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Salt-induced alterations of the fluorescence yield and of emission spectra in *Chlorella pyrenoidosa*

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(1) The addition of salts to the suspending medium induces a decrease in the yield of chlorophyll *a* fluorescence in normal and DCMU-poisoned intact algal cells of *Chlorella pyrenoidosa*. Potassium and sodium acetate cause a pronounced lowering of the fluorescence at relatively low concentrations (0.01–0.1 M). $MgCl_2$ and KCl cause a similar lowering of fluorescence but at much higher concentrations (0.1–0.4 M). In contrast to sodium acetate, ammonium acetate does not cause any significant change in the fluorescence transient.

(2) Unlike the case in isolated chloroplasts, $MgCl_2$ decreases the ratio of short wavelength (mainly system 2) to long wavelength (mainly system 1) emission bands in both DCMU poisoned and normal cells. Since these salt-induced changes do not appear to be related to the redox reactions of photosynthesis, the salts might have caused a decrease in the mutual distance between the two photosystems by changing the microstructure of the chloroplasts in vivo thereby facilitating the spillover of excitation energy from strongly fluorescent system 2 to weakly fluorescent system 1.

(3) The light induced turbidity changes in intact algal cells, as measured by the increase in optical density at 540 nm, is reduced in the presence of these salts. However, $MgCl_2$ produces the greatest reduction while Na acetate the least, even though both of these salts (at the concentrations used) cause large suppression of the fluorescence transient. Moreover, the light induced turbidity changes were, essentially irreversible.

(4) As high concentrations of salts increase the osmotic potential of the bathing medium, it seems that the osmotic changes as well as the ionic changes in the intact algal cells are responsible for the fluorescence quenching and changes in the mode of excitation transfer observed in this study. In the case of low concentrations of salts (e.g., 0.1 M Na or K acetate) the effects are predominantly ionic, and in the case of very high concentrations of $MgCl_2$ (0.4 M), the osmotic effects play a much larger role.

Upon illumination with saturating light, the dark adapted cells of *Chlorella pyrenoidosa* show characteristic fluctuations (labelled $O \rightarrow I \rightarrow D \rightarrow P \rightarrow S \rightarrow M \rightarrow T$) in the yield of chlorophyll *a* (Chl *a*) fluorescence (*1*). Parallel studies of Chl *a* fluorescence and oxygen evolution clearly indicate that the slow SMT type change is not directly dependent on the electron flow of photosynthesis or the redox state of Q (see review of literature in ref. *1*). Similarly, the slow PS decline of Chl *a* fluorescence has been shown to be independent of the redox state of Q (*2*).

Recent studies on light-induced chlorophyll fluorescence indicate that the PSMT fluorescence yield changes are regulated by the conformational state(s)

of chloroplasts associated with the energy conservation processes of photosynthesis (*1*; see ref. *3* and literature cited therein). Murata (*4*) and Bonaventura and Myers (*5*) concluded from their studies with system 1 and 2 preillumination that the slow change in the yield of Chl *a* fluorescence is represented as an adaptation toward an optimal distribution of absorbed photons between the two interacting pigment systems. Duysens (*6*) proposed that this adaptation is brought about by mutual microscopic movements of the two pigment systems.

The Q (redox) independent (DCMU-resistant) changes in Chl *a* fluorescence yield can be altered in isolated broken chloroplasts by the addition of various cofactors and cations (see e.g. ref. *7* and literature cited therein). It has been shown that the ion-induced changes in fluorescence yield in isolated chloroplasts correlate with the structural changes in chloroplast membranes (*8*). Addition of salts of mono and divalent cations are also known to cause both microscopic and macroscopic changes in chloroplast structure in situ (*9*). Thus, it appears that alteration of chloroplast structure regulates the chlorophyll *a* fluorescence (*1*).

In intact algal cells, Papageorgiou and Govindjee (*10*) and deKouchkovsky (*11*) showed that the pH of external medium influences Chl *a* fluorescence yield. These authors suggested that a structural change was involved in the regulation of this type of change in the fluorescence yield. DeKouchkovsky (*11*) and Mohanty and Govindjee (*12*) observed that salts like KCl cause a depression in the fluorescence yield of intact algal cells[1] in contrast to an increase observed in isolated chloroplasts. In this study we have extended these observations by using a variety of salts containing weak acid anions (e.g., acetate) and weak base cations (e.g., NH_4^+) and other ionic species (e.g., Na^+, K^+, Mg^{++}) to alter the ionic environment.

Materials and methods

The cells of the green alga *Chlorella pyrenoidosa* were grown autotrophically in continuous culture as outlined in ref. (*15*). Five or six day old cultures were harvested for use. Cells were washed 3 to 4 times in distilled water and suspended in dilute phosphate buffer (0.5 mM, pH 6.8) or distilled water and were kept stirred in darkness (for about 1 hr) before measurements. Small volumes of concentrated salt solutions (pH adjusted close to neutrality) were added in the dark and were allowed to equilibrate for at least 5–10 min before each measurement.

The details of the spectrofluorometer have been described in ref. (*16*). The procedures for measuring fast and slow fluorescence transients and the methods for the measurement of the emission spectra have also been outlined earlier (*17*, *18*). The emission spectra were corrected for the spectral variation of the spectrofluorometer. Absorption spectra were measured in a Bausch and Lomb Spectronic 505 Spectrophotometer equipped with an integrating sphere. The light-induced turbidity changes were measured as the changes in absorption at 540 nm using the split beam difference spectrophotometer of Sybesma and Fowler (*19*) as described earlier (*20*). Other details are given in the legends of the figures.

[1] The effect of 2–4 M NaCl on Chlorophyll *a* fluorescence was previovsly observed by Giraud (*13*) in *Rhodosorus* cells and of 0.1 to 1.5 M NaCl by Brody et al. (*14*) in *Euglena* fragments.

Results

Salt induced changes in the fluorescence yield

The fast fluorescence transient of *Chlorella* are known to depend on a variety of physiological factors, especially growth conditions of algae (*21*). For the experiments presented in this paper, the cells were grown under almost identical conditions, and for comparison of results with and without salts, identical dark periods were allowed before exposure to light of identical intensity and wavelength. Usually, the peak fluorescence was observed at 0.4 sec, and the S level reached around 3 sec of illumination; the P/S ratio, without salts, varied from 1.3 to 1.5 in different cultures, but had a unique value in one culture from which data of individual experiments were obtained. As the characteristic points (O, P, S) of the fast transient remained fairly constant for hours in one culture, we have presented the effect of salts as percentages of the untreated sample.

Fig. 1 shows the effect of KCl on the amplitude of O, and P and S in one sample. The percentage of quenching at P varied between 10 to 20%, depending on the exact culture conditions. The quenching occurred only at a relatively high concentration of salts. We did not observe any increase in the relative yield of fluorescence at any concentration of salts used. The quenching of Chl *a* fluorescence is greater at P than at S in the presence of KCl. The 'O' level varied very slightly, and in some measurements, it remained constant. NaCl yielded essentially the

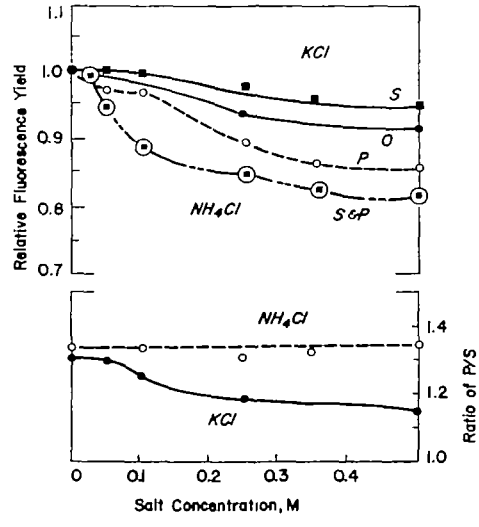


Fig. 1. *Salt induced fluorescence yield changes in* Chlorella: *effect of various concentrations of salts* (*KCl and* NH_4Cl) *on the P and S levels of the fast fluorescence transient.* Upper 3 traces, fluorescence yield changes with KCl at S (solid squares), at O (solid circles), and at P (open circles); curve with large open circles (with small solid squares inside) represents the changes in yield at both P and S with NH_4Cl (O level was not measured). Bottom traces, P/S ratio for KCl (solid circles); and for NH_4Cl (open circles); ordinate scale to the right. Fluorescence yields of cells, without salts, were adjusted to read 1.0 in the upper 4 curves. Excitation, blue light (C. S. 4–72, 3–73); intensity, 11 Kergs cm^{-2} sec^{-1}; λ observation, 685 nm (half band width, 6.6 nm). All additions were made in the dark.

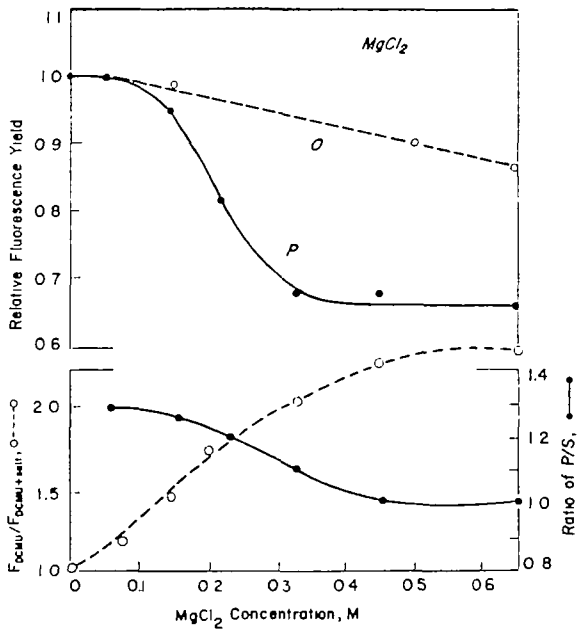


Fig. 2. *Salt induced fluorescence yield changes in* Chlorella: *effect of various concentrations of* $MgCl_2$ *on the O and P levels of the fluorescence transient.* Bottom curves, P/S ratio (solid dots), ordinate scale to the right; $F_{DCMU}/F_{DCMU+salt}$ (bottom curve with dashed line and open circles), ordinate scale to the left. All other details of measurement as in Fig. 1.

same results in the same concentration range. In the case of NH_4Cl, the extent of quenching remained the same at both P and S, i.e., the P/S ratio did not change. (However, in some of the cultures NH_4Cl affected P/S ratio. We have not investigated the cause of such variations.)

Fig. 2 shows the relative changes in the yield at O and at P levels with increasing concentration of $MgCl_2$. The effect of $MgCl_2$ was very similar to KCl but the former was more effective in lowering the yield than the latter. We did not observe any increase in yield at any concentration of $MgCl_2$. The relative yield at O declined only 9 to 10% while the P was suppressed approximately 30 to 35% of the untreated sample. The bottom solid curve shows the decrease in the P/S ratio with increasing concentrations of $MgCl_2$; a complete suppression of P to S decline occurred with 0.35 to 0.4 M $MgCl_2$. (The exact concentration of the salt necessary to suppress this decline varied with different cultures.)

The percent quenching of the variable fluorescence remained constant at all the light intensities used (2–14 Kergs cm^{-2} sec^{-1}) (see Fig. 61 in Mohanty, *22*). We know that the yield at P declines when the intensity is lowered to 40% of the maximum intensity used (*21*, *22*). The intensity independence of quenching by $MgCl_2$ suggests that this lowering of the yield is not associated with the primary acceptor (Q) of PS II. This suggestion is further verified from the observation of quenching in the presence of DCMU which keeps all Q in the reduced state at high intensity of illumination. With 0.35 M $MgCl_2$, we observed

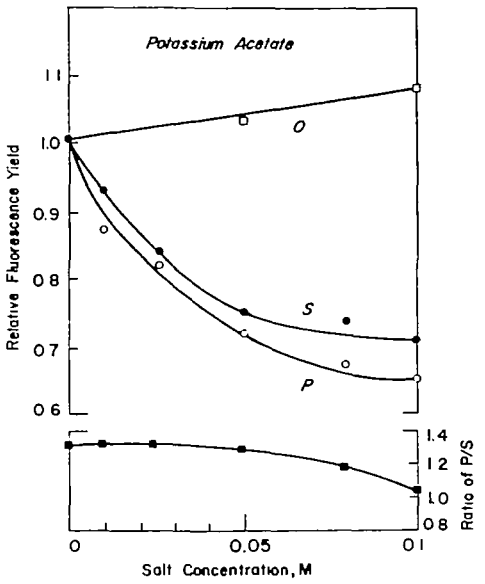


Fig. 3. *Effect of varying concentrations of potassium acetate on the O, P and S of the fast transient in* Chlorella. Bottom curve (solid squares), P/S ratio; ordinate scale to the right. Other details of measurement as in Fig. 1.

a 50–55% quenching. A plot of $F_{DCMU}/F_{DCMU+salt}$ versus the concentration of added $MgCl_2$ (Fig. 2 bottom curve with dashed line) indicated that quenching by $MgCl_2$ is not a simple dynamic quenching since a significant deviation from straight line (as predicted by the Stern-Volmer relationship) was obtained beyond 0.3 M $MgCl_2$. Thus, from our observations of quenching by KCl, NaCl, or $MgCl_2$ in the presence of DCMU, we conclude that fluorescence is not influenced by the reduction level of the primary acceptor Q, and that the quenching by the salts is not of a simple collisional type.

Crofts et al. (*23*), from their studies with various acetates (0.1 M) on isolated chloroplasts, have proposed a molecular mechanism for the structural alterations induced by salts of weak acids. Fig. 3 illustrates the effect of potassium acetate on the amplitudes of various characteristic points of the fast transient in *Chlorella*. Both the fluorescence yields at P and S were lowered, but the yield at P was more suppressed than at S. At 0.05 to 0.1 M K-acetate, the P to S decline was abolished. Injection of 15 μM DCMU to the potassium acetate-quenched samples caused an increase in the fluorescence yield; this yield was, however, always lower than the yield of normal samples treated with DCMU alone. Further addition of K-acetate quenched the fluorescence even in the presence of DCMU.

Fig. 4 shows the effect of 0.1 M NH_4-acetate and 0.1 M K-acetate on the OPS transient. K-acetate completely abolished most of the fast transient while the same concentration of ammonium acetate only hastened the D to P rise. The faster DP rise with NH_4-acetate could be due to a slowing down of the rate of electron transport. It may be argued that a suppression of the development of P by sodium and potassium acetate may be due to an acceleration of electron flow.

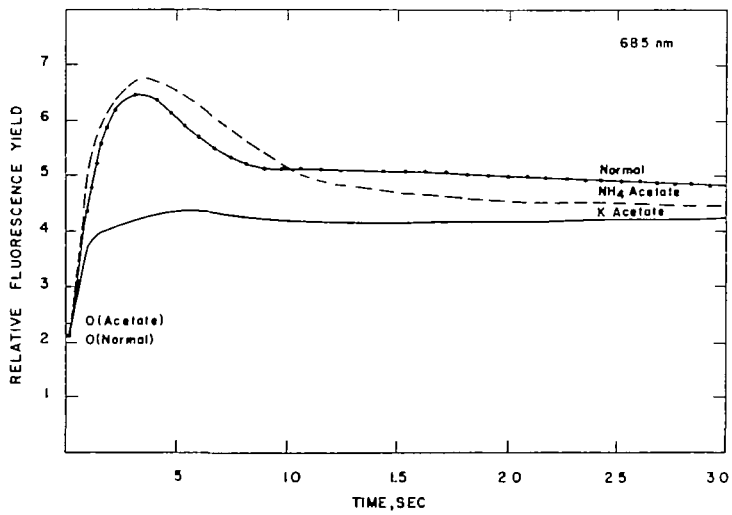


Fig. 4. *Time course of Chl a fluorescence yield of* Chlorella. λ observation, 685 nm (half band width, 5 nm); excitation, blue light (C. S. 4–72 plus C. S. 3–73); intensity, 14 Kergs cm^{-2} sec^{-1}; a C. S. 2–62 filter was used to guard the analyzing monochromator. Curve with solid circles, normal; dashed line, 0.1 M ammonium acetate; solid line, 0.1 M potassium acetate. Dark time, 7 min before illumination.

However, it seems quite unlikely that ammonium and sodium salts of acetic acid would cause entirely opposite effects on the electron flow. NH_4Cl did not alter the rate of DP rise to any measureable extent as compared to KCl (not shown). Furthermore, from the data in Table 1 (top), it is clear that sodium benzoate and sodium acetate also lower the fluorescence yield even in the presence of DCMU although to a lesser extent than $MgCl_2$. Thus, these data indicate that lowering of P by salts of sodium or potassium acetate could not be due to a change in the rate of electron transport. The lowering of fluorescence yield by benzoate, acetate or other salts in the presence of DCMU may also be interpreted as being due to an acceleration of "back" reactions between the oxidized primary donor (Z^+) and the reduced acceptor (Q^-), as these salts are known to stimulate delayed light emission (*24*). But, in these experiments, salts are added after the illumination to cause a shift or jump in the ionic environment between the inside and outside of chloroplast membranes. In our experiments, salts were added in the dark before illumination. We believe that the cause of the fluorescence yield decrease by the addition of salts is not due to a stimulation of back reaction, as the extent of quenching remained the same at all intensities of illumination.

Since the lowering of fluorescence was observed with relatively high concentration of several salts (Table 1, bottom) these observed effects could be ascribed to high osmotic potentials of the bathing medium. The addition of 0.6 M sorbitol ($\sim$ 15 bars) only slightly depressed the yield at P and did not change the PS decline. However, 1.2 M sorbitol ($\sim$ 30 bars) did significantly lower the fluorescence yield and suppress the PS decline (not shown). Thus, the effects of 0.1 M acetate ($\sim$ 9 bars), 0.25 M $MgCl_2$ ($\sim$ 18 bars) and 0.4 M KCl ($\sim$ 20 bars) could not be due to

Table 1. A. *Effect of sodium acetate and sodium benzoate on the steady state fluorescence yield in (10 μM) DCMU poisoned* Chlorella

Concentration	Relative fluorescence yield
10 μM DCMU (control)	1.0
+100 mM Na-acetate	0.94
+200 mM Na-acetate	0.74
+200 mM Na-benzoate	0.83

B. *Effect of the addition of 0.1 M salts on the yield of fluorescence in* Chlorella

Salts	Relative fluorescence yield at *P*	Ratio P/S
No addition	1.00	1.3 to 1.50
KCl	0.97	1.25
NaCl	0.98	1.27
NH_4Cl	0.90	1.30
K-acetate	0.65	1.00
Na-acetate	0.60	1.00
Na-benzoate	0.70	1.00
NH_4-acetate	1.04	1.50

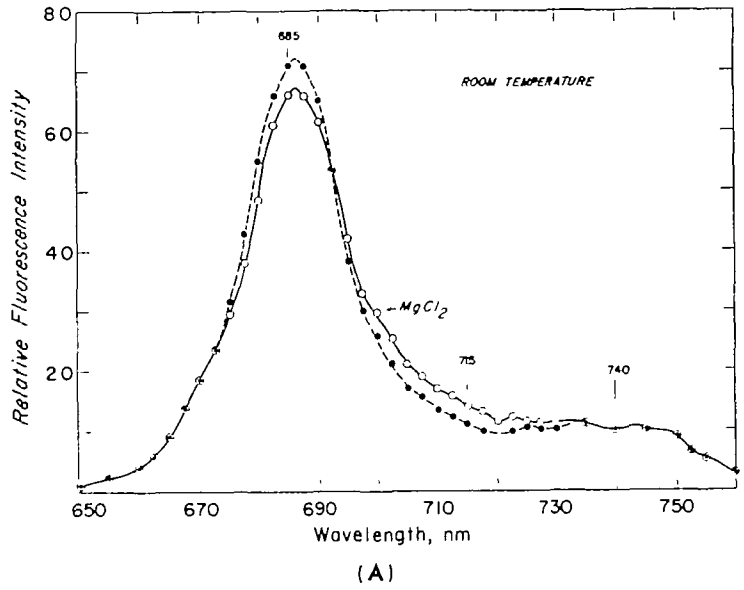
Fluorescence was measured at 685 nm; excitation, broad band blue light with intensity~14 Kergs cm^{-2} sec^{-1}; salts were added 5–7 min before measurements.

osmotic effects, while the effect of 0.4 M $MgCl_2$ (~ 30 bars) could be partly due to these effects.

Spectral changes in the presence of salts

Murata (*25*) and Mohanty et al. (*7*) have shown that, in isolated broken chloroplasts, salt-induced fluorescence yield enhancement is also accompanied by spectral changes in the emission characteristics both at room temperature and at 77°K. The ratio of F720 (Chl a_1)/F685 (Chl a_2) at room temperature was increased by the addition of $MgCl_2$ (Fig. 5A). As Chl a_1 is weakly fluorescent, this causes an overall decrease in the yield. The same ratio at liquid nitrogen temperature increased from 0.9 to 1.4 by the addition of $MgCl_2$. This indicates that $MgCl_2$ increases energy transfer from system 2 to system 1 in vivo.

Fig. 5B shows the emission spectra measured at liquid nitrogen temperature (77°K) with and without potassium acetate; F720 is enhanced with respect to F685 in potassium acetate treated samples. Similar results were obtained with sodium acetate. In these cases, 15 μM DCMU was added to the samples to avoid the complication of spectral changes that may be associated with SMT changes during illumination before freezing. However, we obtained almost similar results when DCMU was omitted. These results indicate that the relative decrease in the yield of Chl *a* fluorescence of system 2 (Chl a_2) may indeed be due to an increase in energy transfer from PS 2 to PS 1.



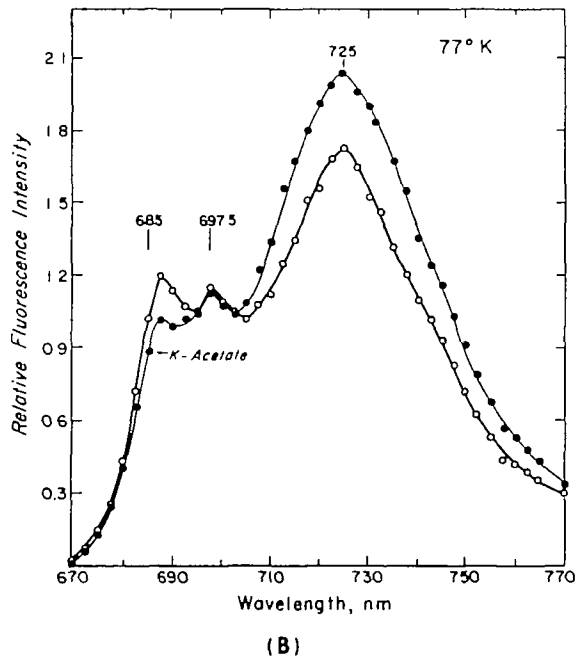


Fig. 5. (A) *Room temperature emission spectra of* Chlorella *with and without* $MgCl_2$ *(normalized at 660 nm).* Solid circles, untreated cells; open circles, 0.3 M $MgCl_2$; excitation, broad-band blue light (C. S. 4–72 plus 3–73); intensity, 14 Kergs cm^{-2} sec^{-1}; λ observation, variable (half band width, 5 nm). A Corning C. S. 2–64 filter was placed before the analyzing monochromator. Fluorescence, measured 5 min after illumination. (B) *Emission spectra of chlorophyll* a *fluorescence of* Chlorella *at 77°K.* Open circles, 15 μM DCMU; solid circles, 15 μM DCMU plus 0.1 M potassium acetate. Both samples were preilluminated before cooling. λ excitation, 436 nm (half band width, 10 nm); C. S. 2–58 filter before the photomultiplier.

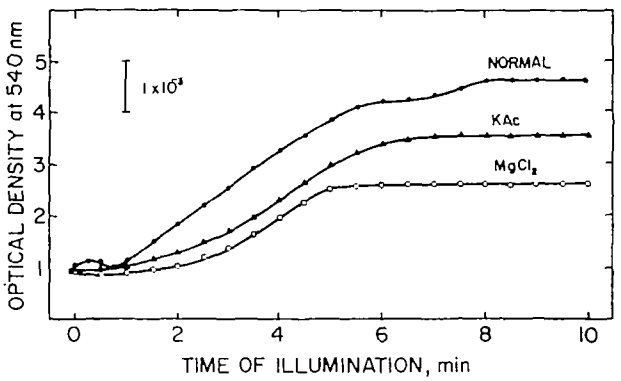


Fig. 6. *Time course for the increase in "optical density" at 540 nm.* Slit width, 6.6 nm; C. S. 4–72 filter before the photomultiplier; sample was illuminated with red actinic light (Schott R. G. 645; intensity, 30 Kergs cm^{-2} sec^{-1}). Dark preincubation times: 10 min with 0.4 M $MgCl_2$ and 60 min with 0.1 M K-acetate. Absorbance of the red chlorophyll *a* peak=0.045.

Changes in "optical density" at 540 nm

Shrinkage (and swelling) of chloroplasts lead to turbidity changes which can be monitored by the changes in absorbance (or transmission) at 540 nm (*9*, *23*). In intact algae, an increase in absorbance (decrease in transmission) has been attributed to shrinkage of the chloroplast in vivo (*26*).

Fig. 6 shows a typical signal for normal *Chlorella* cells and for cells treated with 0.4 M $MgCl_2$ (10 min incubation) and with 0.1 M K-acetate (60 min incubation). All of the salts gave approximately similar kinetics. There was an initial lag for 30–90 sec followed by a slow rise to a final steady state level in 5–6 min. The light induced changes both in the control and treated samples were, for the most part, irreversible. $MgCl_2$ produced the greatest decrease (about 56%) in the 540 nm change. With a 10 min preincubation period 0.1 M K-acetate produced only a small change (5%) in the 540 nm signal, although under the same conditions the effect on fluorescence was relatively large. An increase in K-acetate concentration to 0.4 M still resulted in only a 6% reduction in the signal. However, an increase in the dark incubation time from the usual 7–10 min to 60 min resulted in a 30% reduction in the signal by 0.1 M K-acetate.

For each sample, the signal was adjusted to read zero in the dark; therefore, the effects of salts on the dark level of turbidity could not be reported here.

Discussion

As noted earlier, the additions of 0.25-0.4 M KCl or $MgCl_2$ or 0.1 M K-acetate or Na-acetate cause a lowering of fluorescence yield in *Chlorella* (Fig. 1–4). This result is in contrast to salt-induced increases in fluorescence yield observed with isolated chloroplasts. Unlike the case in isolated chloroplasts (*7*, *25*), low concentrations of KCl or $MgCl_2$, added to the suspending medium, have no effect on the normal fluorescence transient or on the fluorescence yield of DCMU treated intact cells of *Chlorella*. Salts containing weak base cations, such as NH_4-acetate,

did not appreciably alter the fluorescence transient characteristics— slight elevation of the P level was observed (Fig. 4). NH_4Cl lowered the fluorescence yield at P but did not significantly affect the P/S ratio (Fig. 1), although in some cases NH_4Cl did cause a suppression of the PS decline. The ionic make up of the suspending medium, therefore, appears to be important for salt-induced fluorescence yield changes. Apparently, not only the cations but also the anions are responsible for the salt effects.

Although the concentration of salts or ions inside the vicinity of chlorophyll molecules in the chloroplasts is unknown, it must be lower than in the suspending medium. The high salt concentration of the suspending medium must certainly exert large osmotic effects on the cells. However, the changes observed in this study are not entirely due to osmotic effects since changes induced by sorbitol occurred at a concentration (1.2 M) which produces an osmotic potential comparable only with the high $MgCl_2$ concentration (0.4 M).

As shown in Table 1 increase in the Na-acetate concentration induced further lowering of the fluorescence yield even in the presence of DCMU which blocks all electron flow between the two photosystems. Also, the extent of quenching of fluorescence at P by $MgCl_2$ remained independent of the incident light intensities. These results strongly indicate that the salt induced changes of the fluorescence yield and the suppression of the PS decline are not directly related to electron flow and the redox state of Q.

The salt induced lowering of fluorescence yield accompanies a change in the emission spectra (Fig. 5), both with and without DCMU, in the opposite direction to what has been observed with isolated chloroplasts (*25*). The salt induced change may be, at least, in part, due to a change in the mode of excitation energy transfer between the two pigment systems.

In intact *Chlorella* cells a light induced increase in absorbance at 540 nm is reduced upon the addition of salts (Fig. 6). Although the changes are irreversible, they most likely reflect gross structural alterations of thylakoid membranes of the cells. In some cases, salt-induced fluorescence lowering is, roughly, inversely related to these volume changes, but in the case of high concentration of $MgCl_2$ it is not. The intimate conformational changes of the two photosystems, not observable at 540 nm in whole cells, most likely regulate Chl *a* fluorescence.

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