Biochimica et Biophysica Acta, 502 (1978) 309—320

BBA 47493

THE INVOLVEMENT OF THE ELECTRICAL DOUBLE LAYER IN THE QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE BY NEGATIVELY CHARGED SURFACES

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(Received October 27th, 1977)

Summary

The addition of 9-aminoacridine monohydrochloride to carboxymethyl-cellulose particles or azolectin liposomes suspended in a low cation medium results in a quenching of its fluorescence. This quenching can be released on the addition of cations. The effectiveness of cations is related only to their valency in the series of salts tested, being monovalent < divalent < trivalent, and is independent of the associated anions. These results indicate an electrical rather than a chemical effect, and the relative effectiveness of the various cations can be predicted by the application of classical electrical double layer theory. Fluorescence quenching can also be released on protonation of the fixed negatively charged ionisable groups, and the quenching release curve follows the ionisation curve of these groups.

We postulate that when 9-aminoacridine molecules are in the electrical diffuse layer adjacent to the charged surface their fluorescence is quenched, probably due to aggregate formation. As cations are added the 9-aminoacridine concentration at the surface falls as it is displaced into the bulk solution, where it shows a high fluorescence yield with a fluorescence lifetime of 16.3 ns. The fluorescence quenching is associated with an absorbance decrease, which is pronounced with carboxymethyl-cellulose particles and can probably be attributed to self-shielding.

The negative charges carried by lipoprotein membranes are primarily due to carboxyl and phosphate groups. Therefore these results with carboxymethyl-cellulose (carboxyl) and azolectin (phosphate) support our earlier suggestion that 9-aminoacridine may be used to probe the electrical double layer associated with negatively charged biological membranes.

Introduction

Recent papers from Barber and colleagues [1,2] have stressed the importance to the electrical double layer at the chloroplast thylakoid surface in con-

trolling certain membrane-associated phenomena in chloroplasts. Further work from the same laboratory has also shown that the protonated monovalent 9-aminoacridine cation can be used to probe this electrical double layer [3]. It was found that 9-aminoacridine fluorescence was quenched when this molecule was drawn close to the negatively charged membrane surface [4]. The quenching was not associated with any wavelength changes in the absorption or emission spectra and could be reversed in a predictable way by adding other cations to the suspending medium. These results contrast with those for other positively charged dyes which have been shown to interact with artificial polyanions [5—7] and liposomes [8,9] resulting in changes in the absorption and fluorescence emission spectra. To explain these effects it has been suggested that the quenching is due to the formation of non-fluorescent aggregates [10]. Spectral shifts are evidence of hydrophobic or chemical interactions with the surface in addition to electrostatic attraction (e.g. ref. 7). We have postulated from our study of 9-aminoacridine association with the chloroplast thylakoid membrane that this fluorescence probe shows a purely electrostatic interaction with the surface and that it acts as a diffusible cation.

In this paper we extend our previous work [3] by investigating changes of 9-aminoacridine fluorescence using model negatively charged surfaces which, unlike chloroplast membranes, are free of interferring pigments. In particular we have used carboxymethyl-cellulose particles and liposomes of azolectin which possess carboxylate and phosphate groups, respectively, and are therefore comparable with the negatively charged surfaces of biological lipoprotein membranes.

Materials and Methods

Washed thylakoids were prepared from pea or spinach leaves as previously described [3]. Carboxymethyl-cellulose (CM 11) was obtained from Whatman, converted into the sodium form with 0.5 M NaOH and then washed thoroughly with water until the pH of the suspension was 6.0—6.5. The particles were 50—250 μm long and 20—30 μm diameter fibres having a small ion capacity of 0.7 mequiv./gm dry weight. As the carboxymethyl-cellulose tended to settle rapidly the suspension was remixed prior to each fluorescence or absorbance measurement. The carboxymethyl-cellulose equilibrated slowly after salt additions, particularly at low ionic levels, so that several minutes were needed to reach a steady state. Azolectin was obtained from Associated Concentrates Inc., and was dissolved in *n*-heptane. To this heptane solution water was added to give a 1% (w/v) azolectin suspension, which was dispersed by sonication for 30—45 s using a Dawe Soniprobe at power setting 5, while the temperature was kept below 15° C. The treatment gave rise to large multilamellar aggragates estimated to have a range of sizes between 5 and 50 μm. Cation additions were made with salts of at least analytical grade purity and concentrations were checked by estimation of the Cl^- concentration. $LaCl_3$ is not susceptible to hydrolysis in the pH range of the experiments (pH 6.0—6.5) according to Biedermann and Ciavatta [11].

A stock solution of 9-aminoacridine was added to a concentration of 25 μM to a low cation medium, which contained only 0.4 M sucrose and sufficient

tris (hydroxymethyl) aminomethane (Tris) base to bring the pH to 6.0—6.5. To this solution was then added thylakoids, carboxymethyl-cellulose or azolectin. The sample of 3 ml was contained in a 10 × 10 mm quartz cuvette, clear on all four sides, and 9-aminoacridine fluorescence was observed with a Perkin-Elmer MPF3 fluorescence spectrophotometer on excitation at 398 nm (2 nm bandwidth) with the emission monitored at 456 nm (bandwidth 2 nm) at 90° to the excitation beam. In control experiments it was found that neither carboxymethyl-celluose nor azolectin had detectable fluorescence without addition of 9-aminoacridine, and that traces of heptane did not interfere with 9-aminoacridine fluorescence quenching by azolectin. Absorption spectra were measured on an Aminco DW2 double beam spectrophotometer in the split beam mode using a diffusion plate to reduce scattering artifacts. To observe the pH dependence of the carboxymethyl-cellulose- or azolectin-induced fluorescence quenching the pH was adjusted by additions of 0.1 M acetic acid and subsequently 0.1 M HCl. In the case of azolectin 0.1 M Tris base was also added to adjust the pH to alkaline values. The particle electrophoresis of azolectin was performed at the Chester Beatty Institute of Cancer Research using a Zeiss Cytopherometer Ph40 at a constant temperature of 20°C in 0.33 M sorbitol containing 20 mM KCl and a minimal concentration of Tris/maleate buffer to bring the pH to 6. The fluorescence lifetimes were measured at the Royal Institution by time-correlated single photon counting [12] using excitation of 380 nm wavelength and monitoring the emission with a Philips 56 TUVP phototube protected by a Balzer B-40 453 nm interference filter.

Results

Relative effectiveness of cations in releasing 9-aminoacridine fluorescence quenching

Particle electrophoresis studies at pH 6.0 showed that the liposomes of azolectin carried a net negative charge, as expected for a phospholipid mixture. When either carboxymethyl-cellulose or azolectin was added to a solution of 9-aminoacridine fluorescence quenching occurred, and as found in a previous study with thylakoid membranes, this quenching did not involve a change in shape or a measurable wavelength shift of the emission spectrum.

In Fig. 1 it is seen that the quenching of 9-aminoacridine fluorescence induced by the addition of azolectin could be almost completely reversed by the addition of $CaCl_2$. Similar results were found for carboxymethyl-cellulose, and also with other cations. However, if the zwitterion, glycine, was used there was no reversal of the fluorescence quenching even with concentrations as high as 100—150 mM. The relative ability of various salts to release 9-aminoacridine fluorescence quenching is presented in Table I for carboxymethyl-cellulose and azolectin and can be compared with previously published data for thylakoids [3]. It can be seen that the monovalent cations of the alkali metal series are equally effective, having $C_{1/2}$ values (the concentration producing 50% change) of about 5 mM for carboxymethyl-cellulose and 15 mM for azolectin. A value of about 15 mM was previously found for thylakoids [3]. Note that bulky organic molecules which carry one net positive charge, such as lysine and choline, also have $C_{1/2}$ values identical to alkali metal cations. In contrast,

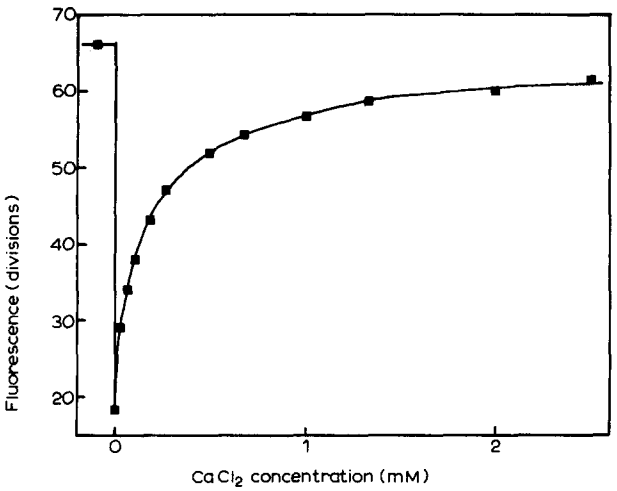

Fig. 1. The release of 9-aminoacridine fluorescence quenching by azolectin in a low cation medium on addition of $CaCl_2$. The fluorescence emission at 456 nm (2 nm bandwidth) is expressed on an arbitrary linear scale.

the divalent cations of the alkaline earth metal series were much more effective at reversing the fluorescence quenching. In this case the $C_{1/2}$ was in the region of 0.12—0.14 mM for both carboxymethyl-cellulose and azolectin and identical to that found for thylakoids [3]. Furthermore the effectiveness of the series Mg^{2+} to Ba^{2+} is constant, and approximately equal to that of L-lysyl-L-lysine · 2 HCl which also carries a net charge of +2 at neutral pH. Table I also shows the effect of several different magnesium salts and emphasises that the nature of the anion is not important in the release of 9-aminoacridine fluorescence quenching. The trivalent cation La^{3+} is more effective than divalent cations by a factor of seven for azolectin and three for carboxymethyl-cellulose which

TABLE I

RELATIVE EFFECTIVENESS OF SALTS IN RELEASING 9-AMINOACRIDINE FLUORESCENCE QUENCHING BY CARBOXYMETHYL-CELLULOSE AND AZOLECTIN

The salt additions were made to the carboxymethyl-cellulose (CMC) particles or azolectin liposomes in low cation medium containing 25 μM 9-aminoacridine (see Materials and Methods). The $C_{1/2}$ values given are the concentration of salt producing 50% release of fluorescence quenching, where the final fluorescence level reached at high salt concentration is taken as 100%.

Addition	$C_{1/2}$ (mM)		Addition	$C_{1/2}$ (mM)	
	CMC	Azolectin		CMC	Azolectin
LiCl	4.8	15.0	$MgCl_2$	0.14	0.13
NaCl	4.8	17.0	$Mg(NO_3)_2$	0.13	0.13
KCl	4.4	18.0	$MgSO_4$	0.13	0.14
RbCl	5.1	15.0	$CaCl_2$	0.13	0.13
CsCl	4.6	13.5	$SrCl_2$	0.12	0.13
Lysine · HCl	4.2	16.5	$BaCl_2$	0.14	0.12
Choline chloride	6.1	18.0	lysyl-lysine · 2HCl	0.19	0.26
$LaCl_3$	0.041	0.021			

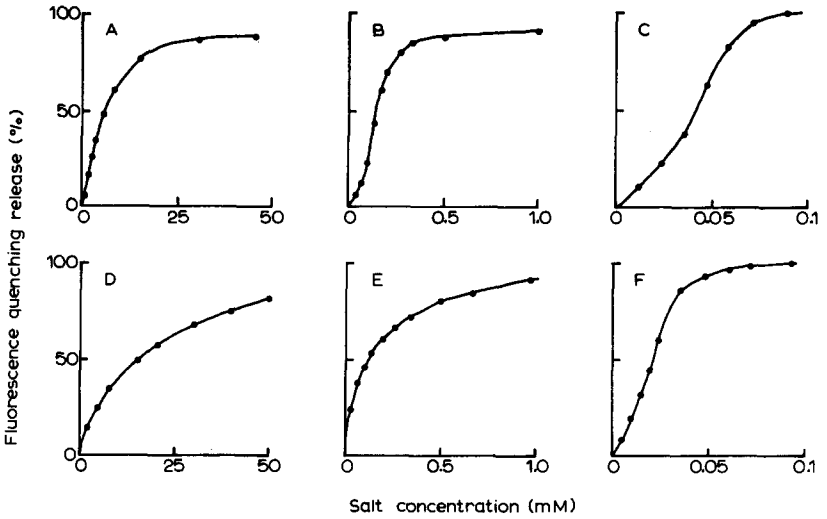

Fig. 2. The relative effectiveness of salts in releasing 9-aminoacridine fluorescence quenching by carboxymethyl-cellulose (A—C) and azolectin (D—F) in low cation medium. The salts added were: A, LiCl; B, $CaCl_2$; C, $LaCl_3$; D, RbCl; E, $BaCl_2$; F, $LaCl_3$.

in both cases is larger than the factor of two to be expected from an effect mediated purely by ionic strength.

Fig. 2. presents some typical concentration curves of the release of 9-aminoacridine fluorescence quenching for carboxymethyl-cellulose and azolectin using mono-, di- and trivalent cations. For both charged surfaces, monovalent cation additions led to a monotonic rise in fluorescence (Fig. 2, A and D). Divalent cations gave a monotonic rise in the case of azolectin (Fig. 2E) as was also seen for thylakoids [3], but with carboxymethyl-cellulose a pronounced biphasic rise curve was observed with all the divalent cations tested (e.g. Ca^{2+}, Fig. 2B). With $LaCl_3$ the fluorescence quenching release curve was biphasic for both carboxymethyl-cellulose and azolectin (Fig. 2, C and F).

pH dependence of 9-aminoacridine fluorescence quenching

Fig. 3 shows the dependence of the degree of 9-aminoacridine fluorescence quenching by carboxymethyl-cellulose and azolectin on the pH of the low cation suspension medium. A change in the degree of protonation of 9-aminoacridine itself can be neglected in the pH range studied. In the case of carboxymethyl-cellulose (Fig. 3a) it is clear that the quenching is dependent upon the fraction of ionised carboxyl groups and that complete suppression of ionisation results in loss of quenching ability. 50% release is found at pH 4.7, and the fluorescence level follows closely the theoretical titration curve for an ionisable group of pK 4.7. A similar observation was noted for the pH dependence of thylakoid-induced 9-aminoacridine fluorescence quenching [3]. For azolectin the pH dependence is more complex (Fig. 3b), but in the pH range used for the experiments presented in Fig. 2 and Table I (pH 5.5—6.5), the 9-aminoacridine fluorescence quenching is independent of pH. The slight increase in quenching at alkaline pH values could be due to the presence of traces of phosphatidic

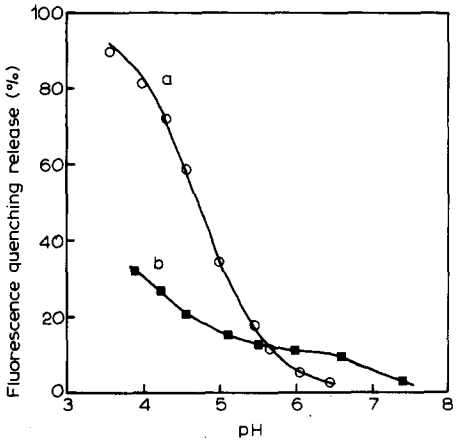

Fig. 3. The pH dependence of 9-aminoacridine fluorescence quenching in low cation medium by (a) carboxymethyl-cellulose and (b) azolectin. The pH was adjusted with acetic acid and HCl, and measured with a glass electrode. The solid line in a is the theoretical titration curve for a group with pK 4.7.

acid in azolectin (a mixture of phospholipids from soya bean). The bulk of the negative charges responsible for fluorescence quenching appear to have a pK lower than 4, as expected for phosphate groups of phospholipids. Control experiments showed that the release of 9-aminoacridine fluorescence quenching induced by salt additions to either carboxymethyl-cellulose or azolectin was not associated with a decrease in the pH of the suspending medium.

9-Aminoacridine absorbance changes

Together with the fluorescence quenching induced by the association of 9-aminoacridine with charged surfaces in a low cation medium, a decrease in 9-aminoacridine absorbance can also be observed, and this is particularly pronounced in the case of carboxymethyl-cellulose (Fig. 4A). It is seen that the absorption spectrum remains essentially unaltered in shape, although the 380 nm peak is depressed slightly relative to the other peaks. This absorbance decrease can probably be attributed to the self-shielding effect described by Duysens [13]. It is completely reversible on addition of salts.

In contrast, the 9-aminoacridine fluorescence quenching induced by azolectin in low cation medium is not associated with a pronounced change in the absorbance of the 9-aminoacridine. Fig. 4B shows that whereas fluorescence can be quenched up to 80% the 9-aminoacridine absorbance is only reduced by 15—20%. Therefore the extent of the fluorescence and the absorbance changes are not necessarily related, and at least in the case of azolectin, self-shielding is not the cause of the lowered fluorescence yield when 9-aminoacridine is closely associated with the charged surface.

As mentioned in our previous paper [3], a 9-aminoacridine absorbance decrease is also observed together with the fluorescence quenching induced by thylakoid membranes. In Fig. 4B the decrease in absorbance with thylakoid membranes is shown to be up to 50%, resembling the situation with carboxymethylcellulose rather than azolectin. It should be noted that experiments with thylakoid membranes could not be carried out at the concentration giving

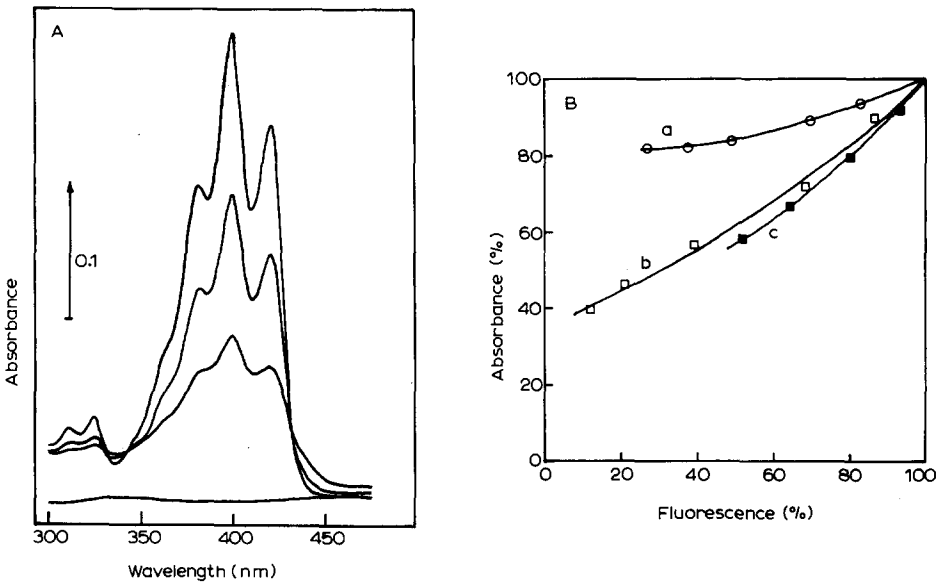

Fig. 4. (A) The 9-aminoacridine absorbance decrease induced by the addition of carboxymethyl-cellulose to a 9-aminoacridine solution in low cation medium. Curve a is for no addition, and curves b and c are for increasing amounts of carboxymethyl-cellulose added. The spectra were taken on an Aminco DW2 spectrophotometer with a diffusing plate to reduce scattering although the upward shift in the baseline at 450 nm indicates that some scattering artifact remains. (B) The relationship between the absorbance decrease at 398 nm and the fluorescence quenching at 456 nm on the addition of increasing concentrations of charged surface to a solution of 9-aminoacridine in low cation medium for (a) azolectin, (b) carboxymethyl-cellulose and (c) thylakoid membranes. The absorbance was read against a reference containing the added charged surface but without 9-aminoacridine. The fluorescence change on addition of thylakoids was calculated from the percentage increase in 9-aminoacridine fluorescence seen when 1 mM $MgCl_2$ was added at each thylakoid membrane concentration.

maximal 9-aminoacridine fluorescence quenching as the fluorescence detected was reduced due to the absorption of excitation light and of the resulting 9-aminoacridine fluorescence by the photosynthetic pigments. With carboxymethyl-cellulose and azolectin however the concentration of charged surface giving maximal 9-aminoacridine fluorescence quenching could be used.

9-Aminoacridine fluorescence lifetime

The fluorescence lifetime of 25 μM 9-aminoacridine was measured both in free solution in low cation medium, and after the fluorescence was quenched 50% by the addition of azolectin. The fluorescence decay curve was indistinguishable in the two cases, being a single exponential with a lifetime of 16.3 ns, with no shorter component being resolved in the decay of the quenched sample. Similar experiments could not be performed using carboxymethyl-cellulose or thylakoids owing to interference from settling during the experiments. This result implies that the fluorescing species is the 9-aminoacridine molecule free in solution, and that the 9-aminoacridine closely associated with the charged surface is essentially non-fluorescent, as a dynamic quenching mechanism would be expected to shorten the overall fluorescence lifetime. The measured fluorescence lifetime is sufficiently long for all solvent relaxation processes to have occurred before emission.

Discussion

All the data presented above support the concept suggested earlier [3] that when 9-aminoacridine is drawn into the diffuse electrical layer adjacent to a negatively charged surface, its fluorescence is quenched. This fluorescence decrease could well be due to the elevated concentration of the dye at the membrane surface since even in free solution 9-aminoacridine becomes essentially non-fluorescent above about 0.5 mM [3]. Such a level is almost certainly exceeded in the diffuse layer when the negative surface potential is high and 9-aminoacridine acts as a major counterion (see Table II).

The exact mechanism of quenching is uncertain and may not necessarily be the same as the concentration quenching observed in free solution [3], but since there are no associated significant spectral shifts, neither for emission nor absorption, it is unlikely that any significant chemical interaction with the surface takes place. This is also supported by the fact that 9-aminoacridine can be readily displaced from the surface simply by increasing the cation level of the suspending medium. Indeed the lack of specificity of cations in a particular valency group (Table I) is indicative of an electrostatic interaction. This is supported by the differential effect seen between cations of different valencies and emphasised by comparison of lysine (monovalent) and its dimer lysyl-lysine (divalent). All these observations can be accounted for in terms of classical electrical double layer theory as already emphasised in an earlier paper in which chloroplast thylakoid membranes were used [3]. For example, the differential effect of cations of different valencies can be predicted from the Gouy-Chapman theory [14,15] applied to solutions of mixed electrolytes [3]. The appropriate equations are: For the addition of monovalent salt $C^+ A^-$ having a bulk concentration C'_b mol/l,

$$4(C_d + C'_b) \sinh^2\left(\frac{F\psi_0}{2RT}\right) - \frac{\sigma^2}{A^2} = 0 \qquad (1)$$

where C_d mol/l is the bulk concentration of 9-aminoacridine together with other monovalent cations initially present in the suspending medium, σ is the surface charge in μCoulombs/cm^2, ψ_0 is the surface potential in V and $A = (RT\epsilon/2\pi)^{1/2}$ (where ϵ is the permittivity of water).

TABLE II

THE DEPENDENCE ON σ AND C_d OF THE CALCULATED 9-AMINOACRIDINE SURFACE CONCENTRATION

The values given are the concentrations of 9-aminoacridine (mol/l) at the charged surface (C_{F_s}) in a low cation medium, calculated using Eqns. 1—4 (see text), where σ is the surface charge density in μC/cm^2 and C_d is the background bulk monovalent cation concentration in mol/l.

C_d	C_{F_s}	
	$\sigma = 1.0$	$\sigma = 2.5$
$1 \cdot 10^{-3}$	$3.1 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$
$5 \cdot 10^{-3}$	$7.7 \cdot 10^{-5}$	$3.9 \cdot 10^{-4}$

For the addition of a divalent salt C^{2+}/A^{2-} having a bulk concentration C_b'' mol/l,

$$2C_b'' \cosh^2\left(\frac{F\psi_0}{RT}\right) + C_d \cosh\left(\frac{F\psi_0}{RT}\right) - \left(2C_b'' + C_d + \frac{\sigma^2}{2A^2}\right) = 0 \quad (2)$$

For addition of trivalent salt C^{3+}/A^{3-} having a bulk concentration C_b''' mol/1.

$$4C_b''' \cosh^3\left(\frac{F\psi_0}{RT}\right) + (C_d - 3C_b''') \cosh\left(\frac{F\psi_0}{RT}\right) - \left(C_d + C_b''' + \frac{\sigma^2}{2A^2}\right) = 0 \quad (3)$$

For a given value of σ and using the above equations, values of ψ_0 can be obtained for various salt additions. Then by using the Boltzmann equation,

$$C_{F_s} = C_{F_b} \exp\left(\frac{-ZF\psi_0}{RT}\right) \quad (4)$$

for any value of ψ_0 the concentration of 9-aminoacridine (a monovalent cation i.e. $Z = +1$) at the surface (C_{F_s}) can be calculated, taking the bulk concentration of 9-aminoacridine (C_{F_b}) as 10 μM. Similar equations were given in ref. 3, but it should be noted that they contained some minor errors.

As Table I shows, the concentration of salt needed to give 50% release of quenching with carboxymethyl-cellulose was about 5 mM for monovalent, 0.14 mM for divalent and 0.04 mM for trivalent cations. Using a computer to solve Eqns. 1—4 we have varied σ and C_d to find which would predict $C_{1/2}$ values close to those seen experimentally and give 9-aminoacridine surface concentrations of about 0.5 mM or more (see Tables II and III). With σ = 2.5 μC/cm^2 (the value also adopted for the thylakoid membranes) [3] and C_d = 5 mM then 50% reduction of 9-aminoacridine at the surface occurs with 5.6 mM for monovalents, 0.26 mM for divalents and 0.013 mM for trivalent cations (Table III).

Although these calculations emphasise the differential effects of cations of different valency, the experimental results cannot be matched completely by theoretical values. This is to be expected since many assumptions have been made in applying the classical Gouy-Chapman theory (see ref. 2). For simplicity we have kept the bulk concentration of 9-aminoacridine constant at 10^{-5} M inEqn. 4, which cannot actually be the case if our concept is correct

TABLE III

THE DEPENDENCE ON σ AND C_d OF THE CALCULATED RELATIVE EFFECTIVENESS OF CATIONS IN RELEASING 9-AMINOACRIDINE FLUORESCENCE QUENCHING

The values shown are concentrations of added cations (mol/l) giving 50% release of quenching ($C_{1/2}$ mol/l) calculated using Eqns. 1—4 (see text) for various values of the surface charge density σ (μC/cm^2) and background bulk monovalent cation concentration C_d (mol/l).

	σ = 1.0		σ = 2.5	
C_d	$1.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$
Monovalent ($C_{1/2}$)	$1.1 \cdot 10^{-3}$	$9.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$5.6 \cdot 10^{-3}$
Divalent ($C_{1/2}$)	$6.5 \cdot 10^{-5}$	$1.4 \cdot 10^{-3}$	$1.0 \cdot 10^{-5}$	$2.6 \cdot 10^{-4}$
Trivalent ($C_{1/2}$)	$4.0 \cdot 10^{-6}$	$3.4 \cdot 10^{-4}$	$1.1 \cdot 10^{-7}$	$1.3 \cdot 10^{-5}$

that an increase in 9-aminoacridine fluorescence represents an increase in the bulk concentration of the dye. Also ideally it would have been more correct to calculate the dye concentration for the whole diffuse electrical layer instead of just at the plane immediately adjacent to the charged surface. A further source of uncertainty arising from the need to use large surface areas relative to the volume of suspending medium is the value of C_d. Thus the need to assume a relatively high background level of monovalent cations in our computer analyses may be related to the release of monovalent cations from the charged surface into the bulk on the addition of salts, which would tend to increase its value above that originally present in the suspending medium. Further, the exact bulk concentration of added cations will be uncertain when a significant proportion of those added becomes closely associated with the surface. Finally, our calculations are for reduction to 50% of the 9-aminoacridine at the surface which does not necessarily relate directly with the fluorescence changes. However, in spite of these assumptions and approximations it is seen that the application of classical electrical double layer theory does in fact predict a relative effectiveness of cations which is in good agreement with observations.

The concentration curves for the reversal of 9-aminoacridine fluorescence quenching shown in Fig. 2 tend to be biphasic in the case of divalent and trivalent cations, as would be expected from Eqns. 1—4 (see Fig. 9 of ref. 3). This should also apply for the addition of monovalent cations but in this case the curves in Fig. 2 do not show an obvious biphasic nature. It is possible that the initial part of the concentration curve is not being observed in these cases.

The finding that the pK of the carboxyl groups providing the negative charge on carboxymethyl-cellulose could be evaluated from the pH dependence of 9-aminoacridine fluorescence quenching is important in relation to similar experiments conducted previously with thylakoid membranes [3]. Thus our interpretation that the pH dependence of 9-aminoacridine fluorescence quenching by thylakoid membranes is due to surface protein carboxyl groups [3] is reinforced and it is this ionisable group which gives rise to the net negative charge carried by the chloroplast membrane.

As mentioned above the fluorescence quenching mechanism which emerges from these studies with charged surfaces in low cation medium is one involving some form of quenching associated with increased levels of the dye at the membrane surface. This may involve the formation of non-fluorescent aggregates, perhaps via vertical stacking of the ring systems of adjacent molecules as seen in the crystal structure of 9-aminoacridine [16]. These aggregates would be in equilibrium with monomeric 9-aminoacridine in the electrical double layer and would readily undergo exchange with the 9-aminoacridine in the suspending medium. The formation of non-fluorescent aggregates might be expected to alter the absorption spectrum, however, a pronounced change is not observed (Fig. 4A). In the case of azolectin-induced quenching, where self-shielding is negligible, a slight shift by 1—2 nm of the entire absorption spectrum between 350 and 450 nm can however be detected together with the flattening of the 380 nm peak noted above (Fig. 4A). This suggests that the non-fluorescent aggregates of 9-aminoacridine have an absorption spectrum which is hardly distinguishable from that of monomeric 9-aminoacridine.

As shown in Fig. 4 the increased levels of 9-aminoacridine in the electrical diffuse layer can lead to self-shielding as seen by a decrease in the absorbance on addition of carboxymethyl-cellulose or thylakoid membranes. However, this absorbance decrease does not correlate with the fluorescence change implying that self-shielding is not the reason for 9-aminoacridine fluorescence quenching. The self-shielding is much lower with azolectin liposomes, and also in free solution 9-aminoacridine shows no absorbance changes in the concentration range where virtually complete fluorescence quenching is observed [17]. The reason for the difference could be a more complicated topography of thylakoid membranes and carboxymethyl-cellulose particles compared to smooth phospholipid spheres.

9-Aminoacridine has been used for estimating light-induced transmembrane pH gradients across thylakoid membranes [18] and as an indicator of the high energy state [19]. The above study does not attempt to give an explanation to the quenching of 9-aminoacridine fluorescence observed on illuminating chloroplasts but simply to emphasise that the fluorescence yield of this dye can be influenced by changes in the electrical properties of charged surfaces. Indeed neutralisation of surface charges by protonation increases the fluorescence yield of 9-aminoacridine (Fig. 3) in contrast to the quenching observed when illuminated chloroplasts take up H^+ from a buffered suspending medium. Qualitatively, the concept that the high-energy-state-induced quenching of 9-aminoacridine fluorescence reflects the accumulation of the protonated dye within the intrathylakoid space [18] does not seem unreasonable. However, in any quantitative analysis it may also be necessary to take account of fluorescence changes due to any light-induced changes in the surface charge density on either side of the thylakoid membrane.

Acknowledgements

This work was supported by grants from the EEC Solar Energy Research and Development Programme and the Science Research Council. We thank Herb Nakatani for the particle electrophoresis and Dr. Godfrey Beddard for the single photon counting. The technical assistance of Elizabeth Dibb and the assistance of Andrew Love with the computer analysis is also gratefully acknowledged.

References

1 Barber, J. and Mills, J. (1976) FEBS Lett. 68, 288—292
2 Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174—181
3 Searle, G.F.W., Barber, J. and Mills, J. (1977) Biochim. Biophys. Acta 461, 413—425
4 Barber, J. (1977) in International Symposium on Membrane Bioenergetics, Spetsai, Greece (Packer, L. and Papageorgiou, G.C., eds.)
5 Dell'Antone, P., Colonna, R. and Azzone, G.F. (1972) Eur. J. Biochem. 24, 566—576
6 Colonna, R., Massari, S. and Azzone, G.F. (1973) Eur. J. Biochem. 34, 577—585
7 Massari, S., Dell'Antone, P., Colonna, R. and Azzone, G.F. (1974) Biochemistry 13, 1038—1043
8 Deamer, D.W., Prince, R.C. and Crofts, A.R. (1972) Biochim. Biophys. Acta 274, 323—335
9 Fiolet, J.W.T., Bakker, E.P. and van Dam, K. (1974) Biochim. Biophys. Acta 368, 432—445
10 Azzi, A. (1975) Q. Rev. Biophys. 8, 237—316
11 Biedermann, G. and Ciavatta, L. (1961) Acta Chem. Scand. 15, 1347—1366
12 Beddard, G.S., Carlin, S. and Lewis, C. (1975) J. Chem. Soc. 71, 1894—1902

13 Duysens, L.N.M. (1956) Biochim. Biophys. Acta 19, 1—12
14 Gouy, M. (1910) J. Phys. (Paris) 9, 457—468
15 Chapman, D.L. (1913) Phil. Mag. 25, 475—481
16 Talacki, R., Carrell, H.L. and Glusker, J.P. (1974) Acta Crystallogr., Sect. B 30, 1044—1047
17 Casadio, R., Baccarini-Melandri, A. and Melandri, A.B. (1974) Eur. J. Biochem. 47, 121—128
18 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64—70
19 Fiolet, J.W.T., van der Erf-ter Haar, L., Kraayenhof, R. and van Dam, K. (1975) Biochim. Biophys. Acta 387, 320—334