (PMCT/C/BIO/156/90). R.A. Videira is a recipient of a grant from JNICT.

5. References

[1] Ware, G.W. (1983) Pesticides, Theory and Application (Zweig, G., ed.), Part 2, pp. 35–67, CRC Press, New York.
[2] Eto, M. (1974) Organophosphorus Pesticides: Organic and Biological Chemistry (Zweig, G., ed.), pp. 123–133, CRC Press, Cleveland, OH.
[3] Antunes-Madeira, M.C. and Madeira, V.M.C. (1979) Biochim. Biophys. Acta 550, 384–392.
[4] Antunes-Madeira, M.C., Carvalho, A.P. and Madeira, V.M.C. (1980) Pestic. Biochem. Physiol. 14, 161–169.
[5] Antunes-Madeira, M.C., Carvalho, A.P. and Madeira, V.M.C. (1981) Pestic. Biochem. Physiol. 15, 79–89.
[6] Antunes-Madeira, M.C. and Madeira, V.M.C. (1982) Pestic. Biochem. Physiol. 17, 185–190.
[7] Antunes-Madeira, M.C. and Madeira, V.M.C. (1989) Pestic. Sci. 26, 167–179.
[8] Chefurka, W., Chatelier, R.C. and Sawyer, W.H. (1987) Biochim. Biophys. Acta 896, 181–186.
[9] Antunes-Madeira, M.C. and Madeira, V.M.C. (1989) Biochim. Biophys. Acta 982, 161–166.
[10] Antunes-Madeira, M.C. Almeida, L.M. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1022, 110–114.
[11] Antunes-Madeira, M.C. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1023, 469–474.
[12] Antunes-Madeira, M.C. Almeida, L.M. and Madeira, V.M.C. (1991) Pestic. Sci. 33, 347–357.
[13] Antunes-Madeira, M.C. and Madeira, V.M.C. (1993) Biochim. Biophys. Acta 1149, 86–92.
[14] Antunes-Madeira, M.C. Almeida, L.M. and Madeira, V.M.C. (1993) Bull. Environ. Contam. Toxicol. 51, 787–794.
[15] Antunes-Madeira, M.C. and Madeira, V.M.C. (1984) Biochim. Biophys. Acta 778, 49–56.
[16] Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394.
[17] Litman, B.J. and Barenholz, Y. (1982) Methods Enzymol. 81, 678–685.
[18] Trotter, P.J. and Storch, J. (1989) Biochim. Biophys. Acta 982, 131–139.
[19] Bittman, R. and Blau, L. (1976) J. Chem. Educ. 53, 259–261.
[20] Jain, M.K. and Wu, N.M. (1977) J. Membr. Biol. 34, 157–201.
[21] Engelman, D.M. and Rothman, J.E (1972) J. Biol. Chem. 247, 3694–3697.
[22] Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340.
[23] Presti, F.T., Pace, R.J. and Sunney, I. (1982) Biochemistry 21, 3831–3835.
[24] Ohki, K. (1988) J. Biochem. 104, 14–17.
[25] Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132.
[26] Silvius, J.R. McMillen, D.A., Saley, N.D., Jost, P.C. and Griffith, O.H. (1984) Biochemistry 23, 538–547.
[27] Houslay, M.D. and Stanley, K.K. (1982) Dynamics of Biological Membranes, pp. 92–138, John Wiley and Sons, Chichester, NY.