ADENOSINE TRIPHOSPHATASE ACTIVITY OF PLASTID MEMBRANE DURING CHLOROPLAST DEVELOPMENT OF KIDNEY BEAN LEAVES

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
CHEN-HSIUNG WU
1974

ABSTRACT

ADENOSINE TRIPHOSPHATASE ACTIVITY OF PLASTID MEMBRANE DURING CHLOROPLAST DEVELOPMENT OF KIDNEY BEAN LEAVES

BY

Chen-hsiung Wu

1. The activity of a Ca^{2+}-dependent, trypsin-activated ATPase from the membranes of kidney bean (Phaseolus vulgaris) leaf plastids has been followed during greening.

2. In continuous light, there was no increase in the total activity per leaf of this etioplast membrane ATPase during the first 6 hours of plastid development. The increase in ATPase activity occurred in the later stages of chloroplast development.

3. The highest specific activity of the ATPase of plastid membrane (based in μmoles phosphate released/mg protein) was obtained from preparations of etioplast membrane. In continuous light, specific activity of etioplast membrane ATPase was constant for 9 hours, then decreased.

4. The relations between chlorophyll synthesis and increase in plastid membrane protein and ATPase activity were investigated under several different light regimes. Chlorophyll and plastid membrane protein could be increased without

concomittant increases in ATPase activity; but the converse could not be obtained.

5. Pre-illumination of intact plants shortened the lag phase of chlorophyll synthesis, and increased the amount of plastid membrane protein and plastid membrane ATPase activity immediately during subsequent continuous illumination.

6. Etiolated plants, treated with repetitive cycles of 5 minutes of light and 8 hours of dark, showed an increase in the total leaf ATPase activity after 5 cycles of light-dark period while chlorophyll increased linearly with the number of cycles and plastid membrane protein increased after two light-dark cycles.

7. The ATPase of the etioplast membranes was more easily inactivated by being kept at 55°C than the ATPase of the chloroplast membrane.

8. ATPase was activated more by a combined treatment of trypsin and dithiothreitol than by the treatment of trypsin and dithiothreitol alone. The maximum activity of the ATPase was obtained by a combined treatment of first dithiothreitol, then trypsin; the reducing agent potentiates the activation by trypsin but the reverse is not observed. This indicates the mechanisms of ATPase activation by trypsin and

dithiothreitol are different.

9. I conclude that plastid membrane development and increase in plastid membrane ATPase activity during chloroplast greening are not limited by chlorophyll synthesis. The binding of this ATPase to plastid membranes appears to change during greening.

ADENOSINE TRIPHOSPHATASE ACTIVITY OF PLASTID MEMBRANE DURING CHLOROPLAST DEVELOPMENT OF KIDNEY BEAN LEAVES

By

Chen-hsiung Wu

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1974

ACKNOWLEDGMENTS

I wish to thank Dr. Kenneth D. Nadler for the inspiration and guidance he provided in the course of these studies as my research supervisor. I would also like to thank Dr. Clifford J. Pollard, Dr. Peter C. Wolk and Dr. Loran L. Bieber for their service on my guidance committee and Dr. Robert S. Bandurski for the use of his facilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The formation of the lamellar system in plastids of dark-grown leaves progresses through a series of light-dependent steps (13, 14). The tubes in the prolamellar body constitute a specific arrangement of the membrane as part of the starting material for the development of the chloroplast lamellar system. When etiolated leaves are illuminated with white light, protochlorophyllide a is reduced photochemically to chlorophyllide a, and the tubes in the crystalline prolamellar bodies are transformed and dispersed into the primary lamellar layers which are parallel to each other (13, 14, 32). The acquisition of photophosphorylation activity and photosynthetic competence all occur within the first few hours of illumination of etiolated leaves (11, 25, 28). As both chlorophyll a and b accumulate, extensive formation of grana takes place. Grana formation is correlated with the rapid phase of chlorophyll synthesis (11, 14). The detailed molecular composition of the membranes of prolamellar body is little known. Little is understood about the changes occuring when the membranes of prolamellar body become

capable of photosynthesis, nor is the role of chlorophyll in this assembly process understood. I decided to study these problems in chloroplast membrane formation using the Ca^{2+}-dependent ATPase activity of these membranes as a marker.

It has been shown that the treatment of chloroplasts or subchloroplast particles with trypsin activates a Ca^{2+}-dependent ATPase. Treatment with dilute EDTA solution solubilizes this ATPase and releases a coupling factor which markedly stimulates photophosphorylation in partially resolved subchloroplast particles (3, 4, 5, 7, 8, 9, 18, 29, 30). Racker and his associates have shown that a Ca^{2+}-activated ATPase activity is associated with spinach chloroplast, and this ATPase can be washed from the membrane by dilute EDTA solution. The chloroplast membrane after extraction by EDTA is incapable of carrying on photosynthetic phosphorylation. The EDTA extract of chloroplast membranes exhibits a trypsin- and dithiothreitol-activated, Ca^{2+}-dependent ATPase activity. When this EDTA extract is added to the depleted chloroplast membrane, the photophosphorylation of chloroplast membrane can be restored (5, 23). Ca^{2+}-activated ATPase activity is also present in etioplast membranes. This etioplast ATPase appears to have identical

properties to the chloroplast ATPase; for example, NaCl-EDTA extracts of etioplasts can restore photosynthetic phosphorylation activity to the depleted green membranes of chloroplasts (15, 19).

These studies were initiated to probe plastid membrane development by relating the development of membrane-bound ATPase activity to membrane protein and chlorophyll content during greening.

The choice of suitable plant leaf tissue for these studies is of some importance. Racker et. al. (23, 30) used spinach for studying properties of chloroplast ATPase; Lockshin et. al. (19) used maize for measuring the specific activity of membrane bound ATPase during chloroplast development. However it is inconvenient to use maize for greening experiments because of the gradient in age of the leaf cells in this plant. While spinach leaves are more uniform, it is difficult to grow large amounts of leaf tissue in the dark. Therefore leaves of kidney beans, which green up synchronously and can be easily grown in the dark were selected as plant material in these experiments.

MATERIALS AND METHODS

Plant Growth

Kidney beans (Phaseolus vulgaris) were sterilized in 1% NaOCl for 15 minutes. After washing out the NaOCl with water, the sterilized seeds were sown in 2.5 liters vermiculite in plastic flats (length 54 cm, width 26 cm, depth 6.5 cm) containing 2.5 liters Hutner's nutrient solution (in appendix). After one week growth in darkness at 25°C, plants were watered with one liter deionized water. Leaves of the etiolated plants were harvested generally at 11 days to 13 days. Before 11 days, the primary leaves were still enfolded within the cotyledons and after 13 days or longer, the etiolated leaves showed long lag phase of chlorophyll synthesis or greened poorly. Greening plants were obtained by putting the dark-grown plants in 430 ft-candles white light-obtained from four 20 W incandesent bulbs and 2 cool white 20 W fluorescent lamps.

Isolation of Plastid Membrane

To obtain plastid membranes, etiolated leaves or

green leaves were ground in a solution of 0.01 M NaCl and 0.05 M Tris-HCl at pH 8.0 by mortar and pestle. Preliminary experiments indicated that more ATPase activity (per mg protein) was obtained by using a grinding medium without sucrose than one with sucrose. This was continued in all experiments described. After filtering the homogenate through 4 layers of cheesecloth and 2 layers of miracloth, plastid membranes were spun down by centrifugation at 3000 x g for 15 minutes. The pellet of plastid membranes was resuspended in solution of 0.05 M Tricine-NaOH and 0.01 M NaCl at pH 8.0 by the mixer. This suspension was used for assaying Ca^{2+}-dependent ATPase activity.

All experimental manipulations in the isolation of intact plastids and plastid membrane were performed at ice temperatures (0 - 4°C).

Assay of ATPase

For assay of ATPase activity, 0.5 ml plastid membrane suspension was added to 2 ml substrate containing 100 μmoles Tricine-NaOH at pH 8.0, 25 μmoles Ca^{2+} and 10 μmoles ATP. The reaction mixture was incubated for 30 minutes at 37°C in a water bath and was then stopped by addition of 1 ml of cold 16% trichloroacetic acid. The phosphate released by

ATP hydrolysis during incubation was determined. After spinning out the TCA precipitate, the phosphate content of the supernatant was measured with the Ames reagent by incubating at 45°C for 10 minutes and then reading the absorbance at 820 nm with Gilford spectrophotometer 240 (1). The non-enzymatically released phosphate was subtracted from all readings.

Protein content was determined by the method of Lowry et. al. (20).

Chlorophyll content in the leaf was measured by the method of Arnon (2).

RESULTS

ATPase of Plastid Membrane of Kidney Bean

The Ca^{2+}-dependence of the ATPase of etioplast or chloroplast membranes of kidney bean leaves is shown in Fig. 1. An optimum Ca^{2+} concentration occurs in the range 5 - 17.5 millimolar Ca^{2+}. There was a slight inhibition at 25 millimolar Ca^{2+} in reaction mixture. The specific activity of the plastid membrane ATPase from 12 days old dark-grown bean leaves as high as 9.4 µmoles ATP hydrolyzed per mg membrane protein in 30 minutes could be obtained by incubating plastid membrane at 37°C with ATP, Ca^{2+} and Tricine-NaOH buffer at pH 8.0. Levels of trypsin-treated ATPase activity could be obtained as high as 20.5 µmoles per mg membrane protein per 30 minutes at the same condition as those without trypsin treated ATPase. This activity is low compared with that from spinach leaves as reported by Racker et. al. (23, 30) and higher than from corn leaves used by Lockshin et. al. (19). The specific activity (on a mg protein basis) of the ATPase of chloroplast membrane was less than that of the etioplast.

Trypsin Activation of the Plastid Membrane Ca^{2+}-dependent ATPase

ATPase activity is linearly dependent on added plastid membrane protein to at least 0.6 mg membrane protein (Fig. 2). Trypsin activates this ATPase activity (Fig. 3). A fifteen-minute incubation at room temperature with 10 μg trypsin yields the maximum ATPase activity. If 10 μg trypsin are added 15 minutes after the first trypsin addition, there is no additional increase in ATPase activity (Fig. 3). In fact, the ATPase activity decreases slightly 10 minutes after the second trypsin addition probably due to trypsin digestion of some active ATPase. This indicates that 10 μg trypsin treatment for 15 minutes at room temperature fully activates the ATPase in 200 μg plastid membrane.

ATPase Activity of Plastid Membrane During Chloroplast Development

The development of twelve-day old dark-grown etiolated bean plants illuminated continuously with white light is shown in Fig. 4. The lag phase of chlorophyll synthesis is ended after 4 hours light. An increase in plastid membrane protein per leaf could be detected only after 6 hours light. Increases in total leaf ATPase activity, either with or

without trypsin activation, could also be detected only after 6 hours light; however specific activity (expressed as μmoles of phosphate released from ATP per mg membrane protein in 30 minutes) decreased after 9 hours light for both trypsin activated ATPase and non-trypsin ATPase.

After 3 days of continuous light, the total ATPase of chloroplast membrane and chlorophyll in the leaf remained constant, but membrane protein of chloroplast still increased for 3 more days in continuous light (Fig. 5). The data of Fig. 5 also show that ATPase of plastid membranes, the plastid membrane protein and chlorophyll all increased maximally between 1 day and 3 days continuous light.

ATPase Activity During Chloroplast Development of the Pre-illuminated Leaves (Which were Treated with 10 Minutes Light and Put in the Dark for 25 Hours)

The lag phase of chlorophyll synthesis can be shortened or abolished by treating the plant with a short light period followed by a long period of incubation in darkness (31, 33). This long incubation in darkness after brief illumination probably permits the enzymatic adaptation of the etioplast; i. e. the brief illumination followed by a period of prolonged darkness results in the synthesis of

some of the enzymes necessary for ALA formation (24) and photosynthesis (21). I therefore studied the effect of pre-illumination on plastid membrane ATPase activity during greening.

The etiolated leaves of 12 days dark-grown plants were illuminated for 10 minutes and put in the dark for 25 hours, then were illuminated with continuous light. Compared to non-preilluminated experiment shown in Fig. 4, this treatment markedly shortens the lag phase of chlorophyll synthesis and results in slight but immediate increases in the membrane protein of plastid per leaf and total ATPase, both trypsin-activated and non-trypsin-activated, activity of plastid membrane per leaf. The specific activity of the ATPase decreases immediately after exposing pre-illuminated plants to continuous light (Fig. 6 and Fig. 7) as opposed to the relatively constant ATPase activity of control plants, not pre-illuminated.

I have repeated this pre-illuminated treatment experiment. The data for plastid membrane protein per leaf, total ATPase activity of plastid membrane per leaf and specific ATPase activity of plastid membrane are represented in Table 2. There is no doubt that plastid membrane protein content per leaf increases and the specific activity of

plastid membrane ATPase immediately decreases after the beginning of the continuous light. Although there is no significant increase in total ATPase activity per leaf during the first two hours of illumination, the total ATPase content of the leaf linearly increases with illumination time beyond 2 hours. I believe that there is an immediate slight increase in total ATPase activity per leaf after the beginning of the continuous light after pre-illumination.

Plastid Membrane ATPase Activity During Repetitive Light-Dark Treatment

If etiolated leaves are repetitively illuminated with 5 minutes light, then returned to darkness for several hours, this treatment can induce the etioplast of the dark-grown leaves partially develop into a chloroplast, although chloroplasts developed under this condition lack grana formation (6, 10, 11, 13, 14, 28). Such chloroplasts show large stacks of the parallel primary thylakoids with very little fusion. These plastids have as much protein as mature chloroplasts. In order to select conditions for the light part of the treatment, I determined how much time was required to convert all proto-chlorophyllide a in etiolated leaves to chlorophyllide. In the 12 days dark-grown

etiolated bean leaves, this required 3 minutes illumination (Fig. 8). In fact, most of protochlorophyllide a was photoconverted to chlorophyll within 1 minute. After 1 minute, there is only a small amount protochlorophyllide a in the etiolated leaves but this small amount of protochlorophyllide a requires an additional another 2 minutes illumination for complete conversion to chlorophyllide.

In order to ensure that all protochlorophyllide a in the etiolated leaves had been converted to chlorophyllide by brief light, five minutes instead of three minutes illumination of the etiolated plants was used. I then determined the length of the dark period needed for resynthesis of the maximum amount of protochlorophyllide a in the etiolated leaves. This required 7 hours darkness (Fig. 9). In the first two hours darkness after the brief illumination, there is little protochlorophyllide a synthesis, as in leaves with a lag phase in chlorophyll synthesis. Eight hours were used for the dark period in this experiment for reasons similar to those mentioned above. Thus, the dark-grown leaves of kidney bean were treated repetitively with cycles of 5 minutes light and 8 hours darkness.

The results of this treatment are indicated in Fig. 10. Leaf chlorophyll content increased linearly for 6

light-dark cycles. This means that the rate of chlorophyll synthesis and therefore protochlorophyllide regeneration within 6 light-dark cycles remains constant. After 6 cycles of light-dark period, the rate of chlorophyll synthesis increases greatly indicating that the rate of ALA synthesis is now greater than that before 6 light-dark cycles. Plastid membrane protein and leaf fresh weight increase after 2 cycles of light-dark period while plastid membrane ATPase increases only after 5 cycles of light-dark period.

The Effect of Dithiothreitol and Trypsin on ATPase Activity of Chloroplast Membrane

The plastid membrane ATPase activity can be activated by several methods; by treatment with trypsin, by heating two minutes at 65°C in the presence of ATP and by sulfhydryl compounds such as dithiothreitol, α-thioglycerol and β-mercaptoethanol (23, 30). The ATPase activity of chloroplast membrane treated by dithiothreitol for 2 hours was much less than those activated by trypsin (Table 1). If ATPase was activated by trypsin for 15 minutes, then activated by dithiothreitol for one hour or two hours, or if the ATPase was activated by dithiothreitol for one hour or two hours, then activated by trypsin for 15 minutes, the

resulting ATPase activity was more than that obtained by trypsin- or dithiothreitol-activation alone. Furthermore, the activity of ATPase first activated by trypsin then by dithiothreitol was about equal to the combined activities of ATPase activated by trypsin and dithiothreitol alone. However the activity of ATPase first activated by dithiothreitol then by trypsin was much more than the activity of ATPase first activated by trypsin then by dithiothreitol. Clearly there is a synergistic effect of dithiothreitol on trypsin activation.

Heat Inactivation of Plastid Membrane ATPase

A change in the thermal stability of a protein indicates a change in protein conformation and/or a change in the environment of a protein. The ATPase of etioplast membranes is rapidly inactivated by 55°C (Fig. 11), while the ATPase of chloroplast membranes is relatively stable at 55°C. Although the ATPase of chloroplast membrane drops to approximately 80% of the control ATPase after 10 minutes incubation, the ATPase activity of etioplast membrane drops to 30% of the control ATPase after 10 minutes incubation. The different heat sensitivity between etioplast membrane ATPase and chloroplast membrane ATPase may indicate that the environment of plastid membrane ATPase changes during chloroplast development.

DISCUSSION

ATPase of crude bean plastid membrane preparation is used for assaying the ATPase activity in these experiments. Mitochondria also have trypsin activated, Ca^{2+}-dependent ATPase (30). Using an isolation medium without sucrose to isolate plastid membranes and using a low speed centrifugation, contamination by mitochondrial membranes was minimized.

Lockshin et. al. measured plastid membrane ATPase activity of maize leaves during greening. ATPase activity per mg membrane protein was relatively constant through 12 hours of illumination. After 12 hours of illumination, the specific activity of ATPase decreased by a factor of about 2 during the next 13.5 hours regardless of whether or not the plants were illuminated. They suggested that most or possibly all of the coupling factor (measured as ATPase activity) of the chloroplast may be present in the etioplast from which it develops. They also reported that the specific activity of the enzyme dropped two fold during the next 13.5 hours due to synthesis of much more membrane but little new synthesis of the coupling factor occurs (19). This does not appear to

be the case in greening bean leaves. The specific activity of the Ca^{2+}-dependent ATPase of bean plastid membranes remains constant in early period of greening because there is neither net membrane protein synthesis nor ATPase synthesis at this time; the specific activity of the ATPase dropped in the later period of chloroplast greening since other membrane proteins of plastids were synthesized much more than the ATPase during this period. The total plastid membrane ATPase of the leaf increases in continuous light, after a lag period, for about 3 days. Horak and Hill (16) also reported that there is a considerable increase in the specific activity of the ATPase in plastids during greening. They measured the activity of the ATPase in EDTA extracts of plastids and found that it increased during greening. The proteins extracted by EDTA from plastid membrane are mainly ATPase with little contamination by other membrane proteins (17).

All the ATPase activity of plastid membranes during the first 6 hours in continuous light or in 5 light-dark cycles is present in the etioplast. If the ATPase of the etioplast membrane is originally in the prolamellar body (19), it is undoubtedly redistributed into lamellae during the first several hours of chloroplast development.

The lag phase of chlorophyll synthesis ends within 4 hours during continuous illumination; ATPase activity of plastid membranes increases only after 6 hours in continuous light. Furthermore, in conditions where etiolated leaves were treated by cycles of 5 minutes of light and 8 hours of the dark, chlorophyll accumulates while the ATPase activity of plastid membrane increases only after 5 cycles of light-dark period. The above experimental results suggest that (a) chlorophyll does not limit the synthesis of plastid membrane ATPase, (b) ATPase synthesis can occur in the dark without simultaneous chlorophyll synthesis (although chlorophyll synthesis must precede it).

There was no chlorophyll formation (except that a small amount of protochlorophyllide a was converted to chlorophyll a within the first 3 minutes of light) in etiolated leaves during the 3 hours lag phase of chlorophyll synthesis. Mego and Jagendorf (22) showed that the etiolated bean plastids had the same amount of chlorophyll formed in one minute or two hours of light· but plastid growth was greater in two hours light than one minute light (22). By the use of red light and far-red light to control enlargement of bean leaf plastids, they also provided evidence that there was no correlation between plastid size and the amount of

chlorophyll development. This suggests that chloroplast membrane development is not controlled by chlorophyll synthesis.

It has been shown by Butler and associates that when the dark-grown bean leaves (Phaseolus vulgaris) were illuminated with white light, photophosphorylation appeared after one to two hours of illumination (11, 28). Using similar plants in these experiments, I have also shown that there is no increase in chlorophyll and plastid membrane protein content as well as ATPase activity in this period. Because the membranes of prolamellar body progress through a series of changes and arrangements that are stimulated by light, I suggest that neither chlorophyll nor plastid membrane protein synthesis but some kind of conformational changes in prolamellar body membrane is required for photophosphorylation development.

Glydenholm and Whatley (12) also reported that cyclic and non-cyclic photophosphorylation were detectable only after 10 hours illumination. In neither report did the development of photophosphorylation parallel the increase of ATPase activity of etioplast membranes during chloroplast development as was shown in my experiment. Even if the ATPase of plastid membranes is related to a coupling factor

of photophosphorylation, the time for the onset of photophosphorylation of etiolated leaves would not correspond to the time for increase ATPase activity. Because there is already a certain amount of ATPase in etioplast membrane, it can be used for the development of photophosphorylation without new ATPase synthesis. Indeed, ATPase synthesis may not commence until the activity of etioplast ATPase becomes limiting for photophosphorylation at later greening stages. So we can not compare the time relationship between photophosphorylation development and the ATPase synthesis to conclude whether the ATPase of plastid membrane is a coupling factor or not.

The Ca^{2+}-dependent ATPase of plastid membrane is a very stable enzyme. For the assay of ATPase activity, the enzyme was incubated at 37°C for 30 minutes. In fact this ATPase, in the presence of ATP, can be heated at temperature as high as 65°C for 2 minutes to increase its activity (23, 30). This high stability of the ATPase of chloroplast membranes may be due to the high thermal stability of chloroplast lamellae. The lamellar proteins and lipids may protect it from denaturation by the environment (5). That the ATPase of etioplast membranes is easier to denature by heat than the ATPase of chloroplast membrane suggests that the synthesis or reorganization of the proteins and lipids

of chloroplast membranes occurs during greening.

Racker et. al. first isolated a coupling factor from chloroplasts. This coupling factor has been shown to stimulate photophosphorylation and to have trypsin-activated, Ca^{2+}-dependent ATPase. They called it chloroplast coupling factor 1 (the abbreviation used is CF1) to distinguish it from mitochondrial coupling factor 1 (the abbreviation used is F1) (23, 30). Recently, Racker et. al. (26, 27) reported that five different polypeptides could be isolated from CF1. These five subunits are referred to as α-, β-, γ-, δ- and ϵ-chains in the order of decreasing molecular weight of 59000, 56000, 37000, 17500 and 13000. They called ϵ subunit a CF1 inhibitor; because it can inhibit ATPase activity. Antibodies against the 59000 and 37000 molecular weight subunit (α and γ component) inhibited cyclic photophosphorylation and phosphorylation coupled to the Hill reaction. They concluded that α and γ subunits of CF1 are intimately involved in the function of the coupling factor and the ϵ subunit is a regulatory subunit of the enzyme. They proposed that CF1 inhibitor is more firmly bound to CF1 at a site required for binding to the membrane. They suggested that trypsin activated the ATPase of CF1 either by destroying the inhibitor or removing it from the active site of the enzyme and dithio-

threitol somehow interferes with the interaction of the inhibitor with the active site of CF1.

In my experiments, plastid membrane ATPase after activation by trypsin can still be activated by dithiothreitol. So, I don't support the suggestion by Racker et. al. that dithiothreitol interferes with the interaction of inhibitor with active site of CF1. Instead the evidence reported here suggest that there are two reactions to activate the enzyme ATPase. One is to reduce disulfide groups(-S-S-) of protein to sulfhydryl groups (-2SH). Another activation is to remove CF1 inhibitor. Thus the enzyme ATPase can enhance its activity either when its disulfide groups are reduced to sulfhydryl groups or when CF1 inhibitor is removed. Thus the enzyme ATPase after trypsin activation can still be activated by dithiothreitol and vice versa. The reason for the enhanced activity of ATPase first activated by dithiothreitol then by trypsin is not clear. Perhaps the protein conformation of the enzyme is affected by this treatment.

Racker et. al. suggest that trypsin activated Ca^{2+}-dependent ATPase of chloroplast was associated with a solubilization of the ATPase (23). If plastid membrane ATPase is first treated by dithiothreitol, many disulfide bonds (-S-S-) are reduced by dithiothreitol to sulfhydryl groups (-2SH).

This reaction many render plastid membrane protein more open to removal of CF1 inhibitor by trypsin; and the maximum activity of ATPase may be obtained.

Racker and coworkers (23) have shown that EDTA extracts of chloroplast (CF1) could stimulate photophosphorylation. But when this extract was treated by trypsin to exhibit ATPase activity, its properties as a coupling factor were lost. Horak and Hill demonstrated that no coupling factor activity could be detected in disc gel electrophoresis zones containing ATPase activity (16). The trypsin-activated ATPase can not function as a coupling factor. The ability to couple requires CF1 inhibitor binding to the ATPase. Both ATPase activity and photophosphorylation could be easily demonstrated in the chloroplasts that have been exposed to dithiothreitol (23). So, dithiothreitol-activated ATPase contains the trypsin-activated ATPase and the CF1 inhibitor.

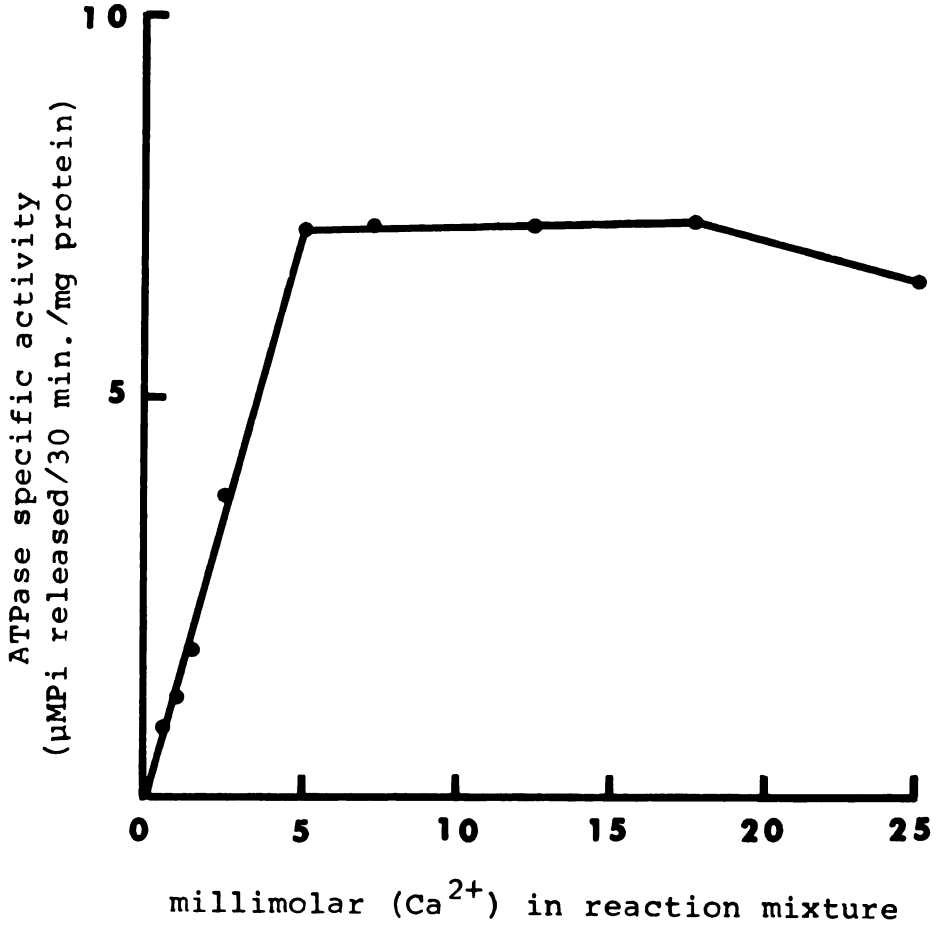


Figure 1. The Ca^{2+}-dependence of the ATPase activity of chloroplast membranes. 190 µg membrane protein of chloroplast were treated by 10 µg trypsin for 15 minutes and trypsin activation was stopped by adding 100 µg trypsin inhibitor. Enzymatic phosphate release was measured as described in Methods except calcium salts were not included in the substrate stock solution.

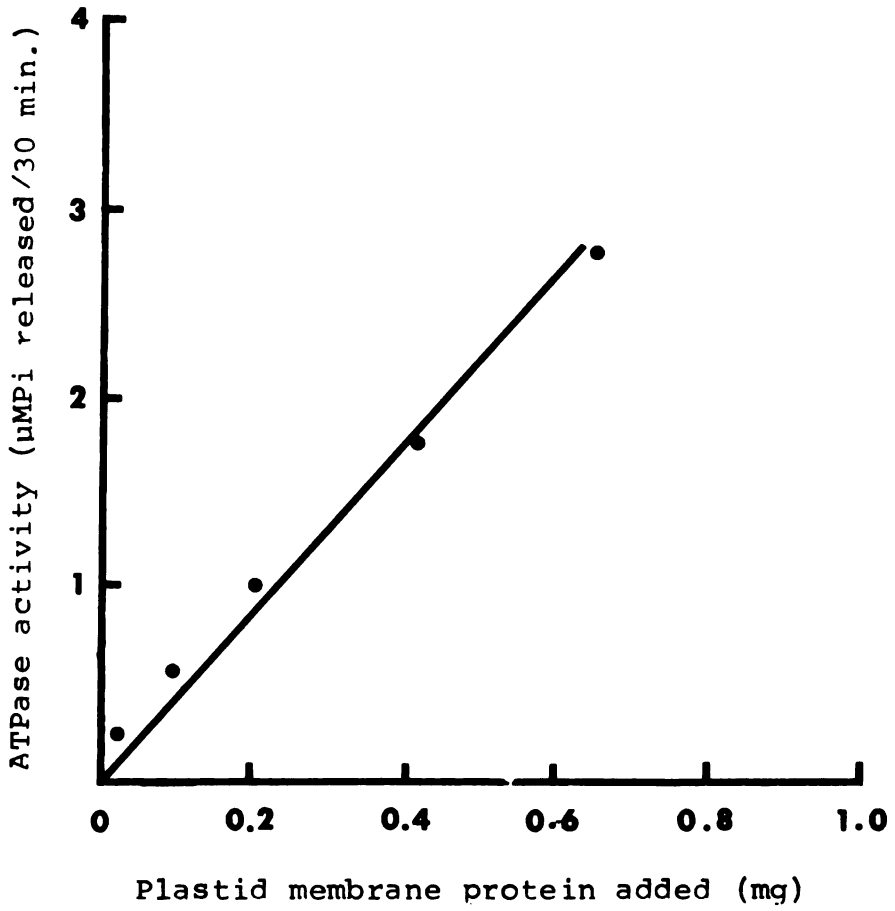


Figure 2. The effect of the amount of etioplast membrane protein on ATPase activity. 30 pairs of primary leaves of 14 days dark-grown plants were ground in 20 ml of 0.05 M Tris-HCl buffer containing 0.01 M NaCl at pH 8.0 with sand in a mortar and pestle. After filtering through 4 layers of cheesecloth and 2 layers of miracloth, plastid membranes were spun down by centrifugation at 3000 x g for 15 minutes. The plastid pellet was suspended in 10 ml of 0.05 M Tricine-NaOH buffer containing 0.01 M NaCl at pH 8.0 by the mixer. Various amount of plastid membrane suspension were assayed for ATPase as described in Methods.

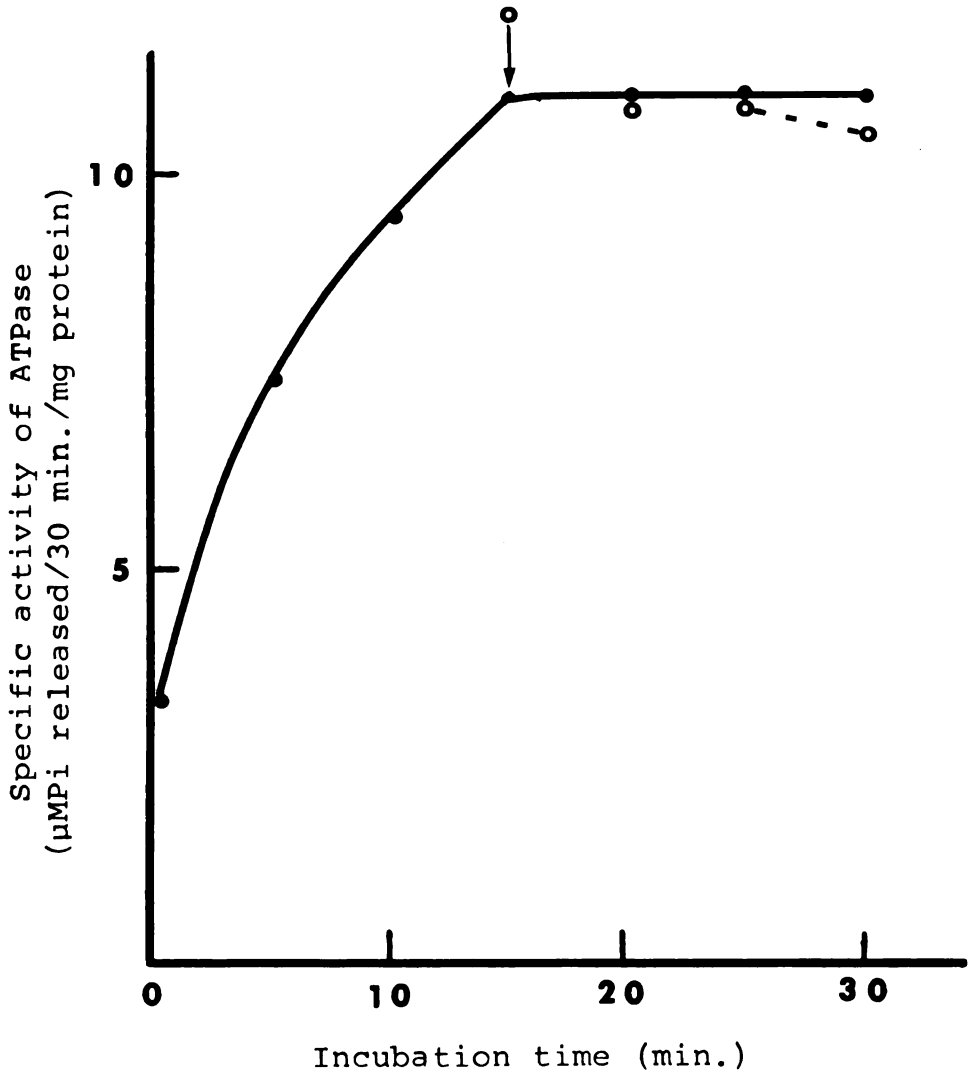
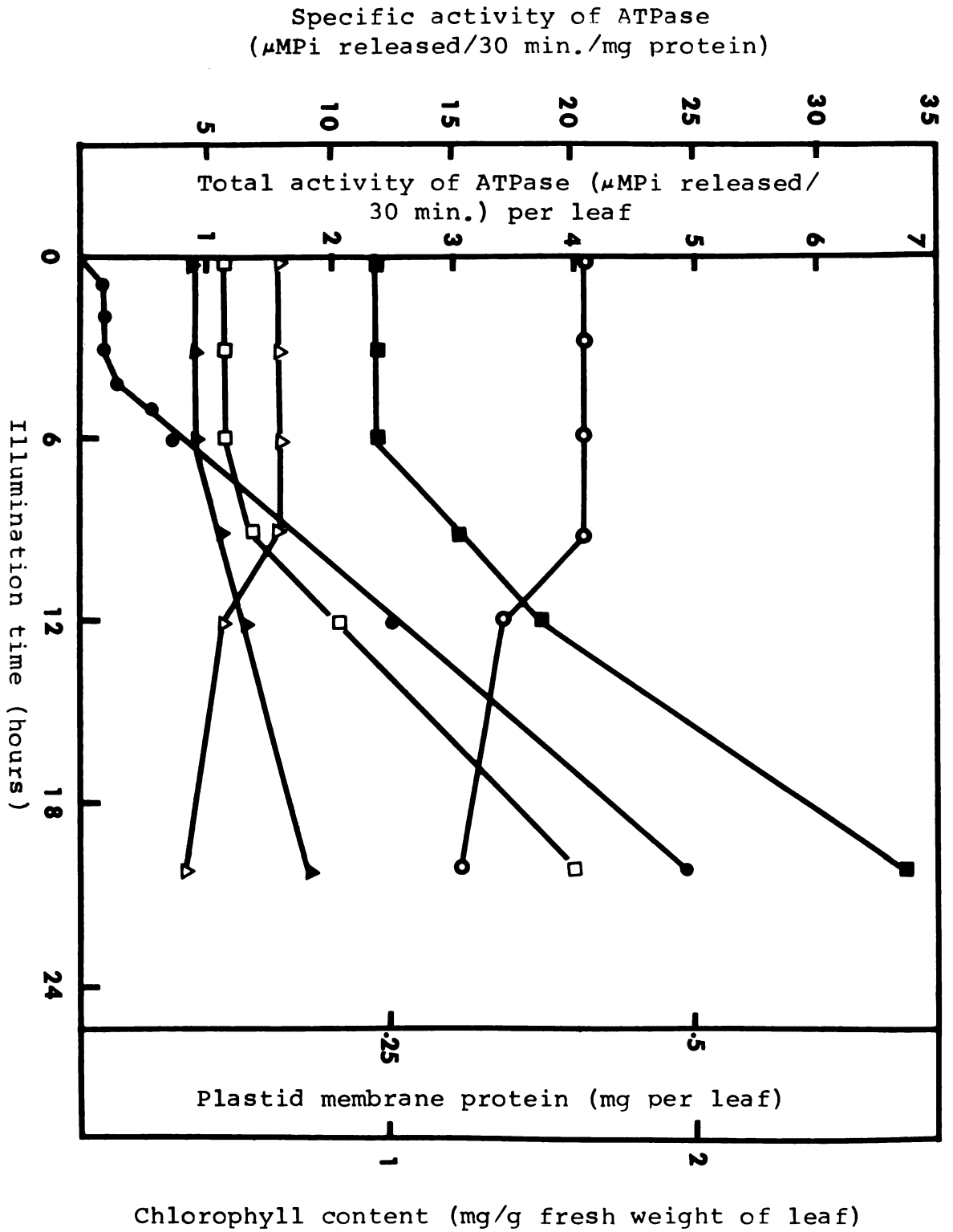


Figure 3. Trypsin activation of etioplast membrane ATPase. 200 μg plastid membrane was activated by 10 μg trypsin and 10 μg trypsin was further added after 15 minutes incubation as indicated by an arrow.

Figure 4. Ca^{2+}-dependent ATPase activity, chlorophyll content and insoluble protein content of plastids during chloroplast development in 12 days dark-grown bean leaves. Plants were illuminated under continuous light. 10 pairs of primary leaf were harvested each time and the plastid membranes were isolated as described in the Methods. 0.05 ml membrane suspension were activated by treatment with 10 μg trypsin for 15 minutes. Trypsin activation was stopped by adding 100 μg trypsin inhibitor. Then, plastid membrane suspension was assayed for ATPase as in the Methods. (●) chlorophyll content. (▲) total ATPase activity of non-trypsin treated plastid membrane per leaf. (■) total ATPase activity of trypsin treated plastid membrane per leaf. (△) specific activity of non-trypsin activated ATPase of plastid membrane. (○) specific activity of trypsin activated ATPase of plastid membrane. (□) total plastid membrane protein per leaf.


Specific activity of ATPase
(μMPi released/30 min./mg protein)
5
10
15
20
25
30
35
Total activity of ATPase (μMPi released/
30 min.) per leaf
1
2
3
4
5
6
7
0
6
12
18
24
Illumination time (hours)
.25
.5
Plastid membrane protein (mg per leaf)
1
2
Chlorophyll content (mg/g fresh weight of leaf)

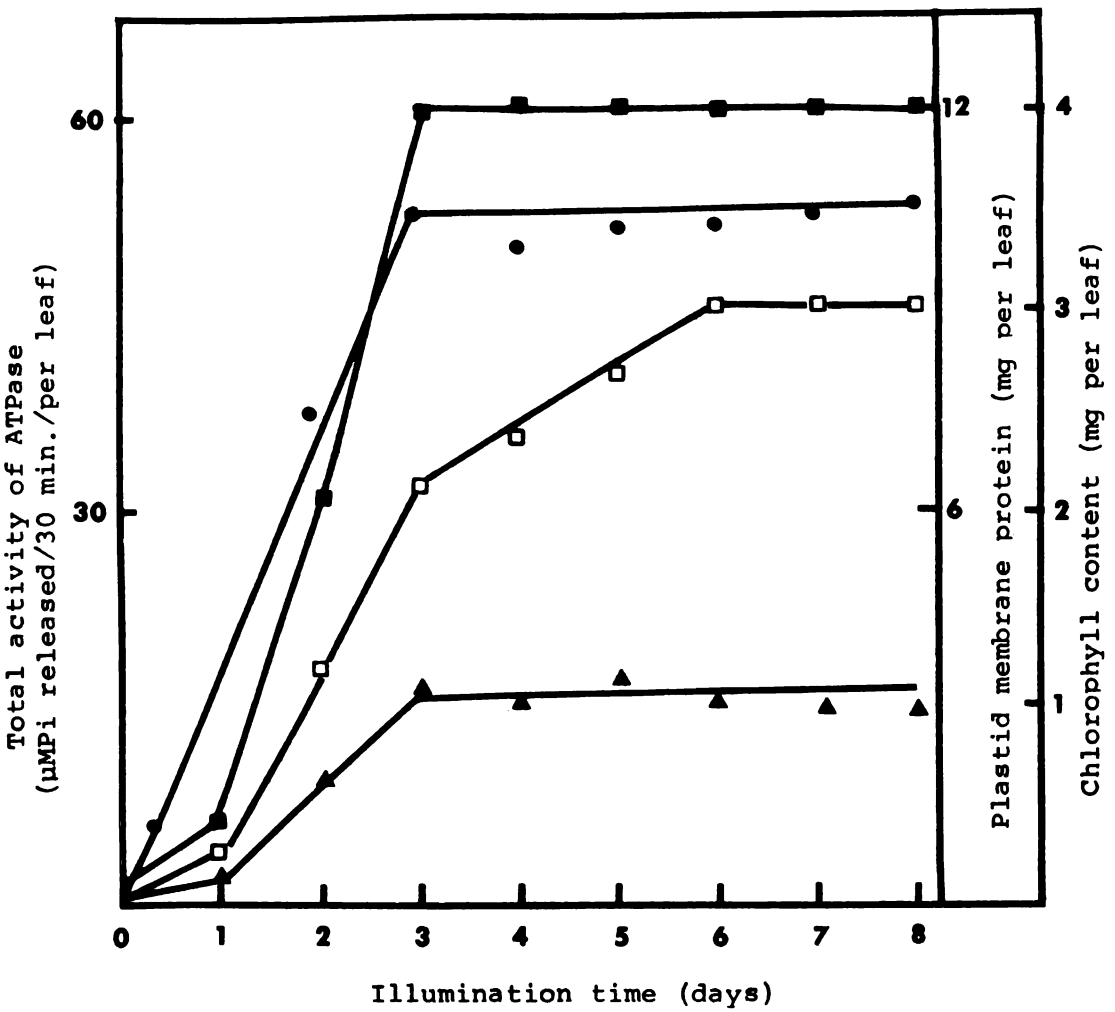


Figure 5. Long-term variations in Ca^{2+}-dependent ATPase activity, chlorophyll content and plastid membrane protein in plastids greening bean leaf. Methods for the experiment are the same as in Fig. 4. (■) total ATPase activity of trypsin treated plastid membrane per leaf. (▲) total ATPase activity of non-trypsin treated plastid membrane per leaf. (□) total protein of plastid membrane per leaf. (●) chlorophyll content.

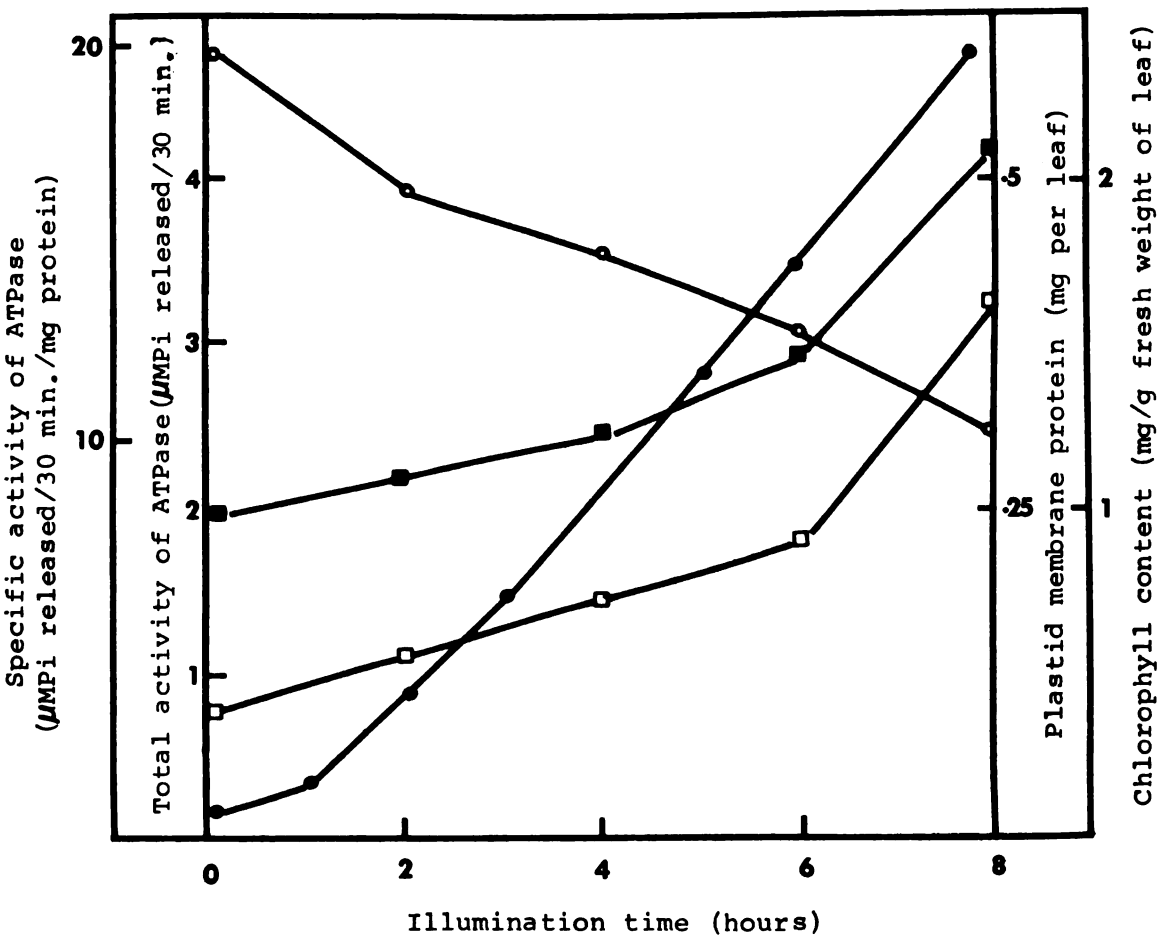


Figure 6. Effect of pre-illumination on the Ca^{2+}-dependent ATPase activity, chlorophyll content and insoluble protein of plastids during chloroplast development. Leaves were illuminated for 10 minutes and placed in darkness for 25 hours, then continuously illuminated for various periods. Methods for the experiment are the same as in Fig. 4. (●) chlorophyll content. (■) total ATPase activity of trypsin treated plastid membrane per leaf. (⊙) specific activity of trypsin activated ATPase of plastid membrane. (□) total protein of plastid membrane per leaf.

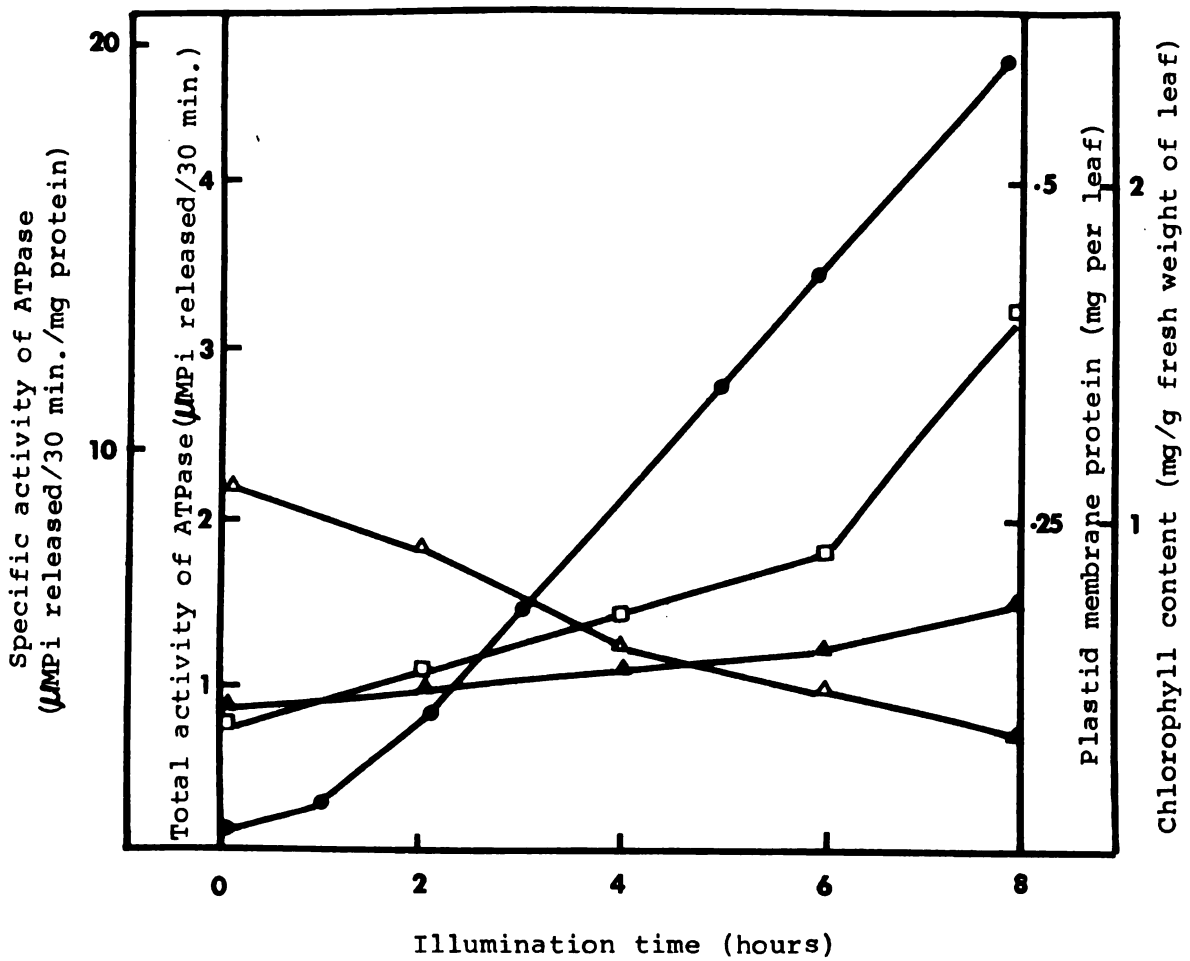


Figure 7. Effect of pre-illumination on the Ca^{2+}-dependent ATPase activity, chlorophyll content and insoluble protein of plastids during chloroplast development. Leaves were illuminated for 10 minutes and placed in darkness for 25 hours, then continuously illuminated for various periods. Methods for the experiment are the same as in Fig. 4. (●) chlorophyll content. (▲) total ATPase activity of non-trypsin treated plastid membrane per leaf. (△) specific activity of non-trypsin activated ATPase of plastid membrane. (□) total protein of plastid membrane per leaf.

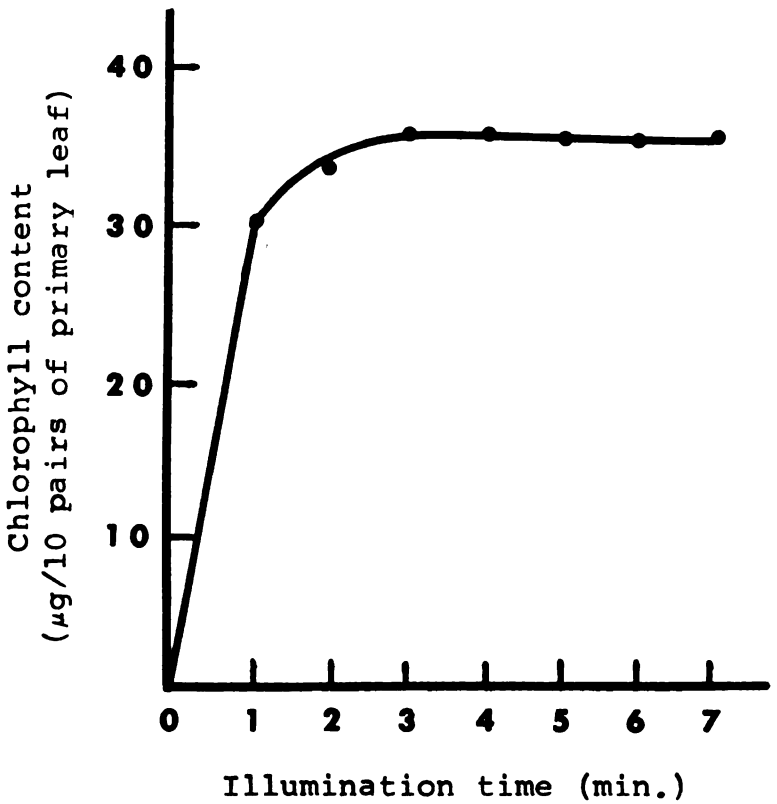


Figure 8. Conversion of protochlorophyllide a to chlorophyll in etiolated leaves. 12 days old etiolated leaves were illuminated and the pigments extracted as described previously.

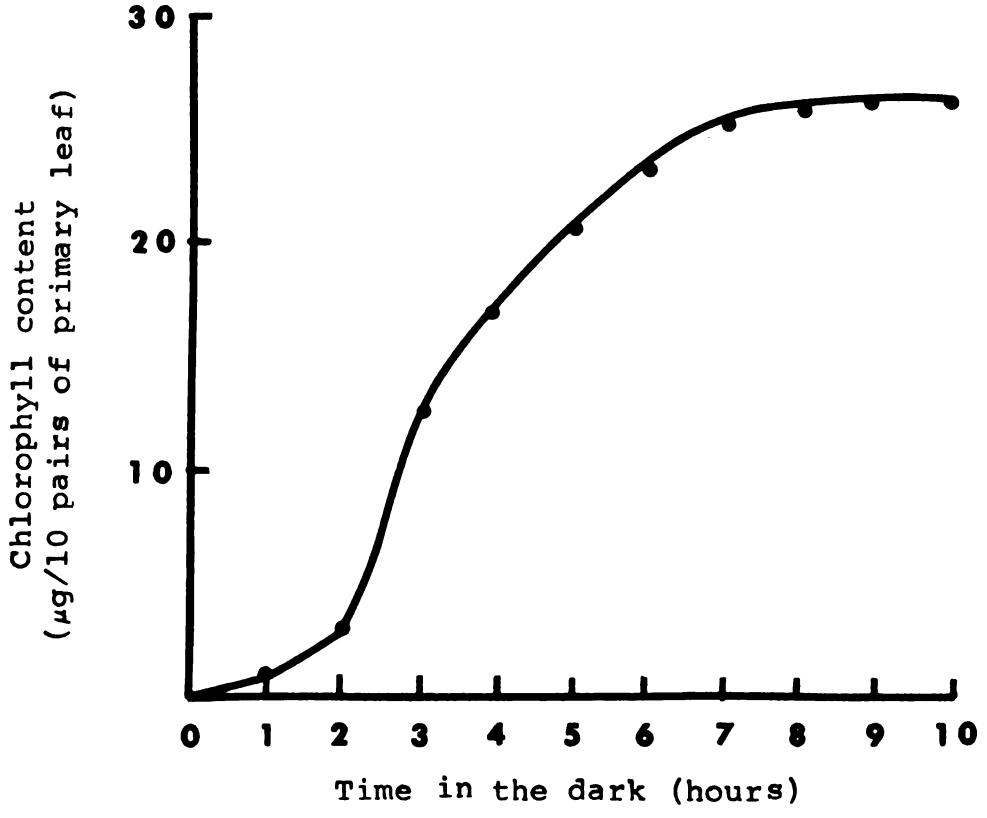
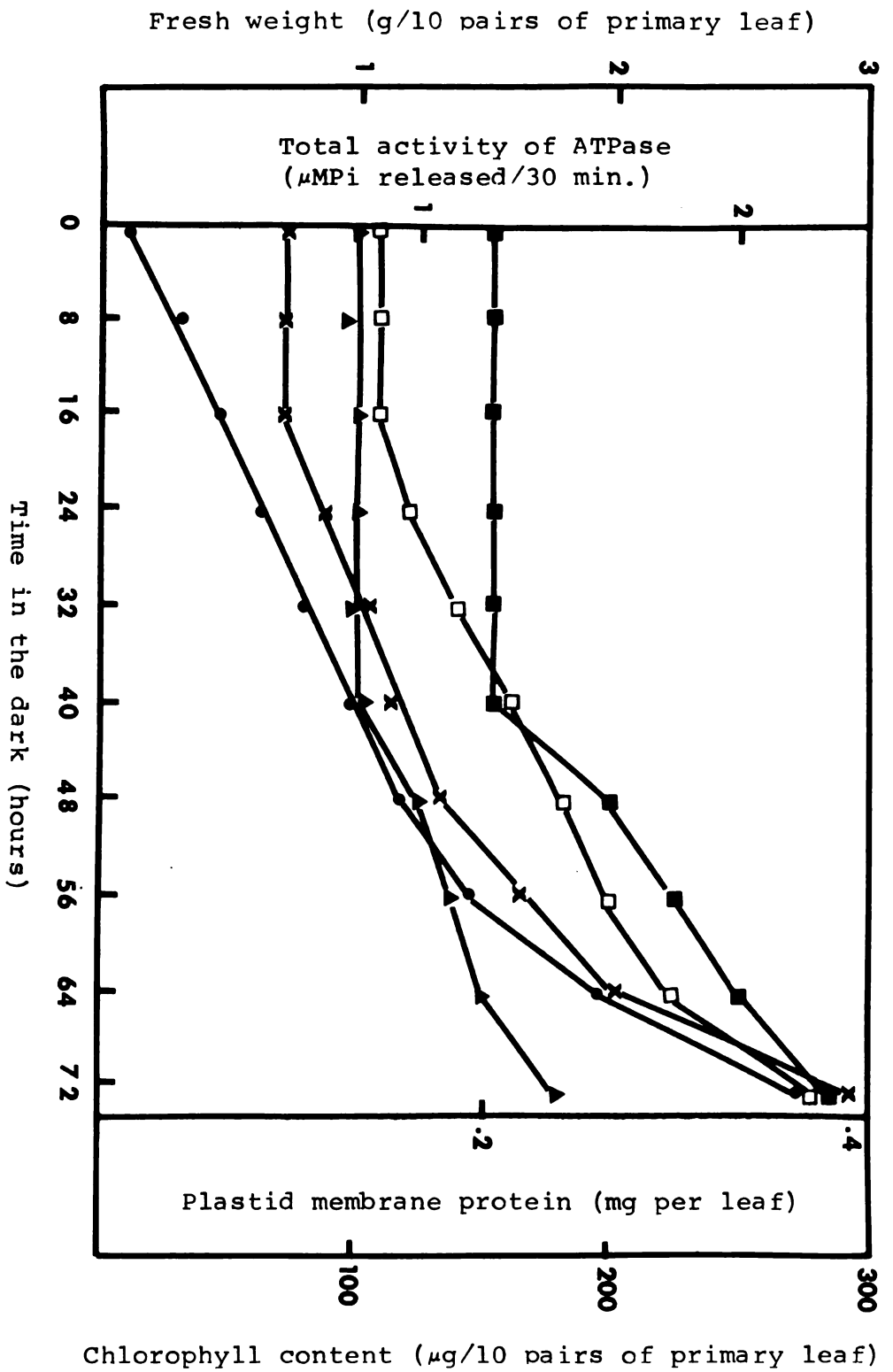


Figure 9. Regeneration of protochlorophyllide a after illumination of etiolated leaves with 5 minutes light. Leaves were put in the dark after this 5 minutes light. leaves were given for 5 minutes light again after every hour in the dark and measurement of the increase in chlorophyll content used as an index of protochlorophyllide a content.

Figure 10. Ca^{2+}-dependent ATPase activity of plastid membrane after several light-dark cycles of 5 minutes light and 8 hours darkness. After each illumination, the leaves were assayed as previously described. (●) chlorophyll content. (▲) total ATPase activity of non-trypsin treated plastid membrane per leaf. (■) total ATPase activity of trypsin treated membrane per leaf. (□) total protein of plastid membrane per leaf. (✖) fresh weight of leaves.


Fresh weight (g/10 pairs of primary leaf)
1
2
3
Total activity of ATPase
(μMPi released/30 min.)
1
2
0
8
16
24
32
40
48
56
64
72
Time in the dark (hours)
.2
.4
Plastid membrane protein (mg per leaf)
100
200
300
Chlorophyll content (μg/10 pairs of primary leaf)

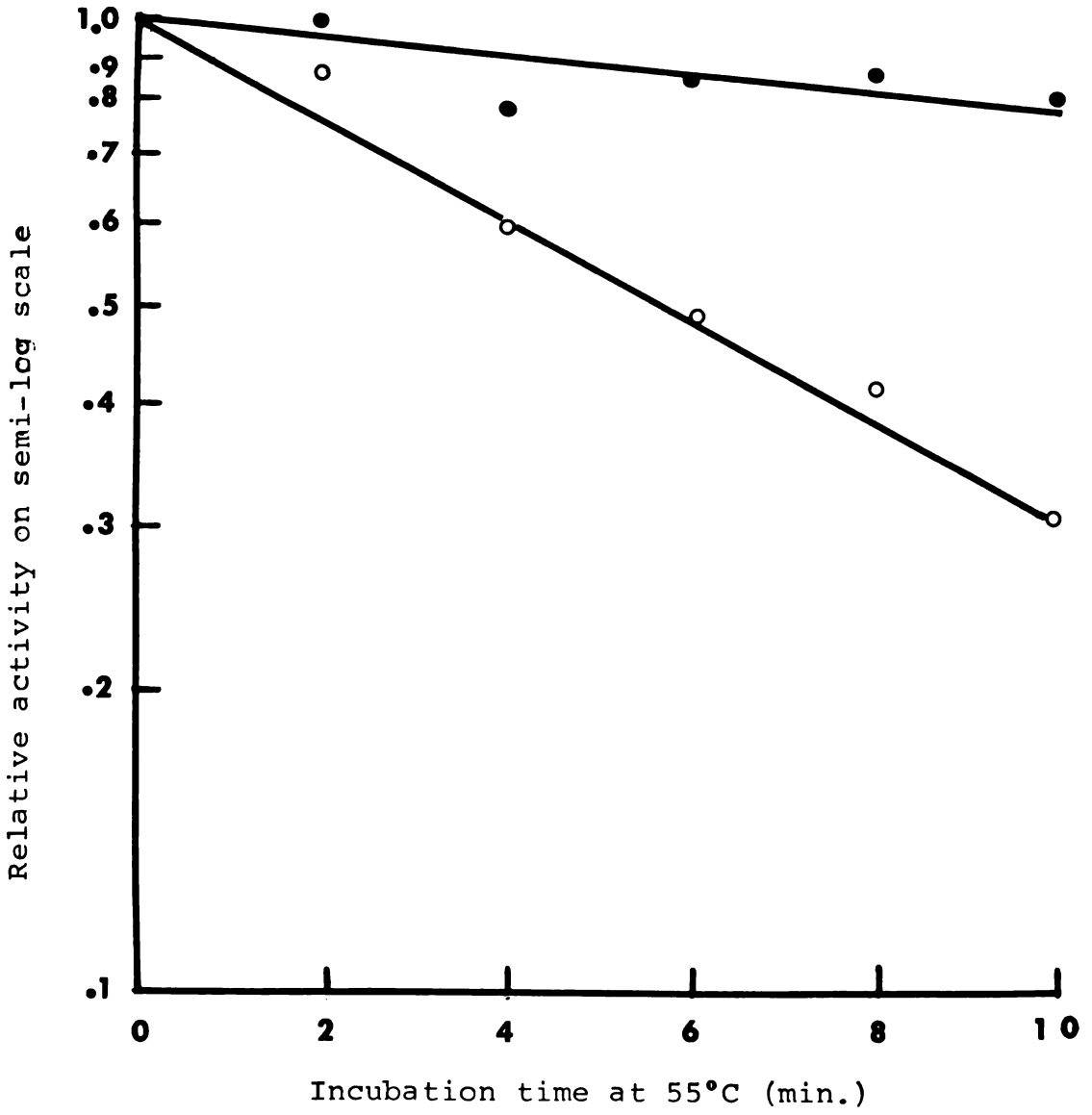


Figure 11. Time course of heat inactivation of trypsin-activated Ca^{2+}-dependent ATPase of etioplast and chloroplast membranes. The plastid suspension in the reaction mixture after heating was rapidly cooled in the cold ice water. Total ATPase activity of etioplast membrane and chloroplast membrane before heating are 2.4 μmoles and 1.8 μmoles phosphate released per 30 minutes respectively. (●) ATPase of chloroplast membrane. (O) ATPase of etioplast membrane.

TABLE 1

The effect of trypsin and dithiothreitol (DTT) on ATPase activity of chloroplast membranes. Thirteen-day old etiolated plants were illuminated for 8 days in continuous light and the leaves were harvested for ATPase assay.

Treatment	Specific activity of ATPase (μMPi/mg protein/30 min.)	% Control
Control without any incubation with trypsin or DTT	1.16	100
Incubation with 0.05 M DTT for zero time	2.41	208
Incubation with 0.05 M DTT for 30 min.	2.80	241
Incubation with 0.05 M DTT for 1 hour	3.35	288
Incubation with 0.05 M DTT for 2 hours	4.30	370
Incubation with 10 μg trypsin for 15 min.	7.30	629
Incubation with 10 μg trypsin for 15 min. then with 0.05 M DTT for 1 hour	10.20	879
Incubation with 0.05 M DTT for 1 hour then with 10 μg trypsin for 15 min.	13.60	1172
Incubation with 10 μg trypsin for 15 min. then with 0.05 M DTT for 2 hours	11.44	986
Incubation with 0.05 M DTT for 2 hours then with 10 μg trypsin for 15 min.	14.55	1254

TABLE 2

Effect of pre-illumination on the Ca^{2+}-dependent ATPase activity and membrane protein of plastids during chloroplast development.

	Time in continuous light (hour)	µg Membrane protein per leaf	Total membrane ATPase (µmoles Pi/per leaf/30 min.) non-trypsin	Total membrane ATPase (µmoles Pi/per leaf/30 min.) trypsin treatment	Specific activity of plastid membrane ATPase (µmoles Pi/mg protein/30 min.) non-trypsin	Specific activity of plastid membrane ATPase (µmoles Pi/mg protein/30 min.) trypsin treatment
Experiment 1	0	190	1.20 ± 0.06	2.76 ± 0.12	6.30	14.50
	2	240	1.36 ± 0.07	2.88 ± 0.10	5.60	12.00
	4	285	1.48 ± 0.15	3.00 ± 0.04	5.10	10.50
	6	345	1.60 ± 0.04	3.48 ± 0.12	4.60	10.08
	8	450	1.80 ± 0.03	4.08 ± 0.11	4.00	9.06
Experiment 2	0	102	0.96 ± 0.08	2.00 ± 0.14	9.41	19.60
	2	132	1.00 ± 0.08	2.20 ± 0.12	7.50	16.60
	4	162	1.04 ± 0.12	2.50 ± 0.08	6.50	15.40
	6	228	1.12 ± 0.06	3.00 ± 0.12	4.80	13.10
	8	438	1.42 ± 0.04	4.48 ± 0.08	3.20	10.20

LIST OF REFERENCES

LIST OF REFERENCES

1. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatase. Methods in Enzymol. VIII: 115-116.

2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts, Polyphenoloxidase in Beta vulgaris. Plant Physiol. 24: 1-15.

3. Avron, M. 1963. A coupling factor for photophosphorylation. Biochem. Biophys. Acta 77: 699-702.

4. Bennun, A. and M. Avron. 1965. The relation of the light-dependent and light-triggered adenosine triphosphatase to photophosphorylation. Biochem. Biophys. Acta 109: 117-127.

5. Bennun, A. and E. Racker. 1969. Partial resolution of the enzymes catalyzing photophosphorylation. J. Biol. Chem. 244: 1325-1331.

6. Butler, W. L., J. De Greef, T. F. Roth, and H. Oelze-Karow. 1972. The influence of carbonylcyanide-m-chlorophenyl hydrazone and 3-(3,4-dichlorophenyl)-1, 1-dimethylurea on the fusion of primary thylakoids and the formation of crystalline fibrils in bean leaves partially greened in far red light. Plant Physiol. 49: 102-104.

7. Carmelic, C. and M. Avron. 1967. A light-triggered adenosine triphosphate-phosphate exchange reaction in chloroplasts. European J. Biochem. 2: 318-326.

8. Carmelic, C. 1969. Properties of ATPase in chloroplasts. Biochem. Biophys. Acta 189: 256-266.

9. Carmelic, C. and Y. Lifshitz. 1969. The requirement of adenosine diphosphate for light-triggered ATPase and ATP-Pi exchange reaction in chloroplasts. FEBS Letters 5: 227-230.

10. Eilam, Y. and S. Klein. 1962. The effect of light intensity and sucrose feeding on the fine structure in chlorophyll content in etiolated leaves. J. Cell Biol. 14: 169-182.

11. Greef, De., W. L. Butler and J. F. Roth. 1971. Greening of etiolated bean leaves in far red light. Plant Physiol. 47: 457-464.

12. Glydenholm, A. O. and F. R. Whatly. 1968. The onset of photophosphorylation in chloroplasts isolated from developing bean leaves. New Phytol. 67: 461-468.

13. Henningsen, K. W. and J. E. Boynton. 1969. Macromolecular physiology of plastids VII. The effect of a brief illumination on plastids of dark-grown barley leaves. J. Cell Sci. 5: 757-793.

14. Henningsen, K. W. and J. E. Boynton. 1970. Macromolecular physiology of plastids VIII. Pigment and membrane formation in plastids of barley greening under low light intensity. J. Cell Biol. 44: 290-304.

15. Horak, A. and R. D. Hill. 1971. Coupling factor for photophosphorylation in bean etioplasts and chloroplasts. Can. J. Biochem. 49: 207-209.

16. Horak, A. and R. D. Hill. 1972. Adenosine triphosphatase of bean plastids. Plant Physiol. 49: 365-370.

17. Howell, S. H. and E. N. Moudrianakis. 1967. Function of the "quantasome" in photosynthesis: structure and properties of membrane-bound particle active in the dark reactions of photophosphorylation. Proc. Nat. Acad. Sci. Wash. 58: 1261-1268.

18. Livne, A. and E. Racker. 1969. Partial resolution of the enzymes catalyzing photophosphorylation. J. Biol. Chem. 244: 1339-1344.

19. Lockshin, A., R. H. Falk, L. Bogorad, and C. L. F. Woodcock. 1971. A coupling factor for photosynthetic phosphorylation from plastids of light- and dark-grown maize. Biochem. Biophys. Acta 226: 366-382.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

21. Margulies, M. 1964. Effect of chloramphenicol on light-dependent synthesis of protein and enzymes of leaves and chloroplasts of Phaseolus vulgaris. Plant Physiol. 39: 579-585.

22. Mego, J. L. and A. T. Jagendorf. 1961. Effect of light on growth of Black Valentine bean plastids. Biochem. Biophys. Acta 53: 237-254.

23. McLarty, R. E. and E. Racker. 1968. Partial resolution of the enzymes catalyzing photophosphorylation. J. Biol. Chem. 243: 129-237.

24. Nadler, K. and S. Granick. 1970. Controls on chlorophyll synthesis in barley. Plant Physiol. 46: 240-246.

25. Nadler, K. and H. A. Herron, and S. Granick. 1972. Development of chlorophyll and Hill activity. Plant Physiol. 49: 388-392.

26. Nelson, N., H. Nelson, and E. Racker. 1972. Partial resolution of the enzymes catalyzing photophosphorylation XII purification and properties of an inhibitor isolated from chloroplast coupling factor 1. J. Biol. Chem. 247: 7657-7662.

27. Nelson, N., D. W. Deters, H. Nelson and E. Racker. 1973. Partial resolution of the enzymes catalyzing photophosphorylation. XIII properties of isolated subunits of coupling factor 1 from spicach chloroplasts. J. Biol. Chem. 248: 2049-2055.

28. Oelze-Karow, H. and W. L. Butler. 1971. The development of photophosphorylation and photosynthesis in greening bean leaves. Plant Physiol. 48: 621-625.

29. Petrack, B.,A. Craston, F. Sheppy, and F. Farron. 1965. Studies on the hydrolysis of adenosine triphosphate by spinach chloroplasts. J. Biol. Chem. 250: 906-914.

30. Vambutas, V. K. and E. Racker. 1965. Partial resolution of the enzymes catalyzing photophosphorylation. J. Biol. Chem. 240: 2660-2667.

31. Virgin, H. I.. 1957. Chlorophyll a content and transpiration of etiolated wheat leaves after pre-treatment with a short light impulse followed by dark periods of varying light. Physiol. Plantarum. 10: 445-453.

32. Weier, T. E. and D. L. Brown. 1970. Formation of the prolamellar body in 8-day dark-grown seedlings. Amer. J. Bot. 57: 267-275.

33. Withrow, R. B., J. B. Wolff, and L. Price. 1956. Elimination of the lag phase of chlorophyll synthesis in dark-grown bean leaves by a pretreatment with low irradiances of monochromatic energies. Plant Physiol. 31: XIII.

APPENDIX

APPENDIX

For 2 liter Hutner's ($-Mg^{2+}$) stock solution:

Solution A: in 400 ml distilled water

$Ca(NO_3)_2$	35.40 g
EDTA(acid)	50.00 g
K_2HPO_4	40.00 g
KOH(85% pellets)	24 - 26 g

Cool with ice.

Solution B: in 300 ml distilled water

$ZnSO_4 \cdot 7H_2O$	6.59 g
H_3BO_3	1.42 g
$Na_2MO_4 \cdot 2H_2O$	2.52 g
$CuSO_4 \cdot 5H_2O$	0.394 g

Add 1 N HCl until cloudiness disappears.

Solution C: in 100 ml

$FeSO_4 \cdot 7H_2O$	2.47 g

Then solution A + solution B + solution C + H_2O ⟶ 2 liters.

"Mg^{2+}" stock solution: 25 g $MgSO_4 \cdot 7H_2O$ per liter

The complete nutrient solution for plant growth:

(1 part Hutner's solution + 1 part "Mg^{2+}" stock solution)

⟶ diluted to 50 times by H_2O