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Interactions of hexachlorocyclohexanes with lipid bilayers

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Quenching of the fluorescence of a hydrophobic analogue of tryptophan incorporated into lipid bilayers has been used to measure partition coefficients for lindane and the α- and δ-isomers of hexachlorocyclohexane. Partition coefficients between water and lipid are comparable to those between water and octanol and exhibit a negative temperature coefficient. Binding to the lipid phase is limited by saturation of the aqueous phase rather than of the lipid phase. The binding of lindane has no detectable effect on membrane fluidity as measured by fluorescence polarisation of diphenylhexatriene, or on the permeability properties of the membrane, as measured by the leak of carboxyfluorescein.

Organochlorine insecticides such as DDT and lindane (the γ-isomer of hexachlorocyclohexane) are small, hydrophobic molecules whose site of action is thought to be in the membrane. Despite their great commercial importance, their exact mode of action is unknown. Poisoning by organochlorines is characterised initially by locomotor instability (knock-down) and hyperexcitability; subsequent paralysis is followed by death of the insect [1]. These symptoms indicate an effect on the neuromuscular system, and it has been suggested that the primary site of action is the synapse [2]. Organochlorine insecticides have been shown to affect a number of transport ATPases including Ca^{2+}-ATPases [3]. Since release of neurotransmitters are often dependent on Ca^{2+}, block of Ca^{2+} transport could lead to an increased rate of neurotransmitter release. Lindane has been shown to have just this effect at the frog neuromuscular junction [4]. Lindane has also been shown to cause extensive myofilament damage in skeletal muscle, an effect which follows from increased levels of internal calcium [5].

In many ways the organochlorine insecticides are reminiscent of the general anaesthetics, whose mode of action is also unclear. Because of their marked hydrophobicity, both groups of compounds are believed to act on the membrane, but whether on the lipid or protein components is not yet clear. The major problem with a lipid site of action is in explaining how binding to the lipid component could produce significant alterations in the properties of the membrane. For the anaesthetics, effects on lipid fluidity appear to be too small to be relevant physiologically [6] and the activities of at least one transport protein (the $Ca^{2+} + Mg^{2+}$)-ATPase from sarcoplasmic reticulum) has been shown to be relatively insensitive to changes

Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DPH, diphenylhexatriene; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulphonic acid; NPTH, *N*-palmitoyl-L-tryptophan *n*-hexyl ester.

in fluidity [7]. A more convincing case can be made for the importance of the thickness of the lipid component of the membrane, and it has been argued that the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase is sensitive to membrane thickness [8]. It has been suggested that a variety of hydrophobic molecules can affect the thickness of lipid bilayers [9] although direct measurements using X-ray techniques have often failed to detect such effects [10].

Direct effects on membrane proteins must also be considered. Thus organochlorines could bind at receptor sites for neurotransmitters in the same way that halothane has been shown to bind at the adenine binding sites in adenylate kinase [11] or xenon to a site on haemoglobin [12]. It is also possible that they bind at the lipid-protein interface of membrane proteins, and the activities of many membrane proteins are known to be very sensitive to the chemical structure of the surrounding molecules [13].

Whatever the site of action of the organochlorines, however, any quantitative study of insecticide activity must take account of lipid binding since the hydrophobicity of the organochlorine insecticides means that much of the insecticide will be bound to the lipid phase of the membrane. In a recent paper, Omann and Lakowicz [14] have shown how to estimate partition coefficients for the organochlorines into lipid bilayers from measurements of the extent of quenching of the fluorescence of derivatives of carbazole. Omann and Lakowicz [14] used fluorescence life-time methods but here we show that steady-state measurements of fluorescence quenching of a hydrophobic analogue of tryptophan can provide comparable information. In the following paper [15] we show how quenching of the tryptophan fluorescence of the $(Ca^{2+} + Mg^{2+})$-ATPase can be used to study binding to protein-containing systems.

Materials and Methods

Lipids were obtained from Lipid Products, and carboxyfluorescein from Eastman. The isomers of hexachlorocyclohexane were a gift from ICI and ran as single spots on thin-layer chromatography. The hydrophobic tryptophan analogue, *N*-palmitoyl-L-tryptophan *n*-hexyl ester (NPTH) was prepared by esterification of tryptophan with thionylchloride in hexanol followed by coupling to palmitic acid with *N*,*N*-dicyclohexylcarbodiimide.

For fluorescence measurements, lipids and probe were dried from methanol solution onto the sides of flasks, to which buffer (40 mM Hepes, 100 mM NaCl, 1 mM EGTA, pH 7.2) was added and the mixture shaken on a vortex mixer. The molar ratio of NPTH to lipid was usually 1 : 30. Organochlorines were added to liposomes from a concentrated stock solution in methanol, the final volume of methanol never exceeding 2% of the total volume. Fluorescence quenching experiments were performed using a Perkin-Elmer MPF44A fluorimeter, exciting at 276 nm and recording fluorescence intensity at 336 nm. Measurements of fluorescence polarisation were made using an Aminco-Bowman fluorimeter equipped with quartz Polacoat filters, samples being contained in a 200 μl quartz microcell. The molar ratio of diphenylhexatriene (DPH) to lipid was 1 : 100. Measurements of light scattering were made recording absorbance at 590 nm or by measuring the intensity of scattered light at 90° to the incident light for liposomes prepared in the absence of probe.

Integrity of liposomes was tested in terms of their ability to retain carboxyfluorescein. Carboxyfluorescein was purified largely as described by Ralston et al. [16] and ran as a single spot on thin-layer chromatography in chloroform/methanol/water (65 : 25 : 4, by volume). Dioleoylphosphatidylcholine (12.5 mg) was dried onto the sides of flasks, buffer (2 ml) containing 100 mM carboxyfluorescein was added and the mixture shaken. Free and entrapped carboxyfluorescein were separated on Sephadex G-75. Leakage of dye from liposomes was monitored by measuring fluorescence intensity at 520 nm, exciting at 492 nm.

Data analysis. Fluorescence quenching may be described by the Stern-Volmer equation:

$$F_0/F = 1 + K_{sv}Q \qquad (1)$$

where F_0 and F are fluorescence intensities in the absence and presence of quencher respectively, Q is the quencher concentration and K_{sv} is the quenching constant. For membrane systems, the relevant quencher concentration is that in the

membrane. In the analysis of Thulborn and Sawyer [17], the membrane concentration Q_m is related to the concentration Q_a in the aqueous phase by a partition coefficient:

$$K_p = Q_m/Q_a \quad (2)$$

where Q_m is expressed as moles per litre of membrane. Since the total number of moles of quencher added must equal the sum of those in the aqueous and membrane phases:

$$V_t Q_t = V_m Q_m + V_a Q_a \quad (3)$$

where V_t is the total volume of the system and V_a and V_m are the volumes of the aqueous and membrane phases, respectively. Quenching can then be expressed as:

$$F_0/F = 1.0 + [K_{sv} K_p V_t Q_t]/[V_a + K_p V_m] \quad (4)$$

In the calculation of V_m, the vesicle density was taken as 1 g/ml [18].

An alternative to the partition model is to describe binding by a Langmuir adsorption isotherm: this approach has been used to describe the binding of a variety of charged molecules to lipid bilayers [28,29]. The maximum extent of binding to the bilayer is then limited, the limit being expressed either in terms of the maximum number of molecules adsorbed per unit area or, equivalently, the maximum number of molecules, N, adsorbed per phospholipid molecule. Binding can then be expressed in terms of a 'site' concentration given by $N \cdot E_t$ where E_t is the total lipid concentration. The concentration of bound quencher, Q_b, is given by:

$$Q_b = Q_a E_f / K_d \quad (5)$$

where E_f is the free site concentration, and K_d is the dissociation constant for binding. In terms of total concentration:

$$Q_b = \left(A - [A^2 - 4NE_t Q_t]^{1/2}\right)/2.0 \quad (6)$$

where

$$A = K_d + NE_t + Q_t \quad (7)$$

and N is the number of binding sites per lipid and E_t is the total lipid concentration. Fluorescence quenching can then be expressed as:

$$F_0/F = 1 + K'_{sv} Q_b/(NE_t) \quad (8)$$

The Stern-Volmer constant in Eqn. 8 is different to that in Eqn. 1 because of the different concentration units. In the region where only a small fraction of the lipid sites are occupied, it can be shown that the two constants are related by:

$$K'_{sv} = K_{sv} N V_t E_t / V_m \quad (9)$$

In some systems it has been found that fluorescence quenching data plotted according to the Stern-Volmer relationship shows upward curvature. It has been suggested that this arises because only a fraction of the fluorophores are quenched by the collisional mechanism (which is the basis of the Stern-Volmer equation), the rest being quenched by a static mechanism. A modified form of the Stern-Volmer equation has then been introduced:

$$F_0/F = [1 + K_{sv} Q] \exp(VQ) \quad (10)$$

where V is a static quenching constant (see Ref. 19).

Results

Fluorescence quenching

Lindane is an effective quencher of the fluorescence of the hydrophobic tryptophan analogue NPTH in free solution in methanol, the quenching data fitting the Stern-Volmer equation with a quenching constant, K_{sv} of 16.8 M^{-1} at 20°C. On incorporation of NPTH into lipid bilayers there is a marked increase in fluorescence intensity and a shift of the spectrum to lower wavelengths compared to spectra in methanol, consistent with incorporation of the probe into a hydrophobic environment within the lipid bilayer [20]. As shown in Fig. 1, addition of lindane to the suspension of liposomes causes marked quenching of NPTH fluorescence. The concentrations of lindane necessary to cause quenching were much lower than those required to cause quenching of solutions of NPTH in methanol, and the required concentrations decreased with decreasing lipid concentration. These

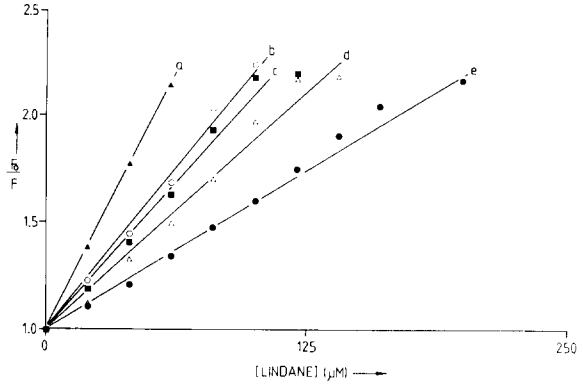

Fig. 1. Quenching of the fluorescence of NPTH in liposomes of dioleoylphosphatidylcholine by lindane at 20°C, at lipid concentrations (μM) of: a, 60; b, 150; c, 210; d, 300 and e, 600.

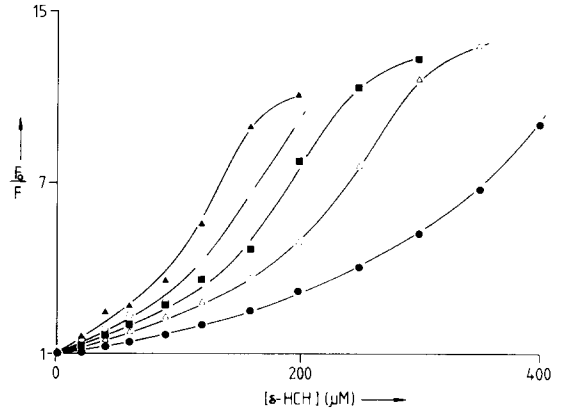

Fig. 3. Quenching of the fluorescence of NPTH in liposomes of dioleoylphosphatidylcholine by δ-hexachlorocyclohexane (δ-HCH) at 20°C. Lipid concentrations as in Fig. 2.

observations are consistent with quenching in the lipid phase. No change in shape was observed for the fluorescence emission spectrum on addition of lindane and the extent of quenching was independent of molar ratio of NPTH to lipid from 1 : 200 to 1 : 10. As shown in Figs. 2 and 3, the α and δ isomers of hexachlorocyclohexane also quench the fluorescence of NPTH in liposomes. Due to its low solubilities in methanol and water, consistent results could not be obtained with the β-isomer.

All the plots exhibit saturation, a concentration of quencher being reached at which further addition of quencher resulted in no further quenching. For lindane, at concentrations below saturation, Stern-Volmer plots were linear, and so could be fitted to a partition model (Eqn. 4). Omann and Lakowicz [14] have presented a graphical method for extracting K_p from the data, and the values that we obtain in this way are given in Table I. An estimate of the accuracy of the extracted parameters was made by comparing the best fit to the data (Fig. 4A) with quenching profiles calculated assuming errors of $\pm 15\%$ in either K_p (Fig. 4B) or K_{sv} (Fig. 4C).

Fluorescence quenching caused by any given concentration of lindane was found to go through a maximum as a function of temperature (Fig. 5). Analysis of quenching data obtained at different temperatures (Table I, Fig. 6) showed that this was due to opposite effects of temperature on K_{sv} and K_p, the former increasing and the latter decreasing with increasing temperature. Parameters obtained for the dipalmitoylphosphatidylcholine system are also given in Table I.

The α-isomer also gave linear Stern-Volmer plots at concentrations below saturation (Fig. 2) and were analysed as for lindane: results are given in Table I. Stern-Volmer plots for the δ-isomer, however, showed marked upward curvature (Fig. 3).

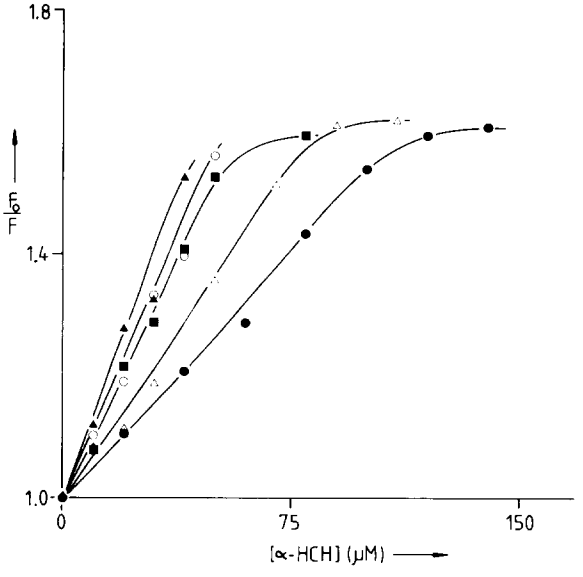

Fig. 2. Quenching of the fluorescence of NPTH in liposomes of dioleoylphosphatidylcholine by α-hexachlorocyclohexane (α-HCH) at 20°C at lipid concentrations (μM) of: ▲, 60; ○, 150; ■, 210; △, 300 and ●, 600.

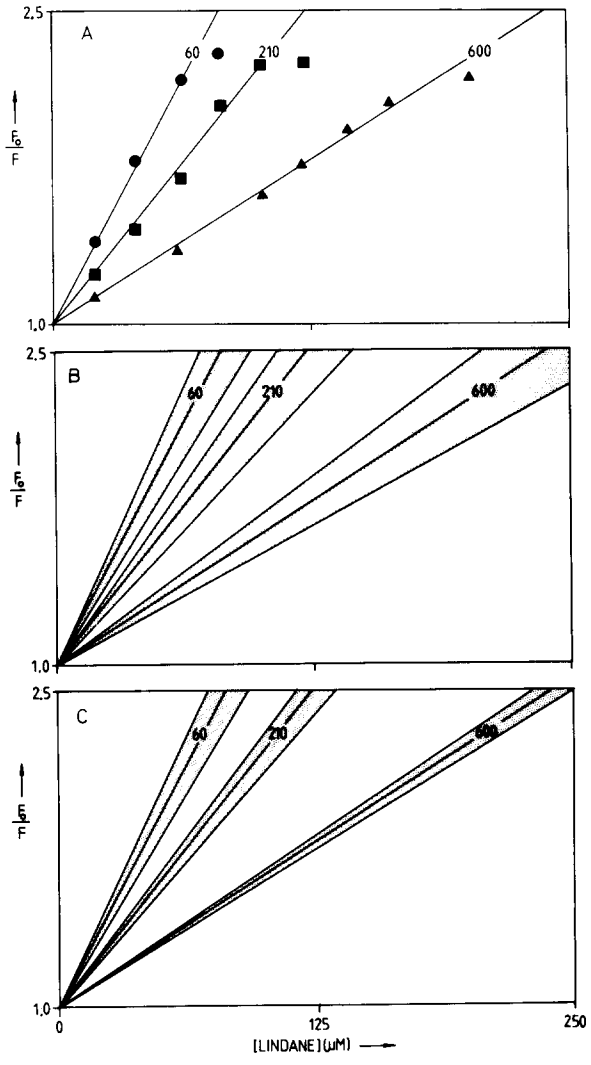

Fig. 4. Simulation of the fluorescence quenching of NPTH in liposomes by lindane, under the conditions of Fig. 1. (A) Best simulation of the experimental data at lipid concentrations of 60, 210 and 600 μM using the parameters given in Table I. (B) Calculated quenching profiles with parameters as in (A), but with a range of K_p values of $\pm 15\%$ (K_p between 5100 and 6900). (C) Calculated quenching profiles with parameters as in (A), but with a range of K_{sv} values of $\pm 15\%$ (K_{sv} between 3.4 and 4.6 M^{-1}).

As shown in Fig. 7, this data could be fitted to the modified Stern-Volmer plot (Eqn. 10) and again the parameters derived are given in Table I.

Effects of bulk lipid properties

No effect of lindane was detected on the fluid-

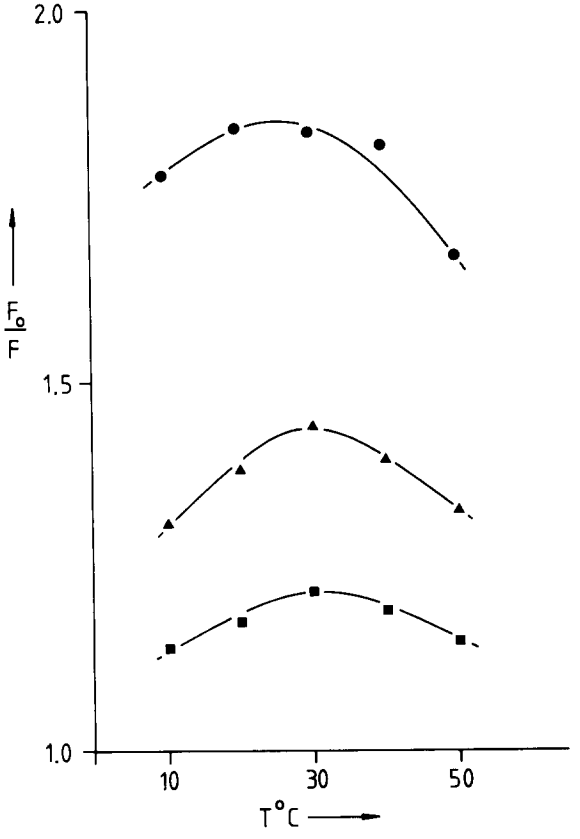

Fig. 5. Fluorescence quenching for NPTH in liposomes of dioleoylphosphatidylcholine (210 μM) as a function of temperature for lindane concentrations (μM) of: ■, 20; ▲, 40; ●, 80.

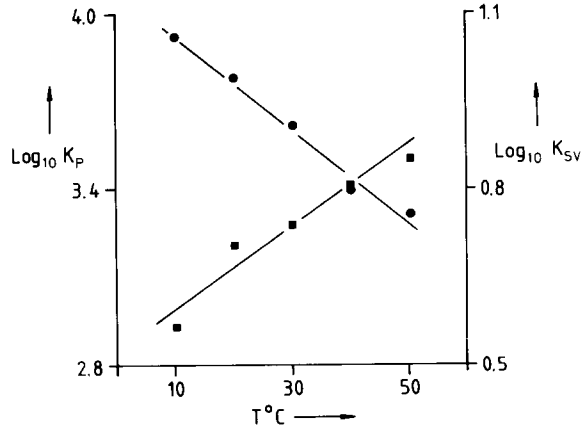

Fig. 6. Effect of temperature on the partition coefficient K_p (●) and Stern-Volmer quenching constant, K_{sv} (■), for lindane in liposomes of dioleoylphosphatidylcholine.

TABLE I

PARTITION COEFFICIENTS AND STERN-VOLMER QUENCHING CONSTANTS FOR HEXACHLOROCYCLOHEXANES

DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

Isomer	Lipid	This study			Omann and Lakowicz [14]	
		Temp. (°C)	K_p	K_{sv} (M^{-1})	Temp. (°C)	K_p
γ	DOPC	10	8200	2.8		
		20	6000	4.0	25	13400
		30	4000	4.3		
		40	2500	5.1		
		50	2000	5.5		
	DPPC	10	3000	4.0		
		30	3200	8.0	25	3800
		50	3600	6.0	50	4200
α	DOPC	10	8000	2.5		
		20	5700	3.2	26	12600
		30	4500	4.0		
		40	2900	4.9		
δ	DOPC	20	5300	2.2 [a]	25	12400

[a] With a static quenching constant of $V = 2.28$.

ity of dioleoylphosphatidylcholine as monitored by the fluorescence polarisation of diphenylhexatriene at up to a 1 : 1 molar ratio of hexachlorocyclohexane (α, γ or δ isomer). Further, addition of lindane to liposomes of dioleoylphosphatidylcholine at a 1 : 4 molar ratio had no significant effect on the rate of release of trapped carboxyfluorescein showing that at these concentrations it did not cause any extensive disruption of the liposome structure.

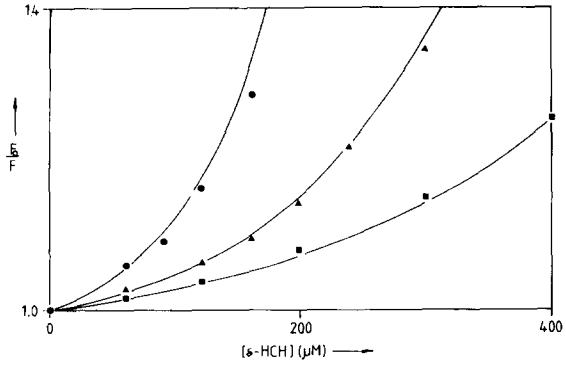

Fig. 7. Simulation of fluorescence quenching data (Fig. 3) for δ-hexachlorocyclohexane (δ-HCH) at lipid concentrations of (●) 60, (▲) 300 and (■) 600 μM, in terms of a mixture of both static and collisional quenching (Eqn. 10).

Discussion

Our experiments show that lindane and the other isomers of hexachlorocyclohexane bind strongly to lipid bilayers, but have no detectable effects on the bulk properties of the membranes. None of the isomers affect the fluorescence polarisation of diphenylhexatriene, suggesting that they have no effect on membrane fluidity. DDT has also been reported to have no effect on lipid fluidity [21]. The lack of effect on the rate of leak of entrapped dye shows that there is no gross disruption of the lipid bilayer, and is consistent with the results reported previously by Omann and Lakowicz [14].

Lindane and the α- and δ-isomers of hexachlorocyclohexane quench the fluorescence of the hydrophobic tryptophan analogue NPTH incorporated into liposomes of dioleoylphosphatidylcholine. The quenching shows saturation, a concentration of quencher being reached beyond which further addition of quencher causes no further quenching. Stern-Volmer plots for lindane and α-hexachlorocyclohexane at concentrations below this saturation point are reasonably linear, and can be analysed to obtain partition coefficients for the

quencher. Stern-Volmer plots for δ-hexachlorocyclohexane are, however, markedly curved and have thus been interpreted in terms of mixed static and collisional quenching. In Table I, the partition coefficients we derive are compared to those reported previously by Omann and Lakowicz [14]. Our values for partition coefficients between water and dioleoylphosphatidylcholine are lower by a factor of about two than those reported by Omann and Lakowicz [14]. This difference could possibly arise because we used multilamellar liposomes and Omann and Lakowicz used sonicated unilamellar vesicles. Our values for the partition coefficients between water and dioleoylphosphatidylcholine are very similar to those between water and octanol for lindane and α-hexachlorocylclohexane whereas our value for the δ-isomer is about half that for water/octanol [22]. This suggests that our value for the δ-isomer could be in error: this isomer gave curved Stern-Volmer plots and were analysed in terms of an equation (Eqn. 10) suggested for mixed static and collisional quenching. However, recent studies have suggested that positive deviations from linearity in Stern-Volmer plots can arise at sufficiently high quencher concentrations, even in the absence of static quenching [26,27]. Unfortunately, the equations derived by Kizer [26] and Peak et al. [27] contain too many parameters that are unknown in our case to allow meaningful fits to our data.

For hydrophobic molecules that mix ideally with lipids, Hill [25] has shown that the maximum aqueous solubility, S, and partition coefficient, P, are related by:

$$P \cdot S = 2$$

when all concentrations are expressed as moles of solute per mole of solvent. For lindane, the product $P \cdot S$ is about 0.3, suggesting that mixing is non-ideal.

For dioleoylphosphatidylcholine, partition coefficients decreased with increasing temperature (Table I, Fig. 6). The negative temperature coefficient for partitioning is consistent with a predominantly hydrophobic interaction [23] and mirrors the negative temperature coefficient of insecticidal potency [1]. The Stern-Volmer quenching constant increases with increasing temperature, as expected for a collisional quenching mechanism. For dipalmitoylphosphatidylcholine, partition coefficients for lindane at 10 and 50° are very similar (Table I). This can be attributed to the effects of the phase transition for dipalmitoylphosphatidylcholine, with partition coefficients being less for lipid in the gel phase than in the liquid-crystalline phase.

The remaining feature of the fluorescence quenching plots is the observation of a maximum level of quenching beyond which further addition of quencher has no further effect (Figs. 1–3). Omann and Lakowicz [14] observed a similar phenomenon in their studies of the effects of hexachlorocyclohexanes on the fluorescence lifetimes of membrane-bound derivatives of carbazoles. They suggested that it could reflect saturation of the lipid phase occurring at a molar ratio of hexachlorocyclohexane to lipid of about 1 to 5 [14], that is, that 5 lipids make up 1 'binding site'. At the saturation point, we observe a large increase in light scatter for the samples, as shown in Fig. 8. This certainly suggests that, at the saturation point, the hexachlorocyclohexane that has not partitioned into the lipid phase is forming scattering suspensions in the aqueous phase. However, saturation does not occur at any unique molar ratio of hexachlorocyclohexane to lipid, the saturation point for lindane being 1.57 : 1, 1 : 1 and

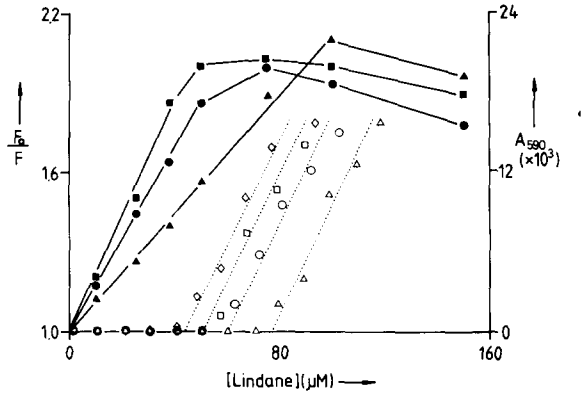

Fig. 8. A comparison of the effects of lindane on the fluorescence intensity of NPTH in liposomes of dioleoylphosphatidylcholine (filled symbols) and on sample turbidity, as measured by absorption at 590 nm (open symbols). Temp. 20°C. Lipid concentration (μM): ■□, 30; ●○, 60; ▲△, 150; ◇, buffer alone.

0.6 : 1 for lipid concentrations of 30, 60 and 150 μM, respectively (Fig. 8).

If saturation of the lipid phase were occurring in these experiments, then a description of binding in terms of a partition coefficient would be inappropriate. The appropriate binding isotherm would be one including saturation of binding sites such as the Langmuir adsorption isotherm. An equation expressing fluorescence quenching for such a binding model can be derived readily (Eqn. 8). However, such an equation does not provide a fit to the experimental data. The problem is illustrated in Fig. 9 which compares the predictions of the partition (Eqn. 4) and saturation (Eqn. 8) models. Data at low concentrations of lindane can be fitted with, for example, $K_d = 61$ μM and 0.3 binding sites per lipid. However, at higher concentrations, the calculated quenching profiles differ markedly from experiment. Alternatively, a much small K_d value of 1 μM gives much sharper changes in slope on approaching saturation (as is seen experimentally) but now the dependence on lipid concentration is too small. We conclude that saturation of the lipid phase cannot explain our experimental data.

An alternative explanation for the saturation phenomenon is that it is not the lipid phase that is saturating but the aqueous phase. Assuming that in a saturated aqueous solution of lindane the aqueous solution is in equilibrium with crystalline lindane, then the chemical potential (μ_w^{sat}) of the lindane in the aqueous phase must be the same as in the crystal ($\mu^{crystal}$). That is,

$$\mu_w^{sat} = \mu_w^o + RT \ln c_w^{sat} = \mu^{crystal} \quad (11)$$

where μ_w^o is the chemical potential in the standard state for the aqueous phase and c_w^{sat} is the saturating aqueous concentration (see, for example, Ref. 30). Similarly, in the presence of lipid, when the aqueous phase is saturated, the chemical potential of lindane in the lipid phase (μ_l^{sat}) must equal those in the aqueous phase and the crystal,

$$\mu_l^{sat} = \mu_l^o + RT \ln c_l^{sat} = \mu^{crystal} \quad (12)$$

where μ_l^o and c_l^{sat} are the standard state potential and concentration for the lipid phase, respectively. Thus we have,

$$RT \ln(c_l^{sat}/c_w^{sat}) = \mu_w^o - \mu_l^o \quad (13)$$

and there is a maximum concentration that can be reached in the lipid phase, determined by saturation of the aqueous phase. We stress that in this case binding to the lipid phase is not limited by saturation of available 'binding sites' in the lipid phase (that is, by occupation of all the lipid binding sites), but by the limited solubility of lindane in the aqueous phase. Under these conditions, not all of the binding isotherm for the lipid phase will be accessable experimentally.

From the light scattering measurements in the absence of lipid shown in Fig. 8, it appears that saturation of the aqueous phase occurs at approx. 45 μM for lindane, which can be compared to the published saturation concentration of 27 μM (7.9 ppm, Ref. 22). Saturation of the aqueous phase at a concentration, Q_{sat} will limit the concentration of lindane in the membrane to (see Eqn. 2):

$$Q_m^{max} = Q_{sat} K_p \quad (14)$$

In the presence of lipid, the concentration Q' of lindane at which saturation of the aqueous phase will occur is then:

$$Q' = Q_{sat} + Q_m^{max} V_m \quad (15)$$

Using the parameters in Table I, saturating concentrations Q' of lindane of 51, 58 and 77 μM are

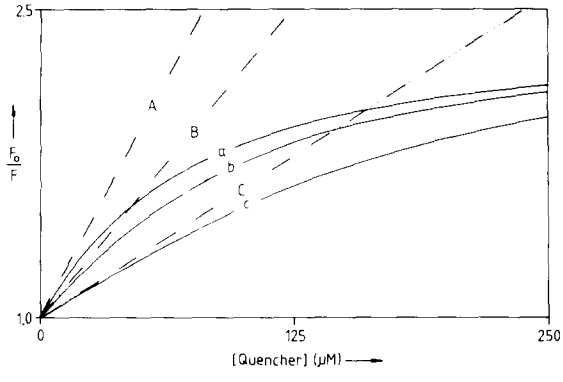

Fig. 9. A comparison of the quenching profiles calculated on the basis of the partition (Eqn. 4) and saturation models (Eqn. 8). Broken lines, partition model with $K_p = 6000$, $K_{sv} = 4.0$ M^{-1}, $V_t = 2.5$ ml. Solid lines, saturation model with $K_d = 61$ μM, $N = 0.29$, $K'_{sv} = 1.45$ M^{-1}. Lipid concentrations (μM): a, 60; b, 210; c, 600.

calculated for concentrations of dioleoylphosphatidylcholine of 30, 60 and 150 μM, respectively, at 20°C, which compare well with the experimental data (Fig. 8).

As reported by Omann and Lakowicz [14] there is no correlation between insecticidal potency and lipid binding, since the α- and δ-isomers of hexachlorocyclohexane are insecticidally inactive [24]. It therefore seems likely that direct interaction with membrane proteins is involved in insecticidal activity. In the following paper we show how quenching of the fluorescence of tryptophan residues in a membrane protein can be used to study interactions of hexachlorocyclohexanes with membrane proteins.

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