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MONOVALENT AND DIVALENT CATION-INDUCED CHANGES IN CHLOROPHYLL *a* FLUORESCENCE AND CHLOROPLAST STRUCTURE

E. L. Gross,† T. Wydrzynski,* D. VanderMeulen* and Govindjee*

†Department of Biochemistry, The Ohio State University, Columbus, and

*Departments of Botany, Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801 (U.S.A.)

Abstract

Monovalent cations were found to decrease both the constant and variable chlorophyll *a* fluorescence. The change in the constant fluorescence may imply a direct effect of the cations on the bulk chlorophyll *a* molecules. The monovalent cations were also found to cause changes in chloroplast structure as measured by light scattering, fluorescent probes and electron microscopic techniques. However, a correlation between larger structural changes and changes in chlorophyll *a* fluorescence was observed only in spinach but not in other plant species (oats, peas and lettuce). It is suggested that the cations may regulate energy distribution between the two photosystems, as reflected by the chlorophyll *a* fluorescence, through small conformational changes in certain key chlorophyll-protein complexes, perhaps in conjunction with larger chloroplast structural changes.

Introduction

Murata[1-3], Murakami and Packer[4], Mohanty, *et al.*[5], and others[6-8] have suggested that divalent cations such as Mg^{2+} and Ca^{2+} control the transfer of excitation energy between the two photosystems in isolated chloroplasts. Murata[3] and Murakami and Packer[4] found that the divalent cation-induced changes in energy transfer could be correlated with divalent cation-induced changes in chloroplast structure.

Gross and Hess[9] and Gross and Prasher[10] found that, in spinach, low concentrations (2-10 mM) of salts of monovalent cations such as Na^{+} caused a decrease

in the steady state chlorophyll a fluorescence (measured at 680 nm) at room temperature and an increase in the ratio of fluorescence emitted at 735 nm to that emitted at 685 nm at 77° K. The effects could be prevented or reversed by low concentrations of salts of divalent cations such as Mg^{2+} or Ca^{2+}. The divalent cation effects on fluorescence were shown to be the result of divalent cation binding to the chloroplast membranes[11]. Assuming that spillover proceeds from Photosystem II (PS II) to Photosystem I (PS I)[12], these results indicated that monovalent cations at low concentrations promote spillover and divalent cations or monovalent cations at much higher concentration can reverse this process. If, on the other hand, spillover proceeds from PS I to PS II[13] via the short wavelength form of chlorophyll a, then the opposite argument can be used. Alternately, the salt effects can be described as influencing the energy distribution in the photosynthetic membrane in favor of either PS I or PS II (cf. ref. 14). In this paper, we summarize observations that include species differences in cation effects on chlorophyll a fluorescence, effects of cations on fluorescence transients and the possible role of structural changes in the regulation of spillover (see VanderMeulen and Govindjee[15] and Wydrzynski et al.[16]).

Results

Comparison of the effects of cations on chlorophyll a fluorescence in oats and spinach

Gross and Hess[9] and Gross and Prasher[10] showed that, in spinach, divalent cations had no effect on chlorophyll a fluorescence when added alone in the absence of monovalent cations, indicating that spillover was maximally inhibited under these conditions so that addition of the divalent cations could have no further effect. Also, addition of the monovalent cations after the divalent cations had no effect.

VanderMeulen and Govindjee[15] examined the effects of salts on chlorophyll a fluorescence changes in other species including lettuce, peas and oats and found that although mono- and divalent cations excite opposite effects, these effects are somewhat different than observed for spinach. Namely, divalent cations added

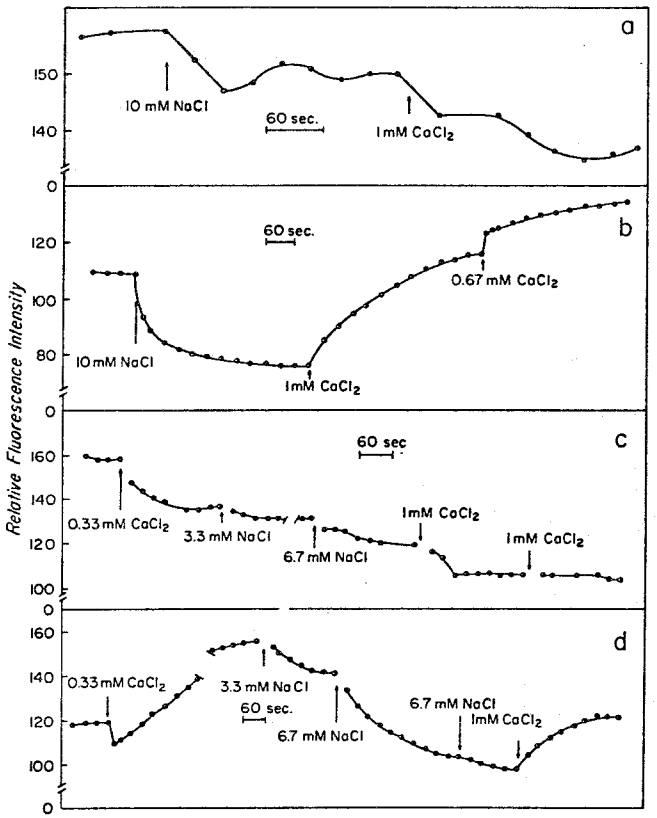

Fig. 1. Time course of cation-induced changes in fluorescence of N-phenyl-1-napthylamine (NPN) (a,c) and chlorophyll a (b,d) in washed oat chloroplasts. Chloroplasts were incubated in 100 mM sucrose, sufficient Tris base (0.17-0.2 mM) to titrate to pH 8, and 7.5 μM DCMU. The probe concentration was 5 μM (after VanderMeulen and Govindjee[15]).

alone cause a transient decrease in chlorophyll a fluorescence which is followed by an increase (Fig. 1d) and monovalent cations can reverse the divalent cation effects. These differences can be explained if spillover is not completely inhibited under low salt conditions in oats and peas as it is in spinach. (For a more complete discussion of these results see VanderMeulen and Govindjee[15].)

The effect of cations on chlorophyll a fluorescence transients

Chlorophyll a fluorescence can be divided in two parts, one of which is constant

and independent of the state of the traps (O level[17]) and another, the so-called variable fluorescence (P minus O level), which depends on the state of the traps and is high when the traps are closed (Q reduced) and low when they are open (Q oxidized). Previous workers[3,5,6] found that when Mg^{2+} was added to chloroplasts incubated in 50 mM Tricine buffer or 1 mM Tris-Cl, there was a large increase in the variable fluorescence but very little effect on the constant fluorescence.

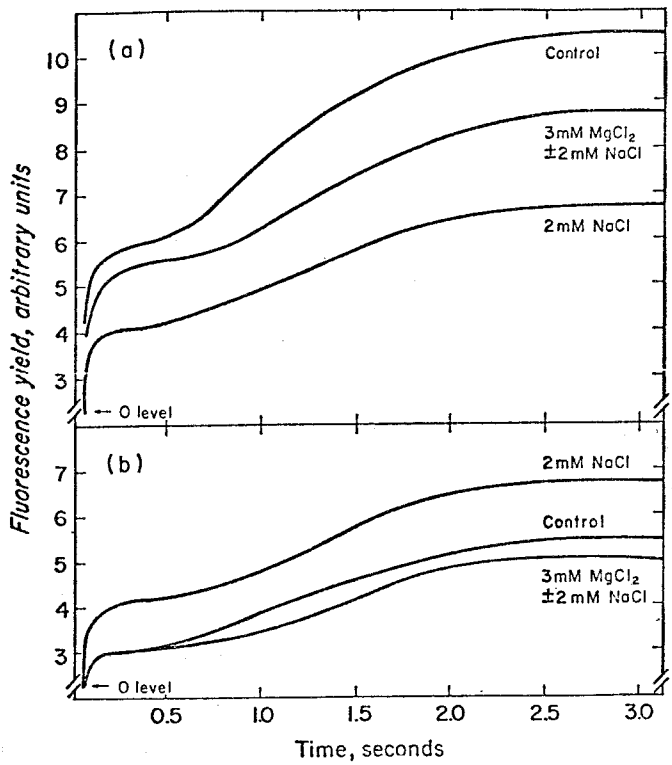

Fig. 2(a). Time course of chlorophyll a fluorescence in sucrose-washed spinach chloroplasts in the presence and absence of sodium and magnesium. Fluorescence was measured at 685 nm (half-band width, 6.6 nm), excitation, broad band blue light (CS 4-96 and CS 3-73); intensity, 1.2 x 10^4 ergs cm^{-2} sec^{-1}. The 4 ml reaction mixture consisted of 5 µg chlorophyll ml^{-1} suspension, 0.2 mM Tris buffer, pH 7.8-8.2, and 100 mM sucrose. Appropriate amounts of 100 mM stock salt solutions were added to give final concentrations of 2 mM NaCl and 3 mM $MgCl_2$; samples were kept dark adapted until the time of measurement. 2(b). Fluorescence transients from Fig. 1 normalized at the O level (after Wydrzynski et al.[16]).

Gross and Hess[9] studied the effects of monovalent cations only on the final steady-state level of fluorescence observed in the presence of DCMU. It was of interest, therefore, to examine the effects of monovalent cations on the fluorescence transients to determine whether they also only effect the variable fluorescence. It was found (Fig. 2) (see Wydrzynski et al.[16]) that both the constant and variable fluorescence was affected. This suggests that the bulk chlorophyll molecules[17] as well as the traps may be involved in the monovalent cation regulation of spillover. Moreover, it shows beyond any doubt that light and light-induced structural changes[18] are not required for the monovalent cation effects. $MgCl_2$ added alone caused a decrease in the P level and a very slight decrease in the O level. When $MgCl_2$ was added in the presence of 2 mM NaCl, the fluorescence transient was exactly the same as when $MgCl_2$ was added alone. When compared with the NaCl trace, this meant an increase in both O and P levels. The change in the O level apparently disagrees with the results of other workers[3,5] but the difference may be due to the actual salt conditions employed. Also, it appears that Mg^{2+} ions do not really "reverse" the NaCl effect but rather "override" it by superimposing their own effect.

An NaCl concentration curve for both the O and P levels[16] (Fig. 3) shows that in both cases the effect is saturated at 2 mM with half-maximal effects at 0.5 mM. In each case, addition of 3 mM $MgCl_2$ "reversed" the NaCl effect to the extent of producing its own effect on the fluorescence level.

The effect of cations on chlorophyll a fluorescence spectra

Emission spectra of chloroplasts at 77° K show three emission peaks at 685, 695 and 735 nm[19]. The 685 and 695 nm peaks are thought to be emitted from the light-harvesting antenna and molecules close to the trap of Photosystem II respectively whereas the 735 nm peak is thought to be emitted by Photosystem I[19-21]. The results[16] (Table I) are presented as the ratio of the fluorescence emitted at either 735 or 695 to that emitted at 685 nm with the values for the control chloroplasts (no salts added) normalized to 1.0. Addition of NaCl caused an increase in the F735/F685 ratio both with and without preillumination

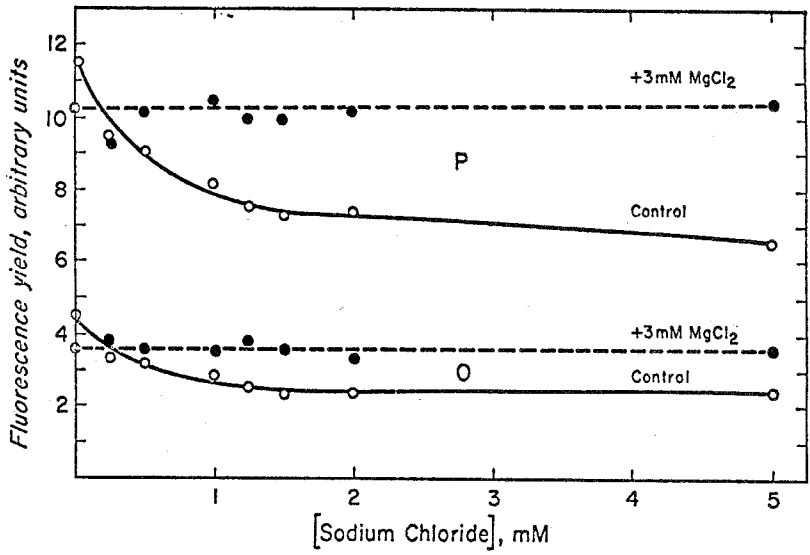

Fig. 3. Concentration curves showing the changes in the O and P level fluorescence as a function of NaCl concentration. O level was measured after 30 msec of illumination. Experimental conditions as in Fig. 2 (after Wydrzynski et al.[16]).

confirming the results of the fluorescence transients that the light harvesting chlorophylls may be involved in causing changes in energy distribution between PS I and PS II. No effect was observed on the F695/F685 ratio indicating that the Photosystem II traps may not be affected.

The Mg^{2+} ions effects are more complex. First, there is an increase in the F695/F685 ratio both in the dark and after preillumination suggesting that Mg^{2+} may affect the transfer of excitation energy from the light harvesting chlorophyll to molecules that are indicators of the Photosystem II traps or increase the number of traps[22,23].

Secondly, the effect on the F735/F685 ratio depends on whether the chloroplasts were preilluminated before freezing or not. In the dark adapted samples the F735/F685 ratio decreased whereas it increased in the preilluminated samples. These results can be explained on the basis that Mg^{2+} ions may have, at least, two effects. One effect is to decrease energy transfer to PS I. This effect could predominate in the dark adapted samples causing the observed decrease in

Table I. Emission Peak Ratios Normalized to a Control Value of 1.00 for Sucrose-Washed Spinach Chloroplasts at 77° K in the Presence and Absence of Sodium and Magnesium (after Wydrzynski et al.[16]).

	F735/F685		F695/F685	
	Dark*	Light**	Dark	Light
Control	1.00	1.00	1.00	1.00
2 mM NaCl	1.22	1.25	1.04	1.02
3 mM $MgCl_2$	0.81	1.17	1.15	1.11
2 mM NaCl + 3 mM $MgCl_2$	0.90	1.14	1.11	1.05

* Samples were kept dark-adapted at all times. Chloroplasts were preincubated with the salts for 3-5 minutes at room temperature before freezing. Exciting light, 435 nm, 6.6 nm half-band width, plus CS 4-96 filter; intensity, 30 ergs cm^{-2} sec^{-1}. Measuring wavelengths, variable, 6.6 nm half-band width; CS 2-61 filter before the analyzing monochromator. Chlorophyll concentration, 10 $\mu g/ml^{-1}$ suspension. Each value represents an average of 6-11 different samples.
**Same condition as above except chloroplasts were preilluminated for 1 minute in strong white light (200 watt incandescent bulb) prior to freezing in the light.

the F735/F685 ratio. The second effect, perhaps, reflected by the change in the F695/F685 ratios, may involve an increase in the energy transfer from bulk Chl a to energy traps of PS II. This effect could predominate in the preilluminated samples causing the observed increase in the F735/F685 ratio.

The relation between structural changes and changes in chlorophyll a fluorescence

Murata[3] and Murakami and Packer[4] showed that structural changes involving stacking of the thylakoid membranes could be correlated with the chlorophyll a fluorescence changes. This appears reasonable since energy transfer between pigment molecules is determined by the distance between them and their mutual orientation[24]. A change in chloroplast structure which could alter the spatial distribution of pigment molecules could change the degree and direction of energy transfer. Alternately, a very minor change in the orientation of a few pigment molecules, such as has been proposed by Seely[25,26], could produce changes in

spillover. Such small changes would not necessarily be reflected in large amplitude membrane alterations. To determine whether structural changes are the cause of the monovalent cation-induced decreases in chlorophyll a fluorescence, three methods for monitoring structural changes were used. These were light-scattering, fluorescent probes and electron microscopy.

The kinetics of 90° light-scattering changes and the corresponding changes in chlorophyll a fluorescence are shown for spinach and oat chloroplasts in Figure 4[15]. In both cases, the addition of NaCl caused a decrease in 90° light-scattering as well as in chlorophyll a fluorescence. The kinetics of the decrease in 90° light-scattering appear to be faster than for chlorophyll a fluorescence. Also, the correlation between the extent of 90° scattering and Chl a fluorescence is

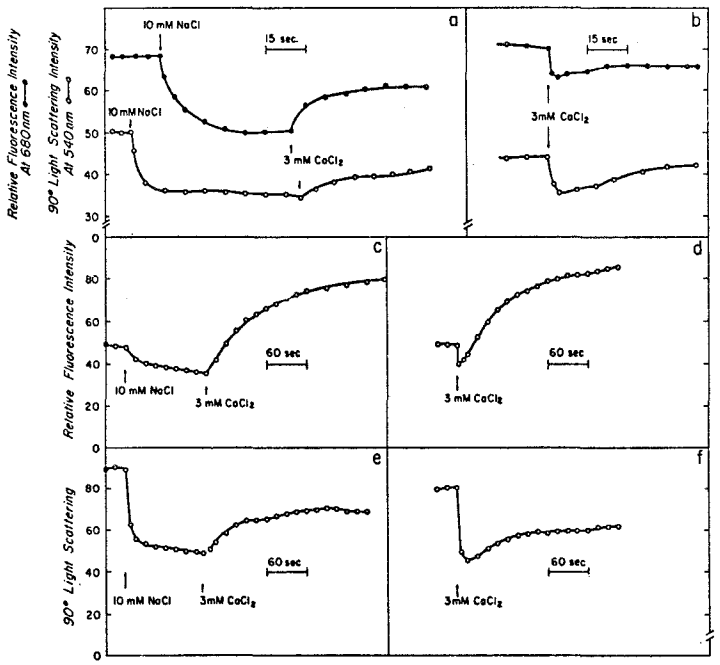

Fig. 4. Time course of cation-induced changes in chlorophyll a fluorescence and 90° light-scattering in washed spinach chloroplasts (a,b) and washed oat chloroplasts (c,d,e,f). Other conditions were as for Fig. 1 (after Vandermeulen and Govindjee[15]).

apparent for spinach (Fig. 4a & 4b) but in oats there are obvious differences (Fig. 4c-4f). Lettuce and pea chloroplasts behaved as oats. In spinach the kinetics of turbidity changes (ΔA_{540}) and chlorophyll a fluorescence changes (Fig. 5) are the same to within the limit of error of making either measurement.

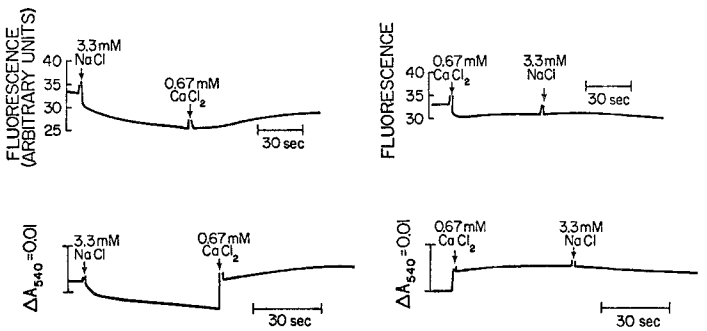

Fig. 5. Time course of cation-induced changes in chlorophyll a fluorescence and turbidity (ΔA_{540}) in spinach chloroplasts. Conditions were as for Fig. 1 (after E. L. Gross and Prasher[10]).

Moreover, in spinach, the NaCl concentration curve is the same for the decreases in fluorescence and turbidity (Fig. 6). These results with spinach may indicate that there is a better correlation between NaCl-induced chlorophyll a fluorescence changes and turbidity measurements whan with 90° light-scattering measurements. However, no data are available for oats at the time of the writing of this report.

Turbidity and 90° light-scattering measure different aspects of chloroplast structure. Turbidity is thought to measure large changes in size and shape[4] whereas 90° light-scattering measures smaller changes in the membranes themselves. It could be that the monovalent cations begin by promoting small, localized

changes in the membranes observable by 90° light-scattering. However, these may not be enough to cause the fluorescence changes. As enough of the small amplitude changes take place they might cause the necessary changes in the chlorophyll bed that lead to fluorescence changes as well as to the overall changes in the shape of the chloroplasts as monitored by turbidity changes. Either the large or small amplitude changes may, in turn, regulate spillover. Alternately, the difference in kinetics of 90° scattering may result from the ambiguity of making light-scattering measurements on heterogeneous samples of large pigmented particles[27,28]. It is also likely that neither turbidity nor 90° scattering change measure the microevents that lead to changes in chlorophyll fluorescence. New approaches may be needed to solve this problem.

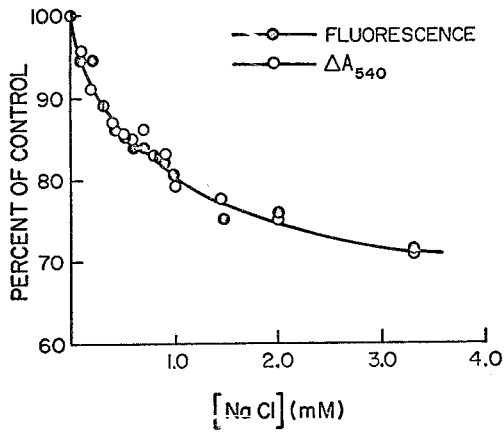

Fig. 6. The NaCl concentration dependence of changes in turbidity and chlorophyll a fluorescence for spinach chloroplasts. Various concentrations of NaCl were added to chloroplast suspensions and the effects on turbidity (ΔA_{540}) and chlorophyll a fluorescence were determined. The final fluorescence or turbidity levels obtained after salt addition were calculated as percent of the initial control level prior to salt addition (after E. L. Gross and Prasher[10]).

For both oat and spinach chloroplasts addition of divalent after monovalent cation increases light-scattering and turbidity as well as fluorescence. Again there are differences in kinetics between the different types of measurements. The fluorescence traces show a slow monotonic increase. The 90° light-scattering

trace also increases but it is slower than the fluorescence trace. The turbidity trace, however, shows a rapid phase which is too fast to measure which is not reflected in the fluorescence trace. It could be that only the slow phase can be "correlated" with the fluorescence changes.

The similarities between the light-scattering (and turbidity) data and the chlorophyll a fluorescence changes leads us to postulate that structural change does cause the observed changes in spillover. The differences may be due to the complexities involved in light-scattering studies. On the other hand, direct and quantitative correlations cannot be made.

The fluorescent probes auramine-O, 1-anilinonapthalene-8-sulphonate (ANS^-) and N-phenyl-1-napthylamine (NPN) were also used to monitor changes in dielectric constant in the vicinity of the probe[4,29]. ANS^- is thought to reflect changes on the membrane surface such as those caused by ion binding[4,30-33], whereas NPN is thought to reflect events in the interior of the hydrophobic part of the membrane[34]. In contrast to their effects on chlorophyll a fluorescence changes (see Figs. 1 and 7), both mono- and divalent cations caused an increase in ANS^- fluorescence. The oppositely charged probe auramine-O responds in an opposite, but analogous manner. Moreover, both caused a decrease in NPN fluorescence (Fig. 1a,c). These results do not agree with those obtained for either chlorophyll a fluorescence or light-scattering but do agree with the cation-induced changes in pigment absorption which are due to absorption flattening[10,35,36] and may reflect cation binding to the membrane surface. Evidence supporting this interpretation include the observations that the cation binding sites on the chloroplast membrane[11] bind both mono- and divalent cations. Also the cation concentrations required for saturation of the probe fluorescence changes (namely 1 mM for divalent cations and 10 mM for monovalent cations[15]) are the same as those required for saturating the binding sites[11].

Electron microscopy was done[10] to clarify the nature of the cation-induced structural changes. It was found (Fig. 8) that spinach chloroplasts incubated in the absence of salts appeared slightly swollen due to the hypotonic medium but

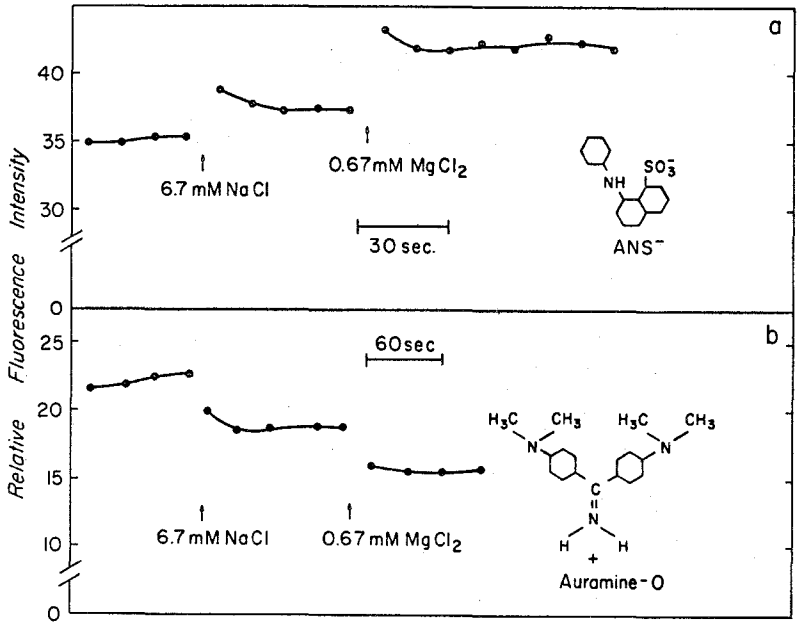

Fig. 7. Time course of the cation-induced changes in 1-anilinonapthalene-8-sulphonate (ANS^-) and auramine-O fluorescence in washed spinach chloroplasts. The probe concentration was 5 μM (after VanderMeulen and Govindjee[15]).

had visible grana stacks. This was surprising since it has been postulated[37] that unstacking of thylakoids occurs in a low ionic strength medium due to charge repulsion.

Addition of 3 mM NaCl caused the grana to unstack; it also promoted spillover. Addition of $CaCl_2$ (either together with (Fig. 9) or after the NaCl (not shown)) caused a restacking of the thylakoids. Unstacking of the thylakoids may bring the two photosystems together, thereby promoting spillover. The differences observed between species may be due to differences in the amount of thylakoid

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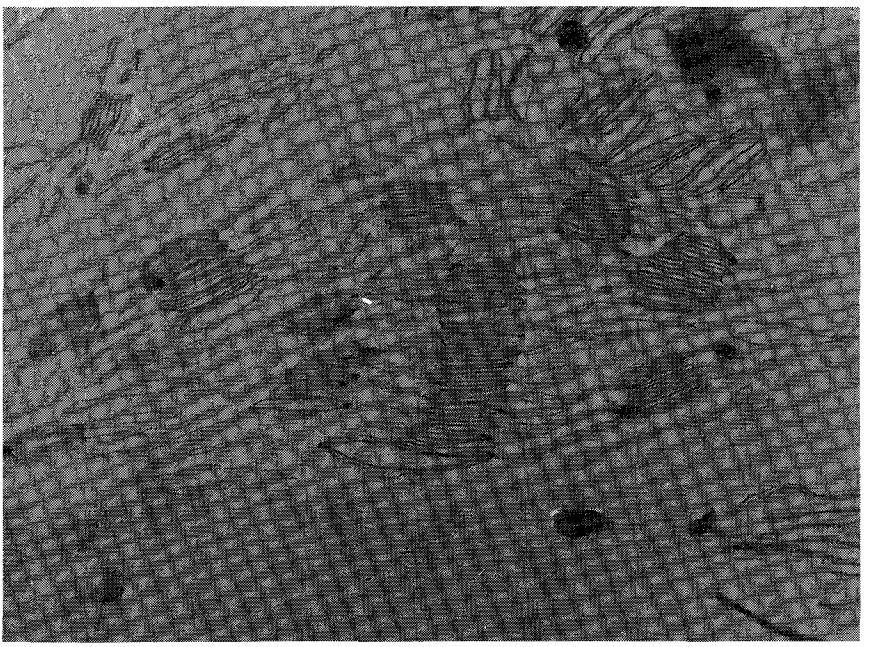
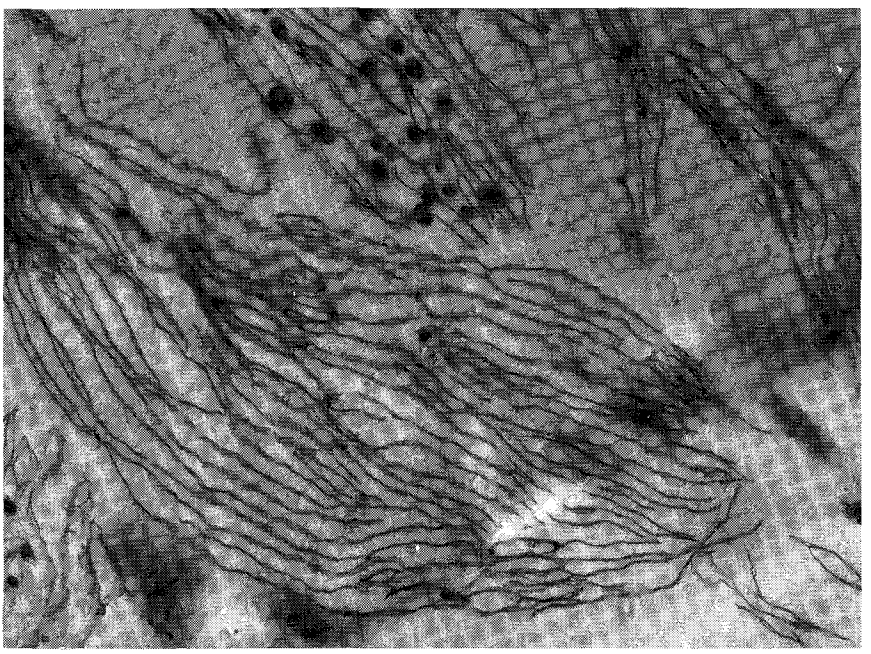
Fig. 8. The effect of NaCl on the ultrastructure of chloroplasts. Chloroplasts were incubated in 100 mM sucrose + 0.2 mM Tris Base (A) and in the same medium + 3.0 mM NaCl (B) prior to fixation with glutaraldehyde. The magnification was 25,000 x.



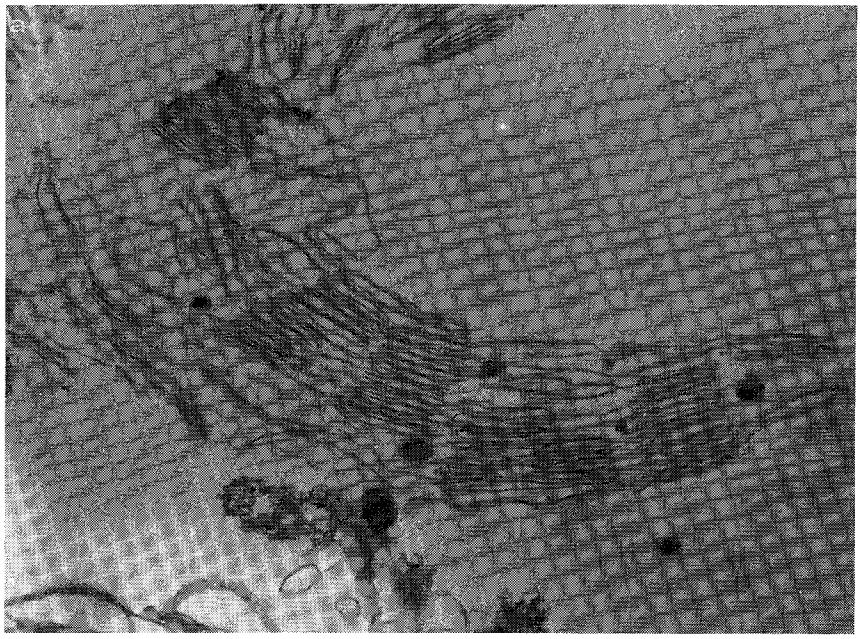


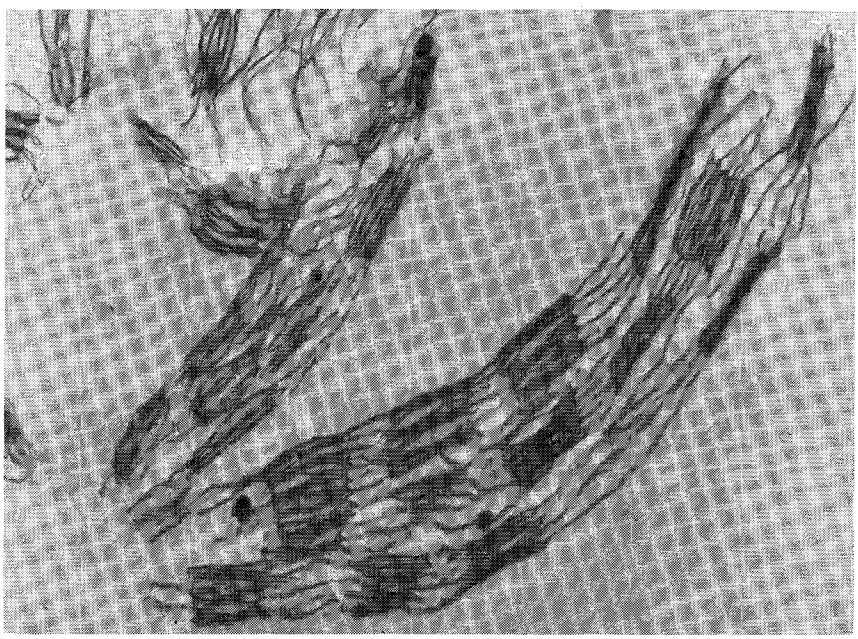
stacking observed under zero salt conditions. Alternately, the mechanism may be more subtle involving slight changes in orientation such as those postulated by Seely[25,26] which may be sufficient to regulate energy distribution. Further work is required to settle this problem.

Concluding Remarks

Since the discovery of Mg^{2+} effect on Chl a fluorescence yield by Homann in 1969 (ref. 6), large numbers of experiments have been done and several ideas have been proposed to explain them. It is now obvious that divalent ions have various effects on chlorophyll a fluorescence, some directly on PS II, and on PS I and some on energy distribution among photosystem I and photosystem II. The work presented here suggests that monovalent ions at low concentration decrease the "O" level, and this effect must be due to effect through bulk chlorophylls, not trap chlorophylls. As it has been difficult to obtain evidence for the motion of PS I and PS II closer or farther from each other to cause changes in energy spillover (in spite of recent evidence for motion of particles upon Mg^{2+} treatment[38]), it may be useful to probe an alternative picture in which light harvesting chlorophyll a molecules (common to both PS I and PS II) feed energy to both PS I and PS II. Monovalent and divalent cations may affect energy distribution by causing conformation changes on chlorophyll-protein complexes that lead to changes in orientation of few key chlorophyll molecules that control energy transfer to PS I and PS II[15].

Fig. 9. The effect of $CaCl_2$ on the ultrastructure of chloroplasts. Chloroplasts were incubated in the presence of 1 mM $CaCl_2$ (A) and 1 mM $CaCl_2$ + 3 mM NaCl (B) prior to fixation. The magnification was 25,000 x.





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