Biochimica et Biophysica Acta, 461 (1977) 413–425

BBA 47343

9-AMINO-ACRIDINE AS A PROBE OF THE ELECTRICAL DOUBLE LAYER ASSOCIATED WITH THE CHLOROPLAST THYLAKOID MEMBRANES

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(Received February 7th, 1977)

SUMMARY

Chloroplasts washed with monovalent cations are found to quench 9-amino-acridine fluorescence after resuspension in a cation-free medium. This quenching occurs in the absence of a high energy state and can be reversed by the addition of salts. The effectiveness of these salts is related to the charge carried by the cations and appears to be essentially independent of the associated anions. The order of effectiveness is polyvalent > divalent > monovalent, and virtually no variation is found within the groups of monovalent cations and divalent cations tested. Furthermore, choline and lysine are as effective as alkali metal cations, and lysyl-lysine is almost as effective as alkaline earth metal cations. These results are consistent with an effect mediated by the electrical double layer at the membrane surface rather than chemical bonding, and can be qualitatively explained in terms of the Gouy-Chapman theory.

It appears that 9-amino-acridine acts as a diffusible monovalent cation which increases its fluorescence when displaced from the diffuse layer adjacent to the negatively charged membrane surface. The 9-amino-acridine fluorescence changes have been experimentally correlated with the cation-induced chlorophyll *a* fluorescence changes also observed with isolated chloroplasts.

INTRODUCTION

Recent work in this laboratory has revealed the importance of the diffuse electrical layer associated with the thylakoid membranes in controlling the level of chlorophyll *a* fluorescence of isolated chloroplasts treated with DCMU [1, 2]. The relative effectiveness of monovalent and divalent cations in bringing about a transition from a low to a high fluorescing state of these DCMU-treated chloroplasts [3–5] is in agreement with the predictions of the classical Gouy-Chapman theory of diffuse electrical layers [6]. Moreover, the antagonism [7] and competition [8, 9] between low concentrations of monovalent and divalent cations are similar to the well established

Abbreviations: 9-AA, 9-amino-acridine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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effects on colloid aggregation [10] and may be explained in terms of changes in the total diffusible positive charge (space charge density) or electrical field immediately adjacent to the thylakoid membrane surface [6].

In this paper we report experiments in which we have used the monovalent fluorescent cation 9-amino-acridine (9-AA) to investigate further the properties of the electrical double layer associated with the negatively charged surfaces of the thylakoid membrane. The introduction of acridines as indicators of the state of energisation of transducing membranes [11] led to the application of 9-AA as a monitor of H^+ concentration gradients [12]. Later reports by Kraayenhof and van Dam and colleagues [13, 14] suggested that the ability of acridines to indicate energisation was closely associated with their interaction with the membrane surface and that transport across the membrane may not be required.

Bose [15] has made the important observation that quenching of 9-AA fluorescence by isolated chloroplasts in the absence of a high energy state can be released by the addition of Mg^{2+}. In the work presented below this approach has been developed and explained in terms of electrical double layer theory. It is shown that 9-AA acts as a diffusible monovalent cationic probe of the electrical layer adjacent to the negatively charged thylakoids and provides a means of investigating the relationship between the ionic concentration in the bulk solution and adjacent to the membrane surface.

MATERIALS AND METHODS

All procedures in the preparation of chloroplasts were carried out at 0–4 °C. Chloroplasts were isolated from spinach, spinach beet or pea leaves by homogenising in 0.4 M sucrose plus 50 mM Tris · HCl buffer, pH 8.0, filtering through a cheese cloth/cotton wool pad and centrifuged at $3000 \times g$ for 1–2 min. The pellet, after washing with 0.4 M sucrose plus 50–100 mM NaCl or/Tris · HCl (pH 8.0), was resuspended in a medium of low cationic strength consisting of 0.4 M sucrose brought to about pH 7.5 with 1 mM Tris base. The suspension was then treated with 1 mM 9-AA, recentrifuged and the 9-AA-loaded pellet washed and resuspended in the same cation-free medium. In other experiments 25 μM 9-AA was added to chloroplasts in the cation-free medium and used directly for experimentation. The chemicals used were all analytical grade reagents. $LaCl_3 \cdot 7H_2O$ was obtained from B.D.H. Chemicals; choline chloride, L-lysine · HCl, L-lysyl-L-lysine · 2HCl and poly-L-lysine hydrobromide (Type II, degree of polymerisation about 9, molecular weight approx. 2000) were obtained from Sigma.

Chlorophyll concentrations were estimated according to Bruinsma [16]. The 9-AA content of the loaded chloroplasts was determined fluorimetrically by addition of small aliquots of standard 9-AA solution to the chloroplasts in dilute solution (< 0.5 μg chlorophyll/ml) and in the presence of 10 mM $MgCl_2$. The molar absorption of 9-AA at 398 nm was taken to be 10 000 [17]. 9-AA fluorescence was measured in a Perkin-Elmer MPF3 fluorescence spectrophotometer with an R106 (Hamamatsu) photomultiplier. In the absence of 9-AA, chloroplasts gave no detectable signal under the conditions used for the cation addition experiments: excitation at 398 nm (band width 2 nm) and observation of fluorescence emitted at right angles at 456 nm (bandwidth 2 nm). No correction was made for photomultiplier sensitivity or monochromator response when emission and excitation spectra were measured.

Absorption spectra were measured on a Unicam SP800 scanning spectrophotometer, with the wavelength scale checked with a holmium filter. The samples were placed close to the photomultiplier to reduce effects of scattering. Simultaneous observations of 9-AA and chlorophyll *a* fluorescence was carried out with a laboratory-constructed fluorimeter using excitation light transmitted by a combination of three filters: Schott UG1 4 mm, BG18 2 mm, BG38 2 mm. Fluorescence from 9-AA was observed at 498 nm (Balzer B-40 498 nm, Schott GG495 4 mm, OY516 4 mm), while chlorophyll fluorescence was observed at 685 nm (Balzer B-40 685 nm, Schott RG665 2 mm). The two EMI 9558B photomultipliers used for detecting the fluorescence were at right angles to the excitation beam.

The sample, volume 3 ml, was placed in a quartz 10×10 mm fluroescence cuvette, and additions of salts were made with Terumo microsyringes. The concentrations of salt solutions were checked by estimation of Cl^- concentration using standard $AgNO_3$, with dichlorofluorescein as indicator.

RESULTS

9-AA fluorescence quenching by thylakoid membranes

As shown in Fig. 1 it was found that concentration quenching of 9-AA fluorescence occurs in aqueous media above 0.1 mM in agreement with Casadio et al. [18] and Albert [19]. Addition of electrolytes to the solution did not relieve this type of concentration quenching.

When broken chloroplasts were added to a 25 μM fluorescing solution of 9-AA in the cation-free medium (see Materials and Methods), fluorescence quenching also occurred. However, in this case when the electrolyte composition was raised the fluorescence quenching was relieved. As Fig. 2A shows, the salt-induced increase in 9-AA fluorescence was not associated with a change in the shape of its emission spectrum. Fig. 2B shows that both in the absence and presence of salt (e.g. 1 mM $MgCl_2$) the level of fluorescence detected decreases with increasing concentrations of

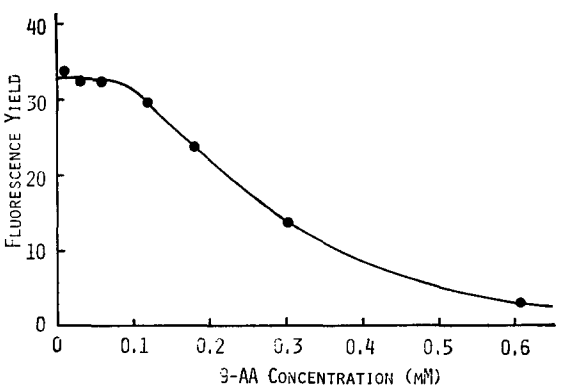

Fig. 1. The concentration quenching of 9-amino-acridine fluorescence in the cation-free medium. The fluorescence was measured at 456 nm, and corrected for the absorption of the 398 nm excitation beam to give an estimate of the relative fluorescence yield. Fluorescence is expressed in arbitrary units.

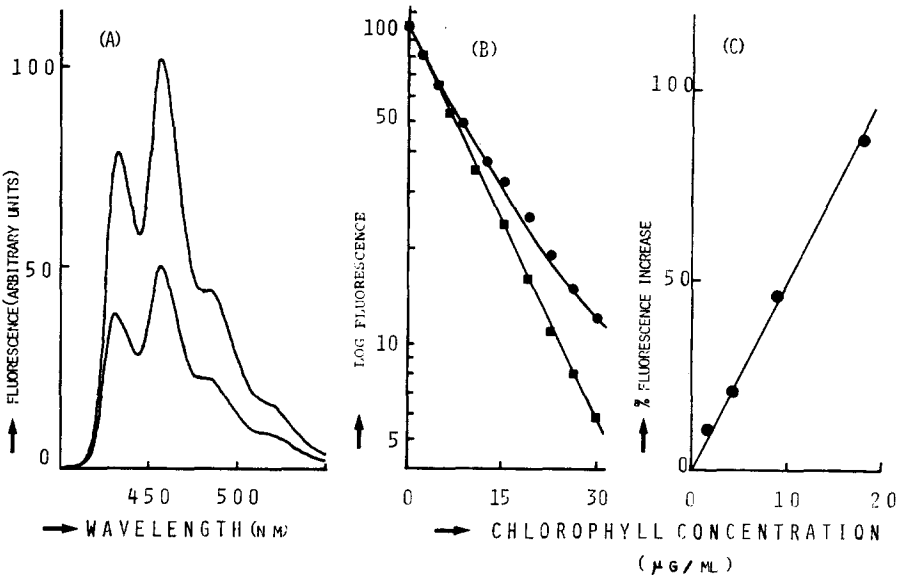

Fig. 2. Characteristics of the salt-induced release of 9-AA fluorescence quenching measured with chloroplasts pretreated with the dye. (A), emission spectrum in cation-free medium (lower curve) and its increase on addition of 1 mM $MgCl_2$ (upper curve). (B), dependence of 25 μM 9-AA fluorescence on the chloroplast concentration in the cation-free medium (■) and on addition of 1 mM $MgCl_2$ (●). In (C) the fluorescence increase on addition of $MgCl_2$ is expressed as a percentage of the fluorescence intensity in the absence of cations, and plotted as a function of chloroplast concentration.

chloroplasts. The quenching observed with high concentrations of chloroplasts reflects the increased absorption of the excitation beam and the reabsorption of the 9-AA fluorescence by the photosynthetic pigments. Nevertheless, as Fig. 2C shows there was a linear relationship between the salt-stimulated fluorescence increase and chlorophyll concentration. It can be seen that in order to obtain an approximate doubling in the fluorescence intensity on adding salt, a chloroplast concentration corresponding to about 20 μg chlorophyll/ml was required. These cation-induced 9-AA fluorescence changes were not dependent on a high energy state, as neither DCMU nor gramicidin had any influence on the fluorescence levels.

Nature of the salt and differential effect of cations

As Fig. 3 and Table I show, the release of the chloroplast-induced 9-AA fluorescence quenching by salt additions is dependent on the charge carried by the cation. With a typical monovalent cation salt, such as KCl, the concentration giving 50 % stimulation of the fluorescence ($C_{\frac{1}{2}}$) was found to be about 13 mM, but with the divalent cations $C_{\frac{1}{2}}$ was close to 0.16 mM, corresponding to nearly a 90-fold difference in sensitivity. Moreover, it was found that La^{3+} was even more effective than divalent cations. Fig. 3 also shows the differential effect of lysine, lysyl-lysine and polylysine, which bear one, two and approximately nine net positive charges per molecule, respectively, at neutral pH.

Changing the charge on the cation is the major influencing factor since varying

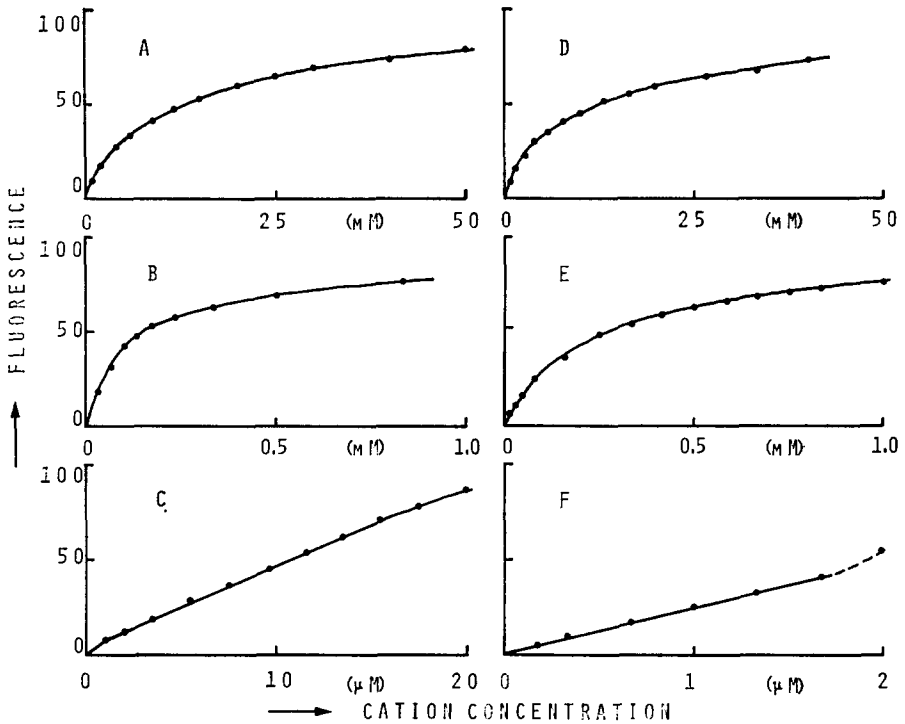

Fig. 3. The effectiveness of mono-, di- and polyvalent cations in releasing the quenching of 9-AA fluorescence by chloroplasts pretreated with 9-AA. In each case the increase above the level found in the absence of added cations is expressed as a percentage of the total increase seen on addition of 20 mM divalent cation. The curves shown were found for (A) KCl, (B) $MgCl_2$, (C) $LaCl_3$, (D) lysine · HCl, (E) lysyl-lysine · 2HCl and (F) polylysine hydrobromide. In the case of polylysine, aggregation of the chloroplasts occurs above about 2.0 μM, resulting in anomalously large increases in fluorescence on further addition of polylysine.

TABLE I

THE EFFECTIVENESS OF VARIOUS CATIONS IN RELEASING THE QUENCHING OF 9-AA FLUORESCENCE BY CHLOROPLASTS IN THE ABSENCE OF A HIGH-ENERGY STATE

100 % release of fluorescence quenching was determined by addition of 20 mM $MgCl_2$.

Addition	Concentration (mM) for 50 % release of quenching ($C_{\frac{1}{2}}$)	Addition	Concentration (mM) for 50 % release of quenching ($C_{\frac{1}{2}}$)
Monovalent		Divalent	
LiCl	12.7	$MgCl_2$	0.16
NaCl	12.3		
KCl	13.2	$Mg(NO_3)_2$	0.18
RbCl	12.6	$MgSO_4$	0.17
Choline chloride	14.8		
Lysine · HCl	13.2	$CaCl_2$	0.16
		$SrCl_2$	0.19
Polyvalent			
$LaCl_3$	0.011	$BaCl_2$	0.16
Polylysine · HBr	0.002	Lysyl-lysine · 2HCl	0.28

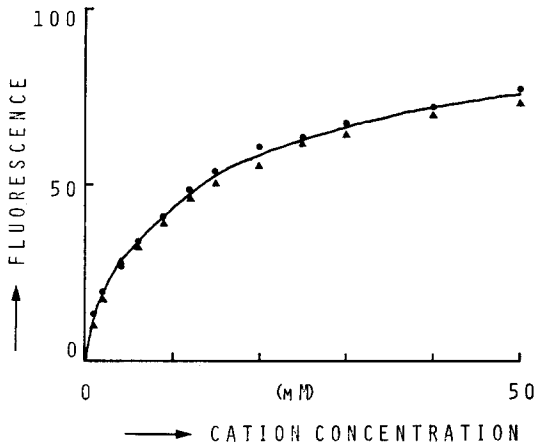

Fig. 4. Comparison of the effectiveness of large and small monovalent cations on the release of 9-AA fluorescence quenching in 9-AA pretreated chloroplasts. ▲, choline chloride; ●, LiCl. Lysine · HCl and KCl (shown in Fig. 3) and NaCl and RbCl (not shown) could also be fitted to the same curve. As in Fig. 3, 100 % fluorescence is the final level found with 20 mM divalent cation added.

the chemical nature of a cation in a particular charged group (see Table I, Figs. 4 and 5) or the associated anions (Table I and Fig. 6) had little or no effect on the ability of the salt to increase 9-AA fluorescence. For example, the monovalent cations lysine and choline were as equally as effective as the alkali metal cations while lysyl-lysine behaved like the alkaline earth metal cations. The stimulation in the emission was not due to osmotic changes in the medium since additions of non-charged solutes, such as glycine, even at high concentrations, had essentially no effect on the 9-AA fluorescence yield.

As will be discussed in more detail later, these three characteristics, a differen-

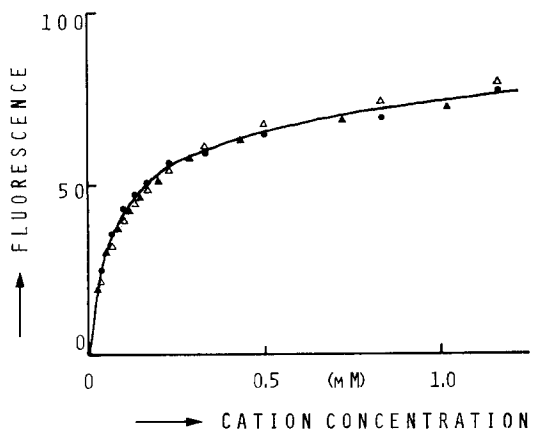

Fig. 5. Comparison of the effectiveness of the alkaline earth metal chlorides on the release of 9-AA fluorescence quenching by 9-AA-pretreated chloroplasts. △, $CaCl_2$; ▲, $SrCl_2$ and ●, $BaCl_2$. $MgCl_2$ (shown in Fig. 3) also fits the same curve. Other conditions as for Fig. 4.

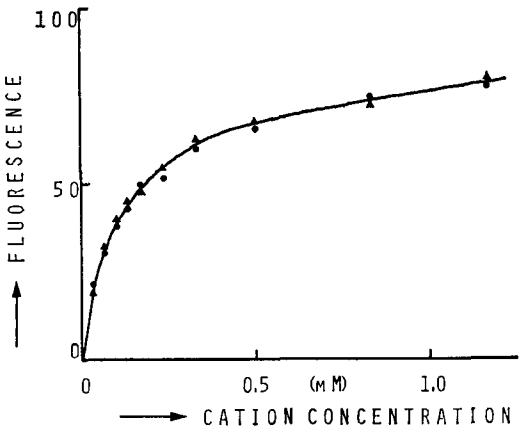

Fig. 6. Comparison of the effectiveness of magnesium salts on the release of 9-AA fluorescence quenching in 9-AA-pretreated chloroplasts. ▲, $MgSO_4$ and ●, $Mg(NO_3)_2$. $MgCl_2$ (shown in Fig. 3) also fits the same curve. Other conditions as for Fig. 4.

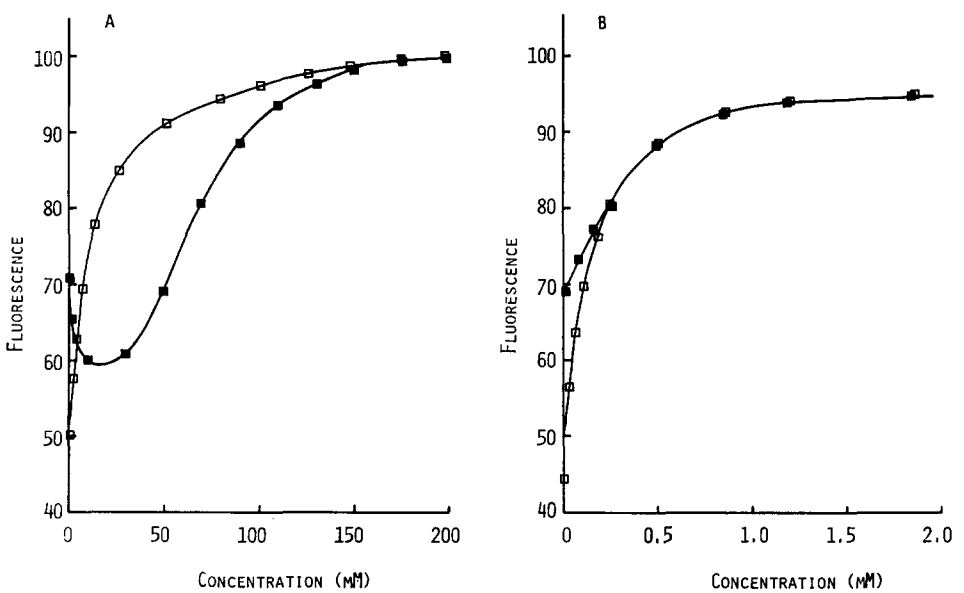

Fig. 7. Simultaneous observation of the changes in 9-AA (□) and chlorophyll *a* (■) fluorescence on the addition of salts to chloroplasts in a low cation medium. Isolated intact chloroplasts (ref. 8) were shocked osmotically in the cuvette and 10 μM DCMU and 25 μM 9-AA were added. 9-AA fluorescence was observed at 498 nm and chlorophyll fluorescence at 685 nm. (A) shows the effects of KCl addition, and (B) that of $MgCl_2$ addition. The fluorescence is expressed as a percentage of the final maximum level reached with either 10 mM $MgCl_2$ or 200 mM KCl.

tial effect based on the charge carried by the cation, a lack of specificity within each valency group and independence from the nature of the anion, are consistent with an electrical rather than a chemical control of the salt-induced 9-AA fluorescence changes.

Comparison of the salt-induced 9-AA and in vivo chlorophyll fluorescence changes

In some respects the above changes of 9-AA fluorescence induced by cations have similar characteristics to the cation-controlled chlorophyll fluorescence changes [1, 6]. Fig. 7 shows simultaneous measurements of chlorophyll and 9-AA fluorescence using DCMU-poisoned chloroplasts. As can be seen when monovalent cations were added to chloroplasts suspended in low cation medium chlorophyll fluorescence decreased on adding low levels of Na^+ but rose to a maximum with increasing Na^+ concentrations ($C_{\frac{1}{2}}$ 67 mM). This biphasic curve obtained for the monovalent cation-sensitive chlorophyll fluorescence was first reported by Gross and Hess [7] and as shown in Fig. 7 was not observed for 9-AA fluorescence where the rise was monotonic with a $C_{\frac{1}{2}}$ of about 10 mM. On the other hand with the addition of Mg^{2+} both the 9-AA and chlorophyll fluorescence rose to a maximum level at about the same Mg^{2+} concentration. As already emphasised [1, 2, 6, 20, 21] the monovalent- and divalent cation-induced changes of chlorophyll fluorescence are also only dependent on the charge carried by the cation rather than on its chemical nature.

Sensitivity of 9-AA fluorescence quenching to pH

Fig. 8 shows the pH dependence of 9-AA fluorescence quenching by chloro-

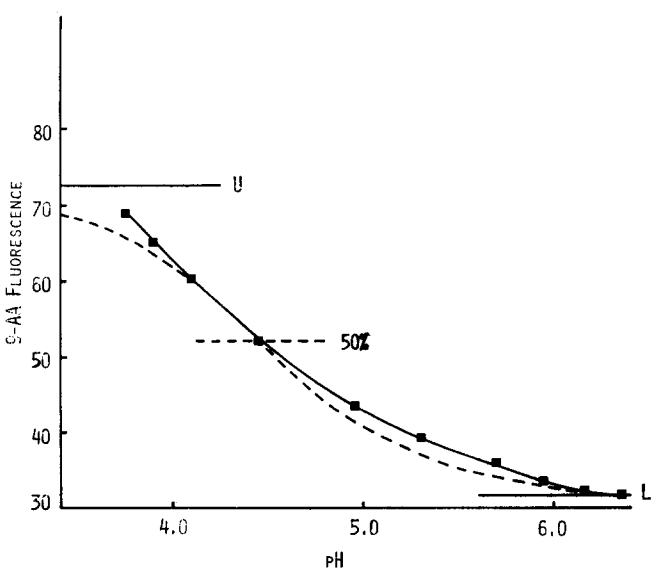

Fig. 8. The effect of pH on the degree of quenching of 9-amino-acridine fluorescence by chloroplasts in a cation-free medium. 9-AA fluorescence is expressed in arbitrary units. The lower limit (L) is found at neutral pH, whereas the upper limit (U) is that seen on complete release of quenching at high salt concentrations. The dashed line is the theoretical pH titration curve for an ionisable group with $pK = 4.5$ (i.e. equal to the pH at which 50 % fluorescence quenching occurs).

plasts in the cation-free medium. When the pH was steadily lowered by additions of acetic acid the fluorescence quenching was released, with 50 % change occurring near pH 4.5. A theoretical curve for the dissociation of an acidic group with pK 4.5 gives a reasonable approximation to the experimental curve. The upper limit (U) of the titrations is obscurred by aggregation of the chloroplasts and this limit has been fixed by the fluorescence level seen on complete release of quenching at high salt concentrations.

DISCUSSION

To interpret the significance of the results presented above it is necessary to realise that the thylakoid membrane is negatively charged [22, 23] and that for the sake of electroneutrality positive ions are drawn into a diffuse electrical layer adjacent to the surface. The relative difference between the concentration of a particular cationic species in the bulk solution and near the surface will vary depending on the composition of the medium and can be estimated by applying double layer theory developed by Gouy and Chapman (refs. 24 and 25, and also see ref. 26). The derivation and applicability of this theory to the thylakoid membrane has already been discussed in some depth in previous papers [1, 2, 6]. The two relevant expressions which are of importance here are:

$$q = \pm \left[\frac{RT\varepsilon}{2\pi} \sum_i C_{i\infty} \left(\exp\left(-\frac{Z_i F\psi_0}{RT}\right) - 1\right)\right]^{\frac{1}{2}} \tag{1}$$

where q is the surface charge density in μC/cm^2, $C_{i\infty}$ is the concentration of ionic species i in the bulk solution in mol/l. ε is the permittivity of water, ψ_0 is the surface potential and the other symbols have their usual meanings.

$$\frac{d\psi}{dx} = \pm 2\left(\frac{2\pi RT}{\varepsilon}\right)^{\frac{1}{2}} \left[\sum_i C_{i\infty} \left(\exp\left(\frac{-Z_i F\psi}{RT}\right) - 1\right)\right]^{\frac{1}{2}} \tag{2}$$

where ψ is the potential at some distance x from the membrane surface. Thus by using the Boltzmann equation

$$C_i = C_{i\infty} \exp\left(\frac{-ZF\psi}{RT}\right) \tag{3}$$

it is possible to calculate the concentration of species i at the surface (C_{i0}) where $\psi = \psi_0$ for any value of $C_{i\infty}$, or the concentration at any point x in the diffuse electrical layer where $\psi = \psi_x$.

Although integration of Eqn. 2 and calculation of the total concentration of the ionic species within the diffuse layer for any particular condition would be perhaps more desirable for the discussion presented below, for simplicity only Eqn. 1 will be used and calculation of surface concentration changes made (when $x = 0$, i.e. C_{i0}).

Eqn. 3 can be integrated for solutions of mixed electrolytes but it requires computer methods (see ref. 6) and as far as the qualitative arguments are concerned little is gained by these additional calculations.

The main reasoning behind the following calculations has already been discussed [1, 2, 6, 20]. It seems that when chloroplasts are carefully isolated so as to retain the envelope intact, and then broken osmotically in cation-free medium, the

surface negative charges are balanced by the presence of divalent cations, probably Mg^{2+} [27]. This is indicated by the existence of a high chlorophyll fluorescing state. Addition of low levels of monovalent cations to the cation-free medium displaces the divalent cations from the surface and the chlorophyll fluorescence is lowered. This effect can partially be seen in Fig. 7A. If sufficient divalent cations are present in the suspending medium no fluorescence lowering is observed. Since high levels of monovalent cations can also induce the high fluorescence state it was argued that the chlorophyll fluorescence changes reflected changes in the space charge density or the electrical field very close to the membrane surface (see ref. 6).

In the experiments presented in Table I and Figs. 2–6, 9-AA was used simply as a monovalent cation. The fluorescence effects observed were not due to proton movements since experiments were conducted at very low measuring light intensities in the absence of an added electron acceptor and furthermore DCMU and gramicidin had no effect. In most cases the chloroplasts were extensively washed with monovalent cations, usually Tris but also sodium. This should replace divalent cations at the surface by monovalent cations which would allow 9-AA better access to the membrane surface when these treated chloroplasts were resuspended in an almost cation-free medium containing the dye. The major monovalent cation in the experimental solution was 10^{-3} M Tris, while fluorescence measurements indicated that the 9-AA concentration in the bulk solution was about 10^{-5} M. In the low cation medium, the level of these cations at the surface would be considerably higher due to a substantial value of ψ_0 but will be lowered as other monovalent, divalent or trivalent cations are introduced into the suspending medium. In order to estimate the extent of the surface concentration changes it is necessary to derive expressions from Eqn. 1 for mixed electrolytes to obtain values of ψ_0. For a mixture of monovalent salts consisting of $C_1^+/A_1^-(C'_{1\infty})$ and $C_2^+/A_2^-(C'_{2\infty})$:

$$4(C'_{1\infty}+C'_{2\infty})\sinh^2\frac{F\psi_0}{2RT}-\frac{q^2}{A^2}=0 \tag{4}$$

where $A=\left[\frac{RT\varepsilon}{2\pi}\right]^{\frac{1}{2}}$

For a mixture of monovalent salt C^+/A^- (C'_∞) and divalent salt C^{2+}/A^{2-} (C''_∞) the expression is:

$$2C''_\infty\cosh^2\left(\frac{F\psi_0}{RT}\right)+C'_\infty\cosh\left(\frac{F\psi_0}{RT}\right)-\left(2C''_\infty+C'_\infty+\frac{q}{2A}\right)=0 \tag{5}$$

For a mixture of monovalent salt C^+/A^- (C'_∞) and trivalent salt C^{3+}/A^{3-} (C'''_∞) the expression is:

$$4C'''_\infty\cosh^3\left(\frac{F\psi_0}{RT}\right)+(C'_\infty-3C'''_\infty)\cosh\left(\frac{F\psi_0}{RT}\right)-\left(C'_\infty+C'''_\infty+\frac{q}{2A^2}\right)=0 \tag{6}$$

The surface charge density (q) has been taken as 2.5 μ C/cm^2 since in previous calculations [1, 2, 6] this value seemed to give calculated effects similar to those experimentally observed. Solutions to these expressions have been obtained using a computer (numerical method for Eqn. 6) and the values of ψ_0 plotted in Fig. 9A. $C'_{1\infty}$ and C'_∞

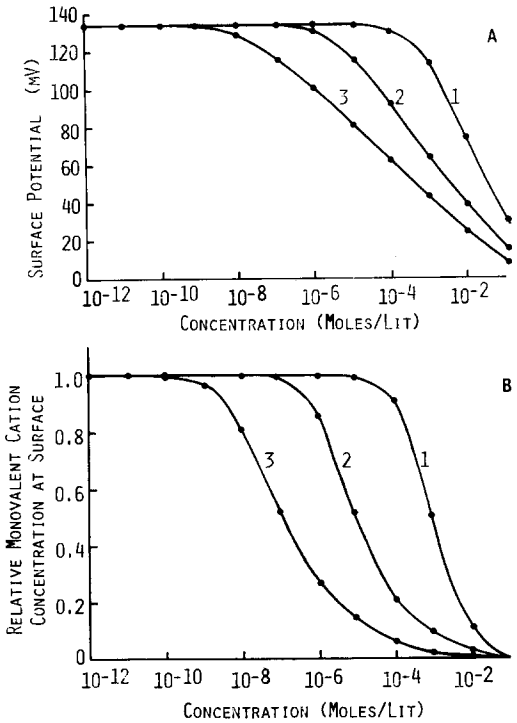

Fig. 9. Theoretical curves of (A) the surface potential (ψ_0) and (B) the relative surface monovalent cation concentration (C'_0) for various additions of monovalent (1), divalent (2) and trivalent (3) cations to chloroplasts suspended in a medium containing an initial bulk concentration of monovalent cations at 1 mM. The chloroplast membranes are assumed to have excess negative charges on their surfaces corresponding to a charge density (q) of 2.5 μC/cm^2. The curves have been calculated using Eqns. 3, 4, 5 and 6 given in the text.

have been taken as 10^{-3} M since this is essentially the cation level of the low cation medium (as the Tris cation). Fig. 9B shows the curves calculated by using Eqn. 3 (Boltzmann equation). The curves have been computed for the displacement of any monovalent cation from the surface. It clearly shows the differential ability of trivalent, divalent and monovalent cations to lower the calculated concentrations of monovalent cations at the surface.

The actual level of 9-AA at the surface can be calculated at an estimated bulk concentration of 10^{-5} M for various values of ψ_0. In low cation medium ψ_0 is calculated to be -134 mV before any significant salt additions were made, corresponding to a concentration at the surface of about 2 mM 9-AA. At this concentration fluorescence quenching will occur (see Fig. 1). Thus the above reasoning suggests that the increase in 9-AA fluorescence observed on adding cations to the chloroplast suspension is due to the release of the dye from the diffuse electrical layer adjacent to the membrane surface. Support for this simple concept not only comes from the expected differential effects associated with increasing the charge carried by the cation but also from the lack of specificity between similarly charged species. Moreover, the salt-induced fluorescence yield changes do not alter the fluorescence (Fig. 2) or absorption (un-

published observations) spectra of the dye. Such spectral changes would be expected to occur if 9-AA interacts more intimately with the membrane such as entering its hydrophobic interior or binding to the surface.

The analysis set out above is in many ways approximate and serves only to emphasise the significance of the charged membrane in controlling the fluorescence of 9-AA. There are a number of intrinsic assumptions in the Gouy-Chapman theory which are not strictly applicable (see ref. 6): the actual value of q is not known, the calculations have assumed for convenience symmetrical electrolytes which in fact were not always used in the experiments, and no provision has been made for the changes in the concentration of 9-AA in the bulk solution. Of more importance, there is no way of quantitatively relating the 9-AA fluorescence yield changes with changes of its concentration within the diffuse electrical layer. Thus it is not surprising that the calculated concentration of monovalent, divalent and trivalent cations for a 50 % reduction in the surface concentration of 9-AA does not compare with the concentrations experimentally observed for a 50 % increase in fluorescence yield. Nevertheless, bearing in mind the problems of knowing accurately the bulk concentrations of salts at very low values in the presence of charged membranes, there is good agreement for the differential effect of cations carrying either one, two or three charges. The pH sensitivity of the 9-AA fluorescence quenching strongly indicates that the release of the dye from the diffuse electrical layer can occur on protonation of groups having a pK of about 4.5, which is consistent with the concept that carboxyl groups are responsible for the excess negative charges on the thylakoid membrane surface at neutral pH [22, 23]. However, carboxyl groups are not specifically required for 9-AA association with membranes since it has clearly been shown that artificial phospholipid liposomes also quench 9-AA fluorescence [14].

The cation-induced changes in chlorophyll fluorescence do not follow changes in ψ_0 as already discussed in previous publications [1, 6] and the above experiments also demonstrate this (Fig. 7A). Chlorophyll fluorescence studies have indicated two types of cation interactions with the thylakoid membrane [20, 21]. Most monovalent and divalent cations can readily be exchanged from the diffuse electrical layer and give rise to "reversible" chlorophyll fluorescence changes. However, polyvalent cations like polylysine and La^{3+} seem to bind to the membrane strongly causing an "irreversible" chlorophyll fluorescence lowering. Although La^{3+} and Mg^{2+} may behave differently at the membrane surface, the initial attraction will be governed by their charges and both would act to displace 9-AA from the surface and bring about an increase in its fluorescence.

The purpose of our work was to use 9-AA as a fluorescent monovalent cationic probe of the electrical double layer associated with thylakoid membranes in order to give qualitative support to concepts presented in earlier papers [1, 2, 6, 21]. However, in conclusion, it is worth emphasising that this view of the interaction of 9-AA with the chloroplast membrane may be important with regard to its use in monitoring the high energy state and gives credibility to the arguments of Kraayenhof and his colleagues [13]. These workers have argued that for some acridines there is significant association with the membrane surface, and certainly significant binding does occur for more hydrophobic acridine derivatives like ACMA (9-amino-6-chloro-2-methoxyacridine) [28]. The local elevated levels of any species drawn to a charged membrane surface may well encourage binding and some account for this should ideally be intro-

duced into the double layer theory (see ref. 29). Finally, light-induced proton pumping will almost certainly change surface charge densities or surface potentials on either side of the thylakoid membrane [30] which will in turn attract or displace 9-AA from the diffuse electrical layers.

ACKNOWLEDGEMENTS

The work was supported by grants from the E.E.C. (Solar Energy Research and Development Programme) and the Science Research Council. The technical assistance of E. Dibb is gratefully acknowledged and the help of Andrew Love with the computer analysis. Thanks also go to Herb Nakatani, Alison Telfer and David Nickson for useful discussion.

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