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PROPERTIES OF ATPASE IN CHLOROPLASTS

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SUMMARY

1. Mg^{2+}-ATPase (light-triggered Mg^{2+}-dependent ATPase) activity in chloroplasts was stimulated by atebrin, NH_4Cl and gramicidin when the uncouplers were added after light triggering. The stimulation was followed in time by inhibition when the reaction took place in the dark.

2. This inhibition of Mg^{2+}-ATPase activity was overcome when the reaction was carried out under continuous illumination.

3. The energy transfer inhibitors of photophosphorylation, phlorizin and Dio-9, inhibited Mg^{2+}-ATPase activity and the extent of inhibition increased with time. The inhibition by Dio-9 was partially reversed by light while that of phlorizin was not.

4. Light-triggered ATP–P_i exchange activity in chloroplasts was inhibited by both atebrin and phlorizin. The extent of the inhibition increased with time.

5. The activity of a soluble Ca^{2+}-ATPase was inhibited by Dio-9, phlorizin, NH_4Cl and atebrin. The kinetics of activity was linear with time, except in the presence of phlorizin.

6. These results are interpreted as indicating a requirement of a high-energy state for triggering and maintenance of Mg^{2+}-ATPase and ATP–P_i exchange reactions. The relation of ATPase activity to the coupling mechanism in chloroplasts is discussed.

INTRODUCTION

A Mg^{2+}-dependent ATPase activity in chloroplasts[12] was shown to be triggered by light in the presence of sulfhydryl reagents. The Mg^{2+}-ATPase (light-triggered Mg^{2+}-ATPase) activity was inhibited by uncouplers of photophosphorylation when the uncouplers were present both during the light-triggering stage and in the following dark reaction stage[5,8,11–13]. However, there are conflicting reports regarding the effect of uncouplers on Mg^{2+}-ATPase activity when added after the light-triggering stage. In some cases uncouplers stimulated[8,10–12] and in others they inhibited[5,13] Mg^{2+}-ATPase activity. Inhibition of Mg^{2+}-ATPase activity by uncouplers in chloroplasts is in contrast with the stimulatory effect of uncouplers on ATPase activity in mitochondria. The difference might be due to the fact that unlike mitochondrial ATPase, light-triggered Mg^{2+}ATPase activity in chloroplasts decays in the dark unless ATP is present[12]. It was previously suggested[12] that the energy of ATP is needed for the maintenance of

Abbreviations: PMS, phenazine methosulphate; PEP, phosphoenolpyruvate.

ATPase activity in the dark. Therefore, it can be assumed that uncouplers inhibit by preventing the energy from ATP from functioning in the maintenance of Mg^{2+}-ATPase activity. This assumption was difficult to prove, because ATP is both the substrate and the energy source for the maintenance of Mg^{2+}-ATPase activity. However, it was thought that under proper conditions light could substitute ATP as a source of energy for the maintenance of Mg^{2+}-ATPase activity in chloroplasts. In a preliminary work[7] it was shown that under continuous illumination uncouplers only stimulated Mg^{2+}-ATPase activity.

A soluble Ca^{2+}-ATPase was shown to be released[10] from light-triggered chloroplasts after treatment with EDTA. It will be shown that in the presence of some uncouplers and inhibitors of photophosphorylation, the kinetics of Ca^{2+}-ATPase differs from that of light-triggered Mg^{2+}-ATPase. It is suggested that the differences in kinetics reflect the fact that, unlike Mg^{2+}-ATPase, Ca^{2+}-ATPase activity was not subjected to decay in the absence of ATP.

MATERIALS AND METHODS

Chloroplasts washed once were prepared from lettuce (*Lactuca sativa* var. *romaine*) leaves as previously described by AVRON[3]. Light-triggered Mg^{2+}-ATPase activity and incorporation of phosphate into ATP were assayed at 22° in a reaction mixture containing: 20 mM Tris–HCl (pH 7.8), 20 mM KCl; 0.28 mM sodium–potassium phosphate (pH 7.8) (containing about 10^6 counts/min of $^{32}P_i$); 9 mM $MgCl_2$; 10 μM phenazine methosulphate (PMS); 5 mM ATP; 10 mM dithiothreitol and chloroplasts containing 60 μg of chlorophyll in a total volume of 1.0 ml. When indicated, 4 mM phosphoenolpyruvate (PEP) and pyruvate kinase, in excess, were added to the reaction mixture. In order to remove $(NH_4)_2SO_4$, 1 ml of pyruvate kinase suspension was dialyzed overnight against 1 l of 5 mM Tris–HCl (pH 7.8) and 0.1 M KCl. Routinely the reaction mixture was illuminated for 5 min, at 70000 lux in the absence of ATP and PEP. ATP and PEP were added immediately after light was either turned off or turned down to an intensity of 10000 lux, as indicated, and the reaction was allowed to proceed for varying intervals of time. It was terminated by the addition of trichloroacetic acid to a final concentration of 3 %. P_i was determined according to the method of AMES[1]. [^{32}P]ATP content was assayed by an isobutanol–benzene extraction procedure[3]. Chlorophyll content was determined according to the method of ARNON[2].

Ca^{2+}-ATPase was activated at 22° in a reaction mixture containing: 20 mM Tris–HCl (pH 7.8); 20 mM NaCl; 2.5 mM $MgCl_2$; 10 mM dithiothreitol; 0.1 mM FMN and chloroplasts containing 0.75 mg chlorophyll in a total volume of 0.1 ml. The reaction was illuminated for 5 min at 140000 lux, and then aliquots of 0.15 ml were transferred into a reaction mixture containing 0.75 mM EDTA (pH 7) in a total volume of 5 ml. After a 5-min incubation in the dark, the mixture was filtered through a Millipore filter having an average pore size diameter of 0.47 μ. Most of the chloroplasts remained on the filter, while the filtrate was pale green. For assay of Ca^{2+}-ATPase activity, aliquots of 0.5 ml were transferred from the mixture, either before or after filtration, into a reaction mixture containing: 50 mM Tris–HCl (pH 7.8); 5 mM $MgCl_2$ or $CaCl_2$; 5 mM [^{32}P]ATP (containing 10^6 counts/min) in a total volume of 1.0 ml. After incubation at 37° for various time intervals, the reaction was terminated by addition of trichloroacetic acid to a final concentration of 3 %. $^{32}P_i$ content of the reaction mixture was

determined by the isobutanol–benzene extraction procedure. Highly labeled [^{32}P]-ATP was prepared by photophosphorylation with the same chloroplasts and isolated by charcoal adsorption[4].

RESULTS

Effect of NH_4Cl on Mg^{2+}-ATPase activity

CARMELI AND AVRON[6] reported that some uncouplers inhibit the light-triggering stage of the ATP–P_i exchange reaction in chloroplasts. In the following experiments the inhibitory effect that uncouplers might have on the light-triggering stage of Mg^{2+}-ATPase activity was avoided by adopting a procedure in which the chloroplasts were light triggered in the presence of dithiothreitol but in the absence of uncouplers. The uncouplers were added together with ATP only after 5 min of illumination. The effect of various concentrations of NH_4Cl on Mg^{2+}-ATPase activity and on phosphate incorporation into ATP is shown in Table I. Without NH_4Cl there were considerable Mg^{2+}-ATPase and ATP–P_i exchange activities when the reaction stage, which followed light triggering, was carried out in the dark. However, there was no net phosphate increase

TABLE I

EFFECT OF NH_4Cl ON Mg^{2+}-ATPASE ACTIVITY AND ON THE INCORPORATION OF PHOSPHATE INTO ATP

Mg^{2+}-ATPase activity was assayed for 10 min without pyruvate kinase as described under MATERIALS AND METHODS. NH_4Cl was added after light triggering.

NH_4Cl (mM)	P_i released*		P_i incorporated*	
	Light	Dark	Light	Dark
0	0	17.2	10.5	7.6
0.15	3.3	21.0	8.7	6.8
1	34.0	41.9	4.3	2.6
3	77.6	60.0	0.6	0.3

*μmoles·mg chlorophyll^{-1}·h^{-1}.

in the medium when the reaction stage took place in the light. Phosphorylation in the light must have completely reincorporated the phosphate released *via* Mg^{2+}-ATPase activity. This is supported by the fact that the rate of phosphate incorporation into ATP was higher in the light than in the dark. This higher rate in the light was probably due to both ATP–P_i exchange and to the resynthesis of ATP from ADP and phosphate by photophosphorylation. NH_4Cl decreased the incorporation of phosphate into ATP and increased phosphate release when the reaction stage took place in the light or in the dark. The increase in phosphate release was much greater than the inhibition of phosphate incorporation into ATP. This stoichiometry indicates that the increase in phosphate release was caused mainly by the stimulation of ATPase activity by NH_4Cl rather than by the inhibition of phosphorylation or ATP–P_i exchange.

Effect of pyruvate kinase

The effect of NH_4Cl on Mg^{2+}-ATPase activity could be separated from its effect on phosphate incorporation into ATP by the addition of pyruvate kinase *plus* PEP.

Pyruvate kinase competes with photophosphorylation for the rephosphorylation of ADP released from ATP by ATPase activity. This was seen by the marked decrease in the incorporation of phosphate into ATP and the increase in the amount of phosphate released when the reaction stage was in the light (Table II). Pyruvate kinase inhibited ATP–P_i exchange (not indicated in the table) but did not stimulate ATPase activity when the reaction stage was in the dark (Fig. 1). NH_4Cl added together with pyruvate kinase caused a further decrease in the incorporation of phosphate into ATP. However, this decrease was very small in comparison with the increase in the release of phosphate from ATP. It can be concluded that the further increase in the release of phosphate was due mainly to the stimulation of ATPase activity by NH_4Cl. Phlorizin, an energy transfer inhibitor in photophosphorylation, inhibited all activities in the light.

Effect of uncouplers on the time-course of Mg^{2+}-ATPase activity

The effect of NH_4Cl on the time-course of Mg^{2+}-ATPase activity is shown in Fig. 1. When the reaction stage was in the dark the rate was first stimulated and later inhibited by NH_4Cl, while the rate of Mg^{2+}-ATPase activity without NH_4Cl was linear. NH_4Cl would stimulate or inhibit ATPase activity, depending on the duration of the assay. This observation could explain why various researchers reported contradicting results regarding the effect of NH_4Cl on Mg^{2+}-ATPase activity. Similar results were obtained with desaspidin[13]. When the reaction was carried out in the light with pyruvate kinase, the rate of Mg^{2+}-ATPase activity was almost linear and of the same rate as when the reaction proceeded in the dark. However, the same concentration of NH_4Cl gave higher rates and stimulated Mg^{2+}-ATPase activity for a larger time in the light than in the dark. The higher rate of activity in the light was not caused by new triggering of Mg^{2+}-ATPase activity by the light during the reaction. Such additional triggering would have caused also an increase in the rate of Mg^{2+}-ATPase activity in the light without NH_4Cl. However, without NH_4Cl the rate was lower and almost linear. The fact that the rate of Mg^{2+}-ATPase activity without NH_4Cl was the same in the light as in the dark also indicates no additional triggering during the light. Atebrin, another uncoupler of photophosphorylation, also gave higher stimulation of the rate of ATPase activity when the reaction took place in the light rather than in the dark (Fig. 2). The stimulation in the dark was smaller with atebrin than with NH_4Cl. Similar re-

TABLE II

EFFECT OF PYRUVATE KINASE, NH_4Cl AND PHLORIZIN ON Mg^{2+}-ATPASE ACTIVITY AND ON THE INCORPORATION OF PHOSPHATE INTO ATP

All additions were made after light triggering. The reaction stage took place in the light for 10 min while other assay conditions were as indicated under MATERIALS AND METHODS.

Additions	*P_i incorporated**	*P_i released**
None	10.5	0
Pyruvate kinase	1.8	17.1
Pyruvate kinase + 3 mM NH_4Cl	0.8	78.1
3 mM NH_4Cl	0.6	77.6
1 mM phlorizin	—	−0.3
Pyruvate kinase + 1 mM phlorizin	0.7	6.9
Dark control	0.1	1.9

* μmoles·mg chlorophyll^{-1}·h^{-1}.

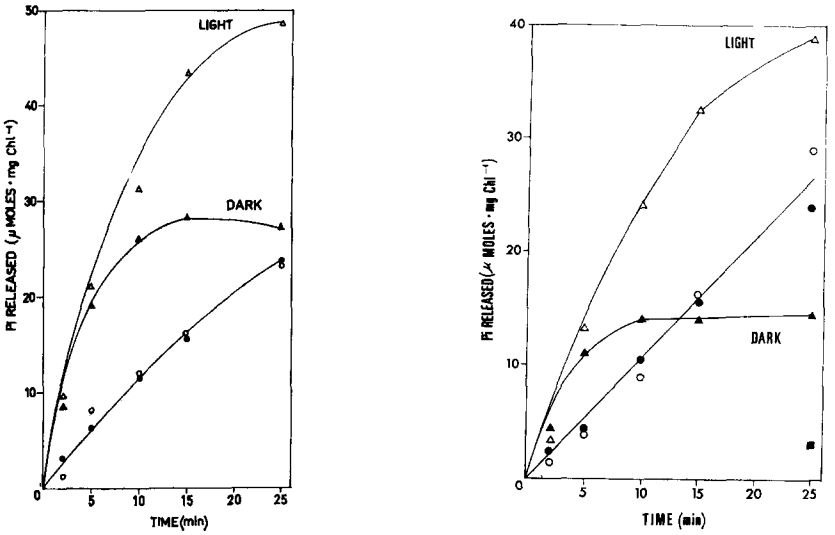


Fig. 1. Effect of NH_4Cl on the time-course of Mg^{2+}-ATPase activity. Chloroplasts were light triggered for 5 min, then, the reaction was carried out in the dark (●—●), in the dark with 3 mM NH_4Cl (▲—▲), in the light (○—○), in the light with 3 mM NH_4Cl (△—△). The reaction medium contained pyruvate kinase and PEP. Other assay conditions were as described under MATERIALS AND METHODS.

Fig. 2. Effect of atebrin on the time-course of Mg^{2+}-ATPase activity. The reaction was carried out in the dark (●—●), in the dark with 20 μM atebrin (▲—▲), in the light (○—○), in the light with 20 μM atebrin (△—△). Other experimental conditions were as described under Fig. 1.

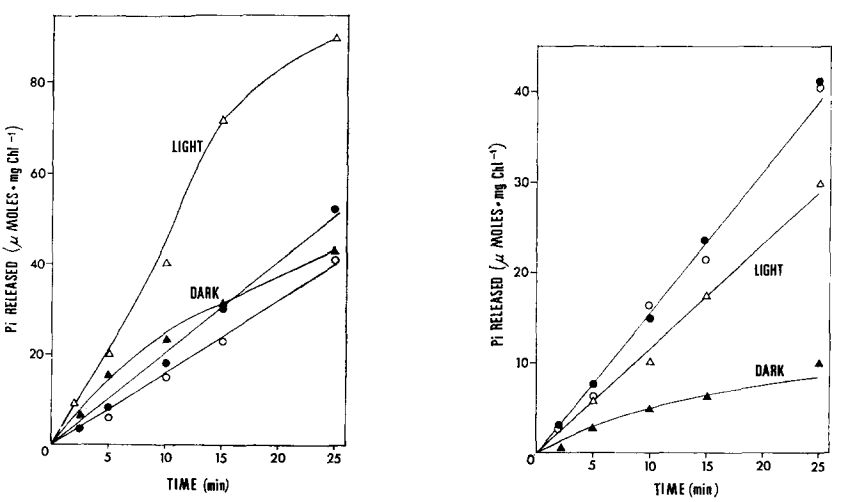


Fig. 3. Effect of gramicidin on the time-course of Mg^{2+}-ATPase activity. The reaction was carried out in the dark (●—●), in the dark with 0.03 μM gramicidin (▲—▲), in the light (○—○), in the light with 0.03 μM gramicidin (△—△). Other assay conditions were as described under Fig. 1.

Fig. 4. Effect of Dio-9 on Mg^{2+}-ATPase activity. The reaction was carried out in the dark (●—●), in the dark with 5 μg/ml Dio-9 (▲—▲), in the light (○—○), in the light with 5 μg/ml Dio-9 (△—△). 5 μg/ml of the Dio-9 used inhibited 75% of cyclic photophosphorylation. Other assay conditions as described under Fig. 1.

sults were obtained with gramicidin (Fig. 3) which is known to uncouple and to affect ion transfer in chloroplasts[14].

Effect of Dio-9 and phlorizin on Mg^{2+}-ATPase

Dio-9, an energy transfer inhibitor of photophosphorylation[10], inhibited Mg^{2+}-ATPase activity in the dark (Fig. 4). The inhibition increased with time and was partially reversed by light. The increase in inhibition with time suggests that the inhibition of the enzyme by Dio-9 also caused inactivation of the triggered state, which resulted in a decrease in the activity with time. Light apparently reversed this inactivation, and thus the activity in the light in the presence of Dio-9 was linear. The linearity of the activity in the light rules out the possibility of photoinactivation of Dio-9, since such inactivation should have caused a decrease in the inhibitory effect of Dio-9 with time. There was no difference in the extent to which Dio-9 inhibited Mg^{2+}-ATPase activity whether the inhibitor was present during light triggering or added during the reaction period (Table III). This result supported the conclusion that Dio-9 was not photoinactivated. The fact that longer light triggering made Mg^{2+}-ATPase activity more resistant to Dio-9 inhibition, probably contributed to the decrease in the inhibition of Mg^{2+}-ATPase activity by Dio-9 in the light. Dio-9 inhibited Mg^{2+}-ATPase activity at approximately the same concentration which inhibited cyclic photophosphorylation.

Phlorizin, another energy transfer inhibitor of photophosphorylation[9], inhibited Mg^{2+}-ATPase activity (Fig. 5). Similar to the inhibition by Dio-9, the extent of phlorizin inhibition of Mg^{2+}-ATPase activity increased in the dark. However, unlike Dio-9, phlorizin inhibition was not partially reversed by light. Data which will be presented later in this paper also indicates partially irreversible inhibition of Ca^{2+}-ATPase activity by phlorizin.

Phlorizin inhibited light-triggered ATP–P_i exchange in a manner which is consistent with the inhibition of ATPase activity. The time-course shows an increase in the inhibition of ATP–P_i exchange reaction with time (Fig. 6). The inhibition of light-triggered ATP–P_i exchange reaction by atebrin also increased with time. This result was consistent with results obtained for Mg^{2+}-ATPase activity if it is assumed that ATPase was in competition with ATP–P_i exchange reaction. It would be expected that atebrin which stimulated Mg^{2+}-ATPase, would inhibit ATP–P_i exchange activity in that case.

TABLE III

THE EFFECT OF PREINCUBATION OF Dio-9 IN THE LIGHT ON ITS INHIBITION OF Mg^{2+}-ATPase ACTIVITY

Light triggering was carried out for 5 or 10 min and 15 μg/ml of Dio-9 were added either before or after triggering as indicated. The reaction was carried out without pyruvate kinase for 20 min in the dark. 15 μg/ml of the Dio-9 used in this experiment gave approx. 50% inhibition of photophosphorylation with PMS. Data are expressed as μmoles $P_i \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$.

Additions	*Time of light triggering*	
	5 min	*10 min*
None	98.5	115.0
Dio-9 in light	19.8	63.4
Dio-9 in dark	20.7	71.5

Ca^{2+}-ATPase

ATPase activity obtained after EDTA treatment of light-triggered chloroplasts was better activated by Ca^{2+} than by Mg^{2+} (Table IV). A much lower Ca^{2+}-ATPase

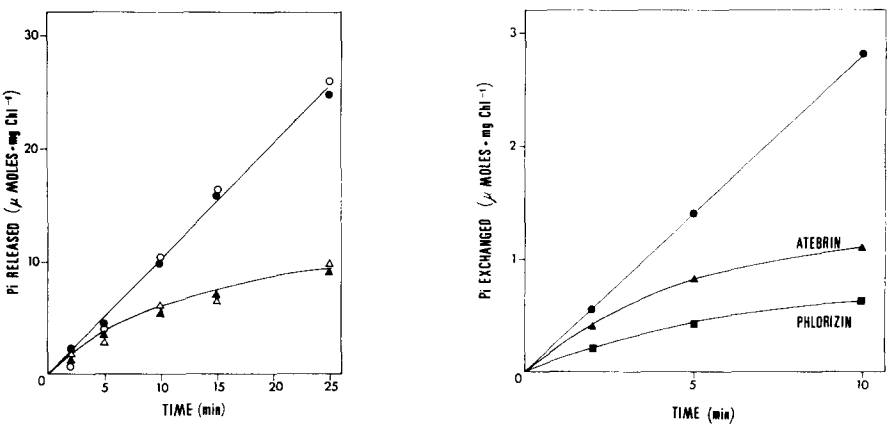


Fig. 5. The effect of phlorizin on Mg^{2+}-ATPase activity. The reaction was carried out in the dark (●—●), in the dark with 1 mM phlorizin (▲—▲), in the light (○—○), in the light with 1 mM phlorizin (△—△). Other experimental conditions were as described under Fig. 1.

Fig. 6. The effect of atebrin and phlorizin on light-triggered ATP–P_i exchange reaction. Chloroplasts were light triggered for 2 min at 70000 lux, then light was turned off and ATP was added either alone (○—○), or together with 7 μM atebrin (△—△), or with 0.5 mM phlorizin. Other assay conditions were as described under MATERIALS AND METHODS.

TABLE IV

ATPASE ACTIVITY AFTER EDTA TREATMENT OF DITHIOTHREITOL-ACTIVATED CHLOROPLASTS

Chloroplasts were activated either in the light or in the dark. ATPase activity was assayed for 20 min with either Ca^{2+} or Mg^{2+} in the medium, before or after filtration as indicated. Other assay conditions as described under MATERIALS AND METHODS. Data are expressed as μmoles $P_i \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$.

Treatment	*Activating ion*			
	Ca^{2+}		*Mg^{2+}*	
	Light	*Dark*	*Light*	*Dark*
Before filtration	33.6	14.1	7.2	5.7
After filtration	32.6	12.5	5.1	3.0

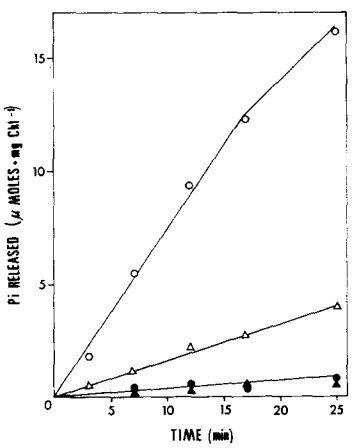
activity was obtained if, instead of light triggering, the chloroplasts were incubated in the dark before EDTA treatment. The fact that most of the Ca^{2+}-ATPase activity remained in the medium after the removal of the chloroplasts by filtration indicated that Ca^{2+}-ATPase activity, which was obtained after EDTA treatment, was mainly in the soluble fraction. Since Ca^{2+}-ATPase is an enzyme which was detached from the chloroplast membrane, it was not expected that uncouplers would stimulate its activity. Indeed, 10 mM NH_4Cl, which stimulated light-triggered Mg^{2+}-ATPase activity, inhibited Ca^{2+}-ATPase activity (Table V). Atebrin, which stimulated Mg^{2+}-ATPase activity at a lower concentration, inhibited both Ca^{2+}- and Mg^{2+}-ATPase activities at a con-

TABLE V

EFFECT OF VARIOUS AGENTS ON Ca^{2+} AND Mg^{2+}-ATPASE ACTIVITIES

The results were collected from several separate experiments in which the rate of activity after 10 min, without any additions, was given the value of 100.5 μg/ml of the Dio-9 used gave 75% inhibition of cyclic photophosporylation. Other assay conditions were as described under MATERIALS AND METHODS.

Additions	*Concn.*	*Rate of reaction (% of control)*	
		Ca^{2+}-ATPase	*Mg^{2+}-ATPase*
None	—	100	100
Phlorizin	1 mM	110	60
Phlorizin	7.5 mM	47	0
Dio-9	5 μg/ml	22	29
NH_4Cl	3 mM	87	250
NH_4Cl	10 mM	27	132
Atebrin	20 μM	100	140
Atebrin	0.25 mM	36	0
Gramicidin	0.03 μM	102	140
Gramicidin	1 μM	99	113



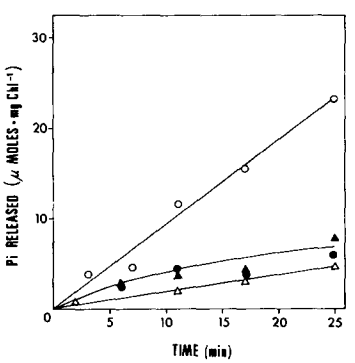


Fig. 7. Effect of Dio-9 on Ca^{2+}-ATPase activity. Assay of activity took place with $CaCl_2$ (○—○), with $CaCl_2$ and 43 μg/ml Dio-9 (△—△), with $MgCl_2$ (●—●), with $MgCl_2$ and 43 μg/ml Dio-9 (▲—▲). Other assay conditions were as described under MATERIALS AND METHODS. 43 μg/ml of the Dio-9 used gave approx. 70% inhibition of the Mg^{2+}-ATPase activity.

Fig. 8. Effect of NH_4Cl on Ca^{2+}-ATPase activity. Assay of the activity was carried out with $CaCl_2$ (○—○), with $CaCl_2$ and 10 mM NH_4Cl (△—△), with $MgCl_2$ (●—●), with $MgCl_2$ and 10 mM NH_4Cl (▲—▲). Other assay conditions as described under MATERIALS AND METHODS.

centration of 0.25 mM (Table V). Gramicidin did not have any effect on Ca^{2+}-ATPase activity even at a concentration of 1 μM, although it greatly stimulated Mg^{2+}-ATPase at 0.03 μM (Table V). Both Dio-9 and phlorizin inhibited Ca^{2+}-ATPase activity, However, higher concentrations of phlorizin were required in order to obtain the same amount of inhibition of Ca^{2+}-ATPase as that of Mg^{2+}-ATPase.

Kinetics of Ca^{2+}-ATPase activity

A kinetic study of the effect of Dio-9 on Ca^{2+}-ATPase revealed that both inhibited and uninhibited activities were linear for 20 min (Fig. 7). The inhibition of Mg^{2+}-

ATPase activity by Dio-9 increased with time, unlike the linear kinetics of inhibition of Ca^{2+}-ATPase activity. The inhibition of Ca^{2+}-ATPase activity by NH_4Cl and by atebrin was also linear (Figs. 8, 9). The differences in the kinetics of the inhibition of Mg^{2+}- and Ca^{2+}-ATPase was related to the fact that unlike the former, the latter activity did not decay in the absence of ATP.

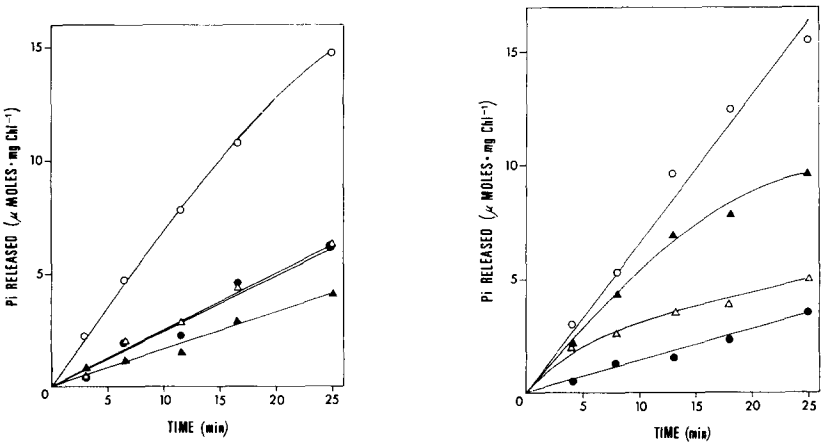


Fig. 9. Effect of atebrin on Ca^{2+}-ATPase activity. Assay was carried out with $CaCl_2$ (○—○), with $CaCl_2$ and 0.25 mM atebrin (△—△), with $MgCl_2$ (●—●), with $MgCl_2$ and 0.25 mM atebrin (▲—▲). Other assay conditions as described under MATERIALS AND METHODS.

Fig. 10. Effect of phlorizin on Ca^{2+}-ATPase activity. The reaction was carried out with $CaCl_2$ (○—○), with $CaCl_2$ and 7.5 mM phlorizin (△—△), with $MgCl_2$ (●—●), with $MgCl_2$ and 7.5 mM phlorizin (▲—▲). Other assay conditions as described under MATERIALS AND METHODS.

Phlorizin affected the kinetics of Ca^{2+}-ATPase activity differently than the other agents which were tested. Phlorizin inhibition increased with time (Fig. 10), while the inhibition of Ca^{2+}-ATPase activity by other agents was linear. Phlorizin inhibition of Ca^{2+}-ATPase activity was rather like its effect on Mg^{2+}-ATPase activity. This pattern of inhibition could indicate a partially irreversible inhibition of ATPase activity by phlorizin. In the presence of phlorizin the enzyme was activated by Mg^{2+} to almost the same level which was obtained when Ca^{2+} was used as an activating ion in the absence of phlorizin. Alteration of the enzyme specificity to ions, beside its inhibitory effect, could explain these results.

DISCUSSION

The stimulation of ATPase activity by uncouplers of photophosphorylation is similar to the effect of uncouplers on ATPase activity in mitochondria. As in oxidative phosphorylation, it can be assumed that uncouplers dissipate an high-energy intermediate or state of phosphorylation and that this breakdown enables ATP to be hydrolyzed *via* a reversal of some of the reactions of phosphorylation. The inhibition which follows the stimulation of Mg^{2+}-ATPase activity in the dark could be explained on the assumption that energy from ATP is required for the maintenance of the activity. The light-triggered state decays in the absence of ATP but the decay is not accelerated by uncouplers[6]. However, the acceleration of the dissipation of an

high-energy intermediate or state of phorphorylation, which is caused by uncouplers and stimulates ATPase activity, also reduced the availability of energy for the maintenance of the activity. The Mg^{2+}-ATPase activity gradually declines and thus, the stimulation by uncouplers is followed by inhibition. The fact that light reverses the inhibition and prolongs the stimulation of Mg^{2+}-ATPase activity supports this assumption. Light serves as an additional source of energy for the maintenance of ATPase activity. Indeed, at the same concentration of an uncoupler, the initial rates of Mg^{2+}-ATPase activity were the same both in the light and in the dark (Figs. 1, 2). However, the rate of Mg^{2+}-ATPase activity in the light changed only slightly when Mg^{2+}-ATPase activity in the dark was completely inhibited. Energy transfer inhibitors such as Dio-9 are believed to affect one of the terminal steps in the reaction sequence of photophosphorylation, possibly the terminal enzyme which catalyses the synthesis of ATP. If so, it is not surprising that Mg^{2+}-ATPase activity was inhibited by Dio-9. The increase in inhibition with time indicates that the decrease in hydrolysis of ATP resulted in a decrease in the level of the energy which was available for the maintenance of Mg^{2+}-ATPase activity. Decrease in the maintenance resulted in gradual decay of Mg^{2+}-ATPase activity and in an increase in the extent of Dio-9 inhibition with time. In the light, sufficient energy was available for maintenance of the light-triggered state which did not decay. Therefore the inhibition of Mg^{2+}-ATPase activity by Dio-9 was lower than in the dark and linear with time. The fact that the inhibition of light-triggered ATP–P_i exchange reaction increased with time is also consistent with the mechanism suggested for ATPase. Atebrin dissipated the energy state or intermediate which was needed both for the energizing and for the maintenance of ATP–P_i exchange reaction. This compound effect resulted in an increase in the inhibition of ATP–P_i exchange reaction with time.

Several observations, including immunological studies[10], indicate that the same enzyme which catalyzes Mg^{2+}-ATPase activity becomes Ca^{2+}-dependent when released from the chloroplast. Removed from the chloroplast and detached from the rest of the coupling membrane, Ca^{2+}-ATPase provides an isolated system by which the effect of various agents on the enzyme itself can be separated from their effect on the coupling mechanism. Indeed uncouplers such as atebrin, NH_4Cl and gramicidin, which stimulated Mg^{2+}-ATPase activity in the chloroplast, did not stimulate Ca^{2+}-ATPase activity. The fact that high concentration of atebrin inhibited Ca^{2+}-ATPase activity indicates that direct interaction of atebrin at this concentration with the enzyme caused the inhibition of Mg^{2+}-ATPase activity. There is no such direct interaction of the enzyme with NH_4Cl, since at concentrations at which Ca^{2+}-ATPase activity was inhibited, Mg^{2+}-ATPase activity was still stimulated. A clear separation of the effect on the coupling membrane rather than on the enzyme was demonstrated in the case of gramicidin which affects ion transfer in membranes and stimulated Mg^{2+}-ATPase activity at 0.03 μM but did not have any effect on Ca^{2+}-ATPase activity even at a concentration of 1 μM.

As was previously discussed, the assay of Mg^{2+}-ATPase in the light provided a system in which the effect of uncouplers and inhibitors on the decay of the activity could be separated from their effect on the activity of the enzyme. Ca^{2+}-ATPase activity, which did not decay in the absence of ATP[10], provided another system in which the effect of these agents on the enzymic activity could be separated from their effect on the decay. Thus, the fact that Dio-9 inhibition of Ca^{2+}-ATPase activity was linear sup-

ports the suggestion that the increase in inhibition of Mg^{2+}-ATPase activity by Dio-9 was due to the decay of the enzymic activity. The linear kinetics of Ca^{2+}-ATPase activity in the presence of atebrin and NH_4Cl is in agreement with the same hypothesis. The increase in the inhibition of Mg^{2+}-ATPase activity by phlorizin with time also seems to be the result of a partially irreversible inhibition of the enzyme. This assumption is supported by the fact that unlike Dio-9 , phlorizin inhibition of Mg^{2+}-ATPase activity was not partially reversed by light, and by the fact that, unlike Dio-9, phlorizin inhibition of Ca^{2+}-ATPase activity increased with time. Ca^{2+}-ATPase gave low activity when Ca^{2+} was substituted by Mg^{2+} as the activating ion. However, in the presence of phlorizin, the activities with Mg^{2+} approached the control activities with Ca^{2+}. It seems that, besides its inhibitory effect, phlorizin also alters the ion specificity of ATPase.

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