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Determination of the partition of the tetramisole derivative (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole into liposomal membranes by fluorescence quenching of the membrane probe 8-(2-anthryl)-octanoic acid

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Abstract. Fluorescence quenching has been used to study the partition of the tetramisole derivative (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole into liposomes, consisting of a mixture of egg L-α-phosphatidylcholine, egg phosphatidylethanolamine and dipalmitoylphosphatidic acid (2:1:0.06 w/w/w). The tetramisole derivative quenched the fluorescence of the intramembrane probe 8-(2-anthryl)-octanoic acid. The quenching process could be rationalized by a model for dynamic quenching in which an intermediate excited-state non-emitting complex $(PQ)^*$ between neutral quencher (Q) and excited probe (P^*) is involved:

$$Q_A \rightleftharpoons Q_L + P_L^* \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (PQ)_L^* \overset{k_i}{\rightarrow} P + Q$$

where k_d, k_{-d} and k_i represent the rate constants of complex formation, dissociation and deactivation, respectively. The subscripts A and L denote the aqueous and lipid phases, and the asterisk indicates the excited state. Linear Stern-Volmer plots were obtained from quenching experiments of fluorescence intensities and fluorescence lifetimes. The slopes of the plots were dependent on the lipid volume fraction of the liposomes. Measurement of the reciprocal of the apparent bimolecular quenching rate constant at various lipid volume fractions yielded the partition coefficient K_p and the overall quenching rate constant $k_q[k_q = k_d k_i/(k_i + k_{-d})]$ of the tetramisole derivative. The steady-state measurements were performed at three different pH-values. Time-correlated single photon counting measurements revealed a single-exponential fluorescence decay for 8-(2-anthryl)-octanoic acid in the presence and absence of quencher. The quenching results were in accordance with the model that only the neutral form is capable of partitioning into the lipid phase. Combined average values of 318 and $6.59 \times 10^8\ M^{-1}\,s^{-1}$ were calculated for the partition coefficient and the bimolecular quenching rate constant, respectively, from the steady-state and time-resolved quenching experiments.

Key words: Fluorescence quenching – (±)-5,6-dihydro-6-phenyl-2-n-propyl-imidazo[2,1-b]thiazole – 8-(2-anthryl)-octanoic acid – Partition coefficient – Large unilamellar liposomes

Introduction

The interaction of hydrophobic and amphipathic molecules with biomembranes and phospholipid bilayers has currently become an important research area. An interesting and very sensitive method for studying such interactions is fluorescence quenching. This technique has been used to determine the partition coefficients and diffusion constants of biologically active molecules, including chlorinated hydrocarbon insecticides (Daems et al. 1988, 1989; Omann and Lakowicz 1982) and local anaesthetics (Sikaris and Sawyer 1982).

In the present paper the liposomal partitioning of an ionizable tetramisole derivative, (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole (Fig. 1) was studied by fluorescence quenching of the intramembrane probe 8-(2-anthryl)-octanoic acid (Fig. 1). Tetramisole is a potent broad-spectrum anthelminthic (Thienpont et al. 1966), the activity of which mainly originates from its laevorotatory enantiomer, levamisole (Van den Bossche and Janssen 1969). Levamisole is also known as an organ-specific inhibitor of alkaline phosphatase (Van Belle 1976) and a stimulator of the immunological system (Symoens et al. 1979). In order to evaluate the hypothesis that only the uncharged from of the quencher is able to accumulate into the hydrophobic compartment of the liposomes, steady-state fluorescence quenching measurements at constant liposomal concentration were carried out at dif-

Abbreviations: PC, egg L-α-phosphatidylcholine; PE, egg phosphatidyl-ethanolamine; DPPA, dipalmitoylphosphatidic acid; DCM, 4-dicyanomethylene-2-methyl-6-*p*-dimethylaminostyril-4*H*-pyran; dimethylPOPOP, 2,2′-*p*-phenylenebis(4-methyl-5-phenyloxazole)

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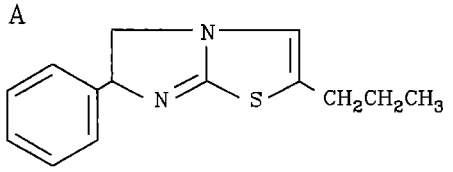
A



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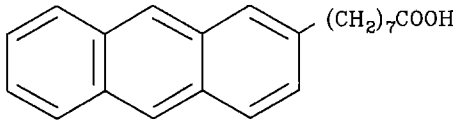


Fig. 1. The chemical structure of **A** (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole and **B** 8-(2-anthryl)-octanoic acid

ferent pH. The membrane partition coefficient of the tetramisole derivative was determined from steady-state and time-resolved fluorescence quenching experiments at several liposomal lipid volume fractions. The steady-state fluorescence quenching experiments were performed at three different pH-values. The results of the fluorescence intensity quenching measurements were analyzed with both Stern-Volmer plots and Scatchard plots.

Materials and methods

Chemicals

(±)-5,6-Dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole cyclohexylaminosulphonic acid was a generous gift from Janssen Pharmaceutica (Beerse, Belgium). Egg L-α-phosphatidyl-choline and egg phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Dipalmitoylphosphatidic acid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Thin-layer chromatography of the phospholipids on silica (solvent chloroform:methanol:water 65:25:4 v/v/v; I_2 staining) showed a single spot. *n*-Octyl-β-D-glucopyranoside was purchased from Boehringer GmbH (Mannheim, FRG). Glycine (gold label) and Tris (gold label) were products of Aldrich Chemie (Brussels, Belgium). Chloroform and methanol (both of fluorimetric grade) were supplied by Merck (Darmstadt, FRG). 8-(2-Anthryl)-octanoic acid was prepared as described by Kaplun et al. (1979). DimethylPOPOP, scintillation grade, was from Eastman Kodak (Rochester, NY, USA). All products were used as received.

Methods

Unilamellar liposomes were prepared by a detergent dialysis method (Zumbühl and Weder 1981; Schwendener et al. 1981). The phospholipid composition of the vesicles was similar to that used for the preparation of vesicle systems with microsome-like properties (Bösterling et al. 1979; Schwarz et al. 1984). A chloroform solution of 38.25 mg of phospholipid (PC:PE:DPPA 2:1:0.06 w/w/w) was dried as a film in a small conical vessel under a stream of argon. Remaining solvent was evaporated in vacuo for 1 h. Micellization was effected by the addition of 75 mg of *n*-octyl-β-D-glucopyranoside in 6 ml of 0.1 M glycine buffer or 0.1 M *Tris*-HCl buffer, each buffer containing 0.2 mM EDTA. To determine the membrane partition coefficient of the tetramisole derivative by steady-state fluorescence quenching, liposomal preparations in 0.1 M glycine buffer at pH 9.0, 9.5 and 10.0 were used. Time-resolved fluorescence experiments were done using liposomes in 0.1 M *Tris*-HCl buffer pH 8.5, containing 0.2 mM EDTA. Clarified lipid suspensions were dialyzed for 16 h at room temperature against 3 l of the solubilization buffer using a Lipoprep device (Diachema, Langnau a.A./Zürich, Switzerland). The vesicle preparation was diluted to the desired lipid concentrations, and the fluorophore, dissolved in a small volume (<10 µl) of methanol, was added to each diluted solution. Uptake of the probe was accomplished by mixing for 15 s on a Vortex. The probe to lipid molar ratio varied between 1:100 and 1:800.

Stock solutions of the tetramisole derivate (250–500 mM) were prepared in 50% aqueous methanol. Microlitre quantities (total volume <20 µl) of quencher were added to the vesicle dilutions with Agla micrometer syringes (Wellcome Research Laboratories, Beckenham, England). The total concentration of methanol in the liposomal suspensions, after addition of probe and increasing amounts of quencher, did not exceed 1% (by volume). At these concentrations, methanol did not have any influence on either the fluorescence spectra or the decay times of the fluorophore.

Phospholipid concentrations were determined by quantitation of inorganic phosphate (Fiske and Subbarow 1925). The density of the vesicles was assumed to be 1 g/ml (Newman and Huang 1975). The average relative molecular mass of the phospholipids was taken as 770.

Negative staining electron microscopy was performed with 1% uranylacetate. For the calculation of the vesicle concentration, it was assumed that the vesicles had a mean diameter of 176 nm (Schwendener et al. 1981) and a bilayer thickness of 4 nm (Israelachvili and Mitchell 1975), and that the entire bilayer volume of the liposomes was available for partition. A vesicle suspension of 1 mg/ml then corresponded to a 4.5×10^{-6} mM solution of vesicles.

The following pK_a-values, determined by potentiometric titration, were communicated by Janssen Pharmaceutica. For (±)-5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole: 8.7 (in water) and 8.2 (in 50% aqueous methanol); for (±)-5,6-dihydro-6-phenyl-2-ethyl-imidazo[2,1-*b*]thiazole: 9.0 (in water) and 8.5 (in 50% aqueous methanol) and for (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo-[2,1-*b*]thiazole: 8.4 (in 50% aqueous methanol). Based on a difference of 0.5 between the pK_a-value in water and the pK_a-value in 50% aqueous methanol for both (±)-5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole and its 2-ethyl derivative, the pK_a-value of the 2-*n*-propyl homologue in water was assumed to be 8.9.

Fluorescence experiments

All fluorescence measurements were performed at 30 °C with 2 ml of liposomal solution in 1 cm × 1 cm cross-section quartz cuvettes. Fluorescence spectra were recorded with a computerized Spex Fluorolog 212/Datamate using double monochromators in both excitation and emission. Excitation was at 360 nm (bandwidth 5 nm). Emission was measured between 370 and 600 nm (bandwidth 2 nm). Fluorescence decays were measured using the time-correlated single photon counting technique (Demas 1983; O'Connor and Phillips 1984; Boens et al. 1987). Sample excitation at 330 nm was achieved with a frequency-doubled, mode-locked, cavity-dumped, synchronously pumped DCM dye laser (Spectra-Physics). The instrumentation has been described in detail elsewhere (Van den Zegel et al. 1986; Boens et al. 1987). The delta function convolution method (Zuker et al. 1985; Boens et al. 1988) was applied to correct for the wavelength dependence of the instrument response function. This procedure requires a reference compound with single exponential kinetics. The decay parameters of a modified sample response function $\tilde{f}_s(t)$ are obtained from the fluorescence decays of sample, $d_s(t)$, and reference, $d_r(t)$, measured under identical instrumental conditions:

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

If the sample's decay $f_s(t)$ is a sum of exponentials, $\tilde{f}_s(t)$ takes on the form:

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

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

DimethylPOPOP was used as reference compound. An iterative reconvolution method based on an algorithm for least-squares estimation of nonlinear parameters (Marquardt 1963) was used to estimate the decay parameters. Several statistical criteria were used to evaluate the goodness-of-fit of calculated decay curves. Briefly, the graphical methods included plots of weighted residuals, the autocorrelation function and the normal probability plot. As numerical tests, the reduced χ^2 and its standard normal deviate Z_{χ^2}, the Durbin-Watson test statistic d and the ordinary runs test statistic Z were calculated. Additionally, the normality of the distribution of the error terms was evaluated by determination of the percentage of the residuals within the [2, −2] interval and the calculation of the mean and the standard deviation of the weighted residuals. A detailed description of the residual analysis has been given elsewhere (Boens et al. 1984; Van den Zegel et al. 1986).

Theory

Fluorescence quenching

The following theory discusses the fluorescence quenching of an intramembrane probe P^* by a dynamic quencher Q, undergoing an acid-base equilibrium of type $QH_A^+ \rightleftharpoons Q_A + H_A^+$ in the aqueous phase (A). If only the neutral form of the quencher partitions into the lipid phase (L) and if the fluorescence quenching occurs via an intermediate non-fluorescent excited-state complex $(PQ)_L^*$, the overall quenching process can be represented by Scheme I:

$$\begin{array}{ccccccc} H_A^+ + Q_A & \overset{K_p}{\rightleftharpoons} & Q_L + P_L^* & \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} & (PQ)_L^* & \overset{k_i}{\rightleftharpoons} & P + Q \\ K_a \uparrow\downarrow & & k_m \downarrow & & & & \\ QH_A^+ & & P_L & & & & \end{array}$$

where K_a and K_p are the dissociation constant and the membrane partition coefficient of the quencher, respectively. The asterisk denotes the excited state. k_d and k_{-d} represent the rate constants of complex formation and dissociation, k_i is the monomolecular rate constant of deactivation of the complex and k_m denote the sum of the rate constants for monomolecular deactivation of the excited singlet state of the fluorophore. The bimolecular quenching rate constant k_q is defined as:

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

According to Scheme I the Stern-Volmer equation for fluorescence quenching is given by (4).

$$I_0/I = 1 + k_q \tau_0 [Q]_L \tag{4}$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively. $[Q]_L$ is the quencher concentration in the lipid phase and τ_0 is the lifetime of the probe in the absence of quencher.

The membrane partition coefficient K_p is defined as:

$$K_p = [Q]_L / [Q]_A \tag{5}$$

with $[Q]_A$ the molar concentration of the quencher in the aqueous phase.

For an ionizable quencher Q dissociating in the aqueous phase, $QH_A^+ \rightleftharpoons Q_A^+ + H_A^+$, the aqueous concentration of non-ionized quencher, $[Q]_A$, is a function of its dissociation constant K_a and of the pH of the aqueous phase:

$$[Q]_A = [QH^+]_A \, 10^{(\mathrm{pH} - \mathrm{p}K_a)}$$

where $[QH^+]_A$ is the aqueous concentration of ionized quencher. The total mass of quencher can be expressed as:

$$[Q]_T V_T = ([QH^+]_A + [Q]_A) V_A + [Q]_L V_L \tag{7}$$

where V_T, V_A and V_L denote the total volume, the volume of the aqueous phase and the volume of the lipid phase, respectively. Introduction of (5) and (6) into (7) yields the quencher concentration in the lipid phase $[Q]_L$:

$$[Q]_L = [Q]_T / \{\alpha_L + (10^{(\mathrm{p}K_a - \mathrm{pH})} + 1) \cdot (1 - \alpha_L)/K_p\} \tag{8}$$

If only the neutral form is capable of partitioning into the lipid phase (see Scheme I), the combination of (4)–(7) leads to the Stern-Volmer equation (9), written as a function of the total quencher concentration, $[Q]_T$

$$I_0/I = 1 + k_q^{\mathrm{app}} \tau_0 [Q]_T \tag{9}$$

with k_q^{app} given by

$$1/k_q^{app} = \left[1/k_q - \frac{10^{(pK_a - pH)}+1}{k_q K_p}\right]\alpha_L + \frac{10^{(pK_a - pH)}+1}{k_q K_p} \quad (10)$$

where $\alpha_L\,(=V_L/V_T)$ is the lipid volume fraction.

Thus, the apparent quenching rate constant k_q^{app} is dependent on the lipid volume fraction α_L. k_q^{app}-values are obtained as the slopes of the Stern-Volmer plots, divided by τ_0. By plotting $1/k_q^{app}$-values versus the lipid volume fraction α_L, the partition coefficient K_p and the overall quenching rate constant k_q can be calculated from (10).

The fluorescence δ-response function $f_s(t)$ which can be derived for the photophysical processes depicted in Scheme I, is given by (Birks 1970):

$$f_s(t) = \alpha_1 \exp(-\lambda_1 t) + \alpha_2 \exp(-\lambda_2 t) \quad (11)$$

with

$$\alpha_1/\alpha_2 = (X-\lambda_2)/(\lambda_1 - X) \quad (12)$$

$$\lambda_{1,2} = 0.5\{X + Y \mp [(X-Y)^2 + 4k_d k_{-d}[Q]_L]^{1/2}\} \quad (13)$$

$$X = k_m + k_d[Q]_L \quad (14)$$

$$Y = k_i + k_{-d} \quad (15)$$

α_1 and α_2 are pre-exponential factors, λ_1^{-1} and λ_2^{-1} are decay times, and X^{-1} and Y^{-1} represent the lifetime of the probe P^* and of the complex $(PQ)^*$, respectively.

If $k_{-d} \ll k_i$, (3) reduces to $k_q = k_d$ and thus the rate of quenching is determined by the diffusion step. The fluorescence δ-response function $f_s(t)$ then simplifies to a single exponential decay function given by

$$f_s(t) = \alpha \exp(-Xt) \quad (16)$$

The Stern-Volmer relation for the lifetime of the locally excited singlet state is given by

$$\tau_0 X = I_0/I = 1 + k_d \tau_0 [Q]_L = 1 + k_d^{app} \tau_0 [Q]_T \quad (17)$$

The expression for the apparent diffusion rate constant k_d^{app} (18) is obtained from (10) by replacing k_q by k_d:

$$1/k_d^{app} = \left[1/k_d - \frac{10^{(pK_a - pH)}+1}{k_d K_p}\right]\alpha_L + \frac{10^{(pK_a - pH)}+1}{k_d K_p} \quad (18)$$

Apparent diffusion rate constants k_d^{app} are derived as the slopes of Stern-Volmer plots (17), divided by τ_0. By plotting the $1/k_d^{app}$-values versus the lipid volume fraction α_L, k_d and the partition coefficient K_p can be calculated from (18). If the probe decays mono-exponentially, the diffusion rate constant is obtained from both time-resolved and stationary quenching measurements.

Partition versus binding model

An alternative treatment to analyze fluorescence quenching data (Encinas and Lissi 1982; Blatt et al. 1984) allows one to distinguish between partition and/or binding of a quencher. The method is based on the assumption that I_0/I at a particular quencher concentration is only dependent on the average number of quenchers per vesicle, $\langle Q \rangle$, regardless of the quenching mechanism. Scheme II describes the distribution of a quencher between an aqueous phase and a lipid bilayer:

$$\begin{array}{ccc} Q_a + v & \overset{K_{eq}}{\rightleftharpoons} & Q_l \\ K_a \downarrow\uparrow H^+ & & \\ QH_a^+ & & \end{array}$$

where Q_a and QH_a^+ pertain to the neutral and ionized form of the quencher in the aqueous phase, respectively, Q_l represents neutral quencher in the lipid phase, v denotes vesicle and K_{eq} is an equilibrium distribution constant given by

$$K_{eq} = \frac{[Q_l]}{[Q_a][v]} \quad (19)$$

$[Q_l]$, $[Q_a]$ and $[v]$ are defined with respect to the total volume V_T. The dissociation constant K_a relates to the aqueous volume V_A. Presuming that $V_A \approx V_T$, one obtains

$$[QH_a^+] = [Q_a]10^{(pK_a - pH)} \quad (20)$$

The average number of quencher molecules per vesicle, $\langle Q \rangle$, is expressed as

$$\langle Q \rangle = [Q_l]/[v] \quad (21)$$

The total concentration of quencher $[Q]_T$ is given by

$$[Q]_T = [Q_a] + [QH_a^+] + [Q_l] \quad (22)$$

Substitution of (20) and (21) into (22) gives

$$[Q]_T = \{\langle Q \rangle \cdot (1 + 10^{(pK_a - pH)})/K_{eq}\} + \langle Q \rangle [v] \quad (23)$$

If binding and partition of neutral quencher occur simultaneously, the equilibrium distribution constant is given by (Blatt et al. 1984)

$$K_{eq} = \frac{\langle Q \rangle}{[Q_a]} = \bar{V}_L K_p + \frac{n K_b^{app}}{1 + K_b^{app}[Q_a]} \quad (24)$$

where n represents the number of equivalent binding sites, K_b^{app} is the apparent binding constant and $\bar{V}_L$ is the molar volume of the vesicles. For an ionizable quencher, the binding of both the ionized and the neutral form should be considered.

The analysis of fluorescence quenching data proceeds as follows. From Stern-Volmer plots measured at several lipid (vesicle) concentrations, sets of pairs of quencher concentrations and vesicle concentrations corresponding to given I_0/I levels are obtained. K_{eq} and $\langle Q \rangle$ can then be obtained by plotting $[Q]_T$ against $[v]$, each particular level of I_0/I yielding one pair of these values, secondary plots (K_{eq} versus $\langle Q \rangle$) of which are, in fact, Scatchard plots. These make it possible to discriminate between binding and/or partition. If K_{eq} is independent of $\langle Q \rangle$, the quencher partitions into the vesicles while a linear relationship with a negative slope indicates binding. When both binding and partition occur, K_{eq} decreases with increasing $\langle Q \rangle$ asymptotically approaching a constant value of $\bar{V}_L K_p$.

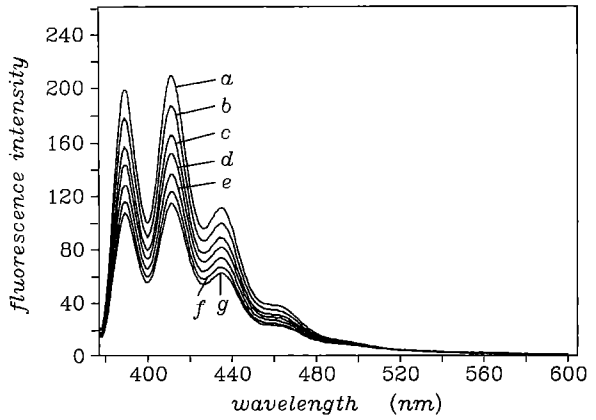


Fig. 2. Quenching of the fluorescence spectrum of 8-(2-anthryl)-octanoic acid solubilized in liposomes of PC:PE:DPPA (2:1:0.06 w/w/w) by the tetramisole at total concentrations of: (*a*) 0 mM; (*b*) 0.25 mM; (*c*) 0.5 mM; (*d*) 0.75 mM; (*e*) 1 mM; (*f*) 1.25 mM and (*g*) 1.5 mM. The vesicle suspension had a phospholipid concentration of 2.26 mg/ml and a lipid to probe molar ratio of 400. Excitation was at 360 nm. 0.1 mM glycine buffer pH 9.5, containing 0.2 mM EDTA, was used for the measurements

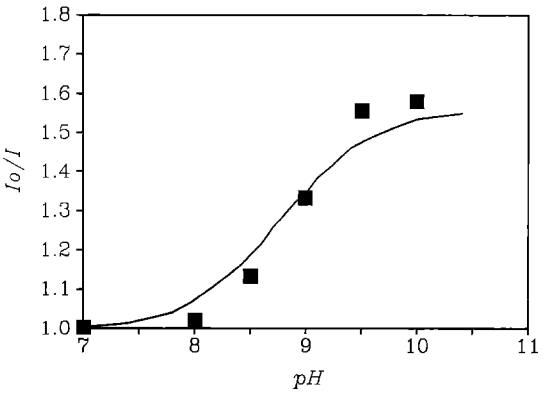


Fig. 3. pH dependence of the quenching of 8-(2-anthryl)-octanoic acid by (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole (1 mM) in PC:PE:DPPA (2:1:0.06 w/w/w) vesicles (1 mg of phospholipid/ml solution) at 30 °C. The measurements of the fluorescence quenching (■) were performed, using 0.1 M *Tris*-HCl buffer pH 7.0, 8.0 and 8.5, and 0.1 M glycine buffer pH 9.0, 9.5 and 10.0, each buffer containing 0.2 mM EDTA. The titration curve (—) was calculated from (4) accepting τ_0 and k_q-values of 3.5 ns and $6.59 \times 10^8\ M^{-1}\ s^{-1}$, respectively. Local quencher concentrations in the lipid phase $[Q]_L$ were obtained from (8)

Results

Choice of the test material

The liposomes obtained by removal of *n*-octyl-β-D-glucopyranoside were unilamellar vesicles with diameters varying between 150 and 250 nm, as judged by negative staining electron microscopy (results not shown).

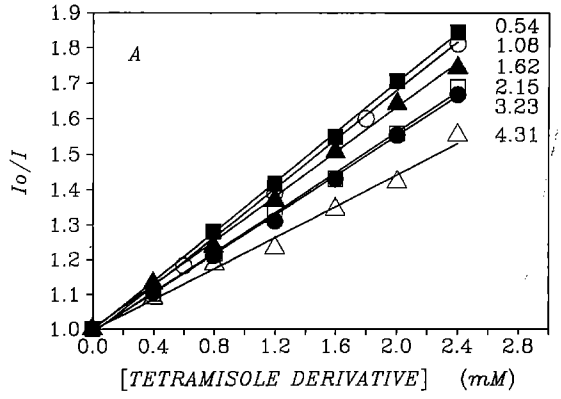
8-(2-Anthryl)-octanoic acid was found to be a suitable fluorescent probe to study the distribution of (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole between buffer and a liposomal membrane. The highly lipophilic character of the anthracene probe favoured its complete incorporation into the interior core of the membrane. No excimer formation was observed when the probe: lipid molar ratio varied between 1:100 and 1:800. The fluorophore was efficiently quenched by the tetramisole derivative (Fig. 2) with no evidence of exciplex emission. No detectable photo-decomposition of the probe occurred in either the presence or absence of quencher during the experiment.

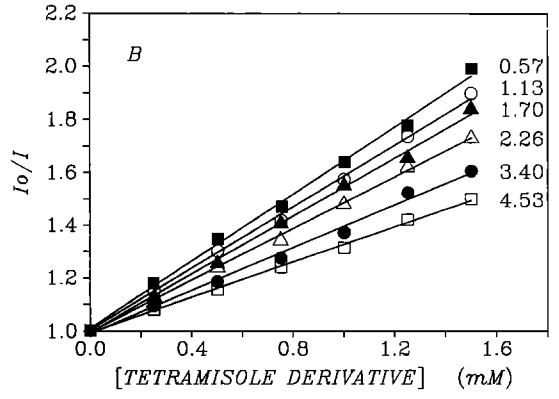
pH dependence of quenching

Equation (10) is valid provided that only the neutral form of the quencher partitions into the liposomal membrane. In order to confirm this assumption, a pH titration of the quenching of 8-(2-anthryl)-octanoic acid in liposomes was performed with 1 mM tetramisole derivative. The results are shown in Fig. 3. Quenching did not occur at pH 7.0 but increased with the decreasing concentration of ionized tetramisole molecules as the pH increased. The titration curve calculated according to (4) based on the assumption that only the uncharged form of the quencher partitions into the hydrophobic part of the liposomal bilayer, fitted well the experimental I_0/I data points (Fig. 3), supporting the veracity of the assumption made. To investigate if the quenching activity originated solely from the neutral form of the tetramisole compound, a quenching experiment was performed in methanol/0.1 M phosphate buffer pH 6.0 (1:1 v/v). Methanol was included to ensure that both fluorophore and quencher remained soluble. Quenching occurred at pH 6.0 which indicated that both the neutral and the cationic form of the tetramisole derivative were capable of quenching. The pK_a-value derived from the fluorescence quenching results was 8.9 which is in agreement with the value assumed on the basis of the potentiometric titrations.

Determination of the partition coefficient

Fluorescence intensity measurements. Fluorescence intensities of 8-(2-anthryl)-octanoic acid in liposomes of PC:PE:DPPA (2:1:0.06 w/w/w) were measured at different lipid volume fractions. Figure 4 A, B and C shows the Stern-Volmer plots based on fluorescence intensities measured at pH 9, 9.5 and 10, respectively. Linear plots indicated that dynamic fluorescence quenching occurred. The k_q^{app}-values decreased as the amount of lipid was increased (Table 1). The quenching efficiency was smallest at pH 9.0. For each experiment, a linear relationship was found between the reciprocal of the apparent quenching rate constant $(k_q^{app})^{-1}$ and the lipid volume fraction α_L, implying a true partitioning process (Fig. 5). The values of K_p and k_q, calculated according to (10), and their respective standard deviations are given in Table 2. Both the partition coefficients K_p and the quenching rate constants k_q obtained from the steady-state quenching experiments at three different pH-values were identical within experimental error (<20%).





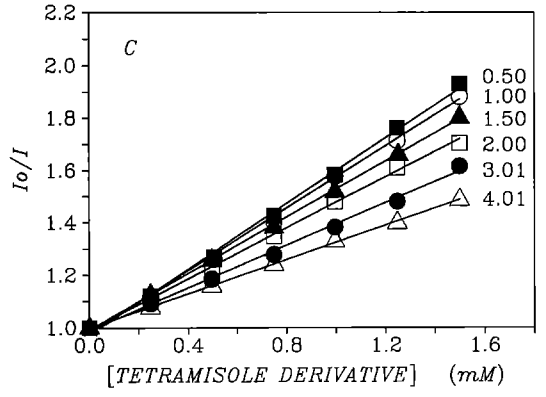


Fig. 4 A–C. Stern-Volmer plots for the quenching of fluorescence spectra of 8-(2-anthryl)-octanoic acid in vesicles of PC:PE:DPPA (2:1:0.06 w/w/w) by (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole at different lipid volume fractions. The numbers represent the phospholipid concentration of the liposomes in mg/ml. The measurements were performed, using 0.1 M glycine buffer containing 0.2 mM EDTA at: **A** pH 9.0; **B** pH 9.5 and **C** pH 10.0

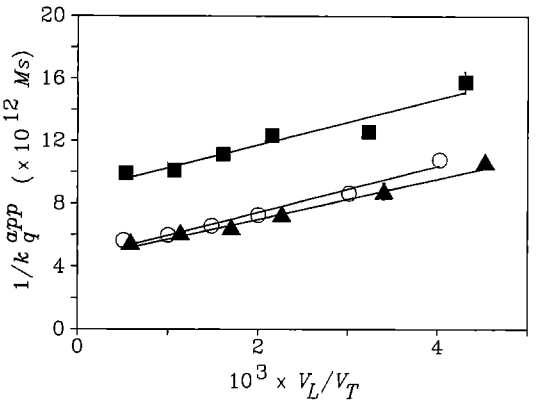


Fig. 5. Dependence of the reciprocal of the apparent bimolecular quenching rate constant $(k_q^{app})^{-1}$ on the lipid volume fraction α_L of the liposomes. The apparent quenching constants were calculated from the data in Fig. 4. The vesicle density was assumed to be 1 mg/ml. The symbols represent the fluorescence intensity measurements at: pH 9.0 (■); pH 9.5 (▲) and pH 10.0 (○)

Table 1. Apparent bimolecular quenching rate constants ($\times 10^{-10}$ M^{-1} s^{-1}) at different lipid volume fractions α_L ($\times 10^3$) for the quenching of 8-(2-anthryl)-octanoic acid by the tetramisole derivative, obtained from Stern-Volmer plots of intensity (k_q^{app}) and decay time (k_d^{app}) measurements

pH 9.0		pH 9.5		pH 10.0		pH 8.5	
α_L	k_q^{app}	α_L	k_q^{app}	α_L	k_q^{app}	α_L	k_d^{app}
0.54	10.06	0.57	18.22	0.50	17.90	1.0	3.43
1.08	9.88	1.13	16.90	1.00	16.98	2.0	3.10
1.62	8.99	1.70	15.77	1.50	15.28	3.0	2.80
2.15	8.11	2.26	14.04	2.00	13.87	4.0	2.91
3.23	7.96	3.40	11.64	3.01	11.57		
4.31	6.34	4.53	9.48	4.01	9.27		

Table 2. Membrane partition coefficients $K_p \pm$ standard deviation of (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole and quenching rate constants $k_q \pm$ standard deviation, obtained by both steady-state and time-resolved fluorescence quenching measurements of 8-(2-anthryl)-octanoic acid in liposomes of PC:PE:DPPA (2:1:0.06 w/w/w)

pH	K_p	$k_q \times 10^{-8}$ (M^{-1} s^{-1})
Fluorescence intensity measurements		
9.0	303 ± 59	6.74 ± 0.94
9.5	365 ± 38	7.75 ± 0.55
10.0	360 ± 47	6.71 ± 0.51
Fluorescence lifetime measurements		
8.5	243 ± 118	5.15 ± 2.13

Fluorescence decay measurements. In the absence of quencher, the fluorescence decay of 8-(2-anthryl)-octanoic acid incorporated into liposomes of PC:PE:DPPA (2:1:0.06 w/w/w) could be fitted to a single exponential decay function with a lifetime $\tau_0 = 3.5 \pm 0.03$ ns. No change in lifetime τ_0 was observed for the different probe:lipid molar ratios, giving further evidence for the absence of excimer formation. The fluorophore's decay remained single exponential upon addition of tetramisole derivative (Fig. 6). The fact that only one lifetime contributed to the fluorophore's decay is an indication that the probe was completely solubilized in the hydrophobic region of the membrane. Fluorescence lifetimes decreased in the presence of quencher. Figure 7 shows the Stern-Volmer plots based on fluorescence lifetimes for different lipid volume fractions α_L. The plot of the reciprocal of the

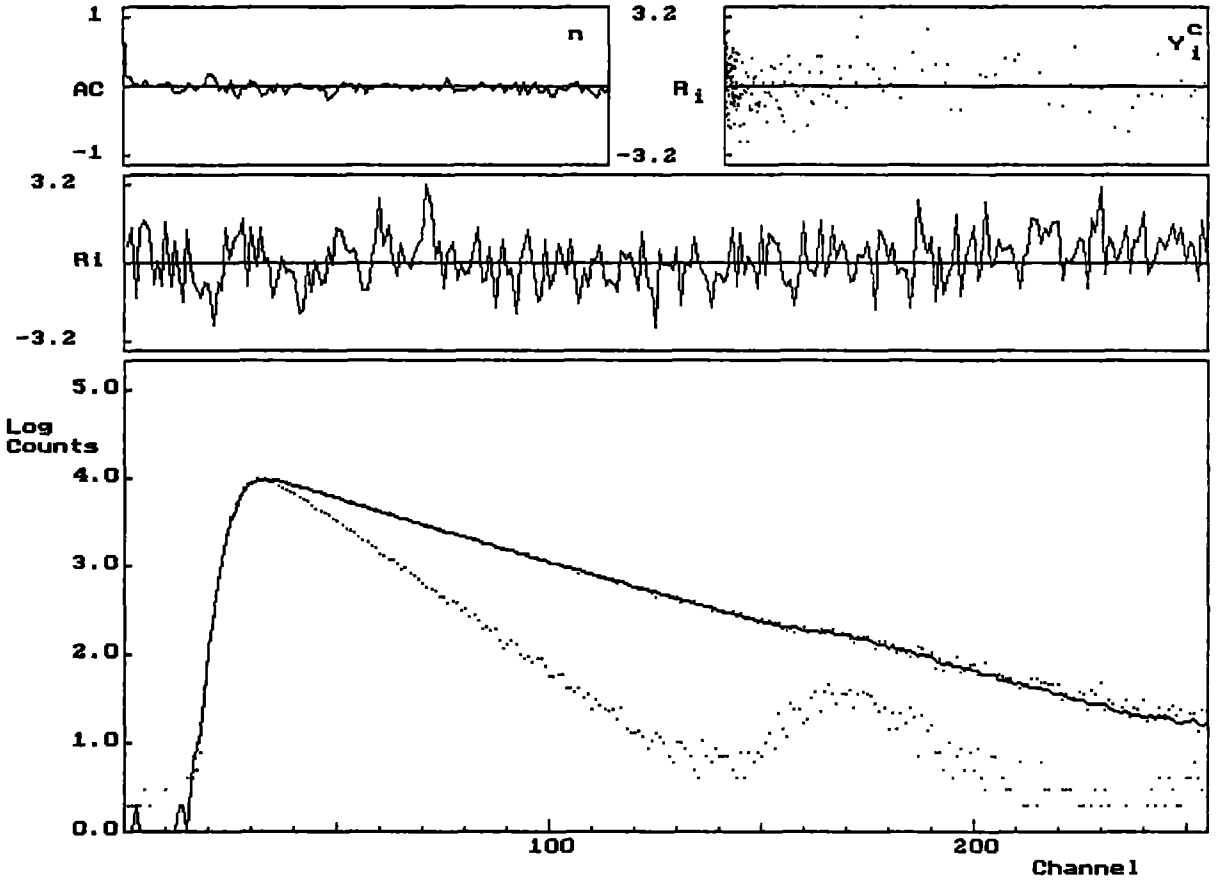


Fig. 6. Fluorescence decay ($\lambda_{ex} = 330$ nm; $\lambda_{em} = 405$ nm) of 8-(2-anthryl)-octanoic acid labelled vesicles of PC:PE:DPPA (2:1:0.06 w/w/w) in 0.1 M *Tris*-HCl buffer pH 8.5 containing 0.2 mM EDTA in the presence of (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]-thiazole. The experimental decay (point plot) was fitted to a single-exponential decay function (solid line) between channels 1 and 255 (channel width 0.098 ns). The measured fluorescence decay of the reference (dimethylPOPOP in isooctane) is depicted as a point plot. The autocorrelation function, AC, and the weighted residuals, R_i, versus channel number i and versus calculated values y_i^c are given at the top of the figure. Estimated decay parameters were: $\alpha = 0.84$, $\tau_s = 3.10$ ns and $\tau_r = 1.47$ ns. Goodness-of-fit statistical parameters: $\chi^2 = 1.08$, $Z_{\chi^2} = 0.84$, ordinary runs test statistic $Z = -2.54$, Durbin-Watson test statistic $d = 1.78$, 95.34% of weighted residuals in $[-2, 2]$ interval with 0.07 as mean and 1.03 as standard deviation

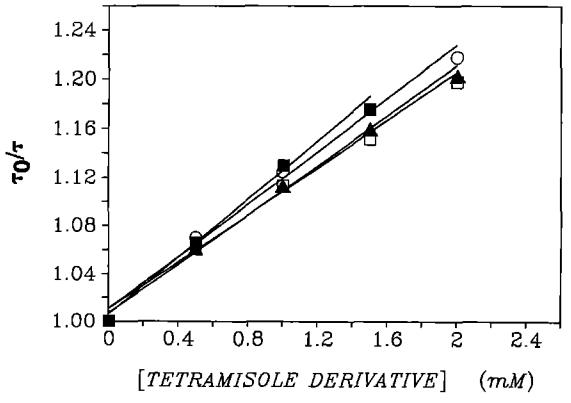


Fig. 7. Stern-Volmer plots for the quenching of fluorescence lifetimes of 8-(2-anthryl)-octanoic acid by (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole in liposomes of PC:PE:DPPA (2:1:0.06 w/w/w) at different lipid volume fractions α_L: 1×10^{-3} (■); 2×10^{-3} (○); 3×10^{-3} (□) and 4×10^{-3} (▲). The measurements were performed, using 0.1 M *Tris*-HCl buffer pH 8.5, containing 0.2 mM EDTA, at 30 °C

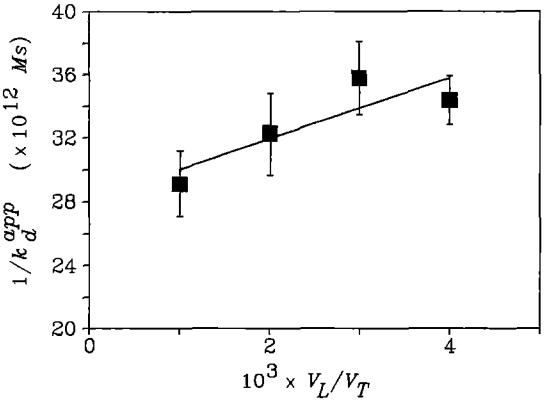
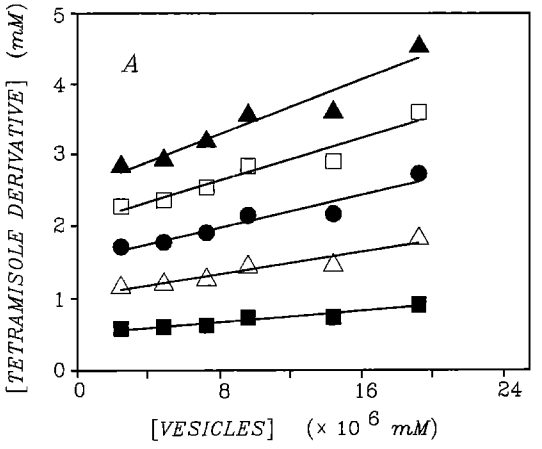
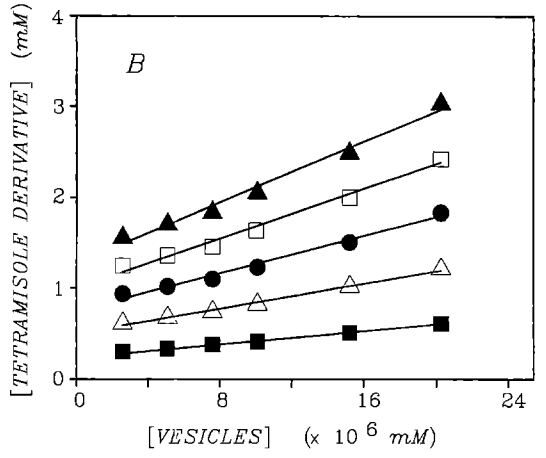


Fig. 8. Effect of the lipid volume fraction α_L of the liposomal suspension on the reciprocal of the apparent bimolecular diffusion rate constant $(k_d^{app})^{-1}$. The apparent rate constants for diffusion were calculated from the slopes of the Stern-Volmer plots shown in Fig. 7. The error bars represent ± one standard deviation





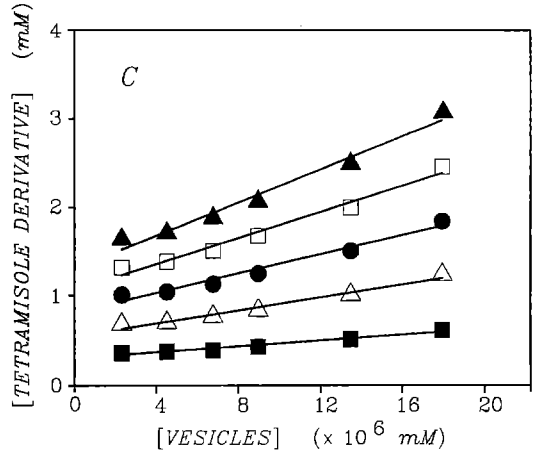


Fig. 9 A–C. Quenching of 8-(2-anthryl)-octanoic acid by the tetramisole derivative at: **A** pH 9.0; **B** pH 9.5 and **C** pH 10.0. The data were plotted according to (23) at I_0/I levels of 1.2 (■); 1.4 (△); 1.6 (●); 1.8 (□) and 2.0 (▲)

apparent diffusion rate constant $(k_q^{app})^{-1}$ vs. the lipid volume fraction α_L (Table 1 and Fig. 8) could be fitted by a straight line ($r = 0.86$) with a slope of 1912 (S.D. = 806) and an intercept of 28.1 (S.D. = 2.2). The values of the partition coefficient K_p and of the diffusion rate constant k_d, calculated from the time-resolved fluorescence measurements, were within the experimental error (Table 2) in accordance with the K_p and k_q values obtained from

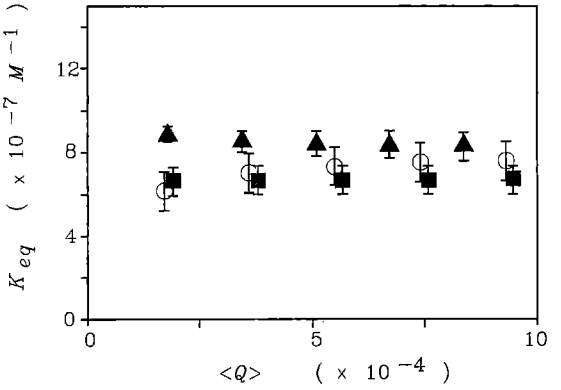


Fig. 10. Scatchard plot for the quenching of 8-(2-anthryl)-octanoic acid by the tetramisole derivative in PC:PE:DPPA (2:1:0.06 w/w/w) vesicles at: pH 9.0 (■); pH 9.5 (▲) and pH 10.0 (○). The error bars represent ± one standard deviation

Table 3. Average number of quenchers $\langle Q \rangle$ per vesicle ± standard deviation at different levels of I_0/I for the quenching of 8-(2-anthryl)-octanoid acid measured at pH 9.0, 9.5 and 10.0

I_0/I	$\langle Q \rangle$ ($\times 10^{-4}$)		
	pH 9.0	pH 9.5	pH 10.0
1.2	1.9 ± 0.3	1.8 ± 0.04	1.7 ± 0.2
1.4	3.8 ± 0.5	3.4 ± 0.2	3.6 ± 0.3
1.6	5.7 ± 0.8	5.1 ± 0.3	5.5 ± 0.5
1.8	7.6 ± 1.0	6.7 ± 0.4	7.4 ± 0.6
2.0	9.5 ± 1.3	8.4 ± 0.5	9.3 ± 0.7

steady-state measurements. The standard deviations (40–50%) were, however, much higher than those obtained from steady-state measurements. Averaging the results in Table 2 gave mean values of 318 and 6.59×10^8 M^{-1} s^{-1} for K_p and k_q, respectively.

Analysis of the steady-state fluorescence quenching data with the partition versus binding model

Figure 9 shows plots of $[Q]_T$ versus the vesicle concentration $[v]$ at several I_0/I levels for the 8-(2-anthryl)-octanoic acid quenching. Values of the average number of quenchers $\langle Q \rangle$ per vesicle (Table 3) and of the equilibrium distribution constant K_{eq} were determined using (23). In each quenching experiment, K_{eq} was independent of $\langle Q \rangle$ (Fig. 10) which indicated that the tetramisole partitions into the membrane with no binding. The values calculated for K_{eq} at a level of $I_0/I = 2.0$ ($6.7 \pm 0.7 \times 10^7$ M^{-1}, $8.3 \pm 0.7 \times 10^7$ M^{-1} and $7.6 \pm 0.9 \times 10^7$ M^{-1} for pH 9.0, 9.5 and 10.0, respectively) were identical within experimental error.

Discussion

Liposomes with diameters greater than 100 nm are considered as large unilamellar vesicles (Szoka and Papahadjopoulos 1980). The preparations produced for the quenching studies, using the dergent dialysis technique,

are of this type. Similar large vesicle sizes were reported by Schwendener et al. (1981) and Schwarz et al. (1984) for liposomal preparations produced from *n*-octyl-β-D-glucopyranoside:phospholipid mixed micelles. The use of large unilamellar vesicles avoids problems of increased lipid disorder or vesicle instability, which are inherently associated with small liposomal systems (Hope et al. 1986).

Anthroyloxy fatty acids have frequently been used as membrane fluorescent probes (Thulborn and Sawyer 1978; Sikaris and Sawyer 1982; Rooney et al. 1983; Chatelier et al. 1984). We used 8-(2-anthryl)-octanoic acid to probe the hydrophobic core of liposomal membranes. The assumption of complete uptake of the probe in the lipid bilayer phase is strongly supported by its single exponential fluorescence decay.

From quenching studies with *N,N*-dimethylaniline (Sikaris et al. 1981) and local anaesthetics (Sikaris and Sawyer 1982) it was concluded that ionized molecules are unable to partition into neutral bilayers. The pH titration of quenching of 8-(2-anthryl)-octanoic acid suggested that a similar situation occurred with the tetramisole derivative. However, it was still possible that both charged and neutral forms partition but only neutral molecules were capable of quenching. The experiments in methanol/phosphate buffer showed that the latter assumption could be excluded and that therefore only the neutral species was taken up into the hydrophobic region of the bilayer.

Divergence from linearity of the Stern-Volmer relationship for fluorescence intensities is observable: (1) if the quencher forms a non-fluorescent complex with the ground state of the fluorophobe, (2) if a significant fraction of the probe molecules is not accessible to the quenchers, or (3) if there is partition of the fluorophore or quencher molecules between different phases of the membrane. None of the above situations occurred for the quenching of 8-(2-anthryl)-octanoic acid by the tetramisole derivative. The linear Stern-Volmer plots obtained from both steady-state and fluorescence decay measurements indicated that dynamic quenching occurred with formation of a transient intermediate excited-state non-emitting complex, consistent with scheme I. The complex was non-fluorescent since the presence of quencher only induced a decrease of the intensity of the fluorescence spectrum but no change in spectral distribution.

Two types of association have been considered to describe the interaction of lipid soluble compounds with biomembranes and phospholipid bilayers, namely, partitioning and binding. Neglecting the anisotropic structure of a lipid bilayer, partitioning implies a constant ratio, at equilibrium, between the solute's concentrations in the membrane and the aqueous phase. In the case of a binding process, the association can be described by a binding equilibrium constant. The latter process frequently occurs in vivo when positively charged compounds interact electrostatically with negatively charge phospholipid head groups. Analysis of the quenching results revealed that, under the experimental conditions, the tetramisole derivative partitions into the PC:PE:DPPA liposomes rather than binding to them. Several findings support this conclusion. First, at each pH, linear Stern-Volmer plots were obtained and the reciprocal of the apparent quenching rate constant was linearly dependent on the lipid volume fraction of the liposomes. Second, the Scatchard plots of the equilibrium distribution constant K_{eq} were independent of the average number of quenchers per vesicle. Further evidence for the partition equilibrium comes from the pH titration of quenching. Although charged tetramisole molecules were capable of quenching in an isotropic solution, the fluorophore was not quenched in liposomes at a pH $\leq$ 7.0, indicating that no measurable electrostatic interaction occurred between ionized quenchers and the negatively charged bilayer. This was probably due to the low charge density of the vesicles since DPPA constituted only about 2% of the phospholipid material, and to the relatively high ionic strength of the buffers, reducing the negative surface potential of the liposomal membranes.

Time-correlated single photon counting has become a standard technique to measure fluorescence decays. Application of the technique revealed that the fluorescence decays of 8-(2-anthryl)-octanoic acid in the presence and the absence of tetramisole derivative could be adequately described by a single exponential decay law.

The membrane partition coefficient of the tetramisole derivative was determined from steady-state (10) and time-resolved (18) measurements. In each steady-state experiment, the standard deviation of the K_p-value did not exceed 20%, a percentage indicative for the accuracy of the steady-state fluorescence quenching technique. A higher standard deviation (about 50%) was calculated for the partition coefficient obtained from time-resolved experiments. The values for the partition coefficient K_p of the tetramisole derivative, determined from both types of fluorescence measurements at different pH's were comparable. The similarity between the different K_p-values was in agreement with the meaning of partition. Indeed, a partition coefficient is assumed to be constant and should thus be independent of the pH of the aqueous medium.

The values of the quenching rate constant k_q, obtained from the intensity measurements, were realistic and their standard deviations were less than 15%. For the diffusion rate constant, a standard deviation of about 40% was determined. The k_d-value and the different k_q-values were identical within the experimental error which was in accordance with the mono-exponentiality of the fluorescence decay. The good agreement between the values of K_p and k_q, respectively, measured at different pH-values, further confirmed that the model for the partitioning of ionizable quenchers is applicable to describe the association of (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole with liposomal membranes.

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