BBA 46286

EFFECTS OF P_i AND ADP ON ATPase ACTIVITY IN CHLOROPLASTS

C. CARMELI AND Y. LIFSHITZ
Department of Biochemistry, Tel-Aviv University, Tel- Aviv (Israel)
(Received September 6th, 1971)
(Revised manuscript received December 7th, 1971)

SUMMARY

1. The rate of decay of the light-triggered state of ATPase in chloroplasts was decreased in the presence of P_i and accelerated by ADP in a phosphateless medium. Phosphate and arsenate inhibited the effect of ADP.

2. Compared with other dinucleotides, ADP was found to be highly specific for the acceleration of the decay, having an apparent K_m of $1.1 \cdot 10^{-6}$ M. A lower apparent K_m for ADP was obtained in the presence of phosphate. The apparent K_m for the inhibition of the effect of ADP by P_i was lowered with increased concentrations of ADP.

3. These and other effects on ATPase and ATP–P_i exchange activities were discussed in terms of changes in the permeability of ADP and P_i across the membrane of the chloroplast.

INTRODUCTION

Chloroplasts can be triggered by light in the presence of sulfhydryl reagents to catalyze, in the following dark reaction period, ATPase[1-4] and ATP–P_i[5-7] exchange activities. The light-triggered state of ATPase decays in the dark unless ATP is present. It was suggested that energy from light or from ATP is required to maintain the light-triggered state[1,8]. Phosphate stabilized and ADP accelerated the rate of the decay of the light-triggered state. These and other data presented here on the effects of ADP and P_i on ATPase and ATP–P_i exchange activities are interpreted by us to be indicative of P_i and ADP movement across the thylakoid membrane. Such movement of metabolites of photophosphorylation could have been expected since phosphorylation requires an intact thylakoid membrane. However, movement of phosphate, phosphate esters and nucleotides was demonstrated in "whole" chloroplasts[9-11] which retain an intact outer membrane but not in "broken" chloroplasts (the outer membrane of which is not intact) which were used in these experiments.

MATERIALS AND METHODS

Chloroplasts once washed were prepared from lettuce leaves as previously described[12]. Light triggered ATPase activity was assayed at 22 °C in a reaction mixture containing: 30 mM Tris–HCl (pH 7.8); 35 mM KCl; 10 mM $MgCl_2$; 5 mM

[^{32}P]ATP (containing 10^6 cpm); 50 μM phenazine methosulfate; 5 mM dithiothreitol and chloroplasts containing 50 μg chlorophyll in a total volume of 1.0 ml. Routinely, the reaction mixture was illuminated for 5 min at 10^6 ergs·cm^{-2}·s^{-1} in the absence of ATP, ATP was added immediately after light was turned off and the reaction was allowed to proceed for 10 min in the dark. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 3 %. $^{32}P_i$ content was assayed according to the isobutanol–benzene extraction procedure[12]. Highly labeled [^{32}P]ATP was prepared by phosphorylation with the same chloroplasts and isolated by charcoal adsorption[13]. Chlorophyll content was determined according to the method of Arnon[14].

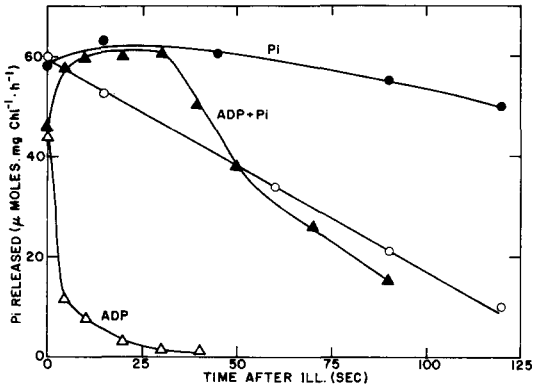

Fig. 1. Effect of ADP and P_i on the decay of the light-triggered state of ATPase. The reaction mixture and experimental conditions were as described under Materials and Methods. After 5 min of illumination, light was turned off and either 0.2 mM ADP (△—△) or 0.2 mM ADP *plus* 2 mM P_i (▲—▲) or 2 mM P_i (●—●) were added, or no additions (○—○) were made. In each treatment [^{32}P]ATP was added at various time intervals as indicated and the reaction was allowed to continue for 10 min.

The decay of the light-triggered state of ATPase was measured in the same reaction mixture as used for the measurement of ATPase activity. The reaction mixture was illuminated for 5 min and ATP was added at varying time intervals after light was turned off. In each experiment, the reaction was allowed to proceed for 10 min in the dark after [^{32}P]ATP was added. The reaction was terminated and $^{32}P_i$ content was measured as described above.

RESULTS

Effect of ADP and P_i on the decay of the light-triggered state of ATPase

The decay of the light-triggered state of ATPase activity was considerably stabilized by addition of phosphate to the reaction medium (Fig. 1). It can be assumed that the phosphate which was added to the medium reached a site where it could effect the stability of the triggered state. It can also be assumed that the level of P_i at that site determined the degree of stability of the triggered state. It is possible that the concentration of P_i in isolated chloroplasts was not optimal for the stabilization of the triggered state. Therefore, an increased P_i concentration rendered the light-triggered state more stable.

TABLE I

THE EFFECT OF ADP, P_i AND ARSENATE ON THE LIGHT-TRIGGERED STATE OF ATPase

0.5 mM P_i, 0.2 mM ADP and 0.5 mM arsenate were added alone or in combinations, either before light was turned on thus present during all the stages of the reaction, or immediately after light was turned off as indicated in the table. [^{32}P]ATP was added either alone or with other additions 20 s after light was turned off. Other reaction conditions were as indicated under Materials and Methods.

Additions	*P_i released μmoles·mg^{-1} chlorophyll·h^{-1}*		
	Before illumination	*Immediately after illumination*	*20 s after illumination (with ATP)*
None	—	—	82.1
ADP	3.0	8.4	81.0
P_i	100	97.0	98.5
(ADP added after illumination) P_i	103.0	90.4	22.0
(ADP added before illumination) P_i	93.2	92.0	18.5
(ADP added before illumination) arsenate	91.5	90.3	19.3

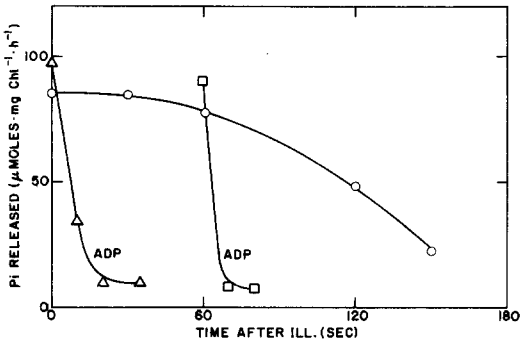

Fig. 2. Time dependence of the effect of ADP on the decay of the light-triggered state of ATPase. The reaction mixture and experimental conditions were as described under Materials and Methods. After 5 min of illumination, light was turned off and 0.1 mM ADP was added either immediately (△—△), or after 60 s (□—□); or no ADP was added (○—○). [^{32}P]ATP was added at various time intervals as indicated.

Addition of ADP to a phosphateless medium greatly accelerated the decay of the triggered state. The effect of ADP was almost completely eliminated when it was added together with P_i.

From Table I, it can be concluded that the presence of ADP after light triggering was sufficient to cause acceleration of the decay. The rate of the decay was increased whether ADP was present during or after the light-triggering stage. However, ADP did not affect ATPase activity when it was added together with ATP. Phosphate inhibited the effect of ADP whether it was added before or after illumination as long as it was present together with ADP after light triggering. No reversal of the effect of ADP by phosphate occurred when P_i was added together with ATP after illumination.

Arsenate, an analogue of phosphate, had the same effect as phosphate. Since arsenate forms a highly unstable ester, it would be unlikely to suggest that the effects of phosphate were due to a formation of a stable phosphate ester.

It is of some interest to note that ADP accelerated the decay whether added at the end of, or 1 min after, light triggering (Fig. 2). Since the high-energy state or intermediate of phosphorylation which is formed in the light decays in less than 1 min[15], none of it is available for phosphorylation 1 min after the end of illumination. Therefore, the effect of ADP on the light-triggered state is unlikely to be a result of the utilization of the high-energy state for the phosphorylation of ADP. Unlikely phosphate and arsenate, other anions such as acetate, sulfate and nitrate did not reverse the effect of ADP (not shown).

TABLE II

EFFECT OF NUCLEOTIDES ON THE LIGHT-TRIGGERED STATE OF ATPase

Various nucleotides were added immediately after light was turned off, either alone or together with ADP, when recovery from the inhibition by ADP was tested. [^{32}P]ATP was added 20 s after light was turned off. The activity without any nucleotide was taken as 100% when inhibition was assayed, and with ADP as zero when recovery was tested. Other experimental conditions and the reaction mixture were as described under Materials and Methods.

Nucleotide	*Concn (mM)*	*ATPase activity*	
		Inhibition (%)	*Recovery from inhibition by ADP (%)*
ADP	0.01	92.6	0
GDP	0.01	c	28
GDP	0.1	45.0	6
UDP	0.01	0	0
UDP	0.1	34.0	0
IDP	0.01	31.0	0
IDP	0.1	59.0	5
CDP	0.01	0	0
CDP	0.1	0	0
α,β-Methyleneadenosine diphosphate	0.01	0	0
α,β-Methyleneadenosine diphosphate	0.1	0	24
β,γ-Methyleneadenosine triphosphate	0.01	0	0
β,γ-Methyleneadenosine triphosphate	0.1	26.0	0
ATP	0.01	0	3
ATP	0.1	0	77

Nucleotide specificity

Acceleration of the decay was highly specific for ADP when compared with other dinucleotides. IDP, which was more effective than the other nucleotides was still more than 3 times less efficient than ADP (Table II). The specificity of the nucleotides did not follow the exact order of their affinity in phosphorylation[16] except for CDP which shows the lowest effect both in phosphorylation and in the acceleration of the decay. The analogues of ADP and ATP α,β-methyleneadenosine diphosphate, and β,γ-methyleneadenosine triphosphate, had little effect on the rate of the decay. Some recovery from the effect of ADP was obtained when GDP and α,β-methyleneadenosine diphosphate were added together with ADP. ATP was most effective in reversing the effect of ADP. The effect of ADP and P_i on the light-triggered state of ATP–P_i exchange activity was similar to their effect on the light-triggered state of ATPase activity (Fig. 3).

The rate of ATPase, which was measured 20 s after light triggering, depended on the concentration of ADP which was added immediately after light triggering

(Fig. 4). The rate of ATPase activity, measured after triggering, depended on the rate of the decay of the light-triggered state. Therefore, it can be concluded from Fig. 4 that there was an increase in the rate of the decay of the light-triggered state when the ADP concentration was increased. An average of five experiments gave an apparent K_m of $1.1 \cdot 10^{-6}$ M. This value was much lower than the K_m of $6 \cdot 10^{-5}$ M, obtained for the phosphorylation of ADP[16]. In the presence of phosphate, only partial inhibition of ATPase was obtained, even at very high concentrations of ADP. Although the decrease in the apparent K_m of ADP in the presence of P_i (Fig. 4, top) is rather small, it could provide a clue for the mode of action of ADP and P_i.

The inhibition of the effect of ADP on the light-triggered state depended on the concentration of P_i which was added together with ADP (Fig. 5). At optimal concentration, P_i completely reversed the effect of ADP. The apparent K_m for P_i varied with the concentration of ADP. At 4 μM and 30 μM ADP, the apparent K_m for P_i was $2.6 \cdot 10^{-5}$ M and $1.7 \cdot 10^{-5}$ M, respectively.

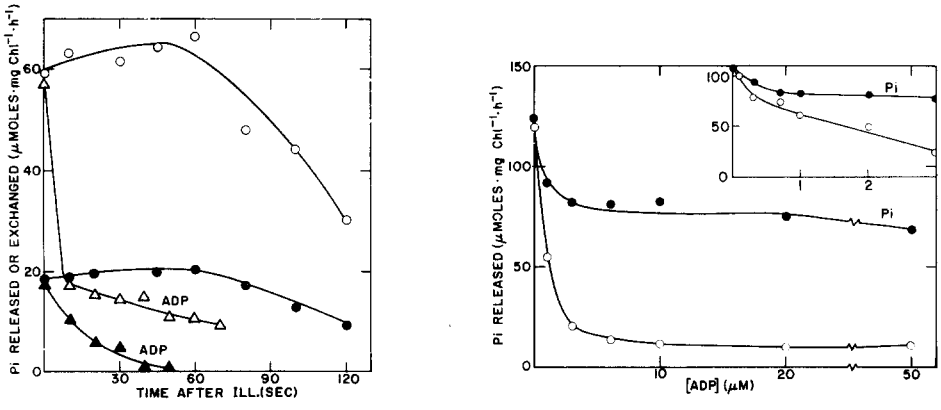

Fig. 3. The decay of the light-triggered states of ATPase and ATP–P_i exchange. The reaction mixture and experimental conditions were as described under Materials and Methods. Immediately after illumination either 5 μM ADP was added (△—△, ▲—▲) or no additions were made (○—○, ●—●). For assay of ATPase activity 5 mM [^{32}P]ATP was added (○—○, △—△) and for assay of ATP–P_i exchange (●—●, ▲—▲) 5 mM ATP and 2 mM [^{32}P]P_i were added at various time intervals as indicated.

Fig. 4. The effect of the concentration of ADP on the acceleration of the decay of the light-triggered state of ATPase. The reaction mixture and experimental condition were as described under Materials and Methods. Immediately after turning the light off, either various concentrations of ADP (○—○) or ADP *plus* 50 μM P_i (●—●) were added. [^{32}P]ATP was added 20 s after light was turned off.

Competition between ADP and ATP

ATP reversed the effect of ADP on the light-triggered state of ATPase (Table I). In these experiments, non-labeled ATP was added together with ADP after triggering while substrate amounts of [^{32}P]ATP were added 20 s after triggering. Thus, the rate of ATPase which was determined by measuring the $^{32}P_i$ released, had already been corrected for the small amounts of P_i released during the first 20 s. From the Lineweaver–Burk plot in Fig. 6, it can be seen that ATP competitively reversed the effect of ADP. The K_m of $4 \cdot 10^{-6}$ M ATP obtained here was

lower than the K_m of $9 \cdot 10^{-5}$ M obtained for the hydrolysis of ATP by ATPase[16]. The lower K_m indicates that the site of binding for hydrolysis has lower affinity for ATP than the site where ATP reverses the effect of ADP on the light-triggered state of ATPase.

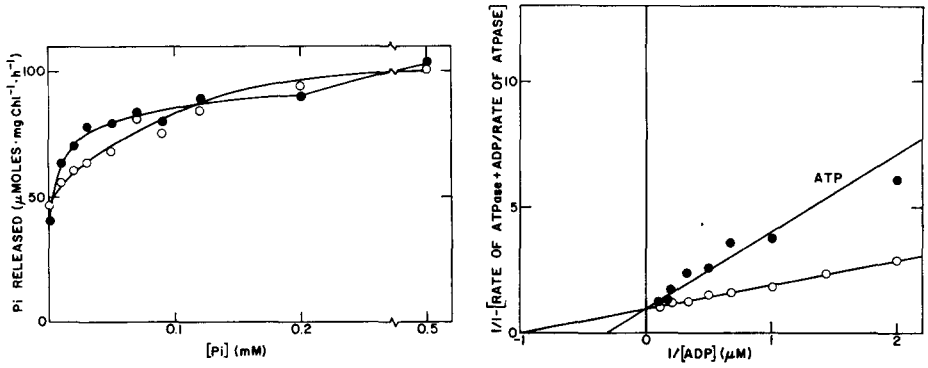

Fig. 5. P_i inhibition of ADP effect on the light-triggered state of ATPase. Immediately after light was turned off, either various concentrations of P_i *plus* 4 μM ADP (○—○) or P_i *plus* 30 μM ADP (●—●) were added. [^{32}P]ATP was added 10 s after light was turned off. The reaction mixture and experimental conditions were as described under Materials and Methods.

Fig. 6. Competition between ADP and ATP for the decay of the light-triggered state of ATPase. Immediately after light was turned off either various concentrations of ADP (○—○) or ADP *plus* 10 μM non-radioactive ATP (●—●) were added. [^{32}P]ATP was added 20 s after light was turned off. Other experimental conditions and the reaction mixture were as described under Materials and Methods.

The effect of uncouplers and energy transfer inhibitors

The light-triggered state of ATPase was shown to be stabilized by P_i and to be maintained by a high-energy state or intermediate. Phosphorylation of ADP would remove P_i and utilize energy. Thus, phosphorylation would be expected to stimulate the decay of the triggered state. Uncouplers and inhibitors of phosphorylation were used to test this possibility. It can be seen that phlorizin and *N,N'*-dicyclohexylcarbodiimide which inhibited, and gramicidin and NH_4Cl (Fig. 7) which stimulated ATPase activity did not affect the half-life of the decay of the light triggered state in the presence of ADP (Table III). The slight apparent decrease in the half life of the decay without ADP did not seem to be a result of the effect of the uncoupler on the triggered state since the same results were obtained when NH_4Cl was added with ATP. Since uncouplers and energy transfer inhibitors inhibited phosphorylation, it is unlikely that the phosphorylation of ADP caused acceleration of the decay of the light-triggered state of ATPase. The half life of the light-triggered state in the presence of ADP was similar to that of the high-energy state of phosphorylation. However, they are different since only the high-energy state of phosphorylation was affected by uncouplers.

The effect of P_i and pyruvate kinase on ATPase activity

The observed stimulation of ATPase activity by P_i[17] is difficult to explain. As seen from Table IV ATPase activity is stimulated not only by addition of P_i but also by the removal of ADP, which is produced during ATPase activity, by

rephosphorylating it with pyruvate kinase. It can be speculated that not only pyruvate kinase but also P_i stimulated ATPase activity by removing ADP from the site of ATP hydrolysis.

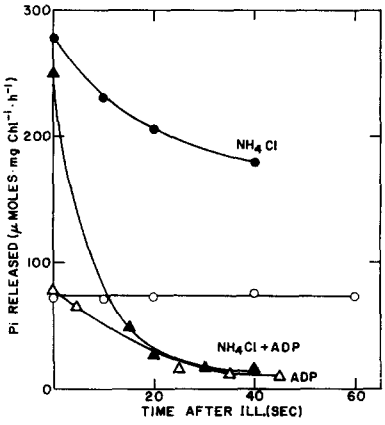

Fig. 7. The effect of NH_4Cl on the decay of the light-triggered state of ATPase. Immediately after light was turned off, either 5 μM ADP (△—△) or 5 μM ADP *plus* 3 mM NH_4Cl (▲—▲) or 3 mM NH_4Cl (●—●) were added, or no additions (○—○) were made. [^{32}P]ATP was added at various time intervals after light was turned off as indicated. Other experimental conditions and the reaction mixture were as described under Materials and Methods.

TABLE III

EFFECT OF INHIBITORS OF PHOTOPHOSPHORYLATION ON THE LIGHT-TRIGGERED STATE OF ATPase

Inhibitors were added together with ATP immediately after light triggering when their effect on the rate of ATPase was tested. For measurements of their effect on the decay of the light-triggered state, inhibitors were added immediately after light triggering together with ADP. [^{32}P]ATP was added at various time intervals after triggering. Other experimental conditions and the reaction mixture were as described under Materials and Methods.

Inhibitor	*Concn (M)*	*Rate of ATPase (% of control)*	*Half-life of decay (s)*		
			ADP	*ADP plus inhibitor*	*No ADP or inhibitor*
Phlorizin	$1 \cdot 10^{-3}$	23	3.5	4	> 90
N,N'-Dicyclohexyl-carbodiimide	$5 \cdot 10^{-5}$	30	10	9.7	>150
Gramicidin	$3 \cdot 10^{-8}$	370	5	5	90
NH_4Cl	$3 \cdot 10^{-3}$	410	10	10.2	>120

DISCUSSION

No direct evidence for the nature of the changes which occur during light triggering is available. However, the requirement for dithiol reagents suggests that alteration of the enzyme may be involved. The high-energy state or intermediate produced by light may provide the necessary conditions under which the dithiol reagent can act, probably on S–S bond of the protein. The resulting changes decay, unless energy from light or from ATP is provided for their maintenance[1,8].

When no energy is provided, the binding of P_i to the enzyme can be assumed

TABLE IV

STIMULATION OF ATPASE ACTIVITY BY P_i AND IN THE PRESENCE OF PYRUVATE KINASE
Experimental conditions and the reaction mixture were as described under Materials and Methods except for the indicated reagents which were added together with [^{32}P]ATP immediately after light triggering.

Additions	*ATPase activity* (μ*moles* P_i *released*·mg^{-1} *chlorophyll*·h^{-1})
None	33.6
2 mM phosphoenolpyruvate + 3 I.U. pyruvate kinase	64.3
0.5 mM P_i	47.0

to control the stability of the triggered state of the ATPase. Isolated chloroplasts contain P_i and it is reasonable to assume that the endogenous concentration of P_i controls the degree of stability of the light triggered state. The fact that added phosphate decreased the rate of the decay indicates that P_i controls the activity and that the concentration of the endogenous P_i was not optimal for the stabilization of the light-triggered state. It is unlikely that either endogenous or added P_i stabilized the light-triggered state by the formation of ATP since uncouplers, which inhibit phosphorylation, did not accelerate the decay and arsenate, which forms a highly unstable ester, had the same effect as P_i.

Several mechanisms for the acceleration of the decay of the light-triggered state by ADP could be proposed. Phosphorylation of P_i to ATP could decrease the concentration of endogenous P_i thus resulting in a faster decay. However, uncouplers and energy transfer inhibitors which inhibit phosphorylation did not prevent ADP from accelerating the decay of the light triggered state. It is, therefore, unlikely that phosphorylation of ADP caused the acceleration of the decay. The fact that ADP accelerated the decay of the light-triggered state even when added 1 min after light was turned off, which is long after the high-energy state of phosphorylation decayed[15], also supports the suggestion that the effect was not a result of phosphorylation of ADP.

The competitive inhibition of ATPase by ADP[16] has a K_i which is approx. 14-fold higher than the K_m for the effect of ADP on the light-triggered state. This inhibition was not reversed by P_i. It would therefore be difficult to assume that the acceleration of the decay of the light-triggered state is a result of a competitive inhibition of ATPase by ADP.

It can also be assumed that the binding of ADP to the enzyme caused an allosteric effect which reversed the effect of P_i. The binding of P_i and ADP should be on different sites of the enzyme since no competition between the two could be shown. It should be further assumed that saturation of all sites with P_i prevented ADP from functioning, since complete reversal of the effect of ADP was achieved at higher P_i concentrations. At a given P_i concentration, only enzymes which do not have any P_i bound to them will be affected by ADP. The fact that only in the absence of P_i ADP completely inhibited the activity would indicate that the endogenous P_i in the chloroplast is not bound to the enzyme, which is hard to assume. It is also difficult to explain why ADP should decreases the K_m of P_i since it was

shown that ADP cannot effect enzymes to which P_i is bound. The effects of P_i on the rate of ATPase activity is also difficult to explain by its allosteric effect on the light-triggered state.

Most of the effects can be better explained if it is assumed that the site of action of P_i is located behind a barrier (possibly the thylakoid membrane or a hydrophobic space in the enzyme) which is relatively inaccessible to P_i and to ADP and that the mobility of each of these two compounds is changed in the presence of the other. In a phosphateless medium, the endogenous P_i tends to become diluted once its mobility was increased by the ADP added. Thus, a decrease in the endogenous concentration of P_i will result in a faster decay. At a given P_i concentration, addition of ADP will result in a fast equilibrium between the inside and outside concentrations of P_i and the rate of decay of the light-triggered state will be controlled by the concentration of the P_i added. The decrease in the apparent K_m for P_i with increased concentrations of ADP and in the apparent K_m for ADP in the presence of P_i is small but may be significant. It can easily be explained if it is assumed that each of these two compounds increases the mobility of the other. Thus, the actual inside concentration of ADP will be higher in the presence of P_i and *vice versa*.

The stimulation of ATPase activity by P_i could also be explained by the same mechanism. It can be assumed that ADP, which was produced during ATPase activity, did not move freely to the medium. Accumulation of ADP at the site of hydrolysis would inhibit the activity since ADP is a competitive inhibitor of ATPase[16]. Added P_i will increase the mobility of ADP which will move to the medium according to the concentration gradient. At lower ADP concentration, the intrinsic inhibition will be released and there will be an apparent stimulation of ATPase activity.

The fact that trapping of the ADP, which is produced during hydrolysis by its phosphorylation with pyruvate kinase stimulated the activity also supports the suggested mechanism. It can be speculated that the effects of P_i and ADP on the light-triggered state of ATPase and ATP–P_i exchange activities is indicative of their movement across a permeability barrier, possibly the thylakoid membrane. This would imply that ATP is synthesized inside the chloroplast membrane. However, a more direct evidence is needed in order to show that a movement of these compounds does occur and by what mechanism it operates. When considering the possibility of compartmentation, it should be remembered that electron microscopy[18] and immunology studies[19] indicated that the ATPase is projected from the membrane into the outside medium. However, since ATPase is located in the membrane, it can be affected by P_i which is present inside the chloroplast[20]. The recent finding that under certain conditions ATPase binds ADP[21] could be related to the observed effect of ADP on ATPase.

REFERENCES

1 B. Petrack, A. Carson, F. Sheppy and F. Farson, *J. Biol. Chem.*, 240 (1965) 906.
2 C. Hoch and I. Martin, *Biochem. Biophys. Res. Commun.*, 12 (1963) 223.
3 L. Packer and R. Marchent, *J. Biol. Chem.*, 239 (1963) 2061.
4 C. Carmeli and M. Avron, *Biochem. Biophys. Res. Commun.*, 24 (1966) 923.
5 C. Carmeli and M. Avron, *Eur. J. Biochem.*, 2 (1967) 318.
6 R. E. McCarty and E. Racker, *J. Biol. Chem.*, 243 (1968) 129.

7 K. G. Rienits, *Biochim. Biophys. Acta*, 143 (1967) 595.
8 C. Carmeli, *Biochim. Biophys. Acta*, 198 (1969) 256.
9 J. A. Bassham, M. Kirk and R. G. Jensen, *Biochim. Biophys. Acta*, 153 (1968) 211.
10 H. W. Heldt and L. Rapley, *FEBS Lett.*, 7 (1970) 139.
11 J. M. Robinson and C. R. Stocking, *Plant Physiol.*, 43 (1968) 1597.
12 M. Avron, *Biochim. Biophys. Acta*, 40 (1960) 257.
13 M. Avron, *Anal. Biochem.*, 2 (1961) 535.
14 I. D. Arnon, *Plant Physiol.*, 24 (1949) 1.
15 G. Hind and A. T. Jagendorf, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 715.
16 A. Bennun and M. Avron, *Biochim. Biophys. Acta*, 109 (1965) 117.
17 G. E. Skye, N. Shavit and P. D. Boxer, *Biochem. Biophys. Res. Commun.*, 28 (1967) 724.
18 S. H. Howell and E. N. Moundrianakis, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1261.
19 R. E. McCarty and E. Racker, *Brookhaven Symp. Biol.*, 19 (1966) 202.
20 K. A. Santarius and U. Heber, *Biochim. Biophys. Acta*, 102 (1965) 39.
21 H. Roy and E. N. Moundrianakis, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 464.