Comparison of 9-Aminoacridine and Atebrine Induced Changes in Optical, Electrical and Mechanical Characteristics of Lipid Bilayers

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Abstract. The effects of fluorescent probes 9-aminoacridine (9AA) and atebrine (AT) on physical properties of liposomes and planar bilayer lipid membranes (BLM) were studied. The method of fluorescence spectroscopy and the electrostriction method based on measurement of higher current harmonics were used. At low concentrations ($10^{-5} - 5 \times 10^{-5}$ mol/l), 9AA increased fluorescence intensity, while in liposomes from soybean phosphatidylcholine fluorescence quenching occurred at higher probe concentration. Fluorescence quenching occurred over the entire concentration range tested ($10^{-5} - 10^{-4}$ mol/l) in liposomes made from a mixture of egg phosphatidylcholine and cardiolipin. In contrast to 9AA, AT, thanks to its hydrophobic chain, penetrates deeper into the hydrophobic membrane moiety; thus, immobilization of the molecule and an increase in fluorescence intensity was always observed. Probes adsorbed to membranes, leaving their electric capacitance effectively unchanged. Adsorption of charged dye particles induced small changes in transmembrane potential. In the presence of 10^{-5} mol/l AT, the modulus of elasticity $E_\perp$ increased somewhat for soft membranes ($E_\perp \sim 2.5 \times 10^7$ Pa), whereas it decreased for hard membranes ($E_\perp \sim 5 \times 10^7$ Pa). pH gradient present on the membrane affected the ability of the dyes to incorporate into the membranes. Our results provide evidence against the proposed model of the quenching mechanism introduced by Rottenberg and Lee (1975).

Key words: Atebrine — 9-Aminoacridine — pH dye — Lipid bilayer — Membrane potential — Modulus of elasticity — Conductance

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Introduction

Fluorescent probes have a significant place among indicators of intracellular pH in small cells or intracellular structures (e.g. mitochondria). Aminoacridine derivatives, in particular 9-aminoacridine and atebrine (Fig. 1*a*), are pH probes that have been most widely employed in studying organelles. Typically, these compounds quench their fluorescence upon energization in membrane systems (organelles, chloroplasts) (Lee 1971; Azzi et al. 1971; Azzi 1975; Kraayenhof et al. 1976; Kraayenhof 1977; Konishi et al. 1986). Lee (1971), Deamer et al. (1972) and Massari et al. (1974) concluded that it is the generation of a pH gradient on membranes that is the major driving force of fluorescence quenching with the above compounds. Two possible mechanisms were studied in this respect: dye transition across membranes and probe binding to membranes. Rottenberg and Lee (1975) proposed a model of the quenching mechanism based on the following assumptions: membranes are permeable to uncharged acridine molecules only; membranes do not bind the dye; optical changes are due to intravesicular changes in the dye concentration; the molecules that have entered vesicles do not contribute to fluorescence. However, several discrepancies between the proposed model and the experimental results occurred: nonlinearity of fluorescence quenching in dependence on pH at low 9-aminoacridine concentrations (Deamer et al. 1972); inapplicability of the model equation for diamines (in particular atebrine); concentration dependence of fluorescence quenching; lack of effect of osmotic particle volume on fluorescence quenching; dependence of the slope of pH/fluorescence quenching curve on membrane surface charge (Fiolet et al. 1974; Massari et al. 1974). In addition, experiment-based estimates of osmotic volumes supply no physically possible results. It can be concluded therefore that the above model does not reflect the exact mechanism of quenching. Generally, the following effects should be considered (Vladimirov and Dobretsov, 1980; Kicq et al. 1987; Grzesiek and Dencher 1988; Grzesiek et al. 1989; Hianik et al. 1990): changes in probe fluorescence induced by probe binding to membranes and to vesicle contents, although quenching only concerns a portion of the bound probe molecules; fluorescence quenching depends on the charge composition of the membrane (no quenching occurred in the presence of a pH gradient with zwitterion lipids and 9-aminoacridine, (Grzesiek and Dencher 1988)); quenching can also be due to the dye accumulating in the near-membrane region because of a negative charge present on the membrane (Grzesiek and Dencher 1988, Grzesiek et al. 1989)

According to an analysis by Tikhonov and Blumenfeld (1985) of the reported data concerning ΔpH determination obtained using fluorescent dyes, e.g. in pH determination inside chloroplasts, show some discrepancies. One reason might be a change in parameters of fluorescent dyes due to their interaction with the membrane. Moreover, 9AA and AT bind to the chloroplast membranes (Krendeleva et al. 1976, Searle et al. 1977). This binding allowed the use of 9AA and AT probes

for the investigation of the structural state of chloroplasts (see, e g Kuznetsova and Kukushkin 1981) The exact mechanism of interaction of fluorescent probes with membrane systems remains unclear It is not clear how membrane physical properties change upon the incorporation of a fluorescent probe or how they depend on electrolyte pH and on the structural state of the lipid bilayer The understanding of these issues is of a great significance for bioenergetic investigations as pH changes play an important role in mechanisms of energy transformation

In particular, changes in fluorescent intensity of probes have to be compared with changes in physical parameters directly related to the possible interaction of the dye with the membrane The appropriate parameters include membrane conductance, capacitance, potential and modulus of elasticity in the direction perpendicular to the membrane plane, $E_\perp$ Membrane potential and capacitance are useful for monitoring adsorption of either charged or large molecules, when some alteration of electric charge or membrane thickness could be expected Membrane conductance reflects either flow of charged particles through membrane or adsorption of particles resulting in membrane deterioration $E_\perp$ is very sensitive to structural changes and to the degree of ordering in the membrane hydrophobic part Thus, this variable could effectively be used to detect interactions of small neutral molecules with membranes BLM consist of lipids and hydrocarbon solvent, and represent a suitable model of cell membranes (Tien 1974) To check the possible effects of dye interaction with lipid bilayers, we used fluorescent spectroscopy, measured of BLM conductance, and employed electrostriction to measure $E_\perp$ electrical capacity, C, and membrane potential, V_m , under various conditions As a model of chloroplast membrane, bilayers from negatively charged lipids - soybean phosphatidylcholine and egg phosphatidylcholine+cardiolipin mixture, were used For comparison, also egg phosphatidylcholine and cholesterol mixture (0 11 mole fraction of cholesterol) was used The values of pH and ΔpH chosen were close to physiological conditions typical for energized chloroplasts pH_e 5 6, pH_i 8 3, ΔpH 2 7 (Tikhonov and Blumenfeld 1985) Measurements of the above parameters were performed under symmetrical (identical pH on both membrane sides) and asymmetrical conditions (formation of a ΔpH gradient)

Materials and Methods

Chemicals

BLMs were formed according to Mueller et al (1962) A solution of soybean phosphatidylcholine (SBP) (Sigma) and egg phosphatidylcholine + cholesterol (EPX) (Kharkov, Plant of Chemical Preparations, Ukraine) (0 11 mole fraction cholesterol) in *n*-heptane (40 mg/ml) (Fluka) was dropped on an aperture (0 3 or 0 5 mm in diameter) in the wall of a teflon cup The electrolyte (0 1 mol/l NaCl + 10 mmol/l Tris-HCl in redistilled water) pH was adjusted with HCl or NaOH to yield values required The fluorescence probes atebrine (AT) and 9-aminoacridine (9AA) (Reanal) dissolved in ethanol were added into

the solution in the frontal compartment under continuous stirring with a magnetic stirrer The concentration of ethanol in the electrolyte did not exceed 2% The same volume of electrolyte was added to the other compartment to compensate for hydrostatic pressure The temperature of the cup was kept at 20 °C

Liposomes were prepared by ethanol injection (Vladimirov and Dobretsov 1980) from egg phosphatidylcholine and cardiolipin mixture (EPC) (mass ratio 50 3 3) (Kharkov, Plant of Chemical Preparations, Ukraine) and from SBP The final liposome concentration in the electrolyte was 2 1 mg/ml All reagents were of analytical grade

Measurements of fluorescence intensity

Fluorescence measurements were performed with a spectrofluorimeter described elsewhere (Kuznetsova and Kukushkin 1981) Fluorescence was excited by light at $\lambda = 360 - 440$ nm (filters FS-1 and FS-6) and recorded at $\lambda = 510$ nm (filters SES-8 and ES-8)

Measurements of BLM conductance

Membrane conductance g was determined using the method described by Hladky and Haydon (1972) Transmembrane current was detected at constant direct voltage with an amplitude of 70 mV (applied through calomel electrodes), using an electrometric amplifier type WSH 223 (Dostál 1981) The apparatus allowed recording of membrane currents of very small amplitudes ($\sim 10^{-13}$ A) The currents were recorded on a line recorder TZ4100 (Laboratorní přístroje Prague, Czechoslovakia)

Measurements of modulus of elasticity $E_\perp$ and transmembrane potential V_m

Membrane elasticity was determined using the method developed by Passechnik and Hianik (1977) According to this method, alternating voltage $V = V_0 \sin(2\pi ft)$, where V_0 is the voltage amplitude, f is the frequency, and t is time, is applied to a membrane, producing electrostatic pressure $p = C_s V_0/2h$, where C_s is the specific capacitance of the membrane and h is its thickness An equivalent electric scheme of the membrane can be represented by a capacitor with a capacity C, connected in parallel with a resistor R Under the conditions of the experiments the values of physical quantities were $C \sim 1$ nF, $f = 1$ kHz, $R \sim 10^8 - 10^{11}\Omega$, $R \gg 1/2\pi fC$ The elasticity modulus of the BLM was measured only if the above condition was fulfilled E g , in the presence of a periodic electric field the resulting ohmic current flowing through EPX membranes after the last addition of the dye (at 7×10^{-5} mol/l) was typically in the range 0 1–100 pA, which corresponds to a 1–1000-fold decrease of membrane resistance Thus, resistance will not affect the capacity current flowing through the circuit Because of electrostriction the membrane is attenuated and its electrical capacitance increases Due to the nonlinear dependence of membrane capacitance on voltage amplitude, this is associated with modulation of the alternating current flowing through the membrane The current with frequency f and amplitude A_1 contains a component with frequency $3f$ and amplitude $A_3 \ll A_1$ The changes in membrane thickness in response to external pressure are characterized by modulus of elasticity $E_\perp = -p/(\Delta h/h)$, where $\Delta h/h$ is the relative change in membrane thickness The parameter $E_\perp$ is determined from (Passechnik and Hianik 1977)

$$E_\perp = 3C_s V_0 A_1/4hA_3 \qquad (1)$$

Upon adsorption to a membrane of charged particles, transmembrane potential V_m is generated Then, the second current harmonics with amplitude A_2 and frequency $2f$ is generated (Carius 1976) The parameter V_m can be determined as

$$V_m = V_0 A_2/4A_3 \tag{2}$$

This means that it is sufficient to measure amplitudes A_1, A_2 and A_3 only This can be done with the use of a standard electronic apparatus including resonance amplifiers (Passechnik and Hianik 1977)

In our experiments, voltage with amplitude $V_0 = 100$ mV and frequency 1kHz was applied The following values were used in calculating the values of $E_\perp$ $C_s = 3\,4 \times 10^{-3}$ F/m^2, $h = 5\,5$ nm (Hianik et al 1984) (BLM from EPX and those from SBP have similar values of C_s and h)

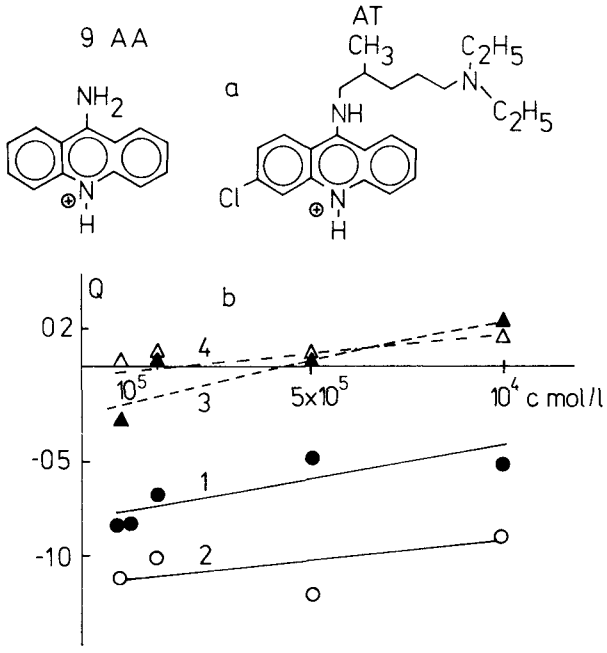

Figure 1. *a* Structural formulae of 9-aminoacridine (9AA) and atebrine (AT) *b* The dependence of the relative change in fluorescence intensity $Q = (F_0 - F)/F_0$ on AT (curves 1,2) and 9AA (curves 3,4) concentration in the liposome solution, liposomes from SPC (curves 1,3) and those from EPC (curves 2,4) F and F_0 are fluorescence intensities in the presence and absence of liposomes, respectively Electrolyte 0 1 mol/l NaCl + 10 mmol/l Tris-HCl, pH 5 6, T = 20 °C

Table 1. The dependence of relative changes in fluorescence intensity Q of dyes 9AA (Q_{9AA}) and AT (Q_{AT}) in SPC liposome suspension on electrolyte pH Dye concentration 10^{-5} mol/l, e and i stay for the external and internal environment of liposomes respectively The dependence of the value of $E_\perp$ SPC BLM on electrolyte pH Standard errors are shown as evaluated from the values of Q and $E_\perp$ from 3 liposome samples and 10 BLMs in each series Statistically significant results at $p > 95\%$ (1,2), $p > 99\%$ (3), 4-insignificant results (Student's t test)

pH		Q_{9AA}	Q_{AT}	$E_\perp, 10^7$ Pa
e	i			
5 6	5 6	$-0\ 36 \pm 0\ 07^{1\,2}$	$-0\ 82 \pm 0\ 12^{1\,3}$	$3\ 05 \pm 0\ 39^4$
8 5	8 5	$0\ 04 \pm 0\ 08^1$	$-0\ 18 \pm 0\ 02^3$	$3\ 12 \pm 0\ 23^4$
8 5	5 6	$0\ 04 \pm 0\ 06^2$	$-0\ 36 \pm 0\ 10^1$	$3\ 22 \pm 0\ 42^4$

Results

Fluorescence intensity

Fig 1*b* illustrates the dependence of the relative change in fluorescence intensity Q on the concentrations of the probes 9AA and AT (liposomes from SBP and EPC, electrolyte pH 5 6) At low concentrations, 9AA increased fluorescence intensity ($Q = (F_0 - F)/F_0 < 0$) over the entire concentration range tested ($10^{-5} - 10^{-4}$ mol/l) in SBP liposomes (curve 3) only Fluorescence quenching ($Q > 0$) occurred at higher probe concentrations ($c > 5 \times 10^{-5}$ mol/l) In EPC liposomes, quenching was observed practically over the entire range of concentrations tested (curve 4) In principle, atebrine acted in the same manner on both liposome types In both cases AT increased the fluorescence intensity (curves 1, 2) The above results were obtained at fixed electrolyte pH (pH 5 6) Under this condition the dyes 9AA and AT are in protonated form A pH-dependence of the effectivity of the dye interaction with lipid bilayer is expected To check this the pH dependence of the value of Q was measured for 9AA and AT after their addition into the suspension of SPB liposomes at 10^{-5} mol/l under both the symmetrical and the asymmetrical condition The pH value required was obtained by the addition of small amounts of 0 1 mol/l NaOH or HCl The experimental results are shown in Table 1 It is obvious that absolute values of Q_{AT} and Q_{9AA} decrease with the increasing pH Under asymmetrical condition, Q_{AT} has an approximately intermediate value and lies between the values of Q_{AT} (pH 5 6) and Q_{AT} (pH 8 3) The value of Q_{9AA} (pH 8 3/pH 5 6) is the same as that for Q_{AT} (pH 8 3) Under these conditions the structural state of non-modified membranes, as determined by the value of $E_\perp$ is practically independent of electrolyte pH The changes of Q_{AT} agree with the pK values for the dye The protonated form of AT interacts stronglier with the

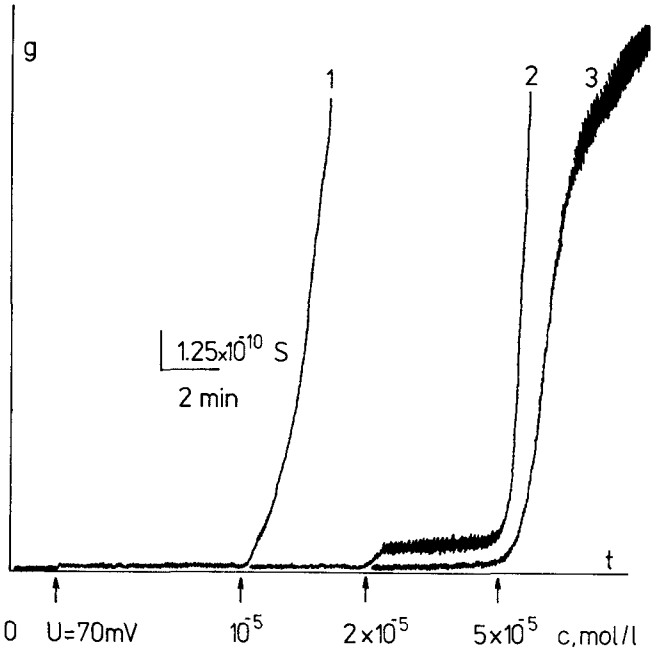

Figure 2. Time dependence of SPC BLM conductance in the presence of pH probes: 9AA (pH 5.6; curve 1), AT (pH 8.5; curve 2) and AT (pH 5.6; curve 3). All measurements were performed under symmetrical conditions (pH values relate to both membrane sides). Arrows indicate application of constant voltage and/or dye additions.

membranes. No similar correlation could be observed for the 9AA.

Conductance measurements

The original idea concerning the quenching mechanism of pH probe fluorescence was based on dye diffusion into membrane structures (Rottenberg and Lee 1975). To check this possibility, the effect of dye addition on membrane conductance was studied. Fig. 2 shows an example of the time course of conductance changes induced by AT and/or 9AA on SBP BLMs. The addition of small amounts of 9AA into the electrolyte, up to 10^{-5} mol/l, resulted in large changes in BLM conductance (curve 1). During the first 2.5 min the conductance changed more than one order of magnitude. No statistically significant pH dependence of changes in relative values of conductance $\Delta g/g_0 = (g - g_0)/g_0$ could be observed (see Table 2). Unlike 9AA, atebrine induced changes in BLM conductance occurred at higher concentrations ($2 - 5 \times 10^{-5}$ mol/l). In contrast, the relative changes in membrane conductance upon AT addition (up to 2×10^{-5} mol/l) depended on electrolyte pH. During the first 2.5 min these changes reached considerably lower values than those induced by 9AA in the same period and at twice higher concentrations. The increasing

Table 2. The dependence of the relative change in BLM conductance $\Delta g/g_0 = (g-g_0)/g_0$, at time $t = 2.5$ s after the addition of 9AA (final concentration 2×10^{-5} mol/l) and/or AT (final concentration 2×10^{-5} mol/l) into the electrolyte, on electrolyte pH. The dyes were added to the outer membrane side. The indices e and i stay for the external and internal BLM side respectively. Three membranes in each series. * - statistically significant results at $p > 99\%$ (Student's t-test).

pH		$(\Delta g/g_0)_{9AA}$	$(\Delta g/g_0)_{AT}$
e	i	$c = 10^{-5}$ mol/l	$c = 2 \times 10^{-5}$ mol/l
5.6	5.6	47.3 ± 18.5	$0.4 \pm 0.2^*$
8.5	8.5	23.5 ± 11.8	$3.8 \pm 0.7^*$
8.5	5.6	74.9 ± 55.7	3.0 ± 1.4

conductance frequently resulted in membrane destruction; not infrequently, the membrane was destroyed immediately after the addition. Owing to the presence of cholesterol, membranes from EPX showed increased stability; however, with the increasing conductance they were destroyed even more rapidly than SPB bilayers. The results of measurements of $\Delta g/g_0$ on SPB BLMs are shown in Table 2. It is obvious that with the increasing electrolyte pH the value of $\Delta g/g_0$ increases almost one order in magnitude upon AT addition.

Direct diffusion of the probes across the membrane would be expected to be associated with smaller changes of BLM conductance at the added dye concentrations. Probably, the dyes induced certain structural transitions in the membrane because of the formation of defects in the membrane structure. Evidence for such a possibility is that, upon addition of 9AA and/or AT into the electrolyte at $10^{-5} - 2 \times 10^{-5}$ and 5×10^{-5} mol/l respectively, the BLM conductance continously increased and did not saturate. Also, in some cases BLM got destroyed. Immediately prior to BLM destruction, membrane conductance fluctuations were considerably enhanced. Thus, the dyes could have induced structural changes of BLM. Such changes can be detected by measurements of mechanical parameters of BLM.

Capacitance measurements

With the method of higher current harmonics, BLM capacitance is given by equation

$$A_1 = 2\pi f C V_0 \tag{3}$$

In many membranes, the value of A_1 did not change during the measurements. Based on this it can be concluded that within the interval of volume concentrations tested (10^{-5} mol/l $- 7.5 \times 10^{-5}$ mol/l) the dye adsorption did not change

membrane capacitance. Thus, eq. (1) can be used. Changes in membrane capacitance might reflect changes in parameters C_s and h in eq.(1), and could in addition introduce an error in determining capacitance in the presence of a significant contribution of the conductance component. But, A_1 showed only random changes that frequently occurred after repeated additions. These changes were induced by membrane destabilization due to changes in hydrostatic pressure during dye addition (membrane bulging due to the osmotic pressure of the added dye), and due to the increased membrane conductance (see previous paragraph). Usually, at dyes concentrations of $c > 7.5 \times 10^{-5}$ mol/l, the value of A_1 increased (membrane area extension and attenuation). Membrane bulging was most marked with AT at pH 5.6; at this pH, the prevailing part of the dye molecules carry two positive charges, and this creates some electroosmotic pressure. At large changes in A_1 ($\Delta A_1/A_1 > 0.3$) membranes burst. Change in A_1 were mostly of a saturating nature. Continuous changes in A_1 after dye additions were typically observed in measurements with ΔpH generated across the membrane. These changes unequivocally reflect an increase of membrane conductance under these conditions. Sometimes, growth of A_1 (at A_3 = const) was observed, especially when a pH gradient was present during the measurements. This unequivocally reflects an increase in membrane conductance.

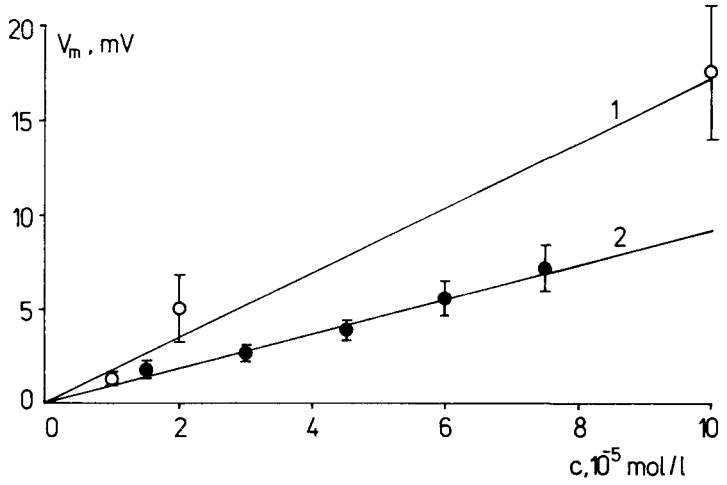

Figure 3. The dependence of transmembrane potential (as determined from parameters A_1 and A_3) on AT concentration in the solution, for SPC membranes (curve 1) and those from EPX (curve 2). S.E. calculated from 3–5 membranes in each series are shown.

Transmembrane potential measurements

Fig. 3 shows the dependence of transmembrane potential on AT concentration (electrolyte pH 5.6) for membranes formed from SBP (curve 1) and for those from EPX (curve 2). Evidently, changes in transmembrane potential, and thus adsorption, are larger for SBP bilayers, due to the larger negative charge of the membrane and to the absence of cholesterol. If AT adsorbs only on one side of the membrane and both the surface and the dipole lipid potentials are negligible, the resulting change in transmembrane potential equals the surface potential of the adsorbed dye. Based on this assumption, transmembrane potential values correspond to a surface charge density $\sigma \sim (1.6 - 27.2) \times 10^{-3}$ C/m^2 and $\sigma \sim (2.6 - 10.6) \times 10^{-3}$ C/m^2 for SBP and EPX membranes respectively. Considering that the phospholipid head area is approx. 0.7 nm^2 (Ivkov and Berestovsky 1981) and if the dye does not lose charge (AT^{2+}), the molecular lipid . dye ratio changes within approx. (330:1, 22:1) and (235:1, 54:1) for SBP and the EPX given concentration ranges respectively.

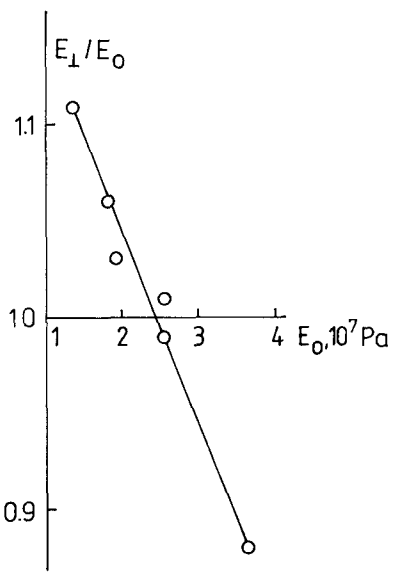

Figure 4. The dependence of the relative modulus of elasticity $E_\perp/E_0$ on the initial value E_0 in the presence of AT (final concentration 10^{-5} mol/l) in the electrolyte in contact with one BLM side BLM from SPC.

The dependence of dye-induced changes in modulus of elasticity on the initial value of the parameter

Membranes prepared by the method according to Mueller et al. (1962) contain various solvent concentrations; consequently, they differ in their physical characteristics. Due to differences in initial ordering, the addition of identical amounts of any of the dyes tested may produce different changes in modulus of elasticity

Fig. 4 illustrates the relationship of the ratio $E_\perp/E_0$ and the initial value of E_0, ($E_\perp$ is the modulus of elasticity in the presence of 10^{-5} mol/l AT). Parameter $E_\perp$ increases ($E_\perp > E_0$) for somewhat soft membranes with $E = 2.5 \times 10^7$ Pa, whereas it decreases ($E_\perp/E_0 < 1$) for hard membranes. The effects of incorporation of AT molecules are similar to that of cholesterol (Ivkov and Berestovsky 1981). At small values of modulus of elasticity, the membrane is little ordered. The dye addition results in an increased ordering of the membrane, associated with an increase in modulus of elasticity. At high values of modulus of elasticity, the dye adsorption disturbs the ordering, resulting in a decrease of $E_\perp$.

The dependence of modulus of elasticity on dye concentration and electrolyte pH

Membranes prepared from EPX (0.11 mole fraction cholesterol) with high values of modulus of elasticity (approx. 5×10^7 Pa) were used for the investigations of the direct effects of dye concentration and electrolyte pH on membrane modulus of elasticity. Only experiments showing small relative changes of parameter A_1 ($< 3\%$) were included in the analysis. Figs. 5*a* and 5*b* show the results for relative changes of elasticity modulus $\Delta E_\perp = (E_\perp - E_0)/E_0$ ($E_\perp$ is the elasticity modulus of BLM after the addition of the dye, E_0 is the elasticity modulus of non-modified BLM, in the absence of dye). Under symmetrical conditions, atebrine-induced changes in $\Delta E_\perp$ increased with the increasing pH (Fig. 5*a*, curves 1,2), due to the growth of the negative charge on the membrane, to the presence of the neutral form in the solution, and to the possible titration of the positive charge on the membrane. The dye thus can incorporate deeper into the BLM. In the presence of a pH gradient, adsorption was strongly blocked by the electrical field (at pH 8.5/5.6, the initial membrane charge was approx. 20 mV), due to the proton gradient which acts against the incorporation into the membrane of the positively charged form of the atebrine molecule. Therefore, the change $\Delta E_\perp$ is considerably smaller in the presence of a pH gradient of 8.5/5.6 (curve 3) than that for the symmetrical case of pH 8.5/8.5 (Fig. 5a, curve 2).

9-Aminoacridine shows a largely inverse behaviour. Its single charge is compensated for higher pH. The uncharged form of the molecule has but a weak affinity to the membrane, and its effect on modulus of elasticity weakens with the increasing pH. The 9AA-induced changes in modulus of elasticity are slightly stronger in the presence of a pH gradient 8.5/5.6 (Fig. 5*b*, curve 3) than under symmetrical conditions. These changes may reflect enhanced formation of dimers on the membrane or in the near membrane region (Grzesiek et al. 1989). The pH gradient acts against the "anchoring" of 9AA in the membrane, the dye molecules "float" on the BLM surface and form dimers with other molecules. Interaction of these dye aggregates with the membrane may result in a slightly greater decrease of $E_\perp$ comparing to that under symmetrical pH conditions (Fig. 5*b*). 9-Aminoacridine shows saturation ($\Delta E_\perp = \Delta E_\perp(c)$) in the presence of a pH gradient. The behaviour

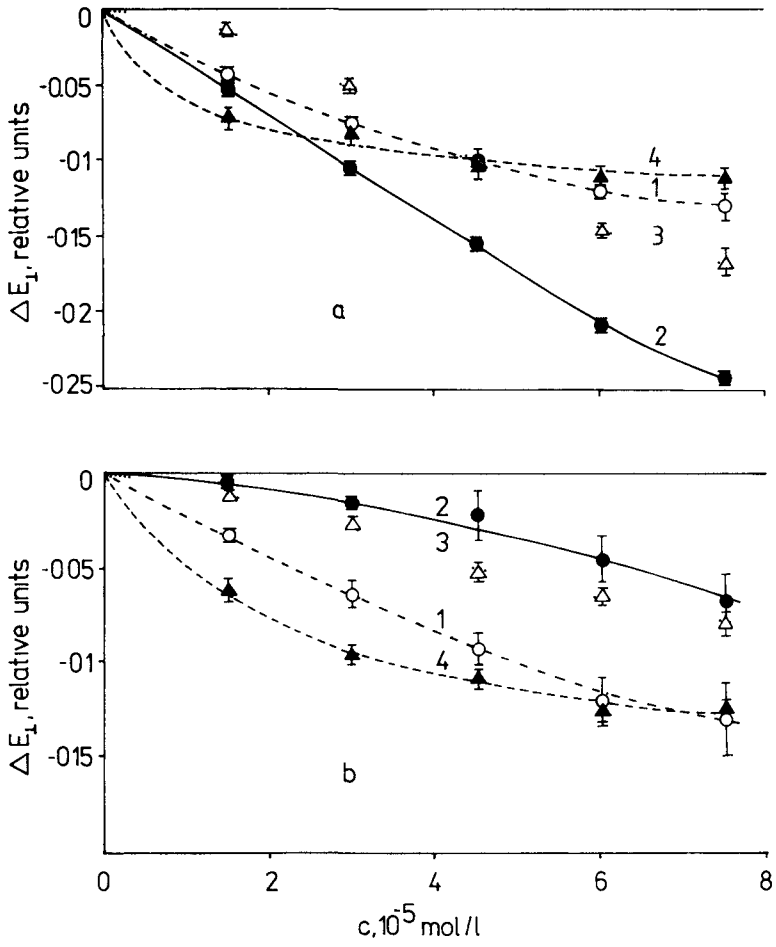

Figure 5. The dependence of the relative change in modulus of elasticity $\Delta E_\perp = (E_\perp - E_0)/E_0$ on the dye concentrations in the electrolyte a AT, b 9AA pH values 1 – 5 6/5 6, 2 – 8 5/8 5, 3 – 8 5/5 6, 4 – 5 6/8 5 The first figure always refers to the membrane side to which the dye was added Membranes from EPX S E calculated from 3–5 membranes in each series are shown

of this probe under the above condition is thus very similar to that of AT Both dyes show approximately identical concentration dependencies in the presence of own inverse pH gradient (Fig 5*a*,*b*, curve 4) The electrical field across the membrane has opposite direction and helps positively charged particles adsorb to the membrane (rapid saturation of the concentration dependence) The limit values are close to those measured in symmetrical conditions at pH 5 6/5 6 (Fig 5*a*, *b*)

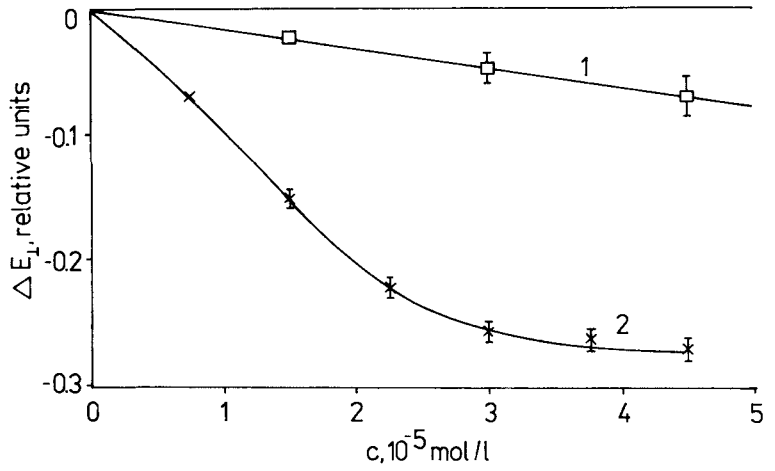

Figure 6. The dependencies of the relative change in modulus of elasticity $\Delta E_\perp = (E_\perp - E_0)/E_0$ on 9AA (curve 1) and AT (curve 2) concentration in the electrolyte at symmetrical pH 10/10. Membranes from EPX. S.E. calculated from 3–5 membranes in each series are shown.

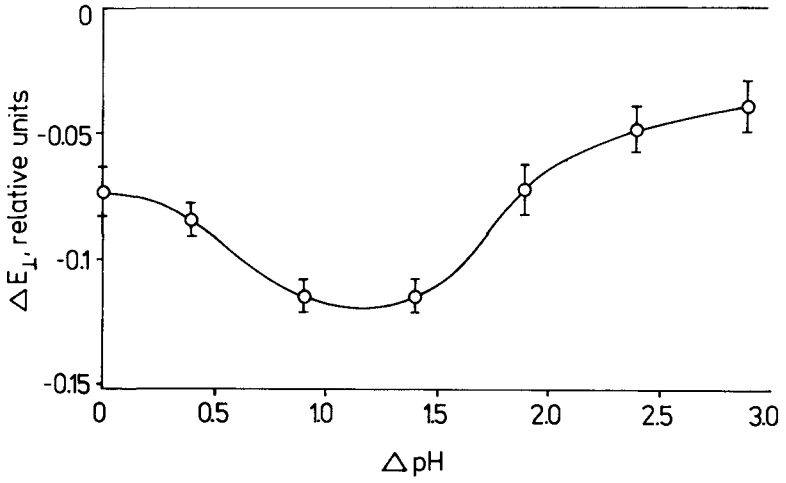

Figure 7. The dependence of the relative change in modulus of elasticity on the pH gradient on the membrane in the presence of 2×10^{-5} mol/l AT in the electrolyte. The pH gradient was created by the addition of 0.1 mol/l NaOH into the compartment containing the dye. Initially, both compartments had identical pH of 5.6. Membranes from EPX. S.E. calculated from 3–5 membranes in each series are shown.

(identical number of adsorption sites). Further increasing of pH in symmetrical conditions (at pH 10/10), Fig. 6, curve 2) results in increasing of $\Delta E_\perp$ in the presence of AT. The enhanced adsorption leads to saturation of the concentration dependence of the change in modulus of elasticity. The absence of a hydrophobic tail of 9AA is associated with the dyes being inable to change the ordering of the BLM hydrophobic part. 9AA-induced changes in $E_\perp$ are thus considerable smaller than those induced by AT (Fig. 6, curve 1).

To study the effects of adsorption of both charged forms of AT (AT^+ and AT^{2+}), electrolyte pH was gradually increased, and modulus of elasticity of the membrane with adsorbed AT was monitored (Fig. 7). At low pH, AT adsorption is partially blocked by competing protons present in the solution, and by the weak negative charge on the membrane. With the increasing pH, the number of competing H^+ decreases, the membrane surface turns increasingly negative, and thus adsorption is enhanced. Gradually however, one positive charge is compensated for ($pK_{AT} = 7.92$) and adsorption is limited again (the minimum in Fig. 7). This result compares well with AT fluorescence measurements on egg lecithin liposomes (Deamer et al. 1972). These authors studied the effect of pH between the internal and external solution, and observed a maximum of fluorescence in the same region.

Discussion

As already noted in Introduction, many unexplained questions exist concerning the interaction of pH sensitive fluorescent probes with biological membranes. A complex study of the mechanisms of interaction of 9AA and AT with liposomes and BLMs allowed us to demonstrate that both dyes interact with lipid bilayers. This is reflected in changes in fluorescence intensity of the dyes, in BLM conductance, membrane potential as well as in mechanical properties of BLMs. The results of fluorescence experiments showed dependences of fluorescence intensity on dye concentration, electrolyte pH and ΔpH. Fluorescence quenching for 9AA was observed only in the region of somewhat higher concentrations of the probe ($c > 5 \times 10^{-5}$ mol/l), whereas fluorescence intensity was observed to increase at lower concentrations. An increase in fluorescence intensity for AT occurred over the entire range of concentrations and pH values tested. Our results agree with those reported by Fiolet et al. (1974). These authors used liposomes from egg phosphatidylcholine and dicetylphosphate, and obtained similar results. The differences in fluorescence intensity changes observed between 9AA and AT are due to the differences in chemical structure of the dyes (Fig. 1*a*). The hydrophobic chain of atebrine allows the molecule to penetrate deep into the hydrophobic membrane moiety. It leads to immobilization of the molecule and, as a result, in increased fluorescence intensity. The differences between curve 3 and 4 (Fig. 1) for 9AA can be associated with differences in the amounts of negative charge on the membrane (Kraayenhof 1977;

Sokolov et al. 1980). Then, fluorescence quenching can be due to accumulation of the particles in the near-membrane region, or to adsorption of dye aggregates. Unlike 9AA, the interaction of atebrine with liposomes was practically the same for SBP and EPC. The reason for this effect were obviously different structural states of the hydrophobic parts of lipid bilayers of SBP and EPC. Taking the value of elasticity modulus $E_\perp$ as a physical characteristic of the structural state of the bilayer, we found a considerable difference. So, for 0.1 mol/l NaCl (pH 5.6), the following values of E_0 were obtained for SBP BLM: $E_0 = (3.05 \pm 0.39) \times 10^7$ Pa, and $E_0 = (6.02 \pm 2.0) \times 10^6$ Pa for EPC BLM. This means that SPB lipid bilayers are more ordered than EPC bilayers. This fact may be essential for the interaction of AT with membrane. Due to the hydrophobic tail the depth reached by AT in the bilayer will be dependent not only on the quantity of negative charge but also on the structural state of the membrane. It is obvious that dyes with less ordered bilayers will interact more easily.

The pH dependent changes in fluorescence intensity (see Table 1) agree with pK values for AT polar groups ($pK_{AT} = 7.92$). The protonated groups of the dye interact with the membranes more strongly than do neutral ones. Such a correlation was not observed for 9AA ($pK_{9AA} = 9.99$). Around pH 5.6–8.3, 9AA is in protonated form. The observed changes in fluorescence intensity, Q_{AT} and Q_{9AA}, dependent on dye concentration and electrolyte pH, can be brought into association with at least two mechanisms: changes in BLM conductance, and interaction of the dye with the lipid environment. Our results provide evidence for considerable changes in BLM conductance to occur in dependence on dye concentration and electrolyte pH. In view of the considerable dispersion of the values, it can be concluded that the changes in membrane conductance were primarily due to interaction of the probes with defects present in the membranes. Probe diffusion across the membrane itself is thus improbable. The process was marked with 9AA, and this suggests that the probe forms clusters in the near membrane region. Formation of similar near membrane aggregates, although at higher dye concentrations, can also be expected to occur with AT.

Additional evidence in support of the absorbtion of dyes onto BLM surface and the formation of aggregates is the generation of membrane potential V_m . However, it is difficult to decide whether the dyes are distributed evenly in the membrane or whether they form clusters. Nevertheless, a number of compounds are known that can communicate with each other at molecular ratios as low as approx. 100 : 1 (e.g. gramicidin). When clusters are formed, the surface charge density σ, determined by Gouy-Chapman equation, may not be true, due to the discrete charge effect (e.g. Kozlov et al. 1983). With AT at higher pH values and with 9AA, the changes in parameter A_2 were very small (approx. 1 mV) and did not allow reliable evaluation. However, small values of V_m correspond well with the electrical characteristics of the dyes (Vladimirov and Dobretsov 1980). In this case,

the considerable dispersion of potential values was due to voltage responses to the individual additions which may have induced changes in electrode potentials (in view of this, electrodes are sometimes short-circuited during chamber washouts), and may have been sensed by membrane itself. As a result of electrical pulses, structural transitions and charge redistribution may have been produced. Most importantly however, the above processes did not have any qualitative effect on changes in modulus of elasticity $E_\perp$.

The changes in elasticity modulus $E_\perp$ induced by AT and 9AA confirm the influence of these dyes on the ordering of lipid bilayers. The changes in $E_\perp$ depend on the structural state of the membranes (and can vary also during physiological processes taking place in biomembranes, e.g., upon energization of chloroplasts) as well as on electrolyte pH and ΔpH.

In analyzing the shapes of the dependences shown in Figs. 5*a*, 5*b*, and 6, it can be concluded that they represent different types of flow functions. Their time courses up to saturation could be viewed as "phase transitions" between pure membrane and "membrane+dye" system. Two types of cooperative processes are in place: cooperativity of dye adsorption onto the membrane (convexities in the lower parts of the curves and saturation in the upper parts), and cooperative effect of the dye on the modulus of elasticity (initial enhancement of membrane destruction - the convex part of the curve and saturation in the upper part, e.g. rapid saturation of $E_\perp$ with AT at pH 10/10, Fig. 6). In the latter case, saturation is reached due to the formation of a new membrane structure with overlapping regions of adsorbed dye; thus, the attachment of further molecules will not significantly alter the value of modulus of elasticity. The formation of dimers plays a significant role in the cooperative action of the probe on modulus of elasticity. Dimers may accumulate in the near-membrane region, instead of entering deep into the membrane. The results obtained show that in 9AA /AT - membrane systems the dye interaction with the lipid bilayers is the predominating processes. Probably, this is the main reason underlying the changes of the parameters studied. Our results thus provide evidence against the hyphothesis of Rottenberg and Lee (1975). This means that whenewer fluorescent dyes are used as pH indicators the degree of their interaction with the membranes and the dependence of this interaction on the structural state of the bilayer as well as on the external conditions must be known.

Exact modelling of probe interactions with the membrane requires detailed knowledge on the formation and nature of clusters of optical probes and their concentration dependencies. Similar data can possibly be obtained by determining the fractal dimensions from fluorescence spectra (see e.g. Dewey and Data 1989).

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