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Partition of lindane in synthetic and native membranes

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Partition coefficients of the insecticide γ-1,2,3,4,5,6-hexachlorocyclohexane (trivially, lindane) were determined in model and native membranes. Partition in egg phosphatidylcholine bilayers decreases linearly with temperature, over a range (10–40°C) at which the lipid is in the liquid-crystalline state. Addition of 50 mol% cholesterol dramatically decreases partition (2100 falls to 100, at 10°C) and abolishes the temperature dependence. First-order phase transitions of dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholines (DMPC, DPPC and DSPC) are accompanied by a sharp increase in lindane partition. Apparently, the insecticide is easily accommodated in bilayers of short-aliphatic-chain lipids, since the partitions were 2450, 600 and 50 in DMPC, DPPC and DSPC, respectively, at temperatures 10 Cdeg below the midpoint of their transitions. The lindane partition sequence in native membranes is as follows: mitochondria, sarcoplasmic reticulum, myelin, brain microsomes and erythrocytes. This sequence correlates reasonably well with the relative content of cholesterol and is similar in liposomes of total extracted lipids, although the absolute partitions showed decreased values. Therefore, the presence of proteins in native membranes contributes to the insecticide partition, probably by favouring its interaction with lipids.

Introduction

Organochlorinated insecticides are highly lipophilic, persistent in food chains and, consequently, easily accumulated by top predators, including man [1–3]. Therefore, great efforts have been made to establish the extent of undesirable toxic effects in useful insects, other animals and man himself.

Pyrethroids and DDT analogs apparently alter specific properties of ion channels in axon membranes responsible for delayed repolarization of the action potential [4–9]. Cyclodienes and lindane presumably act as presynaptic modulators of acetylcholine release [4,7–10]. However, in spite of the attempts carried out to understand the physiological effects, the precise biochemical mechanism of these compounds are still poorly understood.

Most organochlorinated insecticides are highly lipophilic, and therefore they are likely to accumulate in membrane lipid moieties en route to their target sites. Consequently, their toxic effects may be partially related with the accumulation in cell membranes and organelles. Since biomembranes are candidates for target sites of immediate and also delayed insecticide action, the interaction of these xenobiotics with biomembranes has been studied in our laboratory in the past few years [11–13], as an attempt to characterize perturbations induced in basic membrane mechanisms. It has been shown that lindane affects molecular interactions in model and native membranes and that these effects induce perturbations of membrane permeability [11–13]. Interaction of lindane

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Abbreviations: Lindane, γ-1,2,3,4,5,6-hexachlorocyclohexane; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane.

with membrane components has also been increasingly emphasized in other laboratories [14–16].

To characterize further the membrane mechanisms affected by lindane, we report here its partitioning in several native membranes and also model membranes used as operational systems to aid the interpretation of results in clear terms. The results will allow elucidation of the distribution of the compound in affected organisms.

Materials and Methods

Membrane preparations

Liposomes. Multilamellar liposomes were prepared as previously described [12], except that the hydration buffer contained 50 mM KCl and 10 mM Tris-maleate (pH 7.0). Cholesterol incorporation was accomplished by supplementing original lipid solutions with appropriate amounts of cholesterol. Liposomes of mitochondrial lipid extracts were prepared at pH 8.5, vortexing five times for 30 s each time, at room temperature, as indicated elsewhere [17].

The lipids from native membranes were extracted as previously described [18,19]. Phospholipids were quantitated by measuring inorganic phosphate [20] released after hydrolysis of extracts at 180°C in 70% $HClO_4$ [21]. Cholesterol in lipid extracts was assayed by the Lieberman-Bürchard method [22] and expressed in relation to the phospholipid content.

Native membranes. The mitochondrial fraction from rat liver was isolated according to Hogeboom [23]. Sarcoplasmic reticulum was obtained from white muscles of rabbit as described elsewhere [19,24]. Brain microsomes and crude myelin were obtained from sheep brain according to Hajós [25]. Ghost membranes from pig erythrocytes were prepared by the method of Schneider and Kirschner [26], as modified by Buckley and Hawthorne [27] and Buckley [28]. Protein concentrations were determined by the biuret method [29] calibrated with serum albumin. Membrane suspensions were rapidly frozen in liquid nitrogen and kept at −80°C.

Determination of partition coefficients

The partition coefficients of [^{14}C]lindane were determined as previously described for [^{14}C]-parathion [30], with appropriate modifications. The period of equilibration of [^{14}C]lindane with membrane suspensions in buffer was about 1 h. The concentrations of the insecticide and of membrane lipid were 0.25 and 100 μM, respectively.

Radioactive counting was performed as described previously [31]. The partition coefficients, K_p, were calculated from the fraction of insecticide retained in the membrane (p) according to the equation [32]

$$p = \frac{K_p(V_l/V_a)}{K_p(V_l/V_a)+1}$$

where V_l and V_a are the volumes of lipid and aqueous phases, respectively. In our particular experimental conditions, the equation can be rewritten as:

$$K_p = \frac{p}{1.22L(1-p)}$$

where L is the amount of lipid in nmol.

Reagents

Egg phosphatidylcholine (type V-E), cholesterol, DMPC, DPPC and DSPC, at least 98% pure, were obtained from Sigma. [^{14}C]Lindane (82.3 mCi/mmol) was obtained from Amersham International, U.K.

Results

Partition coefficients of [^{14}C]lindane in egg phosphatidylcholine membranes

The organochlorinated insecticide, lindane, concentrates 2100–1300-fold in egg phosphatidylcholine bilayers relative to the buffer phase, over the temperature range from 10 to 40°C (Fig. 1). Over this range, egg phosphatidylcholine bilayers are in the liquid-crystalline state, since the phase transition is centered at −5°C [33]. Partition of lindane decreases dramatically to about 100 when 50 mol% cholesterol is incorporated in bilayers (Fig. 1). This finding prompted us to study the effects of moderate concentrations of this sterol in lindane partitioning. An apparent inverse linear relationship between the partition of lindane and the molar ratio of cholesterol was found (Fig. 2). This linear dependence has proven to be statistically

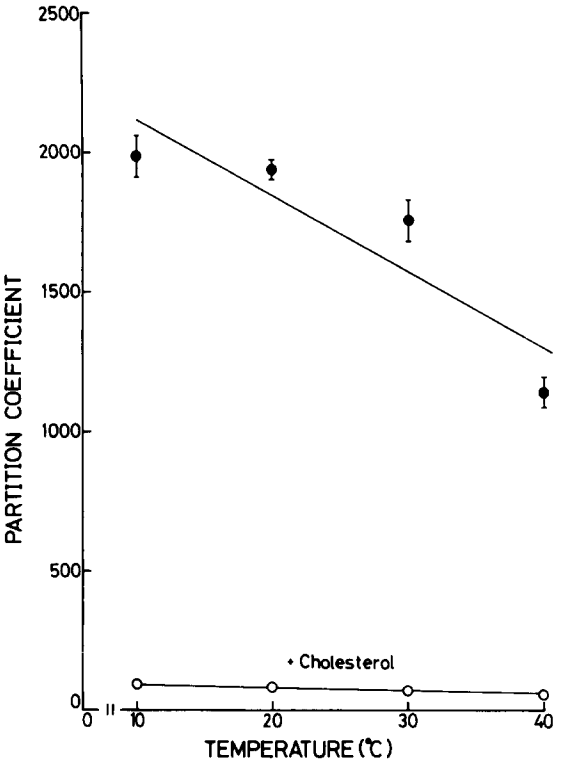

Fig. 1. Membrane/buffer partition coefficients of lindane as a function of temperature for egg phosphatidylcholine membranes. Partition is dramatically decreased when cholesterol (50 mol%) is incorporated in membranes (open symbols). The regression line was calculated by the least-squares method; each point represents the average of at least six independent measurements (vertical lines indicate ±S.D.). Note that the partition has a negative dependence on temperature. Correlation coefficient is −0.91.

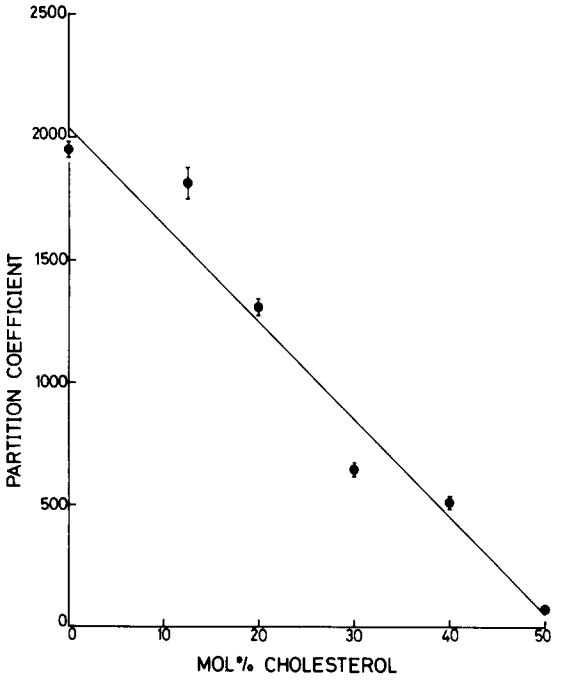

Fig. 2. Effect of cholesterol on lindane partition in egg phosphatidylcholine bilayers. The regression line was calculated as indicated for Fig. 1. The correlation coefficient is −0.98. Note that partition would theoretically approach zero for 51 mol% cholesterol.

significant, with correlation coefficients exceeding −0.98, corresponding to absolute coefficients better than 0.96. The coefficients were determined by the least-squares method analysis of linear regressions. Extrapolation of the curve to the abscissa predicts a zero partition at 51 mol% cholesterol.

Partition coefficients of [^{14}C]lindane in model membranes of DMPC, DPPC and DSPC

In order to understand the dependence of lindane partitioning on the lipid composition and fluidity, we have studied its incorporation in model membranes of DMPC, DPPC and DSPC, i.e., lipids with the same headgroup, but differing in aliphatic chain lengths. In all cases, lindane incorporates maximally within the temperature range of cooperative phase transition. Maximal partitions were 3700, 2700 and 1100 for DMPC, DPPC and DSPC, respectively (Fig. 3). Within the range of the phase transition, gel and liquid-crystalline phases coexist and the constant oscillation between the two phases creates boundaries with packing defects [34,35] which presumably favour the incorporation of lindane. The data of Fig. 3 clearly indicate that, independently of temperature, lindane incorporates better in bilayers of short-aliphatic-chain lipids. Thus, an increase in chain length by two carbon atoms results in a partitioning quenching of about 1000. Since short-chain lipids produce membranes with higher fluidity relative to those formed by long-chain species [36], membrane fluidity likely modulates the partitioning of lindane. This interpretation is reinforced by the observation that lindane is drastically excluded if the temperature falls far below the transition midpoint. Thus, complete exclusion of insecticide is observed in bilayers of DPPC and DSPC at temperatures 10 Cdeg below the midpoint of their cooperative phase transi-

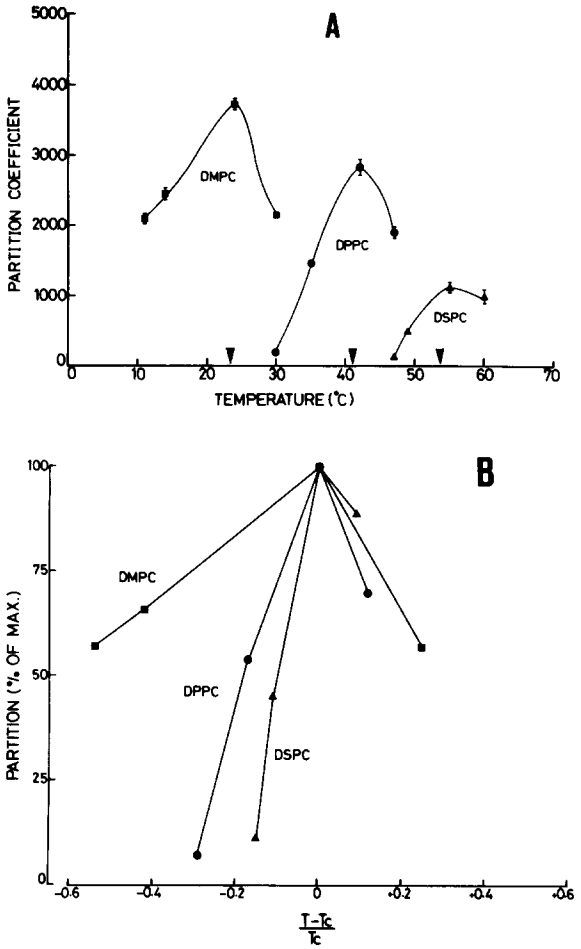

Fig. 3. Partition coefficients of lindane in artificial membranes of DMPC (■), DPPC (●) and DSPC (▲) as a function of temperature. The midpoint temperatures of thermotropic phase transitions (T_c) of DMPC, DPPC and DSPC are 24, 41 and 54°C, respectively, as indicated by the arrow-heads. Maximal partitions are observed at these temperatures. Lindane partitioning decreases sharply above and below the range of phase transition. Furthermore, incorporation of lindane is abolished in DPPC and DSPC when the temperature falls 10 Cdeg below the midpoint transition. Normalized data are replotted in part B on a 'reduced temperature' scale. Lowering temperature below T_c affects lindane incorporation as a function of lipid chain length. As it increases incorporation dramatically decreases.

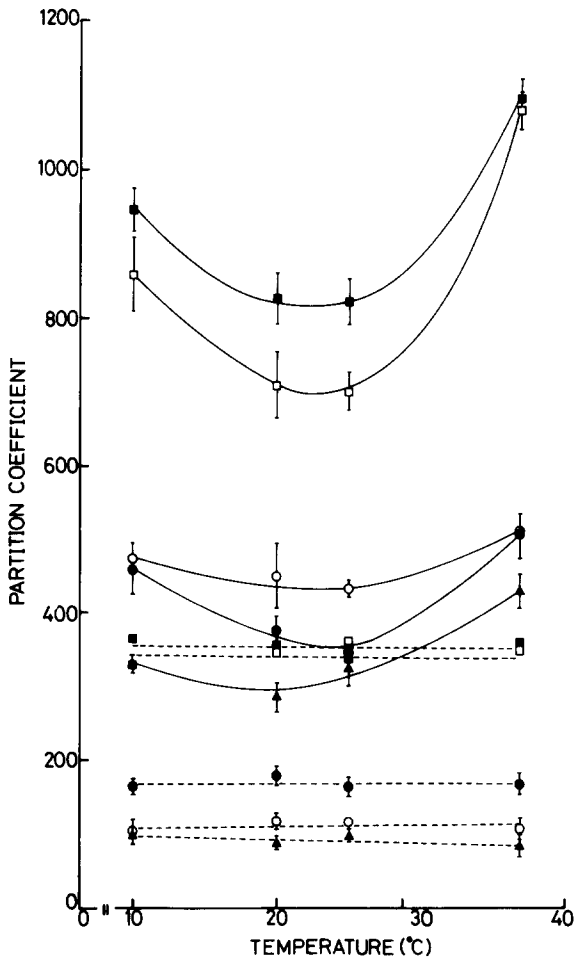

Fig. 4. Partition coefficients of lindane into native membranes (———) and representative phospholipid dispersions (— — —) as a function of temperature. ■, mitochondria; □, sarcoplasmic reticulum; ●, microsomes; ○, myelin; ▲, erythrocytes. Dependence of partition on temperature changes at 24°C from a negative to a positive slope.

tions, i.e., when the bilayers are ordered in the gel phase. Thence, it follows that the almost perfect molecular packing of individual lipids leaves no free volume for lindane accommodation. Thus, these physical constraints squeeze the insecticide, excluding it from interaction with the hydrophobic membrane moiety. However, factors other than fluidity also contribute, since significant partitions are observed in the gel phase of DMPC bilayers and partition in egg phosphatidylcholine shows negative dependence on temperature. Therefore, we tentatively advance the theory that geometrical complementarity resulting from the relative molecular shapes of individual lipids and lindane may also modulate incorporation.

Partition coefficients of [^{14}C]lindane in native membranes and their lipid models

The partition of lindane was studied in a variety

of native membranes, namely, mitochondria, sarcoplasmic reticulum, brain microsomes, myelin and erythrocytes, and in model membranes obtained from their extracted lipids. It should be emphasized that these membrane types were deliberately chosen, since they can be easily isolated in a pure state, therefore avoiding contaminants which would lead to misinterpretations. Therefore, these well-defined systems may be taken as general prototypes of biomembranes.

Lindane incorporates poorly in erythrocytes, myelin and brain microsomes as compared with sarcoplasmic reticulum and mitochondria (Fig.4). Interesting also is the fact that lindane incorporation undergoes a change of temperature dependence over the temperature range from 10 to 37°C. Thus, the dependence of partition on temperature clearly changes at about 24°C from a negative to a positive slope, observed more clearly for mitochondrial and sarcoplasmic reticulum membranes.

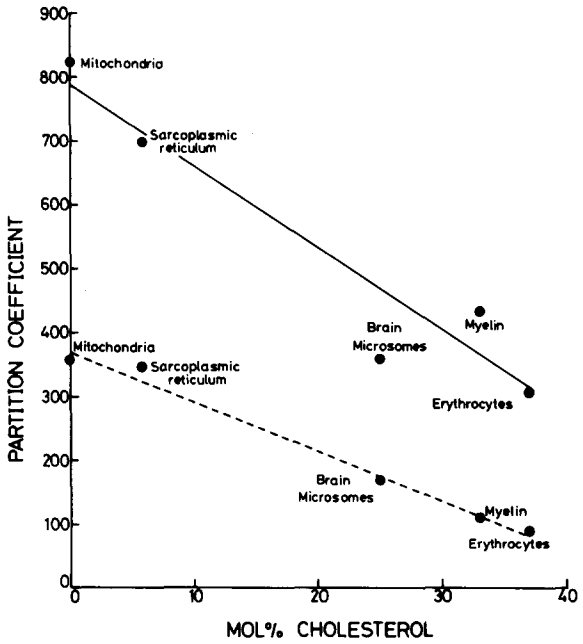

Fig. 5. Partition coefficients of lindane in native membranes (———) and representative phospholipid dispersions (— — —) as a function of intrinsic cholesterol content. These data were taken from Fig. 4 (partitions at 24°C). Regression lines were calculated as described in Fig. 1. The correlation coefficient in native membranes is −0.95 and in lipid dispersions in −0.99. The partition coefficient would theoretically approach zero for about 47 mol% and 61 mol% cholesterol in phospholipid dispersions and native membranes, respectively.

Partition of lindane in dispersions of total extracted lipids exhibits a distribution similar to that found for native membranes. Likewise native membranes, mitochondrial and sarcoplasmic reticulum lipids incorporate considerable concentrations of lindane as compared with relatively poor incorporations observed for the other membrane lipids. Nonetheless, the absolute partitions are reduced below one-half relative to those determined for native membranes. Furthermore, no dependence on temperature was observed in lipid dispersions; the partition profiles remain flat over the entire temperature range (Fig. 4).

Lindane partitioning, either in native membranes or in total lipid dispersions, decreases linearly with the increase of the relative content of intrinsic cholesterol (Fig. 5), similarly to the observations in artificial membranes of egg phosphatidylcholine supplemented with increasing concentrations of cholesterol (Fig. 2). Correlation coefficients of linear regressions in native membranes and related lipid dispersions are −0.95 and −0.99, respectively. The plots extrapolated to the abscissa (zero partition) give cholesterol concentrations of about 61 and 47 mol% for native membranes and lipid dispersions, respectively. Therefore, withdrawal of lindane by cholesterol is more effective in lipid bilayers than it is in native membranes containing proteins.

Discussion

Several factors control lindane incorporation in artificial and native membranes, namely, temperature, lipid composition, cholesterol content, membrane order, presence of proteins and geometrical parameters. The various factors can be individually controlled in model membranes, thus offering advantage over native membranes containing complex mixtures of lipids and proteins. Though the conclusions in artificial models cannot be readily extrapolated to real membranes, they facilitate the interpretation of results when careful assays are carried out in parallel. Studies carried out with these considerations in mind showed that tempera-

ture is certainly a parameter which consistently controls lindane incorporation in membranes. Nevertheless, no unique mode of action was found, since lindane incorporation displays either negative temperature dependence in egg phosphatidylcholine bilayers or mixed negative and positive profiles in native membranes over the temperature range 10–37°C. On the other hand, no dependence was observed for bilayers prepared with lipids extracted from native membranes. Therefore, the structural order of lipids modulated by temperature appears to affect incorporation of lindane optimized at a certain degree of order dependent on lipid composition. Similar conclusions have been forwarded for the incorporation of chlorinated hydrocarbons [37,38].

Data obtained with synthetic phosphatidylcholines (DMPC, DPPC and DSPC) indicate that lindane incorporation decreases below and above the midpoint temperature of the phase transition. Additionally, it appears that lindane is excluded from the gel phase, as better observed in DPPC and DSPC bilayers (Fig. 3B). However, the insecticide is incorporated into DMPC bilayers, either in the gel or the fluid phase (Fig. 3B). Therefore, the apparent discrepancies in the effect of temperature may result from thermotropic geometrical factors imposed by the molecular structure of lipids according to the 'shape hypothesis', as suggested by Hornby and Cullis [39] for the interaction of anesthetics with membranes. In conclusion, membrane order and general fluidity dependent on temperature, as well as thermotropic geometrical parameters, control lindane incorporation in lipid bilayers.

Studies with synthetic phosphatidylcholines having the same headgroup but differing in aliphatic chain composition additionally suggest that lindane accommodates between the acyl chains of the phospholipids, i.e., a localization compatible with its hydrophobic nature. This conclusion is reinforced by the fact that cholesterol effectively withdraws lindane from the membranes, suggesting that the insecticide locates at the cooperativity region of the bilayer. Nevertheless, lindane does not extensively perturb the cooperativity, since only a small shift in transition temperature accompanied by a slight broadening was observed in DMPC bilayers (not shown). This effectless action may be related to the small size (4 Å, Ref. 40) and chair structure of lindane as opposed to the extensive planar rigid cyclopentanephenanthrene structure of cholesterol. The effects on membrane fluidity and geometry [41] also deserve attention in the interpretation of cholesterol-induced lindane withdrawal from the membrane. This effect of cholesterol assumes a general character, since it has been observed previously for lindane (indirect technique, Refs. 37, 38), parathion [30] and other drugs [42–45].

The sterol content is the major factor controlling partitioning of lindane in native membranes, since a linear decrease of partition is observed with an increase of cholesterol molar concentration, in either intact membranes or their lipid representatives. These exhibit a behaviour similar to that of egg phosphatidylcholine bilayers, showing complete exclusion of lindane at cholesterol concentrations of about 50 mol%. However, native membranes only show complete exclusion of lindane (theoretically) at 61 mol% cholesterol. Moreover, native membranes incorporate an excess of lindane over the related lipid membranes. Therefore, extra free volume is available in native membranes for lindane incorporation. This free volume provided by the lipid-protein interfaces is accessible to lindane, but not to cholesterol, which appears to be excluded from direct contact with integral membrane proteins [46–48].

Our values partially agree with those recently reported for DPPC based on indirect calculations from fluorescence quenching of a tryptophan analogue [50]. However, these authors failed to detect the effect of the phase transition, reporting partitions of about 3000 at 10, 30 and 50°C. Our values are calculated from direct measurements, as opposed to the use of fluorescent probes often subjected to artifacts resulting from probe location and behaviour in the membrane. Also, the high values recently reported for sarcoplasmic reticulum [51] are in conflict with our measurements, although a negative temperature dependence was also noticed over the low-temperature range. These calculations derived from quenching of ATPase tryptophyls do not account for their relative accessibility to the quencher lindane. Furthermore, the distribution of lindane in the lipid annulus sensed by fluorescence quenching is presumably different

from the general distribution in the membrane phase as reflected by our results.

It turns out that accumulation of lindane in biological structures cannot be estimated on the basis of the classical octanol/water partitioning (5200, cf. Ref. 37). Our studies indicate that partitioning in biomembranes is affected by several physical parameters, including the nature of the membrane itself. This variability indicates that distribution of lindane in poisoned animals is rather heterogeneous, with preferential accumulation in functional membranes of organelles, e.g., mitochondria and microsomes. Similar results were previously obtained with the organophosphorus insecticide parathion [30], but its partition is lower than that of lindane, either in model or native membranes. However, functional perturbations induced by parathion are much more extensive [11–13,49], in accord with its higher toxicity [1]. Therefore, the toxicity of these compounds in terms of functional perturbations is related not only to the incorporation but also to physicochemical properties of the molecules themselves.

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