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SALTS AND CHLOROPLAST FLUORESCENCE*

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SUMMARY

Chloroplast fluorescence was excited by a weak measuring beam. A time-separated actinic light was used to modify the redox states of Q which in turn induced a change in the fluorescence yield. In salt-depleted chloroplasts, fluorescence saturated at a low actinic light intensity. $CaCl_2$ increased the "variable" fluorescence as well as the rate of ferricyanide–Hill reaction. With Tris-washed chloroplasts, Photosystem II donor couple, phenylenediamine and ascorbate, did not increase the fluorescence to a large extent without the presence of $CaCl_2$. It is suggested that salt-depletion inactivates the Photosystem II reaction center of chloroplasts.

INTRODUCTION

Homann [1] showed that cations increased the variable fluorescence. Then, on the basis of divalent ion-enhanced dichlorophenol indolephenol–Hill reaction, fluorescence, and ratio of low temperature fluorescence peaks at 685 and 730 nm, Murata [2] postulated that divalent cations acted on steps which regulated energy spilling over from Photosystem II to Photosystem I. Sun and Sauer [3] supported his model and added a revised one which supposed $MgCl_2$ induced a spillover of energy from System I to II. However, the observation of Ruranski et al. [4] that $MgCl_2$ improved the short wavelength quantum yield of NADP reduction could not be explained by the spillover hypothesis. It seems to us salt may act on other sites in chloroplasts as well.

We have studied the salt effects on fluorescence with a phosphoroscope in which white actinic flashes are provided to modify the redox state of Q, fluorescence is excited with a weak monitoring beam and observed between flashes. And it is found in salt-depleted chloroplasts, Q cannot be reduced by actinic flashes. The results are interpreted in terms of changed properties of the System II centers in the absence of salts.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholine)ethanesulfonic acid; Q, hypothetical primary electron acceptor of Photosystem II.

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MATERIALS AND METHODS

Chloroplasts isolated from 12–14 day old oat plants were used. The isolation method of Izawa and Good [5] consisted of disrupting the leaves with a blender in a medium consisting of ethylenediamine tetraacetic acid (1 mM), NaCl (400 mM), and sodium phosphate, pH 7.3 (50 mM). After removal of cell debris by straining through cheesecloth, the chloroplasts were sedimented by low speed centrifugation and "washed" with sodium tricine, pH 7.3 (50 mM) containing sucrose (150 mM). The reaction mixtures are described in the legends of figures and tables.

Chlorophyll concentration was determined spectrophotometrically by the method of Mackinney [6].

Measurement of fluorescence emission

The phosphoroscope instrument described by Hoch [7] was used. In this instrument a monochromatic beam (480 nm) of 15 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} was incident on the sample. This beam was obtained from a monochromator supplemented with auxiliary blue glass filters. The emission from the sample (contained in a 1-cm^2 quartz cuvette) was measured by a photomultiplier tube through a 680-nm interference filter. The emission was viewed at 180° to the exciting beam; however, the chloroplast suspensions used were optically thin, so that self-absorption of the emitted fluorescence was minimal.

Actinic light was supplied to the sample through the phosphoroscope disk as flashes at a frequency of 1080 flashes per s. The disk shuttered the photomultiplier tube during these flashes and opened between them. Hence, the emission from the sample was recorded from about 0.3 to 0.7 ms after each flash. This emission consisted of fluorescence from the measuring beam and delayed emission from the sample caused by the actinic light. Neither the actinic light, nor the fluorescence from this light, was recorded. The delayed emission could be recorded by turning off the monochromatic fluorescence exciting beam. The fluorescence was then obtained by subtracting this from the total photosignal. Thus the fluorescence signal represented changes in the fluorescence yield of the measuring beam caused by actinic light. Emission measured in the presence of actinic light is defined as light fluorescence (fl). Except otherwise mentioned, all values of fluorescence were taken at 4 min after the onset of actinic light. Light fluorescence did not change much with time. Emission measured in the absence of actinic light is defined as dark fluorescence (fd). Two types of dark fluorescence were measured: dark fluorescence before turning on actinic light (fdb) and dark fluorescence after turning off the actinic light (fda), since some time was required for fluorescence to relax to a dark steady-state level, the emission at 10 s after the cessation of actinic light was chosen as the value of dark fluorescence after turning off the actinic light.

Hill reaction

Ferricyanide reduction rates were measured with the same instrument by observing the changes in transmission at 420 nm. All rates were computed at about 1 min after the onset of illumination. Low intensity illumination was provided by the time-separated actinic light. Scattering effects of the reversible volume change might be minimized by the alternating dark and light periods. Strong illumination was

provided by an auxiliary light source which was color separated from the measuring beam. System II light was isolated by a wideband interference filter transmitting between 530 and 650 nm. Scattering effects were not eliminated; however, the $CaCl_2$ effects on the Hill rates were confirmed by measuring O_2 evolution as well as proton production as a result of ferricyanide reduction according to the following equation;

$$2H_2O + 4Fe(CN)_6^{3-} \xrightarrow[\text{chlorophyll}]{h\nu} O_2 + 4Fe(CN)_6^{4-} + 4H^+ \quad (1)$$

RESULTS

(*A*) *Fluorescence and* $CaCl_2$

Fig. 1 shows, at the origin, the dark fluorescence is slightly increased by the addition of $CaCl_2$. In the absence of salt, the light fluorescence saturates at low light intensity and at a low level. A 4-fold increase of light fluorescence results from the same actinic illumination in the presence of $CaCl_2$. This increase is almost completely suppressed by the addition of ferricyanide. The suppression indicates that both salt and closed reaction centers (in this case induced by actinic illumination and lack of Hill oxidant) are necessary for a large increase in fluorescence yield, hence almost all of the $CaCl_2$-induced fluorescence increment belongs to the variable fluorescence [8]. The actinic light intensity dependence of the yield in the absence of ferricyanide may result from a limited use of oxygen as a Hill oxidant. It is interesting to note that $CaCl_2$ is both capable to promote the photochemistry (Fig. 4) and, at the same time, to intensify the light fluorescence (Fig. 1).

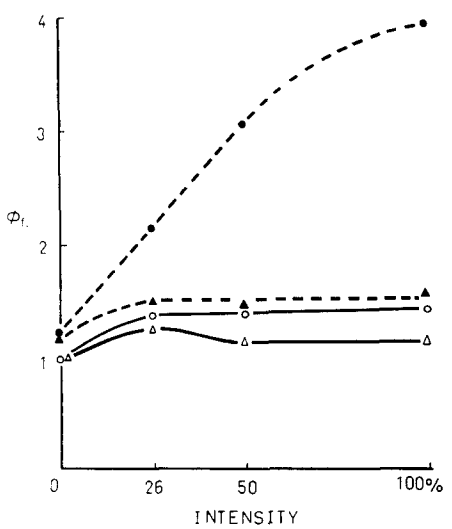

Fig. 1. Relative light fluorescence yield as a function of actinic light intensity. The reaction m xture contained: chloroplasts at a concentration of 5 μg chlorophyll per ml; sucrose, 150 mM; HEPES, 50 mM (pH 8.05). The measuring beam intensity was 15 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} and the actinic light was $2 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. The wavelength of the measuring beam was 480 nm and white light was used for actinic illumination. ○—○, no addition; △—△, ferricyanide (0.1 mM); ●—●, $CaCl_2$ (1.25 mM); ▲—▲, $CaCl_2$ (1.25 mM) plus ferricyanide (0.1 mM).

The important observation is that in salt-depleted chloroplasts, the light fluorescence fails to respond to the increase of light intensity which indicates that light cannot keep a large amount of Q in a reduced state. Nor can the System II inhibitor DCMU keep Q reduced in a large amount in the absence of salt (Fig. 2).

Fig. 3 shows that the addition of 20 mM NaCl increases the light fluorescence some, however, additional effect is due to Ca^{2+} which saturates at about 1.4 mM.

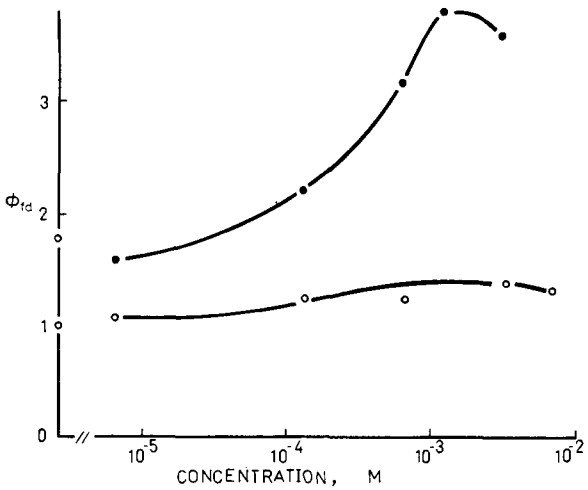

Fig. 2. Relative dark fluorescence yield as a function of $CaCl_2$ concentration. The reaction mixture contained: chloroplasts at a concentration of 8 μg chlorophyll per ml; sucrose, 150 mM; Tris–HCl, 38 mM (pH 7.7). The measuring beam intensity was 15 ergs · cm^{-2} · s^{-1}. The wavelength of the measuring beam was 480 nm. ○—○, no addition; ●—●, DCMU (5 μM).

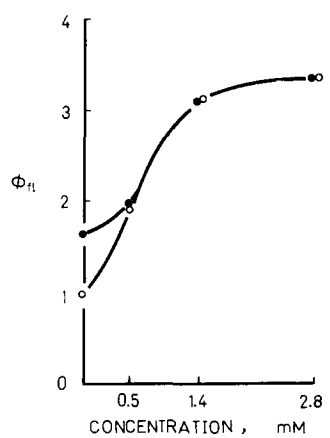

Fig. 3. NaCl, $CaCl_2$ and light fluorescence. The reaction mixture contained: chloroplasts at a concentration of 5 μg chlorophyll per ml; sucrose, 150 mM; HEPES, 50 mM (pH 8.05). The measuring beam intensity was 15 ergs · cm^{-2} · s^{-1} and actinic light was $2 \cdot 10^4$ ergs · cm^{-2} · s^{-1}. The wavelength of the measuring beam was 480 nm and white light was used for actinic illumination. ○, no addition; ●, 20 mM NaCl. The concentrations of $CaCl_2$ are indicated on the abscissa.

TABLE I

EFFECT OF DIVALENT IONS ON FLUORESCENCE

The reaction mixture contained: chloroplasts at a concentration of 5 μg chlorophyll per ml; sucrose, 150 mM; MES, 50 mM (pH 5.9). The low pH used in this experiment is to avoid precipitation of certain salts. The illumination conditions are the same as described in Fig. 1. fdb, dark fluorescence before illumination. fda, dark fluorescence after illumination. fl, light fluorescence.

Salts	Concentration (mM)	fdb	fda	fl	
				1 min	4 min
$Cr(NO_3)_2$	2.5	0.86	1.16	1.88	1.7
$Pb(NO_3)_2$	2.5	0.86	0.69	0.89	0.79
$ZnSO_4$	2.5	0.87	0.84	1.77	0.99
$Cd(NO_3)_2$	2.5	0.93	0.89	1.54	1.02
$Co(NO_3)_2$	2.5	0.94	1.38	2.92	2.54
No addition		1	1.14	1.5	1.43
$CaCl_2$	2.3	1	1.4	3.31	3.45
$MnCl_2$	2.3	1.01	1.38	3.03	3.11
$MgSO_4$	3.3	1.13	1.6	2.6	2.5
$MgCl_2$	2.1	1.16	1.6	3.37	3.37
$FeSO_4$	1.9	1.28	1.38	2.28	1.77

The effect is not Ca^{2+} specific, Co^{2+}, Mn^{2+}, Mg^{2+} are also efficient. The combination of chloride with one of these divalent cations seems to be most efficient (Table I).

(B) Hill reaction and $CaCl_2$

The quantum yield of ferricyanide reduction in System II light was greatly increased by the addition of salt to the reaction mixture, hence the high fluorescence in salt-supplemented chloroplasts was not due to retarded photochemistry. Fig. 4 shows that about a 5-fold increase in the slope of the velocity vs intensity plot was observed after salt addition. It appears as if the saturation rate was also increased by the salt.

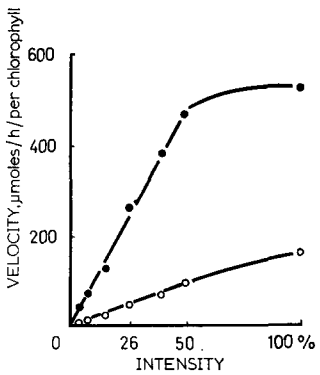

Fig. 4. Effect of $CaCl_2$ on yield of the ferricyanide–Hill reaction. The reaction mixture contained: chloroplasts at a concentration of 12.5 μg chlorophyll per ml; sucrose, 150 mM; HEPES, 50 mM (pH 8); ferricyanide, 0.2 mM. Illumination of 650 nm was employed and the intensity reduced by neutral density screens. The time course of the reaction was followed for 2–3 min. ○ — ○, no addition; ● — ●, 2.5 mM $CaCl_2$.

(C) $CaCl_2$ effects on Tris-washed chloroplasts

With low fluorescence and low photochemistry, the salt-depleted chloroplasts are similar to the Tris-washed chloroplasts which may have impaired water splitting system [9]. To differentiate the site of action of the salt-depleted chloroplasts from that of Tris-washed chloroplasts, we tested the calcium effects on the Tris-washed chloroplasts in the presence of chloride. Table II shows that the fluorescence yield of Tris-washed chloroplasts depends again on the presence of Ca^{2+}. The low fluorescence observed in divalent cation-depleted and Tris-washed chloroplasts is not primarily due to damaged water splitting system, because it is calcium, not Photosystem II donor couple, phenylenediamine and ascorbate, which induces a large fluorescence increment. Maximal fluorescence is only possible in the presence of both Ca^{2+} and an electron donor. This observation shows that divalent ion may act at a site where both water and artificial donors supply their electrons. The Photosystem II reaction center is a possible site where Ca^{2+} acts.

TABLE II

$CaCl_2$ EFFECTS ON THE FLUORESCENCE YIELD OF TRIS-WASHED CHLOROPLASTS

Chloroplasts are suspended in a medium contained: sucrose, 150 mM; NaCl, 10 mM; HEPES, 50 mM (pH 8) and reagents as mentioned in the table. The illumination conditions are the same as described in Fig. 1. fdb, dark fluorescence before illumination. fl, light fluorescence.

Addition	Concentration (mM)	fdb	fl
No addition		62	72
$CaCl_2$	1.14	65	110
Phenylenediamine, ascorbate	0.02 0.2	62.5	84
Phenylenediamine, ascorbate, $CaCl_2$	Same as above	65	144

DISCUSSION

The dark fluorescence emitted from chloroplasts with open state photosynthetic units, because of its independence of the presence of Hill acceptors (Fig. 1) may be identified with the constant fluorescence of Lavorel [8]; the photochemistry-dependent light fluorescence may be the sum of the constant and the variable fluorescence. The non-proportional $CaCl_2$ effects on the light and on the dark fluorescence and on the emissions at different wavelengths (less effective on long wavelengths, unpublished results) favors a combination of the two yields and the two emission hypotheses of Lavorel [8] proposed to handle the presence of two fluorescence components, a variable and a constant fluorescence in organisms like *Chlorella*. $CaCl_2$ may affect only one of the two types of emitters, and it changes the emitter's property.

Undoubtedly, the low fluorescence and photochemistry in salt-depleted chloroplasts is due partially to the Cl^- deficiency which may interrupt the electron transport between water splitting system and Photosystem II reaction center [10]. The divalent cation is not likely acting at the same site as the Cl^- because Photosystem II donors do not release the detrimental effect of salt depletion on the chloro-

plast fluorescence (unpublished results). Other site of action of divalent cations must be considered.

The fact that the increased actinic light intensity fails to increase the light fluorescence is not in accord with the "spillover" hypothesis of salt effect. The spillover hypothesis states that in the absence of salt, part of the energy absorbed by System II spills over to System I; salt prohibits the spillover, therefore, more energy can be utilized by System II for fluorescence or for photochemistry. In the kind of experiment shown in Fig. 1, the spillover hypothesis predicts that an increase in light intensity should compensate the spillover effect on the chloroplast fluorescence in the absence of salt. The idea is more expressively shown in Eqn 2:

$$E_{II} = C \cdot \text{light intensity } (\alpha_{II} - \alpha_{II} \cdot S) \tag{2}$$

where E_{II} is the amount of energy flows to System II trap and utilized for the reduction of Q, C is a collective term of all other factors which determine the amount of energy arriving and utilized at System II traps, α_{II} is the amount of light absorbed by System II chlorophylls and S, the fraction of light absorbed by System II and spillovers to System I. It is obvious that E_{II} can be increased by either an increase in light intensity or a decrease in S. However, Fig. 1 shows, in the absence of Hill acceptors, a quadruple increase of light intensity produces no effect on light fluorescence at all. Apparently, the "spillover" hypothesis cannot explain the low fluorescence yield of salt-depleted chloroplasts.

The observation that $CaCl_2$ simultaneously stimulated both the rate of the ferricyanide–Hill reaction and the fluorescence yield indicates the necessity of a modification of the law of "fundamental alternative" [11], which proclaims the existing of a conservative linear relation between the photochemical yield ϕ_{ps} and the fluorescence yield ϕ_f,

$$\phi_{ps} + \phi_f/\beta = 1 \tag{3}$$

the fluorescence is assumed to be a constant proportion (β) of all non-photochemical losses. However, the observed parallelism of photochemistry and fluorescence demands a change of the value of β, if Eqn 3 remains to be valid under both salt-depleted and salt-supplemented conditions. It then follows that fluorescence is not a constant proportion of all non-photochemical losses; in salt-depleted chloroplasts β is small. Ironically, because of the fluorescence yield in the absence of photochemistry is a measure of the life time of the chlorophyll's first excited states, the correspondence of low photochemistry with a low fluorescence yield, together with the finding that, under any particular set of condition, the photochemistry and the fluorescence are still competitive as shown by the quenching of light fluorescence with ferricyanide (Fig. 1, triangles), the parallelism is actually reinforcing the well documented assumption that photochemical conversion (System II) is competing with the natural decay of the first excited state of chlorophyll, on which Eqn 3 rests; it also shows the generality of Eqn 3, in spite of the difficulty it imposes on the latter. In salt-depleted chloroplasts, the low fluorescence suggests a dominance of an intersystem crossing of the excited energy into triplet state over fluorescence or photochemistry. The possibility of reversing the order of the energy levels of the n–π and π–π singlets makes a change in the proportion of intersystem crossing feasible. We may envision that, in salt-depleted chloroplasts, the first excited n–π singlet of most of

the reaction center chlorophylls lies below the π–π singlet level, consequently, the excitation will be transferred to the n–π singlet which increases the chance of triplet state and transforms the center into a wasteful (?) energy sink ("inactivation" hypothesis). In salt-supplemented chloroplasts, the trapped quantum will either photoreduce Q or, in a closed unit, fluoresce at the center, or transfer to other units, or fluoresce from bulk chlorophylls. The inactivation may not be an all or none phenomenon; depending on the relative levels of the n–π and π–π singlets, which may govern the lifetime of the singlet, the quantum yields of chemistry or fluorescence may be affected in varied degrees ("centerinactivation or -partial inactivation" hypothesis or "inactivation" hypothesis for short). Franck [12] gave an analysis of the relationship between chlorophyll fluorescence and its n–π electronic system and then suggested that external agents induced changes of water content of the protein in the chloroplast membrane might cause changes in quantum yields of photosynthesis and fluorescence. Ruranski et al. [4] reached the same conclusion from their NADP reduction and fluorescence studies. The concept of activation was previously discussed by Joliot [13] and by Bannister and Rice [14] concerning the induction phenomena in algae.

The suggestion that the observed phenomena are mainly controlled by the reaction center chlorophylls is based on two reasons: firstly, the dark fluorescence emitted from open units, which may reflect the properties of both the Photosystem I and Photosystem II bulk chlorophylls, representing the inevitable loss of energy due to imperfect energy transfer among bulk chlorophylls, is only slightly increased by adding salts, while the variable fluorescence and photochemistry which are functions of both the energy flow of the bulk chlorophylls and the activities of reaction centers are strongly affected by the addition of salts; secondly, the reaction center may be more susceptible to the environmental changes.

The salt-induced far red light enhancement phenomenon in isolated chloroplasts observed by Sun and Sauer [3] can also be understood on the basis of salts-activated System II units. In red light, the salt-depleted chloroplast activities are limited by both a lack of active System II units and System I activities. Far red light will not enhance the overall photosynthetic activities because the System II activities are low. Salts increase the number of active System II units and render the System I as the remaining limiting factor in red light. Far red light releases this bottle-neck and makes enhancement possible.

In conclusion, it is proposed that the major effect of salts in salt-depleted chloroplast is to activate the Photosystem II centers; salts may also change the spillover properties [2, 3]. The salt effects on electron transport we observed here are not likely related to the finding of McEvoy and Lynn [15] that divalent ions increase the internal concentration of oxidant, for the present data show that both electron transport rate and the fluorescence intensity are simultaneously increased by salts. Evidently, salts affect chloroplasts in more than one way, the nature of their effects may depend on the states of the photosynthetic apparatus. To understand and to relate the diversified salt-dependent phenomena such as the coupling state of chloroplasts [16–21] the electron transport-dependent conformation changes [5, 22, 23] and the fluorescence changes [1, 2, 23–25] may be helpful for the understanding of the nature of Photosystem II and, perhaps, the mechanism of photophosphorylation.

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