

Quenching of fluorescence of pyrene-substituted lecithin by tetracyanoquinodimethane in liposomes

Helge Lemmetyinen, Marjo Yliperttula, Jukka Mikkola, and Paavo Kinnunen
Department of Membrane Physics, KSV Research Laboratories, SF-00381 Helsinki, Finland

ABSTRACT In this work we have applied a kinetic scheme derived from fluorescence kinetics of pyrene-labeled phosphatidylcholine in phosphatidylcholine membranes to explain the fluorescence quenching of 1-palmitoyl-2-(10-[pyren-1-yl]decanoyl)-*sn*-glycero-3-phosphatidylcholine (PPDPC) liposomes by tetracyanoquinodimethane (TCNQ). The scheme was also found to be applicable to neat PPDPC and the effect of the quencher could be attributed to certain steps of the proposed mechanism. The

TCNQ molecules influence the fluorescence of pyrene moieties in PPDPC liposome in two ways. Firstly, an interaction between the quencher molecule and the pyrene monomer in the excited state quenches monomer fluorescence and effectively prevents the diffusional formation of the excimer. Secondly, an interaction between the quencher molecule and the excited dimer quenches the excimer fluorescence. The TCNQ molecule does not prevent the formation of the excimer in pyrene moieties

aggregated in such a way that they require only a small rotational motion to attain excimer configuration. The diffusional quenching rate constant is calculated to be $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for the pyrene monomer quenching and $1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the pyrene excimer quenching. The diffusion constant of TCNQ is $1.5 \times 10^{-7} \text{ cm}^2\text{s}^{-1}$ for the interaction radii of 0.8–0.9 nm. The TCNQ molecules are practically totally partitioned in the membrane phase.

INTRODUCTION

Fluorescence quenching studies have been widely used in the application of fluorescence to biochemical problems because of the favorable properties of the process of quenching. Among the most common of these are collisional or dynamic quenching, where the quencher diffuses to the fluorophore during the lifetime of the excited state, and static quenching, where a complex between the fluorophore and the quencher is formed. The primary requirement for quenching is that the fluorophore and quencher must be in contact. Fluorescence quenching measurements can be used to study the accessibility of fluorophores to quenchers, as well as diffusion rates and distances of quenchers.

Fluorescence probes are widely used in biological systems to investigate the structural and dynamic properties of membranes. Studies of the quenching of fluorescence with quenchers of known location can be used to determine the location of fluorophores relative to lipid-water interfaces. Quenching of membrane-bound fluorophores gives information concerning the diffusion and partitioning of small quenching molecules in membranes. Interpretation of the quenching data requires the separation of the quencher diffusion rates from the effects of lipid-

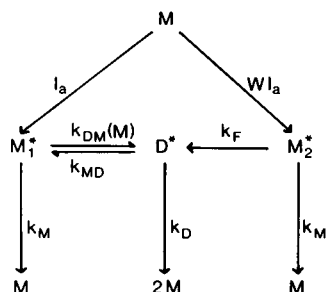
water partitioning. Data so obtained can be used to estimate approximate partition coefficients for the system.

Dynamic fluorescence quenching in isotropic solutions is usually described by the Stern-Volmer equation, in which the reciprocal of the fluorescence quantum yield, or the ratio of the fluorescence intensities in the absence and presence of quencher, is presented as a function of the quencher concentration. The slope of the straight line obtained for this plot is related to diffusion coefficient. Thus, the diffusion coefficient may be measured using this method. A linear Stern-Volmer plot is generally indicative of a single class of fluorophores. A nonlinear deviation toward the concentration axis indicates the presence of two fluorophore populations, one of which is not accessible to quencher. Nonlinearity is also produced by simultaneous dynamic and static quenching. In this case an upward curvature is observed. It is important to note that a linear Stern-Volmer plot does not prove that dynamic quenching of fluorescence has occurred, as static quenching also results in a linear plot. Discrimination between the two mechanisms can be achieved by use of lifetime measurements. In static quenching the ratio of the lifetimes in the presence and absence of quencher is constant and differs from the ratio of fluorescence intensities. In dynamic quenching the ratios of lifetimes and intensities are equal and increase with the concentration of quencher.

A number of studies have been reported, in which the biomolecular fluorescence quenching method has been

Dr. Lemmetyinen's and Dr. Yliperttula's present address is Department of Physical Chemistry, University of Helsinki, Meritullinkatu 1 C, SF-00170 Helsinki, Finland.

applied to membrane systems (1–14). In this paper, we report the fluorescence quenching of pyrene lecithin in liposomes by tetracyanoquinodimethane (TCNQ), which is well known to be an effective fluorescence quencher. Pyrene-labeled lecithins form liposomes which exhibit both the monomer and excimer emissions. In our earlier studies (Lemmetynen, H., M. Yliperttula, J. Mikkola, J. Virtanen, and P. K. J. Kinnunen, submitted for publication) it was observed that the fluorescence kinetics of 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphatidylcholine (PPDPC), at concentrations <10 mol% in 1,2-dipalmitoylphosphatidylcholine (DPPC) matrices and at temperatures above the phase transition, can be explained by a rather simple kinetic scheme (Scheme 1).



SCHEME 1

The rate parameters in Scheme 1 were estimated (Lemmetynen, H., M. Yliperttula, J. Mikkola, J. Virtanen, and P. K. J. Kinnunen, submitted for publication).

One of the aims of this study was to attribute the quenching effects of TCNQ to certain steps in Scheme 1 and in this way explain the dynamics of fluorescence quenching, as well as the location and diffusion of the quencher in the membrane.

THEORY

Scheme 1 involves two different paths for excimer formation. The first path, indicated by the rate constant k_F , represents a configuration of pyrene moieties in the lattice where only a small motion is required to attain the excimer configuration. The opposite reaction is thought to be slow when compared with other dissociation processes. The second, path, indicated by the rate constants k_{DM} and k_{MD} , may be diffusional and reversible for excimer formation. I_a is the excitation light intensity. W is the weighting factor representing the ratio of conformer M_1 to M_2 ready to form M_1^* and M_2^* . k_{DM} and k_F are rate constants for formation of excimer, k_{MD} is rate constant of excimer dissociation, $k_D = k_{FD} + k_{ID}$ is the sum of excimer

fluorescence and nonradiative decay parameters, and $k_M = k_{FM} + k_{IM}$ is the sum of the monomer fluorescence and the nonradiative decay parameters. It is still assumed that k_M is equal for both the initial excited species M_1^* and M_2^* .

Defining the parameters $X = k_M + k_{DM}[M]$, $Y = k_D + k_{MD}$, and $Z = k_F + k_M$, one can write for the measureable rate parameters k_1 , k_2 , and k_3 ,

$$k_{1,2} = (1/2)\{(X + Y) \pm [(X - Y)^2 + 4k_{MD}k_{DM}(M)]^{1/2}\} \quad (1)$$

$$k_3 = k_F + k_M. \quad (2)$$

In the presence of the quencher Q, new parameters X' and Y' can be written as $X' = X + k_{QM1}[Q]$, $Y' = Y + k_{QD}[Q]$, and $Z' = Z + k_{QM2}[Q]$, where k_{QM1} , k_{QD} , and k_{QM2} are the rate constants for quenching of M_1^* , D^* , and M_2^* , respectively. The observable rate parameters are now

$$k_{1,2} = (1/2)\{X + Y + (k_{QM1} + k_{QD})[Q] \pm \{(X - Y + (k_{QM1} - k_{QD})[Q])^2 + 4k_{MD}k_{DM}[M]\}^{1/2}\} = 1/t_{1,2} \quad (3)$$

and

$$k_3 = Z + k_{QM2}[Q] = 1/t_3. \quad (4)$$

If a linear dependence between the concentration of Q and the rate parameters k_1 and k_2 is observed it is reasonable to assume that $\{X - Y + (k_{QM1} - k_{QD})[Q]\}^2 \gg 4k_{MD}k_{DM}[M]$. Thus the equations for k_1 and k_2 can be written as

$$k_1 = X + k_{QM1}[Q] = 1/t_1 \quad (5)$$

and

$$k_2 = Y + k_{QD}[Q] = 1/t_2. \quad (6)$$

For a hydrophobic quencher and a membrane-bound fluorophore the dynamic quenching constant reveals the collisional frequency between the probe and quencher in the membrane. The collisional frequency is proportional to the local quencher concentration which makes it possible to estimate the quenching rate constant and lipid-water partition coefficient (8).

The partition coefficient is defined as the ratio of the concentration of quencher in the water (w) and the membrane (m).

$$P = [Q_m]/[Q_w]. \quad (7)$$

If V_m and V_t represents the volumes of the membrane and total systems, respectively, the volume fraction of membrane phase is

$$a_m = V_m/V_t \quad (8)$$

and

$$[Q_m] = P[Q_t]/[Pa_m + (1 - a_m)], \quad (9)$$

where $[Q_t]$ is the total concentration of the added quencher. By substituting this expression for the membrane concentration of quencher in the Stern-Volmer equation, we obtain

$$\begin{aligned} 1/t &= 1/t_0 + \{P[Q_t]/[Pa_m + (1 - a_m)]\}k_m \\ &= 1/t_0 + k_{app}[Q_t], \end{aligned} \quad (10)$$

where k_m is the bimolecular quenching constant for membrane-bound fluorophore. The apparent quenching constant k_{app} is given by

$$1/k_{app} = a_m [(1/k_m) - (1/k_m P)] + (1/k_m P). \quad (11)$$

The fraction of the quencher bound to the membrane can be defined as

$$f_m = Pa_m/[Pa_m + (1 - a_m)] \quad (12)$$

The rate of the diffusion-controlled reaction, where the limiting step is the rate of the reactants may be calculated using the Smoluchowski equation,

$$k = 4\gamma\pi(R_q + R_f)(D_q + D_f)N/1,000, \quad (13)$$

where γ is the quenching efficiency of the fraction of collisional encounters that are effective in quenching, D_q , D_f , R_q , and R_f are the diffusion coefficients and molecular radii of the quencher and fluorophore, and N is Avogadro's constant. Substituted fluorophores can be considered to be stationary. Thus the only diffusion apparent is that of the quencher with a coefficient D to the collision distance. The collision radius is assumed to be the sum of the radii of fluorophore and quencher.

The static component of quenching is not necessarily due to the complexing of the fluorophore and quencher, but rather to quencher molecules being adjacent to fluorophores at the moment of excitation. If there exists a sphere of volume V within which the probability of quenching is unity, the observable fluorophores are those for which there are no adjacent quenchers. A modified form of Stern-Volmer equation can be used to describe this situation

$$F^0/F = A \exp ([Q_m]VN), \quad (14)$$

where A is a constant due to the diffusional part of quenching, F^0 and F are the fluorescence intensities in the absence and presence of quencher, $[Q_m]$ is the concentration of quencher in the membrane and N is Avogadro's constant.

MATERIALS AND METHODS

PPDPC was provided by the Department of the Chemistry of Liquid Crystals of KSV Chemical Corp., (Helsinki, Finland). TCNQ was purchased from the Eastman Kodak Co. (Rochester, NY) and used without further purification.

Multilamellar liposomes were prepared according to conventional methods. PPDPC and TCNQ stock solutions were made in chloroform. The solutions were mixed to obtain the desired concentration ratios. The mixtures were first dried under a stream of nitrogen and then further dried for several hours under reduced pressure. The dry lipid was then dispersed in nitrogen-saturated water by vortexing or by sonication in a nitrogen atmosphere at 45°C. The total volumes of the samples were 3 ml with lipid concentrations of 10, 20, and 40 nmol corresponding to concentrations of 3.33, 6.67, and 13.3×10^{-6} M. Four different TCNQ concentrations were used at each lipid concentration ranging from 3.0 to 20×10^{-6} M. Before the fluorescence measurements, the liposome solutions were deaerated by flushing nitrogen for 10 min through the samples. During the measurements the sample compartments were purged with nitrogen gas. All spectroscopic measurements were performed at a temperature below (10°C) and above (30°C) the phase transition temperature, which for neat PPDPC is 15°C (15). All the samples were at the desired temperatures for at least 20 min before the spectroscopic and lifetime measurements.

Steady-state fluorescence spectra were obtained with an SLM 4800S spectrofluorometer whose polarizers had been removed. The sample intensity was recorded relative to a triangular cuvette containing a reference solution of rhodamine B. The samples were stirred during the measurements. The excitation wavelength was 343 nm (2-nm slit width), and the emission was scanned from 360 to 600 nm with increments of 1 nm. Each data point was an average of 10 measurements.

The fluorescence decay curves and time-resolved spectra were measured by a single-photon-counting method with a model 199 fluorescence time-domain spectrometer (Edinburgh Instruments, Edinburgh, Scotland) using a coaxial flashlamp (FWHM time of 1.5 ns). The excitation was 343 nm and the monomer and excimer emissions were 377 and 470 nm, respectively. The number of counts per channel was at least 10^4 at the maximum for every measurement. Appropriate low-cut, low fluorescent optical filters were used to suppress second-order and stray light from the excitation monochromator. Fluorescence decay curves were analyzed using a nonlinear least-squares iterative deconvolution method with a PDP-11 processor. The values obtained for the goodness (chisq in Fig. 3) of the applied triple exponential fits were between 1.0 and 1.3, which are well acceptable (16). Analysis by double or quadruple exponential fits produce poorer or similar goodness as analysis by triple exponential fits.

RESULTS

Rate parameters below the phase transition

The fluorescence intensity-time profiles of the excited monomer and excimer can be fitted by triple-exponential functions in neat PPDPC (Fig. 1) at temperatures below the phase transitions. The rate parameters k_i and k'_i , for monomer and excimer decays, respectively, were different, apart from the values of k_3 and k'_3 , which were equal in magnitude, being $0.6 \times 10^9 \text{ s}^{-1}$, within experi-

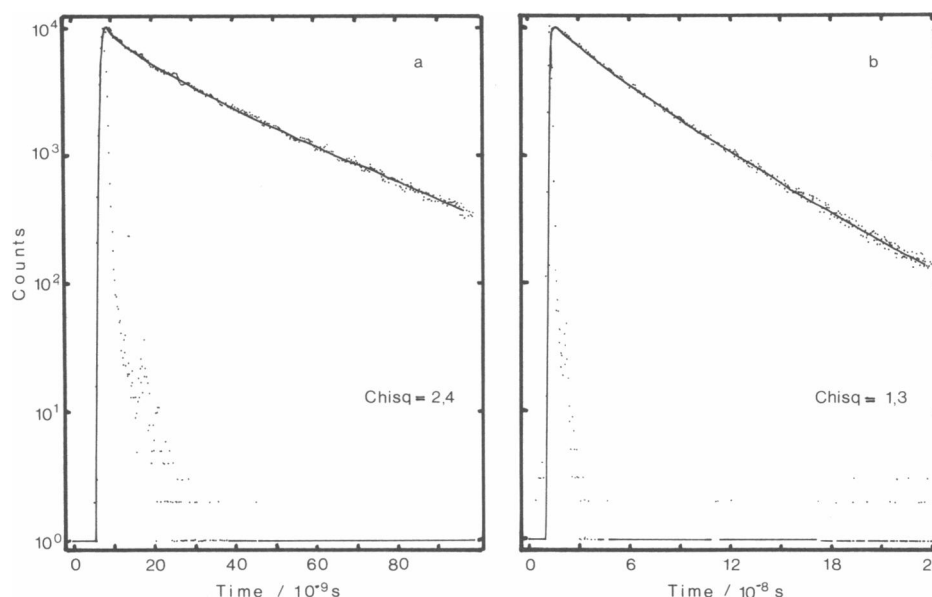


FIGURE 1 Fluorescence decay curves of PPDPC-liposome (40 nmol/3 ml) in the absence of TCNQ at 10°C. The decay curves have been fitted to three exponentials. (a) Monomer (399 nm); rate parameters of $1/k_1 = 9.1$ ns, $1/k_2 = 33.4$ ns, and $1/k_3 = 0.9$ ns. (b) Excimer (470 nm); lifetimes of $1/k'_1 = 26.3$ ns, $1/k'_2 = 58.6$ ns, and $1/k'_3 = 0.5$ ns (formation of the excimer).

mental accuracy. k'_3 had a negative preexponential factor. This indicates the formation of excimer. The negative preexponential factor is not observed in the presence of TCNQ, however, the decay is still triple-exponential. These facts indicate that more than three excited species are present in the system at temperatures below the phase transition.

The Stern-Volmer plots (Eqs. 5 and 6) for lifetimes $1/k_1$ and $1/k_2$ for the monomer and excimer decays at different lipid concentrations are presented in Fig. 2. Nonlinear plots and irregular dependencies on lipid concentrations show that the quencher molecules have an influence on the liposome structure and the diffusional processes are reduced at low temperatures. TCNQ molecules are most probably aggregated in membranes. A more detailed kinetic study below the phase transition temperature would probably be extremely complicated.

Application of the quenching data to the kinetic scheme above the phase transition

It is possible to interpret the data obtained in the framework of the mechanism (Scheme 1) suggested for the fluorescence kinetics of PPDPC in a DPPC matrix at temperatures above the phase transition. For this reason a scheme with three observable transient species was formulated (Lemmettyinen, H., M. Yliperttula, J. Mikkola, J. Virtanen, and P. K. J. Kinnunen, submitted for publi-

cation). At 30°C the fluorescence intensity-time profiles of the excited monomer and excimer can be fitted by triple-exponential functions (Fig. 3). In the absence of TCNQ one of the preexponential factors is negative, indicating the formation of excimer. This formation cannot be detected in the presence of TCNQ, which indicates that the slow diffusional path of the excimer formation is hindered by the quenching effect of TCNQ on one of the precursors of the excimer. In the presence of TCNQ the excimer decay is still triple-exponential. The quickest rate parameters for monomer and excimer decays k_3 and k'_3 , respectively, are independent of TCNQ concentration and equal in magnitude, being $\sim 1.0 \times 10^9 \text{ s}^{-1}$, within experimental accuracy.

The Stern-Volmer plots (Eqs. 5 and 6) for the rate parameters k_1 and k_2 for monomer and parameters k'_1 and k'_2 for excimer decays as a function of TCNQ concentration are presented in Fig. 4. k_1 increases at low quencher concentrations but obtains a constant value at high concentration (Fig. 4 a). This is consistent with Scheme 1, where two different types of excited monomeric species are proposed. One is quenched by TCNQ but the other has a constant value at all TCNQ concentrations. The quenched species is that which forms excimer by a slow diffusional process. Thus, TCNQ interacts with the M_1^* state in Scheme 1.

The plots k'_1/k_1^0 vs. quencher concentration (Eq. 5) are linear at all lipid concentrations (Fig. 4 c), but the slopes of the curves increase with lipid concentration indicating

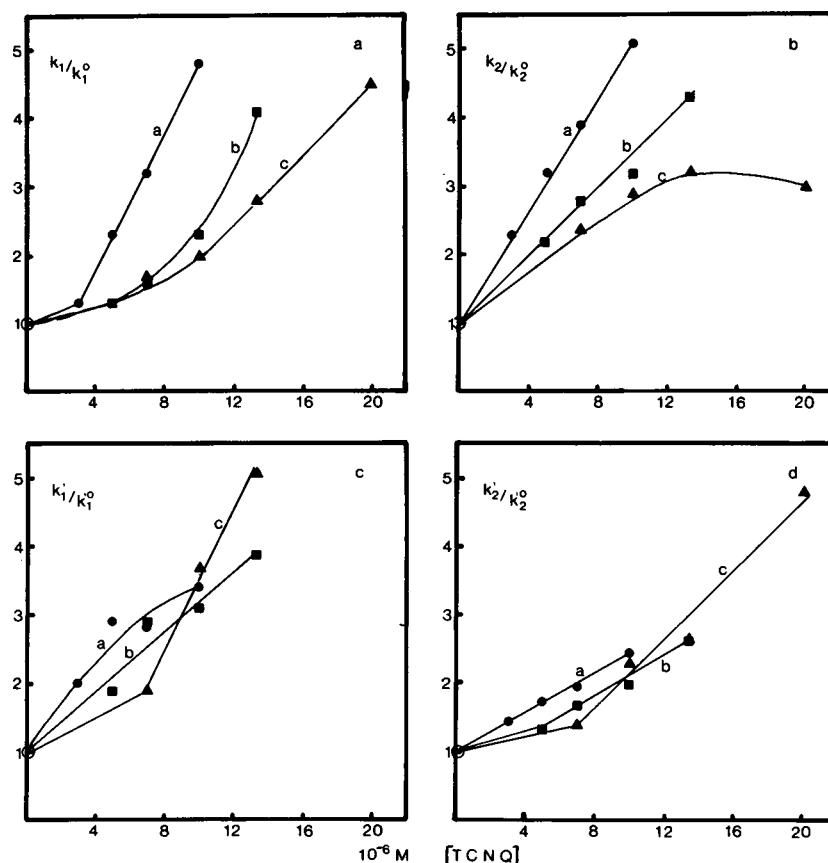


FIGURE 2 Stern-Volmer plots for fluorescence quenching of PPDPC-liposomes by TCNQ at a temperature of 10°C. (a) Monomer decay for k_1 ($1/k_1^0 = 9.1$ ns); (b) monomer decay for k_2 ($1/k_2^0 = 33.4$ ns); (c) excimer decay for k'_1 ($1/k'_1 = 26.3$ ns); (d) excimer decay for k'_2 ($1/k'_2 = 58.6$ ns). Concentrations of the quencher are concentrations in the total volume. Liposome concentrations (in 3 ml) are indicated by letters $a = 10$ nmol, $b = 20$ nmol, and $c = 40$ nmol.

more effective quenching. This kind of Stern-Volmer plot is typical of dynamical quenching. The value obtained for $1/t_1$, $75 \times 10^6 \text{ s}^{-1}$, is in good agreement with the values calculated for $X = k_M + k_{DM}[M]$ in the PPDPC/DPPC system, $83 \times 10^6 \text{ s}^{-1}$ (Lemmettyinen, H., M. Yliperttula, J. Mikkola, J. Virtanen, and P. K. J. Kinnunen, submitted for publication). Thus the rate parameters k'_1 can be considered to be a characteristic of the quenching of the monomeric species M_1^* . The Stern-Volmer constants calculated are 0.36, 1.10, and 1.85 M^{-1} corresponding to rate constants of k_{QM1} , 0.27, 0.83, and $1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and lipid concentrations of 10, 20, and 40 nmol/3 ml. These values are permissible for diffusion-controlled reactions in membranes.

The plots k_2/k_2^0 and k'_1/k'_1^0 vs. TCNQ concentration (Eq. 6) also show linear dependences on the quencher concentration (Fig. 4, b and d). The lifetimes $1/k_2^0$ and $1/k_{20}$ are 2.2×10^7 and $1.8 \times 10^7 \text{ s}^{-1}$, respectively, for monomer and excimer decays. They correspond well to the value of

$Y = k_D + k_{MD} = 2.3 \times 10^7 \text{ s}^{-1}$ calculated (Lemmettyinen, H., M. Yliperttula, J. Mikkola, J. Virtanen, and P. K. J. Kinnunen, submitted for publication) for the decay of PDPC in a DPPC matrix. Thus the rate parameter $k_2 = Y + k_{QD}[Q]$ can be considered to correspond to the process, where the fluorescence of the dimeric species D^* is quenched by TCNQ. The values for k_{QD} , calculated from the slopes, are between 0.6 and $1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, which are permissible for diffusion-controlled reactions in membranes. The partitioning of the quencher between the membrane and water phases must, however, be taken into consideration for more accurate determination of the rate constants.

It is interesting to note that no interaction between the TCNQ molecule (Eq. 4) and the monomeric species M_2^* was observed, that is, k_3 is independent of TCNQ. Moreover, a constant rate parameter $1/k_1$, probably corresponding to the lifetime of M_2^* , was measured (Fig. 4 a) at rather high TCNQ concentrations. A constant value for

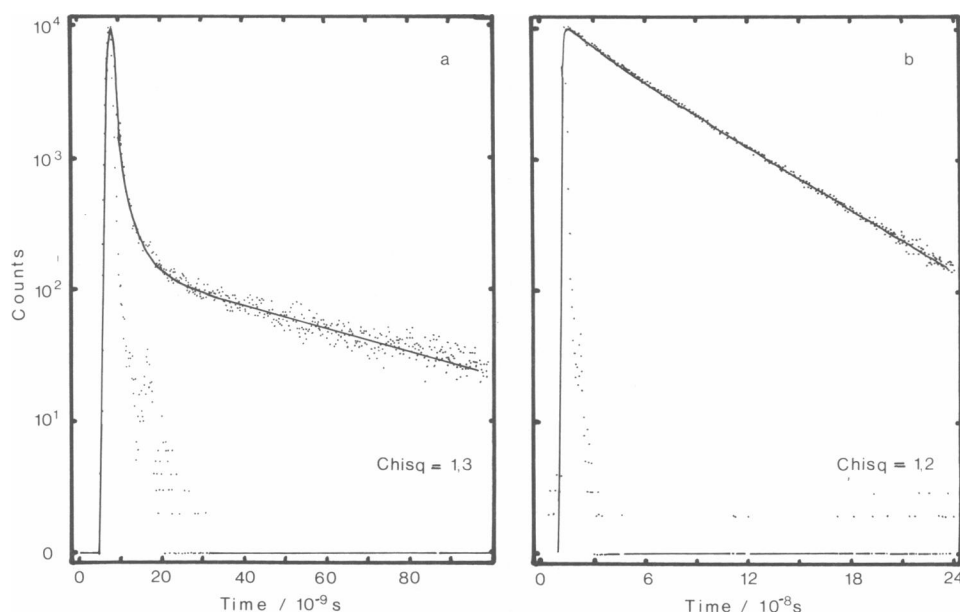


FIGURE 3 Fluorescence decay curves of PPDPC-liposome (40 nmol/3 ml) in the absence of TCNQ at 30°C. The decay curves have been fitted to three exponentials. (a) Monomer (399 nm); rate parameters of $1/k_1 = 2.9$ ns, $1/k_2 = 46.2$ ns, and $1/k_3 = 0.6$ ns. (b) Excimer (470 nm); rate parameters of $1/k'_1 = 13.2$ ns, $1/k'_2 = 55.3$ ns, and $1/k'_3 = 0.6$ ns (formation of the excimer).

the ratios of monomer fluorescence intensities, in the presence and absence of quencher, was also observed. These observations indicate that the rate constant k_F (Scheme 1) is much larger than $k_{QM2}[Q]$.

Steady-state fluorescence intensities

The excimer to monomer fluorescence intensity ratio depends strongly on temperature. Thus the monomer emission dominates at temperatures below, and the excimer emission at those above the phase transition temperature (15°C). It was observed that at 10°C monomer emission is dependent on, and excimer emission independent of, TCNQ concentration. At 30°C monomer emission is independent of, but excimer emission is dependent on, the concentration of TCNQ. The ratios of the fluorescence intensities in the absence (F^0), to that in the presence (F) of quencher are presented in Fig. 5 for the monomer at 10°C (Fig. 5 a) and for the excimer at 30°C (Fig. 5 b). The intensity-concentration curves at 30°C show a regular dependence on lipid concentration for excimer emission, however a substantial upward curvature is apparent. This is probably due to the location of the quencher, which is adjacent to the fluorophore at the moment of excitation. This produces an immediate deactivation.

Positive deviations from the Stern-Volmer equation at

low temperatures, for monomer emission (Fig. 5 a), are observed as a result of static quenching. Irregular behavior with respect to lipid concentration is evidence for nonuniform distribution of the quenching molecules in the membrane below the phase transition.

Quenching sphere of action

The ratios F^0/F for excimer emission of PPDPC system as a function of TCNQ concentration in the total volume, $[Qt]$ at 30°C are presented in Fig. 5 b. The upward curvature of the curves indicates the presence of static quenching. The values of $\ln(F^0/F)$ as a function of TCNQ concentration (Eq. 14) partitioned in the membrane, $[Q_m]$, are presented in Fig. 6. From the slopes of the straight lines the radius of the sphere of action can be calculated at different lipid concentrations. They are 7.9, 8.6, and 9.4 Å corresponding to lipid concentrations of 3.3 , 6.7 , and 13.3×10^{-6} M. The average value is 8.6, which is about the same as the sum of the radii of the fluorophore and quencher.

Separation of the quencher diffusion and partition coefficients

The rate parameters k_2 and k'_2 , which correspond to excimer fluorescence quenching of the species D^* in Scheme 1, depend on the concentrations both of TCNQ

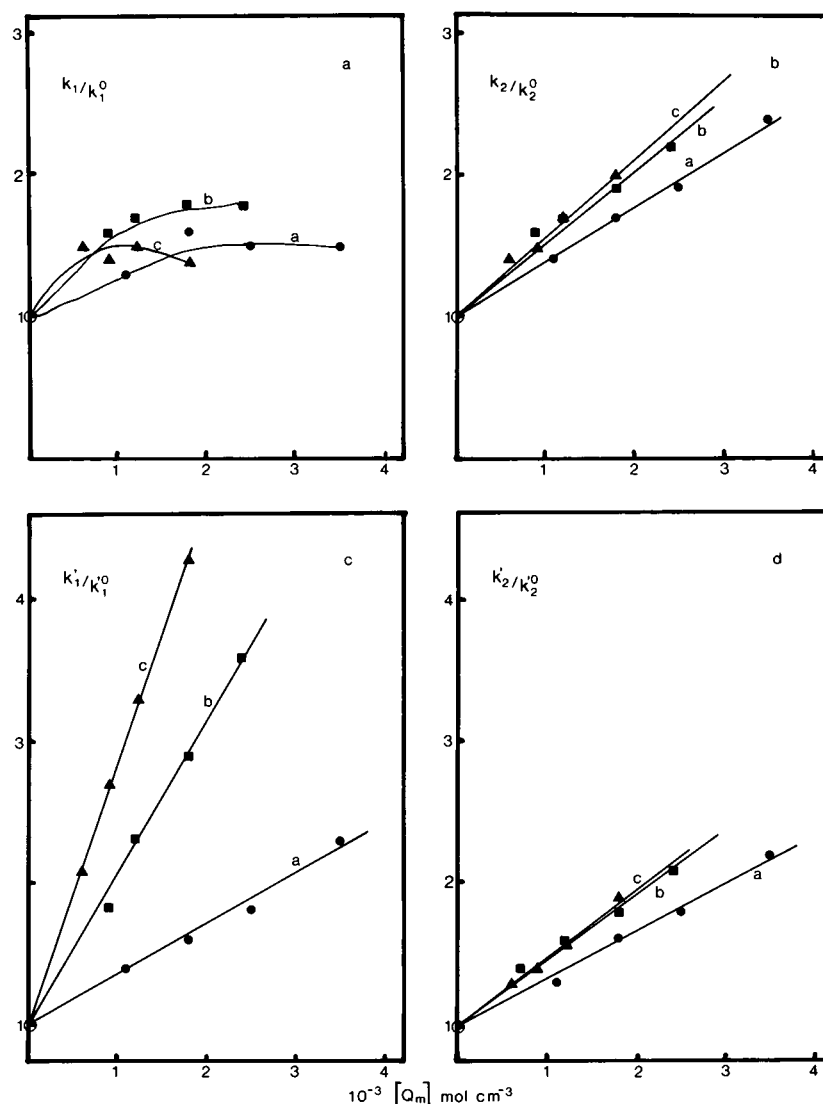


FIGURE 4 The Stern-Volmer plots for fluorescence quenching of PPDPC-liposomes by TCNQ as a function of the quencher concentration in the membrane phase at 30°C assuming all TCNQ dissolved in the membrane phase. (a) k_1/k_1^0 for monomer quenching ($k_1^0 = 3.4 \cdot 10^8 \text{ s}^{-1}$); (b) k_2/k_2^0 for monomer quenching ($k_2^0 = 0.22 \cdot 10^8 \text{ s}^{-1}$); (c) $k_1'/k_1'^0$ for excimer quenching ($k_1'^0 = 0.75 \cdot 10^8 \text{ s}^{-1}$); and (d) $k_2'/k_2'^0$ for excimer quenching ($k_2'^0 = 0.18 \cdot 10^8 \text{ s}^{-1}$). Liposome concentrations (in 3 ml) are indicated by letters a = 10 nmol, b = 20 nmol, and c = 40 nmol. The concentrations of TCNQ in the membrane were estimated in moles per gram lipid, assuming a lipid density of 1.0 g cm^{-3} .

and PPDPC (Fig. 4, b and d). The efficiency of quencher increases with increasing lipid concentration. This kind of behavior indicates that quenching can be dynamic in nature, and that the quencher is partitioned between lipid and water. The separation of the quencher diffusion rates from the effects of lipid-water separation is required for kinetic interpretation to be made.

The apparent quenching constants, k_{app} , can be obtained from Fig. 7, where the rate parameters k_2 and k_2' from monomer and excimer decays are presented as a function of quencher concentration (Eq. 10) in the total

volume of the system. Plotting the values of $1/k_{app}$ as a function of the volume fraction (Eq. 11) of the membrane phase, a_m , produced straight lines (Fig. 8), with an intercept of $0.15 \times 10^{-12} \text{ Ms}$ and slopes of 7.0 and $9.6 \times 10^{-8} \text{ Ms}$ from monomer and excimer decays, respectively. These values correspond to rate constants and partition coefficients of $1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $440,000$ calculated from the monomer decay and $1.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $600,000$ from the excimer decay. It is reasonable to assume that the averages of these values, $1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $520,000$, can be considered to be the rate

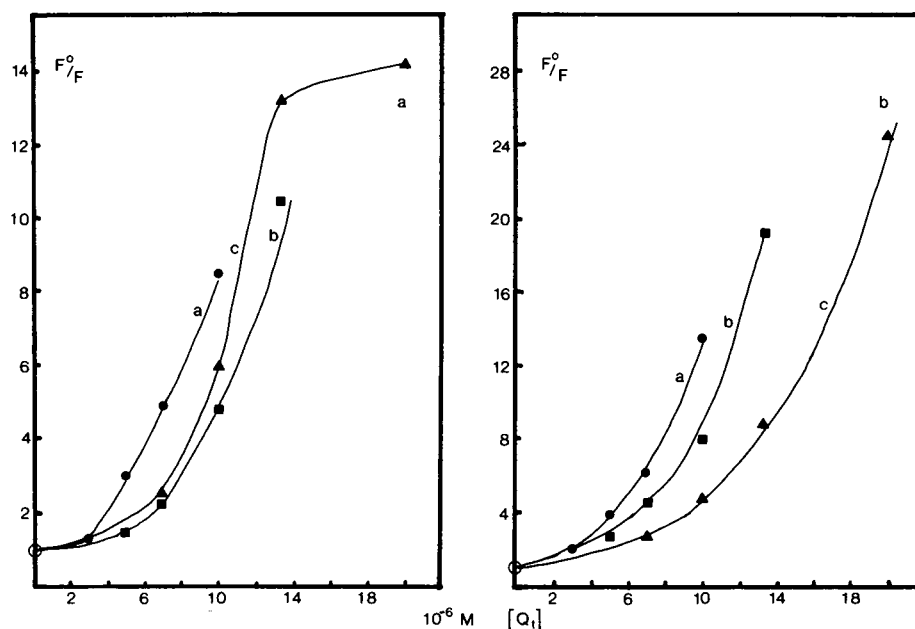


FIGURE 5 The ratios of the fluorescence intensities in the absence, F^0 , to that in the presence, F , of the quencher as a function of the total quencher concentration. (a) F^0/F for the monomer at 10°C, and (b) F^0/F for the excimer at 30°C. The liposome concentrations (in 3 ml) are indicated by letters $a = 10$ nmol, $b = 20$ nmol, and $c = 40$ nmol.

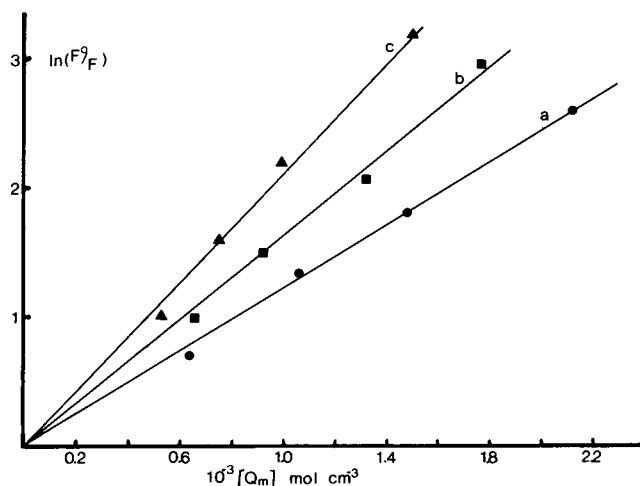


FIGURE 6 $\ln(F^0/F)$ as a function of the quencher concentration partitioned in the membrane phase. Concentrations of TCNQ in the membrane phase were calculated by multiplying the amount of TCNQ by the fraction of the quencher bound to the membrane ($f_m = 0.60$, 0.75 , and 0.85 at lipid concentrations of 10 , 20 , and 40 nmol in 3.0 ml, respectively), and dividing the product by the volume of the membrane. The liposome concentrations (in 3 ml) are indicated by letters $a = 10$ nmol, $b = 20$ nmol, and $c = 40$ nmol.

constant for the pyrene excimer fluorescence quenching by TCNQ, and the partition coefficient of the TCNQ partition between the membrane and water.

By a similar way, using the rate parameter k'_1 (Fig. 4 c), which corresponds to the monomer fluorescence quenching of the species M_1^* , values of $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for the bimolecular quenching constant and $400,000$ for the partition coefficient can be calculated.

The fraction of the quencher, f_m , bound to the membrane can now be calculated by using Eq. 12. In the present case, when the partition coefficient of $520,000$ is used, the values of f_m obtained are 0.60 , 0.75 , and 0.85 at lipid concentrations of 3.3 , 6.7 , and $13.3 \times 10^{-6} \text{ M}$.

Lateral diffusion coefficients

The average value of 8.6 \AA was calculated (Eq. 14) for the radii of the sphere of action between fluorophore and the quencher. A value of $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ was estimated (Eq. 11) for the diffusion-controlled rate constant for pyrene monomer fluorescence quenching by TCNQ. If it is assumed that the quenching efficiency (γ in Eq. 13) of TCNQ for the monomer quenching is one, a value of $1.5 \times 10^{-7} \text{ cm}^2\text{s}^{-1}$ is obtained for the diffusion constant of TCNQ in PPDPC membranes. The diffusion coefficient calculated in this way is the smallest that can possibly occur.

As the substituted pyrene moieties can be considered to

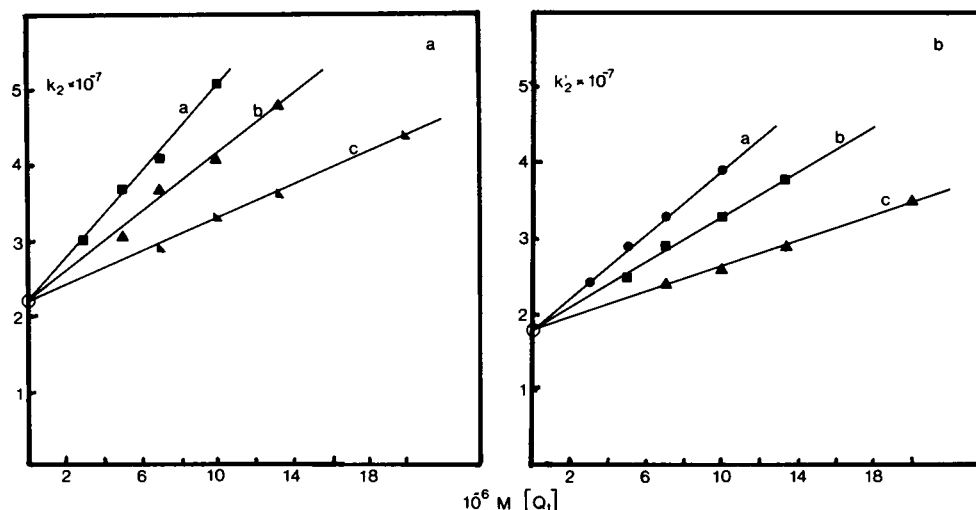


FIGURE 7 The fluorescence quenching rate constants k_2 as a function of the quencher concentration in the total volume at 30°C for calculating the apparent quenching constants k_{app} . (a) The monomer decay, and (b) the excimer decay. Liposome concentrations (in 3 ml) are indicated by letters $a = 10$ nmol, $b = 20$ nmol, and $c = 40$ nmol. Estimated values are 2.94 , 1.98 , and $1.12 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$ for monomer decays and 2.07 , 1.49 , and $0.85 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$ for excimer decays lipid concentrations of 10, 20, and 40 nmol in 3 ml, respectively.

be stationary, the only diffusion apparent is that of the quencher. It is reasonable to assume that all other parameters in the Smoluchowski equation (Eq. 13) apart from their quenching efficiencies which differ from each other, are the same for the pyrene monomer and excimer quenching. A rate constant of $1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ was

calculated for pyrene excimer fluorescence quenching by TCNQ. On substituting this value in the Smoluchowski equation a value 0.13 is obtained for the quenching efficiency of the pyrene excimer quenching by TCNQ.

DISCUSSION

The fluorescence behavior of pyrene-labeled lecithin can obviously be divided into two categories, i.e., fluorescence below and above the phase transition. At lower temperatures, in the crystal state, a regular behavior of the fluorescence as a function of temperature and pyrene concentration cannot be observed. Furthermore, the variation of the TCNQ-liposome concentration ratio at low temperatures indicates a heterogeneous distribution of the quencher molecules in the bilayer.

From the point of formal kinetics, the most significant observation at temperatures below the phase transition is that none of the measured rate parameters of the triple-exponential decay were the same for the monomer and excimer decays. This is evidence for an existence of more than three fluorescing transients.

At temperatures above the phase transition in the liquid crystal state, the kinetics of the monomer-excimer fluorescence behavior changes considerably. At low concentrations of pyrene moieties the fluorescence time-profiles can be analyzed by triple-exponential functions. The rate parameters measured were the same for the monomer and excimer. Furthermore, two of the preex-

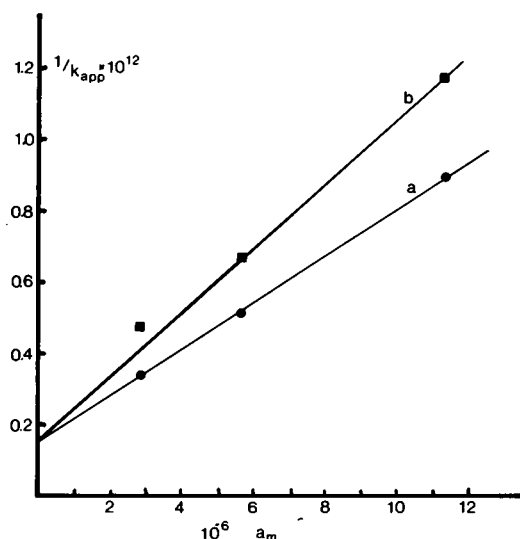


FIGURE 8 The inverse values of the apparent quenching constants, $1/k_{app}$, as a function of the volume fraction of the membrane phase, a_m , at 30°C. (a) Monomer decay, and (b) excimer decay. The average value of slopes of monomer and excimer decays is $7.7 \times 10^{-8} \text{ s}^{-1} \text{ mol}^{-1} \text{ dm}^3$ and the intercept $0.15 \times 10^{-12} \text{ s}^{-1}$.

ponential factors of the excimer fluorescence time-profiles were negative in value and the sum of these two values was equal to the value of the third preexponential factor. These experimental observations point to the presence of three kinetically distinguishable fluorescing species, which are in equilibrium with each other.

The kinetic model used in this work is based on the most simple kinetic treatment possible. The experimental observations at temperatures above the phase transitions do not unequivocally rule out the existence of more complicated models, but all the observed results can be explained well enough by the simple model used. Regrettably the model used does not identify which measured rate parameter corresponds to any given species, and it does not describe the effects of quencher on each state. This information may be obtained by setting up a kinetic model, and deriving fluorescence intensity-time profiles separately for the monomer and excimer fluorescences. The preexponential factors and rate parameters thus derived are solely functions of rate constants of the model. The measured values can be assigned to the rate constants belonging to any species in the model by fitting measured rate parameters and preexponential factors to those derived from the model. It is essential to note that only one combination from the six possible resulted in rate constants that are physically permissible.

In the present work it was observed that the rate constants k_1 and k_2 were linear functions of the quencher concentration, but the rate parameter k_3 had a constant value at all quencher concentrations. These observations essentially simplified the equations for k_1 and k_2 (Eq. 3) to the forms

$$k_1 = X + k_{QM1}[Q] = 1/t_1 \quad (5)$$

and

$$k_2 = Y + k_{QD}[Q] = 1/t_2, \quad (6)$$

thus separating monomer and excimer quenching.

So the effect of the quenching may be related to the rate parameters k_1 and k_2 and to the species M_1^* and D^* , respectively, but not to the rate parameter k_3 and to the state M_2^* . As k_3 is independent of quencher concentration, it is reasonable to assume that formation of excimer from the configuration M_2^* is faster than interaction between the quencher molecule and species M_2^* .

Assuming that all the quencher molecules are in the membrane phase, we calculated values between 0.27 and $1.4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and 0.6 and $1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for k_{QM1} and k_{QD} at different lipid concentrations. If on the other hand it is assumed that the quenching molecules may be partitioned between the water and membrane phases and that quenching may only occur in the lipid phase, bimolecular quenching constants in the membrane phase were

calculated to be $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for pyrene monomer quenching and $1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for pyrene excimer quenching. These values are about the same as the rate constants obtained assuming a perfect partition of the quenchers to the membrane phase. This means that the assumption that all the quencher molecules are in the membrane phase is probably correct.

The lower value of the rate constant for the pyrene excimer quenching can be better explained by a lower efficiency of the quenching process due to resonance stabilization rather than by a faster diffusion of TCNQ molecule. It should be noted that the values for the quenching rate constants were calculated from the decay rate parameters measured separately for the monomer and excimer decays (Figs. 4 and 6). This increased the reliability of the calculations.

The value of $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ obtained for the quenching rate constant of pyrene monomer is about the same order of magnitude, $1-7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (13), as the estimated values for fluorescence quenching of anilino-naphthalenesulfonic acid by a nitroxide doxyl group substituted stearic acid in phosphatidylcholine-cholesterol membranes, and the value of $10^8 \text{ M}^{-1}\text{s}^{-1}$ (14) calculated for pyrene fluorescence quenching by plastoquinone and plastoquinol. The observed quenching rate constant is about one order of magnitude less than the quenching rate constants obtained for fluorescence quenching of 9-anthroxystearic acids by ubiquinones in phospholipid membranes (17).

Partition coefficients between 40×10^4 and 60×10^4 were estimated using the modified Stern-Volmer equation (Eq. 11). The accuracy of these values is very sensitive to the slope and intercept of the Stern-Volmer equation. Thus, the different values obtained for the monomer and excimer can be considered to be the same within experimental accuracy. Values between 1.2 and 7.2×10^4 were obtained for the partition coefficient of nitroxide doxyl group substituted stearic acid in egg yolk phosphatidylcholine membrane (18). It is reasonable to expect the hydrophobic TCNQ molecule to have a much higher partition coefficient. High partition coefficients, from 10 to 60×10^4 , were also observed for ubiquinone analogues in different types of membranes (17).

The calculated value for the diffusion coefficient of the TCNQ molecule was $1.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. This value is about the same order of magnitude as those obtained for the diffusion of quinones in phosphatidylcholine liposomes (14) and of 1-pyrenedecanoic acid in a dipalmitoyl-phosphatidylcholine bilayer (19), but about one order of magnitude less than the values for the diffusion of ubiquinones in phospholipid membranes (17).

There is no actual observable proof of the existence of a heterogenous distribution of the quencher molecules above the phase transition, but this possibility is not ruled

out. On the other hand, the appearance of local islands of the quencher molecules would lead to an increase in the diffusion constant, because the concentration of the quencher molecules would decrease outside of the islands. The observed diffusion constant $1.5 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ is, however, low rather than high, as it would be if local islands existed.

It was concluded from IR-spectra measured in anhydrous lipid/TCNQ complexes (Lotta, T. I., A. P. Tulkki, J. A. Virtanen, and P. K. J. Kinnunen, submitted for publication), that TCNQ has only a small perturbation effect on the hydrocarbon chains but interacts with the polar headgroup regions of the lipids. The results of this work in the aqueous phase showed a rather close interaction between the pyrene moieties and TCNQ molecules. Interaction radii of $\sim 1 \text{ nm}$ also rule out the applicability of the Förster theory (20) as an energy transfer mechanism in this case.

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