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THE INFLUENCE OF MAGNESIUM ON THE CHLOROPHYLL FLUORESCENCE YIELD OF ISOLATED CHLOROPLASTS

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SUMMARY

Isolated chloroplasts of *Euglena* show the slow increase of fluorescence yield upon magnesium addition frequently reported for higher plant chloroplasts.

1. This response does not appear to be associated with either the increased Photosystem II electron transport induced by magnesium ions, or the magnesium-induced changes in light scattering, as both processes have a higher magnesium requirement than the fluorescence response.

2. The full magnesium effect was observed in the presence of sufficient dithionite to reduce the electron transport components, indicating that electron transport is not necessary.

3. 3-(3,4,-Dichlorophenyl)-1,1-dimethylurea induced a slow decline in fluorescence which was stimulated by similar concentrations of magnesium as are required to induce the maximal fluorescence stimulation.

4. Both trypsin and glutaraldehyde treatment prevented the magnesium effect, indicating that protein structural changes are involved.

5. Removal of coupling factor from spinach chloroplasts did not influence the magnesium response.

6. Fluorescence induction kinetics indicate a close relationship between the pool of reduced primary electron acceptor, Q, and the fluorescence response to magnesium. Only when Q was substantially reduced, either chemically or photochemically, was the fluorescence effect evident.

It is concluded that the increased fluorescence yield is not due to magnesium interfering with the "spillover" between Photosystem II and Photosystem I, but is probably caused by an increase in Photosystem II fluorescing units, which is brought about by magnesium-induced protein structural changes when the primary electron acceptor pool is reduced.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

INTRODUCTION

In recent years, considerable interest has been shown in the suggestion, first put forward by Murata [1], that divalent cations, and particularly Mg^{2+}, may have an important function in regulating the energy "spillover" between Photosystem II and Photosystem I. Murata, in particular, working with spinach chloroplasts, suggested that magnesium decreases the rate constant for energy transfer between Photosystem II and Photosystem I, thus giving rise to an increased Photosystem II fluorescence yield. Support for this interpretation came from the observation that Mg^{2+} decreased the reduction of NADP with reduced 2,6-dichlorophenolindophenol (DCIP) as the electron donor increased the reduction of ferricyanide, and at liquid nitrogen temperature the PSII fluorescence yield was enhanced, while Photosystem I fluorescence decreased. Furthermore Murata [2] suggested that the light scattering changes observed upon the addition of Mg^{2+} are indicative of structural changes associated with this putative interruption of "spillover". Support for this general interpretation has recently come from Mohanty et al. [3], who reported that Mg^{2+} failed to influence the fluorescence yield of Photosystem II particles. However Rurainski and Hoch [4] do not favour this interpretation, largely on the basis of the constancy of the apparent fluorescence relaxation time in the presence and absence of Mg^{2+}, in pea chloroplasts. They consider that this argues against an increased velocity for the System II reactions, required by the Murata model, and instead favours the possibility that Mg^{2+} increases the number of fluorescing units. Moreover, we have recently demonstrated in *Euglena* chloroplasts, which display the characteristic fluorescence rise in response to Mg^{2+}, [5] that under certain conditions, Photosystem II-absorbed quanta are efficiently utilised in the Photosystem I reduction of methyl viologen with the DCIP–ascorbate electron donor system. Despite this efficient "spillover", however, Mg^{2+} did not influence the rate of methyl viologen reduction, and it was concluded that the fluorescence rise was not related to any interruption of "spillover" [5].

In this paper we report further experiments on the Mg^{2+}-induced fluorescence rise in *Euglena* and spinach chloroplasts, which suggest that while electron transport from Photosystem II is not necessary for the response, the primary electron acceptor, Q, needs to be in a reduced state.

METHODS

Class II chloroplasts were extracted from *Euglena*, cultured in the manner previously described [5]: cells were broken in a French press at 1000 lb/inch^2 in a 0.05 M Tricine buffer, pH 7.8, containing 0.1 M NaCl and 0.2 M sucrose. The resulting solution was centrifuged briefly at approximately $300 \times g$ to remove unbroken cells and the supernatant was then spun at $1500 \times g$ for 5 min to precipitate the chloroplasts, which were resuspended and washed once in a 0.05 M Tricine buffer pH 7.6, containing 10 mM NaCl and 0.4 M sucrose, and finally resuspended in the same buffer. All reactions were performed in this buffer. Continual microscope checks were made to ensure that almost all unbroken cells were removed.

Fluorescence measurements were routinely made in a Perkin–Elmer MPF-3 spectrofluorimeter with 440 nm exciting light of approximately 10 000 ergs $\cdot$ cm^{-2} $\cdot$

s^{-1} intensity and monitored at 688 nm. Steady-state fluorescence measurements were made with rapid stirring from above. The chlorophyll concentration of the chloroplast suspension was usually between 1 and 2 μg/ml. Fluorescence induction experiments were made with another instrument, in which an oscilloscope equipped with memory storage was attached to the photomultiplier, which was situated at an angle of 90° to the direction of the exciting beam. Exciting light was filtered through a Corning CS 4-96 filter and gave an intensity of 16 000 ergs · cm^{-2} · s^{-1}. Fluorescence was measured at 681 nm with a Balzers interference filter placed against the photomultiplier. The time constant of the instrument was 3 ms, which is the opening time of the shutter.

Light scattering measurements were made in the same instrument as the fluorescence induction kinetics, except that 535 nm Balzers filters were used for both incident and measurement light beams.

For experiments involving EDTA uncoupling, spinach chloroplasts were used. They were extracted and treated with 1 mM EDTA as described by McCarty [6], except that NaCl was omitted from the buffer during EDTA treatment. The final suspension was placed in a buffer containing 0.05 M Tricine, pH 7.8, and 0.01 M NaCl, and all reactions were performed in this medium.

Reduction of oxidised *p*-phenylenediamine was measured indirectly as oxygen evolution with a Clark-type oxygen electrode. Phenylenediamine was present at a concentration of 0.5 mM and ferricyanide at 1 mM. Ferricyanide reduction was measured as the decrease in absorbance at 420 nm, at a ferricyanide concentration of 1.4 mM. Chlorophyll was measured according to Arnon [7].

RESULTS

When Mg^{2+} is added to a stirred suspension of *Euglena* chloroplasts, a slow fluorescence rise occurs over a period of between 2 and 5 min, depending on the sample. This rate is dependent on the Mg^{2+} concentration (Fig. 1) and probably reflects the uptake of Mg^{2+} into the sensitive site. It occurs in the dark as well as in the light. While the rate of attainment of the maximal effect increases with increasing Mg^{2+} concentration up to about 10 mM, the maximal total response is usually saturated by a Mg^{2+} concentration between 1 and 2.5 mM (Fig. 1). The fluorescence emission spectrum measured at room temperature shows the maximal increase in yield upon Mg^{2+} addition to occur in the 670–690 nm region of Photosystem II fluorescence, in confirmation of the data of Murata [1] at liquid nitrogen temperature. Mohanty et al. [3] made a similar observation at room temperature upon the addition of Ca^{2+} to maize chloroplast fragments.

From Fig. 1, it can be seen that Mg^{2+} stimulated the rate of *p*-phenylenediamine reduction, a Photosystem II-mediated reaction in *Euglena* as well as in higher plants [8, 5], thus resembling the Mg^{2+} stimulation of ferricyanide reduction which Murata [1] reported for spinach chloroplasts. However, maximal stimulation of this reaction required a somewhat higher concentration of Mg^{2+} than did the fluorescence yield effect. Similar results have been obtained for phenylenediamine reduction with spinach chloroplasts prepared in the same buffers as are used for *Euglena*.

As Mg^{2+}-induced light scattering increases have been suggested by Murata [2] to be associated with the fluorescence increase, we investigated the effect of vary-

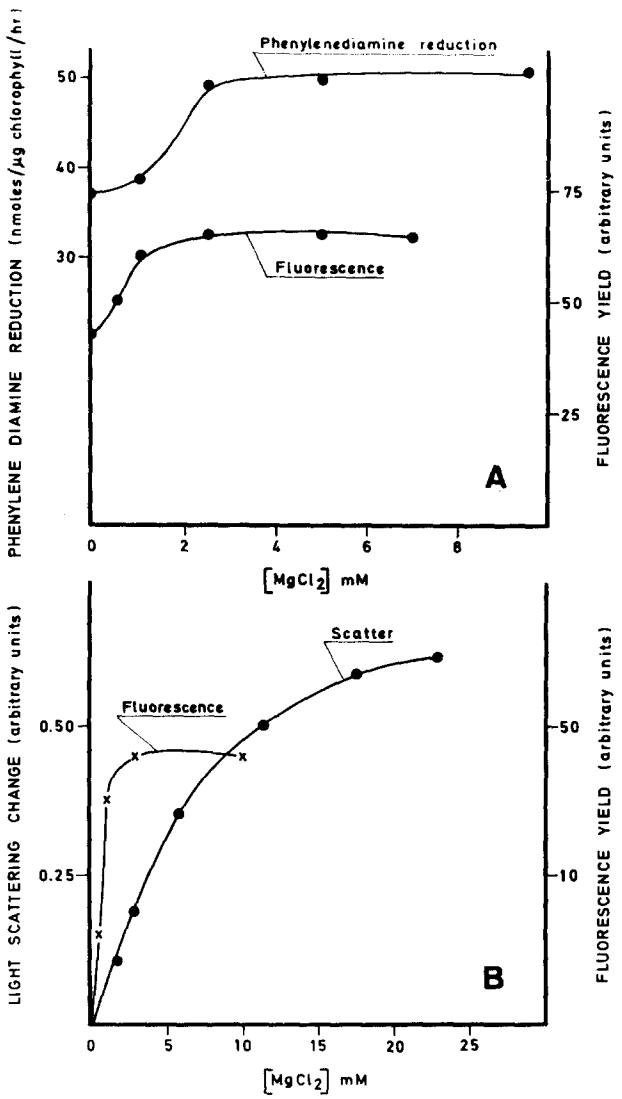

Fig. 1. (A) Dependence of rate of *p*-phenylenediamine reduction and fluorescence yield on Mg^{2+} concentration. (B) Dependence of fluorescence yield and light scattering on Mg^{2+} concentration. Light scattering is expressed as the increment of scatter induced upon addition of Mg^{2+} (For conditions, see Methods).

ing the Mg^{2+} concentration on both parameters (Fig. 1B). Clearly they differ greatly in Mg^{2+} requirement, with 20 mM not completely saturating the light scattering increase, whereas 2.5 mM produced the maximal fluorescence response on this occasion. The two processes were also easily distinguishable kinetically, thus differing from the situation in spinach chloroplasts reported by Murata [2].

When 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to block electron transport, a substantial Mg^{2+} stimulation of fluorescence emisssion could

TABLE I

INFLUENCE OF DCMU AND DITHIONITE ON THE STIMULATION OF FLUORESCENCE YIELD IN *EUGLENA* CHLOROPLASTS BY 5 mM $MgCl_2$

DCMU ($3.0 \cdot 10^{-6}$) was added in a small volume of methanol, which did not influence fluorescence, and a few grains of dithionite were added to saturation of the response.

Treatment	Fluorescence (relative units)		
	Normal chloroplasts	DCMU-treated	Dithionite-treated
None	34	40	45
$MgCl_2$ (5 mM)	51	53	69
Ratio +Mg/−Mg	1.50	1.32	1.54

be subsequently detected, though this was somewhat less than the stimulation without added DCMU when expressed in terms of the basal level of fluorescence before the addition of Mg^{2+} (Table I). When electron transport was prevented by reduction of the electron transport chain with dithionite, however, the full effect was observed, thus indicating that DCMU interfered with the Mg^{2+} stimulation in some manner which did not involve the inhibition of electron transport. We investigated this further by adding DCMU and dithionite to chloroplasts after the Mg^{2+} effect was evident (Fig. 2), and it can be seen that subsequent to the initial sharp rise in fluorescence upon addition of either compound (due to Q reduction in both cases),

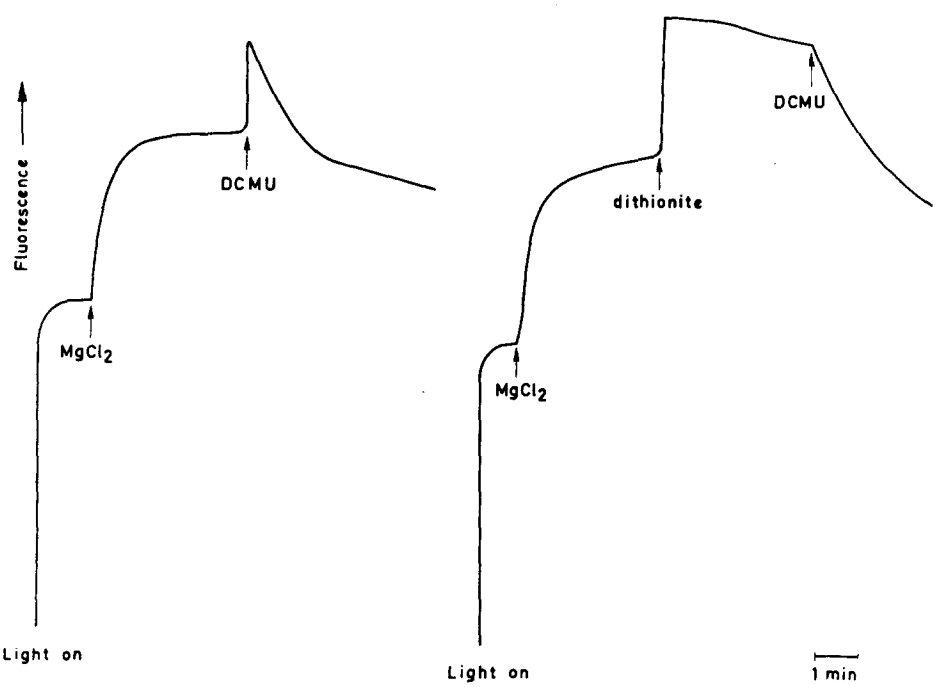

Fig. 2. Effect of DCMU and dithionite on the fluorescence yield of *Euglena* chloroplasts treated with 5 mM $MgCl_2$. Experimental conditions were as described in Table I.

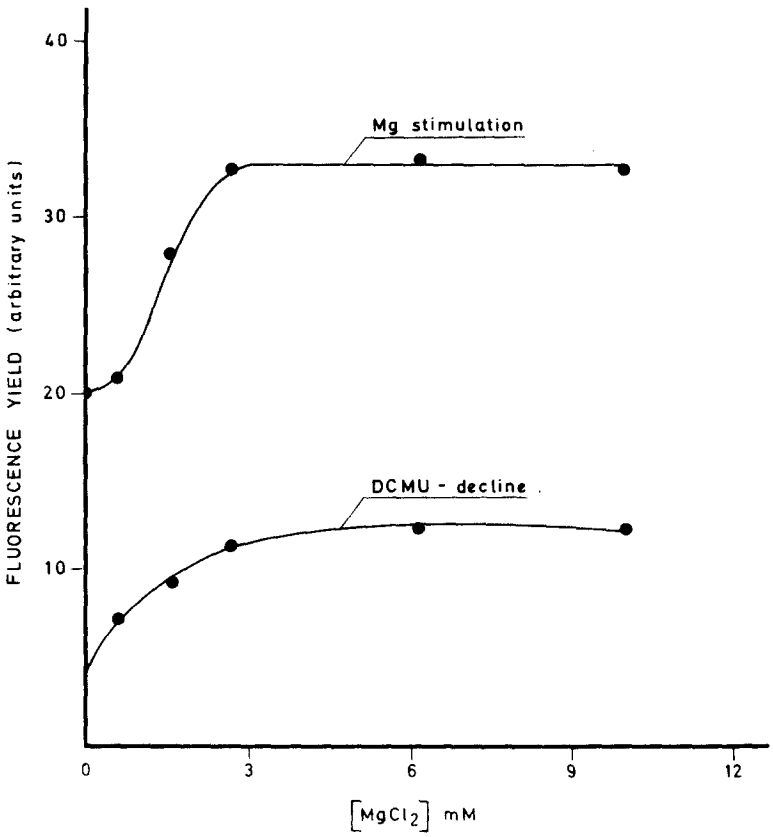

Fig. 3. Dependence of the DCMU-induced decline in fluorescence yield and of the Mg^{2+} stimulation on Mg^{2+} concentration. The experiment was performed in the same manner as that described in Fig. 2, where DCMU ($3.5 \cdot 10^{-6}$ M) was added after the maximal Mg^{2+}-induced stimulation was manifest. The extent of the decline is that measured 10 min after the addition of DCMU.

TABLE II

EFFECT OF TREATMENT OF *EUGLENA* CHLOROPLASTS WITH GLUTARALDEHYDE AND TRYPSIN ON THE Mg^{2+}-STIMULATED FLUORESCENCE INCREASE

Chloroplasts were incubated with 0.25 % glutaraldehyde at 0 °C for 60 min before testing. Trypsin (Sigma, Type I) at a concentration of 0.5 mg/ml was added to the chloroplasts at room temperature 7 min before testing with Mg^{2+}. Trypsin-treated chloroplasts still showed a fluorescence rise in response to dithionite, of about 30 % that of normal chloroplasts, and so presumably were still capable of electron transport, though at a diminished rate.

Treatment	Fluorescence (relative units)		
	Normal chloroplasts	Glutaraldehyde-treated	Trypsin-treated
None	23.5	22	23
$MgCl_2$ (5 mM)	35	22	23.5

DCMU treatment caused a marked fluorescence decline, while this was slight or totally absent in dithionite-treated chloroplasts. Addition of DCMU to chloroplasts already treated with Mg^{2+} and dithionite also stimulated this decline in yield. In Fig. 3, it can be seen that the DCMU-stimulated decline in fluorescence occurred without the addition of Mg^{2+}, but was greatly stimulated by the cation and this response was saturated by a similar concentration as the fluorescence stimulation. We, therefore, feel that DCMU is able to interact with the Mg^{2+} sensitive site.

To determine whether protein structural alterations are involved, we have examined the effect of trypsin and glutaraldehyde on the Mg^{2+} response. In both cases prior incubation with these compounds completely prevented the Mg^{2+} effect (Table II). To eliminate the possibility that this was due to a prevention of Mg^{2+} uptake, both compounds were added to chloroplasts in which the Mg^{2+} effect was evident. This resulted in a gradual reversal of the Mg^{2+}-induced fluorescence yield over a period of 3–5 min, thus indicating that protein structural changes are required.

While electron transport is clearly not required for the Mg^{2+} effect, the possibility remained that somehow the coupling factor might be involved in a structural role in view of its well known Mg^{2+} requirement [9, 10]. We therefore treated spinach chloroplasts with EDTA, a procedure known to remove coupling factor from chloroplast membranes [6]. From Table III it is clear that the EDTA treatment resulted in a complete uncoupling of the chloroplasts, presumably by removal of the coupling factor from the membranes. It can also be seen that this treatment did not alter the effect of Mg^{2+} on fluorescence yield in any way, thus eliminating the possible involvement of coupling factor.

Fig. 4A and B, shows oscilloscope pictures of fluorescence kinetics of *Euglena* chloroplasts in the absence and presence of Mg^{2+}, respectively. The induction pattern is typical of Class II chloroplasts. It is clear that Mg^{2+} only influenced the variable component, a phenomenon already noted by Murata [1] and Mohanty et al. [3]. The stimulation by Mg^{2+} developed progressively with time and it was not until approximately 500 msec after the onset of illumination that the maximal increment was recorded. By this time, approximately 90% of the primary electron acceptor pool was reduced, where this is estimated from the area above the induction curve. The possibility was therefore investigated, that the primary electron acceptor pool was required to be in the reduced state for Mg^{2+} stimulation of fluorescence to occur. Fig. 4, C and D, shows induction curves in the presence of dithionite with (Fig. 4D)

TABLE III

Effect of uncoupling spinach chloroplasts with EDTA on the rate of electron transport to ferricyanide in the presence or absence of NH_4Cl (3 mM), or phosphorylation reagents (5 mM $MgCl_2$; 1 mM KH_2PO_4, 1 mM ADP), and the influence of this uncoupling on the Mg^{2+}-induced fluorescence rise.

Treatment	Ferricyanide reduction (nmoles/μg chloroplasts per h)			Fluorescence	
	None	NH_4Cl	Phosphorylation reagents	$+MgCl_2$	$-MgCl_2$
None	154	449	210	42	29
EDTA	463	421	452	69	47

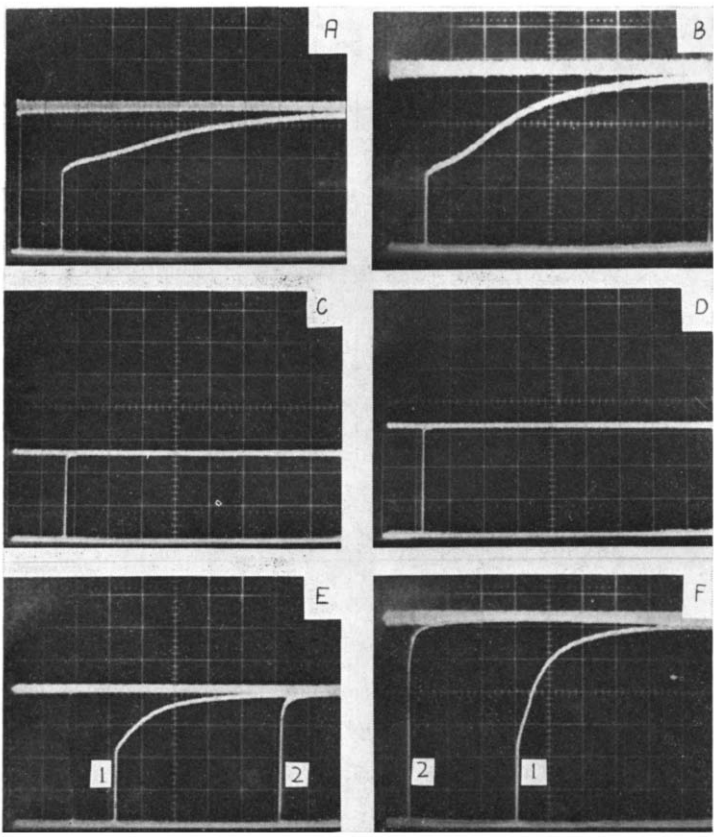

Fig. 4. Oscilloscope pictures of fluorescence induction kinetics of *Euglena* chloroplasts. A, no additions; B, incubated for 2 min with $MgCl_2$ in the dark; C, incubated with dithionite for 2 min in the dark; D, incubated with dithionite plus 5 mM $MgCl_2$ for 2 min in the dark; E. no additions; Trace 1, incubated in dark for 2 min; Trace 2, same sample as for Trace 1 but after 30 s light, and then 1 s dark. F. Same treatment as E except that 5 mM $MgCl_2$ was added 2 min before the initial illumination. A, B, C, and D were illuminated with 16 000 ergs · cm^{-2} · s^{-1} of blue light (Corning CS 4-96 filter) and the time scale is 100 ms per division. E and F were illuminated with 160 000 ergs · cm^{-2} · s^{-1} and the time scale is 50 ms per division.

or without (Fig. 4C) added Mg^{2+}. Reduction of the primary electron acceptor pool by dithionite leads to the expected drastic shortening of the induction time, and in the presence of Mg^{2+}, the stimulated fluorescence yield was observed with no lag.

This experiment eliminated the possibility that there was any light requirement other than that for primary electron transfer processes. Fig. 4, E and F, shows the data for a similar experiment in which the primary acceptor pool was initially reduced by a 30 sec period of illumination. This was followed by a 1 s period of dark followed by another illumination. It is indicated by the shorter induction period of the second illumination (Trace 2), that Q was in a substantially reduced state, and, as expected under these circumstances, the Mg^{2+} stimulation of yield occurred at a corresponding rate. Further information on this point is presented in Table IV, but from the opposite

TABLE IV

EFFECT OF THE *p*-PHENYLENEDIAMINE-FERRICYANIDE ELECTRON ACCEPTOR COUPLE ON THE Mg^{2+}-STIMULATION OF FLUORESCENCE

Fluorescence was excited with 650-nm light of about 5000 ergs · cm^{-2} · s^{-1} intensity and measured at 700 nm.

Treatment	Fluorescence (relative units)	
	None	Phenylenediamine–ferricyanide
None	42	25
$MgCl_2$ (5 mM)	77	32
Ratio +Mg/−Mg	1.83	1.28

experimental situation, in which the primary acceptor pool was kept in a predominantly oxidised state. Thus when the experiment was performed in the presence of the *p*-phenylenediamine–ferricyanide acceptor couple, which would be expected to maintain Q in a more oxidised state than usual in the light, the Mg^{2+} increment in fluorescence was reduced. The primary acceptor pool is also largely oxidised during illumination with extremely low intensity light, and under this condition, the Mg^{2+} effect was greatly reduced (Fig. 5). However, this decreased stimulation at low light was eliminated by the addition of dithionite.

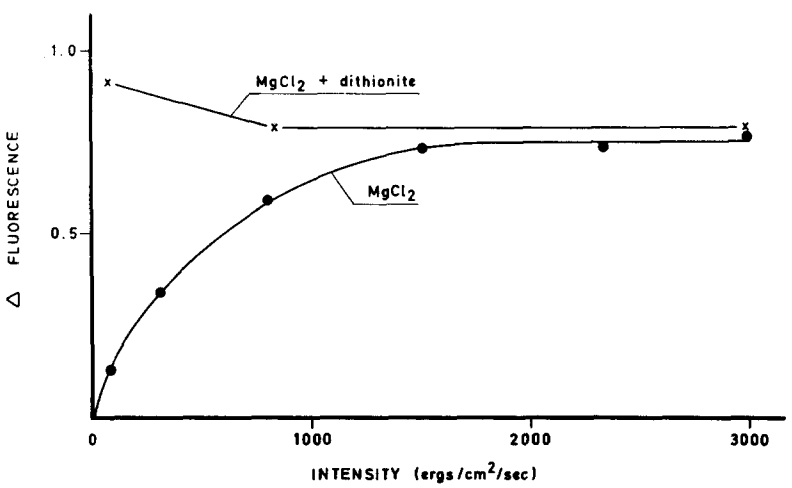

Fig. 5. Dependence of the Mg^{2+}-stimulated fluorescence yield on light intensity in the absence and presence of dithionite. The data are expressed as the increase in fluorescence of chloroplasts treated with 5 mM $MgCl_2$.

DISCUSSION

The currently accepted interpretation that divalent cations interfere with the "spillover" transfer of energy between Photosystem II and Photosystem I, put forward by Murata and co-workers [1, 2, 11] and supported by Mohanty et al. [3], is not corroborated by the data presented in this paper and elsewhere [5]. We have

previously [5] demonstrated that the Photosystem II absorbed quanta which participate in the Photosystem I-mediated reduction of methyl viologen with the DCIP–ascorbate donor couple, was not influenced by Mg^{2+} though a large increase in fluorescence yield was encountered under the same conditions upon addition of the cation. Furthermore, we feel that the Mg^{2+} stimulation of Photosystem II electron transport reported by Murata [1] as supporting his hypothesis may not have been associated with the fluorescence phenomenon, as our data indicate that in both *Euglena* and spinach chloroplasts the Mg^{2+} requirement for the two processes seems to differ. It should be mentioned that Rurainski and Hoch [4] demonstrated a Mg^{2+} stimulation of the reduction of NADP with water as the electron donor, in a reaction not involving P_{700}, to have a similar Mg^{2+} requirement to the fluorescence phenomenon, and so these two processes may be related. However, it is not clear whether this NADP reduction was mediated solely by Photosystem II, owing to the doubt that P_{700} is the sole Photosystem I reaction centre component, and so a detailed interpretation of this observation is impossible at the moment.

Murata [2] working with Photosystem I-enriched particles in which there was no response to Mg^{2+}, and grana stacks containing both photosystems which were responsive to the cation, suggested the necessity for both photosystems to observe the stimulation. Recently, Mohanty et al. [3] arrived at a similar conclusion with Photosystem II-enriched particles which displayed no Mg^{2+} response. We feel that such experiments are difficult to interpret owing to the rather drastic processes involved in membrane fractionation, which could easily be expected to inactivate the protein structural changes, which experiments with trypsin and glutaraldehyde have demonstrated to be necessary (Table II).

The data presented here strongly support the idea that for the Mg^{2+} effect to occur, the primary electron acceptor for Photosystem II, Q, needs to be in a reduced state. This is apparent from the close correlation between Q reduction and the Mg^{2+} response during fluorescence induction, in the presence and absence of dithionite and also after Q was predominantly reduced by a preillumination period. Additionally, when Q was maintained in a predominantly oxidised state during electron transport to the phenylenediamine-ferricyanide couple, and at very low light intensities, the response to Mg^{2+} was greatly reduced. In the latter instance, a maximal response was obtained under the low light conditions by chemically reducing Q with dithionite. It is possible that the slow reduction of the steady-state fluorescence yield by DCMU, which is stimulated by similar concentrations of magnesium as are required to induce the fluorescence increase, involves the interaction of DCMU with Q, in some manner inimical to the fluorescence stimulation. The site of inhibition of electron transport by DCMU is generally considered to be at the level of Q re-oxidation [12] and so such a possibility is entirely feasible.

Mohanty et al. [3], on the basis of experiments with Tris-washed chloroplasts, have suggested that it is of no consequence whether Q is reduced or oxidised for the Mg^{2+} effect. They observed that though Tris washing severely inhibited electron transport from water to DCIP [13], an inhibition partly overcome by the addition of the electron donor diphenyl carbazide, the Mg^{2+} effect was scarcely affected. On the basis of the argument that Tris treatment inhibits on the oxidising side of Photosystem II [14], they suggested that Q should not be reduced under these conditions. However, their experiments also demonstrated that DCMU was equally effective

in increasing the fluorescence yield in Tris-washed chloroplasts, thus indicating that Q was most probably extensively reduced under these experimental conditions even after Tris treatment.

Our results with *Euglena* do not corroborate these of Murata [2] with spinach, that light scattering changes induced by Mg^{2+}, presumably due to chloroplast volume changes, reflect the same phenomenon as the fluorescence stimulation. In *Euglena*, the two processes have greatly different Mg^{2+} requirements and are also kinetically distinct, though with spinach chloroplasts we have noted data more closely agreeing with those of Murata. That protein structural changes are involved in *Euglena* chloroplasts, is demonstrated by the inhibition of the fluorescence increase by both trypsin and glutaraldehyde treatment, whether added before or after the Mg^{2+}. A similar effect of glutaraldehyde was previously shown by Mohanty et al. [3].

These changes are in no way connected with the coupling factor, as the full Mg^{2+} effect was observed even after complete uncoupling with EDTA in spinach chloroplasts, a treatment which removes the coupling factor from the chloroplast membranes [9]. This result, however, is in direct contradiction to a similar experiment performed by Mohanty et al. [3] also with spinach chloroplasts. However, these workers presented no control data to demonstrate that their chloroplasts were in fact uncoupled by EDTA, and in fact their electron transport rates are substantially lower than those they encountered in other experiments and are certainly not uncoupled rates. We feel that their failure to observe a Mg^{2+} stimulation may have been due to the use of damaged chloroplasts. In our hands the procedure for EDTA uncoupling described by McCarty [6] and which was used by Mohanty et al. [3] was not effective until NaCl was omitted from the buffer during EDTA treatment, an observation also reported by Jagendorf and Neumann [15].

To sum up, our data are suggestive of the interpretation that Mg^{2+}, in conjunction with reduced Q, induces a structural change involving protein (s) which leads to an increase in the Photosystem II fluorescence yield. This may be achieved by increasing the yield of an already fluorescing bed of chlorophyll. In view of the data of Mohanty et al. [3] in which the fluorescence lifetime increases proportionally with the fluorescence emission on the addition of Mg^{2+}, we feel that this may be achieved by diverting energy from non-radiative methods of dissipation to fluorescence.

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