*Biochimica et Biophysica Acta*, 684 (1982) 83–95
Elsevier Biomedical Press



BBA 71011

# INTERACTIONS OF CHLORINATED HYDROCARBON INSECTICIDES WITH MEMBRANES

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(Received August 17th, 1981)

*Key words: Insecticide-membrane interaction; Chlorinated hydrocarbon; Fluorescence quenching*

**Chlorinated hydrocarbon insecticides quench the fluorescence of *N*-alkyl derivatives of carbazole. We used phospholipids with covalently attached carbazole as probes for the interactions of chlorinated hydrocarbon insecticides with lipid bilayers, the object being to understand better the toxicities of chlorinated hydrocarbons. Fluorescence quenching measurements revealed the lipid-water partition coefficients of the chlorinated hydrocarbons, their diffusion coefficients in the membranes, and the binding capacities of the membranes for the chlorinated hydrocarbons. Active insecticides were compared with inactive analogues to test whether activities correlated with chlorinated hydrocarbon-membrane interactions. Thus DDT and methoxychlor were compared with inactive DDE, and insecticidal γ-lindane was compared with three less active stereoisomers. The partition coefficients, diffusion coefficients and membrane saturation capacities did not correlate with insecticidal potency. The partition coefficients of these chlorinated hydrocarbons were larger in bilayers containing unsaturated fatty acyl chains as compared to bilayers containing saturated fatty acyl chains. Interestingly, neural membranes are known to contain a large percentage of unsaturated lipids. Our results indicate that the activities of chlorinated hydrocarbons are not a result of specific interactions of these compounds with the lipids of membranes. However, the neurotoxicity of chlorinated hydrocarbons may be amplified by selective partitioning in the unsaturated neural membranes.**

## Introduction

The symptoms of chlorinated hydrocarbon toxicity, hyperexcitability, tremors, convulsions, and paralysis [1], are evidence that chlorinated hydrocarbons exert physiological effects by interaction with components of the nervous system. However, the mechanism of neurotoxicity is not known at the molecular level. Several investigations implicate chlorinated hydrocarbons as effectors of membrane-bound proteins and enzymes. For example, DDT affects the $Na^+$ and $K^+$ permeabilities of neural axons during propagation of the action potential [2], and lindane may affect the $Ca^{2+}$ permeability of synaptic membranes [3]. DDT also affects the activity of membrane-bound ATPase [4]. Michaelis-Menton kinetic studies re-

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The following abbreviations are used for the chlorinated hydrocarbons. DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene; MeOC= methoxychlor, 1,1,1-trichloro-2,2-bis-(*p*-methoxyphenyl)-ethane; α-, β-, δ-, and γ-HCH are the α, β, δ, and γ isomers of 1,2,3,4,5,6-hexachlorocyclohexane. γ-HCH, lindane.

The abbreviations used for the phospholipids are DOPC, DMPC and DPPC for dioleoyl, dimyristoyl and dipalmitoyl-L-α-phosphatidylcholine, respectively; POPC, palmitoyl oleoyl-L-α-phosphatidylcoline; SM, sphingomyelin.

The abbreviations used for the fluorescent probes are CUA, PC, β-(11-(9-carbazole)undecanoyl)-L-α-phosphatidylcholine and CPA-PC, β-(3-(9-carbazole)propionyl)-L-α-phosphatidylcholine.

0005-2736/82/0000–0000/$02.75 

vealed that DDT is a non-competitive inhibitor of lobster peripheral nerve ATPases [5] and rat brain ($Na^+ + K^+$)-ATPase [6], and an uncompetitive inhibitor of flounder sarcoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)-activated ATPase [7]. Probably, DDT does not act at the active sites of these enzymes. Schneider [6] has suggested that the alteration of ATPase function by DDT may be due to DDT-induced alterations of the lipid phase of the membrane, which in turn interferes with allosteric transitions of the ATPase. Although alteration of ATPase activity is not believed to be the primary mechanism of DDT neurotoxicity, a similar inhibition mechanism may operate for proteins mediating sodium and potassium permeability of the axon during impulse transmission [6]. DDT also has been shown to antagonize the effect of valinomycin on synthetic membranes [8]. In particular, valinomycin increased the permeability of the membrane to potassium, but DDT blocked the induced permeability. This was not due to complexing of DDT with valinomycin [9]. In total, these studies imply that lipid-DDT interactions may be fundamental to the mechanism of DDT action. That is, the DDT may alter the lipid phase of the membrane such that proteins which are dependent upon the lipid phase for activity may be affected. The insecticidal chlorinated hydrocarbons in general may be toxic via a similar mechanism.

Information on the interactions of chlorinated hydrocarbons with the lipids of membranes is sparse. Using NMR techniques, it has been shown that DDT and a number of its structural analogues bind to phospholipids in chloroform solution [10] and to aqueous phospholipid dispersions [11,12]. These studies showed that DDT and DDE were incorporated into the hydrophobic region of the bilayer, and maximum binding of DDE to egg phosphatidylcholine (PC) occurred at a lipid to chlorinated hydrocarbon ratio of 10:1 [12]. The effects of DDT on bilayers have been studied using the fluorescent probe *N,N*-di(octadecyl)oxycarbocyanin [13]. DDT had no effect on the diffusion coefficient of this fluorescent probe in flat bilayers prepared from *Torpedo electroplax* membrane lipids. This result suggested that DDT did not alter membrane fluidity. Thus there is evidence that CH-lipid interactions may be important in chlorinated hydrocarbon toxicity, but there appear to be no studies which have correlated these interactions with chlorinated hydrocarbon toxicity.

In earlier reports we described fluorescence quenching methods for studies of chlorinated hydrocarbon-membrane interactions [14–16]. Many chlorinated hydrocarbons are dynamic quenchers of the fluorescence of carbazole. The carbazole moiety was covalently attached to phospholipids [15], and these labeled phospholipids incorporated as probes in model membranes. Fluorescence lifetime measurements of the *N*-alkyl carbazole residues reveal the diffusion coefficients, lipid-water partition coefficients, and the membrane binding capacities for the chlorinated hydrocarbons.

The fluorescent phospholipid probes used in this study were β-(11-(9-carbazole)undecanoyl)-L-α-phosphatidylcholine (CUA-PC) and β-(3-(9-carbazole)propionyl)-L-α-phosphatidylcholine (CPA-PC). Their structures are shown in Fig. 1. These probes were synthesized as described previously [15], and are suitable for studying chlorinated hydrocarbon-membrane interactions for the following reasons: (1) Covalent attachment of the carbazole moiety to phospholipids does not alter the quenching efficiencies of the chlorinated hydrocarbons and carbazole moiety. (2) Quenching efficiencies can be determined by the use of

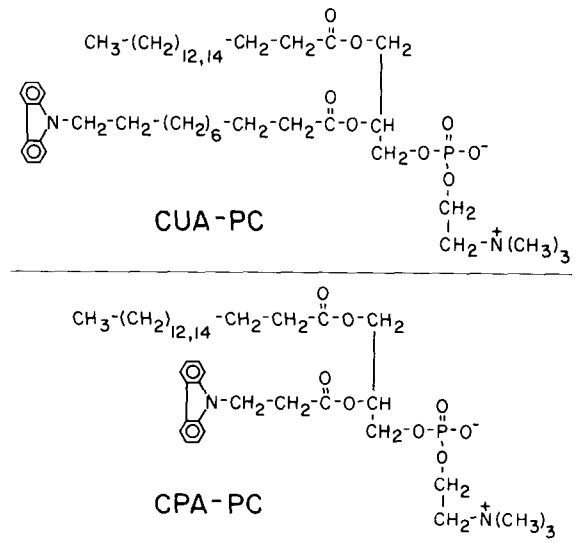


Fig. 1. Carbazole-labeled phospholipids.

the readily obtainable model compound *N*-ethylcarbazole. (3) Variation of the probe concentration over a reasonable range does not alter the fluorescence emission spectra or lifetimes, indicating reabsorption of the emission is minor. (4) Variation of the lipid-to-probe mole ratio from 50:1 to 1000:1 does not alter the spectral properties or the quenching rates, indicating that excimer and exciplex formation in the membrane is not significant. (5) And finally, the carbazole moiety is inaccessible to water-soluble quenchers, indicating that it is localized within the hydrophobic region of the membranes.

If chlorinated hydrocarbon-lipid interactions are important for chlorinated hydrocarbon toxicity, we may expect the insecticidal chlorinated hydrocarbons to behave differently in membranes than their non-toxic structural analogues. For example, if chlorinated hydrocarbon toxicity were dependent upon specific binding of the chlorinated hydrocarbon to lipids, then we expect the diffusional rate of the toxic chlorinated hydrocarbons to be slower than the inactive structural analogues. Alternatively, toxicity could result from the ability of the insecticidally active chlorinated hydrocarbons to reach higher membrane concentrations than the inactive analogues. Thus, we might expect the partition coefficients and/or saturation levels of the membrane to be higher for the insecticidally active chlorinated hydrocarbons. In this paper we report measurement of the diffusion rates, partition coefficients, and saturation limits of chlorinated hydrocarbon insecticides and their inactive structural analogues (Table I).

TABLE I

TOXICITIES OF CHLORINATED HYDROCARBONS USED IN THIS STUDY

| Compound | Rat $LD_{50}$ (mg/kg)'[a] | House fly (Relative topical)[b] |
|---|---|---|
| DDT | 100–400 | 1 |
| DDE | Nontoxic | Nontoxic |
| Methoxychlor | 6000 | 1[c] |
| γ-HCH | 125 | 3.3–17 |
| β-HCH | 6000 | Nontoxic |
| α-HCH | 500 | 0.0003 |
| δ-HCH | 1000 | 0.001 |

[a] From Melnikov [51].
[b] From Brooks [52].
[c] From Matsumura [1].

## Materials and Methods

DDT, DDE, lindane and gardona were obtained from Chemical Service, Inc. and were 99% pure. Uniformly labeled [$^{14}$C]lindane (48 Ci/mol or 164 μCi/mg (99%)) was from Amersham, Searle. α-HCH from ICN-Life Sciences Group and β-HCH from RFR Corporation were 99% pure. Technical grade methoxychlor (MeOC) was obtained from Sigma Chemical Co. and was recrystallized twice from ethanol. δ-HCH was purified from a mixture of hexachlorocyclohexane isomers which was prepared by bubbling chlorine through benzene in the presence of ultraviolet light [17,18]. The δ-HCH was partially purified by fractional precipitation of the mixture and further purified by column chromatography on silica gel G using hexane/ether (20:1, v/v) as a solvent. The final product was recrystallized from chloroform, then methanol. Using NMR analysis and thin-layer chromatography, the product was identified by comparison with a sample of δ-HCH which was obtained from the Environmental Protection Agency. *N*-ethylcarbazole was obtained from Aldrich Chemical Co. and recrystallized six times from ethanol. Synthetic DMPC, DOPC, and DPPC were obtained from Sigma Chemical Co. and were 98% pure. Cholesterol and type I sphingomyelin from bovine brain were also from Sigma Chemical Co. DPPC ether and POPC, 99% pure, were obtained from Serdary Research Laboratories.

Fluorescence measurements were performed using either a spectrofluorometer or a subnanosecond phase fluorometer obtained from SLM Instruments, Inc. Fluorescence lifetimes were measured by either the phase shift or demodulation method [19] using a modulation frequency of 10 MHz. Differences in lifetimes determined by the demodulation and phase method were less than 11%. Unless otherwise indicated, the excitation wavelength and filter were 297 nm and Corning 7-54, respectively, and the emission filters were Corning 0-52 and 7-60 for lifetime measurements. Quenching efficiencies for the chlorinated hydrocarbons and *N*-ethylcarbazole and proof of dynamic quenching were determined as previously

described [14,15]. Single lamellar bilayer vesicles were prepared as previously described [15], except that the sonication was done under argon flow to minimize oxidation of the phospholipids. Liposomes were prepared by mixing the lipid and probe in a vial and evaporating the solvent as for the vesicle preparation. Buffer was added, the vial capped, and the sample sonicated in a Cole-Parmer Model 8845-2 sonifier bath for 30 min. The maximum temperature of the sample was 45°C. These samples were not annealed or centrifuged. Phospholipid and cholesterol concentrations were determined as described by Kates [20].

The lipid-water partition coefficients were determined fom the equation [14,15]:

$$\frac{1}{k_{app}} = \frac{1}{k_{qm}P} + \left(\frac{1}{Pk_{qm}} - \frac{1}{k_{qm}}\right)\alpha_m \quad (1)$$

where $k_{qm}$ is the quenching constant of the chlorinated hydrocarbon in the membrane, $P$ is the lipid-water partition coefficient, $\alpha_m$ is the volume fraction of the membrane, and $k_{app}$ is the apparent quenching constant. $\alpha_m$ was determined from the lipid concentration and the density of phospholipid vesicles. Lifetimes of the carbazole-labeled vesicles were measured as the samples were titrated with microliter additions of chlorinated hydrocarbon in ethanol as previous described [14,15]. $k_{app}$ was calculated from the following equation:

$$\frac{\tau_0}{\tau} = 1 + \tau_0 k_{app}[CH]_{added} \quad (2)$$

where $\tau_0$ and $\tau$ are the lifetime in the absence and presence of chlorinated hydrocarbon (CH).

The diffusion coefficient is related to $k_{qm}$ by the relationship [14,15]:

$$k_{qm} = \gamma 4\pi\sigma_{fq} D_{qm} N/1000 \quad (3)$$

where $\gamma$ is the quenching efficiency, $\sigma_{fq}$ is the sum of the molecular radii of the quencher and fluorophore, $D_{qm}$ is the diffusion coefficient of the quencher in the membrane, and $N$ is Avogadro's number. The temperature dependence of the diffusion coefficient of the chlorinated hydrocarbon in the membrane was studied by measuring the apparent quenching constant at high lipid concentration where $k_{qm} = k_{app}\,\alpha_m$ [14,15]. Membrane binding capacities for the chlorinated hydrocarbons were obtained from these same data. A stock solution of chlorinated hydrocarbon in ethanol was mixed and specified amounts measured into a series of vials. The ethanol was evaporated and 3 ml of CUA-PC labeled vesicles added to each vial. The lipid probe ratio was typically 200:1. Samples were sonicated for 1.5 h in a Cole-Parmer Model 8845-2 sonifier bath, then incubated at 45°C for at least one hour prior to measurement. The excitation wavelength used was 297 nm except for experiments with DDE, where the excitation was 325 nm. The carbazole fluorescence was sufficiently intense in the DDE-containing samples so that Raman scatter of water at 363 nm was not significant. The lifetimes were measured as the temperature was incrementally decreased from 45°C to 5°C, allowing 30 min equilibrium time at each new temperature. Data for samples incubated for one hour did not significantly differ from data collected after 30 min, indicating that equilibrium had occurred within the first half hour of incubation.

To confirm the fluorescence data for saturation of the membranes with chlorinated hydrocarbons, saturation was measured using [$^{14}$C]lindane. Samples were prepared as above, except that [$^{14}$C]lindane was included (0.01 $\mu$Ci/mg lindane). Lifetimes were measured at 5°C and the samples centrifuged at 5°C and 48000 × $g$ for one hour to sediment the chlorinated hydrocarbon crystals. The lifetimes of the supernatants were measured again at 5°C. One ml of each supernatant was counted in 10 ml of scintillation fluid to determine the lindane concentration remaining in each sample. The lipid concentration of each sample also was determined.

## Results

*Determination of quenching efficiencies*

The chlorinated hydrocarbons used in this study were shown to be dynamic quenchers of *N*-ethylcarbazole in homogenous solution by the equivalence of $F_0/F$ and $\tau_0/\tau$ (Fig. 2). In addition, the spectral distribution of *N*-ethylcarbazole fluorescence was not altered upon quenching (data not shown). Thus the chlorinated hydrocarbons did

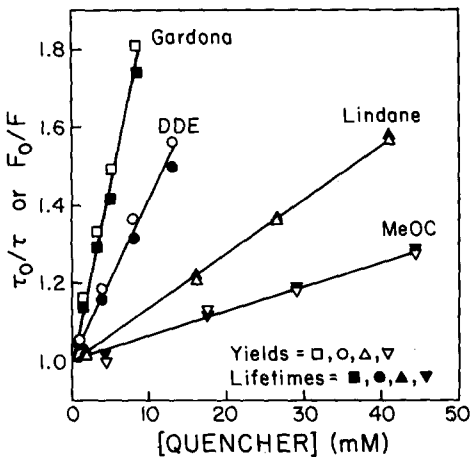


Fig. 2. Quenching of *N*-ethylcarbazole by chlorinated hydrocarbon. Fluorescence lifetimes and yields are shown for DDE, lindane and methoxychlor in ethanol, and for gardona in cyclohexane, at 25°C.

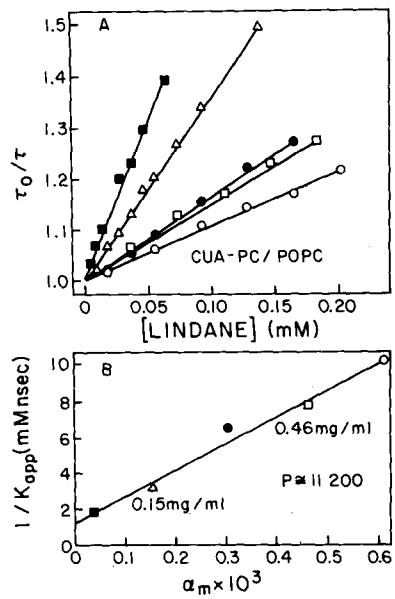


Fig. 3. Lindane partitioning into POPC vesicles at 25°C. (A) Quenching of CUA-PC-labeled POPC vesicles by lindane. Samples of various lipid concentrations were titrated with microliter additions of lindane in ethanol and lifetimes measured as described in Materials and Methods. (B) Dependence of the apparent quenching constant on lipid concentration. The apparent quenching constants were calculated from the data in A using Eqn. 2. $\alpha_m$ was calculated assuming the vesicle density to be 1 g/ml [21]. The partition coefficient was calculated using Eqn. 1. POPC concentrations were 0.61 mg/ml (○), 0.46 mg/ml) (□), 0.31 mg/ml (●), 0.15 mg/ml (△), and 0.04 mg/ml (■).

not complex with *N*-ethylcarbazole such that the spectral properties of the *N*-ethylcarbazole were altered. Bimolecular quenching constants, and hence quenching efficiencies, were determined from the slopes of the Stern-Volmer plots of data similar to that in Fig. 2 [14,15]. The calculated quenching efficiencies are estimated to contain a possible error of up to a factor of two [15] because of uncertainties in the calculated diffusion coefficients of the probe and quenchers.

*Measurement of lipid-water partition coefficients*

Lindane quenching of CUA-PC labeled POPC vesicles is shown in Fig. 3A. Increasing lindane concentrations result in decreasing lifetimes. In addition, greater decreases in lifetime are observed at lower lipid concentrations. The lipid concentration dependence of quenching allows the lipid-water partition coefficient to be calculated, as is shown in Fig. 3B. We assumed the vesicle density to be 1 g/ml [21]. A plot of $(k_{app})^{-1}$ versus the volume fraction of the membrane ($\alpha_m$) yield both the bimolecular quenching constant ($k_{qm}$) and the partition coefficient ($P$), as indicated from Eqn. 1. Lindane was found to concentrate about 11000-fold in POPC vesicles.

Table II compares the partition coefficients of lindane in vesicles of various lipid compositions. In general, the partition coefficients correlate with

TABLE II

PARTITION COEFFICIENTS OF LINDANE IN VARIOUS LIPIDS DETERMINED BY FLUORESCENCE QUENCHING

Partition coefficients were determined as described in Materials and Methods. The data were calculated from demodulation lifetimes using a weighted least squares fit. The partition coefficients are estimated to be accurate within ±20%.

| Lipid | Temp. (°C) | $P$ |
|---|---|---|
| DPPC | 25 | 3800 [a] |
| DPPC | 50 | 4200 [a] |
| DPPC-cholesterol | 25 | 800 [a] |
| DPPC ether | 26 | 5400 |
| DPPC ether | 46 | 4100 |
| SM | 25 | 2700 |
| DOPC | 25 | 13400 [a] |
| DMPC | 37 | 10500 [a] |
| POPC | 25 | 11200 |

[a] From Lakowicz and Hogen [15].

membrane fluidity. Partitioning of lindane into vesicles of unsaturated phospholipid composition (DOPC and POPC) was higher than partitioning into highly saturated membranes (DPPC and DPPC ether). At 25°C DOPC and POPC vesicles are in the fluid liquid-crystalline state whereas DPPC and DPPC ether are in the more rigid gel phase [22,23]. In addition, partitioning into sphingomyelin, which is composed of highly saturated fatty acyl chains [24] and is below its phase transition temperature at 25°C [25], was low. At 37°C DMPC vesicles are in the fluid liquid-crystalline state [22], and partitioning was high in these vesicles. Addition of cholesterol to DPPC vesicles lowered the lindane partition coefficient 4-fold. Below the phase transition temperature, cholesterol has been shown to increase [26,27] or decrease [28,23] the fluidity of synthetic phospholipid vesicles depending upon the probe and method used for measuring the membrane structure. It seems reasonable that cholesterol-dependent increases in the packing density of the membrane could result in a decreased partitioning of lindane into the membrane. DPPC ether has its fatty acid chains attached to the glycerol backbone via ether linkages rather than the usual ester linkages. The similarity of partitioning of lindane between DPPC and DPPC ether suggested that the structure of the glycerol backbone region of the bilayer does not interact with lindane and does not alter the lindane partition coefficient.

Table III lists the partition coefficients of various chlorinated hydrocarbons into DPPC and DOPC vesicles measured by the quenching method. Also shown are the octanol-water partition coefficients which are frequently used to estimate the bioaccumulation potential of the chlorinated hydrocarbon [29]. The values obtained by the fluorescence method are in good agreement with the *n*-octanol-water estimates, indicating that our method gives reasonable estimates for partition coefficients. However, we did not expect nor find precise agreement between lipid-water and octanol-water partitioning.

Examination of Table III shows that for the four chlorinated hydrocarbons we investigated partitioning into DOPC was greater than into DPPC. This suggests a possible explanation for the selective action of chlorinated hydrocarbons on

TABLE III

PARTITION COEFFICIENTS OF CHLORINATED HYDROCARBONS (CH) IN DPPC AND DOPC VESICLES COMPARED TO OCTANOL:WATER

| CH | Temp. (°C) | *P* | | |
|---|---|---|---|---|
| | | DPPC [a] | DOPC [a] | *n*-octanol/water |
| γ-HCH | 25 | 3800 [e] | 13400 [e] | 5200 [b] |
| | 50 | 4200 [e] | – | – |
| α-HCH | 26 | 2900 | 12600 | 6400 [b] |
| | 46 | 9400 | – | – |
| δ-HCH | 25 | 4800 | 12400 | 13730 [b] |
| | 45 | 9000 | – | – |
| β-HCH | 25 | – | – | 6250 [b] |
| MeOC | 25 | 28300 | >130000 | 15000 [c] |
| DDT | 25 | – | – | $1.5 \cdot 10^{6}$ [c] |
| DDE | 25 | – | – | $5 \cdot 10^{5}$ [d] |

[a] The data were calculated from demodulation lifetimes using a weighted least squares fit. Because of its low quenching efficiency and its low solubility, partitioning of β-HCH was not measurable. The partition coefficient of δ-HCH and γ-HCH are estimated to be accurate within 20%. The partition coefficient of α-HCH is estimated to be accurate to within 50%.

[b] From Kurihara et al. [53].

[c] From Veith et al. [54].

[d] From Leo et al. [55].

[e] from Lakowicz and Hogen [15].

TABLE IV

SATURATION OF VARIOUS MEMBRANES BY LINDANE

Saturation limits were determined as described in Materials and Methods. Lipid concentrations were: DPPC, 6.0 mM; DPPC-cholesterol (mole ratio 3:1), DPPC, 18.8 mM; DOPC, 7.6 mM; sphingomyelin, 6.5 mM; and DPPC liposomes, 6.8 mM.

| Lipid | Lipid:lindane (mole ratio) | | |
|---|---|---|---|
| | 45°C | 25°C | 5°C |
| DPPC | 4.5 | 5.5 | 10.0 |
| DPPC-cholesterol (3:1) | 7.0 | 17.0 | 19.0 |
| DOPC | 3.5 | 3.5 | 6.0 |
| Sphingomyelin | 7.0 | 7.0 | 7.0 |
| DPPC liposomes | 4.0 | 13.0 | – |

the nervous system. Neural plasma membranes are known to contain high amounts of unsaturated phospholipids [30–33]. Thus it is possible that chlorinated hydrocarbons selectively exert their effects on neural plasma membranes because partitioning is higher in these membranes.

Partitioning of lindane and two of its inactive analogues, α-HCH and δ-HCH were similar in DPPC at 25°C and DOPC at 25°C, but α-HCH and δ-HCH partition coefficients were approximately 2-fold higher than the lindane partition coefficient above the phase transition temperature of DPPC. It is not clear why the temperature dependence of lindane partitioning differs from that of the inactive analogues. However, it seems unlikely that this difference is related to lindane toxicity. If partitioning were a crucial factor in lindane toxicity, we would expect lindane partitioning to be greater than the other isomers. Our data indicated that this was not the case.

Attempts to measure the partitioning of methoxychlor revealed an unforeseen limitation to the quenching method. Fig. 4 shows the quenching data for methoxychlor in DPPC vesicles. The diffusional rate of methoxychlor in these membranes was similar to that of the hexachlorocyclohexanes. However, the partition coefficients were higher. Since the intercept was determined by $1/k_{qm}P$, order of magnitude increases in the partition coefficient resulted in order of magnitude decreases in the intercept. As a result, the $y$-intercept for methoxychlor was close to the origin and difficult to estimate accurately. Table III lists the estimated partition coefficients calculated for methoxychor. The value for methoxychlor in DOPC is the lower limit estimated from the standard error of the intercept. Because of this limitation, no attempts were made to measure partitioning of other DDT-type compounds which are estimated to have higher partition coefficients than methoxychlor (Table III). However, the slope of the $(k_{app})^{-1}$ vs. $\alpha_m$ plot can still be used to estimate $k_{qm}$ values.

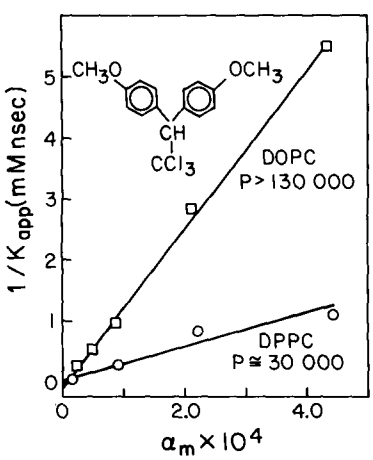


Fig. 4. Partitioning of methoxychlor into DPPC and DOPC vesicles. The $y$-intercept is 0.04 mM·ns for DPPC and −0.06 mM·ns for DOPC.

*Diffusion coefficients of chlorinated hydrocarbons in membranes*

Diffusion coefficients of several chlorinated hydrocarbons in membranes were determined by measuring $k_{qm}$ at high lipid concentrations. The lipid concentrations used for measurement of $k_{qm}$ were sufficiently large such that at least 92% of the chlorinated hydrocarbon was bound to the membrane at 25°C, and a 2-fold increase in the partition coefficient would have resulted in less than a 4% change in the membrane concentration of chlorinated hydrocarbons. Fig. 5 shows the quenching of DOPC and DPPC by lindane at various temperatures. For DOPC vesicles the bimolecular quenching constants, represented by the initial slopes of the curves in Fig. 5, decreased with decreasing temperature. However, a more complex relationship was found for DPPC vesicles. The $k_{qm}$ values determined from the initial slopes were used to calculate the diffusion coefficients of lindane at various temperatures. Fig. 6 shows Arrhenius plots for the temperature dependence of lindane diffusion coefficients. These coefficients were corrected for the quencher concentration in the membrane from the known partition coefficient at 25°C and the lipid concentration.

The apparent diffusional rate of lindane reached a minimum near the phase transition temperature of the lipids (Fig. 6). DOPC, which is above its phase transition temperature throughout the temperature range studied, gave a straight line with an activation energy of 5.5 kcal/mol. Fig. 7 shows the Arrhenius plots for the diffusion of a number of chlorinated hydrocarbons in DPPC and DOPC. Again, for all chlorinated hydrocarbons tested, the apparent diffusion coefficient reached a

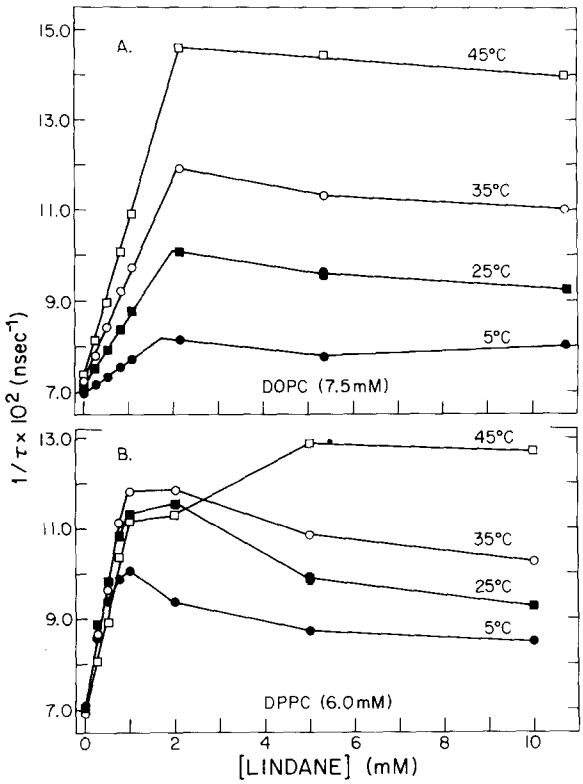


Fig. 5. Effect of temperature on lindane quenching of CUA-PC -labeled DOPC (A) and DPPC (B). Samples were prepared and measurements made as described in Materials and Methods using a lipid-to-probe ratio of 100:1. Fluorescence lifetimes were determined by the demodulation method. The standard deviation in the lifetime measurements is estimated to be 0.1 ns.

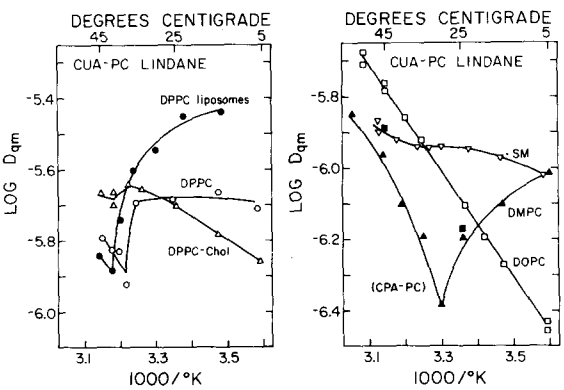


Fig. 6. Temperature dependence of the diffusion coefficient of lindane in phospholipid vesicles. Diffusion coefficients were calculated as described in the legend of Fig. 7. The lipid phase transition temperatures are: DPPC, 41°C, and DMPC, 23°C [49]; DOPC, <0°C [50]; and sphingomyelin, 32°C [25]. The probe CPA-PC was used in DMPC vesicles. All other lipids were studied with CUA-PC. Data are also shown for POPC (■) which has a transition temperature near 4°C [22].

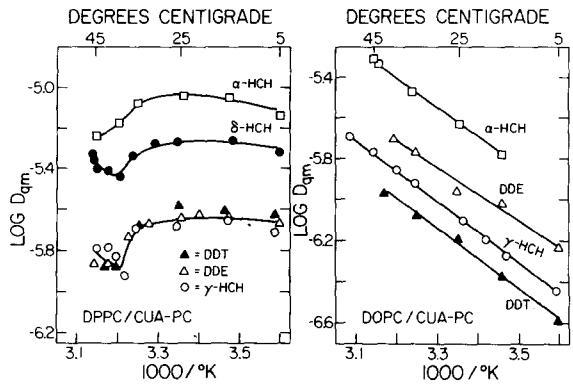


Fig. 7. Diffusion coefficients of chlorinated hydrocarbons in phospholipid vesicles. Samples were prepared and measurements made as described in Fig. 5. Diffusion coefficients were calculated from Eqn. 3 using the following data. Quenching efficiencies were 0.20 for lindane, 0.037 for α-HCH, 0.020 for δ-HCH, 0.21 for DDT and 0.20 for DDE. Molecular radii were 0.41 nm for *N*-ethylcarbazole, 0.37 nm for γ-, α-, and δ-HCH, and 0.56 nm for DDT and DDE.

minimum near the phase transition temperature of DPPC. In addition, diffusion was slower in the more fluid DOPC vesicles than in DPPC vesicles. The reason for observing this anomalous diffusion behavior is not known. It is unlikely that this effect was due to artifacts in the quenching methodology because a similar temperature dependence was observed for measurement of depolarizing rotations of diphenylhexatriene in lipids [23,34]. In addition, Sakanishi and coworkers [35] observed that the ultrasonic attenuation of DPPC vesicles reached a minimum at the phase transition temperature. It seems apparent that the diffusive rates of small foreign molecules in membranes is more complex than expected on the basis of simple microviscosity considerations. Alternatively, at temperatures below the transition temperature, the probe and the chlorinated hydrocarbon may be concentrated in residual regions of the membrane which remain in the fluid state.

Within our limits of error, DDT, DDE, and lindane diffusion was similar in either DOPC or DPPC (Fig. 7). The diffusion coefficients of α- and δ-HCH were significantly higher, as was the diffusion coefficient of methoxychlor ($8.49 \cdot 10^{-6}$ cm$^2$/s and $2.04 \cdot 10^{-6}$ cm$^2$/s in DPPC and DOPC at 25°C, data not shown). It should be noted that the

quenching efficiencies for α-HCH, δ-HCH, and methoxychlor (0.037, 0.020, and 0.060, respectively) were significantly less than that of DDT, DDE, and lindane (0.21, 0.20, and 0.20, respectively). These quenching efficiencies, which were used to calculate the diffusion coefficients in membranes, were obtained from homogeneous solution data where diffusion is three-dimensional. In the membrane, diffusion may be two-dimensional. In two dimensions, the probability of multiple fluorophore-quencher encounters is higher than in three dimensions. This effect is not significant for 100% efficient quenchers, since the first collision results in quenching. However, for low efficiency quenchers this effect can be important because the quencher may collide several times with the fluorophore before the fluorophore returns to the ground state. Thus the probability of quenching for a low efficiency quencher may be greater in two dimensions than in three dimensions. As a result, the calculated diffusions coefficients for the low efficiency quenchers may be overestimated. If we assume diffusion in the membrane is the same for all these compounds and is equal to the lindane diffusion coefficient, our data suggest that the enhancement of α-HCH and δ-HCH quenching in the membrane is 4- and 3-fold, respectively. At present we have no way of knowing whether alterations of the effective quenching can reach this magnitude. However, in view of the anamolous temperature dependence of the chlorinated hydrocarbons diffusion in membranes, it seems inappropriate to assume that a three-dimensional model is valid or that the apparent differences between the diffusion coefficients of the chlorinated hydrocarbons are significant.

*Chlorinated hydrocarbon saturation capacities of membranes*

Quenching data, such as in Fig. 5, were used to estimate the amounts of chlorinated hydrocarbons which could saturate the membranes. For DOPC (Fig. 5A), the fluorescence lifetimes ceased to change above 2 mM lindane. Above 2 mM lindane a precipitate was present in the sample, indicating saturation of the membranes with lindane. At low temperatures, the fluorescence lifetimes were at a minimum in the lindane concentration range where the bilayer became saturated, and then increased to slightly longer lifetimes at concentrations above the saturation limit. The occurrence of this saturation maximum was observed for all chlorinated hydrocarbons tested and was most pronounced for DPPC below its phase transition temperature (Fig. 5B). Alternately incubating the samples at 5°C and 45°C resulted in repeated occurrence and disappearance of the saturation maximum. Thus the temperature effect was reversible.

To confirm the results of our fluorescence method, membrane saturation was also measured by determining the amount of [$^{14}$C]lindane bound to the membrane after crystalline lindane was removed by centrifugation. Excellent agreement between the two methods was observed (data not shown). The radio tracer data also showed a saturation maximum.

We questioned whether the high concentration of chlorinated hydrocarbon in the membranes could disrupt the bilayer structure, and thereby lower the chlorinated hydrocarbon binding capacity of the lipids. Such disruption would result in release of small ionic molecules trapped in the interior aqueous volume of the vesicles. We assayed the integrity of the vesicles by their permeability to umbelliferyl phosphate [36], and examined both saturating and subsaturating concentrations of chlorinated hydrocarbons. DOPC and DPPC vesicles with trapped umbelliferyl phosphate were stable for at least 4 days. Vesicle disruption was not observed at 25°C with subsaturating or saturating concentrations of lindane α-HCH, δ-HCH, DDT, DDE, or methoxychlor. Nor was disruption evident in DPPC at 39°C in the presence of lindane, DDT, DDE, or α-HCH. These chlorinated hydrocarbons do not appear to disrupt the bilayer structures of DOPC and DPPC vesicles. At this point we are uncertain of the reason for the saturation maximum, however, it is possible that it is related to supersaturation of the membrane and aqueous phases.

The saturation point was approximated as the concentration at which the lifetime first ceased to decrease with increasing chlorinated hydrocarbon concentration. Table IV shows the saturation of various lipids by lindane. Saturation of the various membranes is similar, and cholesterol lowers the saturation limit of the membrane by a factor of two. More chlorinated hydrocarbon is taken up

TABLE V

SATURATION OF DPPC AND DOPC BY CHLORINATED HYDROCARBONS (CH)

Saturation limits were determined as described in Materials and Methods. Lipid concentrations were 5 mg/ml for the hexachlorocyclohexanes and 1 mg/ml for DDT and DDE.

| CH | Lipid : CH (mole ratio) | | | | | |
|---|---|---|---|---|---|---|
| | DPPC | | | DOPC | | |
| | 45°C | 25°C | 5°C | 45°C | 25°C | 5°C |
| γ-HCH | 4.5 | 5.5 | 10.0 | 3.5 | 3.5 | 6.0 |
| α-HCH | 13 | – | 17.0 | 9.0 | 18 | – |
| δ-HCH | 2.0 | 3.5 | 4.5 | – | – | – |
| β-HCH | 30 | – | 80 | – | – | – |
| DDE | 8.0 | 12 | 24 | – | 8.5 | – |
| DDT | 10 | 10 | 14 | – | 8.5 | – |

into the membrane at higher temperatures. Table V shows similar saturation data for DPPC and DOPC with several chlorinated hydrocarbons. The saturation of vesicles by the HCH isomers varies considerably, and is of the order $\delta > \gamma > \alpha > \beta$, which is also the order of their solubilities in many organic solvents [17].

How do these saturation limits of the membrane compare with the levels of chlorinated hydrocarbon in the membrane at toxic doses? Assume that a toxic dose of chlorinated hydrocarbon is 100 mg/kg ($LD_{50}$ for rats), the % lipid of an organism is about 2%, and the chlorinated hydrocarbon and lipid molecular weights are 320 and 740. Then at 100 mg/kg, the molar ratio of lipid to chlorinated hydrocarbon is about 86. This estimate probably overestimates the actual concentration of chlorinated hydrocarbon in the membrane because it does not take into account metabolism and elimination of the chlorinated hydrocarbon enroute to the membrane. The toxicity of lindane to insects is about 5 mg/kg [37]. Using the assumptions stated above, lindane toxicity would occur at a lipid to chlorinated hydrocarbon mole ratio of 1700 to 1. Since our studies showed that all four of the HCH isomers can obtain even higher concentrations in the membrane, it is unlikely that differences in HCH toxicity are due to differences in their membrane saturation limits. Moreover, DDT and DDE saturate the membrane at approximately the same level (Table V).

*Comparison of DPPC vesicles and liposomes*

The lindane binding capacity of DPPC liposomes and vesicles at 45°C is similar. However, below the phase transition temperature, less lindane can be taken up into liposomes than vesicles (Table IV). The diffusion coefficients measured above the phase transition temperature are similar for liposomes and vesicles, but below the phase transition temperature difusion is much higher in liposomes (Fig. 6). It has been shown that the apparent molar volume of DPPC vesicles is greater than that of liposomes below the phase transition temperature, although above the phase transition temperature the apparent molar volumes of vesicles and liposomes are equal [38]. Similarly, measurement of the anisotropy of diphenylhexatriene in DPPC vesicles and liposomes showed that the anisotropy is the same above the phase transition temperature, but the anisotropy of liposomes is higher than vesicles below the phase transition temperature [22]. Our data support the observation that the membrane structure is similar for liposomes and vesicles above the phase transition temperature, but below the phase transition temperature the bilayer structure is more ordered in liposomes. The larger apparent diffusion coefficients of chlorinated hydrocarbon in liposomes as compared to vesicles could be a result of concentration of probe and quencher in regions of disorder. At temperatures below the transition temperature there are probably fewer such regions in liposomes than in vesicles.

## Discussion

Our data suggest that chlorinated hydrocarbons may preferentially exert their effects on neural membranes because they partition more strongly into unsaturated phospholipid membranes. However, partitioning of the HCH isomers did not correlate with toxicity. Thus the toxicity of active chlorinated hydrocarbons is not only a result of their ability to partition into membranes. Within the limitations of our method, the diffusion data do not indicate any specific chlorinated hydrocarbon-membrane binding for active analogues as compared to inactive analogues. However, other brain lipids, such as the gangliosides and cerebrosides, need to be studied. The order of the mem-

brane saturation capacities for the HCH isomers was $\delta > \gamma > \alpha > \beta$, which is also the order of their solubilities in a number of organic solvents [17]. This suggests that the nature of binding of the chlorinated hydrocarbon to the membrane is that of dissolution in the membrane rather than binding to specific receptor sites. The occurrence of supersaturation of the membrane also supports this hypothesis since we would not expect to observe supersaturation of specific binding sites. However, our results do not discount the likelyhood that specific receptors are involved in chlorinated hydrocarbon toxicity. The order of toxicity of the hexachlorocyclohexanes is $\gamma > \alpha > \delta > \beta$. The lack of biological activity of the $\alpha$- and $\beta$-isomers could be explained by their inability to reach sufficiently high concentrations in the membrane. However, this does not explain why $\delta$-HCH is not toxic. DDT and DDE also saturate the membrane at about the same level. Thus differential toxicity cannot be explained by different saturation capacities of the membranes. In addition, toxic doses of these insecticides probably are lower than the amounts needed to saturate the membranes.

How does our information compare with the known interactions of other small molecules in membanes? Table VI compares information for local anesthetics and chlorinated hydrocarbons. The anesthetics also are thought to exert their effects via lipid interactions. In several respects the membrane interactions of chlorinated hydrocarbons and these anesthetics are similar. Cholesterol decreases the partition coefficients and saturation capacity of anesthetics and chlorinated hydrocarbons in membranes. Partitioning is greater in membranes with phospholipids that have unsaturated fatty acyl chains. In addition, both anesthetics and chlorinated hydrocarbons have been shown to cause membrane expansion. However, anesthetics affect the nervous system by blocking neural transmission whereas the chlorinated hydrocarbons act by stimulating the nervous system.

Several mechanisms have been proposed for the action of anesthetics on membranes. These include (a) alteration of the density of fixed charges on the surface of the membrane, (b) expansion of some volume of the membrane that is critical for conduction, and (c) interaction with some specific receptor in the nerve membrane [39]. These mechanisms may also be operative for chlorinated hydrocarbons. It seems unlikely that chlorinated hydrocarbons would alter the charge distribution at the surface of the membrane since they tend to partition into the hydrophobic area of the membrane. Since both anesthetics and DDT cause membrane expansion but result in different neurological symptoms, it seems unlikely that membrane expansion is involved in the mechanism of action. Clearly, the different mechanisms of action of these two classes of compounds cannot be attributed only to their interactions with lipids, since they appear to behave similarly in 'lipid mem-

TABLE VI

COMPARISON OF ANESTHETIC-MEMBRANE AND CHLORINATED HYDROCARBON-MEMBRANE INTERACTIONS

| Property | Chlorinated hydrocarbon (CH) | Anesthetic |
|---|---|---|
| Membrane saturation | lipid : CH = 5–10 : 1 | lipid : anesthetic = 5 : 1 [56] |
| Membrane active concentration | lipid : CH = 86 : 1 | lipid : anesthetic = 10 : 1 for local anesthetic, 100 : 1 for general anesthetics [56] |
| Cholesterol in phospholipid | decrease *P*, decreases saturation limit of membrane | decreases P for steroids, decreases binding of some steroids [57] |
| Nature of membrane lipids | partitioning and binding is less in phospholipid vesicles with saturated fatty acyl chains | hydrogenation of endoplasmic reticulum membranes decreases binding of amine anesthetics [56] |
| Membrane expansion | occurs in phospholipid films for DDT, DDE, HO-DDT [9] | occurs in biological membranes and phospholipid films [56] |

branes'. In addition, it is unlikely that all of the chlorinated hydrocarbons act via the same biochemical mechanism. It is possible that the lipid phase is important in solubilizing the xenobiotic and making it available for specific interaction with other membrane components.

The effect of anesthetics in neural membranes is to block the sodium currents in nerves [40], whereas the reported effect of DDT is to prevent the sodium gates from closing after the action potential [2]. Thus these molecules apparently act at different sites on the sodium channel in the membranes. One can speculate as to the type of specific interaction involved. DDT has been shown to alter enzyme functioning, although it does not interact at the enzyme site of action [5–7]. many membrane proteins are known to have an annulus of bound lipid which is necessary for protein activity [41]. It is possible that chlorinated hydrocarbons (and possibly the anesthetics) interact specifically with the bound lipid of the proteins and thus alter the functioning of the protein by altering its immediate environment. The differential effects of xenobiotics on different membrane proteins may be dictated by the specific composition of the protein-bound lipid. Alternatively, the chlorinated hydrocarbons may alter the functioning of proteins by interacting directly with lipophilic regions of the protein other than the active sites.

It is interesting to note the numerous studies which attempt to correlate the structure of the chlorinated hydrocarbon with its insecticidal activity. These studies indicate that the toxicity of a chlorinated hydrocarbon depends upon its ability to fit into an active site of particular size and shape [42–46]. Soloway [45] has suggested that the distribution of electronegative centers within cyclodiene and lindane molecules is also important in their toxicity, and Holan [47] has suggested that the magnitude of the $\pi$-electron density in the phenyl rings is important for toxicity of DDT analogues. Clearly, the size, shape, and dipolar nature of the chlorinated hydrocarbon could be important in determining the location and strength of binding to proteins or annular bound lipid. In this respect it is interesting to note that the dipole moment of lindane in benzene is 2.9 Debye units and that of the inactive $\beta$-HCH is 0 [48]. The first requirement of chlorinated hydrocarbon toxicity appears to be high lipophilicity. This allows the compound to solubilize in the hydrophobic region of the membrane. The chlorinated hydrocarbon must then have the appropriate characteristics for interacting with other functional components in the hydrophobic region of the membrane. These interactions may be indirect via the bound lipids of the protein or direct via protein binding.

Extensive study of other chlorinated hydrocarbons was not possible because of their low quenching efficiencies and/or solubilities in the membranes. This work can be extended by the development of other probes for which chlorinated hydrocarbon quenching is more efficient. 9-Amino -*N*-ethylcarbazole shows potential in this respect. This compound is quenched with essentially 100% efficiency by lindane, endrin, DDT [16], $\alpha$-HCH, $\beta$-HCH, dieldrin, and aldrin (Omann, G.M. and Lakowicz, J.R., unpublished data). The use of amino carbazole derivatives would eliminate the ambiguities in comparing calculated diffusion coefficients for quenchers with low quenching efficiencies. In addition, the lifetime of this fluorophore is near 27 ns. This increased lifetime would provide even greater sensitivity to low chlorinated hydrocarbon concentrations, and the number of chlorinated hydrocarbons which could be studied would be greatly increased. Thus our quenching methodology may be useful for further studies of the interactions of chlorinated hydrocarbons with membranes.

## Acknowledgements

This work was supported by grant ES02624 from the National Institute of Environmental health Sciences. J.R.L. is an Established Investigator of The American Heart Association.

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