ΔpH-induced fluorescence quenching of 9-aminoacridine in lipid vesicles is due to excimer formation at the membrane

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ABSTRACT The fluorescence of 9-aminoacridine (9-AA) is quenched in vesicular suspensions containing negatively charged lipid headgroups (e.g., phosphatidylserine) upon imposition of a transmembrane (inside acidic) pH-gradient. It is shown that this fluorescence loss is accompanied by the formation of 9-AA dimers that undergo a transition in the dimer excited state to a dimer-excimer state. This result has been obtained on the basis of the specific dimer fluorescence excitation and hypochromic absorbance spectra that are redshifted by maximally 275 cm^{-1} (4.4 nm) with respect to the corresponding monomer spectra, as well as by the detection of the characteristic broad excimer emission band, centered at 560 nm. The existence of the spectrally distinct dimer-excimer is further corroborated by fluorescence lifetime measurements that indicate an increased lifetime of up to 24 ns for this complex as compared with the normal monomer fluorescence lifetime of 16 ns. The formation of this dimer-excimer complex from the monomers can be reversed completely and the original monomeric spectral properties restored after the abolishment of the electrochemical proton gradient. In addition to the ΔpH-induced dimer redshift in absorbance and fluorescence excitation, a further small redshift in monomer absorbance, fluorescence excitation, and emission spectra is observed due solely to the presence of the negatively charged phospholipid headgroups.

INTRODUCTION

When a pH-difference (inside acidic) is imposed across the membrane of negatively-charged phospholipid vesicles, the fluorescence of externally added 9-aminoacridine (9-AA) becomes partially quenched (Deamer et al., 1972; Casadio and Melandri, 1977; Fiolet et al., 1974; Grzesiek and Dencher, 1985, *a* and *b*). This effect has been used to justify (Casadio and Melandri, 1977) the application of 9-AA as a ΔpH-probe as proposed by Schuldiner et al. (1972) in a variety of biological systems, e.g., photosynthetic bacteria (Casadio et al., 1974; Casadio and Melandri, 1985), chloroplast thylakoids (Hope and Matthews, 1985; Haraux and De Kouchkovsky, 1980; Pfister and Homann, 1986), gastric microsomal vesicles (Lee and Forte, 1978), skeletal muscles (Vigne et al., 1984), mesophyll vacuoles (Weigel and Weis, 1984), mast cells (De Young et al., 1987), submitochondrial particles (Huang et al., 1983), and also in studies of the proton/hydroxide ion permeability across vesicular lipid membranes (Deamer and Nichols, 1983). A model mechanism (Schuldiner et al., 1972) has been introduced in order to calculate pH-differences across the membrane from the measured fluorescence changes. This model is essentially based on an assumed high permeability of the unprotonated (neutral) 9-AA (pK 9.99 [Albert, 1966]) and the assumed low permeability of the protonated acridinium ion. According to this scheme, in the presence of an appropriate transmembrane pH-difference, the dye accumulates in the intracellular compartment where it is further assumed to lose its fluorescence completely. Although many different quenching mechanisms have been taken into consideration (Deamer et al., 1972; Huang et al., 1983; Kraayenhof et al., 1976; Searle and Barber, 1978) such as screening of the dye molecules, protonation and pK shifts, interactions with membrane bound molecules, concentration (collisional) quenching and aggregation, as well as excimer formation, no direct physical justification for any of these possibilities has been reported. Furthermore the quenching in the different systems mentioned above may be due to different mechanisms. In this chapter experimental evidence is given that ΔpH-induced 9-AA fluorescence quenching in phospholipid vesicles is due to excimer formation at the membrane level. This result has far-reaching implications for the use of 9-AA and related compounds as probes for quantitative determinations of pH-gradients in artificial and natural membrane systems.

EXPERIMENTAL

Materials

Phosphatidylserine (PS) (bovine brain, Avanti Polar Lipids Inc., Birmingham, AL), 9-aminoacridine-hydrochloride (Fluka, Neu-Ulm, FRG), valinomycin (Boehringer Mannheim Diagnostics Inc., Houston,

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TX), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Aldrich Chemical Co., Inc., Milwaukee, WI), sodium phosphate and potassium sulphate (E. Merck Darmstadt, FRG) were used without further purification. Throughout all preparations deionized water ($\kappa \leq 0.1$ μS/cm, Seradest, Ransbach-Baumbach, FRG) and chloroform (fluorescence spectroscopy grade, E. Merck) were used. Double distillation of the deionized water lead to the same results in fluorescence lifetime experiments.

Liposome preparation

Aliquots of phospholipid (PS) were taken from stock solutions in chloroform and dried under vacuum (1 mbar) for at least 12 h at room temperature after solvent evaporation. The dried lipid film was then hydrated in an aqueous solution of 50 mM K_2SO_4 and 10 mM sodium phosphate at pH 5.7 to a final lipid concentration of 10 mg/ml. This sample was sonicated to clarity, i.e., 30 min., with a bath-type sonifier (Sonorex RK 100H, 35 kHz, Bendelin Electronics, Berlin, FRG) at room temperature. Vesicles obtained by this procedure were then further diluted in the same buffer to a final lipid concentration of 0.5 mg/ml. They have a mean diameter of ~60 nm as judged by negative-stain electron microscopy. In order to establish pH-differences across the vesicular membrane, 0.5 ml of the liposome suspension was mixed with 0.5 ml of 50 mM K_2SO_4, 100 mM sodium phosphate and 30 μM 9-AA at pH 8.0 and the obtained sample was placed in a 4 × 10 mm^2 quartz fluorescence cuvette. This final sample has a bulk pH-value (outside the vesicles) of 7.6, whereas the inner vesicular volume is assumed to be still at pH 5.7 immediately after mixing. The final 9-AA concentration of 15 μM was chosen in the range where maximal relative quenching occurs (Grzesiek and Dencher, 1988) and is well below the bulk concentration where dimerization occurs ($k_D = 10^3$ M^{-1} [Gangola et al., 1981]).

Optical measurements

Absorbance and fluorescence spectra have been recorded on a UV-Visible recording spectrophotometer UV 240 equipped with a light-scattering compartment and a spectrofluorometer RS-540 (Shimadzu Scientific Instruments Inc., Columbia, MD), respectively. If necessary, appropriate glass cut-off filters (Schott Optical Glass Inc., FRG) were placed in the fluorescence beam in order to prevent scattered excitation light from passing through higher harmonics of the fluorescence detection monochromator. Fluorescence lifetime measurements were carried out on a conventional single-photon counting apparatus with a photomultiplier (R955, Hamamatsu, Japan) and Ortec Electronics (Oak Ridge, TN). The light source was a nanosecond-gated flashlamp (Edinburgh Instruments Ltd., Edinburgh, Scotland). The lamp was run at 6 kV, 40 kHz with H_2 gas at a pressure of 0.4 bar, and a spark gap of 1 mm. The excitation wavelength was selected by a monochromator (Jobin Yvon), the emission wavelength by low fluorescent cut-off filters (Schott Optical Glass Inc.) as indicated. Data analysis were performed on a HP 1000 computer (Hewlett-Packard Co., Palo Alto, CA) interfaced to the multichannel analyzer, using a Marquardt nonlinear least squares algorithm with χ^2_v to test the goodness of the fit. All experiments were performed at room temperature, i.e., ~23°C.

RESULTS

pH-gradients across the membrane of PS-vesicles were imposed as described. Directly after mixing, the aqueous phase inside the vesicles consisted therefore of 10 mM sodium phosphate, 50 mM K_2SO_4 pH 5.7, whereas the outside vesicular aqueous medium was 55 mM sodium phosphate, 50 mM K_2SO_4, and 15 μM 9-AA at pH 7.6. Under these conditions, 9-AA fluorescence excited at 400 nm and detected at ≥420 nm becomes quenched by ~75% with a time constant of 0.6 s at 25°C, when mixing is accomplished and followed in a stopped-flow apparatus (Grzesiek and Dencher, 1988). The extent and kinetics of 9-AA fluorescence quenching are not altered in a significant way, when the sodium phosphate concentration in the outside medium is changed to 155 mM (not shown). Therefore, a major effect of the difference between extra- and intravesicular ionic strength on the quenching is excluded. This quenching as well as optical spectra and fluorescence lifetimes remain without change for at least 30 min after the initial fluorescence decrease, which is time enough to carry out all the measurements. It must be emphasized that this is not the case for liposomes of other phospholipid composition, e.g., partially purified soybean phospholipids or dipalmitoylphosphatidylglycerol, where 95% of the original fluorescence reappears within a few minutes (Grzesiek and Dencher, 1988). As, in strongly (≥1 mM) buffered vesicular systems with a high transmembrane pH-gradient (ΔpH ≈2), the collapse of the pH-gradient is severely restricted by the simultaneous development of a proton diffusion potential (Bramhall, 1987), the slow reappearance of 9-AA fluorescence in PS vesicles might be an indication for a very low permeability of PS-vesicles not only to proton/hydroxide ions but also to other ions (sodium, potassium) that are involved in dissipating the diffusion potential. However, 9-AA fluorescence in PS-vesicles can be restored with a time constant of ~100 s to more than 90% by the addition of the potassium carrier valinomycin (4 μM), which collapses the diffusion potential in the presence of potassium ions (100 mM), whereas the addition of the proton carrier CCCP (5 μM) is not effective in restoring the original fluorescence.

It is therefore concluded that either 9-AA fluorescence quenching is due to the membrane diffusion potential itself or that it is in fact caused by the pH-gradient and that the dissipation of the pH-gradient is severly hindered by the membrane potential. Furthermore, also some (10%) unspecific quenching induced by CCCP (5 μM) without any vesicles (solution as Fig. 1 *A*, sample [III], 15 μM 9-AA) was observed, presumably due to complexation of 9-AA with the negatively charged CCCP.

Absorption, fluorescence excitation, and emission spectra as well as fluorescence lifetime measurements were recorded in the different states of the vesicle suspensions, i.e., with transmembrane pH-gradient and quenched 9-AA fluorescence (I), with the fluorescence of (I) restored by 4 μM valinomycin (II), without imposed pH-gradient (IV), and also of the mixed buffer solutions of (I) without

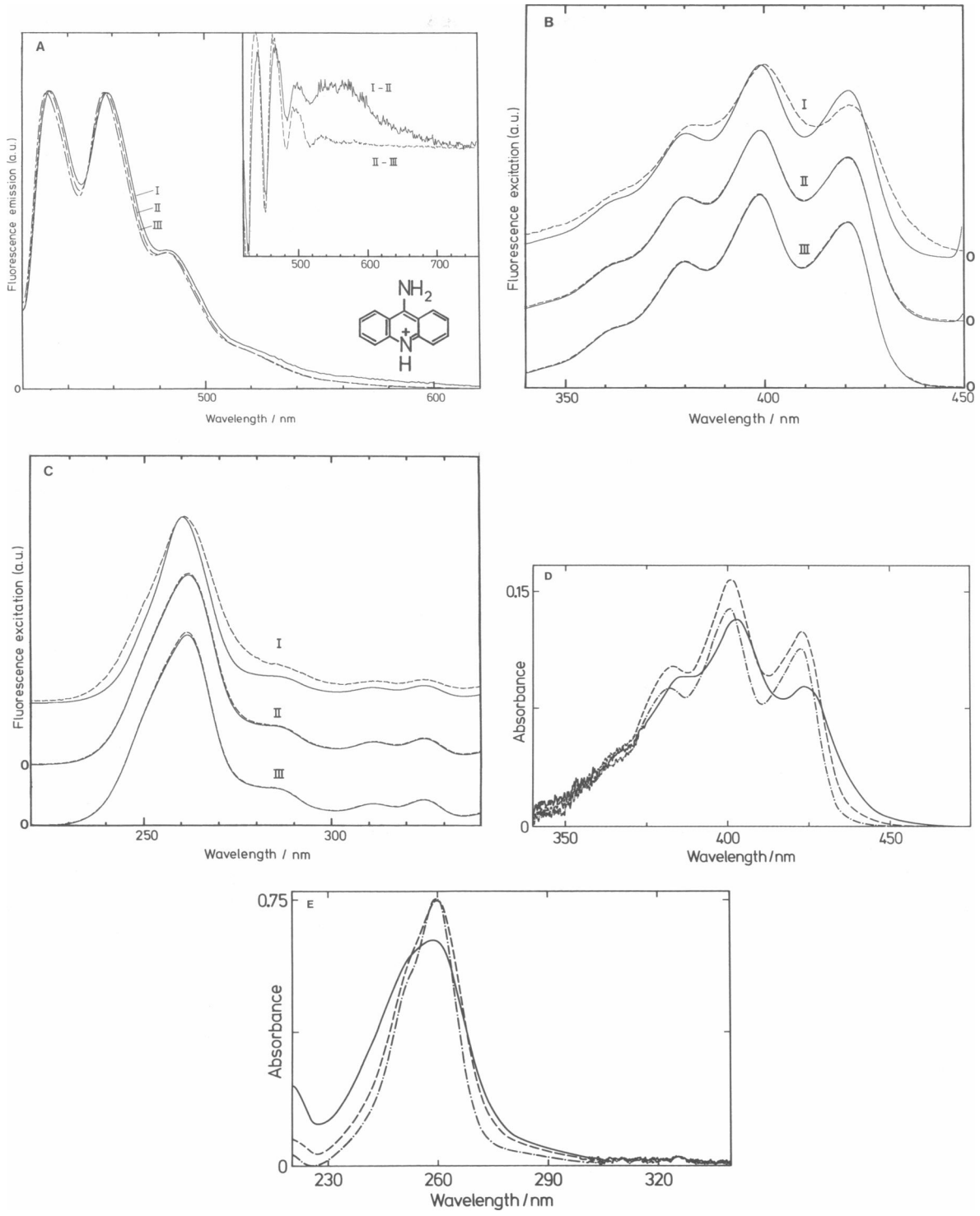

FIGURE 1 (*A*) 9-AA fluorescence emission spectra excited at 400 nm (23°). Sample (I): (——), 15 μM 9-AA in: 0.5 ml 0.5 mg/ml PS-vesicles, 10 mM sodium phosphate, 50 mM K_2SO_4 pH 5.7 mixed with 0.5 ml 100 mM sodium phosphate, 50 mM K_2SO_4 pH 8.0. Sample (II): (---), same as (I) + addition of 4 μM valinomycin in ethanol. Sample (III): (—·— or — - —), same as (I) without presence of phospholipid. Spectra are normalized at the 460 nm peak intensity. True intensity ratios are (I): (II): (III) = 0.18: 0.75: 1. (*Inset*) difference emission spectra: (——) = (I) - (II), (---) = (II) - (III) (*B*) Visible fluorescence excitation spectra of samples (I), (II), (III) as in Fig. 1 *A*, monitored at emission wavelengths of 460 nm (*solid lines*) and 560 nm (*broken lines*). Spectra are normalized at 400 nm peak intensity. (*C*) UV fluorescence excitation spectra: same as Fig. 1 *B*. Spectra are normalized at the 260 nm peak intensity. (*D*) Visible absorbance spectra of sample (I): (——), sample (II): (---), and sample (III): (—·— or — - —) as Fig. 1 *A*. 1 cm optical pathlength. (*E*) UV absorbance spectra of sample (I), (II), and (III).

vesicles (III). (For reaction conditions see Fig. 1 *A*.) However, as there were no differences detectable in any of the measured 9-AA properties between vesicle suspension without imposed pH-gradient (IV) or vesicle suspensions with imposed pH-gradient and valinomycin restored fluorescence (II), only the data for the valinomycin treated sample (II) are shown.

Fluorescence emission

Fig. 1 *A* depicts 9-AA fluorescence emission spectra excited at 400 nm. Clearly observable, there is a redshift of the whole 9-AA emission spectrum from the pure buffer sample (III) to the vesicle sample (II). A further redshift occurs in the state of quenched fluorescence (I). In this state (I), the shift amounts to $\sim 100\ cm^{-1}$ (i.e., ≈ 2 nm) for the 460 nm peak with respect to the pure buffer solution (III). To a smaller extent, such a shift is also observed in the fluorescence excitation and the absorbance spectra (see below). A striking feature of the ΔpH-induced quenched fluorescence emission spectrum is the appearance of a broad structureless band centered at ~560 nm, which is shown as a difference spectrum in the inset of Fig. 1 *A*. This effect can be further enhanced choosing appropriate experimental conditions (Fig. 2). The broad emission at 560 nm was described for concentrated aqueous 9-AA solutions as resulting from ground state dimers that undergo either thermally activated radiationless excimer conversion or a geometrical rearrangement in the excited state (Gangola et al., 1981; Pant et al., 1986). Fluorescence emission occurs therefore from this altered excited state of the dimer, and the electronic Frank-Condon ground state, reached after emission, is repulsive between both monomers. Such a transition has a continuous energy spectrum. The proper dimer fluorescence emission of the unaltered Frank-Condon excited state is, however, not observed in aqueous solution at room temperature (Gangola et al., 1981). Such a dimer-excimer transition is not an uncommon phenomenon and has also been described for other systems in detail (Ferguson, 1966; Mataga and Kubota, 1970; Saigusha and Itoh, 1985). This is in contrast to the more widely known excimer mechanism of ground state monomers (e.g., pyrene) undergoing dimerization only in the excited state. In the latter case, the excitation spectra of excimers and monomers are identical, contrary to the experimental data described below for 9-AA.

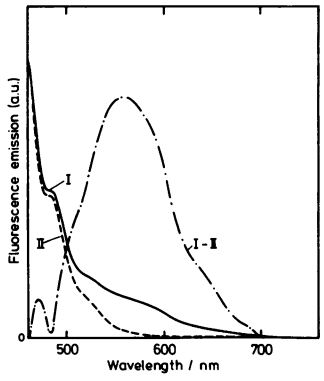

FIGURE 2. Selective excitation of excimer emission. Reaction conditions as Fig. 1 *A*. (——): sample (I) excited at 440 nm, (---): sample (II) excited at 400 nm, (—·—·—) difference of (——) and (---) scaled by a factor of 8.

Fluorescence excitation and absorbance (visible)

The existence of the spectrally distinct dimer excimer complex can be further corroborated in the fluorescence excitation (Figs. 1, *B* and *C*) and in the absorbance spectra (Figs. 1, *D* and *E*). Except for a small redshift of $\sim 40\ cm^{-1}$ (≈ 0.6 nm) when passing from the pure buffer solution (III) over the vesicle solution with restored fluorescence (II) to the vesicle solution with quenched fluorescence (I), the visible fluorescence excitation spectra (Fig. 1 *B*), of all three samples can be completely superimposed, when fluorescence is monitored at 460 nm (monomer emission, *continuous lines*). The transition excited at ~400 nm has been assigned to be the ^1L_a-band (Zanker and Schiefele, 1958; Zanker and Wittwer, 1960) according to the Platt nomenclature. However, when emission is monitored at 560 nm (excimer emission and some contributions of monomer fluorescence, Fig. 1 *B*, *broken lines*), the dimer excitation spectrum (Gangola et al., 1981) redshifted by $\sim 80\ cm^{-1}$ (≈ 1.2 nm) with respect to the corresponding monomer spectrum (Fig. 1 *B*, $\lambda_{em} = 460$ nm, *continuous line*) becomes clearly visible in the sample with quenched 9-AA fluorescence (I). For each of the other two samples without ΔpH-induced quenching (II) and (III), no difference at all is detected between the corresponding excitation spectra monitored at 460 or at 560 nm. These features of the visible excitation spectra are completely reproduced and verified in the absorbance spectra (Fig. 1 *D*); i.e., a small redshift due to the presence of phospholipids (valinomycin restored fluorescence (II) as compared with the pure buffer sample (III), the appearance of the hypochromic dimer absorbance with broader bands, and some further redshift ($155\ cm^{-1}$, 2.5 nm) in the ΔpH-quenched vesicle sample (I). The discrepancies in dimer redshift between excitation and absorbance spectra as well as the value of $275\ cm^{-1}$ (4.4 nm) reported by Gangola et al. (Gangola et al., 1981) for the pure dimer are easily explained by the still appreciable monomeric components in the recorded

spectra. The contributions of the monomer may be different in fluorescence excitation and absorbance.

Fluorescence excitation and absorbance (UV)

The interpretation of the UV fluorescence excitation (Fig. 1 *C*) and absorbance spectra (Fig. 1 *E*) seems somewhat more complicated. At first, the 325 nm weak ^1L_b-transition (Zanker and Schiefele, 1958; Zanker and Wittwer, 1960) does not exhibit any marked differences for all three samples at both monitoring wavelengths. A slight redshift, as for the ^1L_a-band, in the phospholipid samples (I) and (II) might however be undetectable due to the low experimental resolution. Again there are not either any differences in the spectral form of the strong 265 nm ^1B_b-transition (Zanker and Schiefele, 1958; Zanker and Wittwer, 1960) for both monitoring wavelengths and for the two samples without ΔpH-induced quenching (II) and (III), except for a slight redshift (≤80 cm^{-1}, 0.5 nm) in the excitation spectrum of the vesicle sample with valinomycin restored fluorescence (II). There are, however, marked differences between those two samples, (II) and (III) and the vesicle sample with ΔpH-induced fluorescence quenching (I): the maximum of the ^1B_b-band of the quenched sample (I) monitored at 460 nm (monomer emission, *continuous lines*) is shifted to higher energies by ~140 cm^{-1} (≈1 nm) as compared with the buffer solution spectrum (III). For this emission wavelength in sample (I), the slight shoulder in the ^1B_b-band at 250 nm is also much reduced as compared with samples (II) and (III). Monitored at 560 nm (*broken line*), the maximum of the ^1B_b-band in sample (I) is redshifted in comparison with the 460 nm monitoring wavelength, but still slightly blueshifted with respect to both other samples. The band is now broadened on both sides which suggests the existence of at least one underlying second band. This line-broadening and blueshift in vesicle solution (I) can also be detected in the absorbance spectrum (Fig. 1 *E*), where a hypochromism of ~17% is present. It is not attempted to interpret the behavior of the ^1B_b-band, albeit it would be conceivable that the ^1B_b-state (probable higher permanent dipole moment) is much more sensitive to distortions of the structure surrounding the chromophore than the ^1L_a- or ^1L_b-states.

Selective excitation of excimer emission

As excimer and monomer emission exhibit different excitation spectra (Figs. 1, *B* and *C*), the effect can be used to enhance the relative weak excimer emission intensity at 560 nm with respect to the underlying monomer fluorescence. Therefore, in the emission spectra of Fig. 2, sample (I) (ΔpH-quenched 9-AA fluorescence, *continuous line*) is excited at 440 nm, where the monomer excitation is rather weak (Fig. 1 *B*). In comparison, sample (II) (9-AA fluorescence released by valinomycin, *broken line*) is excited at 400 nm, where dimers and monomers have similar absorbances (Gangola et al., 1981). Both emission spectra are normalized to each other at 480 nm, a wavelength where no excimer emission is expected. As a result, the excimer emission band at 560 nm is now clearly visible in sample (I) as superimposed to the monomeric emission in sample (II). The difference between both spectra gives a much improved signal of the pure excimer emission as compared with the inset of Fig. 1 *A* where all spectra are excited at the same wavelength (400 nm).

Fluorescence lifetimes

Strong evidence for the existence of excimers induced by the pH-gradient across the vesicular membrane is further given by the results of fluorescence lifetime measurements (Table 1). For almost all samples a two-exponential fit to the fluorescence decay proved to be significantly better than the one-exponential fit as judged by chi-square (improvement 1.2- to fourfold) and the structure of the residuals.

For aqueous samples with and without buffer, containing no phospholipids and only low 9-AA concentrations (≤1 mM) (Table 1, Nos. 1–6) essentially no differences in lifetimes and amplitudes are observed for all excitation and monitoring wavelengths used: (λ_{ex} = 400 nm, $\lambda_{em} \geq$ 418 nm): mostly monomer emission and (λ_{ex} = 440 nm, $\lambda_{em} \geq$ 500 nm): excimer emission (if any) and monomer emission. The predominant (0.8–0.9 relative amplitude) fluorescence lifetime in these samples has values of 15.8–16.2 ns and is in good agreement with the results of Pant et al. (1986) (16.6 ns, water), Searle and Barber (1978) (16.3 ns, low cation aqueous medium), Casadio and Melandri (1985) (16 ns, aqueous buffer), and Marty and Viallet (1985) (15.2 ± 0.5 ns, water-ethanol). There is, however, also a minor component in the decay process (0.1–0.2 relative amplitude) with lifetimes of 4–6 ns. Both, lifetimes and amplitude ratio were not altered, when double-distilled water and three times recrystallized 9-AA were used instead of deionized water and the commercially obtained dye. (On the thin-layer chromatography, the recrystallized 9-AA did not show any impurity.) The cause for the fast fluorescence decay process in the pure water-dye system is not understood at present. Due to its small amplitude and short lifetime, it might have been easily overlooked in previous investigations. Its appearance could be due to a more complicated decay pattern involving, e.g., solvent-cage relaxation in the excited state. It was not possible to obtain higher than 1 mM 9-AA concentrations in the phosphate buffer

TABLE 1 Amplitudes (A_1, A_2) and lifetimes (τ_1, τ_2) for exponential fits to 9-AA fluorescence decay

No.	[9-AA]	Medium	λ_{ex}	λ_{em}	τ_1	A_1	τ_2	A_2
			nm	*nm*	*ns*		*ns*	
1	15 μM	H_2O	400	≥418	6.3 ± 0.4	0.149 ± 0.007	16.1 ± 0.1	0.851 ± 0.001
2	15 μM	H_2O	440	≥500	4.9 ± 1.7	0.108 ± 0.020	16.2 ± 0.2	0.892 ± 0.003
3	15 μM	Buffer	400	≥418	5.4 ± 0.5	0.118 ± 0.030	15.8 ± 0.2	0.882 ± 0.005
4	15 μM	Buffer	440	≥500			15.3 ± 0.3	1.000
5	1 mM	Buffer	400	≥418	6.0 ± 0.5	0.127 ± 0.006	16.0 ± 0.1	0.873 ± 0.001
6	1 mM	Buffer	440	≥500	4.4 ± 0.8	0.186 ± 0.015	15.8 ± 0.2	0.814 ± 0.004
7	10 mM	H_2O	400	≥418	3.8 ± 0.2	0.583 ± 0.008	23.5 ± 0.4	0.417 ± 0.006
8	10 mM	H_2O	440	≥500	5.1 ± 1.0	0.192 ± 0.015	23.8 ± 0.3	0.808 ± 0.003
9	50 mM	H_2O	400	≥530			23.4 ± 0.3	1.000
10	15 μM	Ves ΔpH Val	400	≥418	3.5 ± 0.3	0.226 ± 0.007	15.2 ± 0.1	0.774 ± 0.002
11	15 μM	Ves ΔpH Val	440	≥500	2.6 ± 1.8	0.120 ± 0.040	14.0 ± 0.2	0.880 ± 0.040
12	15 μM	Ves ΔpH	400	≥418	3.8 ± 0.4	0.330 ± 0.010	15.5 ± 0.2	0.670 ± 0.010
13	15 μM	Ves ΔpH	440	≥500	3.7 ± 0.3	0.480 ± 0.010	18.8 ± 0.3	0.520 ± 0.010

Ves ΔpH: same as Fig. 1 *A:* sample (I). Ves ΔpH Val: same as Fig. 1 *A:* sample (II). Buffer: same as Fig. 1 *A* without phospholipids: sample (III). Values reported are means and SD of 2-3 independent experiments.

solutions. Therefore, at 10 mM and 50 mM 9-AA, lifetimes are only reported for pure H_2O solutions. At 10 mM 9-AA (Table 1, Nos. 7 and 8), the excimer relaxation time (Pant et al., 1986) of 23.5–23.8 ns appears at the expense of the monomer lifetime of 16 ns. Its excitation follows the expected wavelength dependency and a short lifetime (4–5 ns) attributed to the rest monomer fluorescence still exists. However, at 50 mM (Table 1, No. 9), this monomeric component has completely vanished and only excimer emission (τ = 23.4 ± 0.3 ns) is observed.

The interpretation of the 9-AA fluorescence decay in the presence of phospholipid vesicles (Table 1, Nos. 10–13) is now obvious. In the vesicle suspensions with the valinomycin restored 9-AA fluorescence (II) (Table 1, Nos. 10 and 11; Fig. 3 *A*), no dependency of the lifetimes on excitation or emission wavelengths is observed. In the ΔpH-quenched sample (I) monomer fluorescence ($\lambda_{em} \geq$ 418 nm) can still be excited at 400 nm (Table 1, No. 12), with the two lifetimes of 15.5 ± 0.2 ns and 3.8 ± 0.4 ns (relative amplitude of 0.33, slightly more than in low concentration 9-AA aqueous solution). However, the fluorescence excited at 440 nm and observed at $\lambda_{em} \geq$ 500 nm (Table 1, No. 13 and Fig. 3 *B*) shows a significantly different decay: the 15.5 ns lifetime has increased to 18.8 ± 0.3 ns (relative amplitude of 0.52) and the short lifetime of 3.7 ± 0.3 ns is present with an increased intensity (relative amplitude of 0.48). This behavior clearly establishes the presence of a second fluorescence emitting species, presumably the excimer. The fact that the observed lifetime of this species does not amount to the 24 ns of the excimer must be attributed to the underlying still unquenched monomer fluorescence. In the vesicle suspension with ΔpH-induced quenching certainly at least three different 9-AA populations are present: the 9-AA monomers still in the aqueous bulk

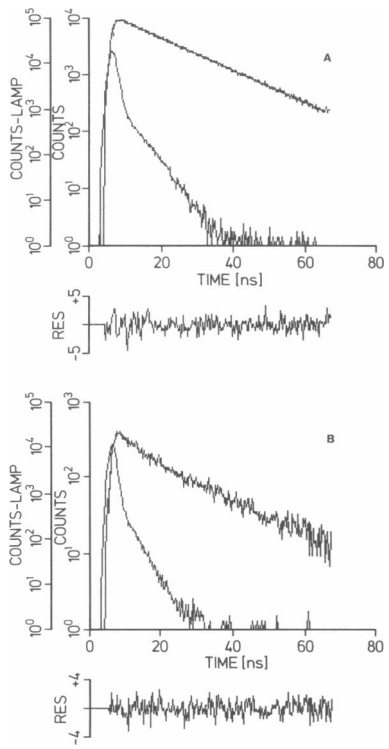

FIGURE 3 Fluorescence decay of 9-AA in vesicle suspensions. The fast decaying curve represents the emission of the excitation lamp. (*Bottom*) residuals of two-exponential fits. (*A*) Sample (II) λ_{ex} = 400 nm, $\lambda_{em} \geq$ 418 nm; 15 μM 9-AA in: 0.5 ml 0.5 mg/ml PS-vesicles, 10 mM sodium phosphate, 50 mM K_2SO_4 pH 5.7 mixed with 0.5 ml 100 mM sodium phosphate, 50 mM K_2SO_4 pH 8.0 + addition of 4 μM valinomycin in ethanol. Parameters of two-exponential fit: $A_{1,2}$ = 0.226 ± 0.007, 0.774 ± 0.002, $\tau_{1,2}$ = (3.54 ± 0.26) ns; (15.2 ± 0.2) ns. (*B*) Sample (I) λ_{ex} = 440 nm, $\lambda_{em} \geq$ 500 nm (same as *A* but without valinomycin, i.e., in the presence of a pH-gradient). Parameters of two-exponential fit: $A_{1,2}$ = 0.493 ± 0.014, 0.507 ± 0.011, $\tau_{1,2}$ = (3.80 ± 0.42) ns; (19.3 ± 0.5) ns.

phase which emit normal monomer fluorescence, 9-AA monomers somehow bound to the negatively charged membrane with probably modified monomer fluorescence and quenched collisionally in part by the dimer-excimer complexes, and finally the dimer-excimer complexes themselves. The fluorescence decay time of 18.8 ns is therefore understood as a mixing of the normal 16 ns monomer fluorescence lifetime and the 24 ns dimer-excimer fluorescence lifetime. A 3-exponential fit possibly could reveal the pure 24 ns component representing the excimer in addition to the 16 ns and 3.7 ns components. However, the lower signal-to-noise ratio for the present experiments with vesicles prevents such a separation.

DISCUSSION

By applying a careful analysis to 9-AA's optical spectra and its fluorescence lifetimes, it was possible to show that the ΔpH-induced 9-AA fluorescence quenching in negatively charged phospholipid vesicles is accompanied by the formation of excimers resulting from excited ground state dimers. The changes in the optical spectra of the quenched state, i.e., the dimer absorbance redshift and the excimer emission band at 560 nm (if not enhanced by selective excitation), are however rather small, so that they may have been easily overlooked or not taken into account in previous studies of the quenching phenomenon (Casadio and Melandri, 1977; 1985).

Apart from the dimer induced redshift in excimer luminescence excitation and absorbance spectra (the dimer is nonfluorescent at room temperature, Gangola et al., 1981), there is also a redshift in the normal, monomer fluorescence emission spectra as well as in the monomer absorbance and fluorescence excitation spectra, induced only by the presence of the negatively-charged phospholipid vesicles without any pH-gradient applied across the membrane. This monomer redshift is easily understood on the basis of the results of Marty and coworkers (Marty and Viallet, 1982; 1983) who have applied the empirical theory of Kamlet and colleagues (Minesinger et al., 1977; Kamlet et al., 1977) to the interactions of 9-AA with the surrounding solvent molecules. According to this theory, the positional shifts in the absorbance and fluorescence emission spectra of a solute in a certain solvent are given by a linear relation depending on three solvent parameters: the polarity and the abilities either to donate hydrogen bonds to or accept them from the solutes. It turns out that redshifts in 9-AA absorbance as well as emission spectra can either be induced by a higher polarity of the solvent or a higher tendency of the solvent to accept hydrogen bonds from the 9-aminoacridinium ion. The latter possibility would clearly point to the negatively-charged phospholipid headgroups as hydrogen bond acceptors. In fact, a very similar redshift is observed when the neutral 9-AA base is formed (pH ≥ 9.99) from the 9-AA cation. This redshift was explained on the basis of stabilization of the cation ground state by the 9-iminoacridan structure (Zanker and Wittwer, 1960; Capomacchia et al., 1974).

The proper dimer-excimer formation seems independent of this solvent-induced redshift. According to the results of Pant and coworkers (Gangola et al., 1981; Pant et al., 1986), the following picture emerges of the quenching mechanism in liposomes (refer to Fig. 4). In the presence of negatively charged phospholipid vesicles without pH-gradient, 9-AA molecules are attracted to the charged headgroups and exhibit the solvent induced redshift. If the attracting forces are strong enough, e.g., in a medium of low ionic strength, dimerization and subsequent excimer formation already takes place without applied pH-difference. The accompanying fluorescence quenching has been reported in a number of biological and artificial membrane systems (Searle and Barber, 1978; Handa et al., 1983; Theuvenet et al., 1984) and in part also the corresponding dimer absorbance redshift and hypochromism have been observed. As the dimerization constant is in the order of $10^3\ M^{-1}$ in aqueous solution of 288 K (Gangola et al., 1981), it seems easily possible to achieve the necessary dimerization concentration at the membrane surface in the presence of a proper electrochemical surface potential, even if the 9-AA bulk concen-

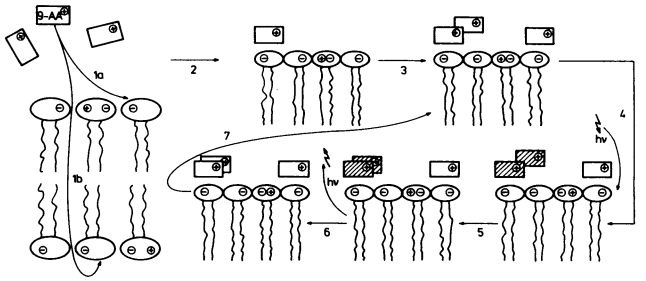

FIGURE 4 Scheme of ΔpH-induced 9-AA dimer-excimer formation in the vesicular membrane with negatively charged lipid headgroups. Upon imposition of a transmembrane pH-gradient, positively charged $9\text{-}AA^+$ monomeric bulk ions are concentrated either near the outer (1*a*) or near the inner (1*b*) negatively charged headgroups as monomers (2). At this water-membrane interphase the (lipid. 9-AA) complexes aggregate as dimers (3), if their concentration is sufficiently large. The dimers can be excited by light according to their specific 9-AA dimer absorbance to a dimer excited state (4). This dimer excited state undergoes a radiationless geometrical rearrangement (attraction between both 9-AA monomers) to the dimer-excimer state (5). The dimer-excimer state can emit light (6) and reach a Frank-Condon ground state (6) of the dimer which is repulsive between both monomers. This dimer ground state is not in equilibrium, but finally undergoes a thermal relaxation to the normal dimer ground state (7).

tration is in the micromolar range (Dell'Antone et al., 1978). However, if a pH-gradient is additionally applied across the vesicular membrane, either due to the mentioned Schuldiner mechanism (Schuldiner et al., 1972) or due to the action of the diffusion potential, still more 9-AA molecules are interacting with the vesicle and are enriched at the membrane-water interphase. The concentration at this interphase therefore passes the dimerization level and membrane-bound dimers as well as monomers will be present. In this context, it is interesting to mention that the ΔpH-induced quenching does not take place in vesicles made of electrically neutral phospholipids (Grzesiek and Dencher, 1985*a*, 1988). Probably the action of the pH-gradient alone is not sufficient to concentrate the monomers up to the dimerization level and a further attraction by the negative phospholipid headgroups (or negative amino-acids of membrane proteins) at the membrane-water interphase is required.

The loss of fluorescence can be due in part to the lower quantum yield of the dimer-excimer complex. On the other hand, also a direct dynamical quenching of the membrane-bound monomers by the excimers occurs, as is expressed by the 3.7 to 3.8 ns component in fluorescence decay, which results from the quenched monomer fluorescence. The same mechanism has been described for the concentration quenching in aqueous solution (Pant et al., 1986).

It is not attempted to explain all of the observed other 9-AA quenching phenomena in biological systems, e.g., in energized small mitochondrial particles and chloroplasts or in 9-AA bound to DNA, by the same dimerization and excimer mechanism. There could also be quenching induced by binding to negatively charged π-bonding complexes that are exposed at the surface of such particles. Yet there is some experimental evidence that dimer formation also happens in those systems. For example, Casadio and Melandri (1985) reported a deviation from single-exponential behavior as well as a shortening of the halftime of 9-AA fluorescence decay accompanied by a 50% fluorescence quenching in the presence of irradiated bacterial chromatophores. This could well be due to an experimentally unresolved bi-exponential decay with enhancement of the fast decay component as shown in Table 1 (Nos. 12 and 13). Similarly, Fornasiero and Kurucsev (1985) have observed directly the dimer absorbance spectrum of 9-AA bound to calf thymus DNA where the fluorescence quenching is nearly complete. In addition, dimerization of other, chemically related compounds, e.g., neutral red (Dell'Antone et al., 1972), acridine orange (Dell'Antone et al., 1972; Kraayenhof, 1977), methylene blue (Handa et al., 1983), 3,3'-dipropyl-2,2'-thiadicarbocyanine (Handa et al., 1983), 9-amino-3-chloro-7-methoxyacridine (Torres-Pereira et al., 1984) has been reported in the presence of negative surfaces. Therefore, such two-dimensional aggregations of positively charged dyes are much more general, although the resulting changes in the molecular photophysics during aggregation may be specific to every dye under investigation.

In summary, it is concluded that interactions of 9-AA and related compounds with biological surfaces can be studied much more precisely, if the specific photophysical properties (e.g., dimer-excimer formation) of such dyes are taken into account, rather than by relying on fluorescence quenching phenomena of unknown origin. Furthermore, as the ΔpH-induced aggregation of 9-AA is caused mainly to transmembrane ion fluxes and potential differences and not to the interaction with specific protein components (Grzesiek and Dencher, 1988), 9-AA and its derivatives seem applicable, in a more refined way than the original Schuldiner model (Schuldiner et al., 1972), to the study of bioenergetic surface phenomena.

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