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THE INTERACTION OF AN AMPHIPATHIC FLUORESCENCE PROBE, 2-*p*-TOLUIDINONAPHTHALENE-6-SULPHONATE, WITH ISOLATED CHLOROPLASTS

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Summary

The amphipathic fluorescence probe, 2-*p*-toluidinonaphthalene-6-sulphonate has been used to investigate the surface electrical properties of chloroplast thylakoid membranes. The fluorescence yield of 2-*p*-toluidinonaphthalene-6-sulphonate in aqueous solution increases on addition of hypotonically shocked chloroplast, and the emission maximum shifts towards the blue to 440 nm, although the emission spectrum is somewhat distorted by chloroplast pigment absorption.

The intensity of 2-*p*-toluidinonaphthalene-6-sulphonate fluorescence is further increased on adding salts to the membrane suspension, and changes of >100% are routinely observed. Similar observations have also been made with soya bean phospholipid (azolectin) liposomes. The magnitude of the fluorescence increase is dependent on membrane concentration, being more pronounced at high surface area/suspending volume ratios. The effect of salt addition appears to be that of shielding the fixed negative charges on the membrane surface, thus increasing the fraction of 2-*p*-toluidinonaphthalene-6-sulphonate molecules at the surface, where the 2-*p*-toluidinonaphthalene-6-sulphonate has a higher fluorescence yield than in free aqueous solution. This concept is supported by the fact that the effectiveness of salts in increasing 2-*p*-toluidinonaphthalene-6-sulphonate fluorescence is as predicted by classical electrical double layer theory: governed mainly by the charge carried by the cation with an order of effectiveness $C^{3+} > C^{2+} > C^{+}$, and not by the chemical nature of the cation or by the nature of its co-ion.

Abbreviations: TNS, 2-*p*-toluidinonaphthalene-6-sulphonate; ANS, 1-anilinonaphthalene-8-sulphonate; DCMU; 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tris, Tris(hydroxymethyl)aminoethane.

It has been argued that the chlorophyll fluorescence yield, controlled by the cation composition of the suspending medium follows the total diffusible positive charge density at the thylakoid membrane surface (Barber, J., Mills, J. and Love, A. (1977) Febs. Lett. 74, 174—181). Although the cation induced 2-*p*-toluidinonaphthalene-6-sulphonate and chlorophyll fluorescence yield changes show similar characteristics, there are also distinct differences between the two phenomena particularly when cations are added to chloroplasts initially suspended in a virtually cation-free medium. Therefore it is concluded that although both 2-*p*-toluidinonaphthalene-6-sulphonate and chlorophyll fluorescence yields are governed by the electrical properties of the thylakoid membrane surface, the mechanism controlling their cation sensitivity is not the same.

Introduction

Chlorophyll fluorescence from the chloroplast can be used as an intrinsic probe of conformational changes taking place in the thylakoid membrane [1]. In particular the effect of addition of cations of different valency on chlorophyll fluorescence yields from broken chloroplasts suspended initially in a low salt medium has been studied [2]. It was found that classical electrical double layer theory could adequately describe the relative effectiveness and the observed competition between mono- and divalent cations [3].

We have also used the extrinsic hydrophilic fluorescence probe 9-aminoacridine, which acts as a highly diffusible monovalent cation, and shown that the fluorescence quenching induced at membrane surfaces having a large negative surface potential, can be released by the addition of cations in accordance with double layer theory [4]. Although the fluorescence from the intrinsic (chlorophyll) and extrinsic (9-aminoacridine) probes showed a similar dependence on added cations when the thylakoid membrane was pretreated with monovalent cations, this was not true when divalent cations were initially present at the membrane surface [5].

In order to understand the processes which control chlorophyll fluorescence it would be valuable to identify an artificial probe whose fluorescence in the presence of chloroplasts is influenced in an identical manner to that of chlorophyll by the ionic composition of the suspending medium. For the present work we have chosen the monovalent anion 2-*p*-toluidinonaphthalene-6-sulphonate (TNS), a member of the anilinonaphthalene sulphonate group of molecules, as these have an amphipathic character, which should allow close interaction with the surface of the thylakoid membrane, and thus possibly mimic the chlorophyll fluorescence changes.

The addition of different cations to 1-anilinonaphthalene-8-sulphonate (ANS) in the presence of chloroplasts has been shown to give rise to fluorescence intensity changes of the probe, however, these effects are small [6—9]. We report here that TNS on the other hand shows relatively large fluorescence changes when associated with the thylakoid membrane, and that these changes moreover can be accounted for, qualitatively at least, by electrical double layer theory. Haynes has reported similar observations using ANS with phospholipid liposomes [10].

Materials and Methods

Chloroplasts with the outer envelope intact were isolated from young pea leaves as described previously [11], and washed with 0.33 M sorbitol containing Tris base to bring the pH to 7.5. Chloroplasts were usually shocked hypotonically in 1.5 ml water directly before use and 1.5 ml buffered sorbitol (low salt medium) added subsequently after about 30 s. However, in some experiments, where it was necessary to have no chloroplasts present initially, chloroplasts were added to the medium buffered with 2 mM Tris-HCl, pH 7.8 but containing no sorbitol. To avoid changes in chlorophyll fluorescence due to changes in the redox state of the Photosystem II reaction centre, 10 μM 3-(3′, 4′-dichlorophenyl)-1,1-dimethylurea (DCMU) was present in all samples. The addition of DCMU should also block generation of the high energy state and inhibit any possible light-induced effects on TNS fluorescence. Washed carboxymethyl cellulose particles and soya bean phospholipid (azolectin) liposomes were prepared as previously [4]. 2-*p*-Toluidinonaphthalene sulphonic acid (Eastman Kodak) was purified by recrystallising twice from distilled water [12], and dissolved in ethanol/water (1 : 1, v/v). It was stored as a 1% solution in the dark, as room lighting can produce fluorescent impurities [12]. Because of its acidity, solutions of TNS can aggregate chloroplasts if sufficient buffering capacity is not present. Salt additions were made with analytical grade reagents.

TNS fluorescence was measured with a Perkin-Elmer MPF 3 fluorescence spectrophotometer. The sample, in a 10 X 10 mm fluorescence cuvette was excited at 320 nm (the maximum of the excitation spectrum for TNS fluorescence in the presence of chloroplasts) with a band width of 10 nm, and the emission monitored at 90°. For most measurements the emission monochromator was set at 456 nm (the emission maximum in the presence of chloroplasts) with a bandwidth of 2—3 nm. No corrections were made for the wavelength-dependence of the xenon lamp emission or the detector sensitivity. As TNS fluorescence is very dependent on temperature [12], the sample temperature was also controlled (25°C).

Chlorophyll fluorescence was measured in a laboratory-constructed apparatus equipped with broad-band blue-green illumination (Schott BG18, 2 mm and BG38, 2 mm), intensity about 50 $W \cdot m^{-2}$. The emission was monitored at 90° by an EMI 9558B photomultiplier (S20 sensitivity) protected from excitation light by a Balzer B-40 695/Schott RG 695 filter combination. Chlorophyll concentrations were measured according to Bruinsma [13]. In some experiments TNS and chlorophyll fluorescence were measured simultaneously and details are given in the legend to Fig. 5.

Results

When chloroplasts are added to a solution of TNS in low salt medium the fluorescence intensity increases (Fig. 1A), and moreover the intensity rises further as salt additions are made (there is no associated change in the fluorescence excitation spectrum). The fluorescence increase is completely dependent upon the presence of chloroplasts, as TNS solutions are unaffected by salt additions alone (Fig. 1B).

At a constant concentration of TNS the intensity of the TNS fluorescence is dependent upon the chloroplast concentration, passing through a maximum before decreasing again at higher chloroplast concentrations in low salt medium (Fig. 2, curve a). The decrease is due to the absorption of the excitation light and the TNS emission by the chloroplast pigments; excitation spectra show that no significant excitation energy transfer occurs between TNS and chlorophyll. The relative increase in TNS fluorescence seen on addition of salt (cf. curves a and b of Fig. 2) is also dependent on the chloroplast concentration, as might be anticipated for different membrane surface area/suspending volume ratios (see Discussion). The salt-induced increase in fluorescence of TNS associated with chloroplasts was seen throughout the range of TNS concentrations tested (10—100 μM).

Experiments were also carried out with thylakoid membranes which had been well washed with 0.33 M sorbitol/100 mM NaCl/2.5 mM Tris-HCl, pH 7.8, and subsequently twice with 0.33 M sorbitol/2.5 mM Tris-HCl, pH 7.8. These washed membranes also cause an increase in TNS fluorescence which is stimulated by the addition of salt in an identical manner to that noted with hypotonically broken whole chloroplasts. This indicates that the fluorescence changes are not due significantly to stromal components released into the measuring cuvette.

Characteristics of TNS fluorescence in the presence of chloroplasts or liposomes

The increase in fluorescence yield seen on adding chloroplasts to a solution

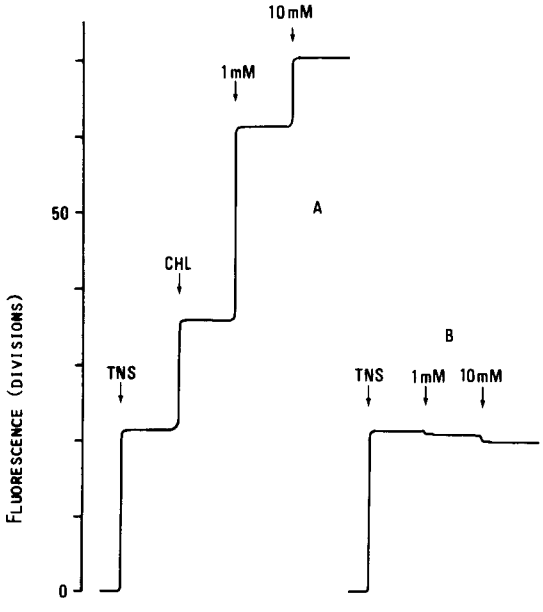

Fig. 1. The effect of addition of $MgCl_2$ on the fluorescence of TNS initially present in a low salt medium, in the presence (A) and absence (B) of chloroplasts. TNS fluorescence is measured at 440 nm and is expressed in arbitrary units. CHL: chloroplasts added at a final concentration of 4 μg chlorophyll/ml. The final TNS concentration is 20 μM.

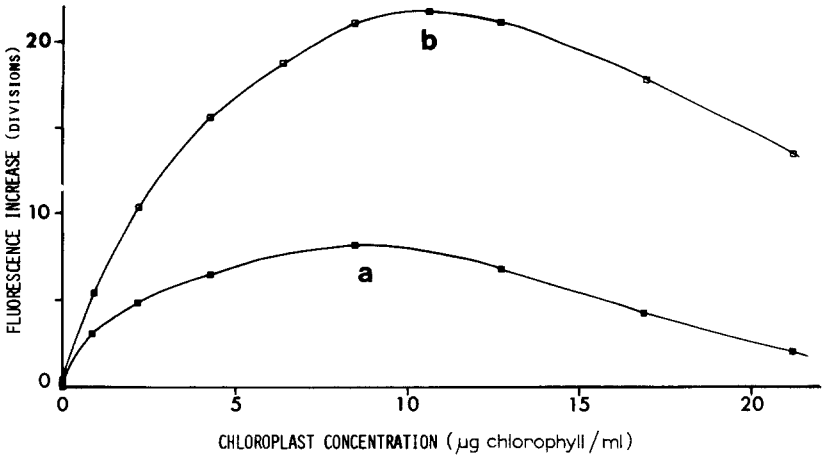

Fig. 2. The increase in TNS fluorescence induced by the addition of chloroplasts. TNS (50 μM) is initially present in low salt medium (curve a), to which is added 100 mM KCl (curve b). The increase in fluorescence, measured at 456 nm, on the addition of chloroplasts at varying concentrations, is expressed in arbitrary units. The level of TNS fluorescence in the absence of chloroplasts is 11 divisions.

of TNS is accompanied by a change in the wavelength of maximum emission: the emission maximum for TNS with chloroplasts is near 440 nm (Fig. 3A), compared to 500 nm in water (ref. 12; in the present work this peak was not clearly detected due to the very low fluorescence yield of TNS in aqueous solu-

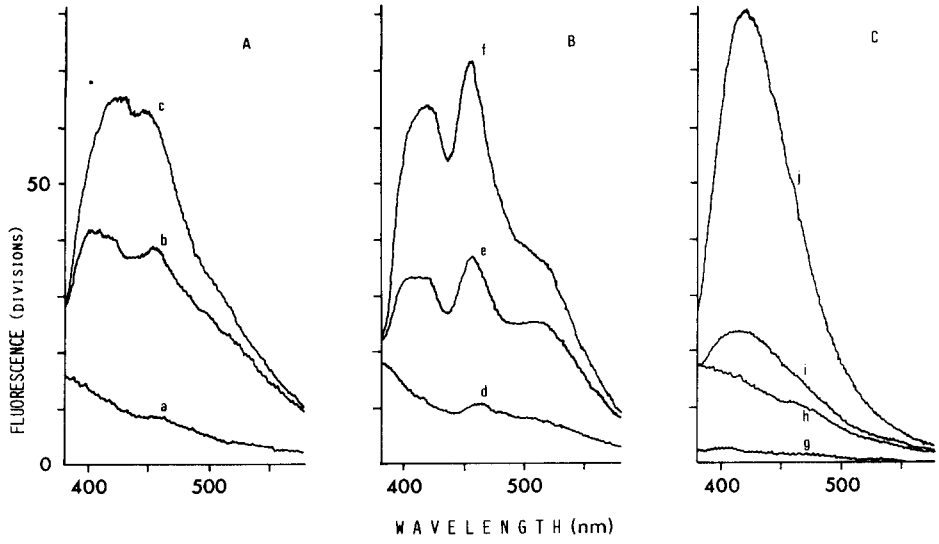

Fig. 3. Fluorescence emission spectra of TNS in the presence of chloroplasts (A and B), compared to that with azolectin liposomes (C). In (A) the chloroplast concentration is 4 μg chlorophyll/ml, and in (B) 15 μg chlorophyll/ml. In (A) and (B): curves (a) and (d) are for chloroplasts suspended in low salt medium; curves (b) and (e) are found after addition of 20 μM TNS; and curves (c) and (f) show the further increase on subsequent addition of 1 mM $MgCl_2$. In (C): curve (g) is the emission spectrum for 20 μM TNS in water, curve (h) is found for liposomes in low salt medium without any additions, curve (i) is seen after addition of 20 μM TNS to liposomes, and curve (j) shows the large further increase found on subsequent addition of 1 mM $MgCl_2$. The spectra are uncorrected for wavelength dependent variations in instrument sensitivity, and the fluorescence is expressed in arbitrary units. Excitation wavelength 320 nm (9 nm bandwidth).

tion and the instrumental baseline distortion). However the emission spectrum is distorted by chloroplast pigment absorption, as seen at a higher chloroplast concentration in Fig. 3B. Similar blue-shifts of the fluorescence maximum have been reported for TNS associated with proteins or in solution in organic solvents [12].

When azolectin liposomes are used instead of chloroplasts in identical experiments, the TNS emission intensity is also increased by liposome addition, and moreover the emission maximum also shifts considerably to the blue (about 425 nm, see Fig. 3C). Again salt addition causes a large increase in fluorescence intensity.

However, an aqueous suspension of carboxymethyl cellulose particles does not produce an increase in TNS fluorescence either in the absence of presence of salt, which suggests that a more hydrophobic surface than that of cellulose is required in order to induce a TNS fluorescence increase.

Relative effectiveness of salts

The relative effectiveness of various salts in causing the increase in TNS fluorescence in the presence of chloroplasts was tested, and the results are presented in Table I. The monovalent cations of the alkali metal series are equally effective within experimental error, having a $C_{1/2}$ value (the concentration giving 50% increase in TNS fluorescence) near 50 mM. The divalent alkaline earth cations also show little specificity but are considerably more effective than monovalent cations, with a $C_{1/2}$ value near 0.45 mM. The trivalent cation tris(ethylenediamine)Co^{3+}, introduced recently as a very effective mediator of the transition from the low to the high chlorophyll fluorescing state in chloroplasts [14], is also much more effective than divalent cations in causing an increase in TNS fluorescence ($C_{1/2}$ = 0.02 mM).

Fig. 4 illustrates these differences between cations of different valency. The final fluorescence level reached at saturating concentration of salt is comparable for all the salts tested, even though the concentrations required (300 mM for mono-, 5 mM for di-, and 0.1 mM for trivalent) are quite different. It should be noted that some variation of the $C_{1/2}$ values, for say Mg^{2+}, was seen between different chloroplast preparations. The dependence of $C_{1/2}$ values on both chloroplast and TNS concentrations was tested and it was found that the

TABLE I

THE RELATIVE EFFECTIVENESS OF SALTS IN INCREASING TNS FLUORESCENCE INTENSITY IN THE PRESENCE OF CHLOROPLASTS

$C_{1/2}$ is the concentration of salt producing an increase in TNS fluorescence which is 50% of that obtained with saturating amounts of salt.

Addition	$C_{1/2}$ (mM)	Addition	$C_{1/2}$ (nm)
$MgCl_2$	0.43	NaCl	52
$CaCl_2$	0.46	KCl	45
$SrCl_2$	0.46	RbCl	48
$Mg(NO_3)_2$	0.45		
$MgSO_4$	0.47	TEC *	0.02

* TEC = tris(ethylenediamine)cobaltic trichloride.

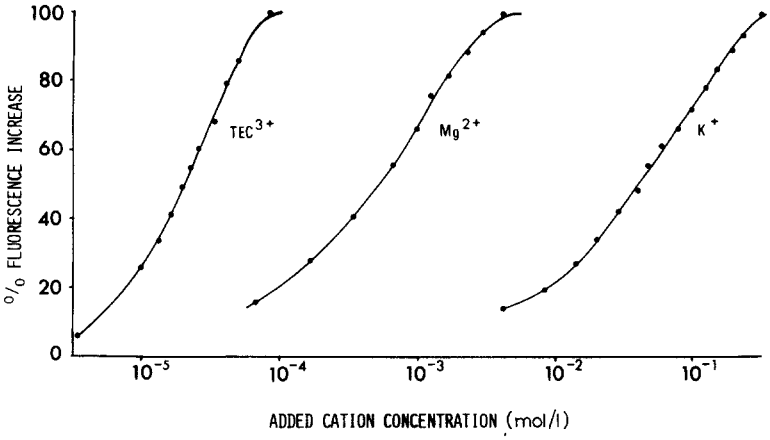

Fig. 4. The relative effectiveness of cations of different valency in producing an increase in TNS fluorescence intensity in the presence of chloroplasts. Fluorescence is measured at 456 nm, and the % fluorescence increase at a given concentration of cation added to the chloroplasts, initially in a low salt medium, is calculated as $[(F - F_0)/(F_m - F_0)] \times 100$. Where F, F_0 and F_m represent the TNS fluorescence intensity at the given cation concentration, the initial fluorescence intensity in low salt medium, and the final fluorescence intensity at saturating salt concentration, respectively. The TNS concentration was 50 μM, and the chloroplast concentration was 14 μg chlorophyll/ml. TEC^{3+}: tris(ethylenediamine)cobalt^{3+}. All cations were added as the chloride salts.

$C_{1/2}$ for Mg^{2+} approximately doubled as the chlorophyll concentration was increased from 7 to 28 μg/ml, and also doubled approximately when the TNS concentration was increased from 10 to 250 μM.

Table I also shows that the three magnesium salts tested were equally effective, indicating that the effectiveness in bringing about a TNS fluorescence increase is independent of the nature of the anion. It is clear that the effectiveness is related to the valency of the cation and that the chemical nature of the ion is not important for species within a given valency group. In a control experiment it was found that variation of the concentration of sorbitol in the medium between 0 and 300 mM had practically no effect on TNS fluorescence in the presence of chloroplasts, indicating that the cation induced increase does not involve effects associated with changes in osmotic strength. Much higher concentrations of sugar have been reported however to cause an increase in fluorescence yield of TNS in free solution [12].

Comparison of salt effects on chlorophyll and TNS fluorescence

When chloroplast thylakoid membranes are suspended in a low salt medium, the fluorescence emission from both chlorophyll and from TNS are susceptible to control by the ionic composition of the suspending medium. In Fig. 5, addition of divalent cations to chloroplasts suspended in a low salt medium (containing 5 mM monovalent cation) is seen to produce a similar increase in both TNS and chlorophyll fluorescence. Also, for the trivalent cation, tris-(ethylenediamine)Co^{3+}, the TNS and chlorophyll fluorescence increases are similar: 0.02 mM causes the transition to the high chlorophyll fluorescing state [14], and the same concentration gives 50% increase in TNS fluorescence (Table I).

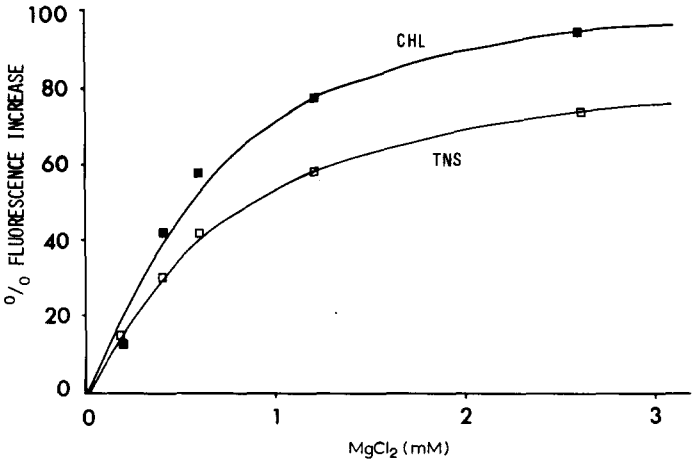

Fig. 5. Simultaneous measurements of chlorophyll and TNS fluorescence changes induced by $MgCl_2$ addition to chloroplasts in the low chlorophyll fluorescence state. The fluorescence increase is expressed as a percentage of the final increase seen at saturating concentrations of $MgCl_2$. Chloroplasts were intially present in a medium containing 0.33 M sorbitol and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid brought to pH 7.5 with 5 mM KOH. 10 μM DCMU was also present. The sample was illuminated with ultraviolet light (Schott UG5, 4 mm and BG38, 2 mm), and the emission observed through a Balzer B-40 458 nm/Schott BG18 (2 mm) filter combination for TNS fluorescence; and a Balzer B-40 695 nm/Schott RG695 (2 mm) filter combination for chlorophyll fluorescence. ■, chlorophyll; □, TNS fluorescence. TNS concentration 50 μM; chloroplast concentration, 10 μg chlorophyll/ml.

However, the similarity between the effects of salt addition on the two types of fluorescence is not noted in other experiments, where the cation-free medium used poises the chloroplasts initially in the high chlorophyll fluorescing state (Fig. 6). Addition of monovalent cations (e.g. K^+) in this case produces a decrease followed by an increase in chlorophyll fluorescence, as first reported by Gross and Hess [15], whereas TNS fluorescence rises monotoni-

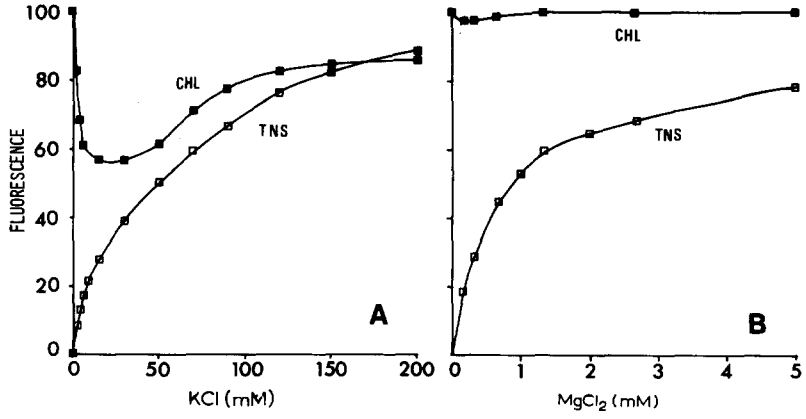

Fig. 6. Changes in TNS and chlorophyll fluorescence induced by the addition of KCl (A) and $MgCl_2$ (B) to chloroplasts in the high chlorophyll fluorescing state. The fluorescence is expressed as a percentage of the maximum value reached for each curve. Chlorophyll and TNS fluorescence levels were measured separately, but on the same preparation of chloroplasts. ■, chlorophyll; □, TNS fluorescence. TNS concentration, 50 μM; chloroplast concentration 11 μg chlorophyll/ml. 10 μM DCMU was present.

cally (Fig. 6A). Addition of Mg^{2+} gives virtually no change in the chlorophyll fluorescence level, but again the TNS fluorescence increases monotonically (Fig. 6B). It appears that whereas chlorophyll fluorescence is probably controlled by the total positive diffusible charge density adjacent to the membrane surface [3], the TNS fluorescence yield is probably a function of the surface potential (see also ref. 16).

A further distinction between TNS and chlorophyll fluorescence changes is the time required for salt-induced increases to occur: TNS fluorescence changes occur within the time resolution of the fluorimeter; whereas chlorophyll fluorescence changes can require several minutes for completion, as also reported by Murata et al. [17].

Discussion

We have recently reported experiments using the fluorescence probe 9-aminoacridine to study the surface electrical properties of the thylakoid membrane, and shown it to act as a freely diffusible monovalent cation in the electrical double layer [5]. We found that although 9-aminoacridine and chlorophyll fluorescence changes induced by cation additions were sometimes comparable they did not follow each other under all conditions. As 9-aminoacridine is hydrophilic and probably shows no interactions with the membrane other than electrostatic, a better correspondence might be expected from a molecule such as TNS, which can be adsorbed onto the membrane surface via hydrophobic interaction [18].

Both 9-aminoacridine and TNS show changes in fluorescence yield in the presence of thylakoid membranes or negatively-charged phospholipid liposomes when the cation composition of the suspending medium is varied. However, the mechanisms bringing about the fluorescence changes are different. 9-aminoacridine fluorescence is quenched by some form of concentration quenching in the diffuse layer when the negative surface potential is large, but the quenching is released on the addition of cations due to the displacement of the probe from the surface [4,5]. The monovalent anion TNS, on the other hand, shows a large increase in fluorescence yield when associated with membrane surfaces together with a blue-shift of the emission maximum, compared to a solution in water. The interpretation of these changes as indicative of a more nonpolar environment of the probe has been questioned [19], and they could also reflect instead the restricted mobility of molecules in the more structured environment at the membrane surface [20]. Certainly our observation that the cation induced TNS fluorescence yield changes do not occur when carboxymethyl cellulose particles are used does support the requirement that the negatively charged surface has to have a degree of hydrophobicity.

In the case of the negatively charged thylakoid membrane the effect of cation additions is to shield the fixed negative charges and thus reduce the negative surface potential. This has clearly been shown by carrying out particle electrophoresis studies [21] and by measuring changes in cation levels associated with membranes under different ionic conditions [22]. Thus increasing the cations in the suspension medium allows TNS anions to approach the thylakoid membrane surface and increase its fluorescence yield. The observed

differences in effectiveness of mono-, di- and trivalent cations (Fig. 4) is predicted by the classical electrical double layer theory of Gouy and Chapman [23,24]. In Fig. 7 we have calculated, using the above theory, how the concentration of a freely diffusible monovalent anion would increase at the membrane surface as the salt level of the suspending medium is raised. The equations used have already been given [4] and the calculations have been made using a surface charge density of 2.5 $\mu C/cm^2$ and ionic concentrations applicable to the experimental conditions (e.g. as for Fig. 4). Even though TNS cannot be regarded as a diffusible anion because it almost certainly interacts with the membrane surface, the calculation does serve to demonstrate the relative effectiveness of cations of different charge groups to decrease the negative surface potential and allow a negatively charged anion like TNS to approach the membrane surface. Of course this approach is only illustrative, making no allowances for assumptions about ion activities, the molecular size of TNS, the effect of a negatively charged species binding to the surface and thereby increasing the surface charge density, or for changes in pH at the membrane surface induced by adding salts [22]. A more rigorous treatment has been adopted by McLaughlin and Harary [25]. Nevertheless, it is clear that the TNS fluorescence yield is controlled by the electrical properties of the thylakoid membrane surfaces in agreement with similar conclusions of Haynes for ANS/liposome systems [10].

The lack of correspondence between TNS and chlorophyll fluorescence changes shown in Fig. 6 emphasises that the chlorophyll fluorescence does not follow the surface potential, a conclusion already reached by other approaches [3,5,22]. It is likely that the positive space charge density immediately adjacent to the membrane surface is the controlling factor for the cation-induced chlorophyll fluorescence changes as proposed elsewhere [2,3]. This parameter undergoes changes similar to the chlorophyll fluorescence and possibly regu-

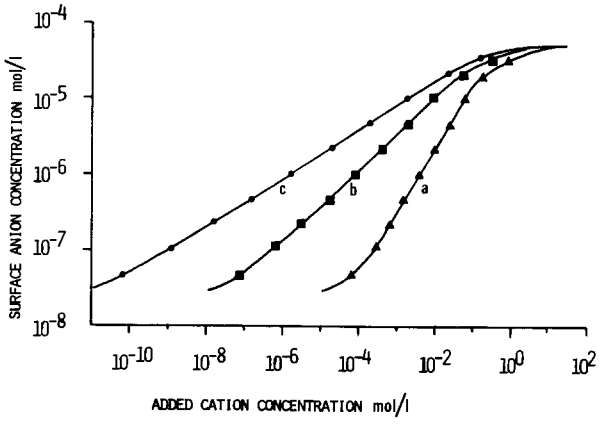

Fig. 7. The theoretical surface concentration curves for a freely diffusible, non-interacting, monovalent anion at a negatively-charged surface, as a function of added cation concentraton (see text). The bulk concentration of the anion is assumed to be constant at 50 μM, the surface charge density is taken as 2.5 $\mu C \cdot cm^{-2}$. The surface is initially suspended in a medium containing 10^{-4} M monovalent cation, and the additions made are (a) monovalent, (b) divalent and (c) trivalent cations.

lates the interactions of fluorescing and nonfluorescing chlorophyll-protein complexes, as discussed in detail recently [26].

The low fluorescence yield of TNS in solution in water [12] allows the increase in fluorescence induced by association with the thylakoid membrane to be seen against a low background level. In this respect TNS is to be preferred to ANS, as ANS in solution in water (emission maximum 525 nm) has a higher fluorescence yield which makes observations of membrane-induced changes (maximum at 460—480 nm) less easy, and accounting for the relatively small effects of cation addition reported for ANS (Searle, G.F.W. and Barber, J., unpublished results and also refs. 6—9).

TNS therefore appears to be a useful fluorescence probe for investigating the properties of the thylakoid membrane surface, and in particular its fluorescence yield appears to follow surface potential changes. However, it does not follow the cation-induced chlorophyll fluorescence changes under all circumstances. Therefore, it seems worth investigating other types of molecules in order to identify an extrinsic fluorescence probe which mimics chlorophyll fluorescence, possibly one which penetrates further into the membrane interior than does TNS.

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