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DIFFUSION AND PARTITIONING OF A PESTICIDE, LINDANE, INTO PHOSPHATIDYLCHOLINE BILAYERS

A NEW FLUORESCENCE QUENCHING METHOD TO STUDY CHLORINATED HYDROCARBON-MEMBRANE INTERACTIONS

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Summary

Chlorinated hydrocarbons, such as the pesticide lindane (γ-hexachlorocyclohexane), quench the fluorescence of carbazole. The observed quenching is a result of the molecular contacts which occur upon diffusional collisions. Because the amount of quenching depends upon the collisional frequency between carbazole and pesticide, this phenomenon provides a measure of both the diffusional rate of lindane and its local concentration. The carbazole fluorophore is localized within phosphatidylcholine bilayers by cosonicating the lipid with a newly synthesized phospholipid, β-(11-(9-carbazole)-undecanoyl)-L-α-phosphatidylcholine. Using this probe in dimyristoyl-L-α-phosphatidylcholine vesicles, and the above mentioned quenching phenomena, we determined the lindane diffusion rate within the bilayer to be $5.7 \cdot 10^{-7}$ cm^2/s at 37°C. Measurement of the apparent quenching constant at various dimyristoyl phosphatidylcholine concentrations yielded a lipid-water partition coefficient for lindane of 9500, which is in agreement with the value of 8980 obtained by our equilibrium dialysis experiments. Vesicles of dimyristoyl-L-α-phosphatidylcholine become saturated with lindane at a pesticide to lipid molar ratio of approx. 0.28.

These results demonstrate the possibility of using the quenching of carbazole fluorescence to investigate the transport and partitioning of pesticides within biological membranes. This ability should prove useful in studies of the interactions of chlorinated hydrocarbons with cell membranes.

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Introduction

The biological uptake and transport of small foreign molecules such as pesticides, polychlorinated biphenyls and toxic metal alkyls is presently of great interest because of the need to understand their bioaccumulation in the food chain. The process of chlorinated hydrocarbon bioaccumulation involves a number of more fundamental processes such as:

1. Uptake of the chlorinated hydrocarbon from the surrounding environment by an organism.
2. Diffusion of the chlorinated hydrocarbon across cell membranes within the organism.
3. Transport mediated by body fluid components such as the serum lipoproteins.
4. Concentration of the chlorinated hydrocarbon in non-polar biological structures.

The process of bioaccumulation thus involves both kinetic (diffusion) and equilibrium (partitioning) processes.

Collisional or dynamic quenching of fluorescence provides a unique method of examining the diffusional transport of chlorinated hydrocarbons within cell membranes and the binding isotherms of them to cell membranes. Dynamic quenching, being a result of diffusional collisions between a fluorophore and quencher, depends upon both the mutual diffusion coefficients of the fluorophore and quencher, and the local quencher concentration. If a suitable fluorophore can be localized within a lipid bilayer, quenching of this fluorophore reveals both the diffusional rate of the chlorinated hydrocarbon within the bilayer, and its lipid-water partition coefficient. Herein lies the usefulness of collisional quenching by chlorinated hydrocarbons.

Many reports have appeared in the literature indicating that halogens such as I^- [1] and BrO_3^- [2] and halogenated compounds such as bromobenzene [3] act as quenchers of fluorescence. Unfortunately, quenching by most organochlorine compounds is inefficient for most commonly used fluorophores [4]. After testing about twenty-five fluorophores, we found that carbazole and its derivatives are efficiently quenched by many commonly used pesticides and chlorinated hydrocarbons. The carbazole fluorescence is quenched via diffusional collisions with mirex, gardona [5], CCl_4, DDT, endrin, methoxychlor [4], and by methylmercuric chloride (Rakow, S. and Lakowicz, J.R. (1977), unpublished).

In this paper we describe the use of β-(11-(9-carbazole)-undecanoyl)-L-α-phosphatidylcholine (Lakowicz, J.R. and Hogen, D., in preparation), a carbazole labeled phospholipid, to determine (a) the diffusion coefficient of a pesticide, lindane, in bilayers of dimyristoyl-L-α-phosphatidylcholine; (b) the lipid-water partition coefficient of lindane; and (c) the lindane binding capacity of dimyristoyl-L-α-phosphatidylcholine bilayers.

Materials and Methods

Dimyristoyl-L-α-phosphatidylcholine is a synthetic lipid obtained from Sigma and used without further purification. Chromatography of the phospho-

lipid on silica using chloroform/methanol/water (65 : 25 : 4) and ethyl ether/benzene/ethanol/acetic acid/H_2O (40 : 40 : 20 : 8 : 4) showed a single spot by both phosphate and char staining. No significant fluorescent impurities were observed in any of the unlabeled vesicles used as controls.

The dimyristoyl phosphatidylcholine vesicles were prepared by weighing the lipid in a tared stainless-steel beaker. The carbazole labeled phospholipid was added at a lipid to lipid-probe ratio of 200 : 1, and these materials were dissolved in benzene to facilitate mixing and uniform probe distribution. The benzene was evaporated by gently warming the solution while maintaining a constant flow of argon over the materials.

Buffer (0.01 M Tris, 0.05 M KCl, pH, 7.5) was added to the dried lipid to establish a concentration of 10 mg of lipid per ml of buffer. Sonication was effected in the stainless-steel beaker using a Heat Systems sonicator at 200 W using a 0.5-inch diameter tip. The temperature of the solution was maintained at 40°C during the 15-min sonication period. This preparation was annealed for 1 h at 4°C and then centrifuged at 40°C, 48 000 $\times g$, for 90 min. The centrifugation removes titanium particles and large lipid aggregates as a pellet. Phospholipid concentrations were determined for all samples using the procedure of Bartlett, as described by Kates [6].

The lindane was obtained from the Chemical Service Company. Lindane was added to 3-ml vesicle samples by the addition of μl quantities of 10 and 100 mM solutions of lindane in ethanol. Mixing was effected by purging the samples with argon. All samples were purged with either argon or nitrogen for about 3 min prior to the fluorescence measurements in order to remove dissolved oxygen. The amount of ethanol added did not exceed 2% by volume. This amount was found to have no effect on the fluorescence lifetimes or spectra of carbazole undecanoyl phosphatidylcholine labeled phospholipid vesicles. Additionally, 2% ethanol did not affect the fluorescence lifetimes of vesicles containing non-saturating levels of lindane. This result indicates that these small amounts of ethanol do not affect the lindane lipid-water partition coefficient. Changes in fluorescence lifetimes and yields resulting from lindane additions were complete within the sample mixing time of approximately 1 min. No further changes were observed over a period of several hours, nor after heating to 50°C and recooling to 37°C. Thus, we conclude that lindane equilibration between the lipid and water phases is rapid.

The lipid-water partition coefficient for lindane was also determined by equilibrium dialysis. [^{14}C]Lindane was obtained from Amersham/Searle (batch number 16928). 2% ethanol did not alter the partition coefficient observed by dialysis.

The synthesis and characterization of the carbazole-labeled phospholipid will be described in detail elsewhere (Lakowicz, J.R. and Hogen, D., in preparation). The carbazole undecanoic acid was a gift from Molecular Probes, Roseville, Minn. Carbazole undecanoyl phosphatidylcholine, containing about 50% palmitoyl and 50% stearoyl residues in the α-acyl position, was synthesized using the procedure of Hubbell and McConnell [18]. Purification was effected by preparative thin-layer chromatography using $CHCl_3$/methanol/water, (65 : 25 : 4). The product migrated as a single spot, as observed by phosphate staining, charring with dichromate in H_2SO_4, and by using silica plates with a fluo-

rescent indicator. This result was consistent in both the solvent used for purification and ethyl ether/benzene/ethanol/acetic acid/water, (40 : 40 : 20 : 8 : 4). All resonances shown by the 270 MHz proton NMR were assigned and found consistent with the structure indicated in Fig. 2.

All fluorescence measurements were obtained using either a spectrofluorometer or a subnanosecond phase fluorometer, both obtained from SLM Instruments, Inc. Fluorescence lifetimes were measured by either the phase shift or demodulation method [7], using a modulation frequency of 10 MHz. Differences in lifetimes determined by the phase and demodulation methods were generally less than 11%.

Molecular radii were calculated from known molecular weights and densities.

Theory

Diffusional quenching of fluorescence is described by the Stern-Volmer equation [8]:

$$\frac{1}{\tau} = \frac{1}{\tau_0} + k[Q] \tag{1}$$

where τ_0 and τ are the fluorescence lifetimes in the absence and presence of quencher, respectively. [Q] is the quencher concentration and k is the bimolecular quenching constant. The subscript s will be used to indicate homogeneous solution and m to indicate the membrane phase. The bimolecular quenching constant is given by the Smoluchowski equation [9]:

$$k_s = \gamma 4\pi\sigma_{pq} N(D_{ps} + D_{qs})/1000 = A(D_{ps} + D_{qs}) \tag{2}$$

where γ is the quenching efficiency, σ_{pq} is the sum of the molecular radii of the probe (p) and the quencher (q), and D_p and D_q are the diffusion coefficients of the probe and quencher, respectively. N is Avogadro's number, and A is the product $\gamma 4\pi\sigma_{pq}N/1000$. Generally, quenching is not solvent-dependent (Table I, see also ref. 5). Thus, we assume equal quenching efficiencies in either the solvent or membrane phase. Hence:

$$k_m = A(D_{pm} + D_{qm}) \tag{3}$$

Covalent attachment of the fluorophore to a phospholipid restricts probe diffusion. Therefore:

$$k_m \simeq A \cdot D_{qm} \tag{4}$$

Measurement of k_s, and estimates of D_{qs} and D_{ps} from the nomogram of Othmer and Thakar [10] allows calculation of constant A. Thus, measurement of k_m permits D_{qm} to be calculated.

The measurement of k_m for fluorescent probes in lipid bilayers poses a problem not encountered in homogeneous solution. It requires knowledge of the quencher concentration in the lipid bilayer. At low quencher concentrations it

appears likely that [Q] in each phase is described by a lipid-water partition coefficient.

$$P = [Q]_m/[Q]_w \tag{5}$$

The total (t) concentration of $[Q]_{added}$ partitions between the water (w) and membrane phases

$$[Q]_{added} V_t = [Q]_m V_m + [Q]_w V_w \tag{6}$$

By defining

$$\alpha_m = V_m/V_t \tag{7}$$

to be the volume fraction of the membrane and substituting $[Q]_w = [Q]_m/P$, we obtain

$$[Q]_m = \frac{P[Q]_{added}}{P\alpha_m + (1-\alpha_m)} \tag{8}$$

Substituting this expression for $[Q]_m$ into Eqn. 1 the observed quenching in the membrane is thus described by

$$\frac{1}{\tau} = \frac{1}{\tau_0} + \frac{k_m P[Q]_{added}}{P\alpha_m + (1-\alpha_m)} = \frac{1}{\tau_0} + k_{app}[Q]_{added} \tag{9}$$

Thus, the apparent quenching constant is dependent upon P, α_m and k_m.

$$\frac{1}{k_{app}} = \alpha_m\left(\frac{1}{k_m} - \frac{1}{k_m P}\right) + \frac{1}{k_m P} \tag{10}$$

A plot of k_{app}^{-1} versus α_m allows the diffusion and partition effects on the observed quenching to be separately determined.

The density of phospholipid vesicles is near 1 g/ml [11]. In these studies we assumed the entire volume of the bilayer is available for lindane partitioning. Thus a 1 mg/ml lipid suspension corresponds to $\alpha_m = 0.001$.

Results

Fig. 1 shows the fluorescence liftetimes and yields of *N*-ethyl carbazole in the presence of increasing concentrations of lindane. The equivalent fractional decrease in both the lifetimes and yields proves that the quenching by lindane is a result of diffusional collisions and not a result of complex formation [19]. Similar results were obtained for the chlorinated hydrocarbons, mentioned in Introduction, and methylmercuric chloride.

Fig. 2 shows the fluorescence emission spectrum of dimyristoyl phosphatidylcholine vesicles labeled with the carbazole-containing phospholipid. Lindane decreases the quantum yield without significant changes in the shape of the emission spectrum; however, a small blue shift can be observed when these spectra are normalized. This effect may result from a decrease in polarity near the carbazole moiety in the presence of lindane or from incomplete relaxation of polar groups around the carbazole moiety, whose fluorescent lifetime has

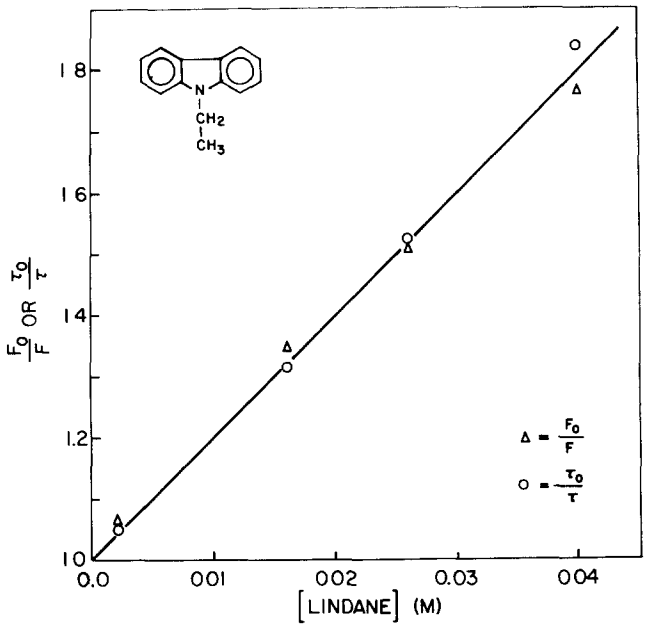

Fig. 1. Quenching of *N*-ethyl carbazole by lindane. Fluorescence lifetimes and yields. Experimental conditions are described in the legend of Figs. 2 and 3, except that the solvent was cyclohexane and the temperature was 25°C.

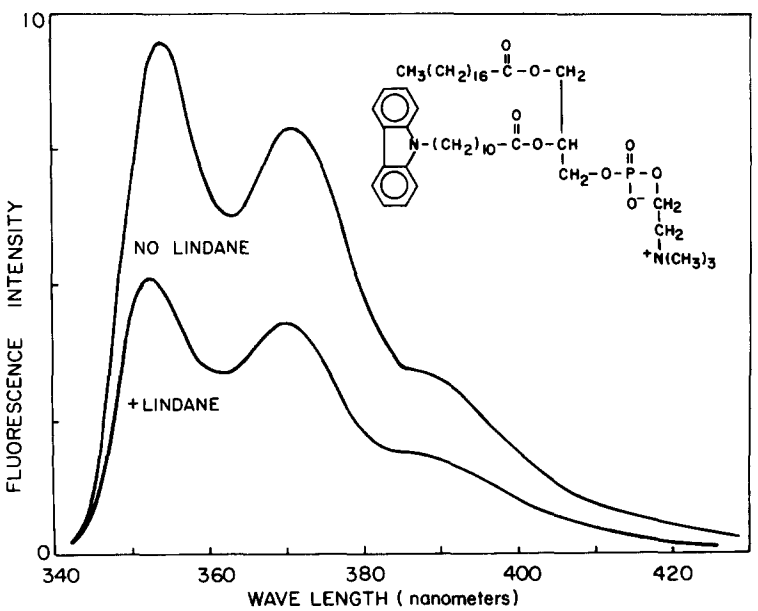

Fig. 2. Fluorescence emission spectra of carbazole undecanoyl phosphatidylcholine-labeled dimyristoyl phosphatidylcholine vesicles. The ratio of lipid to probe is 100. The excitation and emission bandpasses were 8 nm and 2 nm, respectively. Excitation wavelength, 297 nm, 36°C, labeled dimyristoyl phosphatidylcholine, 0.1 mg/ml; $Lindane_{added}$, 0.33 mM, excitation filter, Corning 7.54.

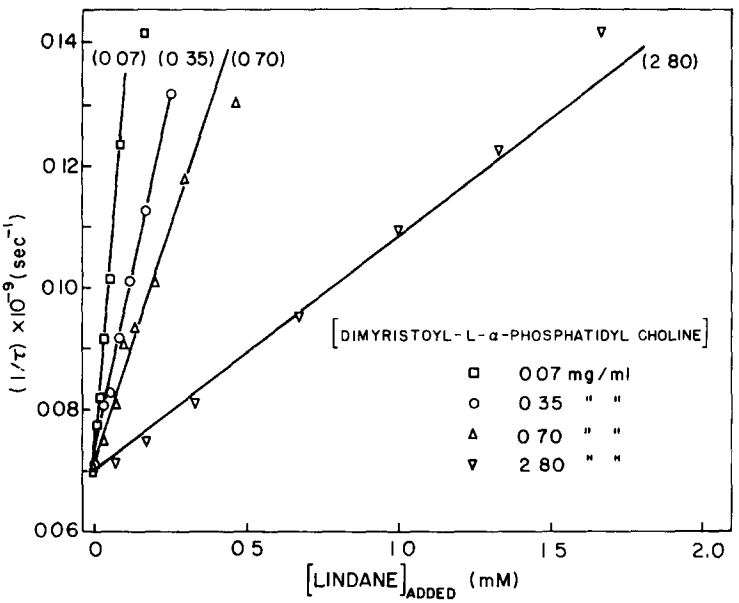

Fig. 3. Fluorescence lifetimes of β-(11-(9-carbazole)-undecanoyl)-L-α-phosphatidylcholine-labeled dimyristoyl phosphatidylcholine in the presence of lindane. Lifetimes were measured by the modulation method, using a frequency of 10 MHz. The emission was observed through a pair of Corning glass filters, 0-52 and 7-60. The numbers represent the concentration of dimyristoyl phosphatidylcholine in mg/ml. See legend to Fig. 2 for additional details.

been decreased from 12.1 to approx. 8 ns [12]. Ethanol, in amounts equivalent to that added with lindane, had no effect on the fluorescence emission spectrum, yield, or lifetime.

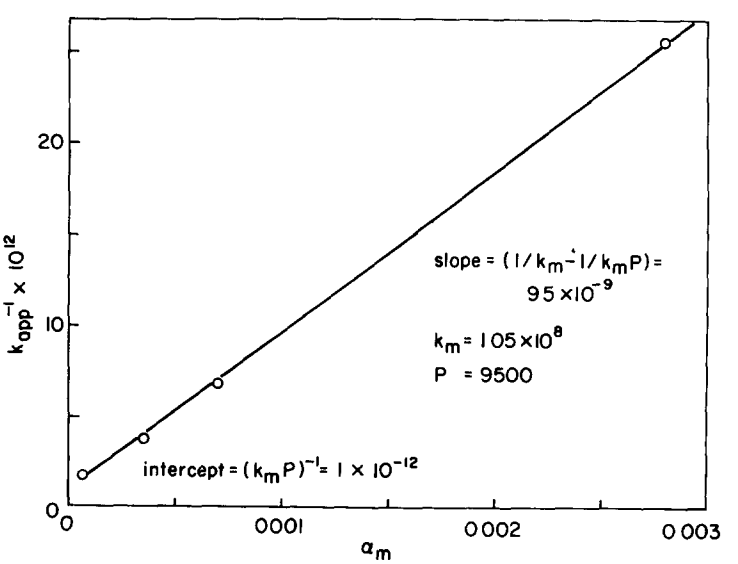

Fig. 4. Separation of diffusion and partition contributions to the observed quenching of carbazole undecanoyl phosphatidylcholine-labeled dimyristoyl phosphatidylcholine by lindane. See legends to Figs. 2 and 3 for additional details.

TABLE I

QUENCHING CONSTANTS AND DIFFUSION COEFFICIENTS FOR LINDANE QUENCHING OF CARBAZOLE FLUORESCENCE

The following information was used in calculating the diffusion coefficients according to the method of Othmer and Thakar [10] The viscosities of ethanol and cyclohexane at 20°C were taken to be 1 194 and 0 96 cP, respectively The heats of vaporization of ethanol, cyclohexane and water were taken to be 9 22 7 19 and 9 72 kcal/mol, respectively The molecular volumes of *N*-ethyl carbazole and lindane were calculated from the molecular weights and crystal densities, 184 and 155 cm^3/mol, respectively

The quenching efficiencies were calculated using the diffusion coefficients and molecular radii shown below, and eqn. 2 All the data are for 37°C

Quencher of probe/solvent	k (M^{-1} s^{-1})	τ_0 (ns)	Quenching efficiency (γ)	Diffusion coefficients [10] $D \times 10^5$ (cm^2/s)	Molecular radius (Å)
N-Ethylcarbazole/ ethanol	1 3 10^9	12 8	0 13	0 76	4 2
N-Ethylcarbazole/ cyclohexane	2 0 10^9	14 7	0 18	0 87	
Lindane/ethanol				0 84	
Lindane/cyclohexane				0 98	3 9
Lindane/water				1 0	

	k (M^{-1} s^{-1})	P	D_{qm} (cm^2/s)
Lindane/carbazole undecanoyl phosphatidylcholine/dimyristoyl phosphatidylcholine	1.1 10^8	9500	5 7 10^{-7}

Fig. 3 shows the dependence of the fluorescence lifetimes of carbazole undecanoyl phosphatidylcholine-labeled dimyristoyl phosphatidylcholine vesicles upon the lindane and lipid concentrations. The apparent quenching constant is highly dependent upon lipid concentration. This dependence was used to

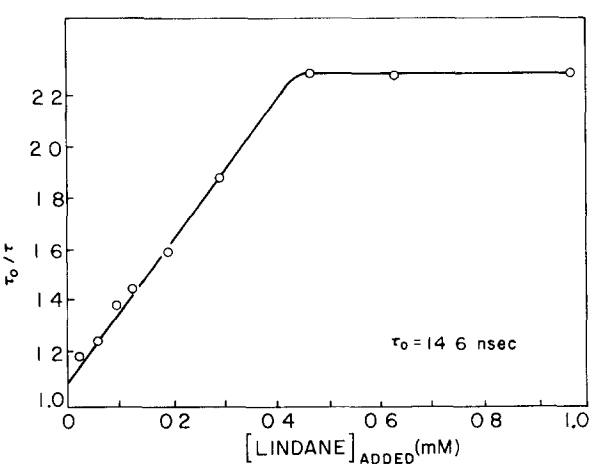

Fig. 5. Saturation of dimyristoyl vesicles with lindane as measured by quenching of carbazole undecanoyl phosphatidylcholine. T = 37°C. Lifetimes were measured by the phase shift method, at 10 MHz. The lipid to probe ratio was 200. Labeled dimyristoyl phosphatidylcholine, 1 mg/ml. All other details the same as Figs. 2 and 3.

separate the diffusion and partition contributions to the observed quenching (Fig. 4). This analysis, in conjunction with the data listed in Table I, makes it possible for us to calculate that lindane concentrates 9500-fold in dimyristoyl phosphatidylcholine bilayers over the water phase and also that it diffuses in the bilayer at approximately 6% of the rate at which it diffuses in ethanol or benzene.

We noted in our studies with dimyristoyl vesicles that the fluorescent lifetime of the carbazole undecanoyl phosphatidylcholine probe stopped decreasing when the lindane additions caused solution turbidity to become apparent. This turbidity is probably a result of lindane crystals forming when the bilayer became saturated (Fig. 5). The bilayer becomes saturated with lindane when there is 1 lindane molecule per 3.5 molecules of phospholipid.

Discussion

The permeability (T_m) of a polymeric sheet to a non-electrolyte is given by [13]:

$$T_m = \frac{PD_m}{\Delta x} \tag{11}$$

where P is the partition coefficient of the non-electrolyte between the polymeric and aqueous phases, D_m is the diffusion coefficient of the non-electrolyte in the sheet, and Δx is the thickness of this sheet. The permeability of an equivalent thickness (Δx) of water is given by

$$T_s = \frac{D_s}{\Delta x} \tag{12}$$

The diffusion coefficient of lindane in dimyristoyl phosphatidylcholine vesicles is 20-fold less than in water, whereas the lipid-water partition coefficient is 9500. Thus, according to Eqns. 11 and 12, lipid bilayers of dimyristoyl phosphatidylcholine pose no permeability barrier to lindane.

Several factors may affect the accuracy of calculated lindane diffusion coefficient in the lipid bilayer. We assumed the lateral diffusion of the labeled phospholipid is not significant when compared with the lindane diffusion. The lateral diffusion rate of dimyristoyl phosphatidylcholine is in the order of $8 \cdot 10^{-8}$ cm^2/s at 37°C [20]. Assuming that this is a reasonable estimate for the diffusion coefficient of the carbazole probe in the membrane, and setting the sum of D_{pm} and D_{qm} to be $5.7 \cdot 10^{-7}$ cm^2/s, the diffusion coefficient of lindane is thus calculated to be $4.9 \cdot 10^{-7}$ cm^2/s. Phospholipid probe diffusion may result in an overestimate of the lindane diffusion coefficient by 14%.

Collisions of lindane with the ethyl moiety of *N*-ethyl carbazole are unlikely to result in quenching since this region is not in electronic conjugation with the aromatic rings. Thus, an increase in the length of this side chain to undecanoyl is unlikely to significantly alter the effective collision radius of *N*-alkyl carbazole and lindane. This is the reason we chose *N*-ethyl carbazole, rather than carbazole itself, as the model compound for homgeneous solution studies. If the increased size of the alkyl side chain of carbazole has any effect it is most

likely to decrease the quenching of the phospholipid derivative, resulting in an underestimate of D_{qm}.

The calculation of the diffusion coefficient of lindane in the lipid bilayer assumes that the quenching efficiency in the bilayer is the same as in ethanol or cyclohexane. Since the quenching efficiency is the same in these two solvents (Table I), it appears likely that the efficiency will remain the same in the lipid bilayer. Additionally, the quenching of *N*-ethyl carbazole by mirex, DDE and gardona is not solvent-dependent (see Table I of ref. 5). Thus we feel that changing the medium from these solvents to the dimyristoyl phosphatidylcholine bilayer should not result in a significant change in quenching efficiency.

The interpretation of our quenching data is also subject to several assumptions concerning the diffusion of lindane in lipid bilayers. First, we have assumed that the lindane is diffusing in three dimensions, and that the quenching we observe is not simply a result of two-dimensional diffusion across the surface of the lipid bilayer. Second, the observation of a lipid concentration dependence on the apparent quenching rate (k_{app}) indicates that the majority of quenching encounters between carbazole and lindane result from lindane molecules which are already partitioned into the bilayer at the moment of carbazole excitation.

The diffusional transport of small non-polar molecules within lipid bilayers poses a difficult theoretical problem. Owen [14] has solved the diffusion equations for a quenching molecule within a bilayer. His solution assumes that the quencher originates in the membrane and does not cross the lipid-water interface at rates sufficient to quench the fluorescence. This assumption appears to be consistent with our partition studies. Unfortunately, this theory does not take into account the anisotropic nature of lipid bilayers. Other workers have examined the theory of two-dimensional diffusion across the surface of lipid bilayers [15—17]. These theories describe the movements of lipid and lipid-like species which are almost equivalent in size to the phospholipids themselves, and are often charged. Because lindane is neutral, and of small size (4 Å) relative to the phospholipid bilayer (70 Å), we do not feel these two-dimensional theories are applicable. It appears unlikely that the anisotropic nature of the quenching medium would alter the quenching efficiency. However, this anisotropy may cause the lateral and transverse diffusion rates to be unequal. Until our understanding of the passive transport properties of lipid bilayers is more complete, we feel the simplest interpretation of the quenching data will provide the most insight. Thus, we have assumed that lindane undergoes diffusion equally in three dimensions, that is, both parallel and normal to the plane of the bilayer.

With the above discussion in mind, we find that lipid bilayers of dimyristoyl phosphatidylcholine do not pose a permeability barrier to lindane. Rapid equilibration of the membrane with added lindane indicates that the lipid-water interface is not a significant barrier to lindane uptake. Our data demonstrate the ability to study chlorinated hydrocarbon membrane interactions using fluorescence quenching of carbazole. With presently available instrumentation, partition coefficients ranging from 40 to 20000 have already been measured and values as high as 50000 should be easily measurable. These measurements are rapid, and we do not foresee any insurmountable difficulties in extending this technique to natural membranes, or even whole cells. Thus, we feel that the

fluorescence-quenching technique provides a valuable new method for investigating the interactions of chlorinated hydrocarbons with cell membranes.

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