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The 'ΔpH'-probe 9-aminoacridine: response time, binding behaviour and dimerization at the membrane

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The fluorescence quenching of 9-aminoacridine (9-AA) after imposition of a transmembrane pH gradient (inside acidic) in liposomes has been investigated for a number of different lipid systems. The initial fluorescence decrease after a rapid pH jump, induced in the extravesicular medium by a stopped-flow mixing technique, was ascribed to a response of 9-AA to the imposed pH gradient and not to changes in the vesicular system itself. Time constants for this fluorescence quenching are in the range of several hundred milliseconds at 25 °C. Fluorescence recovery which should be correlated to the dissipation of the pH gradient occurs in the 100 s time range and is 10–30-times faster than the ΔpH decay monitored with the entrapped hydrophilic pH-indicator dye pyranine. The quenching was severely hindered below the lipid phase transition of dipalmitoylphosphatidylglycerol. No ΔpH-induced quenching was obtained in lipid vesicles containing only zwitterionic, net uncharged phosphatidylcholine headgroups. For the occurrence of quenching, the presence of negatively charged headgroups, i.e. phosphatidylglycerol or phosphatidylserine, was necessary. The extent of quenching, at a specific pH difference applied, had a cooperative dependency (Hill coefficient ≈ 2) on the number of negative headgroups in the membrane and on the concentration of unquenched (unbound) 9-AA molecules. The concentration of quenched 9-AA molecules was furthermore proportional to the number of dimer-excimer complexes of 9-AA which are formed during the quenching process.

Introduction

ATP synthesis, transport of metabolites and ions, and rotation of bacterial flagella are powered by transmembrane electrochemical proton gradients generated via photosynthesis and respiration. Therefore, knowledge of the protonmotive force, Δp, existing across membranes of cells, organelles, and reconstituted lipid-protein vesicles is of fundamental importance for bioenergetics. The determination of the protonmotive force requires the measuring of two separate quantities, the electrical membrane potential, $\Delta\psi$, and the concentration gradient of protons between both membrane sides, ΔpH. For convenience, often solely the difference in pH is determined while the electrical potential difference, $\Delta\psi$, is abolished by ionophores. During the past 1 1/2 decades, the fluorescent probe, 9-aminoacridine (9-AA) and its derivatives became one of the most popular tools for quantitative determination of transmembrane pH gradients (inside acidic), because of ease in application and the assumption that ΔpH can be calculated directly [1] from the measured loss in 9-AA fluorescence (quenching), which occurs in

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the presence of such pH gradients. Although neither the postulated model mechanism [1] for the response of 9-AA to the pH gradient (see Results) has been verified nor the photophysics of the fluorescence quenching had been known, the data obtained by this methodology for the magnitude of ΔpH and protonmotive force had and still have a great impact on basic bioenergetic problems, such as the efficiencies in energetic conversion of photosynthesis and oxidative phosphorylation. Only occasionally, the model proposed by Schuldiner et al. [1] and the applicability of 9-AA as a quantitative ΔpH-indicator have been challenged, e.g., by the careful investigations of Kraayenhof and co-workers [2–6].

Because of the frequent application of 9-AA, even nowadays, and the implications of the determined ΔpH values on bioenergetics, we have conducted experiments on liposomes with imposed well-defined transmembrane pH differences in order to elucidate the underlying molecular processes involved in ΔpH sensing and fluorescence quenching. Several of the results obtained, e.g., the strict requirement of net-negatively charged lipids for the occurrence of quenching, as well as the cooperative dependence of the extent of quenching on the number of negative charges, are incompatible with the previously proposed reaction mechanism and with the applicability of 9-AA as a reliable, quantitative reporter of transmembrane ΔpH. Although the response time of this indicator is in the subsecond range, it cannot be used for monitoring the time-course of ΔpH changes since no kinetic correlation between ΔpH decay and 9-AA fluorescence recovery was found. In addition, experimental evidence is presented that ΔpH-induced quenching of 9-AA fluorescence is accompanied by the formation of 9-AA dimer-excimers at the membrane surface.

Experimental

Materials. Soybean phospholipids were purified according to the procedure of Kagawa and Racker [7]. Dimyristoylphosphatidylcholine (DMPC) (Fluka), dipalmitoylphosphatidylcholine (DPPC) (Fluka), dipalmitoylphosphatidylglycerol (DPPG), phosphatidylserine (PS) (bovine brain), diphytanoylphosphatidylcholine (DPhPC) (all Avanti Biochemicals), 9-aminoacridine hydrochloride (9-AA) (Fluka), 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (pyranine) (Eastman-Kodak), valinomycin (Boehringer), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (Aldrich) were used without further purification. Deionized water ($\kappa \leqslant 1$ μS/cm) and chloroform (fluorescence spectroscopy grade, Merck) were used throughout all preparations.

Methods. Sonicated phospholipid vesicles were prepared as described previously [8,9]. Briefly, the phospholipids were dissolved in a small volume of chloroform and spread as a thin film on the wall of a glass tube by evaporation of the solvent. For the preparation of vesicle membranes containing a mixed population of DPhPC and PS, appropriate aliquots of both lipids were dissolved in the same volume of chloroform. Residual traces of solvent were further removed under vacuum ($\leqslant 100$ Pa, 5–20 h) at room temperature. The dried lipid film was then hydrated in 10 mM sodium phosphate, pH 5.7, with further addition of 50 mM K_2SO_4 or 100 mM KCl, if indicated. This sample with a final lipid concentration of 10 mg/ml was sonicated to clarity, i.e. 30 min, in a bath-type sonicator (Sonorex RK 100 H, Bendelin electronic, F.R.G., 35 kHz) at room temperature for soybean phospholipid and DPhPC/PS, or at 30 °C (46 °C) in the case of DMPC (DPPC, DPPG). When pyranine was used as an indicator of the intravesicular pH, 2.2 mM of this dye was added to the lipid suspension prior to the sonication step and removed from the extravesicular bulk phase after sonication by dialysis (3×6 h, 4 °C). A very low leakage of this dye from anionic liposomes has been reported (1% per day [10]). Prior to the measurements, the vesicle suspension was diluted to a final lipid concentration of 0.5 mg/ml.

The average diameter of these vesicles has been determined previously by electron microscopy [9] for soybean phospholipid (29.6 nm) and DPhPC (64.4 nm). No significant changes in the vesicle size were observed as the PS content of the mixed DPhPC and PS vesicles was varied (0–100%).

Fast alterations of pH in the external medium of the vesicle suspension were induced by addition of 200 μl 100 mM sodium phosphate pH 8.0 (+50 mM K_2SO_4 or 100 mM KCl, if indicated) (+9-AA from a 2.5 mM ethanolic stock solution as indi-

cated) to 200 µl of the vesicle suspension in a thermostated (±0.1° C) stopped-flow unit (Sigma Instrumente, Berlin, $2 \times 2 \times 16$ mm^3 fluorescence or absorption cuvette). The mixing and overall dead time of this instrument was found to be less than 5 ms [9].

The kinetics of the optical changes (fluorescence), induced in the vesicle suspensions after the establishment of a pH gradient across the vesicular membrane, were followed in the stopped-flow unit with an attached dual-wavelength photometer (Sigma Instrumente, Berlin, ZWS-II). Fluorescence signals from the photometer were stored via an analog-digital-converter into an Apple II system which could generate two different time-bases in consecution. Data analysis was performed by a Marquardt procedure [11] which could fit up to two exponential functions to the time-dependent signals. Fluorescence spectra were recorded by a Shimadzu Spectrofluorometer RS-540. pH-measurements were carried out with a Beckmann Model 4500 digital pH meter connected to a combined pH electrode.

Results

Kinetics of ΔpH-induced 9-AA fluorescence quenching and recovery

After the rapid establishment (≤ 5 ms) of a pH-difference (inside acidic) across the membrane of soybean phospholipid vesicles in the stopped-flow apparatus, a fast ($\tau_1 \approx 0.2$ s, 25° C) quenching (Fig. 1A) of 9-AA fluorescence and a slow ($\tau_2 = 31$ s, 25° C) subsequent reappearance (Fig. 1B) of fluorescence are observed. Both relaxation processes can be reasonably well described by one exponential function each, and only within the first 50 ms after mixing, a small deviation with negative amplitude (approx. 15% of the whole fluorescence quenching, Fig. 1A) is detected. The quenching phenomenon has been described quantitatively in a model by Schuldiner et al. [1] based on the following unproven assumptions: the uncharged (deprotonated) 9-AA base (pK 9.99 [12]) is freely permeable across the membrane of the vesicles and is the only 9-AA form that crosses the membrane. As the vesicle interior is more acidic than the outside medium (i.e., $pH_i < pH_0 \ll pK$), the positively charged (protonated) 9-AA

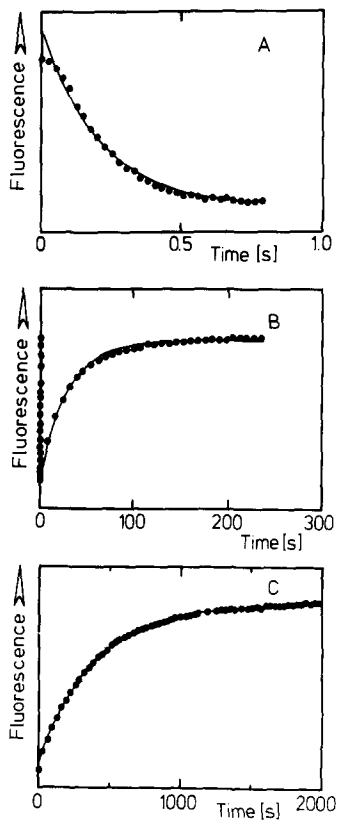

Fig. 1. Comparison of different 'pH indicators': rapid mixing (1:1) of 0.5 mg/ml soybean phospholipid vesicles, 10 mM sodium phosphate, 100 mM KCl (pH 5.7) with 100 mM sodium phosphate, 100 mM KCl (pH 8.0) (25° C). Experimental data are depicted as filled circles and the one- (C) or two- (A, B) exponential fits are drawn as continuous lines. (A, B) Mixing buffer solution of pH 8.0 contained additionally 5 µM 9-AA. Measurement of 9-AA fluorescence ($\lambda_{ex} = 400$ nm, $\lambda_{em} \geq 450$ nm) in a single experiment with two split time-bases. (C) 2.2 mM pyranine was entrapped into the soybean phospholipid vesicles as described in Materials and Methods. Measurement of pyranine fluorescence at $\lambda_{em} \geq 500$ nm ($\lambda_{ex} = 460$ nm).

is accumulated in the vesicle interior where it loses completely its fluorescence. No physical mechanism for this fluorescence quenching was established. According to this model, the time constant of fluorescence decrease, τ_1, corresponds to the accumulation process, i.e., permeation of the 9-AA base, and represents the response time of the indicator. The slow fluorescence recovery time, τ_2, should reflect the dissipation of the pH-gradient caused by the permeation of proton/hydroxide ions. According to Schuldiner et al. [1], the pH-

difference ΔpH across the membrane is calculated from the relative fluorescence loss Q:

$$\Delta pH = pH_o - pH_i = \log_{10}\left(\frac{V_o}{V_i}\right) + \log_{10}\left(\frac{Q}{1-Q}\right) \quad (1)$$

where V_i and V_o represent the inner and outer bulk volume of the vesicle suspension, respectively.

The temperature dependence of both relaxation times for soybean phospholipid vesicles is depicted in Fig. 2 and the corresponding values as well as activation energies are listed in Table I. In the experiments, soybean phospholipid vesicle suspensions in 10 mM phosphate buffer (pH 5.7) were mixed (1 : 1, v/v) with 100 mM phosphate buffer and 5 μM 9-AA at pH 8.0. Experiments were also performed with solutions containing additionally either 50 mM K_2SO_4 or 100 mM KCl. Directly after mixing the pH of the extravesicular medium has a value of 7.6, whereas the intravesicular pH is assumed to be unchanged at a value of 5.7. As can be seen from Table I, the time constants, τ_1, and activation energies, E_{a1}, of the fluorescence quenching process are not significantly affected by the variation of the ionic strength or the salt composition. This finding and the values of E_{a1} are in general agreement with

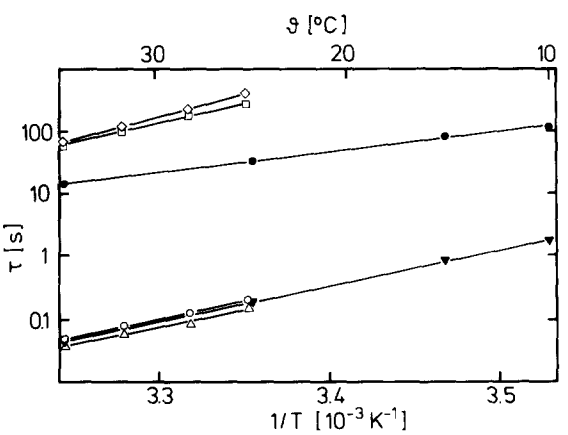

Fig. 2. Arrhenius plot of time constants τ_1 and τ_2 of 9-AA fluorescence signals ($\lambda_{ex} = 400$ nm, $\lambda_{em} \geq 450$ nm) after mixing (1 : 1) of 0.5 mg/ml soybean phospholipid vesicles, 10 mM sodium phosphate (pH 5.7) with 100 mM sodium phosphate, 5 μM 9-AA (pH 8.0). τ_1: ○, no additional electrolyte; △, +50 mM K_2SO_4; ▼, +100 mM KCl. τ_2: □, no additional electrolyte; ◇, +50 mM K_2SO_4; ●, +100 mM KCl.

TABLE I

TIME CONSTANTS AT 25°C AND ACTIVATION ENERGIES ACCORDING TO FIG. 2

Mixing buffer concentrations as in Materials and Methods. Deviations represent S.D. of at least three experiments.

Additional electrolyte:	None	50 mM K_2SO_4	100 mM KCl
τ_1 (ms)	184 ± 10	146 ± 3	182 ± 19
E_{a1} (kJ/mol)	103	94	103
τ_2 (s)	263 ± 21	394 ± 17	31 ± 2
E_{a2} (kJ/mol)	111	134	66

the data ($E_{a1} = 71 \pm 13$ kJ/mol) for bacterial chromatophores reported by Casadio and Melandri [13], who found no significant alteration of activation energies upon addition of 300 mM KCl to a 20 mM buffer solution. Probably therefore, the extent and composition of the electrical double layer have no major influence on the kinetics of this ΔpH-dependent quenching phenomenon. However, the changes induced by salt in the fluorescence recovery time constants, τ_2, and activation energies, E_{a2}, are much more pronounced. Addition of 100 mM KCl reduces τ_2 about eight times at 25°C and reduces the activation energy, E_{a2}, for this process by 45 kJ/mol. This acceleration of τ_2 by KCl can be understood on the basis of an electrosilent HCl flux, which, in addition to the proton/hydroxide ion permeation, dissipates the applied pH gradient. Such a mechanism has been reported for the case of planar lipid bilayers of egg-phosphatidylcholine/decane [14] and also in studies of the proton/hydroxide ion permeability of DPhPC vesicles [9]. The presence of 50 mM K_2SO_4, on the other hand, prolongs the fluorescence recovery by about 50% at 25°C and increases its activation energy by 23 kJ/mol as compared to the experiment where only phosphate buffer was present in the vesicle suspensions.

In this context, it was necessary to establish, whether the exponential fluorescence decrease was really due to the slow response of the dye to the imposed pH gradient or whether it was due to an intrinsic reaction of the vesicle suspension itself, occurring after the mixing step, e.g., the build-up of a diffusion potential. Therefore a pH-gradient across the membrane of 0.5 mg/ml PS vesicles (see below) was established by mixing (like Fig. 2,

50 mM K_2SO_4) without 9-AA in the medium. This vesicular suspension with imposed pH gradient was mixed again (1 : 1) after several seconds with a buffer solution of the same composition as the extravesicular medium, containing additionally 5 μM 9-AA. The same fluorescence quenching kinetics and amplitude were observed as in the case where the imposition of ΔpH and the addition of 9-AA were performed in a single step. This result clearly indicates that the first fluorescence decrease after mixing really represents the 9-AA response.

In order to examine whether the fluorescence recovery of 9-AA is connected to the dissipation of the pH gradient, control experiments were performed using the optical pH-indicator pyranine (absorbance and fluorescence) [8,10]. This dye has a well understood reaction mechanism, i.e., deprotonation of a hydroxyl group (pK 7.2 [10]), and can be used as a reliable reporter of intravesicular pH [8–10] and also as a rapid ($\approx$ 100 μs) indicator of pH changes in flash-photometric experiments [15]. Due to its 3–4 negative charges at pH 7.2, pyranine does not bind to the negative vesicle surface [10] and minimal leakage ($\leqslant$ 1% per day) out of the vesicles was reported [10]. It is therefore possible to enclose this dye into the internal volume of the vesicles as described in Materials and Methods, and to monitor internal pH in a reliable way [8–10]. Using the same reaction conditions as for the other probe, ΔpH decay was observed by pyranine with a time constant of 312 s (Fig. 1C) which is about 10-times slower than the 9-AA signal. The discrepancy became even more pronounced when 50 mM K_2SO_4 was used as an electrolyte instead of 100 mM KCl ($\tau_{2,9\text{-AA}}$ = 394 s, $\tau_{\text{pyranine}} \geqslant$ 11 400 s).

If one assumes that the 9-AA fluorescence quenching is related to the ΔpH in a non-linear manner, as described in Eqn. 1, then the kinetic behaviour of fluorescence and ΔpH are expected to differ to some extent. Nevertheless, it is possible to calculate the time behaviour of 9-AA fluorescence expected from Eqn. 1, if pyranine is taken as the true reporter of internal pH. However, even this calculated fluorescence recovery is about 10-times slower ($\tau_{2,9\text{-AA,calc.}} \approx$ 4000 s) than the observed 9-AA fluorescence recovery for the case of 50 mM K_2SO_4. The possibility that 9-AA acts itself as a ionophore and accelerates ΔpH decay was ruled out in a control experiment where pyranine and 9-AA were used simultaneously. No acceleration of the pyranine signal was found as compared to the experiment without 9-AA.

ΔpH-induced quenching as a function of the physical state and chemical composition of the lipids

If there is really a diffusion of 9-AA through the lipid bilayer in response to the imposed pH gradient, then the phase transition of the lipids should influence the fluorescence quenching amplitudes and/or kinetics. However, using the same reaction conditions as in Fig. 2 (100 mM KCl), but synthetic, zwitter-ionic lipids, neither DMPC nor DPPC vesicles were able to produce any ($\geqslant$ 1%) ΔpH-induced quenching effect on 9-AA. These results were obtained below (15 °C, DMPC) as well as above (27 °C, DMPC) the lipid phase transition temperature (23 °C, DMPC). Pyranine, on the other hand, enclosed in analogous DMPC preparations, was monitoring effectively the decay of the pH gradient, below and above the phase transition temperature. As it was shown by Lee [16], chaotropic anions such as SCN^-, ClO_4^-, I^-, Cl^- are required for the energy-dependent quenching of atebrin fluorescence in submitochondrial particles. The presence of up to 1 mM SCN^- was, however, not effective to produce any ΔpH-induced 9-AA fluorescence quenching in the DMPC vesicle suspensions in our experiments.

As a consequence of these results, the question arose, whether it were the unsaturated side chains or the headgroups of the heterogeneous soybean phospholipid mixture that are responsible for the quenching effect. Therefore, experiments were performed with vesicular preparations of DPPG which differs only in the negatively charged phosphatidylglycerol headgroup, but not in the fatty acid side chains from the net uncharged DPPC. In contrast to DPPC, ΔpH-induced quenching was observed in the DPPG preparations (Fig. 3A/B). Fig. 3A shows the temperature dependence of the amplitudes of fluorescence quenching and recovery, whereas an Arrhenius plot of the corresponding exponential time constant is given in Fig. 3B. Above the lipid phase transition ($\approx$ 39 °C) similar results in the amplitudes as for soybean phospholipid are obtained, but below the phase

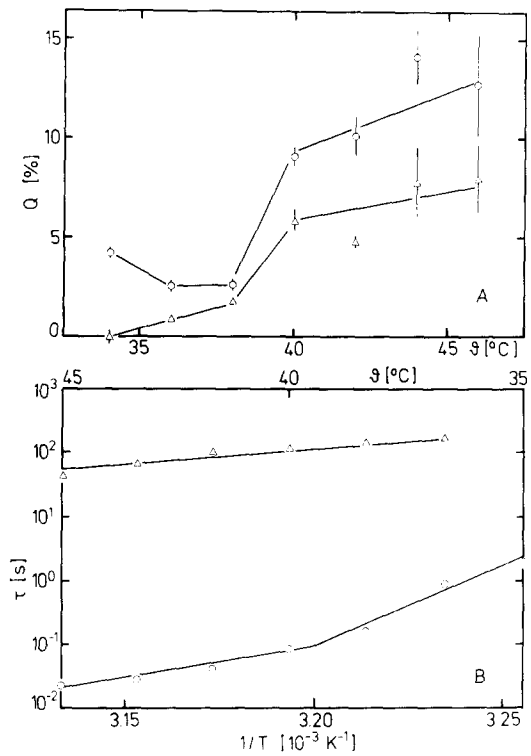

Fig. 3. 9-AA fluorescence signals ($\lambda_{ex} = 400$ nm, $\lambda_{em} \geq 450$ nm) after mixing (1:1) of 0.5 mg/ml DPPG vesicles, 10 mM sodium phosphate, 50 mM K_2SO_4, 274 mM sucrose (pH 5.7) with 100 mM sodium phosphate, 50 mM K_2SO_4, 5 μM 9-AA (pH 8.0). (Sucrose was used to obtain isoosmotic conditions). (A) Temperature dependence of relative fluorescence changes (100% fluorescence immediately, i.e. 5 ms, after mixing). O, amplitude of fluorescence quenching; Δ, amplitude of fluorescence recovery. (B) Arrhenius plot of exponential time constants for the fluorescence changes, O, τ_1 (quenching); Δ, τ_2 (recovery).

transition, fluorescence quenching (open circles) is strongly reduced and the fluorescence recovery (open triangles) after the quenching gradually reaches zero as the temperature decreases. This indicates that 9-AA binds mostly in an irreversible way to the vesicles below the phase transition. The change of quenching amplitudes at the lipid phase transition is accompanied by a sharp break in the Arrhenius plot of the 9-AA response time constant, τ_1: E_{a1} ($\geq 39\,°C$) = 180 kJ/mol, E_{a1} ($\leq 39\,°C$) = 500 kJ/mol). Accumulation of 9-AA by the vesicles is therefore drastically hindered in the gel state of the lipids. The time constant of the fluorescence recovery, τ_2, on the other hand, does not exhibit any marked changes at the phase transition. Its activation energy remains constant at $E_{a2} = 86$ kJ/mol.

As the involvement of the negatively charged headgroups in ΔpH-induced quenching was established quite clearly by the DPPG/DPPC comparison, a variation of the negative lipid headgroup content was performed in order to pin down more precisely the nature of the binding mechanism. For the experiments depicted in Fig. 4A/B, the relative content of the negative PS, $[PS]_r$, was varied in the membrane of vesicles made of DPhPC/PS mixtures. If there was no PS present in the membrane, there was no ($\leq 1\%$) quenching of 9-AA fluorescence detectable after imposition of a pH gradient ($\Delta pH = 1.9$) across the vesicular membrane, and as the PS content of the membrane increased, quenching increased gradually, reaching a maximum of about 60% at 100% $[PS]_r$. Fig. 4A depicts this dependency of relative quenching on the PS content of the membrane. Most interestingly, the binding isotherm is of sigmoidal shape, indicating a cooperative effect of the negative serine lipids on the 9-AA quench-

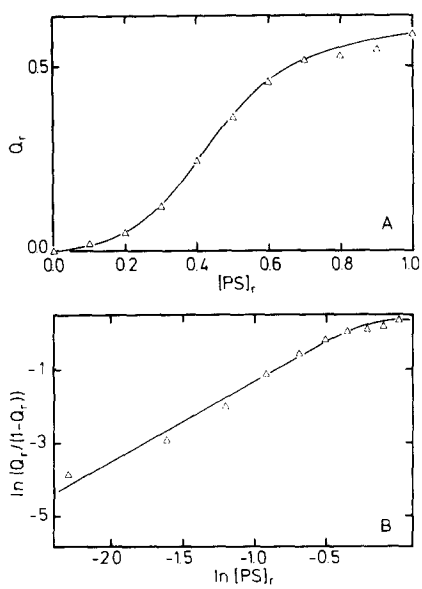

Fig. 4. (A) Relative 9-AA fluorescence quenching ($\lambda_{ex} = 400$ nm, $\lambda_{em} \geq 450$ nm) in vesicles of DPh PC and PS mixtures. Mixing (1:1) of 0.5 mg/ml vesicles, 10 mM sodium phosphate, 50 mM K_2SO_4, 300 mM sucrose (pH 5.7) with 300 mM sodium phosphate, 50 mM K_2SO_4, 5 μM 9-AA (pH 8.0) (25 °C). Control experiments without sugar showed no differences. (B) Hill presentation of the data in (A).

ing. This cooperativity is seen more precisely in a Hill presentation of the data in Fig. 4B. For this Hill presentation serine was regarded as the ligand binding to the 'macromolecule' 9-AA and the ratio of bound to free macromolecules $Q/(1-Q)$ (assuming total fluorescence loss on binding) is plotted in a double logarithmic way as a function of the number of free ligands $[PS]_r$ (assuming a great excess of free over bound ligands). The Hill constant, α_H, which is given by the slope of the straight-line fit of Fig. 4B has a value of 2.2 and the quenching in this concentration range may be described by the following empirical relation:

$$Q = \frac{\left(\frac{[PS]_r}{K}\right)^{\alpha_H}}{1+\left(\frac{[PS]_r}{K}\right)^{\alpha_H}}$$

with $K = 0.67$. This value of the Hill constant indicates that certainly more than one serine headgroup (possibly two or even more, if cooperativity is not perfect) is required for the formation of the non-fluorescent $(9\text{-AA})_x \cdot (PS)_{\geq \alpha_H}$ complex. The number of bound acridine molecules per complex cannot be determined from this experiment, because the absolute concentration of 9-AA binding sites on the vesicle surface is not known. This number will certainly be a function of temperature and most likely of ionic strength. As far as the indicator mechanism is concerned, it will also depend on ΔpH [1] or on a more general function of the electrochemical potential.

In this context, it is interesting that Nichols et al. [17] have used 10 mol% cetyltrimethylammonium bromide (positively charged) in a vesicular membrane made of 98% phosphatidylcholine (probably not synthetical) and 2% phosphatidic acid (negatively charged) and found that 9-AA was quenched by ΔpH. Furthermore also Fiolet and colleagues [18] have mentioned that 9-AA fluorescence was less quenched, but nevertheless quenched, in liposomes positively charged with hexadecyltrimethylammonium bromide. Yet, only nonpurified egg lecithin was used as a basic lipid material. We therefore suggest that in both cases the lipid bilayer contained besides the positive surface charges, net negatively charged lipid headgroups within the membrane. This indicates that the prerequisite for the quenching is the existence of net negative lipid headgroups (or other negative point charges) within the membrane, even if the overall membrane charge is neutral of positive.

In order to clarify this point still further, we have done a control experiment where the positively charged octadecylamine was incorporated (16 mol%) into a net uncharged DPhPC membrane. As in the case of the uncharged DPhPC vesicles (Fig. 4) there was no ($< 1\%$) ΔpH-induced 9-AA fluorescence change under conditions where soybean phospholipid vesicles showed 30% quenching.

The 9-AA quenching kinetics after establishment of a pH gradient were followed in the stopped-flow apparatus for all the lipid mixtures of Fig. 4. At 25°C the fluorescence decay times of the lipid suspensions that showed fluorescence quenching ($\geq 10\%$ PS content) were virtually equal ($\tau_{1,mean} = 0.56 \pm 0.08$ s). For this time constant at 100% PS content, also a temperature dependence was measured in the range of 15°C to 35°C, yielding a linear Arrhenius plot with an activation energy of 100 kJ/mol which is identical to the values for soybean phospholipid vesicles (94–103 kJ/mol, Table I).

After quenching, the fluorescence reappears very slowly in all the DPhPC/PS vesicle suspensions: within 30 min after imposition of ΔpH and after the initial quenching step, there is basically no change at all in fluorescence intensities, and fluorescence recovery is not complete after 24 h. The fluorescence can, however, be brought back, within a few minutes, to about 93% of the initial value before quenching, if micromolar concentrations of the potassium carrier valinomycin are added in the presence of 100 mM K^+. On the other hand, addition of the protonophore CCCP (5 μM) was not effective in abolishing the quenching phenomenon. It is therefore concluded that either 9-AA fluorescence quenching is due to the membrane diffusion potential or that it is in fact caused by the pH gradient itself and that the dissipation of the pH gradient is severely hindered by the membrane potential. Furthermore some (10%) unspecific quenching induced by CCCP (5 μM) without any vesicles (15 μM 9-AA) was observed, presumably due to complexation of 9-AA with the negatively-charged CCCP.

Quenching as a function of 9-AA concentration

As the fluorescence recovery in these PS vesicles is so slow, it is also possible to titrate the fluorescence quenching as a function of 9-AA concentration. Fig. 5 shows the results for these samples, i.e., a pure mixing buffer solution without vesicles (filled circles), a vesicle sample with imposed pH gradient and quenched 9-AA fluorescence (open triangles), and a vesicle sample where a pH gradient had been imposed, but the quenching was abolished by valinomycin (open squares). For low concentrations ($\leqslant 20$ μM 9-AA) there is hardly any difference detectable between the fluorescence ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 484$ nm) of the pure buffer solution and the valinomycin-treated vesicle sample. The double logarithmic presentation of the data gives nearly a straight line in this concentration range, but the slope (0.8) of the straight line is different from 1. Probably, this effect is due to inner filtering of the excitation beam (400 nm) for which no correction had been made. At higher concentrations than 30 μM 9-AA for the buffer sample (20 μM 9-AA for the valinomycin-treated vesicle sample) deviations from linearity become apparent which are due to fluorescence loss resulting from self-aggregation of 9-AA ($k_{\text{dimerization}} = 10^3\ M^{-1}$ for aqueous solutions [19]). This self-aggregation occurs already at lower concentrations in the valinomycin-treated vesicle sample, indicating that the negatively charged membrane surface might either enhance the local concentration of 9-AA or/and lower the repulsive electrostatic forces between two protonated 9-AA molecules. In accordance with the expectation that ΔpH induces quenching, fluorescence intensities of the vesicle sample with imposed pH gradient but without valinomycin are considerably lower than those of the valinomycin-treated sample. However, the relative quenching is not constant at all concentrations, but slowly increases for total 9-AA concentrations of 0.5 to 15 μM and then gradually levels off as the concentration further rises. Presumably, the binding sites for 9-AA at the membrane are all occupied for total 9-AA concentrations higher than 15 μM. This behaviour is better visualized, if the concentrations of free and quenched 9-AA are calculated and plotted. Therefore we assumed that $[9\text{-AA}]_{\text{quenched}} = Q\ [9\text{-AA}]_{\text{total}}$ and that $[9\text{-AA}]_{\text{free}} = [9\text{-AA}]_{\text{total}} - [9\text{-AA}]_{\text{quenched}}$. The result is shown in Fig. 6. Again the binding isotherm is sigmoidal, indicating a cooperative behaviour of 9-AA quenching in dependency of 9-AA concentration.

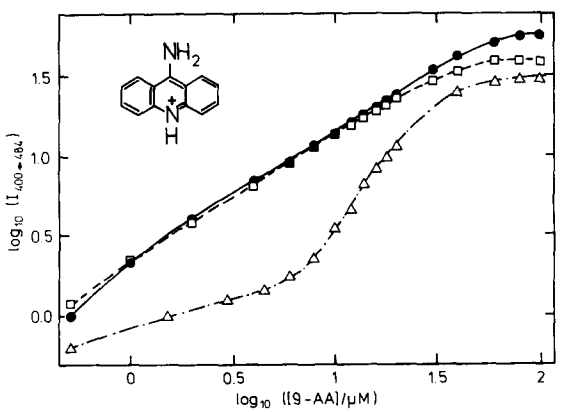

Fig. 5. Concentration dependence of 9-AA fluorescence ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 484$ nm, 25 °C). ●, 10 mM sodium phosphate, 50 mM K_2SO_4 (pH 5.7) mixed (1:1) with 100 mM sodium phosphate, 50 mM K_2SO_4 (pH 8.0). 9-AA concentration as indicated (addition of 9-AA from 1–50 mM aqueous stock solutions). △, same as ●, but pH 5.7 solution contained additionally 0.5 mg/ml PS vesicles. □, same as △, but 8 μM valinomycin was added after establishment of a pH gradient across the vesicular membrane.

9-AA excimer formation

During the course of these investigations, it became evident that ΔpH-induced 9-AA fluorescence decrease is accompanied by the formation

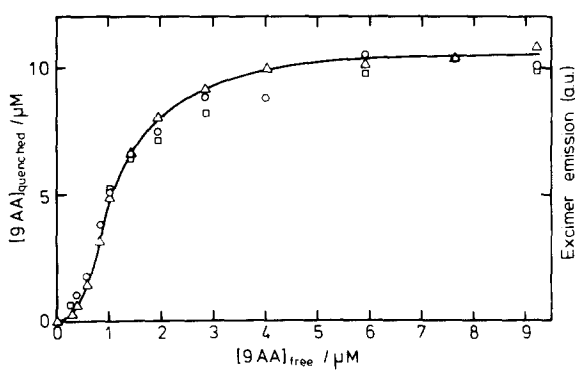

Fig. 6. Reaction conditions as in Fig. 5 (vesicle sample with quenched 9-AA fluorescence). △, dependence of $[9\text{-AA}]_{\text{quenched}}$ on $[9\text{-AA}]_{\text{free}}$ (see text). □, ○, two titrations where excimer emission intensity was determined and calculated as a function of $[9\text{-AA}]_{\text{free}}$ (see text).

of 9-AA excimers [20] which have been described in pure aqueous solutions [19] as resulting from ground state dimers which undergo a thermally activated transition from the excited dimer state to a dimer-excimer state. Excimer fluorescence happens after this transition and the reached Frank-Condon ground state of the dimer is repulsive. The emission of the excited dimer state itself (normal dimer fluorescence) before the excimer transition is not detectable in aqueous solutions at room temperature, but can be seen at liquid nitrogen temperatures [19]. Such dimer-excimer transitions are not uncommon and have been described for several systems in detail [21–23].

The characteristic, structureless, broad band (100 nm FWHM) excimer luminescence of 9-AA is centered at 560 nm [19,20]. This excimer luminescence has an excitation spectrum according to the dimer absorbance spectrum which is redshifted by about 4 nm with respect to the monomer absorbance and excitation spectrum [19,20]. As a consequence, at 440 nm relatively more excimer luminescence, as compared to monomer fluorescence, is excited than at 400 nm. Therefore, it is possible to extract the excimer emission spectrum by selective excitation at these two wavelengths from a single sample. This procedure is depicted in Fig. 7. The resulting two different 9-AA emission spectra are superimposed, after they have been normalized to each other at a wavelength (e.g. 484 nm) where no excimer emission occurs. The difference between both normalized spectra gives the excimer emission band [23]. In order to extract the excimer emission intensity at 560 nm from one sample devoid of the monomeric fluorescence contribution, it is sufficient to measure 9-AA luminescence at the following wavelength pairs ($\lambda_{ex}/\lambda_{em}$): 400/484, 400/560, 440/484 and 440/560 (in nm). Excimer emission intensity at 560 nm, I_{exc}, apart from a constant factor, is then calculated by

$$I_{exc} = I_{440 \to 560} - \frac{I_{440 \to 484}}{I_{400 \to 484}} \cdot I_{400 \to 560}$$

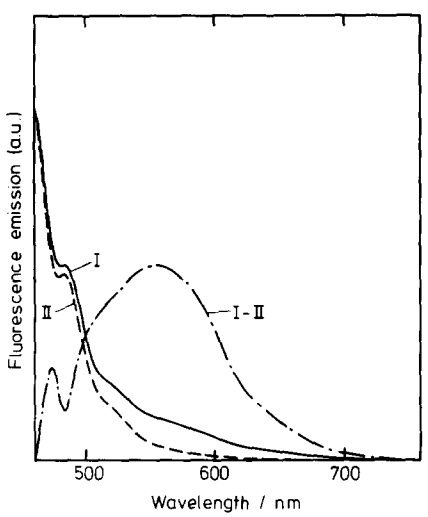

Fig. 7. 9-AA fluorescence spectra of vesicle sample with imposed pH gradient. Reaction conditions as in Fig. 5 △ (15 μM 9-AA). I, λ_{ex} = 440 nm; II, λ_{ex} = 400 nm. I and II are normalized to each other at 484 nm. I–II represents the excimer emission spectrum.

These excimer luminescence intensities have been determined as a function of 9-AA concentration in the vesicles with imposed pH gradient and quenched fluorescence, in the valinomycin treated vesicle sample, and in a control sample of buffer solution without vesicles. To a first approximation at low absorbance ($A \ll 0.4$), they should be proportional to the dimer concentration. Whereas in the vesicle sample with quenched 9-AA fluorescence a steady increase of excimer emission upon addition of 9-AA is observed, in the valinomycin treated vesicle sample and in the control sample of pure buffer without vesicles, no excimer emission (not shown, $\leqslant 20\%$ of the vesicle sample with quenched fluorescence) can be detected. The results of two such excimer titrations for the quenched 9-AA vesicle sample have been drawn into Fig. 6 at the corresponding positions of the free 9-AA concentrations. The excimer luminescence intensities of these quenched samples have been normalized at one point (7.8 μM free 9-AA) to the concentration of quenched 9-AA (Fig. 6). It is clearly evident that this dimer-excimer formation isotherm matches within experimental error the formation of quenched 9-AA. Based on this result, it is concluded that the concentration of the quenched 9-AA is at least proportional, if not equal to the concentration of 9-AA dimers.

The cooperativity seen in the sigmoidal formation isotherm of quenched 9-AA (Fig. 6) can be evaluated in a more quantitative way in a Hill

presentation of the data. For this Hill plot, $[9\text{-AA}]_{free}$ was taken as the free ligand, which can form complexes with the lipids or vesicle surfaces. The concentration of total binding sites on the vesicles was estimated to be 10.5 μM from Fig. 6. Therefore, the ratio of occupied to unoccupied binding sites was calculated as $[9\text{-AA}]_{quenched}/(10.5\ \mu M - [9\text{-AA}]_{quenched})$. A double logarithmic plot of this ratio in dependence of the concentration of free 9-AA is shown in Fig. 8. The result is a rather straight line (correlation coefficient 0.996) with a slope of 2.4, i.e. the Hill constant. Under these experimental conditions, (e.g. ΔpH, lipid composition, ionic strength, temperature) the concentration of quenched 9-AA can therefore be expressed as a function of the free 9-AA concentration in the following way:

$$\frac{[9\text{-AA}]_{quenched}}{10.5\ \mu M} = \frac{\left(\frac{[9\text{-AA}]_{free}}{K}\right)^{\alpha_H}}{1+\left(\frac{[9\text{-AA}]_{free}}{K}\right)^{\alpha_H}}$$

with $K = 1.1\ \mu M$ and $\alpha_H = 2.4$. Again the Hill constant is certainly greater than 1, demonstrating that more than one (possibly two or even more) 9-AA molecules are required to form a non-fluorescent complex of the stoichiometry $(9\text{-AA})_{\geq 2.4} \cdot (PS)_{\geq 2.2}$.

In the light of the proportionality between the concentration of quenched 9-AA molecules and the concentration of the dimer-excimer complexes, it is reasonable to assume as a first approximation that the quenching is due to the formation of a complex $(9\text{-AA})_2 \cdot (PS)_2$ at the membrane surface, which has considerably lower normal monomer fluorescence yield, but exhibits the characteristic excimer luminescence. Nevertheless the possibility for the formation of higher (9-AA · PS) aggregates and excimers of these complexes cannot be completely ruled out, albeit such aggregates have not been observed in aqueous solutions [19].

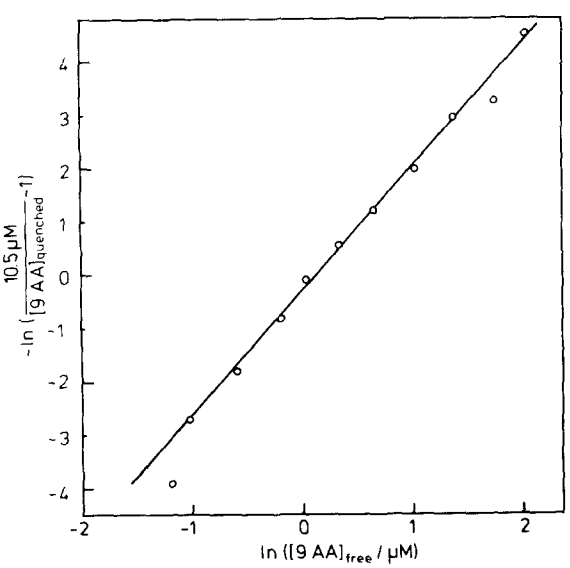

Fig. 8. Hill presentation of the data of Fig. 6 △ (see text).

Discussion

Photophysical mechanisms for the optical changes induced by ΔpH for different cationic dyes

In the present study, it has been shown that the quenching of 9-AA fluorescence due to the imposition of a pH gradient across the membrane of artificial phospholipid vesicles, is accompanied quantitatively by the formation of 9-AA dimer-excimers on the membrane surface. The quenching phenomenon depends in a cooperative manner as well on the 9-AA concentration as on the number of negatively charged phospholipid headgroups. Therefore the formation of $(9\text{-AA})_2 \cdot (\text{negative lipid headgroup})_2$ complexes with a very low monomer fluorescence yield is proposed, which occurs after the imposition of a pH gradient across the vesicular membrane.

The aggregation in the form of dimers or multimers on negatively charged model surfaces [24], model membranes [25], and energized [6,26] or non-energized [26] biological membranes, as well as DNA [27] or RNA [28] strands, has been reported for a number of cationic dyes. It is very important to note that the individual optical response of a certain dye, obviously, depends on its specific photophysical properties, whereas the aggregation phenomenon is determined by exterior properties such as geometry, hydrophobicity, and electrical charges of the dye and of the surface. Nevertheless, it is only by a thorough understanding of the photophysical properties that conclusions are meaningful which relate optical changes of these dyes interacting with the surface to changes within the surface itself. This is the prerequisite for a reliable indicator application.

Whereas quenching of monomer fluorescence during dye interaction with biological membranes is quite a widely reported, but inconclusive phenomenon, aggregation or other binding mechanisms can be deduced on the basis of more specific optical properties. Thus Dell'Antone and coworkers [24] reported a blue-shift and hypochromism in the absorbance spectra of neutral red and acridine orange due to aggregate formation at high concentrations in aqueous buffer solutions or upon binding either to polyanions or to energized submitochondrial particles. Hypochromism in absorbance due to dimerization upon binding to PS-vesicles is also reported [25] for methylene blue, 3,3′-dipropyl-2,2′-thiadicarbocyanine and 9-AA, but whereas dimerization of methylene blue is accompanied by a slight blue-shift, dimerization of the latter two dyes induces a red-shift. 9-Amino-3-chloro-7-methoxyacridine also exhibits some hypochromism upon binding to illuminated chloroplasts, but the shapes of the entire absorbance spectra are rather disturbed during the binding [26]. For some dyes, the fluorescence of the aggregate itself can be detected. This is the case for acridine orange where binding to non-illuminated chloroplast induces dimer fluorescence [26], which is markedly enhanced upon illumination of the chloroplasts [6,26]. The present study deduces the existence of the $(9\text{-AA})_2 \cdot (PS)_2$ complex on the basis of its specific excimer luminescence which differs significantly in shape and position as well as in luminescence lifetimes [20] from the structured monomeric fluorescence bands.

Binding sites on the membrane

Contrary to biological systems (e.g., mitochondria or chloroplasts) containing negative surface charges originating from both proteins and lipids, in our investigation, only negatively charged lipids are present as acceptor sites for the ΔpH-induced formation of the non-fluorescent dye complexes. This is in accordance with the work of Kopacz et al. [29] and Mueller et al. [30] who have covalently bound by photoaffinity labelling the azido analogues of 9-amino-3-chloro-7-methoxyacridine and atebrin to the membrane of non-energized and energized submitochondrial particles. Whereas in the isolated protein-dye fractions no marked optical differences between non-energized and energized particles were found, in the lipid-dye fraction drastic changes, i.e. excitation decrease and redshifts in emission, became visible. Although in these publications no specific lipids were indicated as sites of interaction, the authors gave some reference [31] that the labelling and quenching was more effective in the order phosphatidylcholine $\ll$ phosphatidylethanolamine $<$ cardiolipin. It is therefore reasonable to assume that cardiolipin (4%), phosphatidylinositol (14%), and possibly the large amount of phosphatidylethanolamine (33%), which are present [32] in the soybean phospholipid membrane, constitute the binding sites in the soybean phospholipid vesicles.

So far, only the ΔpH-linked binding to the membranes has been discussed. However, for a long time it has been known that also binding (accompanied by fluorescence quenching) of 9-AA occurs to non-energized, negatively charged membranes in the absence of high ionic strengths [33,34]. This was interpreted as a simple aggregation of the positive dye in the Gouy-Chapman layers adjacent to the negative surface [34], although some deviations from an ideal monovalent behaviour for 9-AA in the Gouy-Chapman theory were reported [35–37]. In fact this quenching has been used to probe the surface potential of yeast cells [36] and mitochondria [35,38]. Such an electrical double layer aggregation can, however, always be abolished by the addition of other ions. Concentrations for a 50% release of 9-AA fluorescence quenching are in the range of 5–20 mM for monovalent salts such as KCl [34,36] or 0.1 to 2 mM for salts of divalent cations such as $MgCl_2$ [34,36]. Therefore in the present study, where at least 100 mM K^+ was used throughout all preparations (except for one sample in Fig. 2), this purely electrical double layer induced aggregation of 9-AA is effectively excluded. In this context, it is also interesting that the presence of Mg^{2+}, on the contrary, enhances the light-induced quenching in chloroplast membranes [39]. On the other hand, quenching due to an artificial pH-gradient in bacterial chromatophores is reduced upon addition of $MgCl_2$ [13]. Casadio and Melandri [13], therefore, concluded that electrostatical forces play a major role in the quenching mechanism. This is certainly in agreement with the long-known fact,

that a net positive charge on the acridine nucleus at the pH of the experiment is a prerequisite for an energy-induced quenching for a great number of acridine derivatives investigated [2,3].

A third quenching phenomenon seems to appear, if, during the working cycle of membrane-bound enzymes, negative amino acid residues are exposed which can act as binding sites for 9-AA. Such observations have been made for a light-dependent nigericin insensitive quenching of 9-AA in chromatophore suspensions [40] and also in suspensions of purple membrane sheets, even in the presence of millimolar Ca^{2+} concentrations, upon excitation of the bacteriorhodopsin photocycle [41,42]. This quenching can be due either to a simple aggregation of 9-AA in the vicinity of the exposed negative amino acids or due to an energy transfer or exciplex formation between 9-AA and specific surrounding protein components.

Mechanisms of 9-AA attraction to the energized membranes

Whereas the photophysical mechanism of the 9-AA fluorescence loss appears now explained, the question remains how the 9-AA molecules are attracted to the vesicle membranes to form the (lipid) · (9-AA) complexes upon imposition of the pH gradient. Intimately related to this problem is the question whether the dye is located at the inner or outer surface of the vesicles in the quenched state [3]. Schuldiner and co-workers [1] had clearly stated their view that the uncharged 9-AA molecules are taken up by the vesicles and become protonated and therefore trapped within the bulk phase of the acidic vesicle interior. On the basis of our study, the Schuldiner mechanism would have to be modified in a way that the concentrated, protonated 9-AA molecules form complexes with the negative lipid headgroups at the inner membrane surface. The attracting force into the vesicles would still be the pH gradient. In accordance with this translocation to the inner surface of the vesicles is the diminuation of quenching and the increase of activation energy for the 9-AA response time in DPPG membranes below the phase transition (Fig. 3). One would expect that the permeation of the uncharged 9-AA is strongly hindered below the phase transition of the lipid barrier. In this context, it is worth mentioning that the presence of membrane proteins in a bilayer of lipids in the liquid-crystalline state also increases the response time of 9-AA [41]. This can be explained by the observed increase in order of the lipids by the interaction with the membrane proteins [43]. The response time of 9-AA to the imposed pH gradients is in the range of several hundred milliseconds (0.2 s for soybean phospholipid and 0.6 s for PS, 25 °C). These long time constants in themselves seem to indicate a slower reaction mechanism than the pure electrostatical attraction of protonated 9-AA to the outer vesicular surface, e.g., migration to buried binding sites in the headgroup layer or translocation across the bilayer.

On the other hand, strong evidence was put forward by Kraayenhof and colleagues that the energy-induced binding of acridine dyes happens at the outside of chloroplasts and that permeation is not required for the light-induced quenching. These authors have bound atebrin derivatives covalently via the 9-amino side chains to membrane-impermeant proteins such as bovine serum albumin [4] or lysozyme [2], and light-induced quenching was still observed in disrupted chloroplasts, i.e. grana particle suspensions. Similarly, such atebrin derivatives were covalently bound to chloroplasts themselves and still preserved their light-induced, uncoupler-sensitive fluorescence quenching [5]. Furthermore, the response of the covalently bound dyes was considerably enhanced and faster (30–60 ms) as compared to control experiments with non-bound dyes [5]. Kraayenhof [4], therefore, proposed the exposure of negative sites on the outside of the grana particles in the energized state. Protein conformation changes with exposure of negative groups are, however, excluded in our experiments where no energized proteins were present. Other attractive forces, therefore, have to be taken into consideration. The most simple attraction mechanism to the outside of the vesicles with artificial pH gradient would be the diffusion potential, $\Delta\psi$, that generates from the more acidic vesicle interior. Certainly, the dependency of quenching on ΔpH (which is proportional to the Nernst potential, if no other than water ion fluxes are involved) as in Eqn. 1 is not in contradiction to this proposal. This linear relation between $Q/(1-Q)$ and ΔpH has been veri-

fied in many experiments [13,44–46], but the volume dependency of Eqn. 1 which is required for the Schuldiner mechanism has been found to be completely incorrect (Refs. 13,47,48 and the present study). In addition, discrepancies between ΔpH decay times as monitored with pyranine or with 9-AA (Fig. 1) contradict a tight relation between ΔpH and the quenching during the complete time course of ΔpH dissipation. As other ions than protons or hydroxide ions must be additionally involved in the dissipation of the transmembrane concentration gradients, certainly $\Delta\psi$ will decay faster than ΔpH. This could be an explanation for the observed faster 9-AA fluorescence recovery kinetics.

Strong arguments, however, contradict this $\Delta\psi$ hypothesis. At first, valinomycin in company with KCl, which abolishes the diffusion potential, enhances the light-induced quenching in bacterial chromatophores [44]. Secondly, the electrical potential difference that generates upon energetization of natural membrane systems (i.e. pumping-in of protons) such as grana particles or submitochondrial particles is in opposite direction to the diffusion potential across the membrane of vesicles where an artificial pH-gradient (inside acidic) has been imposed by mixing.

For the arguments given above, the question whether 9-AA is in fact a ΔpH-probe, remains moot, and the practical uncertainties during its application are considerable. At first, the volume dependency of Eqn. 1 does not hold, a fact which requires a separate calibration for every system under investigation as shown by Casadio and Melandri [13]. Secondly, Eqn. 1 with the corrected volume, in the best case, will work accurately only under steady-state conditions, because a kinetical relation between the ΔpH decay and 9-AA fluorescence recovery could not be established (Fig. 1).

Conclusion

The main results of this investigation may be summarized as follows: (1) Response times of 9-AA fluorescence quenching to artificially imposed pH gradients across vesicular membranes are in the range of several hundred milliseconds and are not strongly affected by the ionic composition of the medium.

(2) 9-AA fluorescence recovery which should indicate the dissipation of the pH gradient, occurs about 10-times faster than the ΔpH decay monitored with the well-behaved pH-indicator pyranine.

(3) Response of 9-AA to imposed pH gradients becomes blocked to a great extent below the lipid phase transition of DPPG.

(4) No ΔpH-induced quenching of 9-AA fluorescence occurs in the case of vesicles made from net-uncharged lipids such as DMPC, DPPC, DPhPC.

(5) ΔpH-induced quenching can be obtained when negatively charged serine lipids are introduced in the membrane of DPhPC vesicles. The quenching amplitude depends in a cooperative way ($\alpha_H = 2.2$) on the number of the serine lipids.

(6) The concentration of quenched 9-AA molecules also depends cooperatively ($\alpha_H = 2.4$) on the concentration of free 9-AA.

(7) The concentration of quenched 9-AA is furthermore proportional to the number of 9-AA dimer-excimer complexes which are formed during the quenching process.

(8) Quantitative determinations of ΔpH across membranes with 9-AA according to Ref. 1 are not recommended.

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