FLUORESCENCE CHANGES IN ISOLATED BROKEN CHLOROPLASTS AND THE INVOLVEMENT OF THE ELECTRICAL DOUBLE LAYER

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ABSTRACT We studied the effects of a variety of cations on chlorophyll fluorescence yield of broken chloroplasts prepared under carefully controlled ionic conditions. In the absence of light-induced electron transport and associated proton pumping, two types of cation-induced chlorophyll fluorescence changes could be distinguished in broken chloroplasts. These are termed "reversible" and "irreversible" fluorescence yield changes. Reversible fluorescence yield changes are characterized by antagonistic effects of monovalent and divalent cations and are prevented by the presence of 5 mM Mg^{2+} in the suspending media. Reversible-type fluorescence yield changes show little or no dependence on the structure, lipid solubility, or coordination number of the cation, but depend strictly on the net positive charge carried by the ion. It is proposed that these fluorescence changes are brought about through the interaction of monovalent or divalent cations with an electrical double layer at the interface of the outer surface of the thylakoid membrane and the surrounding aqueous solution. The results are interpreted in terms of the Gouy-Chapman theory of the diffuse double layer, indicating that the thylakoid outer surface bears an excess fixed negative charge density of about 2.5 $\mu C/cm^2$, or approximately 1 negative charge per 640 $Å^2$ of membrane surface. Chlorophyll fluorescence quenching in isolated broken chloroplasts suspended in media containing 5 mM $MgCl_2$ is also observed on addition of certain polyvalent cations to the medium. This type of cation-induced fluorescence change appears to be largely irreversible and may occur through specific binding of the cation to the thylakoid as a result of the high electrostatic attraction exerted by the negatively charged membrane surface.

INTRODUCTION

It is now well established that in vivo chlorophyll *a* fluorescence may be influenced by factors other than the redox state of the photochemical traps of photosystem II (PSII).[1] In particular, the intensity of room-temperature chlorophyll fluorescence emission from isolated broken chloroplasts (those not retaining functional outer envelopes) is

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[1] Abbreviations used in this paper: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; PS1, Photosystem one; PS11, Photosystem two.

highly dependent on the cation composition of the chloroplast suspending medium (1–9). Gross and Hess (5), confirmed by others (6–8), originally established that broken chloroplasts washed and resuspended in an essentially cation-free medium exhibited a high fluorescence yield, which was reduced to a minimum on addition of monovalent cations at low concentrations (10 mM) to the suspending medium. Further addition of higher levels of monovalent cations (100 mM) or low levels of divalent cations restored chlorophyll fluorescence to the high yield. The fluorescence yield changes observed with these 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated samples could not be related to fluctuations in the redox state of the PSII traps, but appeared to reflect cation-induced changes in relative distribution of incoming light energy to PSII and photosystem I (PSI), as judged by low temperature fluorescence emission studies (5,7). Gross and Hess attempted to explain the antagonistic effects of monovalent and divalent cations on chlorophyll fluorescence from broken chloroplasts in terms of specific binding mechanisms (10).

In contrast to this view, we presented evidence suggesting that cations interact electrostatically with fixed negative charges on the outer side of the thylakoid membrane (8, 11, 12). We suggested that in the absence of added cations, the need to maintain overall electroneutrality at the membrane surface caused residual amounts of divalent cations to remain associated with the thylakoid surface throughout their isolation and washing procedures. These retained divalent cations seem to be sufficient initially to create the high fluorescing state. Subsequently fluorescence changes observed on the addition of other cations to the medium essentially reflected competition effects between monovalent and divalent cations for the fixed negative charges on the thylakoid (8, 11). In this paper we characterize further the properties of these cation-induced chlorophyll fluorescence changes.

MATERIALS AND METHODS

To obtain thylakoid membranes largely free of excess cations, broken chloroplasts were prepared by the following procedure: Intact chloroplasts were isolated from market spinach as previously described (13) and immediately subjected to an osmotic shock in a relatively large volume of distilled water for 2 min. Where indicated, 0.5 mM Na-EDTA, pH 7.5, was also present ("EDTA-washed chloroplasts"). An equal volume of double-strength "cation-free medium" (0.1 M sorbitol containing 0.1–0.2 mM Tris, pH 7) was then added. Washed broken chloroplasts were then obtained by centrifugation and stored on ice in a minimal volume of cation-free medium. For experimentation, broken chloroplasts were suspended in cation-free medium in a 10 × 10 mm cuvette at a chlorophyll concentration of 8–10 μg chlorophyll ml^{-1}, as determined by Arnon's method (14). Chlorophyll *a* fluorescence was detected at right angles to a single actinic beam (Balzers Calflex C, 2 mm Schott BG18, 2 mm Schott BG38 filter combination; Balzers High Vacuum Corp., Santa Ana, Calif.; Schott Optical Glass Inc., Duryea, Pa.) of intensity 80 W m^{-2} at the cuvette surface. The photomultiplier (EMI 9558B, EMI Gencom Inc., Plainview, N.Y.) was screened by a Balzers B40 685-nm interference filter plus sufficient Schott red cut-off filters to completely eliminate scattered actinic light.

All reaction mixtures contained 1.3×10^{-5} M DCMU to eliminate chlorophyll *a* fluorescence yield changes associated with noncyclic electron transport and associated proton pumping.

RESULTS

Effect of Monovalent Cations on Chlorophyll a Fluoresence from Broken Chloroplasts

Fig. 1 *a* shows the characteristic "dip" in chlorophyll *a* fluorescence observed on adding progressively higher concentrations of K^+ to DCMU-treated, broken chloroplasts washed and resuspended in a medium containing low levels of Tris as the only other added cation (cation-free medium). Under these conditions, the photosynthetic traps of PSII are closed, and the fluorescence changes represent cation-induced changes in other rate constants governing chlorophyll *a* singlet de-excitation. It is clear from Fig. 1 and Table I that the effectiveness of monovalent cations in decreasing fluorescence yield at low concentrations (<10 mM) and in increasing this emission at higher concentrations (10–100 mM) is not restricted to small metal cations such as K^+. Both types of fluorescence change were induced by small monovalent cations such as NH_4^+, and larger, bulkier groups such as choline, Tris, and lysine. There seems to be very little structural requirement in the properties of the cation in order to induce this type of

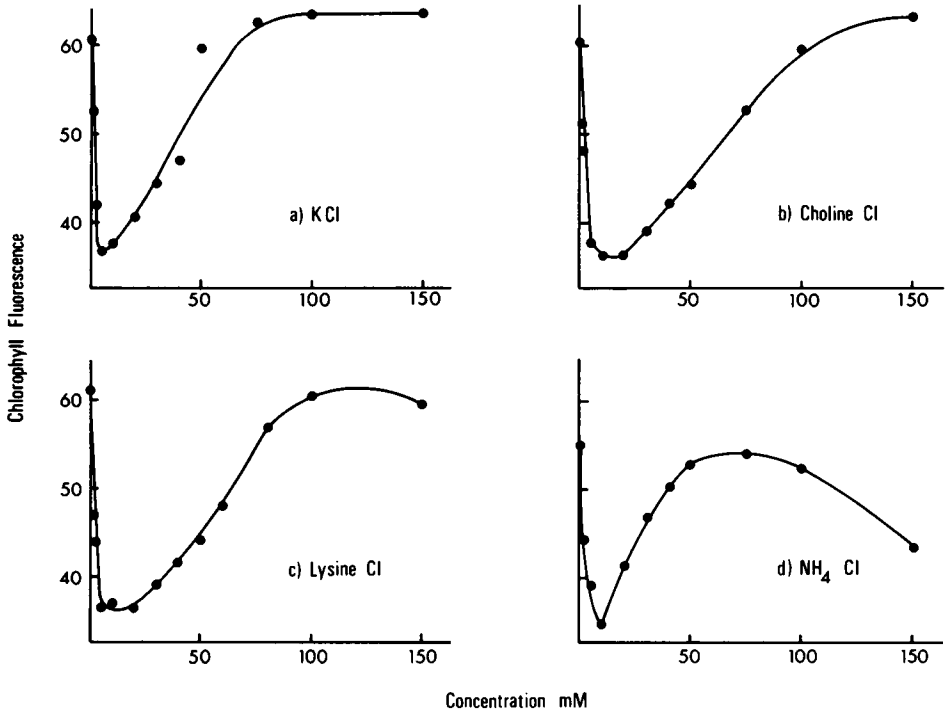

FIGURE 1 Dependence of chlorophyll fluorescence from broken spinach chloroplasts washed and resuspended in cation-free medium on the concentration of monovalent cations added to the medium. Chloroplasts (8 μg chlorophyll ml^{-1}) were preincubated with the appropriate level of cation for 7.5 min before the final steady-state fluorescence was noted. All samples contained 1.3×10^{-5} M DCMU.

TABLE I

RELATIVE EFFECTIVENESS OF DIFFERENT MONOVALENT CATIONS IN BRINGING ABOUT REVERSIBLE CHLOROPHYLL FLUORESCENCE YIELD CHANGES IN BROKEN CHLOROPLASTS SUSPENDED IN A CATION-FREE MEDIUM

Exp.	Salt	$C_{1/2}$ of cation-induced fluorescence change	
		Decrease	Increase
		mM	
1	KCl	1.3 ± 0.3	45 ± 5
	Choline Cl	1.6 ± 0.3	65 ± 5
	NH_4Cl	1.5 ± 0.13	approx. 30
2	Tris Cl	2.5 ± 0.5	approx. 50
3	KNO_3	1.2 ± 0.3	35 ± 5
	Lysine Cl	0.8 ± 0.2	65 ± 5
4	KNO_3	1.3 ± 0.3	41 ± 5
	$TlNO_3$	0.8 ± 0.2	No effect

Conditions are as described in Fig. 1. $C_{1/2}$ refers to the concentration of cation that induced half the maximal effect. Only approximate values for the recorded $C_{1/2}$ of NH_4^+ and Tris-induced fluorescence increases at higher added levels of cation are quoted, because of the obscuring effect of irreversible fluorescence quenching also induced by these cations (see text).

fluorescence yield change. For example, the monovalent zwitterion lysine was observed to be as effective as potassium in bringing about the fluorescence yield responses. On the other hand, the electrically neutral zwitterion HEPES was found to be completely ineffective, and in this respect resembled uncharged molecules such as sucrose (results not shown). For ease of reference, such changes will be termed "reversible" fluorescence yield changes. Monovalent cation-induced reversible fluorescence yield changes are completely prevented by the presence of 5 mM Mg^{2+} in the chloroplast suspending media, and fluorescence is maintained throughout at the high initial yield seen in the absence of added cations.

Fig. 1 shows that certain cations such as Tris and NH_4^+ at high concentrations brought about additional fluorescence quenching of broken chloroplasts apparently superimposed on the reversible changes discussed above. Apart from the concentration dependence, the fluorescence quenching induced by high levels (50–100 mM) of these cations differed from reversible quenching induced by low levels (<10 mM) of added cation in two other respects. The high-cation requiring fluorescence quenching was firstly, not prevented by the presence of 5 mM Mg^{2+} in the chloroplast-suspending medium, and secondly, was markedly dependent on the nature of the monovalent cation used. This type of fluorescence change will be termed "irreversible" fluorescence quenching. Irreversible fluorescence quenching was not observed with K^+ or choline, but was clearly seen with primary amines, such as Tris and NH_4^+. High concentrations of primary amines are known to induce other effects on chloroplasts, such as an inhibition of O_2 evolution (15, 16). It seems possible therefore that specific binding may be involved in their interaction with the thylakoid membrane. The monovalent thallous ion, suggested by Williams as a possible analogue for K^+ (17), appeared to be

particularly effective in irreversibly quenching chlorophyll fluorescence. However, Tl^+ did bring about reversible fluorescence yield changes when low (<20 mM) concentrations of this cation were employed.

Specificity of Divalent Cations on Chlorophyll Fluorescence Yield Changes

It has been shown that divalent cations are much more effective than monovalent cations at inducing the rise from the low to the high fluorescence yield (1, 3, 5, 7, 8). We have previously shown (8) that the low fluorescent conformation state of chloroplasts can also be created by washing the membranes in Na-EDTA before resuspension in an otherwise cation-free medium. We related the formation of the low fluorescence yield to the chelation of membrane localized Mg^{2+} with the EDTA and its replacement by Na^+. Normally this Mg^{2+} is retained when the chloroplasts are washed in cation-free medium not containing EDTA, and under this condition the high fluorescing membrane conformational state exists. Furthermore, when the residually retained Mg^{2+} was removed from the chloroplasts by Na-EDTA, the high fluorescent yield could be regained by addition of very low (100 μM) amounts of Mg^{2+} to the chloroplast suspension medium. Table II shows that in fact, all of the alkaline earth (group IIa) cations are effective at increasing fluorescence of EDTA-washed broken chloroplasts. The recorded $C_{1/2}$'s for this divalent cation-induced fluorescence increase are lower than those previously reported by Murata et al. (3), who had included monovalent cations in the suspending medium. Since it has been shown that monovalent cations competitively inhibit the Mg^{2+}-induced fluorescence rise under these conditions (6, 8),

TABLE II

RELATIVE EFFECTIVENESS OF DIFFERENT DIVALENT CATIONS IN BRINGING ABOUT AN INCREASE IN CHLOROPHYLL FLUORESCENCE FROM NA-EDTA-WASHED BROKEN CHLOROPLASTS SUSPENDED IN CATION-FREE MEDIUM

Exp.	Salt	$C_{1/2}$ for cation-induced fluorescence increase
		μM
1	$MgCl_2$	62 ± 5
	$CaCl_2$	44 ± 5
	$SrCl_2$	42 ± 5
	$BaCl_2$	36 ± 5
2	$MgCl_2$	70 ± 10
	$MnCl_2$	30 ± 10
	$CoCl_2$	approx. 10
	$ZnCl_2$	No effect
3	$MgCl_2$	50 ± 5
	Lysyl-L-lysine	85 ± 10

Conditions as described in Fig. 2. The $C_{1/2}$ given for $CoCl_2$ is very approximate due to obscuring nature of irreversible fluorescence quenching also induced by this cation (see text).

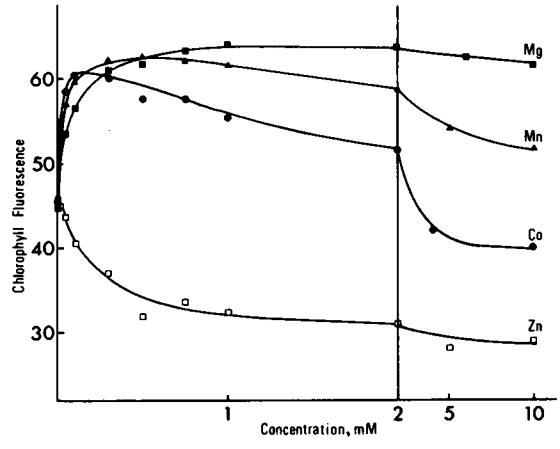

FIGURE 2 Dependence of fluorescence from Na-EDTA-washed broken spinach chloroplasts on the divalent cation content of an otherwise cation-free chloroplast suspending medium. Chloroplast concentration was 10 μg chlorophyll ml^{-1}. All other conditions were as for Fig. 1.

it would appear that the competition between monovalent and divalent cations for the sites controlling fluorescence is a general and nonspecific phenomenon and accounts for the difference in the $C_{1/2}$ values reported in earlier papers.

Fig. 2 shows that the ability to increase fluorescence from EDTA-washed broken chloroplasts suspended in cation-free medium is not restricted to group IIa divalent cations. Both Mn^{2+} and Co^{2+} were effective when added at very low concentrations. It can be seen from Table II that small differences in relative effectiveness could be discerned between divalent cations. These differences were, however, insignificant in comparison to the much higher levels of monovalent cations ($C_{1/2} \sim 40$ mM) required to increase fluorescence yield under these conditions. The divalent cation-induced fluorescence increase will also be termed "reversible" fluorescence yield changes.

In contrast, Fig. 2 also shows that certain divalent cations brought about a decline in fluorescence yield that often required higher concentrations than those which induced the reversible fluorescence increase under these conditions. Like irreversible quenching observed with certain monovalent cations, the divalent cation-induced irreversible fluorescence lowering was not prevented by the presence of 5 mM Mg^{2+} in the chloroplast-suspending medium and varied markedly in extent between different cations. Fluorescence quenching in the presence of certain divalent cations has also been reported by Bazzaz and Govindjee (18, 19).

However, the reversible fluorescence increase brought about by divalent cations showed little specificity on the basis of ionic size and coordination number (cf. Ca^{2+}, Mg^{2+}, ref. 17.). Studies with the dipeptides lysyl-L-lysine serve to illustrate this point. Fig. 3 shows that lysyl-L-lysine was observed to increase fluorescence of EDTA-washed broken chloroplasts with a recorded $C_{1/2}$ only 1.7 times greater than that for Mg^{2+} (Table II). Furthermore, the dipeptide was not observed to bring about fluorescence lowering from the high yield seen with chloroplasts washed and resuspended in

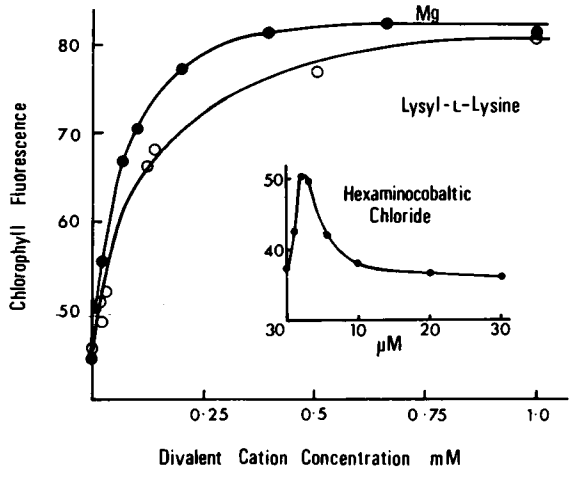

FIGURE 3 Effect of the divalent Mg^{2+} and lysyl-L-lysine and trivalent hexaminocobaltic cations on chlorophyll fluorescence from EDTA-washed broken spinach chloroplasts suspended in an otherwise cation-free medium. Other conditions were as for Fig. 1.

cation-free media from which EDTA had been omitted. Thus, although lysine was shown above to act as a typical monovalent cation, the dipeptide lysyl-L-lysine behaves like a typical divalent cation, even though the distance between the ϵ-amino groups can presumably be quite large. The results clearly suggest that the differential effects of monovalent and divalent cations on fluorescence yield of broken chloroplasts depend mainly on the net positive charge on the cation.

Effect of Polyvalent Cations on Chlorophyll Fluorescence from Broken Chloroplasts

Divalent cations appear to be more effective than monovalent cations in bringing about the high fluorescing conformation of broken chloroplasts. If this increased effectiveness depends only on the net charge on the cation, then it might be expected that polyvalent cations would be more effective than divalent cations. As shown in Fig. 3, the trivalent hexaminocobaltic cation was observed to increase fluorescence from the low yield obtained on suspending EDTA-washed membranes in cation-free media. The maximum stimulation was observed after addition of only 2 μM hexaminocobaltic chloride, although the extent of the fluorescence increase was only approximately one half that seen in saturating concentrations of Mg^{2+}. Further addition of hexaminocobaltic chloride quenched fluorescence to the minimum yield in a manner not prevented by the presence of 5 mM $MgCl_2$ in the medium. It therefore appears that the trivalent hexaminocobaltic cation is capable of increasing the fluorescence yield more effectively on a concentration basis than divalent cations, but that this effect is obscured by irreversible quenching, as shown above for certain monovalent and divalent cations. In fact the trivalent La^{3+} and Ce^{3+} ions were not observed to be at all effective in increasing chlorophyll fluorescence. These latter two trivalent cations only

brought about irreversible fluorescence quenching, which was competitively inhibited, but not prevented, on increasing the level of Mg^{2+} in the medium from 0.1 to 6.7 mM (Fig. 4). Similar results have previously been reported by us (8) using the polyvalent cation poly-L-lysine. The fact that poly-L-lysine only irreversibly quenched chlorophyll fluorescence is in marked contrast to the effects of lysine and lysyl-L-lysine, shown above to act as typical monovalent and divalent cations, respectively.

Effect of Chloroplast Concentration on Cation-Sensitive Fluorescence Yield Changes

The measured $C_{1/2}$ of various cation-induced effects on chlorophyll fluorescence can be equated to apparent binding constants if it is assumed that the interaction between

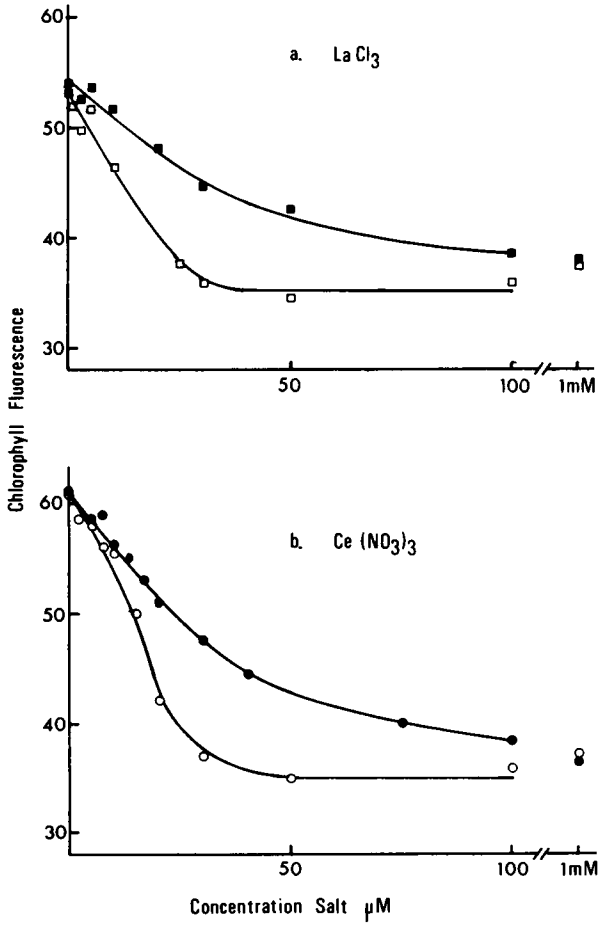

FIGURE 4 Effect of (*a*) La^{3+} and (*b*) Ce^{3+} on chlorophyll fluorescence from broken spinach chloroplasts suspended in cation-free medium containing 100 μM $MgCl_2$ (open symbols) or 6.6 mM $MgCl_2$ (solid symbols). Other conditions were as for Fig. 1.

TABLE III

EFFECT OF CHLOROPLAST CONCENTRATION ON $C_{1/2}$ FOR La^{3+}-INDUCED IRREVERSIBLE CHLOROPHYLL FLUORESCENCE QUENCHING

Chloroplast concentration	$C_{1/2}$ for La^{3+}-induced fluorescence quenching	
	No other additions	+6.6 mM $MgCl_2$
μg chlorophyll ml^{-1}	*μM*	
3.3	15 ± 4	240 ± 40
6.7	32 ± 5	330 ± 50
13.3	92 ± 10	310 ± 50

Conditions are as described for Fig. 1.

cations and the thylakoid membrane can be described by a reversible isotherm. However, such an assumption may not apply to the observations reported here (see Discussion). Also, if a significant proportion of the cations added to the chloroplast suspension become intimately associated with the thylakoid membrane surface, then the $C_{1/2}$ recorded from a concentration curve may be more a measure of the "titratable sites" controlling fluorescence than a description of a reversible binding constant. If the latter situation prevails, a change in the amount of chloroplasts in the suspending medium should significantly alter the recorded $C_{1/2}$. Table III shows that a fourfold increase in chloroplast concentration resulted in an approximately quadrupled increase in the measured $C_{1/2}$ for La^{3+}-induced irreversible fluorescence quenching observed with chloroplasts washed and resuspended in cation-free medium. The results suggest that most of the added La^{3+} becomes closely associated with the thylakoid membranes. From the data of Table III, it can be calculated that the La^{3+} binding capacity of broken chloroplasts under these conditions is approximately 6 ± 1.5 nmol $La^{3+}/\mu g$ chlorophyll. However, this figure varies somewhat with different chloroplast preparations. From the data in Fig. 4 the amount of La^{3+} bound at half saturation corresponds to a full binding capacity of approximately 2 nmol $La^{3+}/\mu g$ chlorophyll. The figure for poly-L-lysine (containing nine lysine residues per molecule), if all of this polycation becomes bound, is approximately 0.1 nmol, or 1.0 neq/μg chlorophyll. This latter figure agrees well with the estimate of Gross and Hess, who found that the monovalent and divalent "binding" capacity of the sites apparently controlling fluorescence was 1.2 neq/μg chlorophyll (10).

In contrast to cation-free conditions, the presence of 6.6 mM Mg^{2+} in the chloroplast-suspending medium caused the relative $C_{1/2}$ for La^{3+}-induced fluorescence quenching to increase, and become insensitive to a change in chloroplast concentration (see Table III). Under these conditions, $C_{1/2}$ may be more related to the binding kinetics of La^{3+}, and not as such a measure of the La^{3+} bound.

Table IV shows that a fourfold change in chloroplast concentration did not significantly affect the concentration at which K^+ brings about reversible fluorescence yield changes in broken chloroplasts. Such a result would be expected from the relatively high $C_{1/2}$ s for monovalent cation-induced fluorescence changes.

TABLE IV

EFFECT OF CHLOROPLAST CONCENTRATION ON $C_{1/2}$ FOR K^+-INDUCED REVERSIBLE FLUORESCENCE YIELD CHANGES

Chloroplast concentration	$C_{1/2}$ for K^+-induced fluorescence changes	
	Decrease	Increase
μg *chlorophyll* ml^{-1}	*mM*	
2.8	1.5 ± 0.2	38 ± 5
5.7	1.7 ± 0.2	36 ± 5
11.3	2.0 ± 0.2	40 ± 5

Conditions are as described for Fig. 1.

In the presence of 10 mM K^+, the $C_{1/2}$ for the divalent cation-induced fluorescence increase of broken chloroplasts was also relatively high and insensitive (within experimental error) to the concentration of chloroplasts in the medium (Table V). However, in the absence of added cations, the $C_{1/2}$ for Mg^{2+}-induced reversible fluorescence yield changes with Na-EDTA washed chloroplasts is relatively low. Under these conditions, a fourfold increase in chlorophyll concentration did increase the recorded $C_{1/2}$ for Mg^{2+}. However, the effect of chloroplast concentration was not as marked as, for example, that observed for La^{3+}-induced irreversible fluorescence quenching in the absence of other added cations. This result may indicate that under otherwise cation-free conditions, some, but not all, of the Mg^{2+} added to the chloroplast suspension becomes closely associated with the membranes.

DISCUSSION

The experiments reported in this paper were designed to clarify the mechanism by which cations bring about changes in chlorophyll *a* fluorescence yield of broken chloroplasts when other factors affecting this emission (changes in the redox state of

TABLE V

EFFECT OF CHLOROPLAST CONCENTRATION ON $C_{1/2}$ FOR THE Mg^{2+}-INDUCED INCREASE IN FLUORESCENCE

Exp.	Chlorophyll concentration	$C_{1/2}$ for Mg^{2+}-induced fluorescence increase	
		No other additions	+10 mM KCl
	$\mu g/ml$	μM	
1	2.2	70 ± 10	480 ± 50
	4.3	180 ± 20	480 ± 50
	8.7	210 ± 20	440 ± 50
2	2.3	40 ± 10	450 ± 50
	4.6	55 ± 10	420 ± 50
	9.3	70 ± 10	490 ± 50

Other conditions are as given for Fig. 3.

the PSII traps, high-energy state quenching) have been experimentally eliminated. Under these controlled conditions, we have distinguished two types of cation-induced fluorescence changes, which we have termed reversible and irreversible. We will initially restrict our discussions to the first of these types of fluorescence change. As shown clearly here and also by others (1–8), monovalent and divalent cations have markedly different effects on chlorophyll a fluorescence. It seems more likely that these effects reflect predominantly electrostatic interactions of cations with the thylakoid rather than specific ion binding mechanisms. Firstly, the effectiveness of a cation was clearly dependent on its net charge (cf. lysine, lysyl-L-lysine, and poly-L-lysine [8]). Secondly, there is remarkably little specificity between cations of the same valency, despite often large differences in ionic radius, lipid solubility, or coordination number. Not only does this indicate that the fluorescence-controlling "sites" on the thylakoid must be relatively exposed, but also that these "sites" exhibit low cation binding selectivity, as would be expected for single weak acid groups (17). However, the existence of such fixed negative groups on the thylakoid surface would result in the formation of an electrical diffuse double layer at the membrane-solution interface, (see 20, 21). The purpose of this discussion is to point out ways in which cation-induced chlorophyll fluorescence changes may merely be reflecting cation interactions via a diffuse double layer at the thylakoid surface. In a previous report, we initially applied the Gouy-Chapman theory of the diffuse double layer in an attempt to understand antagonistic effects of low and high concentrations of monovalent cations on chlorophyll fluorescence. According to this theory (12, 20–22), an electrical potential ψ_0 (relative to bulk solution) exists at the outer surface of the thylakoid. The magnitude of ψ_0 depends on the density of fixed negative charges on the surface (q) and on the concentration of electrolyte in the bathing medium ($C_{i\alpha}$), according to the following expression:

$$q = \pm \left| \frac{RT\epsilon}{2\pi} \sum_i C_{i\alpha}(\exp(-ZF\psi_0/RT) - 1) \right|^{1/2} \quad (1)$$

R, T, Z, and F have their usual meaning and ϵ is the permittivity of water ($= 78.5\ \epsilon_0$ at 25°C, where ϵ_0 is the permittivity of a vacuum). We have shown (8) that thylakoids washed in media containing only low levels of Tris (approximately 10^{-4}M) as added electrolyte apparently retain residual amounts of divalent cation (presumably derived from the stroma). Eq. 1 was therefore written for ψ_0 in the case of a mixture of monovalent (C'_α) and divalent (C''_α) electrolyte in bulk solution:

$$4C''_\alpha \cosh^2(F\psi_0/RT) + 2C'_\alpha \cosh(F\psi_0/RT) - (4C''_\alpha + 2C'_\alpha + q^2/A^2) = 0, \quad (2)$$

where $A = (RT\epsilon/2\pi)^{1/2}$.

To calculate ψ_0 from Eq. 2, it is necessary to make some assumption of the value of q. Since no reliable estimate of q has been experimentally determined, any calculations must be regarded as approximate only. However, by estimating q to be 2.5 μC cm^{-2} (11), it is easy to show that divalent cations are far more effective than monovalent cations in decreasing ψ_0 (screening surface charges) than would be expected on the basis of ionic strength (11, 22). However, this approach does not account for the

quenching of fluorescence by low levels of monovalent cations. It is of course possible that the monovalent cation-induced quenching of fluorescence occurs by a different mechanism unrelated to the subsequent increase in yield obtained on adding higher levels of monovalent or low levels of divalent cations to the medium. However, there is no reason at present to assume that two different mechanisms are involved. On the contrary, we have shown that monovalent cation-induced quenching probably reflects displacement of residual divalent cation from the surface of the thylakoid, an effect also readily predicted by classical double-layer theory (11) and experimentally observed (Nakatani and Barber, unpublished).

An alternative explanation is that the level of total positive diffusible charge in solution at the membrane surface influences the yield of chlorophyll fluorescence (11, 12, 22). The total positive diffusible charge in solution at the membrane surface is calculated as the sum of the concentrations of monovalent (C_0') and divalent (C_0'') cations (in equivalents per liter) at the membrane surface. Since an electrical potential exists at the surface, C_0' and C_0'' are considerably higher than the bulk concentrations, and can be calculated by assuming a Boltzmann distribution, thus:

$$C_{i0} = C_{i\alpha} \exp(- ZF\psi_0/RT). \quad (3)$$

Fig. 5 shows how total positive diffusible charge at the membrane surface varies as a function of bulk concentrations of monovalent and divalent cations. Several important features are evident: (*a*) When divalent cations are held constant at 10^{-6}M, increasing bulk concentration of monovalent cations from 10^{-4}M to 10^{-1}M gives rise to a biphasic curve with a characteristic dip (Fig. 5*a*) (*b*) The dip effect occurs only in the presence of low bulk levels of divalent cations, and is progressively abolished as C_α'' is increased from 10^{-6} to 10^{-3}M. (*c*) When bulk levels of monovalent cations are held constant and the bulk divalent cation concentration varied (Fig. 5*b*), only an increase in positive diffusible charge at the surface is observed. There is no dip effect when divalent cations are increased. (*d*) Increasing the constant level of monovalent cations in the medium competitively inhibits the ability of divalent cations at low bulk concentrations to increase the positive diffusible charge at the membrane surface (Fig. 5*b*).

In fact, from experimental data (see also 11, 12) it seems that reversible fluorescence yield changes correlate well with the expected relative level of positive diffusible charge at the membrane surface. However, it must be stressed that since the value of q is not known, the above calculations are of necessity only semi-quantitative. The actual value of q was selected to give the best fit to experimental data, although recent particle electrophoresis measurements give additional support to the value chosen (Nakatani and Barber, unpublished). Also it is necessary to estimate some value of C_α'' equivalent to the amount of divalent cation residually retained by membranes washed in cation-free media. We assumed the lowest value of C_α'' to be 10^{-6}M so that the calculated total positive diffusible charge at the membrane surface is almost at a maximum for bulk concentrations of monovalent cation equal to 10^{-4}M. This situation is equivalent to washing and resuspending spinach chloroplasts in cation-free medium

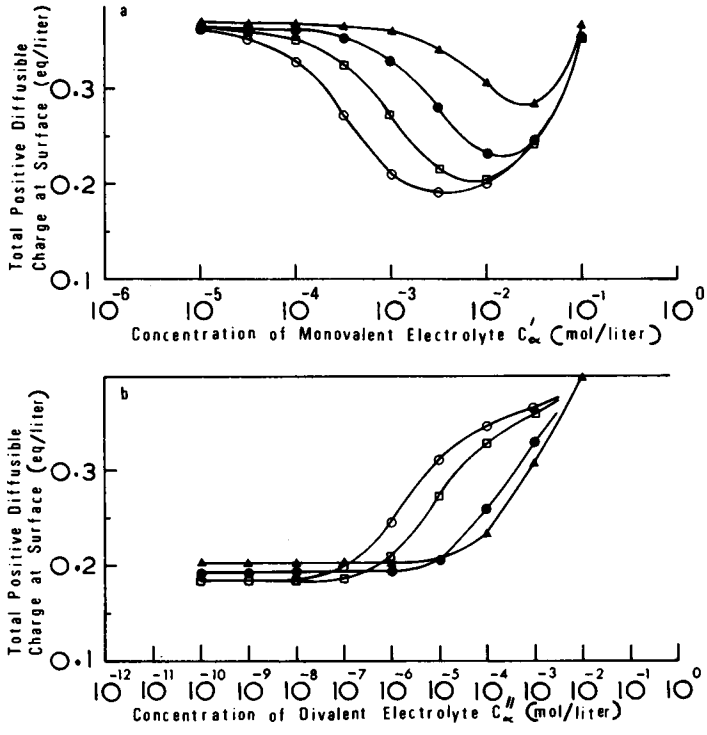

FIGURE 5 Total positive diffusible charge in solution at the membrane surface, as predicted by Eqs. 2 and 3. (*a*) Effect of increasing the constant bulk level of divalent cations (open circles, 10^{-6}M: open squares, 10^{-5}M: closed circles, 10^{-4}M: closed triangles, 10^{-3}M) when the bulk concentrations of monovalent cations vary from 10^{-5} to 10^{-1}M. (*b*) Effect of increasing the constant bulk level of monovalent cation as the concentrations of divalent cations vary from 10^{-10} to 10^{-2}M (open circles, 5×10^{-4}M: open squares, 10^{-3}M: closed circles, 5×10^{-3}M and closed triangles, 10^{-2}M). Calculations were for a negatively charged surface of 2.5 μC cm^{-2}.

and corresponds to a high initial fluorescence yield. It is of interest that Vandermeulen and Govindjee (6) found the initial fluorescence yield of chloroplasts isolated from species other than spinach to be somewhat less than the maximum under similar conditions. This observation can be readily explained by proposing that the residual divalent cation retained by these latter chloroplast preparations was somewhat less than that retained by spinach chloroplasts. Such a situation would arise if, for example, the initial levels of divalent relative to monovalent cations in the stroma are somewhat variable.

The ability of classical Gouy-Chapman theory to qualitatively predict the major effects of monovalent and divalent cations on chlorophyll *a* fluorescence is somewhat surprising, considering the assumptions inherent in the theory. These assumptions have recently been discussed extensively elsewhere (12) and are probably reasonably valid when applied to biological membranes mainly because q is relatively small. It is worth emphasizing here that the theory presumes that only electrostatic forces govern

diffusible ion interactions with the thylakoid (i.e. no specific binding, ion-pairs, or adsorption). It is not difficult to imagine ways in which the concentration of diffusible charge at the membrane surface can influence chlorophyll fluorescence via purely electrostatic forces. For example changes in the repulsive forces between fixed negative charges on the thylakoids would give rise to membrane conformational changes. In fact the "concentration of positive diffusible charge" at the membrane surface is (to a first approximation) equivalent to the space charge density, since the levels of diffusible anions in this region are low (21).

$$d^2\psi_0/dx^2 = (-4\pi/\epsilon)\sum_i Z_i FC_{i0} \approx \text{Total positive diffusible charge at the membrane surface.} \quad (4)$$

Since most of the chlorophyll exists in the form of negatively charged protein complexes (23), it is immediately apparent that the environment of chlorophyll in the membrane can be influenced by electrostatic forces at the membrane surface via reorientation of surface dipoles.

An alternative mechanism that cannot be ruled out is that membrane conformational changes do in fact result from cation binding. However, if this is the case, then cation binding must be nonspecific, and directly proportional to the local concentration of positive diffusible charge at the membrane surface. At this stage, we prefer to think that mainly electrostatic forces are responsible for membrane structural changes, giving rise to reversible-type fluorescence changes.

If this is indeed so, then substantial cation-binding would be an obvious explanation for irreversible cation-induced fluorescence quenching. Not only would specific binding to surface charge groups give rise to the specificity observed between cations of the same valency (see Fig. 2), but the resulting neutralization of surface charge would also account for the observed inhibition of reversible monovalent-divalent cation effects. There are indications that irreversible-type fluorescence changes are also influenced, at least partially, by double-layer effects, since the effectiveness of the cation also depended on its net charge. Those cations which brought about irreversible fluorescence quenching could, in a generalized way, be ranked into the following sequence of decreasing ionic charge and decreasing effectiveness: poly-L-lysine > La^{3+}, Ce^{3+} > Zn^{2+}, Cd^2, Co^{2+} > NH_4^+, Tris. The recognition that electrostatic diffuse double layers exist at the surface of planar lipid bilayers and biological charged membranes is not new (for an excellent review, see ref. 24). We now suggest that the role of the diffuse double layer in controlling ionic interactions with the thylakoid membrane may be more important in photosynthesis than has hitherto been recognized. In this respect it is pertinent that monovalent/divalent cation effects similar to those observed on fluorescence have also been noted in studies on thylakoid stacking (25), light-driven cation fluxes (26), intrathylakoid volume (27), and electron transport reactions (28; see ref. 12 for an extended discussion of some of these studies).

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REFERENCES

1. HOMANN, P. H. 1969. Cation effects on the fluorescence of isolated chloroplasts. *Plant Physiol.* **44:** 932–936.
2. MURATA, N. 1969. Control of excitation energy transfer in photosynthesis. II. Magnesium ion dependent distribution of excitation energy between two pigment systems in spinach chloroplasts. *Biochim. Biophys. Acta.* **189:**171–181.
3. MURATA, N., H. TASHIRO, and A. TAKAMIYA. 1970. Effects of divalent cations on chlorophyll *a* fluorescence in isolated spinach chloroplasts. *Biochim. Biophys. Acta.* **197:**250–256.
4. MURATA, N. 1971. Effects of monovalent cations on light energy distribution between two pigment systems of photosynthesis in isolated chloroplasts. *Biochim. Biophys. Acta.* **226:**422–432.
5. GROSS, E. L., and S. C. HESS. 1973. Monovalent cation induced inhibition of chlorophyll *a* fluorescence: antagonism by divalent cations. *Arch. Biochem. Biophys.* **159:**832–836.
6. VANDERMEULEN, D. L., and GOVINDJEE. 1974. Relation of membrane structural changes to energy spillover in oat and spinach chloroplasts: use of fluorescence probes and light scattering. *Biochim. Biophys. Acta.* **368:**61–70.
7. WYDRZYNSKI, T., E. L. GROSS, and GOVINDJEE. 1975. Effects of sodium and magnesium cations on the "dark" and light-induced chlorophyll *a* fluorescence yields in sucrose washed spinach chloroplasts. *Biochim. Biophys. Acta.* **376:**151–161.
8. MILLS, J. D., A. TELFER, and J. BARBER. 1976. Cation control of chlorophyll *a* fluorescence yield in chloroplasts. Location of cation sensitive sites. *Biochim. Biophys. Acta.* **440:**495–505.
9. BARBER, J. 1976. Ionic regulation in intact chloroplasts and its effect on primary photosynthetic processes. *In* The Intact Chloroplast. J. Barber, editor. Elsevier Scientific Publishing Company, Amsterdam. 89–134.
10. GROSS, E. L., and S. C. HESS. 1974. Correlation between calcium ion binding to chloroplast membranes and divalent cation-induced structural changes and changes in chlorophyll *a* fluorescence. *Biochim. Biophys. Acta.* **339:**334–346.
11. BARBER, J., and J. MILLS. 1976. Control of chlorophyll fluorescence by the diffuse double layer. FEBS (*Fed. Eur. Biochem. Soc.*) Lett. **68:**288–292.
12. BARBER, J., J. MILLS, and A. LOVE. 1977. Electrical diffuse layers and their influence on photosynthetic processes. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **74:**174–181.
13. MILLS, J., and J. BARBER. 1975. Energy-dependent cation-induced control of chlorophyll *a* fluorescence in isolated intact chloroplasts. *Arch. Biochem. Biophys.* **170:**306–314.
14. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Poly-phenol oxidase in *Beta vulgaris*. *Plant Physiol.* **24:**1–15.
15. YAMASHITA, T., and W. L. BUTLER. 1969. Inhibition of the Hill reaction by Tris and restoration by electron donation to PSII. *Plant Physiol.* **44:**435–438.
16. VELTHUYS, B. R. 1975. Binding of the inhibitor NH_3 to the oxygen evolving apparatus of spinach chloroplasts. *Biochim. Biophys. Acta.* **396:**392–401.
17. WILLIAMS, R. J. P. 1970. The biochemistry of sodium, potassium, magnesium and calcium. *Q. Rev. Chem. Soc. Lond.* **24:**331–365.
18. BAZZAZ, M. B., and GOVINDJEE. 1974. Effects of cadmium nitrate on spectral characteristics and light reactions of chloroplasts. *Environ. Lett.* **6:**1–12.
19. BAZZAZ, M. B., and GOVINDJEE. 1974. Effects of lead chloride on chloroplast reactions. *Environ. Lett.* **6:**175–191.
20. GRAHAME, D. C. 1947. The electrical double layer and the theory of electrocapillarity. *Chem. Rev.* **41:** 441–501.
21. DELAHAY, P. 1965. Double layer and electrode kinetics. John Wiley and Sons, Inc., New York.
22. MILLS, J. D. 1976. The role of metal cations in control of *in vivo* chlorophyll *a* fluorescence. Ph.D. thesis, University of London.

23. THORNBER, J. P. 1975. Chlorophyll-proteins: light-harvesting and reaction centre components of plants. *Annu. Rev. Plant Physiol.* **26**:127–158.
24. MCLAUGLIN, S. G. A. 1977. Electrostatic potentials at membrane solution interfaces. *Curr. Top. Membranes Transp.* **9**:71–144.
25. GROSS, E. L., and S. H. PRASHER. 1974. Correlation between monovalent cation induced decreases in chlorophyll *a* fluorescence and chloroplast structural changes. *Arch. Biochem. Biophys.* **164**:460–468.
26. HIND, G., H. Y. NAKATANI, and S. IZAWA. 1974. Light dependent redistribution of ions in suspensions of chloroplast thylakoid membranes. *Proc. Natl. Acad. Sci., U.S.A.* **71**:1484–1488.
27. GROSS, E. L., and L. PACKER. 1967. Ion transport and conformational changes in spinach chloroplast grana. I. Osmotic properties and divalent cation induced volume changes. *Arch. Biochem. Biophys.* **121**:779–789.
28. WALZ, D., S. SCHULDINER, and M. AVRON. 1971. Photoreactions of chloroplasts in a glycine medium. *Eur. J. Biochem.* **22**:439–444.