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Effect of Protons and Cations on Chloroplast Membranes as Visualized by the Bound ANS Fluorescence

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Abstract. Structural changes in the chloroplast membranes caused by acidification and heat-treatment are studied by observing the changes in the fluorescence of ANS* bound to thylakoid membranes. On addition of acids to buffered suspension of isolated pea chloroplasts, the fluorescence intensity of bound ANS shows a sigmoidal rise on reaching a pH value of about 4.5. A part of the fluorescence enhancement of bound ANS brought about by protons is not reversible on back titration with alkali. The reversible part of acid induced rise in ANS fluorescence possibly reflects structural changes expected to be associated with photophosphorylation. Divalent cations enhance the fluorescence of ANS bound to chloroplasts between a pH range 4.5–7.0 but diminish it if the pH is below 4.5.

Addition of acid to heat-treated chloroplasts shows similar sigmoidal rise in ANS fluorescence intensity on lowering the pH to about 4.5. On addition of acid upto a pH of 3.1, the ANS fluorescence is greater than that of untreated chloroplasts, however, at pH below 3.1, the fluorescence of bound ANS is lower than the control chloroplasts. This observation indicates that heat-treatment caused some alteration of the microstructure of thylakoid membranes of chloroplasts besides the usual loss in the O_2 evolving capacity.

This is further confirmed from the studies of Hill-activity and ANS binding to chloroplasts incubated at various temperatures in the absence and presence of aliphatic alcohol. Hill-activity (DCPIP reduction) of chloroplasts incubated at temperatures between 25° C and 55° C first increases reaching a maximum at 45° C and then declines rather sharply, when the chloroplasts are heated beyond 45° C (Tmax). The presence of 200 mM n-butyl alcohol or 40 mM n-amyl alcohol during the warming treatment lowers the temperature by 8° C at which the decline in the Hill-activity is observed. An enhancement in the fluorescence intensity and a blue shift of the emission spectrum of bound ANS are noted if the chloroplasts are heated beyond the Tmax either in absence or presence of alcohol. The changes in the fluorescence of ANS bound to heat-treated chloroplasts

* *Abbreviations:* ANS, 1-anilino-8-naphthalene sulphonate, DCPIP, 2,6-dichlorophenol indophenol

plausibly reflect the nature of the structural changes in chloroplasts during the heating upto 55° C.

Key words: ANS fluorescence – Protonated/heated chloroplasts – Hill-activity.

Introduction

Isolated chloroplasts are known to undergo structural and configurational changes in light, depending on the ionic environment of the medium in which they are suspended [20]. Both protons and metal cations (significantly divalent) govern the structural changes of chloroplasts [4, 8, 18–21]. It has been suggested that the cation-induced structural changes of chloroplasts can also modify the orientation of pigment molecules in the thylakoid membrane and, thereby, regulate the distribution of the absorbed light between the two pigment systems of photosynthesis [13, 15, 21]. The light-induced structural changes, which may be both microscopic and macroscopic, are likely to affect the chloroplast function such as the electron transport and energy conservation processes [5, 20].

Murakami and Packer (1970, 1971) studied the effect of acidification on these structural changes in spinach chloroplasts. They found that protons in dark induce reversible changes in the chloroplast membranes. The present investigation attempted to (1) further characterise the proton-induced structural changes in untreated and heat-treated broken chloroplasts; (2) study the effect of divalent cations on acidified chloroplasts; (3) ascertain, if the heat-treatment causes structural alteration of thylakoid membrane. The fluorescent probe 1-anilino-8-naphthalene sulphonate (ANS) has been used in this study to monitor the alterations in the structure of chloroplast membranes. (Recently, extrinsic fluorescent probes have been used to study the structural changes produced in artificial and biological membranes [1, 6, 7, 12, 22, 23, 25–27].) Our results suggest that the structural changes brought about by lowering the pH to 3.1 (producing maximal alterations) are not fully reversible on back titration with alkali. The divalent cations (Mg^{2+} or Ca^{2+}), which produce an increase in ANS fluorescence upto pH 4.5, cause a quenching of the fluorescence on further lowering the pH of the chloroplast suspensions. The incubation of chloroplast at higher temperatures (upto 55° C) also brings about structural changes in chloroplast membranes, thereby, affecting Hill-activity and coupling of the electron transport chain. The presence of aliphatic alcohol during heat incubation lowers the *T*max for structural alteration as well as loss of O_2 evolution capacity.

Materials and Methods

Isolation of Chloroplasts

Pea (var. Bonneville) plants were grown in continuous white light (~ 2500 lux) at 24 ± 1° C. Leaves grown for two to three weeks were used for chloroplast prepara-

tion. Chloroplasts were isolated by grinding 10 g of leaves with ice-cold buffer using a pre-chilled pestle and mortar. The grinding medium contained 400 mM sucrose, 10 mM KCl and 50 mM Tris-Cl buffer, pH 7.8. The homogenate was filtered through 16 layers of cheese cloth and centrifuged at 2000 × g for 7 min in a Remi T-8 clinical centrifuge. The pellet was once washed in the medium. The chloroplasts were finally collected by suspending the pellet in minimal volume of homogenising medium. Chlorophyll was assayed by the method of Bruinsma (1963). All operations were performed in a cold room maintained at 4° C.

Absorption Spectra

Absorption spectra of chloroplast suspension were measured in a Carl-Zeiss PMQ-II spectrophotometer. The effect of the scattering on absorption by chloroplast was made minimal by aligning the opaque size of matched glass cuvettes in the light path [24]. Chloroplast suspensions having an optical density of approximately 0.5 at the blue peak of the absorption spectrum were used.

Fluorescence Measurements

Fluorescence spectra of ANS were measured in an Aminco-Bowman spectrofluorometer having right angle geometry. The ANS was excited at 390 nm and the emission was measured through a cut off Corning CS 3—73 glass filter. The emission spectra are not corrected for the variation in the lamp and photomultiplier characteristic of the spectrofluorometer.

In our experimental set up, ANS fluorescence was quite linear upto a concentration of 7.6 μM. At this concentration of ANS, chloroplasts containing 5 μg chl/ml gave maximal enhancement of ANS fluorescence. Therefore, in all experiments, we have used chloroplast suspensions containing chlorophyll concentration of 5 μg/ml and ANS concentration of 7.6 μM. All test samples were made by adding small volumes of concentrated solutions of acids or salts such that the volume changes were negligible. In experiments involving the acid titration of chloroplasts, constant volume of different concentrations of acid were added to the 3 ml reaction mixture. As the buffering nature of the chloroplasts is likely to alter the actual proton concentration, we have expressed the amount of H^+ in terms of amounts of acid actually added rather than the final concentration of the acid in the cuvette. All additions were made in darkness and were incubated for at least 5 min before taking the measurements.

Hill Reaction Measurements

The photoreduction of DCPIP was measured spectrophotometrically as described below. Chloroplast sample was illuminated for 30 s with saturating white light (intensity $\sim 7 \times 10^4$ ergs cm^{-2} s^{-1}). The incident light source was a 300 Watt tungsten projection lamp. The incident beam was passed through a water filter and a CS 3-69

Corning cut off glass filter before entering the reaction mixture. The reaction mixture (total volume of 3 ml) contained chloroplasts equivalent to 8–10 μg of chlorophyll (Chl.), 15 μM DCPIP, 100 mM KCl, 1 mM $MgSO_4$ and 10 mM Tris base, adjusted to pH 6.8. The bleaching of DCPIP was measured at 605 nm as described by Mohanty et al., 1971.

Heat-Treatment

Incubation of chloroplast suspensions at different temperatures was similar to that of Mukohata et al., 1973. One ml of the reaction mixture contained 10 mM Tris-Cl (pH 7.8), 10 mM KCl, 400 mM sucrose. Chloroplasts containing 100 μg of chlorophyll were added to it at 0° C. For treatment with alcohols, 200 mM n-butyl alcohol or 40 mM n-amyl alcohol were used. The glass tube containing the sample mixture was first incubated at temperatures ranging from 25–55° C for 5 min in a water bath with mild stirring and then, transferred immediately to an ice bucket. The temperature variation during heat incubation was ± 0.5° C. Assays of all the samples were made at room temperature.

All chemicals used were of analytical grade. 1,8 ANS (Mg salt) was purchased from Serva Fine Chemicals Company (West Germany), and used without any further purification.

Results

Figure 1 shows the emission spectra of ANS in the absence and presence of chloroplasts in the Tris buffer. The inset shows the emission spectrum of ANS in the absence and presence of chloroplasts plotted on a magnified scale. The fluorescence of ANS is slightly enhanced (10–15%) and blue shifted (by 10 nm), when chloroplasts are added to an aqueous solution of the ANS.

Effect of Proton Concentration

Addition of a small amount of the hydrochloric acid to the suspending medium increases the fluorescence intensity of bound ANS by about seven fold and causes a further shift of the emission peak from 510 nm to 480 nm. The fluorescence intensity of free ANS in buffer does not change on addition of the acid. No clumping or deterioration except a little loss of chlorophyll *a* (< 3%) was observed on lowering the pH below 4.5.

Figure 2 shows the effect of the addition of different amounts of HCl on the fluorescence of bound ANS in chloroplast suspensions. The inset of Figure 2 gives the variation of F480/F520 (*f*) as a function of added H^+ concentration. Figures 3A and 3B show the variation of fluorescence intensity of bound ANS as a function of pH. For the data shown in Figure 3A, solutions of different pH (in the range between 2.0 and 7.2) were prepared by titrating Tris with MES or HCl. In Figure 3B, pH values were determined experimentally after the addition of different amounts of

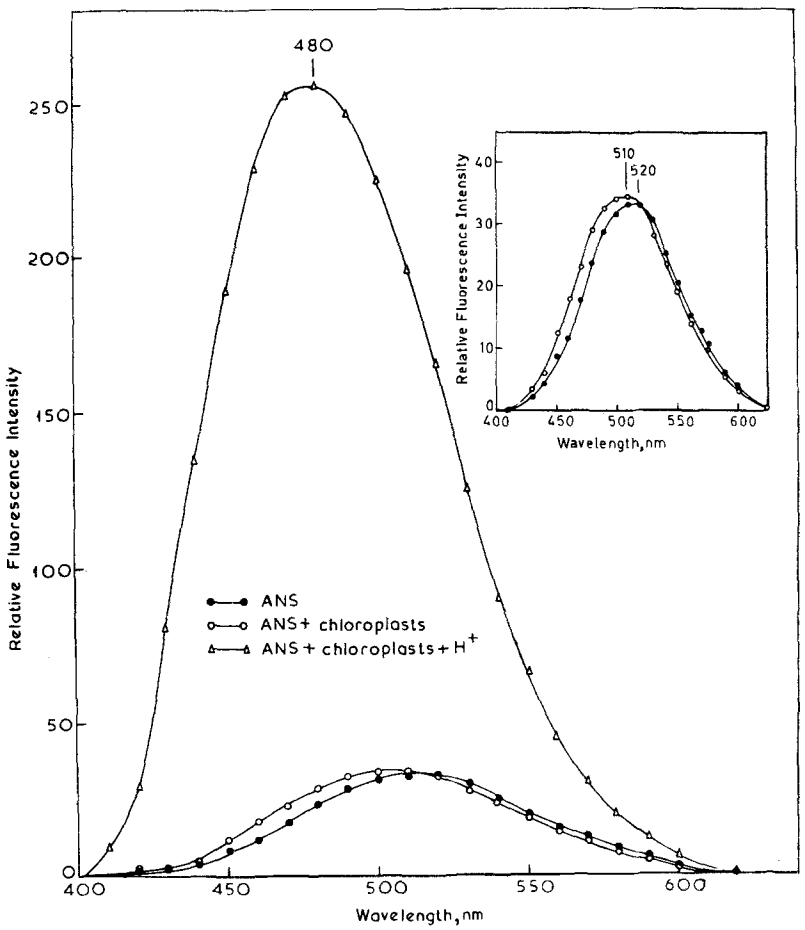

Fig. 1. Emission spectrum of ANS in buffer alone and chloroplast suspensions in the absence and presence of 16.5 μM of added HCl (pH 3.1). The chloroplasts were initially suspended in 50 mM Tris-Cl buffer, pH 7.2. The emission spectrum of ANS in the absence and presence of chloroplasts is plotted on an expanded scale in the inset

HCl. In both the cases (Figs. 3A and 3B), the nature of the fluorescence rise is almost the same. There is a gradual blue shift of the fluorescence peak on addition of increasing amounts of H^+ upto 16.5 μM of added H^+ (Table 1). The observed spectra are probably a superposition of the spectra of bound and unbound ANS.

The rise in fluorescence is gradual upto 15 μM of added H^+ and the fluorescence rise index given by F480/0.5 unit of pH does not exceed the value of 10. On increasing the added amount of H^+ beyond 15 μM (pH < 4.5), a sharp rise in the fluorescence is observed. The fluorescence rise index jumps from 10—196 near the transition point. After the transition is over, the fluorescence level remains almost constant. Addition of alkali to the acidified chloroplasts lowers the fluorescence (F480) of the bound ANS. However, a part of the fluorescence rise induced by acidification is not reversible. The inset of Figure 2 shows the variation of F480/F520 of the ANS

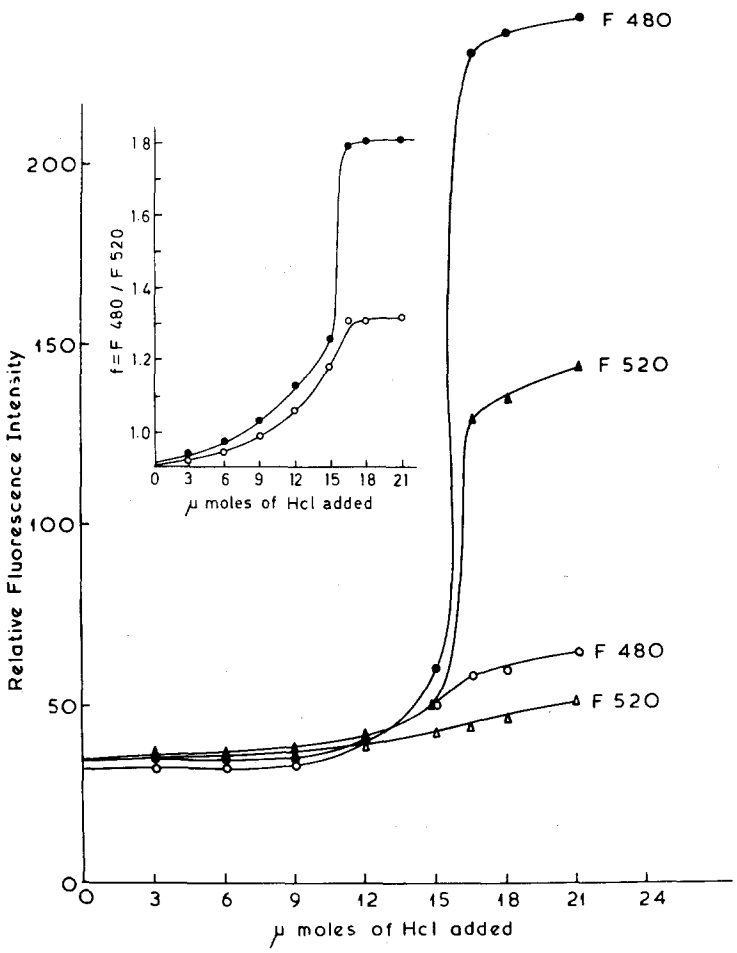

Fig. 2. Variation of fluorescence intensity of ANS at 480 and 520 nm with the concentrations of added HCl and back titration with KOH. The inset shows the variation of the fluorescence ratio (*f*) of ANS (F480/F520) with the concentrations of added HCl and back titration of the acidified chloroplasts with alkali. The medium contained 50 mM Tris-Cl buffer, pH 7.2. ●—● and ▲—▲, in the presence of acid, ○—○ and △—△, after the addition of alkali

fluorescence on addition of H^+, which follows almost the same path as does the fluorescence F480.

Table 1 shows the decrease in the fluorescence intensity of ANS by addition of equimolar amount of KOH to the acidified chloroplast suspensions. Addition of KOH in excess of the amount required to neutralize the added acid does not lower the fluorescence intensity any further. The last column in Table 1 shows the percentage reversibility of the acid induced ANS fluorescence upon addition of equimolar amount of alkali to acidified chloroplasts. The recovery of fluorescence is much less ($< 50\%$) at pH > 4.5 than at pH < 4.5, where it is about 90%. The fluorescence intensity of ANS (F480) shows a similar kind of variation on changing the H^+ concentration by adding succinic acid instead of HCl (data not shown).

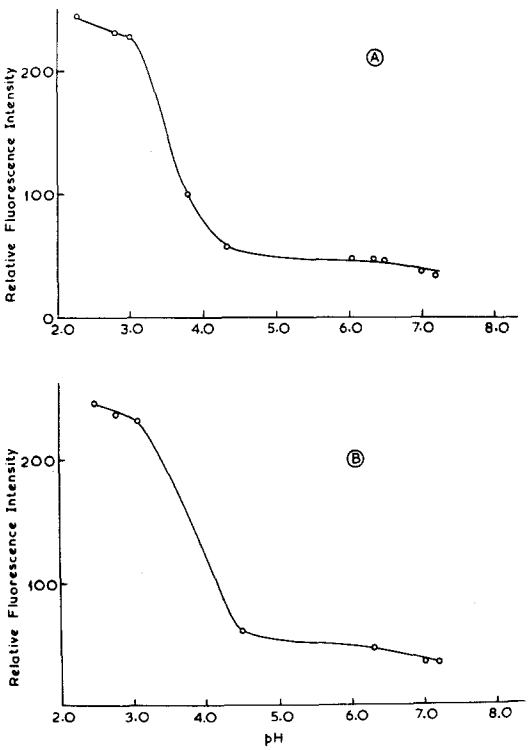

Fig. 3. Variation of fluorescence intensity of ANS at 480 nm with pH. (**A**) pH was varied by titrating Tris with MES or HCl in the range between 2 and 7. (**B**) pH was experimentally determined after each addition of HCl

Table 1. The recovery of initial ANS fluorescence upon addition of alkali to chloroplasts treated with various concentrations of acid

μM of H^+ added	pH	Emission peak (nm)	Fluorescence intensity (F480) in relative units $(x)^a$	F480 after the addition of equimolar amount of KOH $(y)^a$	% reversal of the acid induced fluorescence by alkali $\frac{(x-y)}{(x-x_0)} \times 100$
0	7.2	510	34 (x_0)	34	–
6	7.0	505	35	34	–
12	6.3	500	46	42	33
15	4.5	490	60	49	43
16.5	3.1	480	231	55	89
18	2.8	480	237	56	91
21	2.5	480	245	67	85

[a] Each value of x was measured after adding a given concentration of acid to the chloroplast suspension. The value of y was then determined by adding equimolar concentration of KOH to the acidified suspension

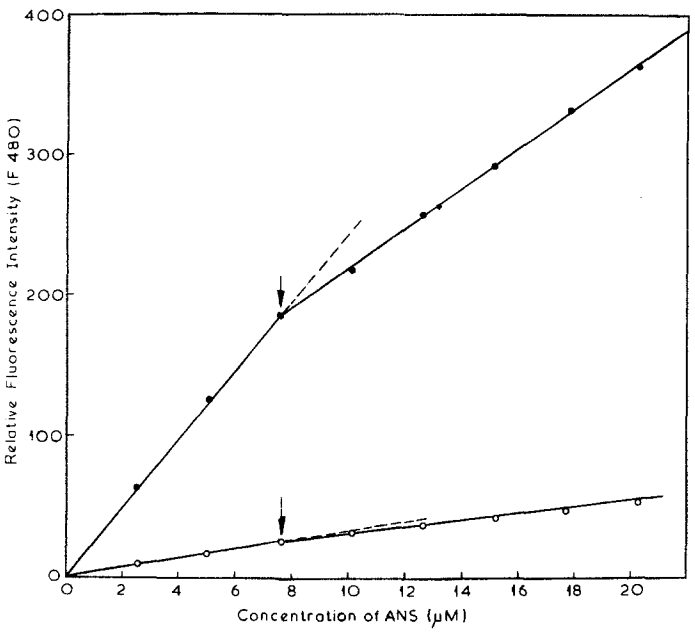

Fig. 4. Relative fluorescence intensity of ANS bound to chloroplasts at 480 nm as a function of the concentration of ANS. O—O, in the absence of acid, ●—● after the addition of 18 μM of HCl. The vertical arrows indicate the concentration of ANS at which a break in the linearity occurs

In order to find the nature of the enhancement of the fluorescence of bound ANS on acidification, titration of chloroplast (containing 5 μg/ml chl) with ANS was done both in the absence and in the presence of a saturating amount of protons (Fig. 4). It is apparent that in absence of acid, the fluorescence intensity (F480) varies almost linearly with ANS concentration. However, in presence of 16.5 μM of added H^+ (pH ~ 3.1), the ANS fluorescence data show two types of proportionalities with break at about 7.6 μM of ANS concentration. This indicates the existence of two types of binding; first type is existing below 7.6 μM and the second type above 7.6 μM of ANS concentration.

Effect of Divalent Cations

Figure 5 shows the effect of Mg^{2+} on the emission spectrum of ANS bound to chloroplasts. Mg^{2+} enhances the fluorescence of bound ANS by about 10% as indicated by the increase in the fluorescence intensity in the blue region of emission spectrum. The effect of Ca^{2+} was found to be the same as that of Mg^{2+} (data not shown). Mg^{2+} causes the small shift (5 nm) in the emission spectrum of bound ANS. The effect of Mg^{2+} or Ca^{2+} on ANS fluorescence saturates at a final concentration of about 3 mM of cations. It is to be pointed out that about the same concentration is also required to saturate the enhancement of Chl *a* fluorescence of chloroplast by salts [21].

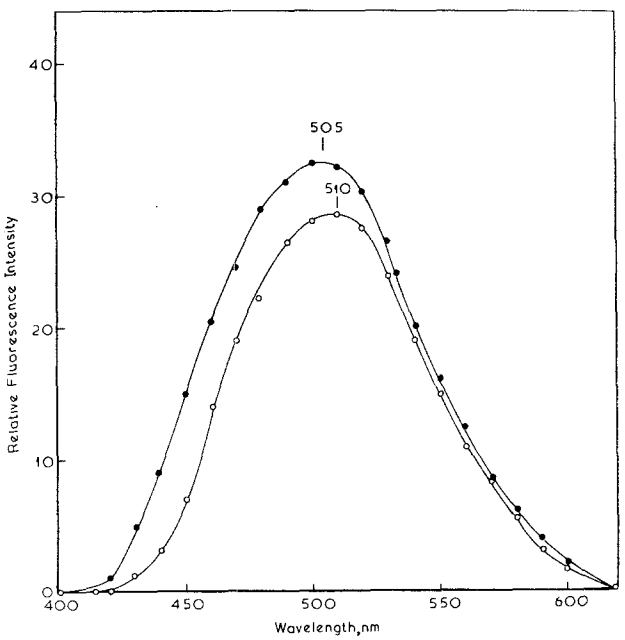

Fig. 5. Salt effect on the emission spectrum of ANS bound to chloroplasts. O—O, no $MgCl_2$, ●—● in the presence of 3 mM $MgCl_2$

Figure 6A shows the effect of addition of Mg^{2+} or Ca^{2+} (3 mM) on the fluorescence of ANS bound to acidified chloroplast suspended in a buffer. Addition of Mg^{2+} or Ca^{2+} to chloroplast suspended in 50 mM Tris-Cl buffer enhances the fluorescence intensity of bound ANS by about 10% if the amount of added H^+ was below 15 μM (pH > 4.5). However, if the amount of added H^+ is higher than 15 μM (pH < 4.5), where a sharp rise in ANS fluorescence is seen, the addition of Mg^{2+} or Ca^{2+} results in the quenching of the fluorescence of ANS by about 10–15%. The final level of the fluorescence in the presence of Mg^{2+} is the same, whether the Mg^{2+} is added before or after the addition of acid. The inset of Figure 6A shows the effect of H^+ in presence of 3 mM of Mg^{2+} on the ratio '*f*' of ANS fluorescence in chloroplast suspensions. Although, the presence of Mg^{2+} caused an enhancement in the ANS fluorescence ratio '*f*', both in the absence or in the presence of added H^+ below 15 μM, no effect on the ratio is observed at higher amounts of added H^+ between acidic and non-acidic suspensions. This suggests that Mg^{2+} in the presence of added amounts of H^+ higher than 15 μM (at pH < 4.5) produces a lowering of fluorescence intensity at both the wavelengths. No further shift in the emission spectrum of bound ANS was observed in the presence of Mg^{2+} at pH $\leqslant$ 3.1.

In order to find out the nature of quenching of ANS fluorescence by Mg^{2+} or Ca^{2+}, we have studied the effect of various concentrations of salts added to acidified chloroplast and the results are given in the form of a Stern-Volmer plot. The plot of F_0/F versus concentration of Mg^{2+}, shown in Figure 6B, is hyperbolic in nature and probably represents the effect on binding of ANS.

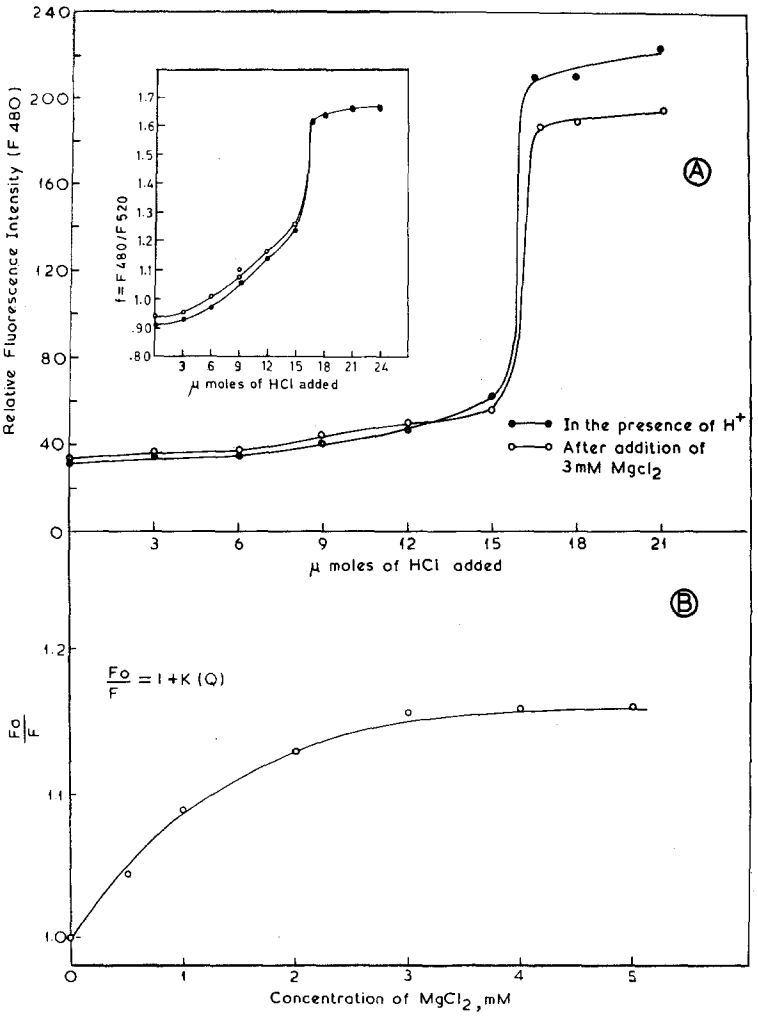

Fig. 6. (A) Effect of 3 mM $MgCl_2$ on the fluorescence intensity of bound ANS at 480 nm at different concentrations of added HCl. The inset shows the effect of $MgCl_2$ on the fluorescence ratio '*f*' at different concentrations of added HCl. **(B)** Plot of F_0/F vs. [$MgCl_2$] in the presence of 18 μM HCl. F_0 and F are respectively the fluorescence intensities of ANS at 480 nm, in the absence and presence of $MgCl_2$

Effect of Heat-Treatment on Hill-Activity and the Fluorescence of ANS

It is well-known that moderate heat-treatment causes uncoupling of phosphorylation and electron transport. Heat-treatment also destroys the ability of chloroplasts to evolve oxygen [3], but it does not affect the activity of photosystem II (PS II) in the presence of artificial donors [28]. The absorption changes of chlorophyll *a* mediated by photosystem I (PS I) also remain unaffected on heat-treatment [30]. Since warming the chloroplasts is expected to alter the microstructure of the chloroplast membranes, we have studied its effect on the fluorescence of ANS bound to heat-treated chloroplasts.

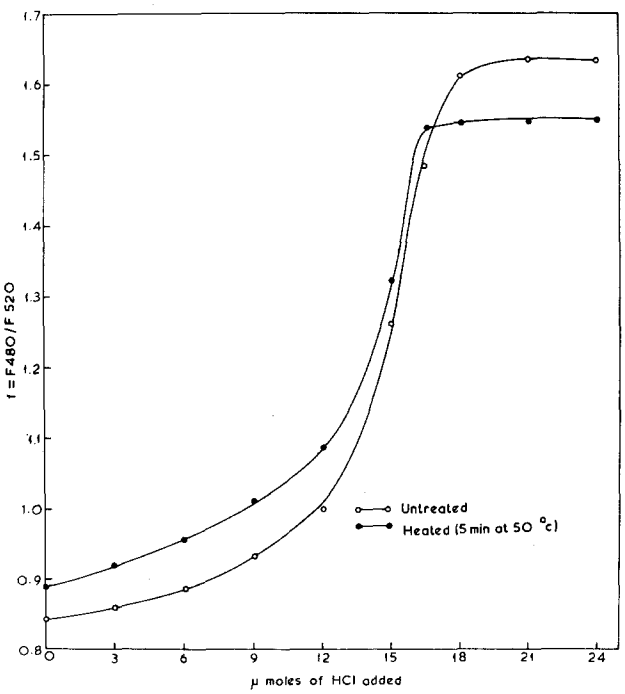

Fig. 7. Variation of the ratio of the fluorescence of ANS, '*f*' with HCl concentration with untreated and heat-treated chloroplasts. Medium as in Figure 1

Figure 7 shows the effect of added protons on the fluorescence of bound ANS in untreated and heat-treated chloroplast suspensions. Two general features are distinct. (I) The ANS fluorescence ratio '*f*' is higher in heat-treated chloroplast suspensions than in untreated suspensions upto the added amounts of H^+ (about 16 μM), which brings the fluorescence ratio '*f*' to the saturation level. However, the level of ANS fluorescence after saturation is higher in untreated than in heat-treated suspensions. (II) The acid titration curves for heat-treated and untreated chloroplasts follow almost the same path.

Hill-Activity

To ascertain the effect of heat incubation on chloroplast activity as well as on the fluorescence yield of ANS, we have measured DCPIP photoreduction (Hill-activity) of chloroplasts and fluorescence of bound ANS as a function of incubation temperature. With rise in the incubation temperature, the rate of DCPIP reduction initially increased reaching the maximum at 45° C and then, it declined as the temperature was increased further. Our values of *T*max for acceleration of photoreduction of DCPIP agree with that of Mukohata et al., who measured Hill-activity with ferricyanide as the electron acceptor. Mukohata et al. have also shown that *T*max (temperature of maximum activation) is shifted to lower incubation temperature if the warming was carried out after addition of some short chain aliphatic alcohol [17].

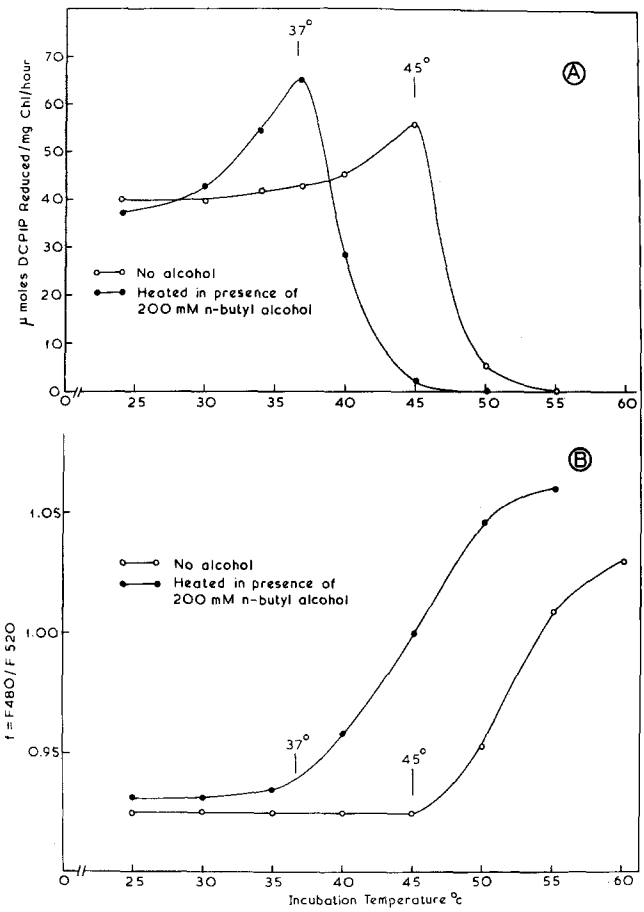

Fig. 8. **(A)** Variation of Hill-activity (DCPIP reduction) with the temperature of incubation of chloroplasts. **(B)** Variation of the fluorescence ratio '*f*' of ANS with the temperature of incubation of chloroplasts treated as in (A)

This is also confirmed by our data of Figure 8A, which shows that in the alcohol treated samples, the *T*max is lowered by 8° C. The lowering of *T*max would imply that the aliphatic alcohols are causing alterations in the lipophilic phase of the membrane to a considerable extent.

Figure 8B shows corresponding experiment on the variation of fluorescence of ANS bound to chloroplast, which were incubated at various temperatures both with and without n-butyl alcohol. Since the ANS fluorescence is high in the presence of $MgCl_2$, we have measured ANS fluorescence in the presence of 3 mM $MgCl_2$ in this experiment. Initially, with rise in the incubation temperature, the fluorescence ratio '*f*' of bound ANS remained almost constant till the *T*max (45° C) was reached. After the *T*max, there was a rise in the fluorescence ratio '*f*' from 0.93–1.05. It has to be noted that the transition of ANS fluorescence is shifted to a lower temperature (i.e., 37° C), when heating was done in the presence of n-butyl alcohol (also see Fig. 8A).

Table 2. Ratios of bound ANS fluorescence measured at different emission wavelengths in heat-treated PEA chloroplasts

Temperature (° C) ature	No alcohol (*T*max = 45° C)			200 mM n-butyl alcohol (*T*max = 37° C)		
	F480/F500 (± 0.02)	F480/F520 (± 0.02)	F470/F520 (± 0.02)	F480/F500 (± 0.02)	F480/F520 (± 0.02)	F470/F520 (± 0.02)
25	0.86	0.92	0.83	0.88	0.93	0.86
30	0.86	0.92	0.83	0.88	0.93	0.86
35	0.86	0.92	0.83	0.92	0.93	0.86
40	0.86	0.92	0.82	0.92	0.97	0.88
45	0.86	0.92	0.82	0.95	1.00	0.90
50	0.90	0.96	0.88	0.98	1.05	0.92
55	0.92	1.02	0.90	1.10	1.07	1.00
60	0.95	1.04	0.97	1.10	1.10	1.16

To ascertain if there is spectral shift in the emission spectrum of ANS bound to heat-treated chloroplasts, the ratio of fluorescence intensities F480/F520, F480/F500 and F470/F520 near the emission peak were calculated. Table 2 shows that these ratios remained constant upto the incubation temperature *T*max and after the *T*max, the values tend to increase. It can be concluded from these results that the enhancement of Hill-activity upto *T*max and of ANS fluorescence after *T*max on incubation above the room temperature would indicate respectively the uncoupling before *T*max (Fig. 8A) and increase in the hydrophobicity after *T*max (Fig. 8B) around ANS molecules bound to chloroplasts.

The fluorescence characteristics of the bound ANS remained the same irrespective of, whether the ANS was added to chloroplast suspensions before or after heating. The presence of ANS in chloroplast suspensions during heat incubation did not affect either the *T*max or the DCPIP Hill-activity. However, the effect of alcohol in shifting the *T*max can only be observed if the alcohol is added prior to heating the chloroplast suspensions [17].

Discussion

The fluorescence intensity of the ANS in chloroplast suspensions is increased by about 10—15%, and its spectrum is shifted from 520 nm to 510 nm. A small increase in the fluorescence of ANS indicates little binding of ANS to chloroplast membranes. A blue shift of 10 nm indicates that the probe is located in polar-apolar mixed phases in the suspensions containing chloroplast membranes.

Chloroplast membranes have sufficient buffering capacity in the pH range between 7 and 4.5 [29]. This buffering capacity amounts to 4.5 μeq protons/mg of chlorophyll. During this range of pH on addition of HCl, protonation of the chloroplast membranes is liable to occur. As shown by Figures 2 and 3, first the increase in ANS fluorescence is gradual on addition of acid till pH 4.5, but afterwards there is a sharp rise. Chloroplast membranes exhibit characteristic behaviour like a polyanion in a solution [14]. Consequently, acidification is likely to neutralize some of the

negative charges of the membrane proteins in thylakoids and thus cause a redistribution of negative and positive charges on the membrane, and also affect the attractive and repulsive forces operative in the maintenance of protein conformation. This may bring about changes in the protein conformation and in the spacing and thickness of the thylakoid membranes. These changes may also give rise to an hydrophobic environment around the binding site. The seven fold increase in the ANS fluorescence (Figs. 1 and 2) and a blue shift of the emission spectrum (Fig. 1) suggest that protonation results in the creation of additional binding sites and hydrophobic environment near the binding sites. The gradual shift of emission peak on protonation upto a pH of 3.1 (Table 1, column 3) should support the contention that binding sites have hydrophobic environment. It also indicates that the emission spectra of ANS observed in suspensions having pH higher than 3.1 is plausibly an overlap of bound and unbound ANS, indicating the involvement of polar-apolar mixed phases. ANS molecule is probably having two types of microenvironment; one very close to aqueous and the other equivalent to 60–80% of ethanol. There could be more microenvironments referring to different Z values (extent of polarity) or dielectric constants. When the concentration of added H^+ is 16.5 μM or more, where the blue shift is maximum, most of the ANS is in the bound state. The sharp rise in ANS fluorescence on addition of 16.5 μM of H^+ could possibly be due to the sharp increase in the binding of ANS due to certain microstructural changes in the membrane. Though, there is no change in ANS fluorescence on addition of similar amount of H^+ in aqueous solutions, one cannot rule out the possibility of an increase in fluorescence efficiency of ANS on binding to thylakoid membrane.

The addition of alkali to the acidified chloroplasts should neutralize the added protons, if protonation is involved in the depolarization of the negative charges on the thylakoid membrane. The lack of restoration on addition of alkali of the fluorescence intensity as well as non-recovery of the peak position of the bound ANS to their original values in the absence of acid suggests that a part of the acid induced structural change of chloroplast membrane is irreversible. The reversible part of the change in the fluorescence of ANS bound to thylakoid membrane may have been caused by the protonation of negative charges of the membrane. This is likely to cause a change in charge distribution on the membrane affecting the forces involved in maintaining the protein conformation. A change in the conformation of the membrane proteins probably is responsible for the increase in binding sites. It is well-known that the treatment of chloroplast first with acid and then, subsequently, with alkali generates ATP in dark [10]. During the acid bath phosphorylation, the chloroplast structure is likely to undergo structural changes very similar to the one that we have described above. The reversible part of ANS fluorescence may, therefore, be indicative of structural alterations associated with phosphorylation.

A 10–15% rise in ANS fluorescence and a blue shift by about 5 nm in the presence of the divalent cations indicate a corresponding increase in the binding of ANS by divalent ions. Protonation, as discussed above, is likely to change the charge distribution on the surface of membrane and if number of ionised groups in the membrane surface changes, effect of Mg^{2+} may also change. Also cations by chelating to lipid phosphate groups may increase the binding of ANS. This could be a reason why different effects above and below pH 3.1 are observed. Acidification may also cause stretching of membrane and may thus hinder the likely formation of

salt bridges of Mg^{2+} between membrane proteins. As a result of this, one might expect no enhancement of ANS fluorescence due to addition of Mg^{2+} above a certain concentration of H^+.

The increase in the fluorescence of bound ANS upon addition of acid upto a pH of 3.1 in heat-treated chloroplast suspensions suggests that heat-treatment probably modifies the degree of binding of ANS molecules. The difference in the level of maximal fluorescence of ANS in untreated and heat-treated chloroplast suspensions could possibly be due to a difference in the extent of stretching brought about by acid in untreated and heat-treated chloroplasts. The rise in the rate of DCPIP reduction is likely to be due to uncoupling of phosphorylation as suggested by Mukohata et al., 1973, but the decline in activity can be attributed to the loss of either Mn^{2+} from photoenzyme or water activating system in chloroplasts [11].

Since warming of chloroplasts in the presence of aliphatic alcohols can produce structural changes in the thylakoid membrane, one can expect these changes to be reflected in the absorption spectra. We could not detect any significant change except a decrease in optical density at 540 nm in the absorption spectra of heated chloroplasts. The scattering by the heated chloroplasts measured at 740 nm, where chloroplast pigments practically do not absorb, decreased by 50% than that of control. These results indicate that heat-treatment makes the chloroplasts swollen and that the swelling is more in presence of the alcohols. However, these gross structural changes do not affect the ANS fluorescence and, therefore, ANS fluorescence only registers the microenvironment of the membranes.

The data presented here have shown that acidification of chloroplast suspensions modifies the structure of the thylakoid membranes. The structural changes may include the opening up of the membrane clefts or interior. Heat-treatment seems to affect both chloroplast activity and structure. Experiments on washed and lipid and associated protein depleted membranes are in progress to know more about the changes in the molecular organization of chloroplast membranes upon acidification.

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References

1. Azzi, A.: The application of fluorescent probes in membrane studies. Quart. Rev. Biophys. **8,** 237–316 (1975)
2. Bruinsma, J.: The quantitative analysis of chlorophylls *a* and *b* in plant extracts. Photochem. Photobiol. **2,** 241–249 (1963)
3. Cheniae, G. M., Martin, I. F.: Photoreactivation of manganese catalyst in photosynthetic oxygen evolution. Plant Physiol. **44,** 261–360 (1969)
4. Deamer, D. W., Crofts, A., Packer, L.: Mechanism of light-induced structural changes in chloroplasts. I. Light scattering increaments and ultrastructural changes mediated by proton transport. Biochim. Biophys. Acta **131,** 81–96 (1967)
5. Dilley, R. A.: Coupling of ion and electron transport in chloroplasts. In: Current topics in bioenergetics, vol. 4 (ed. D. R. Sanadi), pp. 237–271. New York: Academic Press 1971
6. Gulik-Krzywicki, T., Shechter, E., Iwatsubo, M., Rauck, J. L., Luzzati, V.: Correlations between structure and spectroscopic properties in membrane model systems. Tryptophan and 1-anilino-8-naphthalene sulfonate fluorescence in protein-lipid-water phase. Biochim. Biophys. Acta **219,** 1–19 (1970)

7. Haynes, D. H., Staerk, H.: ANS: a fluorescence probe of membrane surface structure, composition and mobility. J. Membrane Biol. **17,** 313–340 (1974)
8. Hind, G., McCarty, R. E.: The role of cation fluxes in chloroplast activity. In: Photophysiology, vol. VIII (ed. A. C. Giese), pp. 113–156. New York: Academic Press 1973
9. Homann, P. H.: Effect of manganese on the fluorescence of chloroplasts. Biochem. biophys. Res. Comm. **33,** 229–234 (1968)
10. Jagendorf, A. T., Uribe, E.: ATP formation caused by acid-base transition of spinach chloroplasts. Proc. Natl. Acad. Sci. USA **55,** 170–177 (1966)
11. Katoh, S., San Pietro, A.: Ascorbate-supported NADP photoreduction by heated *Euglena* chloroplasts. Arch. Biochem. Biophys. **122,** 144–152 (1967)
12. Kraayenhof, R.: Atebrin and related fluorochromes as quantitative probes of membrane energization. In: Fluorescence techniques in cell biology, (eds. A. A. Thaer, M. Sernetz), pp. 381–394. Berlin-Heidelberg-New York: Springer 1973
13. Marsho, T. V., Kok, B.: Photosynthetic regulation by cations in spinach chloroplasts. Biochim. Biophys. Acta **333,** 353–365 (1974)
14. Molotkovsky, Y. G., Dzyubenko, V. B.: Light-induced pH gradient through chloroplast membranes and translocation of cations. Nature (Lond.) **219,** 496–498 (1968)
15. Mohanty, P., Braun, B. Z., Govindjee: Light-induced slow changes in chlorophyll *a* fluorescence in isolated chloroplasts: Effect of magnesium and phenazine methosulfate. Biochim. Biophys. Acta **292,** 459–476 (1972)
16. Mohanty, P., Papageorgiou, G., Govindjee: Fluorescent induction in the red alga *Porphyridium cruentum*. Photochem. Photobiol. **14,** 667–682 (1971)
17. Mukohata, Y., Yagi, T., Matsuno, A., Higashide, M.: Biophysical studies on sub-cellular particles VII. Combined effect of alcohol and heat on photosynthetic activities in isolated chloroplasts. Plant Cell Physiol. **14,** 119–126 (1973).
18. Murakami, S., Packer, L.: Protonation and chloroplast membrane structure. J. Cell Biol. **47,** 332–351 (1970)
19. Murakami, S., Packer, L.: The role of cations in the organization of chloroplast membranes. Arch. Biochem. Biophys. **146,** 337–347 (1970)
20. Murakami, S., Torres-Pareira, J., Packer, L.: Structure of the chloroplast membrane. – Relation to energy coupling and ion transport. In: Bioenergetics of photosynthesis (ed. Govindjee), pp. 555–618. New York: Academic Press 1975
21. Murata, N.: Control of excitation transfer in photosynthesis II. Mg ion dependant distribution of excitation energy between two pigment systems in spinach chloroplasts. Biochim. Biophys. Acta **189,** 171–181 (1969)
22. Radda, G. K.: The design and use of fluorescent probes for membrane studies. In: Current topics in bioenergetics, vol. 4 (ed. D. R. Sanadi), pp. 81–126. New York: Academic Press 1971
23. Radda, G. K. Vanderkooi, J.: Can fluorescent probes tell us anything about membranes? Biochim. Biophys. Acta **265,** 509–549 (1972)
24. Thomas, J. B., Nagaraja, R.: Absorption spectra of algal and chloroplast suspensions: A simple method using conventional spectrophotometers. Ind. J. Biochem. Biophys. **10,** 266–268 (1973)
25. Trauble, H., Overath, P.: The structure of *Escherichia coli* membrane studied by fluorescence measurements of lipid phase transitions. Biochim. Biophys. Acta **307,** 491–512 (1973)
26. Vander Muelen, D. L., Govindjee: 12-9-anthranoyl stearic acid and atebrin as fluorescent probes for energetic states of chloroplasts. FEBS Lett. **45,** 186–190 (1974)
27. Vander Muelen, D. L., Govindjee: 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 (1974)
28. Vernon, L. P., Shaw, E. R.: Photoreduction of 2,6 dichlorophenolindophenol by diphenylcarbazide: A photosystem 2 reaction catalyzed by tris-washed chloroplasts and sub-chloroplast fragments. Plant Physiol. **44,** 1645–1649 (1969)
29. Walz, P., Goldstein, L., Avron, M.: Determination and analysis of the buffer capacity of isolated chloroplasts in the light and in the dark. Eur. J. Biochem. **47,** 403–407 (1974)
30. Witt, H. T.: Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. Results by pulse spectroscopic methods. Quart. Rev. Biophys. **4,** 365–477 (1971)

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