Eur Biophys J (1989) 17:25–36

European Biophysics Journal

Fluorescence quenching with lindane in small unilamellar L,α-dimyristoylphosphatidylcholine vesicles

D. Daems, N. Boens*, and F. C. De Schryver

Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200F, B-3030 Heverlee, Belgium

Received May 31, 1988/Accepted in revised form November 11, 1988

Abstract. The lateral mobility and lipid-water partition of the pesticide lindane was studied by fluorescence quenching of *N*-isopropylcarbazole (NIPC) and L,α-palmitoyl-β-(*N*-carbazolyl)undecanoylphosphatidylcholine (PCUPC) in liposomes of dimyristoylphosphatidylcholine at 50 °C. In isotropic solvents the quenching reaction was highly inefficient. A scheme for dynamic quenching, in which the monomolecular quenching rate constant is small, was valid. In lipid bilayers the same scheme was applied to describe the quenching results but the rate constant of the back-reaction of the excited complex to quencher and excited probe was of comparable magnitude to the monomolecular quenching rate constant. This phenomenon results in biexponential decays of the fluorescent probe in the presence of quencher. All the rate constants of the scheme could be determined. Stern-Volmer plots at different membrane concentrations were obtained from fluorescence intensity and decay time measurements. From these plots the true bimolecular quenching rate constant, k_q, and the rate constant for lateral diffusion, k_d, were determined: $k_{q\,[\mathrm{NIPC}]} = 3.2 \pm 0.5 \times 10^8\ M^{-1}\,s^{-1}$, $k_{q\,[\mathrm{PCUPC}]} = 1.9 \pm 0.4 \times 10^8\ M^{-1}\,s^{-1}$, $k_{d\,[\mathrm{NIPC}]} = 6.6 \pm 0.8 \times 10^8\ M^{-1}\,s^{-1}$. The smaller value of k_q compared to k_d for the quenching reaction of NIPC with lindane indicates that this quenching reaction is not diffusion controlled. The lateral diffusion coefficient D of lindane was found to be $1.7 \pm 0.2 \times 10^{-6}$ cm²/s in dimyristoylphosphatidylcholine vesicles at 50 °C. The partition coefficient of lindane in these lipid bilayers is very high (> 2000).

Key words: Diffusion coefficient, dimyristoylphosphatidylcholine, fluorescence quenching, lindane, partition coefficient

Abbreviations: DMPC: dimyristoylphosphatidylcholine; lindane: 1,2,3,4,5,6-hexachlorocyclohexane (γ-isomer); NIPC: *N*-isopropylcarbazole; PCUPC: L,α-palmitoyl-β-(*N*-carbazolyl) undecanoylphosphatidylcholine; SUV: small unilamellar vesicles

Introduction

The study of the dynamic properties of natural and artificial membranes continues to be an active field of investigation. Interactions of small, biologically important molecules such as anaesthetics, pesticides, steroids, heavy metal ions, etc. with biomembranes, and their diffusion in these membranes can be studied quantitatively by the fluorescence quenching technique (Lakowicz 1983). Indeed, many of these molecules serve as quenchers of suitable fluorescent probes solubilized in the membrane.

Several pesticides, e.g. lindane, Gardona, have been used to quench the fluorescence of a series of carbazole derivatives (Lakowicz et al. 1977; Lakowicz and Hogen 1980; Omann and Lakowicz 1982; Omann and Glaser 1984). For the diffusion coefficient D of lindane in dimyristoylphosphatidylcholine (DMPC) at 37 °C a value of 5.7×10^{-7} cm²/s was obtained (Lakowicz et al. 1977). Values for the diffusion coefficients of lindane in several lipid bilayers ranging from 0.2 to 2×10^{-6} cm²/s were reported by Lakowicz and Hogen (1980). Omann and Glaser (1984) reported a D value for lindane of 1.3×10^{-6} cm²/s in plasma membrane phospholipids of mouse LM cells at 37 °C. Partition coefficients of lindane reported by these authors were always very high. The partition coefficient, however, decreases with increasing length of the aliphatic chain of the lipids (Antunes-Madeira and Madeira 1985).

The present paper describes a fluorescence quenching study of *N*-isopropylcarbazole (NIPC) and L,α-palmitoyl-β-(*N*-carbazolyl)undecanoylphosphatidylcholine (PCUPC) with the hydrophobic quencher lindane. Time-resolved and steady-state fluorescence quenching experiments were carried out to elucidate the quenching mechanism. The diffusion in DMPC membranes and the lipid-water partition of the quencher are studied. These results are in good agreement with earlier experiments with the probe methyl 11-(*N*-carbazolyl)undecanoate (Daems et al. 1988).

Experimental

Chemicals

L,α-dimyristoylphosphatidylcholine (DMPC, Sigma) was used as received. Thin layer chromatography on silica gel (solvent $CHCl_3$:CH_3OH:H_2O 65:25:4; I_2 and phosphate staining) showed a single spot. Under the excitation conditions used in this work no fluorescent impurities could be detected in the unlabeled vesicle solutions. *N*-isopropylcarbazole (NIPC) was synthesized and purified as described elsewhere (Vanendriessche et al. 1984). Its purity was checked by thin layer chromatography on silica gel (solvent *n*-hexane:tetrahydrofuran 95:5). Its fluorescence lifetime in degassed isooctane at 22 °C was found to be 15.0 ± 0.1 ns. L,α-palmitoyl-β-(*N*-carbazolyl)undecanoylphosphatidylcholine (PCUPC, Fig. 1) was synthesized from L,α-palmitoyl-β-lysophosphatidylcholine (Sigma) and 11-(*N*-carbazolyl)undecanoic acid anhydride according to the method of Gupta et al. (1977). 11-(*N*-carbazolyl)undecanoic acid anhydride was synthesized following the method of Selinger and Lapidot (1966). Crude PCUPC was purified by thin layer chromatography on silica gel (solvent $CHCl_3$:CH_3OH:NH_3 62:25:4). The phospholipid was visualized using the Dittmer spray (Dittmer and Lester 1964). The product was further purified by high performance liquid chromatography on a C_{18} reverse phase column type "Ultrapac Column TSK ODS-120 T", 5 μm 4.6–250 mm with CH_3OH:CH_3CN:tetrahydrofuran:H_2O (25:25:32:18) as eluent (McCracken and Holt 1985). The fluorescence decay of PCUPC was found to be monoexponential with a lifetime of 13.7 ± 0.3 ns in degassed methanol at 20 °C. ^1H NMR in $CDCl_3$: 0.9 δ ($C\underline{H}_3-C_{13}H_{26}$); 1–1.9 δ ($-C_{13}H_{26}-$ and $-C_9H_{18}-$), 2.3 δ ($R_1-C\underline{H}_2-C=O$ and $R_2-C\underline{H}_2-C=O$), 3.4 δ [$-N^+(C\underline{H}_3)_3$], 3.9, 4.4 δ ($C\underline{H}_2-C\underline{H}-C\underline{H}_2$), 4.2 δ ($O-(C\underline{H}_2)_2-N$), 5.2 δ ($CH_2-C\underline{H}-CH_2$), 7.2–8.0 δ (carbazole part). Lindane (1,2,3,4,5,6-hexachlorocyclohexane, γ isomer; Aldrich, 97%) was purified by repeated recrystallization from methanol. KCl (Aldrich, Gold Label) was used as received. Chloroform (Fluka spectroscopic grade), methanol, 1-butanol, isooctane (Merck, fluorometric grade) and distilled-deionized water were used as solvents.

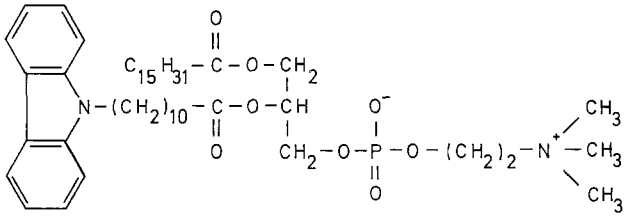


Fig. 1. Chemical structure of PCUPC

Sample preparation

Small unilamellar vesicles (SUV) (Huang 1969; Barenholz et al. 1977) of DMPC were prepared in 50 m*M* KCl solution as reported earlier (Van den Zegel et al. 1984). The phospholipid concentration determined colorimetrically (Stewart 1980) was 6 mg per ml aqueous vesicle solution. This stock solution was diluted to obtain the desired lipid concentration. The necessary amounts of fluorescent probe NIPC and quencher lindane were added to this vesicle solution by injection of microliter quantities from a concentrated solution of NIPC or lindane in methanol. Mixing was complete within ca. 1 min. The total concentration of methanol in the vesicle solution did not exceed 0.5 mole %. This amount did not alter the fluorescence decay times or spectra of NIPC, indicating that the added methanol did not cause the quencher or fluorophore to move in or out of the membrane or change the quenching rate. Therefore, the quencher-membrane interactions were not altered upon addition of small aliquots of methanol. The probe:lipid molar ratio varied between 1:125 and 1:1,000. Alternatively, labeled vesicles were prepared by cosonication of DMPC with NIPC. Both methods gave identical fluorescence quenching results, indicating that this fluorophore was homogeneously distributed in the vesicles when the method of probe injection from a concentrated stock solution was used. For PCUPC the injection method resulted in inhomogeneous vesicle preparations as indicated by deviations from monoexponentiality of the fluorescence decay of PCUPC in DMPC vesicles in the absence of quencher. After sonication of these prepared vesicles for several hours in a bath type sonicator, the deviations from monoexponentiality became smaller but single exponential decays were never obtained. The incorporation of the fluorescently labeled lipid PCUPC into DMPC vesicles was therefore done by the cosonication method. Homogeneously labeled vesicles were obtained by this preparation procedure, as was evident from the single exponential fluorescence decays of PCUPC in the absence of quencher. During preparation, all vesicle samples were kept above the phase transition temperature and they were measured immediately after their preparation. The vesicle samples were not deaerated, as this did not have a measurable effect on the fluorescence measurements. The fluorescence quenching experiments performed in organic solvents were done with solutions that were deaerated by repeated freeze-pump-thaw cycles. A quantitative analysis of fluorescence quenching data in membranes is only possible when the lipid volume fraction is known. Unfortunately, this quantity introduces uncertainties since its value can only be estimated. The volume of the lipid phase of small unilamellar vesicles taken up by 1 g of DMPC was calculated as follows: using a

value of 2,800 (Huang 1969) for the average number of lipid molecules per small unilamellar vesicle, a radius of 12.5 ± 2.5 nm and a thickness of 2 nm in the middle of the bilayer for the region of the membrane where the probe is situated, a specific volume of 0.882 cm^3/g was obtained. This value is somewhat smaller than that estimated by Sikaris et al. (1981) (0.984) and Lakowicz et al. (1977) (1.000). A vesicle suspension of 1 mg lipid/cm^3 solution corresponds to a lipid volume fraction $\alpha_L = 0.882 \times 10^{-3}$ and to a 5.268×10^{-7} molar solution of vesicles.

Fluorescence measurements

All fluorescence measurements of PCUPC labeled vesicles were carried out at 50 ± 0.2 °C. NIPC labeled vesicles were studied as a function of temperature. 4 ml of vesicle solution contained in 1 cm × 1 cm quartz cuvettes were used in all experiments. Fluorescence spectra and quantum yields were obtained at the excitation wavelength of 320 nm with a Spex Fluorolog 212/Datamate. Fluorescence decays were collected using a Spectra-Physics mode-locked, cavity-dumped, synchronously pumped, frequency-doubled R6G (290 nm excitation) or DCM (340 nm excitation) dye laser as excitation source with single photon timing detection (O'Connor and Phillips 1984). The details of the laser system, the associated optical and electronic components, the possibilities and limitations of the time-correlated single photon counting technique are described elsewhere (Van den Zegel et al. 1986; Boens et al. 1987, 1988). We used the reference convolution method (Zuker et al. 1985; Boens et al. 1988) to correct for the wavelength dependence of the shape of the instrument response function. In this correction procedure, the decay parameters of the function $\tilde{f}_s(t)$ are obtained from the measured fluorescence decays of sample, $d_s(t)$, and reference, $d_r(t)$, obtained under identical instrumental conditions:

$$d_s(t) = \int_0^t d_r(s)\, \tilde{f}_s(t-s)\, ds. \tag{1}$$

If the fluorescence δ-response function of the reference is monoexponential and the sample is multiexponential, then $\tilde{f}_s(t)$ is given by

$$\tilde{f}_s(t) = \sum_{j=1}^{k} \alpha_j [\delta(t) + (1/\tau_r - 1/\tau_j) \exp(-t/\tau_j)], \tag{2}$$

where $\delta(t)$ denotes the Dirac δ-function, α_j the pre-exponential of the jth decay component, τ_j the jth decay time, τ_r the reference lifetime, and k the number of exponential terms in the analysis.

An iterative reweighted reconvolution program based on the algorithm of Marquardt (1963) was used to estimate the unknown parameters α_j and τ_j. Several compounds with known fluorescence lifetime τ_r were used as reference. After a model function was fitted to the experimental fluorescence decay data, a careful inspection of the differences between observed and fitted decay data was made. The details of this residual analysis have been reported elsewhere (Boens et al. 1984; Van den Zegel et al. 1986). Briefly, the graphical methods included residual plots, the autocorrelation function and the normal probability plot. The numerical methods used to judge the acceptability of the fit were the reduced χ^2 value and its standard normal deviate Z_{χ^2}, the ordinary runs test statistic Z, and the Durbin-Watson test statistic d. The tests for assessing the normality of the distribution of the error terms included the determination of percentage (%) of the weighted residuals within the $[-2, 2]$ interval, and the calculation of the mean (μ) and standard deviation (σ) of the weighted residuals.

Theory

Fluorescence quenching

If the fluorescence quenching of probe P^* by a dynamic quencher Q in an isotropic solvent occurs via a non-fluorescent intermediate excited state complex $(PQ)^*$, Scheme I describes the photophysical processes.

$$\begin{array}{l} P^* + Q \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (PQ)^* \overset{k_i}{\rightarrow} P + Q \\ \quad\downarrow k_m \\ \quad P \end{array} \qquad \textbf{Scheme I}$$

k_d and k_{-d} denote the rate constants of complex formation and dissociation, k_i represents the monomolecular rate constant of deactivation of the complex, k_m is the sum of the rate constants for monomolecular deactivation of the excited singlet state of the probe, and the superscript * denotes the excited state. The bimolecular quenching constant k_q is defined as

$$k_q = k_d\, k_i/(k_{-d} + k_i) \tag{3}$$

If $k_i \gg k_{-d}$, Eq. (3) simplifies to $k_q = k_d$ and the rate of quenching is limited by the diffusion step. When $k_i \ll k_{-d}$, Eq. (3) becomes

$$k_q = k_d\, k_i/k_{-d} = \beta\, k_d\,. \tag{4}$$

The bimolecular quenching rate constant is then smaller than the diffusion rate constant.

In an isotropic solvent, the dynamic fluorescence quenching can be described by the Stern-Volmer equation:

$$I_0/I = 1 + k_q \tau_0\, [Q]\,. \tag{5}$$

In Eq. (5), I_0 and I denote the fluorescence intensities of the probe in the absence and presence of quencher,

k_q is the bimolecular rate constant of quenching (Eq. (3)), $[Q]$ is the analytical quencher concentration in the solvent and τ_0 is the probe's lifetime in the absence of quencher ($\tau_0 = k_m^{-1}$).

If the probe P is completely solubilized in the lipid bilayer and the quencher Q partitions between the lipid (L) and aqueous (A) phases, and when an intermediate excited state non-fluorescent complex $(PQ)_L^*$ is formed between fluorophore and quencher in the membrane, the fluorescence quenching can be described by Scheme II

$$\begin{array}{l} Q_A \rightleftharpoons Q_L + P_L^* \overset{k_d}{\underset{k_{-d}}{\rightleftharpoons}} (PQ)_L^* \overset{k_i}{\rightarrow} P + Q \\ \qquad\qquad\quad k_m \downarrow \\ \qquad\qquad\quad P_L \end{array} \qquad \textbf{Scheme II}$$

where all the rate constants are associated with the lipid phase. According to this scheme, the Stern-Volmer equation is given by Eq. (6)

$$I_0/I = 1 + k_q \tau_0 [Q]_L = 1 + k_q^{app} \tau_0 [Q]_T \tag{6}$$

where $[Q]_L$ is the quencher concentration in the lipid phase, k_q is the bimolecular quenching rate constant in the membrane, τ_0 is the unquenched lifetime of the probe in the lipid phase, $[Q]_T$ is the total quencher concentration. The apparent bimolecular quenching rate constant is given by Eq. (7) (Lakowicz et al. 1977):

$$\frac{1}{k_q^{app}} = \alpha_L \left[\frac{1}{k_q} - \frac{1}{k_q K_p} \right] + \frac{1}{k_q K_p}, \tag{7}$$

where $K_p = [Q]_L/[Q]_A = n_{Q,L} V_A / n_{Q,A} V_L$ is the lipid-water partition coefficient. Note that $[Q]_L$ and $[Q]_A$ refer to quencher concentrations with respect to the volumes of each phase V_L and V_A. $n_{Q,L}$ and $n_{Q,A}$ are the number of moles of quenchers in lipid and aqueous phases, respectively. $\alpha_L = V_L/V_T$ is the lipid volume fraction. By measuring the apparent bimolecular quenching rate constant k_q^{app} at various lipid volume fractions α_L, k_q and the partition coefficient K_p can be obtained from Eq. (7). k_q^{app} was calculated as the slope of the Stern-Volmer plot (Eq. (6)) divided by the unquenched lifetime of the probe. Under non-stationary light excitation conditions, the following equations are derived from the complex formation Scheme I (Birks 1970):

$$f_m(t) = \alpha_1 \exp(-\lambda_1 t) + \alpha_2 \exp(-\lambda_2 t) \tag{8}$$

with

$$\alpha_1/\alpha_2 = (X - \lambda_2)/(\lambda_1 - X) \tag{9}$$

$$\lambda_{1,2} = 0.5 \{X + Y \mp [(X - Y)^2 + 4 k_d k_{-d} [Q]_L]^{\frac{1}{2}}\} \tag{10}$$

$$X = k_m + k_d [Q]_L \tag{11}$$

$$Y = k_i + k_{-d}. \tag{12}$$

$f_m(t)$ is the δ-response function of the probe's fluorescence, α_1 and α_2 are pre-exponential factors, $1/\lambda_1$ and $1/\lambda_2$ are decay times, X and Y are the sum of the decay rate constants of the monomer and complex excited state, respectively. The rate constants $k_d[Q]_L$, k_{-d} and k_i can be determined from the decay parameters α_1/α_2, λ_1, λ_2 and τ_0. The Stern-Volmer equation for the lifetime of the locally excited singlet state is given by

$$\tau_0 X = 1 + k_d \tau_0 [Q]_L = 1 + k_d^{app} \tau_0 [Q]_T. \tag{13}$$

The apparent diffusion rate constant k_d^{app} can be written in a similar form as k_q^{app} (see Eq. (7)).

$$\frac{1}{k_d^{app}} = \alpha_L \left[\frac{1}{k_d} - \frac{1}{k_d K_p} \right] + \frac{1}{k_d K_p}. \tag{14}$$

By measuring the apparent bimolecular diffusion rate constant k_d^{app} at various lipid volume fractions α_L, k_d and the partition coefficient K_p can be obtained from Eq. (14). k_d^{app} was calculated as the slope of the Stern-Volmer plot (Eq. (13)) divided by the unquenched lifetime of the probe.

Partition and binding

The data analysis method used to distinguish between partition and/or binding is an extension of that proposed by Encinas and Lissi (1982) (Blatt et al. 1984; Blatt and Sawyer 1985). A basic assumption is that the quenching efficiency at a particular quencher concentration will only be dependent on the average number of quenchers per vesicle, $\langle Q \rangle$, irrespective of the quenching mechanism. The distribution of quenchers over the lipid and aqueous phases may be described by Scheme III.

$$Q_A + v \overset{K_{eq}}{\rightleftharpoons} Q_L \qquad \textbf{Scheme III}$$

where v denotes vesicle. An equilibrium distribution constant K_{eq} can be defined (Almgren et al. 1979)

$$K_{eq} = \frac{[Q_L]}{[Q_A][v]}, \tag{15}$$

where $[Q_L]$, $[Q_A]$ and $[v]$ refer to the concentration defined with respect to the total volume V_T. The average number of quenchers per vesicle, $\langle Q \rangle$, is given by

$$\langle Q \rangle = \frac{[Q_L]}{[v]} \tag{16}$$

Since the total quencher concentration $[Q_T]$ is

$$[Q]_T = [Q_A] + [Q_L], \tag{17}$$

equation (17) can be rearranged to

$$[Q]_T = \frac{\langle Q \rangle}{K_{eq}} + \langle Q \rangle [v]. \tag{18}$$

A plot of $[Q]_T$ vs $[v]$ allows the determination of K_{eq} and $\langle Q \rangle$. For fluorescence quenching data, the procedure involves obtaining Stern-Volmer plots at several lipid (vesicle) concentrations. Plots of $[Q]_T$ against $[v]$ can be derived at any level of I_0/I, each different level resulting in one pair of K_{eq} and $\langle Q \rangle$. When partition and binding processes occur simultaneously, $\langle Q \rangle$ is given by (Blatt et al. 1984)

$$\langle Q \rangle = \{V_L K_p + p K_b/(1 + K_b [Q_A])\} [Q_A] = K_{eq} [Q_A] \quad (19)$$

where K_b is the binding constant, p is the number of equivalent binding sites, and V_L is the molar volume of the lipid structure. Secondary plots of K_{eq} vs $\langle Q \rangle$ are Scatchard (1949) plots. Partition occurs when K_{eq} is independent of $\langle Q \rangle$ (horizontal Scatchard plot), and K_{eq} is directly related to K_p by

$$K_p = K_{eq}/V_L = [Q]_L/[Q]_A \quad (20)$$

A linear dependence with a negative slope indicates binding, while a decreasing dependence of K_{eq} with increasing $\langle Q \rangle$ asymptotically approaching a constant value of $V_L K_p$ indicates both partition and binding (Blatt et al. 1984).

Results

Fluorescence quenching of NIPC by lindane in isotropic solvents

The fluorescence quenching of NIPC by lindane in methanol, isooctane and toluene was studied at 20° and 50 °C. Upon addition of quencher, the fluorescence spectra of NIPC decreased in intensity but no additional fluorescence band could be detected. Linear Stern-Volmer plots were obtained from the intensity measurements (Fig. 2), and from their slopes the bimolecular quenching rate constants k_q were calculated (Table 1). The constants are much smaller than the rate constants for diffusion, k_d, in methanol, toluene and isooctane calculated from the Debye equation at temperature T and viscosity η

$$k_d = \frac{8RT}{3{,}000\,\eta}. \quad (21)$$

The results indicate that in those solvents lindane is an inefficient quencher for NIPC, meaning that $k_i \ll k_{-d}$. The monomolecular quenching (k_i) is the rate limiting step in the quenching reaction. From k_q and k_d, the ratio k_i/k_{-d} can be calculated: for toluene at 50 °C $k_i \approx k_{-d}/30$, for toluene at 20 °C $k_i \approx k_{-d}/42$, for methanol at 20 °C $k_i \approx k_{-d}/16$ and for isooctane at 20 °C $k_i \approx k_{-d}/17$.

The fluorescence decays of NIPC were single exponential with lifetimes τ_0 of 14.5 ± 0.1, 15.0 ± 0.1, and 13.0 ± 0.1 ns in respectively methanol, isooctane and toluene at 20 °C. The fluorescence decays remained mono-exponential upon addition of quencher. If $k_d [Q]$, $k_{-d} \gg k_m, k_i$ ('high temperature region') there is a dynamic equilibrium ($K_e = k_d/k_{-d}$) between the excited state complex and the dissociated pair of probe and quencher (Birks 1970). As a result the excited probe and the complex will deactivate mono-exponentially with the same decay rate constant λ, given by

$$\lambda = \frac{k_m + k_i K_e [Q]}{1 + K_e [Q]} \quad (22)$$

If $k_i \ll k_{-d}$, Eqs. (5) and (22) give:

$$\frac{1/\lambda}{\tau_0} \frac{I_0}{I} = 1 + K_e [Q] \quad (23)$$

If $K_e [Q] \ll 1$, then Eq. (23) simplifies to

$$\frac{\tau_0}{1/\lambda} = \frac{I_0}{I} \quad (24)$$

Since k_q values obtained from fluorescence intensities, $k_{q,I}$, and decay times, $k_{q,\tau}$, are within experimental

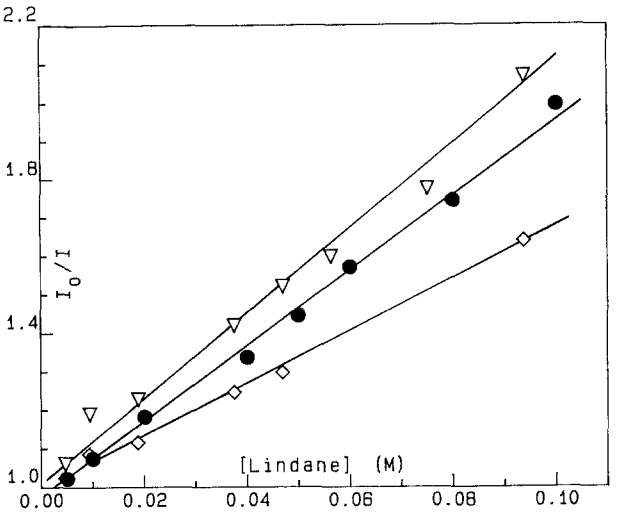


Fig. 2. Stern-Volmer plots of fluorescence spectra of NIPC quenched by lindane in toluene at 20 °C(◇), in toluene at 50 °C(▽), and in methanol at 20 °C(●)

Table 1. Quenching of NIPC by lindane: bimolecular quenching rate constants obtained from the Stern-Volmer plots of fluorescence intensities ($k_{q,I}$) and decay times ($k_{q,\tau}$). Diffusion rate constants k_d are calculated from Eq. (21)

Solvent	T[°C]	$k_{q,I}$(Ms)$^{-1}$ [$\times 10^{-8}$]	$k_{q,\tau}$(Ms)$^{-1}$ [$\times 10^{-8}$]	k_d(Ms)$^{-1}$ [$\times 10^{-10}$]
Toluene	20	5.2 ± 0.5	6.1 ± 0.6	1.7
Toluene	50	8.1 ± 0.8	9.0 ± 0.9	2.4
Methanol	20	6.8 ± 0.7	9.0 ± 1.0	1.1
Isooctane	20	7.6 ± 0.8	12.0 ± 2.0	1.3

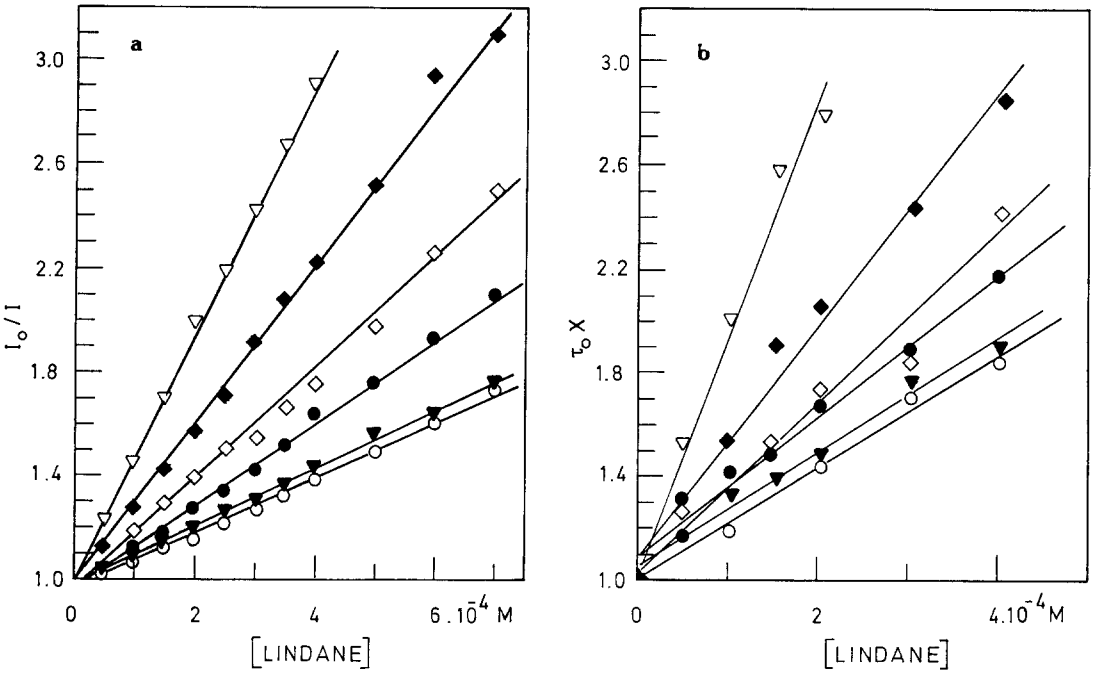


Fig. 3. **a** Stern-Volmer plots of fluorescence spectra of NIPC in the presence of lindane in DMPC vesicles of different lipid volume fractions α_L at 50 °C. **b** Stern-Volmer plots $\tau_0 X$ for the quenching of NIPC with lindane in SUV of DMPC at 50 °C at different $\alpha_L (\times 10^3)$: 3.9 (○), 3.2 (▼), 2.6 (●), 2.1 (◇), 1.3 (◆), and 0.65 (▽)

error equal (Table 1), this indicates that $K_e[Q] \ll 1$. A value of K_e can be estimated from $k_d = 4\pi N R_{AB} D_{AB}$ (Smoluchowski 1917) and $k_{-d} = 3 D_{AB}/R_{AB}^2$ (Eigen 1960)

$$K_e = (4/3)\pi N R_{AB}^3 \tag{25}$$

where $D_{AB} = D_A + D_B$ is the diffusion coefficient of the relative transport of B toward A, and N is the Avogadro number divided by 1,000. Assuming a value of 7 Å for the encounter distance R_{AB} of the excited state complex, a value of $K_e = 0.86\ M^{-1}$ was calculated. In our experiments, quencher concentrations up to 0.1 M were used resulting in a maximum value of $K_e[Q]$ of 0.086. This means that the maximum $\tau_0 \lambda$ value is about 10% lower than its corresponding I_0/I value, a difference within experimental error.

Quenching of NIPC and PCUPC by lindane in DMPC

The fluorescence decays of NIPC in the absence of quencher were single exponential with a lifetime $\tau_0 = 14.4 \pm 0.5$ ns in SUV of DMPC at 50 °C. Since the lifetime of NIPC in water is 10 ns and this value was never obtained from the fluorescence decays of NIPC in DMPC vesicles, this is an indication that the probe is completely solubilized in the hydrophobic region of the membrane. The lifetime of PCUPC in DMPC vesicles at 50 °C is 13.5 ± 0.5 ns.

Fluorescence intensities of NIPC and PCUPC in DMPC vesicles at 50 °C in the presence of lindane were measured at different lipid volume fractions. The fluorescence intensity decreased upon addition of quencher, but no additional fluorescence of the complex was observed. Linear Stern-Volmer plots based on intensities (Eq. (6)) were obtained at the different lipid volume fractions α_L (Figs. 3 a and 4). The slopes of the Stern-Volmer plots were dependent on the vesicle concentration: the apparent quenching constants calculated from the slopes, k_q^{app}, decreased with increasing α_L values (Table 2). The quenching results could be rationalized by Scheme II. The reciprocal of the apparent quenching rate constants was linearly dependent upon the lipid volume fraction α_L (Fig. 5) as predicted by Scheme II and Eq. (7), indicating a true partitioning process. From the plot according to Eq. (7) (Fig. 5), the quenching rate constant k_q was calculated to be $3.2 \pm 0.5 \times 10^8\ M^{-1}\,s^{-1}$ for NIPC:lindane and $1.9 \pm 0.4 \times 10^8\ M^{-1}\,s^{-1}$ for PCUPC:lindane (Fig. 5). The value for the partition coefficient (and its corresponding error) of lindane was found to be 13,000 (300%) from the NIPC:lindane data and 2,040 (90%) from the PCUPC:lindane data.

In the presence of lindane, the fluorescence decays of NIPC and PCUPC in DMPC vesicles were not monoexponential anymore. Satisfactory fits were only obtained when the decay curves were described by a biexponential decay function. A careful residual analysis indicated that two and no more than two exponentials were needed to adequately describe the decays. An example of a biexponential decay analysis is shown

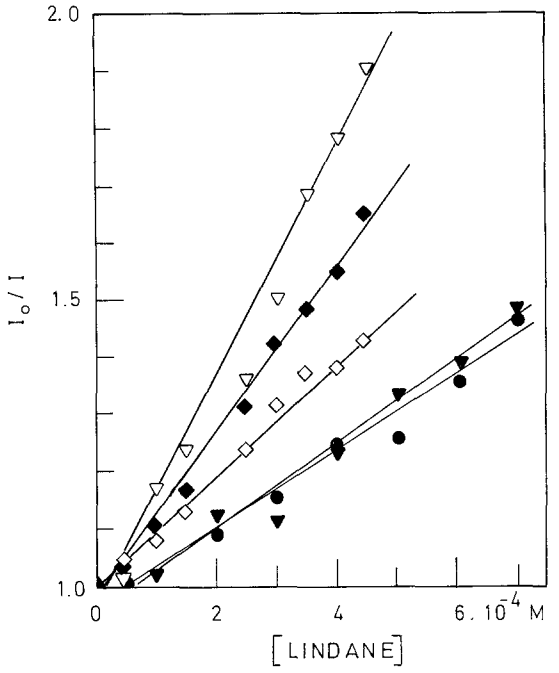


Fig. 4. Stern-Volmer plots of fluorescence spectra of PCUPC in the presence of lindane in DMPC vesicle of different lipid volume fractions α_L at 50 °C. $\alpha_L(\times 10^3) = 3.2(\blacktriangledown)$, 2.6(●), 2.1(◇), 1.3(◆), and 0.65(▽)

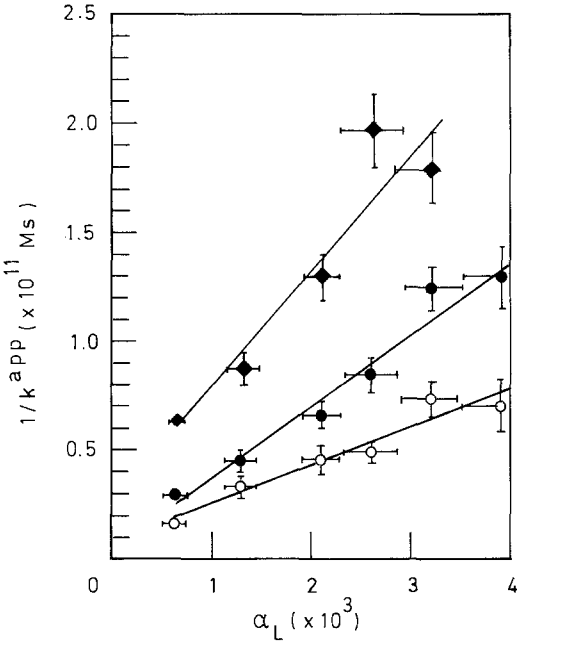


Fig. 5. Determination of the quenching rate constant k_q (Eq. (7)) for NIPC: lindane(●) and PCUPC: lindane(◆), and the diffusion rate constant k_d (Eq. (14)) for NIPC: lindane (○) in SUV of DMPC at 50 °C. The vertical and horizontal lines represent errors on k^{app} and α_L, respectively

in Fig. 6. The two terms in the exponential decay function consist of a rather long decay time λ_1^{-1} with a large contribution dependent on the quencher concentration and a short decay time λ_2^{-1} with a small contribution. The contribution of the short decay time

Table 2. Apparent bimolecular quenching constants ($\times 10^{-10}\ M^{-1}\ s^{-1}$) at different lipid fractions α_L ($\times 10^3$) for NIPC: lindane and PCUPC: lindane, obtained from the Stern-Volmer curves of intensity (k_q^{app}) and decay time (k_d^{app}) measurements. The rate constants are estimated to be accurate within ±15%

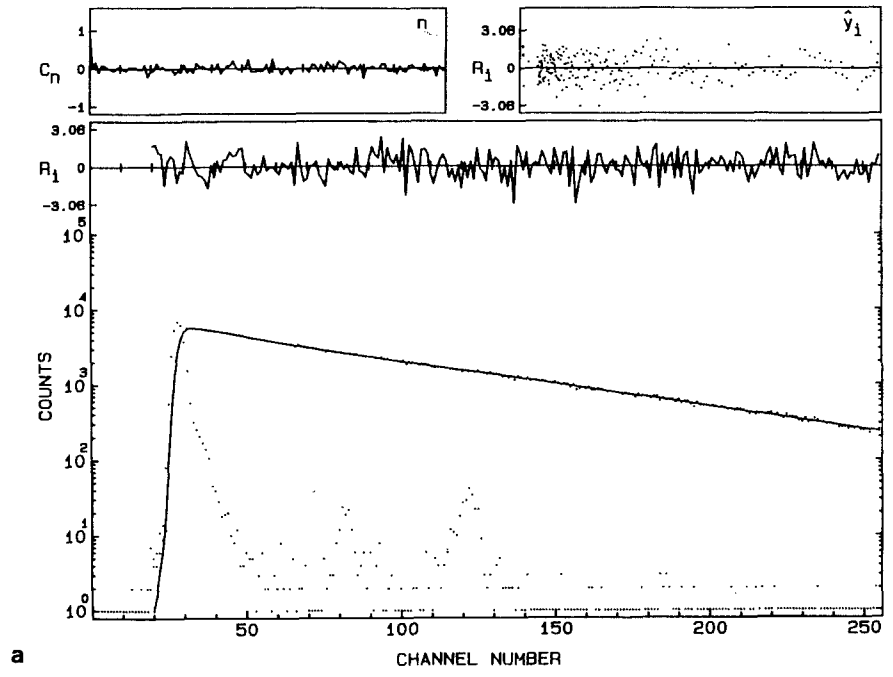
α_L	NIPC k_q^{app}	NIPC k_d^{app}	PCUPC k_q^{app}
3.9	7.7	16	—
3.2	8	16	5.6
2.6	12	22	5.1
2.1	15	24	7.7
1.3	22	33	11
0.65	34	70	16

Table 3. Rate constants and lifetimes of Scheme II calcultated for the quencher concentrations $[Q]_T = 4 \times 10^{-4}\ M$ (part A) and $[Q]_T = 2 \times 10^{-4}\ M$ (part B) at different lipid volume fractions α_L in DMPC vesicles at 50 °C for NIPC: lindane. The errors on X^{-1} are estimated to be approximately 6%. The errors on k_{-d}, k_i and Y^{-1} are ≈ 20%

	α_L $\times 10^3$	X^{-1} [ns]	$k_i\,s^{-1}$ [$\times 10^{-8}$]	$k_{-d}\,s^{-1}$ [$\times 10^{-8}$]	Y^{-1} [ns]
A	3.9	7.3	1.7	1.3	3.4
	3.2	7.1	1.7	1.2	3.6
	2.6	6.2	2.0	1.4	3.0
	2.1	5.5	1.9	1.2	3.2
	1.3	4.7	2.1	1.3	3.0
B	3.9	9.4	1.4	1.2	3.8
	3.2	9.1	1.3	1.2	4.1
	2.6	8.0	1.8	1.9	2.7
	2.1	7.6	1.6	1.4	3.3
	1.3	6.5	2.0	1.7	2.7
	0.65	4.8	2.2	1.1	3.0

increases with increasing quencher concentration. Quantitative measurements of NIPC only are available.

All the quenching results could be rationalized by the complex formation Scheme II. From the decay parameters α_1/α_2, λ_1 and λ_2, the lifetime X^{-1} of the locally excited state of NIPC was calculated (Eq. (11)). The Stern-Volmer curves of $\tau_0 X$ vs $[Q]_T$ (Eq. (13)) were linear (Fig. 3 b) for all measured α_L values. The slopes of the Stern-Volmer plots were dependent on the volume fraction of lipid phase: the apparent diffusion rate constant k_d^{app} decreased with increasing α_L (Table 2). Plotting k_d^{app} as a function of α_L (Eq. (14)) yields a straight line (Fig. 5). From the slope and intercept of this plot a value for the diffusion rate constant $k_d = 6.6 \pm 0.8 \times 10^8\ M^{-1}\ s^{-1}$ was obtained. The value for the partition coefficient K_p was 2,600 with an error of about 90%. From the decay parameters λ_1, λ_2 and X, all individual rate constants of Scheme II were evaluated for all different quencher concentrations and lipid volume fractions investigated. Values are compiled in Table 3 for $[Q]_T = 2 \times 10^{-4}\ M$ and



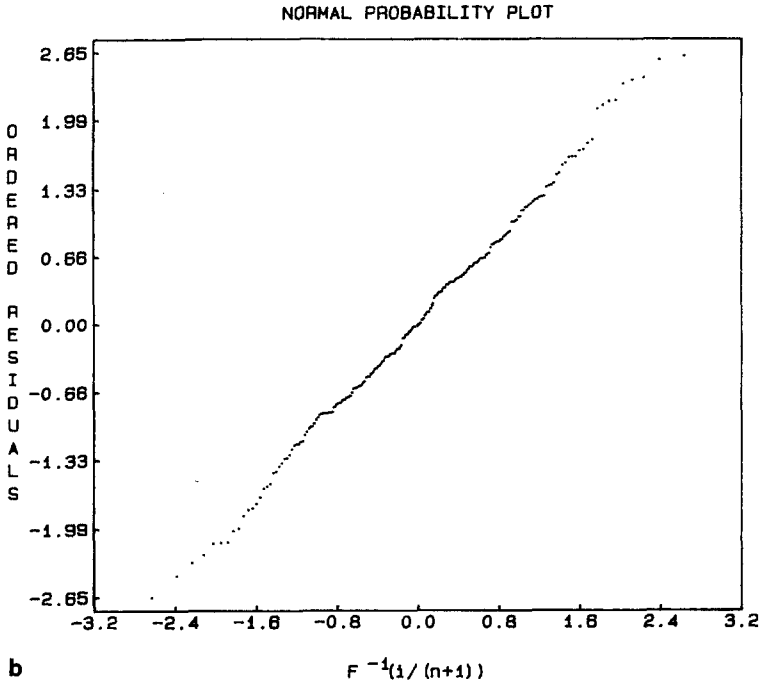


Fig. 6. a Experimental fluorescence decay curve (*point plot*) of NIPC in the presence of lindane in SUV of DMPC at 50 °C. Analysis as a double exponential decay (*solid line*). $\lambda_{ex} = 330$ nm, $\lambda_{em} = 360$ nm, channel width = 142 ps, $\chi^2 = 1.00$, $Z_{\chi^2} = 0.04$, $Z = 0.00$, $d = 2.11$. The reference decay (of *trans*-1-(1-indanylidene) indane; *point plot*) and the residual plots are also shown. **b** Normal probability plot of the weighted residuals of **a**

4×10^{-4} M for different lipid volume fractions. It should be noted that at the lowest quencher concentration ($[Q]_T = 5 \times 10^{-5}$ M), the errors on k_{-d} and k_i become very large (respectively 200% and 600%). As can be seen from Table 3, k_{-d} and k_i are of similar order of magnitude, and k_{-d}, k_i and the lifetime Y^{-1} of the complex remain constant at the different quencher concentrations and volume fractions investigated.

For the diffusion of NIPC:lindane in a two dimensional system the mutual diffusion coefficient D between P^* and Q ($D = D_A^* + D_Q$) is given by (Waka et al. 1980; Kano et al. 1981)

$$D = \frac{k_d}{1.585\,ZN} \tag{26}$$

where Z is the thickness of the two-dimensional system. Taking a value of 2 nm for Z, a value of $3.4 \pm 0.4 \times 10^{-6}$ cm^2/s was obtained for D. If probe and quencher are considered as having the same

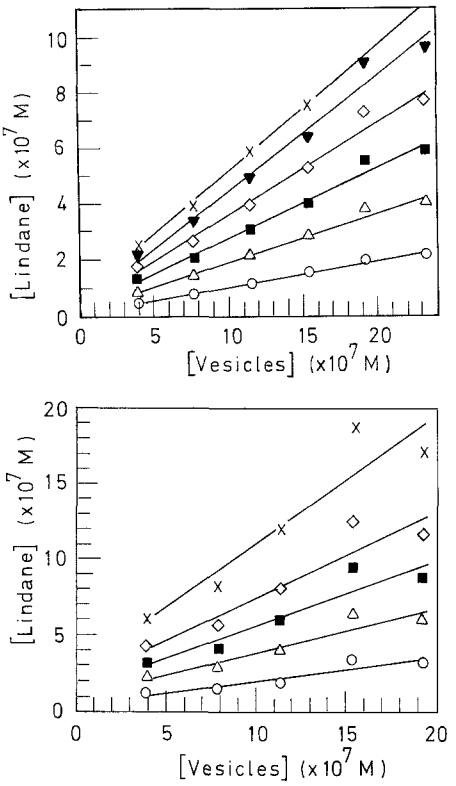


Fig. 7 a and b. Quenching of NIPC (**a**) and PCUPC (**b**) by lindane. Data plotted according to Eq. (18) give an average number of quenchers $\langle Q \rangle$ and an association constant K_{eq} at I_0/I values of 1.2 (○), 1.4 (△), 1.6 (■), 1.8 (◇), 2.0 (▼), 2.2 (×)

Table 4. Average number of quenchers $\langle Q \rangle$ per vesicle ± standard deviation at different I_0/I values for the probes NIPC (1), methyl-11-(*N*-carbazolyl)-undecanoate (2) and PCUPC (3)

$\langle Q \rangle$			
I_0/I	(1)	(2)	(3)
2.2	—	672 ± 184	850 ± 553
2.0	418 ± 102	556 ± 112	711 ± 460
1.8	338 ± 79	446 ± 90	574 ± 374
1.6	258 ± 57	336 ± 75	435 ± 284
1.4	177 ± 35	225 ± 60	296 ± 197
1.2	97 ± 5	115 ± 42	159 ± 113

mobility, the diffusion coefficient of lindane is $1.7 + 0.2 \times 10^{-6}$ cm^2/s.

Plots of $[Q]_T$ against the vesicle concentration $[v]$ at various values of I_0/I for the steady-state fluorescence quenching of NIPC: lindane and PCUPC: lindane are given in Fig. 7. From these plots values of K_{eq} and $[Q]$ were determined (Eq. (18)). The average number of quenchers $[Q]$ per vesicle at different I_0/I values for the probes NIPC, methyl 11-(*N*-carbazolyl) undecanoate and PCUPC are given in Table 4. From these data, it is evident that the bulkier the hydrophobic tails of the probe, the more quenchers per vesicle are needed to

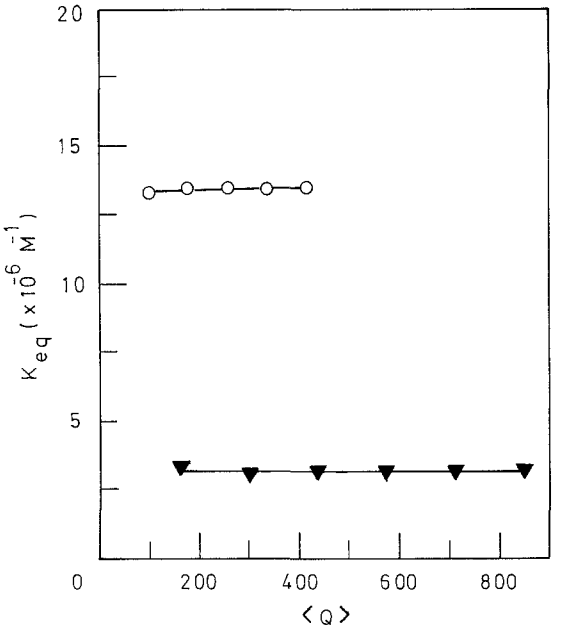


Fig. 8. Scatchard plots for the quenching of NIPC (○) and PCUPC (▼) with lindane in DMPC vesicles at 50 °C

obtain the same amount of quenching. Scatchard plots (K_{eq} vs $[Q]$, Fig. 8) for NIPC: lindane and PCUPC: lindane were horizontal, indicating that only a partition process occurs. So there are no specific binding sites in the DMPC liposomes for the quencher. The values obtained for K_{eq} are 1.35×10^6 *M* for NIPC: lindane and 3.2×10^5 *M* for PCUPC: lindane.

Discussion

The fluorescence decays of NIPC and PCUPC in SUV of DMPC at 50 °C in the absence of quencher were single exponential. In the presence of the quencher lindane they became biexponential. This was also observed for methyl 11-(*N*-carbazolyl)undecanoate: lindane in DMPC vesicles (Daems et al. 1988), but is in contrast with earlier publications from Lakowicz et al. (1977), Lakowicz and Hogen (1980), Omann and Glaser (1984), where a single exponential lifetime was measured by the phase fluorometry technique for a variety of carbazole probes quenched by lindane in liposomes. The limited resolving power of the phase shift-demodulation method using a single modulation frequency can explain the discrepancies between our results and theirs. Deviations of linearity of the Stern-Volmer law for fluorescence intensities are observed when there is a partition of fluorophores (or quenchers) between different phases of the membrane, or if a significant fraction of the fluorophores is not accessible to the quenchers or if static quenching becomes important. The linear Stern-Volmer relationship for

steady-state and transient measurements and the absence of ground-state complexes of the fluorophore with lindane indicate that the above situations did not occur under the experimental conditions used. The observed fluorescence decays of NIPC in the presence of lindane could also not be described by micelle kinetics (Deumié et al. 1981; Kano et al. 1981; Washington et al. 1984).

Collisional quenching can have a complex transient behavior in viscous media like membranes. This effect is described by the Smoluchowski (1917) equation (Eq. (27)) for the rate constant of a diffusion controlled process in three dimensions.

$$k_d(t) = 4\pi N R_{AB} D_{AB} (1 + R_{AB}/\sqrt{\pi D_{AB} t}) . \quad (27)$$

The transient term cannot be neglected at short times after excitation, in viscous media or if the quenching reaction takes place over long distances. For one- or two-dimensional diffusion controlled processes this time-independent term disappears (Gösele 1984). In two dimensions analytical solutions cannot be obtained for the fluorescence decay and the fluorescence quantum yield. Owen (1975) described the two dimensional diffusion process by an equation that is of the same mathematical form as Eq. (27). This equation gives only a rough approximation of two-dimensional diffusion in membranes.

$$k_d(t) = 0.5\, Z D_{AB} N\, (3.17 + 14.18\, R_{AB}/\sqrt{D_{AB} t}) . \quad (28)$$

Qualitatively one can recognize the occurrence of diffusion transients by an upward curvature of the Stern-Volmer plot (Owen 1975; Kano et al. 1981). The intercept will be smaller than unity. When diffusion transients are important the fluorescence decay of the probe changes from mono-exponential in the absence of quencher to non-exponential in the presence of quencher (Eq. (29)).

$$f_m(t) = \alpha \exp[-(At + B\sqrt{t})] . \quad (29)$$

In the case of three-dimensional diffusion A and B are defined as:

$$A = k_m + 4\pi N R_{AB} D_{AB} , \quad (30\,a)$$

$$B = 4\pi N R_{AB}^2 \sqrt{D_{AB}} . \quad (30\,b)$$

For two-dimensional quenching the fluorescence decay is *approximated* by Eq. (29) but the constants A and B have a different meaning. The logarithm of $f_m(t)$ vs t in Eq. (29) has a steep decay at very short times after excitation (Kano et al. 1981). If the quenching reaction is not diffusion controlled (Schemes I and II) the situation is complex. In this case the quenching rate constant is not equal to k_d, but depends also on k_{-d} and k_i. If k_{-d} and k_i are of similar magnitude the fluorescence decay can be described by a bi-exponential. The experimental fluorescence decays of the carbazole probes in the presence of lindane are not single exponential. It is therefore important to determine which model – time-dependent diffusion or reversible quenching – describes the decay data adequately. Quenching studies in solvents (see Results) indicated that in the case of NIPC-lindane the back-reaction is important ($k_{-d} \approx k_i$). Furthermore, in all cases good bi-exponential fits were obtained. The rate constants and lifetimes calculated according to Scheme II were consistent: i. e. k_i, k_{-d} and Ywere independent of $[Q]$, while X decreased linearly with $[Q]$ (see Table 3). Experimentally no upward curvatures were obtained for the Stern-Volmer plots of NIPC in the presence of different quencher concentrations and the intercept with the y-axis was always equal to one. Fitting the data to Eq. (29) did not give consistently acceptable fits.

Our results show that the fluorescence quenching of NIPC (and PCUPC) with lindane is a dynamic or collisional process which takes place via an intermediate excited state non-emitting complex. Electron transfer from the excited carbazole singlet to halocarbon quenchers has been suggested (Ahmad and Durocher 1981) as quenching mechanism for the fluorescence quenching of carbazole with halocarbons. Our data contain no indication that such a mechanism is operative in the fluorescence quenching of MCU by lindane, since the rate of quenching is independent of the solvent polarity. The complex is non-fluorescent since only a decrease of the intensity of the fluorescence spectrum was observed in the presence of quencher but no change in spectral distribution. Scheme II can account for all quenching results.

The rate constants of the bimolecular quenching reaction for NIPC: lindane were calculated from Eqs. (8–12) and are realistic (Table 3). k_i and k_{-d} are constant for all quencher concentrations and lipid fractions. k_i is small ($10^8\ s^{-1}$) and of the same order of magnitude as k_{-d}. This means that the quenching reaction in liposomes occurs in the 'mid-temperature region' (Birks 1970) where bi-exponential decays are oberved. In non-viscous solvents like methanol, isooctane or toluene, where k_{-d} is much higher than k_i ('high temperature region'), the fluorescence decays become single exponential. A similar reaction scheme of exciplex formation in membranes has been used by Kano et al. (1981) and Waka et al. (1980). Kano et al. (1981) have observed exciplex formation for the quenching of pyrene and 11-(1-pyrenyl)undecanoic acid with N,N-dimethylaniline in dipalmitoylphosphatidylcholine above the phase transition temperature. The fluorescence decays were always mono-exponential. This is explained by the fact that k_{-d} is low. Waka et al. observed exciplex fluorescence for pyrene derivatives and N,N-dimethylaniline in DMPC liposomes as well. A biexponential decay was

observed in the monomer region. The long decay time coincided with the decay time in the exciplex region.

Lindane is a membrane soluble quencher as the value of the apparent quenching and diffusion rate constants are very high and the quenching increases at decreasing lipid volume fractions. It was possible to calculate the quenching rate constant k_q and the diffusion rate constant k_d with Eqs. (7) and (14). k_q ($3.2 \pm 0.5 \times 10^8\ M^{-1}\,s^{-1}$) was slightly smaller than k_d ($6.6 \pm 0.8 \times 10^8\ M^{-1}\,s^{-1}$) indicating that the quenching reaction is not completely diffusion controlled. It is in agreement with the fact that k_{-d} and k_i are nearly equal. Quenching constants determined by Kano et al. (1981) for pyrene and 10-(1-pyrenyl)undecanoic acid with *N,N*-dimethylaniline were $8.9 \times 10^7\ M^{-1}\,s^{-1}$ and $5.9 \times 10^7\ M^{-1}\,s^{-1}$, respectively. These authors considered the quencher as completely solubilized in the membrane which can lead to an underestimation of the rate constants.

The diffusion coefficient of lindane is $1.7 \pm 0.2 \times 10^{-6}$ cm^2/s in DMPC vesicles at 50 °C. The values are higher than those determined by Kano for pyrene: *N,N*-dimethylaniline (4.7×10^{-7} cm^2/s) and 10-(1-pyrenyl)decanoic acid: *N,N*-dimethylaniline (3.1×10^{-7} cm^2/s) in dipalmitoylphosphatidylcholine vesicles at 50 °C. Lakowicz and Hogen (1980), and Omann and Glaser (1984) found similar values for the diffusion constant of lindane, namely 1.2×10^{-6} cm^2/s in DMPC vesicles at 50 °C and 1.3×10^{-6} cm^2/s in plasma membrane phospholipids of mouse LM cells at 37 °C. We have no values of diffusion coefficients from the quenching of PCUPC with lindane.

More difficult was the determination of the partition coefficient K_p for lindane. If the apparent quenching rate constants k_q^{app} (Eq. (7)) were used, a value for $K_p = 13{,}000$ with an error of 300% was obtained from the NIPC: lindane data. From the k_d^{app} values a partition coefficient of 2,600 with an error of 90% was calculated. This value is lower than the previous one, but is probably more reliable. Anyhow, these values clearly indicate that lindane is very soluble in lipid membranes.

Acknowledgements. The authors wish to thank Drs. M. Van den Zegel and M. Van der Auweraer for helpful discussions. DD was supported by the IWONL and the K.U. Leuven. NB is "Bevoegdverklaard Navorser" of the "Fonds voor Geneeskundig Wetenschappelijk Onderzoek" (FGWO, Belgium). This work was supported partially by FGWO grant 3.0099.86. The authors thank the FKFO (Belgium) for continuing financial support.

References

Ahmad A, Durocher G (1981) Fluorescence quenching of carbazole by halocarbons in 3-methylpentane solutions at room temperature. Can J Spectrosc 26:19–24

Almgren M, Grieser F, Thomas JK (1979) Dynamic and static aspects of solubilization of neutral arenes in ionic micellar solutions. J Am Chem Soc 101:279–291

Antunes-Madeira MC, Madeira VMC (1985) Partition of lindane in synthetic and native membranes. Biochim Biophys Acta 820:165–172

Barenholz Y, Gibbes D, Litman BJ, Goll J, Thompson TE, Carson FD (1977) A simple method for the preparation of homogenous phospholipid vesicles. Biochemistry 16: 2806–2810

Birks JB (1970) Photophysics of aromatic molecules. Wiley-Interscience, London, pp 301–371

Blatt E, Sawyer WH (1985) Depth-dependent fluorescent quenching in micelles and membranes. Biochim Biophys Acta 822:43–62

Blatt E, Chatelier RC, Sawyer WH (1984) Partition and binding constants in micelles and vesicles from fluorescence quenching data. Chem Phys Lett 108:397–400

Boens N, Van den Zegel M, De Schryver FC (1984) Picosecond lifetime determination of the second excited singlet state of xanthione in solution. Chem Phys Lett 11:340–346

Boens N, Van den Zegel M, De Schryver FC, Desie G (1987) The time-correlated single photon counting technique as a tool in photobiology. In: Favre A, Tyrrell R, Cadet J (eds) From photophysics to photobiology. Elsevier, Amsterdam, pp 93–108

Boens N, Ameloot M, Yamazaki I, De Schryver FC (1988) On the use and the performance of the delta function convolution method for the estimation of fluorescence decay parameters. Chem Phys 121:73–86

Daems D, Boens N, De Schryver FC (1988) Determination of partition and diffusion of lindane in unilamellar L,α-dimyristoylphosphatidylcholine vesicles by fluorescence quenching of the intramembrane probe methyl 11-(*N*-carbazolyl) undecanoate. Anal Chim Acta 205:61–75

Deumié M, Viallet P, Lightcap D, Rhyne RH Jr, Wade CG (1981) Mécanismes spécifiques d'inhibition de la fluorescence dans les membranes d'érythrocytes: étude du pyrène et du benzo(a)pyréne par des inhibiteurs liposolubles. J Chim Phys 78:475–483

Dittmer JC, Lester RL (1964) A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J Lipid Res. 5:126–127

Eigen M (1960) Relaxationsspektren chemischer Umwandlungen (Metallkomplexe und protolytische Reaktionen in wässriger Lösung). Z Elektrochem 64:115–123

Encinas MV, Lissi EA (1982) Evaluation of partition constants in compartmentalised systems from fluorescence quenching data. Chem Phys Lett 91:55–57

Gösele UM (1984) Reaction kinetics and diffusion in condensed matter. Prog React Kinet 13:63–161

Gupta CM, Radhakrishnan R, Khorana HG (1977) Glycerophospholipid synthesis: Improved general method and new analogs containing photoactivable groups. Proc Natl Acad Sci USA 74:4315–4319

Huang C (1969) Studies on phosphatidylcholine vesicles. Formation and physical characteristics. Biochemistry 8: 344–352

Kano K, Kawazumi H, Ogawa T, Sunamoto J (1981) Fluorescence quenching in liposomal membranes. Exciplex as a probe for investigating artificial lipid membrane properties. J Phys Chem 85:2204–2209

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York, pp 257–302

Lakowicz JR, Hogen D (1980) Chlorinated hydrocarbon-cell membrane interactions studied by the fluorescence quenching of carbazole-labeled phospholipids: probe synthesis and characterization of the quenching methodology. Chem Phys Lipids 26:1–40

Lakowicz JR, Hogen D, Omann G (1977) Diffusion and partitioning of a pesticide, lindane, into phosphatidylcholine

bilayers. A new fluorescence quenching method to study chlorinated hydrocarbon-membrane interactions. Biochim Biophys Acta 471:401–411

Marquardt DW (1963) An algorithm for least-squares estimation of nonlinear parameters. J Soc Ind Appl Math 11: 431–441

McCracken MS, Holt NJ (1985) High-performance liquid chromatographic determination of lipids in vesicles. J Chromatogr 348:221–227

O' Connor DV, Phillips D (1984) Time-correlated single photon counting. Academic Press, London

Omann GM, Glaser M (1984) Biosynthetic incorporation of fluorescent carbazolylundecanoic acid into membrane phospholipids of LM cells and determination of quenching constants and partition coefficients of hydrophobic quenchers. Biochemistry 23:4962–4969

Omann GM, Lakowicz JR (1982) Interactions of chlorinated hydrocarbon insecticides with membranes. Biochim Biophys Acta 684:83–95

Owen CS (1975) Two dimensional diffusion theory: cylindrical diffusion model applied to fluorescence quenching. J Chem Phys 62:3204–3207

Scatchard G (1949) The attraction of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672

Selinger Z, Lapidot Y (1966) Synthesis of fatty acid anyhydrides by reaction with dicylohexylcarbodiimide. J Lipid Res 7: 174–175

Sikaris KA, Thulborn KR, Sawyer WH (1981) Resolution of partition coefficients in the transverse plane of the lipid bilayer. Chem Phys Lipids 29:23–36

Smoluchowski M von (1917) Versuch einer mathematischen Theorie der Koagulationskinetik koloider Lösungen. Z Phys Chem 92:129–168

Stewart JCM (1980) Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 104:10–14

Vandendriessche J, Palmans P, Toppet S, Boens N, De Schryver FC, Masuhara H (1984) Configurational and conformational aspects in the excimer formation of bis(carbazoles). J Am Chem Soc 106:8057–8064

Van den Zegel M, Boens N, De Schryver FC (1984) Fluorescence decay of 1-methylpyrene in small unilamellar dimyristoylphosphatidylcholine vesicles. A temperature and concentration dependent study. Biophys Chem 20:333–345

Van den Zegel M, Boens N, Daems D, De Schryver FC (1986) Possibilities and limitations of the time-correlated single photon counting technique: A comparative study of correction methods for the wavelength dependence of the instrument response function. Chem Phys 101:311–335

Waka Y, Mataga N, Tanaka F (1980) Behavior of heteroexcimer systems in single bilayer liposomes. Photochem Photobiol 32:335–340

Washington K, Sarasua MM, Koehler LS, Koehler KA, Schultz JA, Pedersen LG, Hiskey RG (1984) Utilization of heavy-atom effect quenching of pyrene fluorescence to determine the intramembrane distribution of halothane. Photochem Photobiol 40:693–701

Zuker M, Szabo AG, Bramhall L, Krajcarski DT, Selinger B (1985) Delta function convolution method (DFCM) for fluorescence decay experiments. Rev Sci Instrum 56:14–22