Biochimica et Biophysica Acta, 461 (1977) 489–499

BBA 47345

REGULATION OF THE HILL REACTION BY CATIONS AND ITS ABOLISHMENT BY UNCOUPLERS

HANS J. RURAINSKI and GERHARD MADER

Lehrstuhl für Biochemie der Pflanze der Universität, 3400 Göttingen, Untere Karspüle 2 (G.F.R.)

(Received February 14th, 1977)

SUMMARY

Concurrent measurements of *P*-700 turnover and the reduction of $K_3Fe(CN)_6$ revealed an identical relative quantum yield for both reactions in isolated pea chloroplasts as well as chloroplast particles from wild type *Scenedesmus*. On the other hand, chloroplast particles of wild type *Scenedesmus* showed the same relative quantum yield for the Hill reaction as those of the *P*-700-free mutant No. 8, indicating that *P*-700 is not required for ferricyanide reduction.

Several metal ions, such as Mg^{2+}, Ca^{2+}, Na^+ and K^+ stimulated the reduction of $K_3Fe(CN)_6$. In short wavelength light, the stimulation was a function of light intensity, varying in magnitude from an approximate doubling of the yield in low intensities to only a slight increase at light saturation. *P*-700 was totally unaffected by the cations.

The effect of the metal salts was abolished in the presence of uncouplers of photophosphorylation.

The data reconcile several divergent results concerning the effect of divalent cations on the reduction of ferricyanide which have been reported in the recent literature. On the whole the experiments suggest that the Hill acceptor can be reduced at two sites. The stimulation of the Hill reaction by metal ions is proposed to be due to an activation of Photosystem II and a more efficient utilization of quanta at the expense of radiationless de-excitation.

INTRODUCTION

The profound effect of a variety of mono- and divalent cations, notably Mg^{2+}, on isolated chloroplasts is a well-established and amply documented phenomenon (see, for example, refs. 1–6). In addition to stimulating the yield of reduction of several electron acceptors and of chlorophyll *a* fluorescence, cations reportedly decreased the flux through *P*-700 (20 ms component) and the yield of Photosystem I reactions with an artificial electron donor. Mono- and divalent salts were also found to impart

---

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NADP, nicotinamide adenine dinucleotide phosphate; FCCP, carbonylcyanide *m*-fluorophenylhydrazon; DCPIP, 2,6-dichlorophenolindophenol.

Emerson enhancement to chloroplasts [7] and to stabilize the structure of the thylakoid membranes [8]. These effects must be distinguished from other functions of cations such as the involvement of Mg^{2+} in photophosphorylation [1] or of Mn^{2+} in the oxygen-evolving step of photosynthesis [9].

Regarding the mechanism of cation action, the data obtained thus far have been interpreted in terms of several differing models. The basis of most theories was the recognition that after the addition of salts to chloroplasts, their light absorption was little affected. Therefore, the observed changes in the quantum yield of electron transport had to be brought about by quanta which, in the absence of salts, were either inefficiently distributed between the light reactions or which were somehow not utilized at all. The effect of salts was thus postulated as redressing the imbalance of the quantum input into the photosystems by decreased spill-over [3], increased spill-over [7], redistribution of quanta between parallel light reactions [5] and increased use of excitation energy at the expense of radiationless dissipation [10, 11]. Other interpretations included the notions that salts caused an activation of previously inactive photosynthetic units [5] or an increased association of ferredoxin with the multienzyme complex of the electron transport chain [6].

While the experimental observations concerning the effect of cations on several electron transport parameters, especially chlorophyll fluorescence, agree in most reports, differing results were obtained for the reduction of $K_3Fe(CN)_6$. Thus, reports of a marked stimulation [11] have appeared along with those showing little [2] or no effect [10, 12]. Assumedly, these conflicting results may have contributed to the different interpretations outlined above.

In this paper we show that the rate of $K_3Fe(CN)_6$ reduction is stimulated by a variety of salts. The magnitude of the increase is a function of light intensity and further depends on the concentration of some uncouplers present during the measurements. Concurrently measured *P*-700 fluxes are totally unaffected by the cations. Interpreted in the frame work of the currently accepted electron transport scheme, the data are consistent with the idea that salts activate Photosystem II and that quanta are utilized at the expense of radiationless transitions.

MATERIALS AND METHODS

Chloroplasts from peas were prepared as previously described [5]. Unless indicated differently, they were suspended in a basic reaction medium containing 50 mM sucrose, 10 mM NaCl, 15 mM tricine at pH 8.0 and 0.8 mM $K_3Fe(CN)_6$. Chlorophyll concentration was routinely between 18 and 25 μg/ml. Growth conditions for *Scenedesmus* and isolation of chloroplast particles from the algae have been reported elsewhere [13].

Simultaneous measurements of Fe^{3+} reduction and *P*-700 were carried out with a steady-state relaxation spectrophotometer [14, 15] at 420 and 700 nm, respectively. The intensity of the actinic light (530–645 nm) was approx. $2 \cdot 10^4$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$. At high light intensities, ferricyanide reduction was measured in a Zeiss spectrometer. Most experiments were additionally checked by measuring oxygen evolution in the presence of ferricyanide using a Clark-type Pt AgCl electrode.

Reactions rates were calculated with extinction coefficients of 1 $mM^{-1} \cdot cm^{-1}$ for ferricyanide and 65 $mM^{-1} \cdot cm^{-1}$ for *P*-700 [16].

RESULTS

In all experiments reported below, the time-averaged absorption changes at 700 nm and the time courses of absorption decrease at 420 nm were measured in the same sample and under identical conditions. Thus, a direct comparison between the

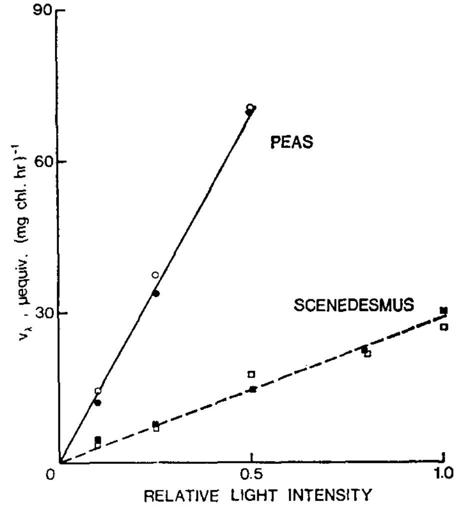

Fig. 1. Relative quantum yields of ferricyanide reduction and *P*-700 flux with chloroplasts from peas and chloroplast particles from wild type *Scenedesmus*. Short wavelength actinic light; modulation frequency 7 cycles/s. Open symbols, ferricyanide reduction (v_{420}); closed symbols, *P*-700 flux (v_{700}).

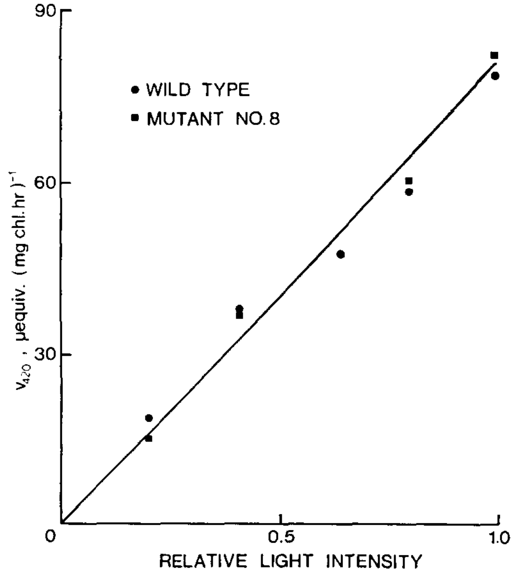

Fig. 2. Relative quantum yields of ferricyanide reduction in chloroplast particles of wild type and mutant No. 8 of *S. obliquus*. Reaction conditions as in Fig. 1.

calculated flux (v_{700}) [14] and the rate of ferricyanide reduction (v_{420}) is possible. Fig. 1 shows that with pea chloroplasts, the relative quantum yield of both reactions is identical. Such a stoichiometry was found in almost all measurements with only occasional small and seemingly random deviations. A stoichiometry of unity was also obtained with chloroplast particles isolated from *Scenedesmus obliquus* although relative to pea chloroplasts, the quantum yield of the particles was considerably lower. Ad hoc these results suggest that *P*-700 is an intermediate of the electron transport pathway leading to the reduction of ferricyanide and that electrons passing through the pigment arrive quantitatively at the site of reduction.

This interpretation may be questionable, however, in view of the data of Fig. 2 which show that chloroplast particles of both the wild type and the *P*-700-free mutant No. 8 of *Scenedesmus* exhibit an identical quantum yield for the reduction of ferricyanide. A similar result has been reported for particles from the wild type and *P*-700-free mutant ac 80 of *Chlamydomonas rheinhardii* [17]. Apparently then the Hill reaction can proceed entirely without involvement of *P*-700.

The following experiments were carried out with pea or spinach chloroplasts

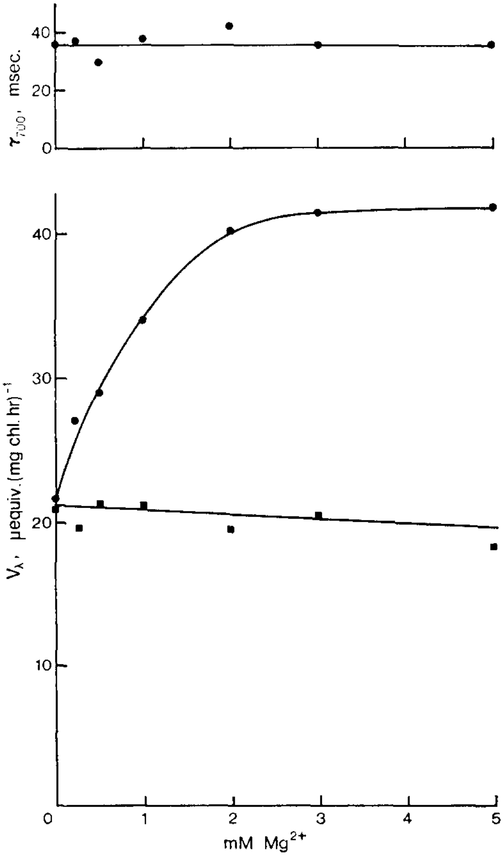

Fig. 3. Effect of Mg^{2+} on the yields of ferricyanide reduction and *P*-700 flux in pea chloroplasts Reaction conditions as in Fig. 1. ●, v_{420}; ■, v_{700}. Upper curve: relaxation times of *P*-700.

and describe the effect of various salts on the Hill reaction and pigment turnover. As is shown in Fig. 3, increasing amounts of Mg^{2+} progressively raise the yield of the ferricyanide reduction. An approx. 2-fold maximum stimulation is obtained with 2 mM salt. The flux through *P*-700 remains constant over the entire concentration range. Also, the relaxation time of the pigment, a measure of the rate of its reduction [15], is unaffected. Thus, the simple stoichiometric relationship between Hill reaction and flux no longer holds in the presence of the salt. This differential effect supports the notion that the two activities are due to different photoreactions.

As was previously shown for the reduction of NADP [5], and the stimulation of fluorescence emission [11], Mg^{2+} can be replaced by a number of other mono- or divalent cations also in Fe^{3+} reduction (Table I). The range of salts that may be used in this case is limited, however, because of chemical interactions between certain salts and the substrate. Addition of Mn^{2+}, for instance, caused appreciable precipitation in the sample. Nevertheless, it is apparent that for an equivalent stimulation, a 10-20-fold higher concentration of monovalent than of divalent cations is required. Cl^- may be substituted by SO_4^{2-} or NO_3^- indicating that the stimulation is non-specific for either cation or anion. Also, none of the salts affected the flux through *P*-700.

In Table II the stimulatory effect of Mg^{2+} is compared in different colors of actinic light. The data represent the slopes of light intensity curves similar to those shown in Figs. 1 and 2 and were arbitrarily normalized for the experiment shown in brackets. Apparently, the highest stimulation of the yield is obtained in short wavelength light which is believed to excite primarily Photosystem II. As the actinic light approaches those wavelength which are mainly absorbed by Photosystem I [18], the degree of stimulation decreases until no further effect is seen. We take these results as an indication that Mg^{2+} produces its effect in the short wavelength light reaction.

The effect of Mg^{2+} on the reduction rate at various intensities of short wavelength light is shown in Fig. 4. The insert indicates that the magnitude of stimulation (i.e. the ratio of rates in the presence and absence of Mg^{2+}, respectively) decreases as the intensity of the actinic light increases. At light saturation only a small effect was seen.

Our observation at saturating intensities agrees with the result of Harnischfeger

TABLE I

EFFECT OF VARIOUS SALTS ON THE REDUCTION OF FERRICYANIDE AND THE TURNOVER OF *P*-700

Reaction conditions as in Fig. 1. Chloroplasts equivalent to 20 μg chlorophyll/ml. Modulation frequency: 8.2 cycles/s; short wavelength actinic light. Results are expressed in μequiv./mg chlorophyll per h.

Salt (mM)	v_{420}	v_{700}
Control	31.6	31.4
$MgCl_2$ (5)	57.7	30.4
$CaCl_2$ (5)	52.2	30.9
NaCl (100)	56.3	31.1
KCl (50)	52.0	28.6
$MgSO_4$ (5)	54.9	32.7
$Ca(NO_3)_2$ (5)	56.3	33.4

TABLE II

EFFECT OF Mg^{2+} ON THE RELATIVE QUANTUM YIELD OF $K_3Fe(CN)_6$ REDUCTION WITH VARIOUS ACTINIC LIGHTS

Reaction conditions as described under Materials and Methods. 5 mM Mg^{2+} was added where indicated. Actinic illumination was obtained by filtering white light through either narrow-banded interference filters with maximum transmission at 649 and 691 nm, respectively, or a steep cut-off glass filter (RG 715) with 50 % transmission at 715 nm. The data represent the slopes of light intensity curves in the linear range of intensity. They were arbitrarily normalized for the sample in brackets.

Actinic light	Relative quantum yields		Stimulation factor
	$-Mg^{2+}$	$+Mg^{2+}$	
IF 649	[1.0]	1.68	1.7
IF 691	0.30	0.46	1.5
RG 715	0.22	0.22	1.0

and Shavit [6] but disagrees with that of Li [11]. The latter author could, however, not exclude the possibility that in his salt-depleted chloroplast preparations, part of the stimulation observed was due to the addition of the Cl^- anion which is required for an efficient Hill reaction. Our data at quantum yield intensities also differ from those of Homann [2] who found a slight stimulation and of Marsho and Kok [12] and Malkin and Siderer [10] who observed no effect due to cations. Since some of these authors included methylamine in their reaction medium, we investigated a possible connection between the presence of this uncoupler and the lack of a cation effect.

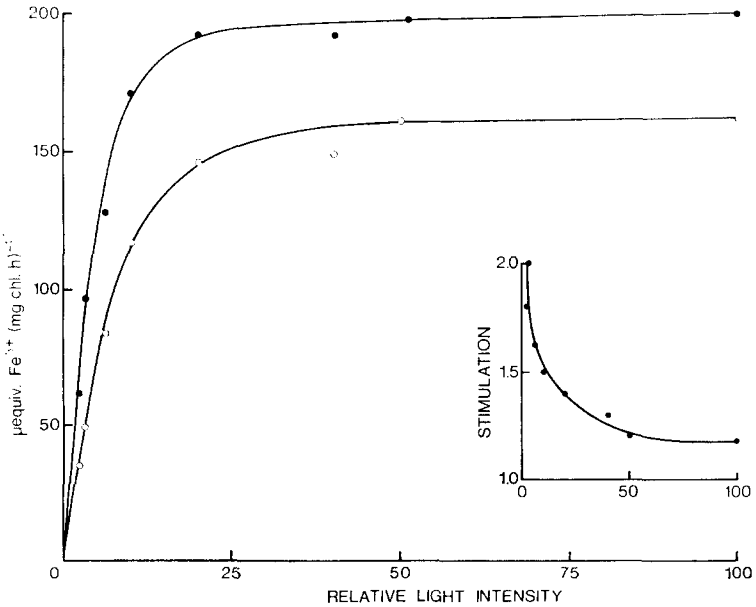

Fig. 4. Stimulation of ferricyanide reduction by Mg^{2+} as a function of short wavelength light intensity. Reaction conditions as described under Materials and Methods. The data were measured in a Zeiss spectrometer using unmodulated light. ○, $-Mg^{2+}$; ●, + 5 mM Mg^{2+}. Insert: ratio of rate ($+Mg^{2+}$) over rate ($-Mg^{2+}$).

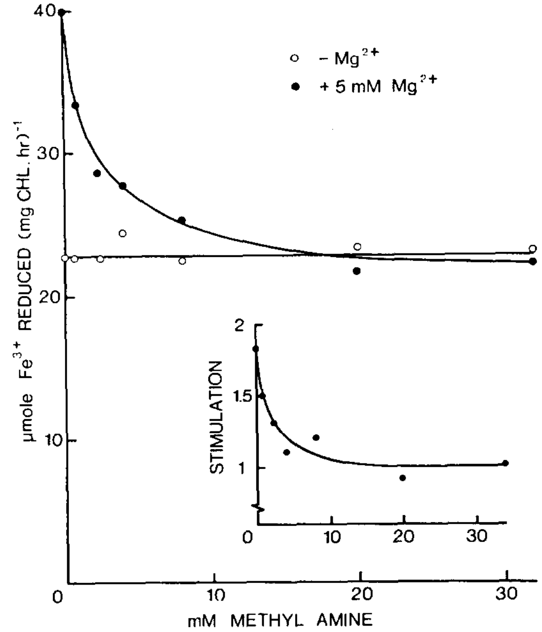

Fig. 5. The effect of methylamine on the quantum yield-stimulating action of Mg^{2+}. Standard reaction conditions. Short wavelength actinic light. Methylamine was added as the hydrochloride. Insert: the stimulation is expressed as the ratio of rates in the presence and absence of 5 mM Mg^{2+}.

Uncouplers of photophosphorylation stimulate the rate of electron transport at high, but not at low light intensities since, in the latter case, the quantum input is the rate-limiting parameter. Therefore, in order to eliminate changes in rates due to the uncoupling effect, most experiments were carried out with limiting intensities. Fig. 5 shows again that in the absence of methylamine, Mg^{2+} increases the yield of ferricyanide reduction nearly 2-fold. As the concentration of the amine is raised, the stimulation progressively decreases until it is ultimately abolished. In the experiment shown, methylamine did not affect the yield in the absence of Mg^{2+}, although occasionally a

TABLE III

ABOLISHMENT OF THE STIMULATING EFFECT OF VARIOUS SALTS BY METHYLAMINE

Standard reaction conditions and short wavelength light. Where indicated, 30 mM methylamine was added as the hydrochloride. Results are expressed in µmol Fe^{3+}/mg chlorophyll per h.

Salt (mM)	−Methylamine	+Methylamine
Control	37.5	34.8
Mg^{2+} (5)	73.2	39.0
Ca^{2+} (5)	66.9	48.0
Na^{+} (100)	62.7	37.6
K^{+} (50)	48.8	22.3

TABLE IV

ABOLISHMENT OF THE CATION EFFECT BY VARIOUS UNCOUPLERS

Standard reaction mixture. Short wavelength actinic light. 5 mM Mg^{2+} was added where indicated.

Uncoupler	μmol Fe^{3+}/mg chlorophyll per h		
	$-Mg^{2+}$	$+Mg^{2+}$	Stimulation
Control	23.7	48.5	2.05
Methylamine, 30 mM	23.0	23.0	1.00
NH_4Cl, 16 mM	11.9	11.3	0.95
NH_4SCN, 15 mM	8.2	8.9	1.09
FCCP, 5 μM	13.0	12.4	0.95
Gramicidin, 40 μM	27.1	51.8	1.91

small inhibition was observed. Even in such cases, the ratio of the rate $+Mg^{2+}$/rate$-Mg^{2+}$ declined in a similar manner as shown in the insert. The effect of the uncoupler on *P*-700, both in the absence and presence of Mg^{2+}, was a decrease of the relaxation time from approx. 35 to 20 ms indicating a faster turn-over. Calculated flux values remained constant (data not shown).

The data of Table III demonstrate that methylamine also abolishes the stimulation due to other mono- and divalent salts. Similar results were obtained with NH_4SCN, NH_4Cl and FCCP (Table IV). Although these uncouplers additionally inhibited the rates of ferricyanide reduction (as is well known [19]) both in the absence and presence of Mg^{2+}, the ratio of these rates decreased steadily as a function of the concentration added. With approx. 15 mM NH_4^+ or 40 μM FCCP, the stimulatory effect of Mg^{2+} was abolished. In contrast, gramicidin which is believed to inhibit the light-induced electrical potential across the thylakoid membrane preserved the effect of the salt even when added in higher concentrations than usually required for complete uncoupling of photophosphorylation [19]. Thus, the lack of a salt effect on the reduction of ferricyanide reported by others may be due to the high methylamine concentrations used in those studies. The results also imply that a less than maximal stimulation of the Hill reaction may be observed if chloroplasts are uncoupled by certain isolation procedures.

DISCUSSION

For a long time, the site of $K_3Fe(CN)_6$ reduction in the overall scheme of photosynthetic electron transport was a matter of controversy. More recently, agreement seems to have been reached that the Hill acceptor is reduced by Photosystem I as well as Photosystem II [20]. Our observation of an identical relative quantum yield in both wild type and Photosystem I-deficient mutant particles supports the idea of a reduction site in light reaction II. This result is not fortuitous since it has also been reported for wild type and *P*-700-deficient *Chlamydomonas* [17]. Moreover, the finding that cations exert an effect on the yield of reduction but not on the flux through *P*-700 also argues for a reduction site in Photosystem II. Taken together, these results suggest that the two reactions are independent.

At the same time, Fe^{3+} is required for efficient *P*-700 turn-over and, in the absence of salts, the reactions occur with unit stoichiometry. (The latter quantitative result depends on the correctness of the extinction coefficient of 65 $mM^{-1} \cdot cm^{-1}$ for *P*-700 [16]). Also, the inhibition of the Hill reaction by DCMU is accompanied by an equivalent inhibition of the flux [21]. Thus, according to these data, the two reactions appear to be linked.

To rationalize all data in terms of the series scheme of photosynthetic electron transport, we must assume that ferricyanide can be reduced at two different sites by a similar mechanism that has previously been proposed for the reduction of DCPIP [26]. Accordingly, in the absence of salt ferricyanide may be reduced by the primary reductant of Photosystem I. This explanation satisfies the observed stoichiometry between v_{420} and v_{700} in both pea chloroplasts and particles of wild type *Scenedesmus*. (Note, incidentally, that these data require the absolute quantum requirement for the reduction of ferricyanide to be $\geqslant 2$ [23]). As salt is added an additional reduction site may be activated which we must place in Photosystem II since the increase in the yield of the Hill reaction is not accompanied by similar changes in the flux through *P*-700. In chloroplast particles of the mutant only this second site would be operating without participation of the pigment. Such a mechanism could also account for the differential effect of methylamine and other amine uncouplers if we postulate that these agents inhibit only reduction at the second site. Moreover, given that the reaction at the Mg^{2+}-activated site has a similar quantum yield as that in Photosystem I (as could be supposed from Fig. 2) but a low saturation rate, we have a possible explanation for the observation that Mg^{2+} nearly doubles the yield at low but not at high light intensities. In Fig. 4 the light intensity curve in the presence of Mg^{2+} would then be a superposition of both reactions. (For an alternative interpretation see below.)

We attempted to obtain further support for this notion by studying the effect of salts on chloroplast particles from wild type and mutant No. 8 of *Scenedesmus*. We failed in this attempt, however, since in order to observe any effect of cations on the reduction of ferricyanide, a certain structural integrity of the chloroplasts seems to be necessary. For example, sonication of pea chloroplasts for only 5 s abolished the stimulation due to cations. Chloroplast particles from the alga did not respond at all to the addition of salts, presumably because they were quite damaged in the isolation procedure. We wish to emphasize that our assumptions concerning two reduction sites for the Hill reaction are unnecessary if our data are interpreted in terms of parallel light reactions (see e.g. ref. 22) in which *P*-700 and the site of ferricyanide reduction operate independently.

A second aspect of this work is concerned with the origin of the quanta which facilitate the increased quantum yield of ferricyanide reduction. Addition of salt has only a marginal effect on the absorption properties of chloroplasts [24]. It follows, therefore, that a portion of the quanta absorbed in the absence of cations must not have been utilized until the salt was added. Murata [3] suggested that Mg^{2+} controls the distribution of excitation transfer between the pigment systems by suppressing the spill-over of excitation energy from pigment system II to pigment system I. This interpretation has been both embraced and challenged (see, e.g. refs. 4, 5, 7, 10–12). Murata's hypothesis was based on the observation that the yield of reduction of DCPIP (supposedly a Photosystem II reaction) increased whereas the yield of reduction of NADP by an artificial donor (supposedly a Photosystem I reaction) decreased slightly. Our results do not support this notion, since we observe no change in the yield of *P*-700 turnover. Moreover, as was shown by Bose [25] and Rurainski and Mader (unpublished results) the slight decrease in the yield of NADP reduction with an artificial donor observed by Murata crucially depends on the pH of the reaction medium. At pH values smaller than 7, Mg^{2+} actually increases the yield substantially.

A different interpretation of the effect of Mg^{2+} was proposed by Sun and Sauer [7]. While maintaining the idea of changes in the spill-over rate between the photosystem, these authors suggested that spill-over occurs in the presence of the cation rather than in its absence. For the same reasons given above, our results do not support this proposition.

Our data are consistent with the interpretations of Malkin and Siderer [10] and of Li [11] that cations activate reaction centers of Photosystem II. In this hypothesis, the distribution of light between the photosystems remains largely unaffected but there is an increased utilization of quanta which, in the absence of salts, were wasted by radiationless transitions. In addition to the arguments discussed above we point out in further support of this postulate that the effect of Mg^{2+} is particularly pronounced in short wavelength light and vanishes in far red light. This idea leads to an alternative interpretation of the observation that the magnitude of cation stimulation is a function of light intensity if we suppose that the efficiency of utilization is variable. This variability may be governed by the concentration of open traps, which can be expected to be highest at low light intensity. Under this condition, the transfer efficiency and therefore stimulation is high. As the intensity increases and, on average, the traps close, transfer of excitation energy vanishes because sufficient excitation is provided by the actinic light directly.

The magnitude of cation stimulation strongly depends on the presence or absence of some uncouplers of phosphorylation in the assay medium. In fact, at high concentration the stimulation due to cations is totally abolished. Marsho and Kok [12] observed such an effect on the kinetics of *P*-700 oxidation in far red light following a pre-illumination with weak short wavelength light. At present, we have no reasonable explanation for the action of uncouplers. The involvement of photosynthetic energy coupling appears indicated. Other conclusions are possible and we are presently investigating this effect further. Nevertheless, the data reconcile several divergent reports in the recent literature by indicating that the results obtained on the effect of cations depend crucially on the assay conditions.

ACKNOWLEDGEMENT

This work was financially supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Shavit, N. and Avron, M. (1967) Biochim. Biophys. Acta 131, 516–525

2 Homann, P. (1969) Plant Physiol. 44, 932–936

3 Murata, N. (1969) Biochim. Biophys. Acta 189, 171–181

4 Avron, M. and Ben-Hayyim, G. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), p. 1185, IUBS Publication, Tübingen

5 Rurainski, H. J. and Hoch, G. E. (1972) in Proceedings of the II International Congress on Photosynthesis (Forti, G., ed.), p. 133, Dr. Junk, N.V., The Hague

6 Harnischfeger, G. and Shavit, N. (1974) FEBS Lett. 45, 286–289

7 Sun, A. S. K. and Sauer, K. (1972) Biochim. Biophys. Acta 256, 409–427

8 Izawa, S. and Good, N. E. (1966) Plant Physiol. 41, 544–552

9 Cheniae, G. M. and Martin, I. (1966) Brookhaven Symp. Biol. 19, 406–417

10 Malkin, S. and Siderer, Y. (1974) Biochim. Biophys. Acta 368, 422–431

11 Li, Y. S. (1975) Biochim. Biophys. Acta 376, 180–188

12 Marsho, T. V. and Kok, B. (1974) Biochim. Biophys. Acta 333, 353–365

13 Rurainski, H. J. and Hoppe, H. J. (1976) Biochim. Biophys. Acta 430, 105–112

14 Hoch, G. E. (1972) in Methods in Enzymology (San Pietro, A., ed.), Vol. 24, p. 297, Academic Press, New York

15 Rurainski, H. J. (1975) Z. Naturforsch. 30c, 761–770

16 Haehnel, W. (1976) Biochim. Biophys. Acta 423, 499–509

17 Givan, A. L. and Levine, R. P. (1969) Biochim. Biophys. Acta 189, 404–410

18 Hoch, G. E. and Martin, I. (1963) Arch. Biochem. Biophys. 102, 430–438

19 Izawa, S. and Good, N. E. (1971) in Methods of Enzymology (San Pietro, A., ed.), Vol. XXIV, p. 355, Academic Press, New York

20 Trebst, A. (1974) Ann. Rev. Plant Physiol. 35, 423–458

21 Rurainski, H. J., Randless, J. and Hoch, G. E. (1970) Biochim. Biophys. Acta 205, 254–262

22 Hoch, G. E. (1964) Rec. Chem. Prog. 24, 165–180

23 Govindjee and Bazzaz, M. (1967) Photochem. Photobiol. 6, 885–894

24 Li, Y. S. (1970) Dissertation, University of Rochester, Rochester, N.Y.

25 Bose, S. (1975) Dissertation, University of Rochester, Rochester, N.Y.

26 Kok, B., Malkin, S., Owens, O. and Forbush, B. (1966) Brookhaven Symp. Biol. 19, 446–459